STEM-SPECIFIC PROMOTERS FROM SORGHUM AND MAIZE FOR USE IN SUGARCANE

By

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Declaration

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Stem-specific promoters from sorghum and maize for use in sugarcane
Abstract

Sugarcane (*Saccharum* spp.) is an important crop which is cultivated worldwide for the high sucrose content in its stem. Conventional plant breeding has proven to be very successful over the years with regard to the enhancement of yield characteristics but due to the exhaustion of genetic potential in the commercial sugarcane germplasm recent progress has been slow. Genetic engineering seems to be a more attractive approach to enhance sucrose content and pest resistance in the stems but requires appropriate transgenes and suitable promoter.

A promoter is essential to drive the transcription of a gene and is therefore critical to the success of transgenic approaches in sugarcane crop improvement. A negligible number of strong stem-specific promoters is available for use in sugarcane and this is one of the major limitations to genetic engineering. The goal of this project was to isolate a stem-specific promoter from maize and sorghum to drive stem-specific transgene expression in sugarcane.

The approach used was to source promoters from non-sugarcane grass species with less complex genomes to simplify isolation and possibly counteract silencing. A cDNA sequence (SS) (EST clone, Accession number AW746904) from sugarcane was shown by Northern and Southern analysis to be stem-specific and to have an appropriately low copy number. The SS gene sequence was not expressed in the leaves of maize, sorghum or the sugarcane cultivars and prominent expression was observed only in the stems of the sugarcane hybrids N19 and 88H0019.

The SS gene sequence was used to isolate its upstream regions from a Lambda genomic library of maize (*Zea mays)* and a sorghum (*Sorghum bicolor)* Bacterial Artificial Chromosome library (BAC). Of the four sorghum and six maize clones obtained in this

study, a 4500 bp maize genomic DNA fragment (λ5) was sub-cloned in three fragments into separate pBluescript vectors using the 'forced' cloning approach for sequence and database (BLASTN) analysis. This revealed the complete SS gene sequence (975 bp), the promoter and a 300 bp intron region.

A stretch of DNA sequence from nucleotides 664-3194 from the maize clone 5 sequence was designated the maize5-pro. Following sequence alignment of the maize and sugarcane promoter regions, significant sequence identity (68%) was observed between nucleotide 1675 and 3194 in maize and nucleotide 1506 and 2947 in sugarcane. The distance between the putative TATA-box and the TSS for this promoter (30 bp) was found to fall within the expected range of 32± 7 bp.

The promoter region was analysed for possible *cis*-acting regulatory elements and revealed several promoter elements that are common in other plant promoters. The comparisons made between the putative transcription factors in maizepro-5 and the sugarcane promoter show that both promoter sequences are very similar as they share ten of the same transcription factors. However, the transcriptional factors WBOX, SRE and SP8BFIBSP8BIB are unique to the maize5-pro and the TAAG motif to the sugarcane promoter.

Primers were designed with appropriate restriction sites and the promoter and intron (2850 bp) region was amplified by PCR (Polymerase chain reaction). The amplified fragment was fused inframe to the GUS reporter gene encoding *ß-glucuronidase* to produce a transformation test vector. This will be used in future work to assess the functionality of the promoter through the production of stable transformants in which GUS activity can be measured in a range of tissues.

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Chapter 1
Introduction

Sugarcane (*Saccharum* spp. hybrids) a member of the grass family, Gramineae, is a crop of major importance for the production of sucrose (Grof *et al.* 2001). Sugarcane is best grown economically in tropical and sub-tropical regions between 15° and 30° latitude (Barnes, 1974) and is cultivated in over 120 countries and is the source for approx. 70% of the world's sugar (South African Sugar Association Industry Directory, 2006). The world sugarcane crop was forecast at a near record of 126.8 million tons in 2001- 2002 (FAS, 2001).

South Africa is the eleventh largest producer of cane sugar, after countries such as Brazil, Cuba, India, Australia, the United States, Philippines and China, with a production of 2.5 million tons of sugar per annum (South African Sugar Association Industry Directory, 2006). About 50% of this sugar is marketed in the Southern Africa Customs Union (SACU) while the remainder is exported to numerous markets in Africa, the Middle East, North America and Asia (South African Sugar Association Industry Directory, 2006).

Sugarcane was first grown in South Africa in 1848 on the Kwa-Zulu Natal North Coast. By 1990 the proceeds of the sugarcane industry exceeded 2 billion Rand for one season (South African Sugar Association Industry Directory, 2006) and in 2003 a record crop of 2763 000 tons of sugar was produced (South African Sugar Association Industry Directory, 2006). Based on the revenue generated through sugar sales, the South African sugarcane industry is responsible for generating direct income totaling 6 billion rand (South African Sugar Association Industry Directory, 2006).

The ideal climate for growing sugarcane is characterised by warm mean day temperatures of 30°C with adequate moisture and high incident solar radiation (Barnes, 1974). It must be noted that sugarcane is grown under fairly adverse conditions in KwaZulu Natal as this area is the furthest south that sugarcane is found in the world (O' Reilly, 1998). Adverse climatic conditions are not the only problems faced by sugarcane farmers; disease and pest damage are also major factors. Since these factors impact directly on the sucrose content of

sugarcane, strategies to solve such production problems are a priority to the sugarcane industry.

The South African Sugarcane Research Institute (SASRI) based in Mount Edgecombe is an institution involved in developing and optimising the productivity of commercial sugarcane cultivars grown in South Africa. These cultivars are selected for producing high sucrose yields, resistance to pests and diseases, herbicide resistance as well as tolerance to environmental stresses. The plant breeding and biotechnology programmes based at the South African Sugarcane Research Institute are both aimed at varietal improvement of the sugarcane plant.

It is not surprising that the genetic improvement of sugarcane has been a major focus of plant breeding efforts and has made sugarcane a prime candidate for the application of genetic engineering (Snyman, 2004). Modern cultivated sugarcane obtained by traditional plant breeding techniques has widely enhanced important economic traits (Liu *et al.,* 2003; Srivastava *et al.,* 1994). These important economic traits encompass reduced disease and pest problems, increased biomass and sugar yields, herbicide resistance and improved adaptability for growth under various stress conditions (Liu *et al.,* 2003; Srivastava *et al.,* 1994).

Although conventional plant breeding has proven to be very successful over the years, the progress made by this method is very slow (Liu, 2003). Significant factors believed to have contributed to this slow progress are the genetic complexity, low fertility, narrow gene pool used in current commercial breeding programs and the long breeding cycles of sugarcane (Ingelbrecht *et al.,* 1999; Yang *et al.,* 2003; Grof and Campbell, 2001 and Roach, 1989). These factors render conventional breeding laborious and make transformation an attractive approach to include for the improvement of sugarcane (Yang *et al.,* 2003).

Genetic engineering by DNA-mediated plant transformation has opened up new avenues to introduce useful genes into sugarcane that otherwise would be difficult or impossible by standard procedures (Hansen and Wright, 1999). The sugarcane industry is under great pressure due to damages caused by insects and pathogens resulting in both sucrose

yield and economic losses (Sharpe, 1998). Resistance to such economically important traits can therefore be conferred to sugarcane via genetic engineering.

To date there has been a number of traits that have been introduced into sugarcane. These include insect resistance via the δ-endotoxin gene from *Bacillus thuringiensis* (Arencibia *et al.,* 1997), proteinase inhibitor genes (Allsopp *et al.,* 1997; Nutt *et al.,* 1999) and mannose-binding lectins (Irvine and Mirkov, 1997), resistance to sugarcane mosaic virus (SCMV) (Joyce *et al.,* 1998; Ingelbrecht *et al.,* 1999), altered sucrose content via down-regulation of pyrophosphate-dependent phosphofructokinase (Groenewald and Botha, 2001), herbicide resistance via the *pat* gene (Leibbrant and Snyman, 2003; Falco *et al.,* 2000) and the modification of carbon partitioning (Grof and Campbell, 2001).

Significant progress has been made in the development of molecular tools for the transformation of sugarcane and other monocotyledonous (Grof and Campbell, 2001) and transformation is now well established for sugarcane (Bower and Birch, 1992). Transformation aims to recover transgenic plants and the target material must be able to regenerate into plants that show efficient uptake of DNA (Franks and Birch, 1991). Particle bombardment and *Agrobacterium tumefaciens* co-cultivation are the two most common methods for the delivery and expression of transgenes (Liu *et al.,* 2003; Yang *et al.,* 2003 and Falco *et al.,* 2000). The most widespread approach for sugarcane transformation is that of particle bombardment using embryogenic callus as target material. An example of transformation success and efficiency using particle bombardment undertaken at SASRI was the genetic engineering of sugarcane with the *pat* gene to confer resistance to the herbicidal compound glufosinate ammonium in Buster® (Leibbrandt and Snyman, 2003; Leibbrandt and Snyman, 2001; Snyman *et al.,* 1998; Synman *et al.,* 2001).

To achieve effective transfer of foreign DNA into sugarcane cells, appropriate genetic constructs need to be made to facilitate integration and expression of the transgene. A typical genetic construct will contain a promoter, a transgene and a terminating signal. The promoter is a major requirement for genetic engineering applications since it offers fundamental control on gene expression therefore a great deal of interest and time is

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invested in isolating and studying plant promoters.

Promoter regions are the key cis-acting regulatory region that controls the transcription of adjacent coding regions into messenger ribonucleic acid which is directly translated into proteins (Datla *et al.,* 1997). A transformation system is only efficient in conjunction with the availability of a range of promoters with varied strengths and tissue specificities (Grof and Campbell, 2001; Yang *et al.* 2003 and Rooke *et al.,* 2000). One major constraint to genetic engineering of sugarcane is the lack of suitable promoters required to drive transgene expression (Liu *et al.,* 2003; Song *et al.,* 2000 and Yang *et al.,* 2003).

There are currently very few promoters that have been shown to be active in sugarcane. These include CaMV 35S promoter which has been widely used for high-level constitutive expression in dicotyledonous (Yang *et al.* 2003). The promoter elements from rice actin (Grof *et al.,* 1996a), rubisco small subunit (Grof *et al.,* 1996b), maize ubiquitin (Gallo-Meagher and Irvine, 1993), CaMV 35S (Elliott *et al.,* 1998) and pEMU (Rathus *et al.,* 1993) show activity in sugarcane although their expression levels are an order lower than the level of transgene expression driven by CaMV 35S in dicotyledonous model plant systems (Grof and Campbell, 2001).

The motivation for this project is based primarily on the lack of suitable promoters and although dicotyledonous promoters can be used in monocotyledonous species, in their native form they are poorly active. A number of constitutive promoters have been described. Constitutive promoters may be appropriate for the expression of certain types of transgene but there is a growing need for promoters that confer unique patterns of transgene expression in sugarcane. A major area of the sugarcane plant where transgene expression is required is in the stem. The stem area is not only the area were the stem borer, *Eldana Saccharina,* enters the plant and lives causing damage but it is also the region of the plant where sucrose is stored.

The isolation of stem-specific promoters to drive key genes that regulate major functional control over metabolic pathways in the stems of sugarcane would prove to be a valuable tool for sucrose manipulation. Moore *et al.,* (1997) has assessed the biophysical

capability of the sugarcane stem to accommodate a significant increase in sucrose concentration, the study shows that sugarcane is capable of storing more than 25% sucrose on a fresh-weight basis (Bull and Glasziou, 1963). Besides having the characteristic of stem-specificity, the isolated promoter should drive strong gene expression. A strong promoter would enable the production of sufficient RNA to activate a turnover mechanism that depends on a threshold RNA concentration (Matzke and Matzke, 1995).

Regulation of specific expression of transgenes in the stem will require stem-specific or stem-preferential promoter elements. This study therefore concentrates on the isolation of a stem-specific promoter for driving transgene expression in sugarcane without encountering promoter inactivation in the plant resulting in gene silencing.

Promoter inactivation leading to transgene silencing has been a major problem encountered in transgenic plant programmes therefore means of avoiding this problem needs to be addressed at an early stage in a transgenic programme. Bhullar *et al.* (2003) have mentioned two ways in which to avoid promoter homology. The first involves the designing of synthetic promoters while the second is more pertinent to this project and involves the use of diverse promoters which have been isolated from different plant and viral genomes. This understanding has been applied to this project by using maize and sorghum, which are close relatives of sugarcane, for isolating promoters to drive transgene expression in sugarcane.

Promoter silencing in transgenic programmes is not the only limiting factor to progress being achieved in this field of study. A limited number of promoters have been isolated for use in sugarcane and other monocotyledonous plants for introducing novel traits in economically important crops. Many of these elements isolated have commercial limitations with respect to their usage. Developing a promoter is considered an invention and is therefore protected by intellectual property laws. Both established and promising promoters already have patents. This causes unnecessary delays in reaching licensing agreements before the promoters can be used in a breeding program. Due to this, renewed efforts for isolating novel regulatory elements from monocotyledonous plants are of major importance. This project therefore sets out to isolate and identify promoters from

monocotyledonous plants to be utilised in the sugarcane breeding programs at SASRI.

The present study was aimed at using a stem-specific and low copy number cDNA clone for the isolation of a stem-specific promoter from maize and sorghum intended to drive high-level transgene expression in sugarcane. This gene was designated the stem-specific gene (SS gene) throughout this study. The SS gene was previously isolated from *Sorghum bicolor* (BAM Potier, SASRI). The specific function and identity of this gene is still unknown to date but preliminary studies carried out by BAM Potier have shown that this gene displays a strong stem-specific expression pattern and a low gene copy number.

The objectives set out in this project were:

- Analysis of the SS gene to confirm tissue-specificity of expression and copy number in maize, sorghum and sugarcane.
- Screening of maize Lambda genomic and sorghum Bacterial Artificial Chromosome libraries with the SS gene as the probe for isolation of candidate clones.
- Ligation of clone fragments into a plasmid vector followed by transformation of *E. coli*. 'Blue-white' screening will be used to select for positive colonies.
- Characterisation of the isolated clone regions by DNA sequence analysis for the identification of promoter elements downstream of the gene sequence.
- Incorporation of the promoter region into test constructs made by fusing the candidate promoters with the reporter gene *GUS.*

The thesis follows a classical structure. The relevant scientific background is summarised in Chapter 2 (Literature Review), techniques and protocols used in the work are described systematically in Chapter 3 (Materials and Methods), research results are presented in Chapter 4 (Results) and the general findings are explored and interpreted in Chapter 5 (Discussion and Conclusion).

Chapter 2
Literature Review

2.1 <u>The genetic complexity of sugarcane</u>

Sugarcane is one of the most important crops in the world, mainly cultivated for the high sucrose content in its stem. The modern sugarcane belongs to the family *Poaceae,* subfamily *Panicoideae* and tribe *Andripogoneae.* The commercial cultivars of sugarcane, as we know them today, have been derived through interspecific hybridisations involving two main species, *Saccha rum officinarum* and *Saccharum spontaneum,* with some contributions from other genera (Babu, 1974). The other genera include *Erianthus, Miscanthus, Sclerostachya* and *Narenga* and are closely related to *Saccharum* and together constitute an interbreeding group which is believed to have been involved in the origin of sugarcane (Daniel and Roach, 1987).

Current cultivated clones are essentially derived from interspecific hybridisation performed between *S. officinarum* (2n = 80) and *S. spontaneum* (2n = 40-128). Up until the 19th century most cultivated sugarcane plants were clones of *Saccharum officinarum*; this is a high sucrose variety which contains 2n=80 chromosomes. Breeding was successful and hybrids between *S. officinarum* and *Saccharum spontaneum* were developed. The breeding system of crossing *S. officinarum* with *S. spontaneum,* and repeatedly backcrossing the hybrids to *S. officinarum* (noble cane) is a process called nobilisation. This process is also commonly referred to as introgression breeding and defined as the transfer of a relatively small number of specific genes from ill-adapted germplasm into current commercial germplasm (Simmonds, 1993).

Cytological studies carried out by Bremer (1961) showed that nobilisation is characterised by asymmetric chromosome transmission. The hybridisation of *S. officinarum* and *S. spontaneum* is characterised by a 2n + n mode of chromosome transmission (Stevenson, 1965). *S. officinarum* generally transmits two haploid

chromosome sets while *S. spontaneum* transmits one causing an increase in the chromosome number of the hybrids. *S. officinarum* has 80 chromosomes consisting of 8 copies of a base set of 10 chromosomes, whilst *S. spontaneum* has 48 to 128 chromosomes made up of 6 to 16 copies of a basic number of 8 chromosomes (Barnes, 1974). The 2n + n mode of transmission is continued to the second backcross therefore modern cultivars have chromosome numbers ranging from 100-130, of which only 5-10% are of *S. spontaneum* origin and the remainder come from *S. officinarum* (D'Hont *et al.,* 1996, Simmonds, 1976). With repeated crossing, *S. spontaneum* chromosomes are preferentially lost.

Recently *in situ* hybridisation analysis of two ribosomal RNA gene families determined that *S. officinarum* has a basic chromosome number of x=10, therefore this plant is octoploid (D'Hont *et al.,* 1998). Using the same method it was shown that *S. spontaneum* has a basic chromosome number of x=8 and that the ploidy level of this species varies between 5 and 16 (D'Hont *et al.,* 1998). These studies established that the difference in chromosome numbers provides two distinct chromosome organisations which co-exist in modern sugarcane cultivars used to date (Grivet and Arruda, 2002). D'Hont *et al.* (1996) and Caudrado *et al.* 2004 used genomic *in situ* hybridisation to demonstrate that modern cultivars contain 15-20% *S. spontaneum* chromosomes of which less than 5% are recombinant or translocated chromosomes.

Commercial sugarcane cultivars thus have complex polyploid, aneuploid genomes and the cytogenetic complexity of interspecific hybrids make classical and molecular genetics as well as breeding studies difficult to interpret considering that the information on the structure and organisation of the genome has been largely speculative (Butterfield *et al.,* 2001).

Polyploidy occurs more frequently in the plant kingdom than the animal kingdom (Pikaard, 2001). Important crops such as banana, canola, coffee, maize, potato, oats, soybean, sugarcane and wheat are polyploids (Wendel, 2000; Osborne *et al.,* 2003). These plants being polyploid have multiple copies of a specific chromosome hence there are multiple copies of a gene present (Wendel, 2000). There are two situations in which

polyploidy could occur; the first involves the combining of two differentiated genomes into a common nucleus (allopolyploidy) and the second the duplication of a single genome (autopolyploidy) in one of the parental cytoplasms. It is thought that both *S. officinarum* and *S. spontaneum* have complex autopolyploid genomes (D'Hont, *et al.,* 1996; Ming *et al.,* 2001).

Understanding the *Saccharum* genome with respect to its evolution and organisation is necessary for making informed decisions in breeding, germplasm introgression and biotechnology. Much has been learned about the sugarcane genome over the past 80 years by implementing cytological studies, breeding experiments and molecular diversity studies (Butterfield and Berding, 2001). Sugarcane has a very complex genome or genetic structure due to the crossing between two or more than two polyploid *Saccharum* spp. (Grivet and Arruda, 2002). Sugarcane cultivars have ploidy levels that range from 5X to 14X (X= 5, 6, 8, 10, 12, or 14) and chromosomal mosaicism has been reported (Burner and Legendre, 1994). Such genetic complexity in the sugarcane genome poses added problems for plant breeding and research.

2.1.1 Comparative genetics of sugarcane, maize and sorghum

Grasses are the members of the family Gramineae or Poaceae and are represented by over 10 000 species including sugarcane, maize and sorghum (Kellogg and Birchler, 1993). The Poaceae includes most of the major food crops, which are well separated from dicotyledonous plants (Bennetzen *et al.,* 2001). Their phylogeny has been extensively studied and is well understood (Kellogg, 2001). Evolutionary divergence of the grass family occurred within the past 50 to 60 million years (Bennetzen *et al.,* 2001).

Amongst cereals, sorghum (*Sorghum bicolor)* is the world's fourth most important crop after rice, wheat and maize (Mutisya *et al.,* 2003). One important finding from mapping studies has revealed that sorghum and maize are very closely related and that large stretches of their genomic DNA are co-linear (Hulbert *et al.,* 1990; Binelli *et al.,* 1992 and Whitkus *et al.,* 1992). Since sorghum is closely related to maize and maize to other grasses (Ahn *et al.,* 1993), genes isolated from sorghum and maize or other

monocotyledonous may be directly transferred into other crops and therefore provide a source of genetic variation for crop improvement (Woo *et al.,* 1994).

Comparative analysis of several grass genomes (Doebley *et al.,* 1990; Binelli *et al.,* 1992; Ahn *et al.,* 1993; Hulbert *et al.,* 1990 and Bennetzen and Freeling, 1993) including maize, rice, sorghum, wheat, and barley have also shown extensive conservation of gene content and order (Gale and Devos, 1998). Molecular systematic studies have revealed that maternally inherited genomes of *Saccharum* and sorghum diverged recently (Al-Janabi, 1994).

There is also a large amount of conservation between the maize and sorghum genomes from detailed comparisons carried out (Whitkus *et al.,* 1992; Ahn *et al.* 1994 and Bennetzen and Freeling, 1993). Maize and sorghum are thought to have diverged before sugarcane diverged from sorghum (Hulbert *et al.,* 1990) which was about 20 million years ago (Bennetzen *et al.,* 2001). Results from Guimaráes *et al.* (1997) showed colinearity between *Saccharum* and sorghum and genetic conservation between these genera. Guimaráes *et al.* (1997) also predict that alleles cloned from sorghum based on map position will usually be orthologous to alleles from *Saccharum.* Considering the amount of genetic conservation between the 3 genera, isolation of promoters from maize and sorghum to drive transgene expression in sugarcane may be more viable than use of a promoter isolated from sugarcane, as gene silencing may be encountered (Potier, personal communication).

2.2 The sugarcane stem

2.2.1 Sugarcane stem morphology and histology

Sugarcane is a tall perennial tropical grass and the basic structure of the sugarcane is closely related to that of other members of the family Gramineae (Barnes, 1964). This grass tillers at the base to produce unbranched stems from 2 to 4 m or more tall, and around 5 cm in diameter (James, 2004). The stem of the plant is of greatest interest to the planter, for it is here that the commercial product sucrose is stored. The aerial part of the sugarcane

consists of a clump of stems which vary in number from variety to variety (Barnes, 1964). The stem is divided into a number of joints, each consisting of a characteristic ring called the node, and an internode.

The stem is a complex organ composed of epidermal, vascular, meristematic and storage parenchyma tissues (Moore, 1995). Each internode has an outer epidermal layer consisting of thick-walled cells which are interspersed with cork and silica cells and stoma, and this surrounds a ring of sclerenchymatous tissue followed by storage parenchymatous cells. The parenchyma cells are large towards the centre of the stem (Babu, 1979) and become lignified at later stages of development. Innumerable vascular bundles permeate the parenchyma tissue and are surrounded by a fibre sheath and sclerenchyma cells. Towards the periphery, the vascular bundles are larger in size (Babu, 1979).

Histochemical and sugar assays carried out by Jacobsen *et al.* (1992) showed changes in the morphological features and sucrose content of the sugarcane stem. An increase of sucrose concentrations down the stem was complemented by an increase in the number of vascular bundles which decreased in size from the core to the peripheral tissues accompanied by an increase in lignification and suberisation. Studies carried out by Moore and Cosgrove (1991) further illustrated the differences between the young and mature internodes since a difference was noted in the length and diameter of the storage parenchyma cells. Investigations carried out by Moore (1995) showed that sucrose accumulation occurs primarily in immature storage parenchyma as opposed to in the mature parenchyma. Apparently, as the parenchyma cells reach maturity they become less 'leaky' and less dependent upon an active transport system for maintaining a high internal sucrose concentration. These cellular differences down the stem can help to interpret the gradient of maturation and sucrose accumulation to a point where full maturity and a stable, high sucrose concentration is reached (Moore, 1995). It is important to consider the specialised function of the different cell-types in the sugarcane stem to understand stem-specific gene expression in this region.

2.2.2 Sucrose accumulation and storage in the stem

The maturation of sugarcane is characterised by the accumulation of sucrose in developing internodes (Moore, 1995) and coincides with a redirection of carbon from water-insoluble components and respiration to produce this sucrose (Botha and Whittaker, 1995). Internode development can be considered in terms of elongation, dry matter accumulation and, more broadly, the directing of sucrose towards either utilisation or storage (Lingle, 1999). Sucrose is sugar which is produced in the leaves by photosynthesis and is translocated in the phloem and exported into sink tissue of the sugarcane stem as a long-term storage molecule (Moore, 1995). Sucrose has three fundamental roles in plants, firstly it is the major product of photosynthesis, secondly it is the main form in which carbon is translocated in plants and thirdly it is one of the main storage sugars occurring in tissues such as stems and leaves.

The understanding of sucrose accumulation began with research carried out by Glasziou and Gayler, 1972. The experiments involved tissue slices suspended in radiolabelled sugar solutions to produce a model of sucrose accumulation. The model claims that sucrose from the phloem diffuses into the apoplastic space and is hydrolysed into glucose and fructose. Both products are taken up into the storage cells by hexose transporters and phosphorylated. The sucrose-phosphate is transported across the tonoplast for storage in the vacuole.

There is also increasing evidence that sucrose is involved in signaling to modulate expression of genes controlling cell division and differentiation, transporters and storage proteins, induction of flowering, differentiation of vascular tissue, seed development and accumulation of storage products (Lunn and MacRae, 2003).

Sucrose accumulation in sugarcane is a complex process, and is probably the result of a number of different processes and enzymes. Sucrose accumulation is simply the difference between the amount of sucrose produced in the leaf by photosynthesis and the amount of this sucrose that is removed by metabolism to produce carbon and energy for growth and other components of the plants (Moore, 2005). Sucrose is produced by photosynthesis in the leaves

and is loaded into the phloem by a proton-sucrose symporter (Gahrtz *et al.,* 1994). Unloading of the sucrose into the stem parenchyma is mediated by cell wall invertase, with sucrose cleavage followed by transport of monosaccharides through the plasma membrane (Roitsch *et al.,* 1995). Within the cell, sucrose is resynthesised and stored in the vacuole.

Sucrose unloaded from the phloem may pass through three cellular compartments, the apoplastic compartment or cell wall and intercellular spaces, the metabolic compartment or cytoplasm and the storage compartment or vacuole (Moore, 2005). There are multiple pathways that use different enzymes which drive either sucrose synthesis or degradation in which there might be rate-limiting physiobiochemical reactions (Moore, 2005). Stem reactions catalysed by key enzymes include membrane transport, sucrose metabolism, carbon partitioning into different pools, remobilization of stored sucrose and translocation to and unloading in various sink tissues including primary storage in parenchyma cells of the stem.

Enzymes controlling sucrose metabolism include sucrose synthase, sucrose phosphate synthase and the various isoenzymes of invertase (neutral invertase, soluble acid invertase and cell wall bound acid invertase). Different invertase isoforms are associated with all three of these metabolic componenets (Lakshmanan *et al.,* 2005). Invertases have been considered as principle regulators of sugarcane growth and accumulation (Gayler and Glasziou, 1972). Work carried out by Ma *et al.* (2000) involving the down-regulation of soluble acid invertase in the vacuole of sugarcane cells in liquid culture increased the concentration of sucrose 2-fold however there was no significant impact on sucrose concentration in the immature internodes of transgenic sugarcane plants after 70% reduction of soluble acid invertase activity (Botha *et al.,* 2001).

A kinetic model of sucrose accumulation was developed to determine which targets when manipulated may increase sucrose accumulation (Botha and Vorster, 1999; Rohwer and Botha, 2001). Rohwer and Botha (2001) determined that cytosolic neutral invertase, the putative vacuolar sucrose import protein and hexose transporters may be promising targets for genetic manipulation to increase sucrose concentration.

Due to differences in morphological features and enzyme activities, the genes are differentially expressed between the different tissue types in the stem and at the different developmental stages. Carson and Botha (2002) have used an expressed sequence tags (ESTs) approach to identify genes preferentially expressed during stem maturation. This research has provided a framework for functional gene analysis in sugarcane sucrose-accumulating tissues. These genes are under the control of specific regulatory elements (promoters) which have a stem-specific expression pattern. The isolation and identification of these specific regulatory elements of stem-specifically expressed genes can be used to regulate transgene expression in the stems with respect to membrane transport, sucrose metabolism, carbon partitioning into different pools and phloem unloading in various sink tissues.

2.3 <u>Genetic engineering of sugarcane</u>

During the past years much effort has been invested in understanding the structure and regulation of plant genes. Both this understanding and the recent advances made in plant molecular biology have provided new strategies to produce genetically engineered plants. Plant molecular biology has also allowed for the development of transfer techniques for precise transfer of new genes from diverse sources into crop plants (Stockmeyer and Kempken, 2004). These diverse sources include plants, animals, bacteria and viruses (Datla *et al.,* 1997). These transfer techniques and tissue culture approaches have been well established.

In principle genetic engineering is the introduction of useful foreign genes into a plant's genome and has potential commercial applications for modifying traits in transgenic plants that were otherwise not possible using other techniques (Datla *et al.,* 1997). Genetic engineering strategies that can be used to modify the function of a plant fall into three categories, (1) up or down regulation of endogenous gene expression, (2) modifications to endogenous genes and their expression and (3) introduction of exogenous genes into the plant (Groenewald *et al.,* 1995).

2.3.1 Requirements of sugarcane genetic engineering

The essential requirements for a gene transfer system for the production of transgenic plants are (a) availability of a target tissue for plant regeneration , (b) genotype specific tissue culture (c) regulator elements (promoters), (d) a method to introduce DNA into the regenerable cells and (e) a procedure to select and regenerate transformed plants (Birch, 1997).

2.3.1.1 *In vitro* culture systems

Sugarcane is vegetatively propagated for commercial use, however a substantial amount of work has been carried out on *in vitro* culture systems for the purposes of somatic cell improvement (Larkin and Scowcroft, 1981), the production of disease-free cells (Irvine and Benda, 1985), *in vitro* micropropagation (Lee, 1987) and genetic transformations (Bower *et al.,* 1996; Gallo-Meagher and Irvine, 1996; Falco *et al.,* 2000). There are two main routes for the regeneration of sugarcane, namely direct and indirect morphogenesis. With respect to direct morphogenesis, plants are regenerated directly from tissue such as immature leaf roll discs while indirect morphogenesis involves the initial culturing of leaf roll sections or inflorescences on an auxin-containing medium to produce an undifferentiated mass of cells, or callus (Snyman, 2004).

With respect to the vegetative propagation of sugarcane for commercial purposes, the termed used is direct organogenesis. This refers to sections of stem or setts which contain a bud and root primordial and are able to give rise to plantlets (Moutia and Dookun, 1999). Direct organogenesis *in vitro* can be achieved by shoot tip culture (Lee, 1987) or shoot multiplication from leaf discs (Irvine and Benda, 1985). Irvine and Benda (1985) first reported the direct somatic embryo formation on sugarcane leaf discs without callus formation *in vitro* during a process of rapid regeneration of plantlets in an attempt to rid sugarcane of sugarcane mosaic virus (SCMV).

The use of embryogenic callus as a target material for microprojectile bombardment has been used by sugar industries worldwide in genetic engineering programmes for the

production of transgenic sugarcane (Snyman *et al.,* 2000). The use of embryonic callus cultures for DNA transfer, particularly by microprojectile bombardment has increased the ability to transform members of the Gramineae (Bower and Birch, 1992). However, it is well known that callus induction and plant regeneration is time consuming and laborious and also causes somaclonal variation (Goldman *et al.,* 2004). In other protocols, freshly isolated immature embryos were used as a target for microprojectile bombardment or for *Agrobacterium* infection (Popelka and Altpeter, 2003).

Studies carried out by Snyman *et al.* (2006) investigated a rapid *in vitro* protocol using direct somatic embryogenesis and microprojectile bombardment to establish the developmental phases most suitable for efficient sugarcane transformation. It was shown that for effective transformation to occur, explants should be cultured for several days to allow initiation of embryo development prior to bombardment.

The first report on the production of transgenic plants without callus formation in monocotyledonous plants was provided by Wang and Ge (2005) using bermudagrass and creeping bentgrass. This study used stolon nodes as explants and successfully bypassed the callus formation phase by direct infection of stolon nodes with *Agrobacterium* followed by direct and rapid regeneration of transgenic plants.

2.3.1.2 Transformation systems and constructs

Plant transformation is a core research tool in plant biology and cultivar improvement since specific genes can be introduced and expressed in plants (Birch, 1997; Koyama *et al.,* 2005). The limiting factor in achieving effective transformation of agronomically important species in the past was the DNA delivery method but now the methods developed include *Agrobacterium*-mediated transformation, particle bombardment, electroporation and microinjection (Songstad *et al.,* 1995; Datla *et al.,* 1997). Transgenic monocotyledonous plants were first obtained by direct gene transfer to protoplasts, then by biolistic transformation, and in more recent years by *Agrobacterium*-mediated transformation.

In vitro single-cell transformation was first documented in 1979 from work carried out by Marton and colleagues. The first experiments used the soil bacterium, *Agrobacterium tumefaciens*. This is a phytopathogen that has evolved a parasitic mechanism allowing it an ecological niche in which to flourish (Smith, 2001). The bacterium transfers a specific region of DNA on its Ti plasmid which would then be incorporated into the plants own genome (Smith *et al.,* 2001).

The use of transformation mediated by the *Agrobacterium* is far more successful for dicotyledonous plants and is now routinely used for many plants including potato (Romano *et al.,* 2000), strawberry (Zhao *et al.,* 2004), tobacco (Roger *et al.,* 2001) etc. Cereals and legumes on the other hand are still rather difficult to engineer (Smith *et al.,* 2001) since they are recalcitrant in tissue culture and their transformation has not been standardized (Popelka *et al.,* 2003). However there are still some reports on the use of *Agrobacterium*-mediated transformation on a range of cereals, these include wheat (Cheng *et al.,* 1997), maize (Ishida *et al.,* 1996), rice (Hiei *et al.,* 1994), barley (Tingay *et al.,* 1997) and sugarcane (Elliott *et al.,* 1998; Arencibia *et al.,* 1998) even though they are outside the host range. Even though there are reports of success with *Agrobacterium tumefaciens*-mediated transformation of sugarcane, it is not used routinely for genetic engineering; rather the technique of microprojectile bombardment is used.

The particle bombardment technology was first described by Sanford *et al.* in 1987 and since then a number of major crop species have been engineered (reviewed by Vain *et al.,* 1993; Hadi *et al.,* 1996; Romano *et al.,* 2000; Breitler *et al.,* 2002; Permingeat *et al.,* 2003; Popelka *et al.,* 2003). Christou *et al.* were the first to report stable transformation events in 1988 in which viable DNA was delivered into immature soybean embryos and stable transformed callus material was isolated from protoplasts. Particle bombardment is the most widely used method of transformation for those plants that are not susceptible to *Agrobacterium* transformation, such as most monocotyledonous and some dicotyledonous plants (Christou, 1995).

Particle bombardment has also proven to be very successful for transformation of sugarcane which has been a very difficult crop plant to transform in the past (reviewed by Bower and Birch, 1992; Sun *et al.,* 1993; Falco *et al.,* 2000; Snyman *et al.,* 2001). Plant regeneration from bombarded embryogenic callus is efficient and simple and since all sugarcane cultivars that have been tested are able to initiate embryogenic callus (Taylor *et al.,* 1992), this method of gene transfer into sugarcane is made feasible (Bower *et al.,* 1996). The use of embryogenic callus as a target material for microprojectile bombardment has been reported by sugar industries around the world. Particle bombardment can be used on any sugarcane cultivar but differences in transformation efficiencies have been reported and this is linked to the cultivar used, genotypic response to hormone treatment and selection regime (Snyman *et al.,* 2000).

This technology uses gold or tungsten particles coated in DNA which are propelled at high speed toward the plant tissue. This therefore allows for the forced penetration of DNA into the cell nucleus where it is integrated into genomic DNA during replication (Smith *et al.,* 2001; Breitler *et al.,* 2002). Its advantage lies in achieving truly genotype-independent transformation since most tissue culture related regeneration problems are avoided (Christou, 1997). Also the DNA of interest can be introduced into regenerable cells, tissues or organs (Christou, 1997).

2.4 **Gene regulation**

The regulation of gene expression in plants is controlled at both the transcriptional and post-transcriptional level. Transcription is divided into three stages namely, initiation, elongation and termination. Initiation begins with the binding of RNA polymerase to the promoter region of each gene. Ribosomal RNA is transcribed by RNA polymerase I, mRNA by RNA polymerase II, and tRNA by RNA polymerase III (An and Kim, 1993). Transcription factors are also required for transcription initiation. Transcription elongation occurs when the RNA moves along the DNA; this extends the RNA chain until RNA synthesis is terminated at the transcription terminator region. At this point the transcription complex dissociates (An and Kim, 1993).

The general steps involved in turning a gene into a protein product, require the gene to be transcribed, spliced and processed to form mRNA which is translated into a polypeptide. All cells contain at least one form of RNA polymerase. This enzyme transcribes DNA into RNA prior to the translation of mRNA into protein. There are additional steps in the process of gene expression found in complex organisms. For example, for many genes, the RNA transcript must be 'spliced', a process that removes unwanted sequences; it must be chemically modified at one or both its ends followed by transportation out of the nucleus for translation (Ptashne and Gann, 2002).

2.4.1 Gene silencing

The inheritance and stable expression of transgenes is an important concern in crop improvement through gene manipulation. Studies have revealed that in some cases transgene expression was lost in a variable proportion of the progeny (Chareonpornwattana *et al.,* 1999). This phenomenon, referred to as 'gene silencing', has been studied most extensively in dicotyledonous plants, such as tobacco, petunia, tomato and buckweed (reviewed in Matzke and Matzke, 1995; Stam *et al.,* 1997). In contrast to the extensive studies on gene silencing in dicotyledonous plants, there have been relatively few studies in gene silencing in monocotyledonous plants or on the mechanisms of transgene inactivation (Cooley *et al.,* 1995).

In plants and animals there is an additional level of gene regulation through the modification of DNA by cytosine methylation (Finnegan, 2001). DNA methylation can inhibit transcription by preventing the binding of the basal transcriptional machinery through the modifications of target sites (Finnegan, 2001). It is thought that this mechanism is important in preventing transcription of genes intended to be permanently turned off. Experiments have shown that a signaling molecule is involved in the systemic spread of gene silencing and resulting resistance of viruses (Palauqui *et al.,* 1997). Both gene silencing and virus resistance have been found to be induced by virus infections of non-transgenic plants (Covey *et al.,* 1997; Morino *et al.,* 2004). Results from these experiments and others have led to the hypothesis that gene silencing is a natural plant defense mechanism.

Homology-based gene silencing (HBGS) is a generic term for the two types of gene silencing recognised, namely transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). Both have been reported to occur extensively in transgenic plants (Meyer and Saedler, 1996; Vaucheret and Fagard, 2001). Both types have been found to lead to reduced or undetected steady state levels of transcripts. It is possible that there are multiple mechanisms for gene silencing therefore several models have been proposed by Matzke and Matzke (1995b) to explain gene silencing. These include the methylation of specific sequences in the promoter region, aberrant RNA production, RNA-DNA pairing and a RNA threshold model.

Furthermore, the phenomenon of gene silencing may be instigated when multiple copies of a gene cassette integrate into plants during transformation (Van der Krol *et al.,* 1990). There is however a vast difference between transcriptional gene silencing and post-transcriptional gene silencing. TGS occurs when interacting genes share sequence homology in promoter regions and inhibits transcription by promoter methylation, whereas post-transcriptional gene silencing requires homology in transcribed sequences in which the transcription rate is not affected but double-stranded RNA induces the degradation of homologous RNA sequences (Mette *et al.,* 1999; Bass, 2000; Fagard and Vaucheret, 2000). Recent studies indicate that TGS and PTGS may be mechanistically and functionally related since they have some of the same events, including changes in DNA methylation (Paszkowski and Whitham, 2001). Since both types of silencing are confined to regions of sequence homology, sequence-specific methylation signals consisting of either DNA-DNA or RNA-DNA associations are believed to be involved (Mette *et al.,* 2000).

A study carried out by Mette *et al.* (1999) demonstrated the effect of sequence homology in the promoter region. The experimental design involved the intentional transcribing of the nopaline synthase promoter (NOSpro) sequences by the cauliflower mosaic virus 35S promoter to produce NOSpro RNAs. The result of this in the tobacco line was the induction of methylation and transcriptional inactivation of homologous NOSpro copies in *trans*.

Both types of gene silencing have been associated with changes in DNA cytosine 5-methylation, which is generally concentrated in promoters for transcriptional gene silencing and at the 3' end of genes for post-transcriptional gene silencing (Mette *et al.,* 1999). This hypermethylation can spread within promoter regions or within transcribed regions, however the extent of spreading is lesser from promoter to adjacent transcribed regions and from transcribed to adjacent promoter regions (Paszkowski and Whitham, 2001).

The sequence homology triggers cellular recognition mechanisms that result in silencing of the repeated genes. One way to prevent silencing of introduced DNA is to develop gene cassettes that contain very little sequence similarity to endogenous sequences or to one another if a number of transgenes need to be introduced (Bhullar *et al.,* 2003). With respect to promoters, homology can be avoided by using diverse promoters isolated from different plant and viral genomes or by designing synthetic promoters (Bhullar *et al.,* 2003).

There have been extensive studies carried out on gene silencing in dicotyledonous plants, however there have been relatively few studies carried out for monocotyledonous plants. When conducting studies involving the isolation of promoters from one plant for use in another, transcriptional gene silencing may place a great limitation. An example of this limitation has been noted when sugarcane promoters, following re-introduction into sugarcane, have lead to transcriptional gene silencing (Birch *et al.,* 1995).

There have been two nuclear proteins identified for TGS, namely the DNA DEMETHYLATION1 GENE (DDM1) and the *Morpheus molecule* (MOM1). DDM1 is a chromatin-remodeling protein belonging to the SNF2/SW12 superfamily. Its impairment releases both TGS and methylation of transgenes and silent retrotransposons (Jeddeloh *et al.,* 1999). The impairment of MOM1, a nuclear protein, releases TGS but not methylation of the transgene, this therefore suggests that TGS could operate through methylation-dependent or –independent pathways (Amedeo *et al.,* 2000). The findings from these two proteins suggest that proteins involved in changes of chromatin structure are

required to ensure the correct levels and allocation of methylation. Furthermore, TGS can also result from expression of dsRNA derived from promoter sequences, suggesting that like PTGS, TGS can be mediated by dsRNA (Chandler and Vaucheret, 2001).

2.5 **Promoters**

A promoter is essential to drive the transcription of a gene since it contains specific sequences recognized by proteins that are involved in the initiation of transcription (Buchanan *et al.,* 2000). This therefore makes promoters critical to the success of transgenic approaches in sugarcane crop improvement. The promoter of a gene contains the information required to direct when, where and to what extent the gene will be expressed (Datla *et al.,* 1997) therefore promoters affect transcription both, quantitatively and qualitatively (Potenza *et al.,* 2004).

2.5.1 Functional and structural organisation of promoters

Transcriptional activation of genes is controlled by both cis-acting DNA sequences and trans-acting factors. The cis-acting sequences comprise the promoter region while trans-acting factors include for example gene-specific DNA-binding proteins. The *cis*-acting sequence of promoters contains two core elements, the "TATA" box and the initiator. These elements are known to function as binding sites for transcription factors and other proteins that are involved in the initiation process (Reese and Eeckman, 1995). The core promoter is located about 40 base pairs upstream of the start of transcription and the upstream promoter region may extend 200 base pairs farther upstream (Potenza *et al.,* 2004).

A TATA box is present in most of the RNA polymerase II mediated promoters and is a highly conserved sequence. It is an AT-rich sequence which is usually located 20 to 35 nucleotides upstream of the transcription start site (Butler and Kadonaga, 2002). The TATA sequence binds to RNA polymerase II through a number of general transcriptional factors (TATA-binding proteins, RNA polymerase and other associated factors) (Butler and Kadonaga, 2002). The TATA element in the promoter region is recognized by this

complex. This element serves as anchor for making contact with gene-specific transcriptional factors to thus activate transcription. Studies carried out by An and Kim (1993) showed that the removal of the TATA box results in a reduced promoter activity. Also for some genes, tissues, organs or environmental conditions, specific expression is determined by the TATA box (Butler and Kadonaga, 2002). Knowledge of TATA-less promoters in plants is limited but it was found that the majority of TATA-less promoters were common to genes associated with photosynthesis (Nakamura *et al.* 2002).

Over the years, numerous promoters have been isolated from a wide variety of organisms and applied to plant genetic engineering systems. Great demand has been placed on increasing the range of available promoters for sugarcane transformation and studies are constantly being carried out to obtain strong promoters. It is known that certain promoters direct gene expression at higher levels than others (Xiao *et al.,* 2005). A good knowledge of the pattern of activity of a promoter is critical to both the design of the transformation experiment and the stability of gene expression (Rooke *et al.,* 2000). An important factor for the expression of a gene is the choice of a suitable promoter that results in the desired amount, location and timing of transgene expression. Promoters that are used and sought after for biotechnology are of different types according to the intended type of control of gene expression. There are two major classifications of promoters; this includes constitutive promoters and tissue-specific promoters.

2.5.2 Constitutive promoters

Constitutive promoters direct expression in virtually all tissues and are largely independent of environmental and developmental factors. The expression of these promoters is normally not conditioned by endogenous factors and therefore can be active across species and even kingdoms (Potenza *et al.,* 2004). However, it does not necessarily imply that this promoter drives the expression of a transgene at the same level in all cell types but rather it expresses the transgene in a wide range of cell types with some variation in expression levels (Bade *et al.,* 2003).

Some examples of constitutive promoters include the nuclear gene promoter from the 3-

subunit of the mitochondrial ATPase complex (Boutry and Chua, 1985), the rice ACT1 promoter (Zhang *et al.,* 1991), the maize Ubi promoter (Christensen and Quail, 1996) and the CaMV 35 S promoter (Fang *et al.,* 1989). The CaMV 35S promoter has been widely used for high-level constitutive expression in dicotyledonous but confers low levels of transgene expression in most of the sugarcane studies reported to date (Lakshmanan *et al.,* 2005). Recently Xiao *et al.* (2005) have isolated and characterised a novel plant promoter, MtHP promoter that drives strong constitutive expression of transgenes in plants. The promoter was isolated from a model legume species, *Medicago truncatula* and was shown to direct higher levels of GUS expression than CaMV promoters in a range of plant species

Although there are some examples of promoters active in dicotyledonous plants also being active in monocotyledonous plants, in many cases the dicotyledonous promoter requires modification in order to show high levels of expression in monocotyledonous species. The maize ubiquitin 1 promoter (Christensen and Quail, 1996) produced significantly higher expression in sugarcane than other tested promoters (Rathus *et al.,* 1993; Gallo-Meagher and Irvine, 1996) such as the CaMV 35S promoter, the synthetic Emu promoter (Last *et al.,* 1991) and the rice actin Act-1 promoter (McElroy *et al.,* 1991). From the current trends obtained from these studies, the Ubi-1 is becoming the promoter of choice for constitutive expression of transgenes in sugarcane.

Studies conducted by Liu *et al.* (2003) showed that the rice ubiquitin promoter RUBQ2 has increased transgene expression by about 1.6 fold over the maize Ubi-1 promoter in sugarcane. From such studies is can be see that there is a constant demand and urgency to obtain promoters for driving transgene expression in sugarcane. But it must be noted that promoter activity in different plant species varies and therefore not all of these promoters can be used for high-level expression in all monocotyledonous species (Wilmink *et al.,* 1995).

Promoter elements from plant viruses have also been investigated for driving high-level transgene expression in sugarcane. Because of the success of the CaMV 35S promoter, other promoters have been developed or are being developed for use. In 2004, Braithwaite *et al.* compared four genomic regions of Sugarcane bacilliform virus shown

to have promoter activity in sugarcane. One of these promoters drove GUS expression in meristems, leaves and roots of glasshouse-grown sugarcane plantlets at levels equal to or higher than those obtained for the maize Ubi-1 promoter. Another study by Schenk *et al.* (2001) showed a promoter isolated from Banana streak virus to be capable of driving green fluorescent protein (GFP) expression up to 3 fold higher than that reported for the maize Ubi-1 promoter. The banana bunchy top virus is another example of a virus from which promoter elements have been isolated and is currently undergoing characterisation in sugarcane in Australian laboratories (Grof and Campbell, 2001).

Another constitutively expressed promoter isolated from a virus was that from the *Cestrum* yellow leaf curling virus (CmYLCV), belonging to the Caulimoviridae family. This promoter was found to be highly active in callus, meristems and vegetative tissues of *Zea mays, Oryza sativa* and *Nicotiana tabacum* (Stavolone *et al.,* 2003). Other viral promoters include those from mirabilis mosaic virus (Dey and Maiti, 1999), cassava mosaic virus (Li *et al.,* 2001), figwort mosaic virus (Sanger *et al.,* 1990) and cotton leaf curl Multan virus (Xie *et al.,* 2003).

2.5.3 Tissue-specific promoters

Tissue-specific promoters drive the expression of a gene in a specific tissue or at certain stages of development. Transgenes driven by these types of promoters will only be expressed in tissues where the transgene product is desired, leaving the rest of the tissue in the plant unmodified by the transgene expression (Stitt *et al.*, 1995; Potenza *et al.,* 2004). Constitutive promoters which have non-specific expression patterns such as CaMV 35 S, maize Ubi 1 and rice actin 1 have a number of potential drawbacks for use in genetic engineering (Gittins *et al.,* 2000). For example the over-expression of a specific transgene in tissues where it is not normally expressed, or at very high levels, can have unexpected consequences on plant growth and development (Potenza *et al.,* 2004).

An example of the value of tissue-specific expression would be the confinement of an

insecticidal transgene product to affected tissues instead of harvestable material (Bucchini and Goldman, 2002). Another advantage is the use of tissue-specific promoters for targeting metabolic processes in a tissue type which could increase yields obtained from commercial crops (Lakshmanan *et al.,* 2005).

Many tissue-specific genes and the corresponding promoters have been isolated. Examples include genes that are specifically expressed in flower (Annadana *et al.,* 2002) pistil (Ficker *et al.,* 1997), anthers (Koltunow *et al.,* 1990), roots (Yamamoto *et al.,* 1991; Nitz *et al.,* 2001; Winicov *et al.,* 2004; Koyama *et al.,* 2005), fruits (Edwards and Coruzzi, 1990), phloem (Zhao *et al.,* 2004) and epidermal cells (Ancillo *et al.,* 2003). The most extensively studied leaf-specific gene is the gene that encodes the small subunit of Rubisco (rbcs) (Fluhr *et al.,* 1986). Sugarcane Rubisco small subunit genes have been isolated, sequenced and the corresponding promoter elements isolated (Tang *et al.,* 1996). The promoter showed expression specifically in leaves and preferentially in bundle sheath. Recently a promoter of a gene for Chrysanthemum Chlorophyll-a/b-binding protein was isolated and expressed a transgene in leaf tissue more efficiently that the 35S promoter (Aida *et al.,* 2004). The promoter of alcohol dehydrogenase expressed during ripening of the grape berry was isolated and characterised as a fruit-ripening promoter (Sarni-Manchado *et al.,* 1997). Another promoter from a gene expressed during grapevine ripening (VvAdh2) has also been isolated and analysed (Verries *et al.,* 2004).

It must be noted that very little is known about tissue-specific promoters in sugarcane, especially strong stem and root specific promoters. However, a sugarcane stem-specific promoter, UQ67P has been isolated and shown to be able to drive reporter gene expression in stem tissue (Hansom *et al.,* 1999). Also Mirkov and his colleagues were successful in cloning two stem-specific promoters from sugarcane; these promoters showed strong reporter gene activity in stem tissues of transgenic sugarcane (Mirkov *et al.,* unpublished results).

2.5.3.1 Regions within the promoter driving tissue-specific expression

There are specific regions of the promoter which are necessary for tissue specific

expression. These regions can be identified by deletion analysis and reporter gene expression studies. It has been found that in most cases the proximal region of the promoter is able to confer specificity of expression (Tyagi, 2001). One of the best characterised and most commonly used seed-specific promoters is the French bean 3-phaseolin gene (van der Geest and Hall, 1997). The -295 bp region of the

β-phaseolin promoter has been identified as containing sequence motifs implicated in directing seed specific expression in transgenic tobacco plants. The two motifs were the legumin box (CATGCATG) and endosperm boxes (TGTAAAGT and RTGAGTCAT). Extensive *in vivo* footprint analysis showed that over 20 cis-elements are present and bind trans-acting factors in seeds. This specific binding mediates expression in various regions of the embryo (Chandrasekharan *et al.,* 2003).

Analysis of the promoters from the 1-aminocyclopropran-1-carboxylate (ACC) gene showed promoter fragments ranging from -1966 to -1159 bp from the start of transcription were able to drive ripening-specific expression of a reporter gene in the tomato fruit, with no activity present in other tissues (Atkinson *et al.,* 1998). An 809 bp region located between -1115 and -306 was found to be responsible for increase in the activity levels of the VvAdh2 promoter from grapevine. Further analysis of this region revealed two putative cis-elements corresponding to enhancer-like elements, respectively positioned at -1781 (TGAAAAAT) and -181 (GTGGATTG) (Verries, *et al.,* 2004). Closer examination of the 809 bp promoter region revealed the presence of an AT-rich region of 60 bp length, with two repeated motifs (TTTTA and TTA respectively). However, these motifs did not correspond to other known cis-elements. But the elements were an interesting putative enhancer motif of the VvAdh2 promoter expression (Verries *et al.,* 2004).

The gSPO-A1 promoter from sweet potato was fused to a reporter gene showing high levels of expression in the stem. Analysis of the promoter region showed two sequence elements; one directs expression to the phloem (-305 and -237 bp) and the other to the pith parenchyma (-192 to -94 bp) (Ohta *et al.,* 1991).

2.5.3.2 The importance of stem-specific promoters to sugarcane

The sugarcane plant comes under great attack from sugarcane pests especially in the region of the stems. Pests such as the eldana borer, which is the larval stage of the *Eldana saccharina* Walker (Lepidoptera: Pyralidae), attack the older cane stems. The borer feeds voraciously on the soft tissue inside the sugarcane stem causing serious loss in yields and cane quality. Up to 14 borers per internode have been recorded and the cane can become completely hollowed out. It has been estimated that, for every one eldana per 100 stems, 0.5 ton cane per hectare is lost (Guidelines and recommendations for Eldana control in the SA sugar industry, 2005).

Another problem faced by the sugarcane stem is the disease Smut caused by the fungus *Ustilago scitaminea.* The symptoms include dark brown, whip-like structures which develop from the tops of infected shoots of stems (Sugarcane Diseases in Southern Africa, 2003). Such problems of pest and diseases have caused major economic losses and these pests and diseases are controlled by integrated pest management (IPM) approaches which involve cultural, biological and insecticidal controls (Allsopp and Manners, 1997; Allsopp and Suasa-ard, 2000). Another technology that can be implemented to maximise and sustain crop productivity is the introduction of novel insecticidal genes by transgenic approaches. Similar studies have been carried out by Liebbrand and Snyman, 2003 in which a *Saccharum* hybrid cultivar Nco310 was transformed with a *pat* gene which confers resistance to the herbicide Buster (glufosinate ammonium; Bayer CropScience Monheim am Rhein, Germany) Therefore the isolation of stem-specific promoters would be very valuable.

Considerable progress has been made to date in targeting pest resistance in the sugarcane stem. A study carried out by Legaspi and Mirkov (2000) observed considerable growth inhibition of sugarcane stem borers when they were fed on transgenic sugarcane engineered with lectin genes. Therefore obtaining stem-specific promoters would provide opportunities to introduce or enhance natural pest resistance (Lakshmanan *et al.,* 2005).

The sugarcane stem is also an important area of the plant with respect to sucrose accumulation. Sucrose cycling and compartmentation are important features for the sucrose accumulation process in the sugarcane stem. However, the regulation of these mechanisms is not well understood (Moore, 1995). The regulations of these mechanisms is controlled by different key enzymes which are expressed in different cell types which are regulated by specific promoters. Therefore the isolation of stem-specific promoters may provide a clear understanding of the sucrose pathway by targeting specific key genes which can be either up-regulated or down-regulated to increase sucrose accumulation in the sugarcane plant using transgenic approaches. It may also help to understand the role of different tissue types involved in sink-to-source interaction in sugarcane.

2.5.4 Enhancer elements

Although initiation of transcription is dependent on sequences found in the core and upstream promoter regions, many other DNA sequence motifs, which occur within the surrounding DNA, are also involved in the regulation of gene expression (Potenza *et al.,* 2004). Much of the fine-tuning of gene expression is controlled by sequence-targeted transcription factors or enhancer-binding proteins (Alberts *et al.,* 2002). Enhancer-binding proteins bind to cis-elements within the promoter of the gene. These cis-elements are also called enhancer elements and are conserved DNA sequence modules that vary in size and show sequence degeneracy which make them difficult to recognize using comparative analysis. A large number of enhancers have been discovered using promoter deletion studies, DNA foot-printing and site-specific sequence mutation (Potenza *et al.,* 2004).

Various strategies can be employed to increase the activity of promoters. One strategy is to add an enhancer element that increases the transcription of a promoter (Last *et al.,* 1991). In plants, extra copies of enhancer elements can enhance the activity of a promoter (Chen *et al.,* 2002). An example would be the anaerobic responsive element to the maize Adh1 promoter (Olive *et al.,* 1990). Multiple copies of enhancer elements have also been employed using the octopine synthase enhancer from *Agrobacterium tumefaciens* to the maize Adh1 promoter (Ellis *et al.,* 1987).

Enhancers can be located hundreds or thousands of base pairs away from the gene they control. They can be found upstream or downstream of the coding region and within the coding or intronic sequences.

In several but not all promoters a CAAT box is contained and seems to play an important role in promoter efficiency, by increasing its strength and functions in either orientation (An and Kim, 1993). There are also hexamer motifs that are found as repeats separated by six to eight nucleotides. A hexamer sequence (TGACGT) is found in most constitutive promoters within a few hundred nucleotides from the transcriptional start site (An and Kim, 1993). These motifs are essential for the transcription activity of the cauliflower mosaic virus (CaMV) 35S, octapine synthase (ocs) and nopaline synthase (nos) promoters.

Several consensus sequence elements, including the G motif, the GATA motif, the GT1 motif and the Z motif, are commonly found in light regulated minimal promoter regions and have been shown by mutagenesis studies to be necessary for high promoter activity in the light (Kehoe *et al.,* 1994). The H-box sequence (CCTACC) together with the G-box sequence (CCACGTGG) is essential for the expression of the bean chalcone synthase gene (Arias *et al.,* 1993). The 3-phaseolin promoter drives expression specifically in transgenic tobacco seeds but it has been found that the inclusion of the matrix attachment regions found flanking the 3-phaseolin gene in the transgenic constructs enhances transcription (van der Geest and Hall, 1997).

An enhancer sequence, TATCCA was found to significantly enhance transcription in the CaMV 35S minimal promoter in rice protoplasts thus serving as a transcriptional enhancer. Also, mutations of the TATCCA element in the promoters of two barley a-amylase genes were found to lower its expression by 20% (Chen *et al.,* 2002). The enhancer sequence, (T/A)AAAG was found to enhance transcription of the C4-type phosphoenolpyruvate carboxylase promoter in *Zea mays* (Yanagisawa and Schmidt, 1999). Another example is the enhancer sequence, GTGAGGTAATAT which enhances the Ribulose-1,5-biphosphate carboxylase promoter in *Pisum sativum* (Green *et al.,*

1987).

2.5.5 Intron-mediated transgene expression

Introns are intervening sequences present in the pre-mRNA but absent in the mature RNA following splicing mechanisms (Bourdon *et al.,* 2001). In plants numerous studies involving introns have focused on their effects when inserted between the promoter region and the coding sequence of the gene. Introns inserted inside the coding region of the reporter gene were shown to enhance gene expression (Tanaka *et al.,* 1990). In plants introns are characterised by their AT content (60%) and their consensus splicing sites (Lorkovic *et al.,* 2000). The AT content of three maize introns in studies carried out by Bourdon *et al.* (2000) were between 54 and 63%. With respect to efficient intron splicing suggestions have been made that the sequences surrounding the introns are important for intron splicing (Matsumoto *et al.,* 1998). Work carried out by Bourdon *et al.* (2001) clearly demonstrated that introns even within the coding sequence are of a major determinant of transgene expression levels. Their sequence and position within any open reading frame could determine how and with what efficiency the ribosomes employ the mRNA once the mRNA has been exported from the nucleus.

The enhancement of gene expression by introns in plants is not a general phenomenon since some naturally occurring genes do not contain introns and are still expressed efficiently therefore intron-mediated enhancement may be a gene dependent process (Rethmeier *et al.,* 1997; Mascarenhas *et al.,* 1990). In plants, enhancement of gene expression is observed only in the more GC-rich genomes, such as those of monocotyledonous plants (Taylor *et al.,* 1993).

In many organisms, a significantly higher level of gene expression is observed from intron-containing transgenes than from intronless constructs (Callis *et al.,* 1987; Duncker *et al.,* 1997). Work carried out by researchers has shown that introns have a strong

enhancing effect on gene expression when inserted downstream of a promoter sequence within the transcriptional unit (Sinibaldi and Mettler, 1992). This effect of introns has also been observed with promoters which, on their own, have weak activity in monocotyledonous, such as the cauliflower mosaic virus (CaMV) 35S promoter (Vain *et al.,* 1996).

The enhancing effect of introns within the transcriptional unit for gene expression in monocotyledonous has also been well documented. This effect seems to be directly related to the splicing mechanism and it is thought that the improved gene expression results from synthesis of a relatively more stable RNA molecule (Sinibaldi and Mettler, 1992). This strategy has been used for the cauliflower mosaic virus (CaMV) 35S promoter. In its native form, the promoter is only poorly active in monocotyledonous, but the addition of an intron taken from Adh1 (Vain *et al.,* 1996), cat1, Ubi1 and Act1 (Vain *et al.,* 1996) dramatically improved promoter activity in maize, rice, barley, wheat and various grasses. Furthermore, the addition of an intron (act1) to the potato pin2 promoter greatly improved expression levels in transgenic rice (Xu *et al.,* 1993).

Although there are examples of a promoter active in dicotyledonous plants also being active in monocotyledonous plants (Verdaguer *et al.,* 1996; Schenk *et al.,* 1999, in many cases the modification of the dicotyledonous promoter is required prior to high levels of expression being achieved in the monocotyledonous species. The most common modification for the enhancement of the promoter activity to obtain high levels of expression being achieved in the monocotyledonous species is the addition of an intron between the promoter and the transgene open reading frame. This strategy has been used for the cauliflower mosaic virus (CaMV) 35S promoter. The native form of this promoter is only poorly active in monocotyledonous but the addition of an intron taken from Adh1 enhances the promoter activity.

Transcription factor binding sites have been found in plant introns. Examples include the

barley HVA22 gene induced by abcissic acid (ABA), in which one of the elements essential for ABA induction is located in the intron (Shen *et al.,* 1993). Another is the intron of the potato sucrose synthase gene (Sus4), which includes a 'promoter-like structure' that regulated the activity of its promoter. Work carried out by Salgueiro *et al.* (2000) showed that the activity of the complete promoter, exon and intron cassette using the maize ubiquitin promoter for driving expression in tritordeum and wheat was up to 20 000-fold higher than background but the maize ubiquitin promoter in isolation had very low activity (Salgueiro *et al.,* 2000). Within the maize ubi intron several 'promoter' sequences were recognized. At position +924 a TATA-box-like sequence (TATAA) and a CAAT box at position +390 (CAAT) was identified. Other known transcription factor binding boxes were located. The intron of maize adh1 showed a TATA motif at position +298, a CAAT sequence at position +274 and an E-box element at position +569.

These elements found within intron regions may play a role in the ability to drive gene expression to a low extent. They may also have a regulatory role when the introns are part of a transcriptional cassette. Results from Salgueiro *et al.* (2000) showed that removing part of the intron and the small exon region resulted in undetectable GUS activity in explants. This may also indicate the regulatory role for the small exon region, as it is known that the 5' - 3'-UTRs can regulate gene expression. Further analysis involving the use of promoterless constructs with only the maize ubiquitin promoter and exon showed expression of GUS and may be due to transcription factors binding to these sequences and initiating a low level of transcription. It was also speculated that some of these binding sites within the intron region may play a role when introns are used as enhancers downstream from weak promoters (Salgueiro *et al.,* 2000).

The promoter of the UDP-glucose dehydrogenase gene was found to be ineffective in driving transgene expression in sugarcane on its own but when fused with an intron (980 bp) derived from the 5'UTR of the same gene the level of transgene expression was increased (van der Merwe *et al.,* 2003). Work carried out by Bourdon *et al.* (2001) showed that the insertion of the intron from maize into the coding region of the luciferase reporter gene produced a significant and reproducible increase in luciferase expression.

2.6 Approaches for promoter isolation

There have been several approaches implemented for the isolation of DNA fragments carrying regulatory elements from plants. Most of the plant promoters isolated during the early 80s used the conventional approach. This approach involved the identification of the cDNA clone of a particular gene, and from its information the corresponding promoter region from a genomic clone was isolated (Datla *et al.,* 1997). The difference between genomic clones and cDNA clones is the lack of introns in cDNA clones thus making them easier to characterise and use as probes (An and Kim, 1993).

The overall approach to evaluating promoter activity involves the determination of the expression pattern of a gene using Northern hybridisation using total RNA and the cDNA as the probe (Yoshida and Shinmyo, 2000). If a gene is found to be expressed specifically in the stem, transcription of the gene might be controlled by a stem-specific promoter. A genomic DNA fragment is cloned from a genomic DNA library using cDNA as a probe. It is possible to amplify the DNA fragment containing the promoter by PCR amplification. The cDNA and the genomic DNA sequences are compared and the 5'- upstream region of the gene is elucidated. The genomic DNA containing the promoter region (500-1000bp upstream of the transcription initiation site) is ligated to a reporter gene such as β-glucuronidase (GUS), luciferase (LUC) or a green fluorescent (GFP) to assess the activity of the promoter activity (Yoshida and Shinmyo, 2000).

Genomic clones carrying a regulatory region can be isolated by screening a genomic library with the cDNA clone used as a probe (Yoshida and Shinmyo, 2000). Transcriptional regulatory regions can be obtained using this cDNA clone to isolate adjacent regions from genomic DNA. The genomic DNA flanking the amino terminus of a cDNA clone should contain the promoter and the region adjacent to the poly A tail contains the terminator region of the gene (An and Kim, 1993).

After the genomic clone is obtained, restriction mapping and Southern blot analysis are used to locate fragments containing the cDNA sequence. A restriction fragment carrying

the region upstream of the transcription start point of the region downstream of the termination site is considered to contain a regulatory region. The length of the regulatory region is difficult to estimate therefore it is best to start with 2 kb fragments for characterisation or utilisation of the promoter (An and Kim, 1993).

This approach for isolating promoters by screening genomic libraries with a cDNA clone has been applied by many researchers. Research carried out by Yang *et al.* (2003) at Texas A&M University used the direct screening of a sugarcane genomic library with a radioactively labeled cDNA probe to isolate constitutive promoters. A total of eleven promoters were isolated with this strategy and two of these promoters driving elongation factor 1ct and sugarcane proline rich protein encoding genes were found to drive β-glucuronidase (GUS) expression in sugarcane callus and wheat embryos at levels equivalent to those obtained with maize Ubi1 promoter (Yang *et al.,* 2003).

Yang *et al.* 2003 implemented four steps in their approach for promoter isolation. First, pooled mRNA from a specific tissue of interest was used as a probe to screen a phage genomic library. Secondly, the coding region of these genomic clones was identified by Southern hybridisation of restriction-enzyme digested Lambda phage DNA clones with pooled first strand cDNA, and corresponding cDNA clones were isolated from a sugarcane cDNA library. Thirdly, the copy number of genes was estimated by Southern analysis and the expression level of genes was confirmed by Nothern analysis. Finally the promoters were characterised by sequencing of the genomic clones, GenBank searches, primer designing and transient expression analysis. Their final result was two genomic clones of the sugarcane proline-rich gene and a sugarcane elongation factor gene, which contained both the promoter and coding regions. These were sub-cloned, sequenced and promoter regions defined by comparison of cDNA and genomic DNA sequences (Yang *et al.,* 2003).

The method implemented by Yang *et al.* 2003 involved isolating strong constitutive promoters from sugarcane but a similar general methodology can be used to identify and isolate tissue-specific promoters as shown by Muhitch *et al.* (2002). This study used the

glutamine synthase (GS) gene found in the maize genome involved in encoding a cytoplasm-localised GS isozyme specifically expressed in the basal maternal tissues of the developing kernel to isolate its corresponding promoter for conferring tissue-specific gene expression in transgenic maize. Another example of this methodology was shown in a study carried out by Opsahl-sorteberg *et al.* (2004) in which a promoter directing aleurone cell specific expression was identified and isolated. In these examples Northern blot analyses were carried out to either determine the level of the gene expression or the tissues in which the gene was being expressed.

A sugarcane stem-specific promoter, UQ67P, has also been isolated and shown to drive reporter gene expression in stem tissue (Hansom *et al.,* 1999) and recently Mirkov and co-workers at Texas A&M University were successful in cloning two stem-specific promoters from sugarcane using a similar methodology implemented in this project. These isolated promoters showed strong reporter gene activity in stem tissues of transgenic plants (Mirkov *et al.,* unpublished results).

Additional studies have been carried out to obtain corresponding promoters for the chitinase C gene in potato (Ancillo *et al.,* 2003), polyubiquitin gene in sugarcane (Garbarino *et al.,* 1995), *Pyk*10 gene in *Arabidopsis thaliana* (Nitz *et al.,* 2001), *MtHP* gene from *Medicago truncatula* (Xiao *et al.,* 2005) and glutamine synthase gene from maize (Muhitch *et al.,* 2002). Studies carried out by Verries *et al.* (2004) involved the screening of a grapevine genomic DNA library for the alcohol dehydrogenase (*Adh*) genes with a cDNA probe. A clone containing the *Adh* gene from this library was selected and sub-cloned into a vector for sequence analysis. The promoter region was identified within this fragment and its activity was analysed by monitoring the Luciferase reporter activity in particle-bombardment experiments on *Vitis vinifera* L. suspension cells (Verries *et al.,* 2004). These are but a few examples of the many promoters currently being isolated.

Other complementary approaches have also been implemented for the isolation of

promoters from plants. These include differential screening, subtractive hybridisation, differential display, promoter tagging and inverse PCR. These approaches do not require prior knowledge of the gene or its product (Datla *et al.,* 1997). The promoter tagging method is a commonly used approach and involves the delivery of promoter-less reporter genes into plant genomes and assaying the transgenics for the activation of the reporter genes. The tagging of plant promoters with reporter genes such as GUS and LUC has an advantage to studying the regulatory properties of the tagged promoter (Springer *et al.,* 1995). The first report on plant promoter tagging employed a promoter-less *npt-II* and positive selection for recovering transcriptional fusions (Teeri *et al.,* 1986).

In another method, plant promoters can be isolated by inserting a random DNA fragment in front of a promoterless reporter gene for the screening of the activation of the reporter gene. A vector that was constructed for this purpose was the pROA97 vector which contains two markers (npt gene and GUS gene). Both markers are placed under the minimal 35S promoter carrying the TATA box region (An and Kim, 1993). Using this method several promoter fragments were isolated from *Arabidopsis thaliana* (Ott and Chua, 1990) and rice (Claes *et al.,* 1991) genomic DNA. Having a completely sequenced genome such as that of the model plant *Arabidopsis,* this facilitates easier promoter identification and comparative analysis. Seki *et al.* (2002) identified promoter regions by comparing data of the 5'- ends of isolated full-length cDNAs, from Arabidopsis plants to genomic sequences of Arabidopsis and constructed a promoter database using data from a plant transcription factor database (Higo *et al.,* 1999).

SASRI has been involved in promoter research programmes since 1990. A vast amount of work has been done in this field to date. The programme is based on targeting differentially expressed genes in plants for expression in specific tissues such as stems, leaves and roots in sugarcane. This would allow for the isolation and characterisation of their respective promoters. The project implements molecular techniques already optimised at SASRI. The most recent promoter project in SASRI is to obtain four types of novel promoters (DNA elements regulating gene expression), from maize and sorghum, for targeted functionality in sugarcane; specifically promoters able to switch on gene expression in leaf, young internodes, mature stem and root (Bernard Potier pers. comm.).

2.7 <u>Genomic libraries</u>

2.7.1 Lambda genomic library

The construction of representative large insert DNA libraries is critical for the analysis of complex genomes. Physical mapping and chromosome walking in complex genomes requires large insert DNA libraries. These libraries are required to be rapidly constructed, easily screened and manipulated (Woo *et al.,* 1994). Most large insert DNA libraries are constructed in bacteriophage, cosmid and yeast artificial chromosome (YAC) cloning vectors.

Large insert libraries have made valuable contributions to genome analysis and molecular genetics (Vanhouten and MacKenzie, 1999). In plants, large insert libraries have been developed for several crop species, such as rice, sugarcane, tomato, soybean, sorghum, lettuce and maize. The clones obtained from these libraries have been used to facilitate gene isolation by positional cloning and promoter isolation, to study the relationship between physical and genetic distance, and to assess genome structure (Vanhouten and MacKenzie, 1999).

2.7.2 Bacterial artificial chromosome library

The most commonly used system for constructing large insert libraries in plants is the bacterial artificial chromosome (BAC) system. The BAC vector is an F plasmid-based vector that is maintained as a single-copy plasmid in recombinant-deficient Escherichia *coli* host to promote sequence stability (Shizuya *et al.,* 1992).

The insert sizes found in most BAC libraries go up to 350 kb, averaging 100-150 kb (Shizuya *et al.,* 1992; Woo *et al.,* 1994). The BAC system offers some important advantages over the YAC system. The BAC insert DNA is easy to isolate and manipulate and clone instability and chimeras (Umehara *et al.,* 1995) have been reported to be low (Bent *et al.,* 1998) or absent (Shizuya *et al.,* 1992; Woo *et al.,* 1992 as compared to the YAC libraries.

There are numerous examples of BAC libraries constructed for monocotyledonous plants. Woo *et al.* (1994) constructed and characterised a BAC library of *Sorghum bicolor* in which the library had an average insert size of 157 kb. This study focused on establishing and implementing efficient map-based gene cloning systems for crop plants for the isolation of important genes. Map based cloning is defined as the isolation of a gene based solely on its position on a genetic map and it includes four basic elements 1) target gene mapping; 2) physical mapping 3) chromosome walking and 4) gene identification (Wing *et al.,* 1994).

Chapter 3
Materials and Methods

3.1 <u>Plant material for Southern and Northern blot analyses</u>

Maize (Mo 17 variety) and sorghum plants were grown in the field at the South African Sugarcane Research Institute (SASRI), Mount Edgecombe. The plants were grown from purchased seeds under non controlled conditions. The five-month old field-grown plants were harvested; the leaves (young and mature), young stems (internodes 1-4), medium stems (internodes 5-9), mature stems (internodes 10-13) (Figure 1) and roots were frozen in liquid nitrogen and stored at -80°C.

Internodes 1 -4   **Internodes 5 -9**   **Internodes 10 - 12**
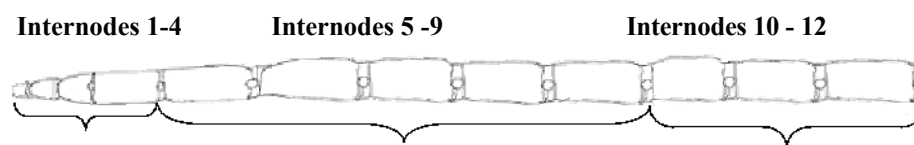
Figure 1: Represents internodal division of the *Saccharum* stem where internodes 1-4 represents the young stem, 5-9 the medium aged part of the stems and 10-13 the mature aged part of the stems.

Ancestral sugarcane cultivars Black Cheribon (*Saccharum officinarum)* and Coimbatore (*Saccharum spontaneum)* were maintained in pots in the glasshouse under controlled glasshouse conditions at SASRI. The commercial variety N19 (Nco376 x CB40/35) and the pre-release variety 88H0019 were grown in the field. The six to eight month old sugarcane plants were harvested between February and May 2004.

3.2 <u>Northern blot hybridisation</u>

3.2.1 Isolation of RNA

Total RNA was isolated from the leaves (young and mature), young internodes (1-4), medium internodes (5-9), mature internodes (10-13) and roots of maize, sorghum,

N19, 88H0019, Black Cheribon and Coimbatore plants according to Bugos *et al.* (1995). Five grams of frozen plant material were ground to a fine powder using a mortar and pestle. The ground tissue was transferred into centrifuge tubes containing 10 ml of TENS homogenisation buffer (0.1M Tris-HCl pH7.5, 1mM EDTA, 0.1M NaCl, 1% SDS and 2-3-mercaptoethanol). One volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the ground tissue and homogenised at high speed for 1-2 minutes. Seven hundred microlitres of 3M sodium acetate pH 5.2 were added and this was placed on ice for 15 minutes. The solution was centrifuged at 12 000 x *g* for 15 minutes at 4°C. The aqueous phase was transferred into a new centrifuge tube and an equal volume of isopropanol was added to precipitate the nucleic acids. The solution was incubated at -70°C for 30 minutes.

The precipitated RNA was recovered by centrifugation at 10 000 x *g* for 10 minutes. The pellet was washed with 70% (v/v) ethanol and briefly dried. The pellet was resuspended in 750 µl deionised water and centrifuged at 10 000 x *g* for 5 minutes at 4°C. The supernatant was transferred to a fresh tube and the RNA precipitated overnight at 4°C by the addition of lithium chloride (LiCl) to a 2M final concentration. The RNA was recovered by centrifugation at 12 000 x *g* for 15 minutes at 4°C. The pellet was washed with 70% (v/v) ethanol and resuspended in 50 µl Diethyl pyrocarbonate (DEPC)-treated deionised water.

3.2.2 Assessment of RNA concentration, purity and integrity

The concentration of the isolated RNA was determined spectrophotometrically. The integrity was verified by the presence of the 18S and 28S ribosomal bands after separation on a 1.2% (w/v) agarose gel and visualised by ethidium bromide staining (0.01 µg/ml) using the AlphaImager[TM] 2200 for visualisation. The RNA concentration was calculated using the equation, (A260*40*Dilution Factor)/1000 (Hillis and Moritz, 1990).

3.2.3 Blotting procedure

Ten micrograms of total RNA per sample were denatured in a buffer of the following composition (20 mM MOPS (pH7.0), 5 mM sodium acetate, 1 mM EDTA, 50% (v/v) formamide and 2.2 M formaldehyde) at 65°C for 5 minutes and followed by immediate quenching of the denatured RNA samples on ice. Total RNA (10 µg per lane) was electrophoretically separated on a 1.2% (w/v) agarose gel and blotted onto a nylon membrane (Hybond-N+ Amersham Pharmacia Biotech, Inc.) by upward capillary transfer according to the protocol described by Sambrook and Russell (2001). Following electrophoresis the RNA was stained for 30 minutes with ethidium bromide (0.01µg/ml) and visualised. The transfer was carried out overnight in a 10X SSC (1X SSC contains 150 mM NaCl and 15 mM sodium citrate) transfer buffer and the RNA bound to the nylon membrane by UV cross-linking (Hoefer Scientific Instruments, San Francisco) at 700 microjoules/cm^2.

3.3 Southern blot hybridisation

3.3.1 Isolation of genomic DNA

Genomic DNA was isolated from 3 g frozen leaf material from the sugarcane cultivars, maize and sorghum using a cetyltrimethylammonium bromide (CTAB) extraction method (Saghai-Maroof et al., 1984). The frozen leaf tissue material was ground into a fine powder using a mortar and pestle. Three grams of the ground tissue were added to 10 ml of warm (65°C) CTAB extraction buffer and mixed several times by gentle inversion. The resulting solution was incubated at 65°C for 1 hour with gentle mixing at 10 minute intervals. The solution was cooled and 5 ml of chloroform:isoamyl alcohol (24:1) were added with gentle shaking for 5-10 minutes.

The contents were spun in an ultracentrifuge (Beckman Avanti^{TM} J-25I) at 12 000 x g for 10 minutes at room temperature. The aqueous phase was transferred to clean tubes to which 5 ml of chloroform:isoamyl alcohol (24:1) were added and gently mixed. This was spun at room temperature for 10 minutes at 12 000 x g. The aqueous phase was transferred

to fresh tubes containing pre-boiled Rnase A to a final concentration of 40µg/ml and mixed by gentle inversion followed by incubation at room temperature for 30 minutes. Six millilitres of isopropanol were added and the sample gently inverted and spun at 1500 x g for 10 minutes. The DNA pellet was dried and resuspended in 1 ml TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8.0). The sample was phenol extracted with 1 ml Tris-equilibrated phenol (pH 8.0). The aqueous phase was transferred to a fresh tube. The DNA was precipitated and resuspended in 1 ml TE buffer.

3.3.2 Assessment of DNA concentration, purity and integrity

The isolated DNA was quantified spectrophotometrically and the integrity was verified by gel electrophoresis on a 1% (w/v) agarose gel. The concentration of the DNA was determined by recording readings at wavelengths of 260 and 280 nm using a spectrophotometer. The equation, (A260*50*Dilution Factor)/1000, was used to calculate the DNA concentration (Hillis and Moritz, 1990).

3.3.3 Blotting procedure

The genomic DNA for each plant was treated separately with each of the restriction endonucleases, *Eco*RI*, Hind*III*, Xba*I and *Xho*I (Fermentas). Ten micrograms of genomic DNA were digested with 10 units of each enzyme in a total reaction volume of 300 µl at 37$^{°}$C overnight. The digested DNA was size-fractionated by agarose gel electrophoresis using a 0.8% (w/v) agarose gel in a 0.5X Tris-borate-EDTA (5X TBE is 445mM Tris base, 445mM borate and 10mM EDTA) running buffer and visualised by ethidium bromide staining (0.01 µg/ml) using the AlphaImager^{TM} 2200 for visualisation. The DNA was denatured for 1 hour in denaturing solution (2M NaOH and 1.5M NaCl) with gentle agitation. Thereafter the gel was rinsed in distilled water to remove residual denaturation solution and submerged in neutralisation buffer (0.5M Tris-HCl and 1.5M NaCl) with constant shaking for 1 hour. The restricted DNA was transferred to a positively charged nylon membrane (Hybond-N+ Amersham Pharmacia Biotech, Inc.) using upward capillary DNA transfer according to Sambrook and Russell (2001) in 10X

SSC transfer buffer (1.5M sodium chloride and 150mM sodium citrate).

The nucleic acids were fixed to the moist nylon membrane by UV cross-linking (Hoefer Scientific Instruments, San Francisco) at a pre-set UV exposure of 700 microjoules/cm^2. Blots were stored dry between Whatman filter paper at 4°C until required.

3.4 <u>SS gene sequence probe</u>

A partial gene sequence (EST clone, Accession number AW746904) isolated from sorghum was designated the SS (stem-specific) gene. The stem-specific expression pattern of this gene allowed for its use in this study. The complete sequence for this gene is shown in Figure 2.

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1      GGCACGAGGATCCATCCCACTCTCAGCTGATCGCTCACTCTTTCAGCTCGATCAGTCTTAGCTCTAGCTC
71     TAGCTAGCCAGCTAGCCAATCCTTCGTGTAGTCGTCATCAGCCTTCTCATCGTCACCAATGGCCACCGCC
141    GAGGTCCAGACCCCGACCGTGGTGGCGACCGAGGAAGCGCCGGTGGTGGAGACCCCGGCGCCGGCCGTCG
211    TGCCCGAGGAGGCTGCCCCCGCCCCCGCCGAGGCTGAGCCGGCCGTGCCAGAGGAGGCTGCCCCCGCCGA
281    GGAGGCCAAGGTGGTGGAGGAGCCAGCTGCCCCGGCGGAGCCCGAGCCTGTCGCCGCTGAGCCTGAGGCC
351    GAGCCTGCCGCCGCGGAGCCGGAGGCGGCACCTGCTGCGGCCGCGGCGGAAGAAGAGGCGCCAAAGGAGG
421    CGGAGCCGGCCGCGGTCGAGGAGGTCAAGGAGGAGGAGGCTGCGGCGCCCGCTGCCGAGACAGAGCCGGC
491    GGCCGCCGAGCCCGAGGCTGCTGCTCCTGCTCCTGCTGCTGCCGAGGAGCCCGCCGCGGCCGAGCCGGCC
561    GCCGAGGAGCCCGAGAAGGCCAGCGAGTGAGGCCTCCTCGGCGGCGGCCAAGGGATCGGAGTGAGATGGC
631    TCATCGTGCGCGCGTACATGCCACGGGCTTTTGCTGTTGAGTACGCTACGGTTACTGGCGTTTCTCTACG
701    TACCTAGCAGTGGCTGTGCGCGTGGACTGTCCGGTGCGGACTGCGAGTGACGTGGGCTAAATAAAGTGAG
771    GGTCGTGTTCGTGTGGCGGGGGGAAGTGGGCCCGGTGTCAGTGGCGCGGTGCCCCATGGCCTGCCACTG
841    CAGCTTGTGTGTCCTTTTGTGTTGTGTTGGTTCCTGTTCTGTGTTCTCTGTCTATCTATGCTCATGTATG
911    TAATGGCAGTTTGGGTATGATGAGTATCAAGCATGTCTGCATTCTCGTCTTCTTTGGTTAAAAAA
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Figure 2: Nucleotide sequence of the cDNA clone (SS gene) used in this study for the isolation of corresponding promoter regions from maize and sorghum.

3.4.1 Isolation and purification of plasmid DNA

The *Sorghum bicolor* cDNA clone in a pBluescript SK(-) plasmid vector was grown in TOP10F' *Escherichia coli* cells. The bacterial cells were grown in Luria Bertani (LB) broth (bacto-tryptone (10 g/l), yeast extract (5 g/l), NaCl (10mg/l) and ampicillin (100µg/ml) at pH 7.5) at 37°C overnight. The plasmid DNA was purified using a Nucleobond AX100 kit (Macherey-Nagel, Germany) and stored at -20°C.

3.4.2 Isolation of the insert clone DNA

The cDNA clone sequence was used to probe Northern and Southern blots. The insert was isolated from the plasmid construct by digestion with the restriction endonuclease *Pst*I (Fermentas) to release a 975 base pair DNA fragment. The digested plasmid was separated on a 0.8% agarose gel and the band corresponding to the insert size was excised. A Qiagen gel extraction kit (Qiagen, Hilden, Germany) was used to purify the DNA from the gel as described by the protocol provided by the manufacturer.

3.4.3 Quantification of the purified insert DNA

The purified insert DNA was separated on a 1% (w/v) agarose gel. Molecular weight marker (EL marker – pEmuLuc restricted with *Pgl*II, *Pvu*II and *BamH*I) was used for visual estimation of the DNA concentration and verification of band sizes.

3.4.4 Radioactive labeling of the DNA fragment

A Megaprime^{TM} DNA labeling systems kit (Amersham Biosciences) was used to radioactively label 25 ng of purified DNA with α-[^{32}P]dCTP (220 Tbq/mmol; Amersham Biosciences). Twenty-five nanograms of template DNA dissolved in deionised water were placed in a microcentrifuge tube, 5 µl primer solution (Random nanomer primers in an aqueous solution) and the appropriate volume of water were added to give a total final volume of 50 µl. The reaction mix was denatured at 100°C for 5 minutes, followed by immediate cooling on ice. Five microlitres labeling buffer (dATP, dGTP and dTTP in

45

Tris/HCl pH7.5, 2-mercaptoethanol and MgCl2) were added. Three microlitres of α-[^{32}P]dCTP were added to the mix, followed by the addition of one unit DNA polymerase 1 Klenow fragment (Fermentas). The solution was gently mixed. This was spun for a few seconds in a microcentrifuge and thereafter incubated at 37°C for 1 hour.

3.4.4.1 Probe purification and denaturation

The labeled probe was purified using a QIAquick PCR Purification kit (Qiagen, Hilden, Germany) to remove unincorporated nucleotides. The purification was carried out according to the protocol provided by the manufacturer and the labeled DNA fragments were eluted in 100 µl elution buffer (10mM Tris-HCl pH8.5). The eluted labeled probe was transferred to a fresh tube and denatured at 100°C followed by immediate chilling on ice for 2 minutes prior to hybridisation.

3.5 **Prehybridisation and hybridization**

The modified Church and Gilbert hybridisation buffer (0.5M phosphate buffer pH7.2, 7% (w/v) SDS and 10mM EDTA) (Church and Gilbert, 1984) was used for the prehybridisation of the membranes for a period of 6 to 8 hours at 65°C. The prehybridisation buffer was preheated to 65°C and 30 ml added to a 300 ml hybridisation bottle (Hybaid^{TM}). To this volume 300 µl of 10 mg/ml denatured herring sperm DNA was added as a blocking agent to minimize non-specific hybridisation. The herring sperm DNA was denatured by boiling for 5 minutes and cooled on ice. The blots were pre-wet in 0.5M phosphate buffer pH7.2 before being placed in the hybridisation bottles. After prehybridisation in a rotating oven, the labeled probe was added directly to the bottle. Hybridisation was carried out for 16 to 18 hours at 65°C. Following hybridisation the radioactive solution was removed from the bottles. The membranes were washed twice with a medium stringency wash solution (1XSSC, 0.1% SDS) at 65°C for 10 minutes.

3.5.1 Image analysis

The membranes were placed in a Hypercassette^{TM} (Amersham, Life Sciences) and exposed to phosphor screens (Cyclone^{TM}) overnight. The phosphor screens were scanned using the Cyclone^{TM} Storage Phosphor System (Packard BioScience). The image was viewed using the OptiQuant^{TM} Image Analysis software.

3.6 <u>Screening of the libraries</u>

3.6.1 Maize Genomic Library

The bacterial host strain used was XLI-Blue MRA' (P2). A single colony was grown in LB broth in a sterile Erlenmeyer flask supplemented with 0.2 % (w/v) maltose and 10mM magnesium sulfate (MgSO$_4$) at 37°C with vigorous shaking (250 rpm) for 4-5 hours. The optical density (OD) was monitored during the course of the bacterial growth. The OD was measured using the spectrophotometer (Beckman DU® 7500, USA) at 600 nm, the ideal OD of the bacteria should be between 0.8 and 1.

The bacteria were centrifuged at 1000 x *g* for 10 minutes. The pellet was gently resuspended in 10 ml of 10mM MgSO$_4$ and diluted to an $_{OD600}$ of 0.5 with 10 mM MgSO$_4$ prior to use.

3.6.1.1 Plating the maize genomic library

The Lambda library has an estimated titre of 1.0x 10^6 pfu/ml (Lambda Library User Manual, Stratagene). Titre estimation at the outset is critical since the number of clones to be screened must be pre-determined. Also the maximum number of clones would provide a good representation of the maize genome for screening thus increasing the probability of locating the desired clones. It must be noted that clones in the library are not unique and therefore screening a large number of clones is also vital in ensuring isolated clones are unique. To obtain single plaque purity three rounds of screening were

required and each round of screening is carried out by hybridisation analysis.

The *Zea mays* genomic library was purchased from Stratagene® and stored at -80°C. From an estimated $1.0x\ 10^6$ pfu/ml in the maize genomic library, 280 000 pfu were plated over 10 agar plates with a lawn of bacterial cells, with approximately 28 000 plaque forming units per plate since between 20 000-30 000 plaque forming units is ideal for a plate size of 150mm^2.

Six-hundred microlitres 0.5OD bacterial host cells were mixed with 28 000 plaque forming units and incubated at 37°C for 15 minutes. Five milliliters of NZY Top agar at 48°C (NaCl (5 g/l), MgSO4.7H2O (2 g/l), yeast extract (5 g/l), amine (casein hydrolysate) (10 g/l) and agarose (7 g/l)) were mixed with the phage-infected bacteria and thereafter poured immediately onto 10 NZY agar plates (NaCl (5 g/l), MgSO4.7H2O (2 g/l), yeast extract (5 g/l), NZ amine (casein hydrolysate) (10 g/l) and agar (15 g/l). The agar was allowed to solidify for 10 minutes and incubated at 37°C.

3.6.1.2 Plaque lifts

After individual plaques were visible, the plates were chilled at 4°C overnight. The plaques were transferred onto the nylon membranes by placing the membranes on the agar surface for 1 minute and for duplicate lifts 2 minutes. The phage DNA was denatured on filter paper pre-soaked with denaturation solution (1.5M NaCl and 0.5M NaOH) for 5 minutes. This was followed by neutralisation of the membranes on filters soaked with neutralisation buffer (1.5M NaCl and 0.5M Tris-HCl pH8.0) for 5 minutes. The membranes were washed in 2X SSC to remove cellular debris followed by UV cross-linking at 700 microjoules/cm^2. The agar plates were stored at 4°C for use after screening.

The positive plaques were located on the agar plates following primary screening by alignment of the autoradiograph film to the corresponding plates and a small plug (diameter ~2-3mm) of agarose and agar from around each positive plaque was cored.

Each plug taken with this diameter was estimated to contain between 5-10 plaques of which each plaque has approximately 1×10^6 pfu. Using this estimation a dilution series was prepared of the plaques to titre the total number of plaques in each agar plug placed in 500 µl SM buffer (100 mM NaCl, 8 mM MgSO4.7H2O, 50 mM Tris-HCl (pH 7.5) and 0.01% (w/v) gelatin). The estimated titre per agar plug assuming each plug contained ten plaques is 10×10^6 pfu/plug, this was used as the basis on which to calculate the dilution factor required to obtain 100 pfu/20µ/ for spotting onto a bacterial lawn to obtain well separated plaques on the agar plates. Twenty thousand plaques were mixed with 100 µl SM buffer and 1 µl of this was used in a 1:50, 1:75 and 1:100 dilution. The number of plaques counted from each dilution gave the titre for each agar plug (Table 4) and this was used to calculate a dilution factor to obtain 100 pfu/20µl for spotting onto an agar plate.

3.6.1.3 Screening of the maize genomic library

The maize blots were screened according to the hybridisation protocol obtained from the Clemson University Genomics Institute (3.5 and 3.5.1). The probed membranes were exposed to phosphor screens and visualised using the Cyclone™ Storage Phosphor System (Packard BioScience). Plaques that showed a strong hybridisation signal were plugged out and transferred to 500 µl SM buffer with 20 µl chloroform. The agar plugs were stored at 4°C to allow the phage to diffuse out of the agar plug into the SM buffer. A dilution series was prepared and a dilution containing ±53 pfu was spotted onto a bacterial lawn. The spots were dried and the agar plates incubated at 37°C overnight. Secondary and tertiary plaque lifts were carried out in order to obtain a single positive plaque that displayed a strong hybridisation signal.

3.6.2 Bacterial Artificial Chromosome library

A *Sorghum bicolor* Bacterial Artificial Chromosome (BAC) library arrayed on membranes was purchased from Clemson University Genomics Institute. The

membranes were screened with the radioactively labelled SS gene sequence according to hybridisation and wash protocols provided by Clemson University. The screened BAC library filters were exposed to high performance autoradiography film (Hyperfilm™ MP; Amersham Biosciences) and autoradiography was carried out for a period of 2 weeks at -80°C.

3.6.2.1 Identification of addresses for hybridising clones

X-rays were aligned to a grid and addresses were allocated to the strongly hybridised clones using the addressing system provided and the clones were ordered. The filter membranes are sub-divided into six fields (Figure 10) and each field contains 384 squares. The 384 squares represent the row and column identification of the BAC. Within each square there are 16 positions where eight clones are spotted in duplicate. The pattern of the spotted clones was used to generate the plate address of the BAC.

3.7 **Isolation of Lambda genomic DNA**

Lambda DNA was isolated using protocols modified from Sambrook and Russell (2001). Six plates were prepared for each isolated phage. Six-hundred microlitres of 0.5 OD bacterial host cells were infected with 20 µl phage (±50 000 pfu) and incubated for 15 minutes at 37°C. The infected bacterial cells were mixed with top agar and poured immediately onto NZY agar plates. The plates were incubated overnight at 37°C. Ten milliliters of SM buffer were poured onto the NZY plates and left at 4°C overnight to allow the diffusion of the phage from the agar into the SM buffer. The bacteriophage suspension from each plate was recovered and pooled into a sterile tube. An additional 5 ml SM buffer was used to further wash and remove the residual bacteriophage. Five milliliters of chloroform were mixed with the phage stock. This was centrifuged at 11 000 x g for 5 minutes and RNaseI (Fermentas) was added to the supernatant to a final concentration of 1µg/ml followed by incubation at room temperature for 30 minutes.

NaCl (2.6 g/50 ml supernatant) was added to the phage supernatant and incubated on ice for 1 hour. The solution was spun at 11 000 x g for 10 minutes to remove the cellular

debris of the bacteria. Polyethylene glycol (PEG) 8000 (4.5 g/50 ml supernatant) was added to the phage supernatant and kept on ice for 30 minutes. This solution was centrifuged at 11 000 x *g* for 10 minutes. The bacteriophage pellet was resuspended in 10 mls SM buffer and one volume of chloroform added. The resulting solution was centrifuged at 3 000 x *g* for 15 minutes. The supernatant was removed and a phenol/chloroform extraction was performed. One volume of isopropanol was added to the supernatant and incubated overnight at -20°C. This was spun at 11 000 x *g* for 10 minutes and the pellet was washed with 80% (v/v) ethanol and air dried. The washed pellet was resuspended in 500 µl deionised water.

3.7.1 Isolation of plasmid DNA

The BAC clones were streaked onto LB plates supplemented with chloramphenicol (Roche, Diagnostics, Mannheim, Germany) to a final concentration of 20 µg/µl. The plates were incubated overnight at 37°C. Fifty milliliters of Terrific broth (Bacto tryptone (12 g/l), Bacto yeast extract (24 g/l), 0.4% (v/v) glycerol) were inoculated with a single bacterial colony for each clone. This was grown to saturation overnight at 37°C after which 1.5 ml of bacterial culture was pelleted by centrifugation at maximum speed for 20 seconds. The BAC plasmid DNA was isolated according to an alkaline lysis procedure described by Birnboim and Doly, 1979. The pellet was resuspended in 100 µl Glucose/Tris/EDTA (GTE), (50mM glucose, 25mM Tris-HCl pH8.0 and 10mM EDTA) solution and allowed to stand for 5 minutes at room temperature. Two-hundred microlitres NaOH/SDS (0.2M NaOH and 1% (w/v) SDS) solution were added to the resuspended mixture and kept on ice for 5 minutes. One hundred and fifty microlitres of 3M potassium acetate solution were added to the lysed bacterial solution. This was placed on ice for 5 minutes and thereafter spun for 10 minutes at maximum speed to pellet the cell debris and chromosomal DNA.

The supernatant was transferred to a clean tube and mixed with 0.8 ml 95% (v/v) ethanol. This was allowed to stand for 2 minutes at room temperature to precipitate the nucleic acids followed by centrifugation for 1 minute at room temperature to pellet the plasmid DNA and RNA. The supernatant was poured off and the pellet washed with 1 ml 70%

(v/v) ethanol and dried. The pelleted plasmid DNA was resuspended in 25 µl deionised water and stored at -20°C.

3.8 <u>Investigation of the isolated clones from both libraries</u>

3.8.1 Agarose gel separation and Southern blot analysis of Lambda and BAC DNA fragments

Southern blot analysis was performed on the restricted isolated clones from both libraries to obtain fragments that were of a suitable size range for cloning into a plasmid vector. Five micrograms of the isolated lambda DNA from the maize genomic library clones were digested with a range of restriction enzymes (*Hind*III, *EcoR*I, *Pst*I and *Xba*I). Five micrograms of the isolated plasmid DNA from the *Sorghum bicolor* BAC library clones were digested using various restriction enzymes (*Eco*01091, *Hind*III, *Xba*I and *Xho*I). The restricted DNA fragments were electrophoretically separated in a 0.8% (w/v) agarose gel and fragments sizes were obtained by comparing to the EL marker. Prior to blotting, the restricted DNA was viewed after ethidium bromide staining (0.01 µg/ml) using the AlphaImager^{TM} 2200 (Alpha Innotech corporation). The restricted DNA was transferred to a positively charged nylon membrane (refer to section 3.2.3).

3.8.2 Cloning of isolated DNA fragments and bacterial transformation

The plasmid DNA pBluescript SK(-) was isolated using a QIAprep® Miniprep kit (Qiagen, Hilden, Germany) and linearised with the respective enzyme. The enzyme was inactivated at 65°C for 30 minutes followed by desphosphorylation with 1 unit shrimp alkaline phosphatase (Fermentas) and 2µl 10x shrimp alkaline phosphatase desphosphorylation buffer (0.1M Tris-HCl pH7.5, 0.1M Mg Cl2) (Fermentas, USA). This was incubated at 37°C for 1 hour and the phosphatase was inactivated by heating at 65°C for 30 minutes.

Two micrograms of lambda DNA and plasmid DNA were digested with the respective enzyme in a reaction volume of 30 µl with 2 units of restriction enzyme and incubated

overnight. The restricted fragments were separated on a 0.8% (w/v) agarose gel and viewed after ethidium bromide staining (0.01µg/ml). The DNA fragments corresponding to the hybridising band were excised from the gel and the DNA purified using a QIAquick® gel extraction kit.

Hybridising fragments of a large size were restricted with an enzyme to obtain a maximum of three smaller fragments (Figure 3). This was verified by separation of the digested insert on a 0.8% (w/v) agarose gel. The plasmid was prepared by restriction with the first enzyme. An aliquot of the digested plasmid was separated on a 0.8% (w/v) agarose gel to verify complete digestion followed by ethanol precipitation overnight. The precipitated plasmid DNA was digested with the second enzyme and complete digestion of the plasmid was verified. The total digested insert mix was used in the ligation reaction and ligation of insert and plasmid was carried out as described in 3.8.3.
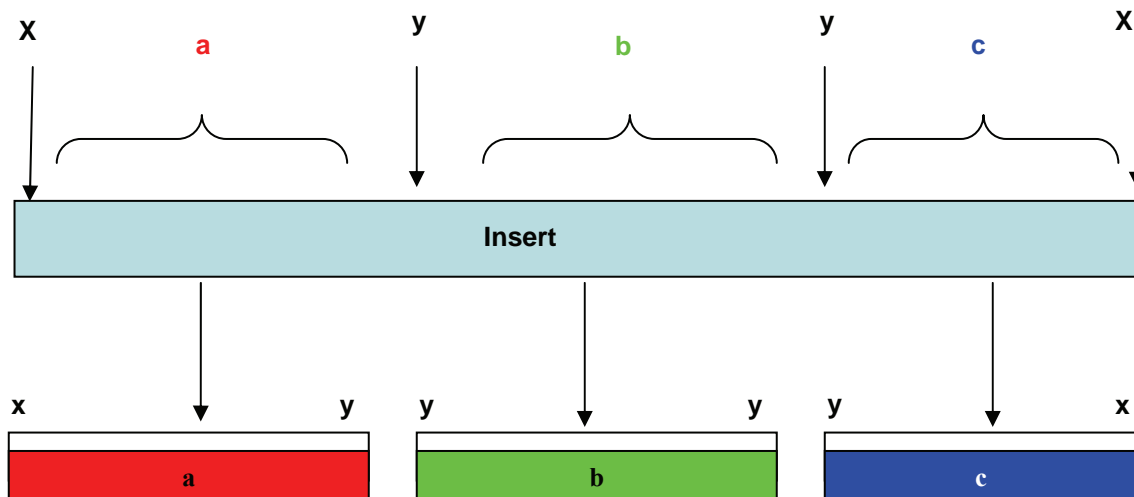
Figure 3: Diagram representing the forced cloning method. The complete insert has the same flanking enzyme (x), and a second enzyme (y) is used to restrict the total fragment into smaller pieces. In this case three fragments were produced (a), (b) and (c). The forced cloning approach limits the plasmid to take up the DNA segment with the (x) and (y) enzymes flanking it since the plasmid has been prepared with these two enzymes.

Ligation was carried out as described by Sambrook and Russell (2001). The ligation was carried out in a reaction volume of 20 µl consisting of 100 ng insert DNA, 50 ng linearised and dephosphorylated plasmid DNA, 5 µl 10X ligation buffer (100 µl of 400mM Tris-HCl , 100mM MgCl2, 100mM DTT and 5mM ATP pH7.8), 1 unit T4 DNA ligase and deionised water overnight at 4°C. The ligation mix was heated at 65°C for 10 minutes to inactivate the T4 DNA ligase.

3.8.3 Transformation of the *Escherichia coli* cells

Transformation was carried out as described by Sambrook and Russell (2001). Two microlitres of ligated plasmid were gently mixed with 50 µl competent TOP10F' *Escherichia coli* cells (Invitrogen) and kept on ice for 10 minutes. This mixture was transferred to pre-chilled cuvettes (BioRad) and electroporated using a Micropulser (BioRad). Immediately after electroporation the cells were mixed with 450 µl LB broth and transferred to clean tubes to be shaken at 240 rpm for 1 hour at 37°C.

The transformation mix (100µl) was plated onto LB agar plates supplemented with 5-Bromo-4chloro-3-indolyl-3-D-galactopyranoside (X-gal) (40 µg/ml)) (Roche Diagnostics, Mannheim, Germany), 0.5mM Isopropyl-3-D-thiogalactoside (IPTG) (Roche Diagnostics, Mannheim, Germany) and ampicillin (100 µg/ml) (Roche Diagnostics, Mannheim, Germany). The white colonies were transferred onto fresh LB agar plates supplemented with X-gal, IPTG and ampicillin. Location was determined using a grid reference. Duplicate plates were also prepared.

3.8.3.1 Colony lifts and hybridisation

The colonies from the plates were transferred onto nylon membranes (Hybond-N+) as described by Sambrook and Russell (2001). The nylon membranes were placed directly over the agar surface and the transfer was allowed to proceed for 2 minutes. The plasmid DNA was denatured following neutralisation as described in section 3.6.1.3. The membranes were allowed to air dry on Whatman filter paper for 30 minutes before UV cross-linking at 700 microjoules/cm^2 using the UV cross-linker.

The colony lifts were screened using the hybridisation and wash procedure carried out in section 3.5. The membranes were probed with a α-[^{32}P]dCTP labeled SS gene sequence as described by the Megaprime^{TM} DNA labeling systems kit (Amersham Biosciences). The blots were exposed to phosphor screens for 1 hour before viewing.

3.9 Plasmid DNA isolation and assessment of concentration and purity

The hybridising colonies were selected from the master plates and used to inoculate 3 ml of LB broth supplemented with ampicillin (100 µg/ml). The bacterial cultures were shaken at 250 rpm overnight at 37°C. The plasmid DNA was purified using a Nucleobond AX100 kit (Macherey-Nagel, Germany) from 6 ml of overnight bacterial culture and eluted in 50 µl deionised water. The concentration was assessed as described in section 3.4.3.

3.10 DNA Sequence analysis

3.10.1 Polymerase chain reaction (PCR)

The cloned fragments were amplified and sequenced using the BigDye® Terminator V3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA) as instructed by the manufacturer. The 20 µl reaction mixture was composed of the following: DNA template (200 ng), either M13Forward- (AACGACGGCCAGTGAATT) or M13Reverse- (GGAAACAGCTATGACCATGAT) primer (3.2 pmol of each primer), 2 µl 5X BigDye Sequencing Buffer, 2 µl 2.5X Ready Reaction mix and sequencing-quality water made to the final reaction volume. Amplification conditions consisted of initial denaturation (96°C for 1 min) followed by 25 cycles (96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min) and a rapid thermal ramp to 4°C in a thermal cycler (GeneAmp® PCR Systems 9700, Applied Biosystems).

The amplified DNA was precipitated using the protocol provided by the manufacturer. Five microlitres of EDTA pH8.0 (125mM) and 60 µl of 100% (v/v) ethanol were added to the PCR mix and left at room temperature for 15 minutes. This was centrifuged at

maximum speed for 20 minutes to pellet the DNA. The pelleted DNA was washed with 60 µl 70% (v/v) ethanol and spun at maximum speed for 20 minutes. The pellet was air dried for 5 minutes and resuspended in 20 µl template suppression reagent (BigDye ® Terminator v3.1 Cycle sequencing kit; Applied Biosystems). The resuspended DNA was denatured by boiling for 2 minutes and then cooled on ice. Sequencing was performed on an ABI Prism™ 310 Genetic Analyser (Applied Biosystems, Foster City, CA).

3.10.2 Sequence analysis

Sequences were edited using a biological sequence alignment program BioEdit (T.A. Hall Software) and used for homology searches on the GenBank database using the BLAST algorithm of the National Centre for Biotechnology Information (NCBI). Restriction maps were constructed for each clone using the restriction map function in BioEdit (T.A. Hall Software). Religations of the plasmid were carried out by restriction with specific enzymes to obtain the complete sequence. Transformation was carried out as shown in section 3.8.3 Single colonies were picked and grown in LB broth and plasmid isolation was carried out using a Nucleobond AX100 kit. The religated plasmid vectors were sequenced as described in section 3.10.1.

3.11 Promoter analyses

A BLAST search was carried on the complete sequence (4500 bp) to determine the location of the SS gene sequence. To identify preliminary proximal promoter regions which included the TATA-box as well as a putative transcriptional start site, the genomic clone sequence was analysed by promoter prediction database software, Neural Network Promoter Prediction (NNPP, http://www.fruitfly.org/seqtools/promoter.html) (Rees and Eeckman, 1995). A BLAST search was carried out using the identified putative promoter region from the NNPP software and maize EST clones in the database. This was carried out to confirm that the coding region of the SS gene sequence was after the putative transcriptional start site.

The Plant Cis-acting Regulatory DNA Elements (PLACE)

(http://www.dna.affrc.go.jp/htdocs/PLACE) (Higo *et al.,* 1998) motif database was used to identify cis-acting regulatory DNA elements. This database contains motifs found in vascular plants only. All sequence similarity search and alignment analyses were conducted using BLAST: http://www.ncbi.nlm.nih.gov/blast/ (Altschul *et al.,* 1990), DNAssist and BioEdit.

3.12 PCR amplification of fragments for cloning into the GUS vector

To clone the genomic sequence immediately upstream of the coding region in the maize genomic clone, primers were designed to carry out polymerase chain reaction (PCR). Based on the sequence obtained, primers were designed to amplify two regions, (i) the promoter region and (ii) the promoter region with the intron. Restriction sites *SacII* and *Spe*I were incorporated at the 5' end of the forward and reverse primers (Table 1) respectively. This was done by PCR amplification of the cloned promoter fragments with primers containing the respective restriction sites.

Table 1: Sequences and positions of PCR primers for cloning (i) Promoter and (ii) Promoter and intron regions into the GUS plasmid. The positions of the 5' end where +1 is the start of the coding region, the corresponding PCR fragment sizes and the regions to be amplified are indicated. The 5' spacer sequences are shown in lowercase.

Primer designed	Primer Sequence (5′-3′)	Position of 5′ end	Region amplified	Fragment size (bp)
ZM51F2	aa CCGCGGGCCTGATATCTG [a]	-2378		
ZM51R2	ttACTAGTGTCTGGACCTGCACAC [b]	+423	Promoter and intron	2815
ZM51R3	ACTAGTATGAGAAGGCTGATGG [b]	+128	Promoter	3183

[a] restriction site underlined

[b] *Spe*I restriction site underlined

The genomic DNA fragments were amplified in a 50 µl reaction solution containing 10 ng of genomic DNA and final concentration of primers (10µM), dNTPs and MgCl2 at 0.2 µM, 40 µM, 3.5 mM respectively. Five microlitres of 10X PCR buffer and 1unit Taq polymerase were added to the PCR. Amplification conditions consisted of initial denaturation (96°C for 2 min) followed by 35 cycles (94°C for 1 min, 55°C for 30 sec and 72°C for 3 min), a final extension at 72°C for 4 min and a rapid thermal ramp to 4°C in a

thermal cycler (GeneAmp® PCR Systems 9700, Applied Biosystems). The amplified fragments were digested with *SacII* and *Spe*I to facilitate cloning into a GUS plasmid prepared with *SacII* and *Spe*I.

The amplified fragments with the flanking SacII and SpeI sites were cloned into the pBScript vector restricted with SacII and SpeI to confirm that the restriction sites were intact. The cloned PCR fragments were cleaved from pBluescript and cloned inframe with the GUS gene in the expression vector pBScr-GUS000. Ligation was carried out as described by Sambrook and Russell (2001). The constructs were subjected to restriction analyses using the enzymes SacII and SpeI.

Chapter 4
Results

4.1 <u>Introduction</u>

A partial gene sequence (EST clone, Accession number AW746904) (975bp) isolated from sorghum was used in this study. This gene sequence had been observed previously to display a stem-specific expression pattern and was designated SS. To date the SS (stem-specific) source gene has an undescribed function. At the outset of this project it was necessary to undertake a preliminary study to verify the expression pattern of this stem-specific gene sequence in maize (*Zea mays),* sorghum (*Sorghum bicolor)* and two ancestral sugarcane cultivars Coimbatore (*Saccharum spontaneum)* and Black Cheribon (*Saccharum officinarum),* the commercial sugarcane hybrid variety N19 and a pre-release sugarcane variety 88H0019 (Section 4.3)

The copy number of the gene was important to the study since a high copy number gene implies numerous genes with similar sequences encoding similar protein families, making the analysis of tissue-specific promoters difficult. Evolutionary modification in gene family sequences over time may render some genes inactive. This does not necessarily imply that the promoter driving the gene is inactive but chances are high that changes have occurred in the promoter as well. Therefore for high copy number genes the probability of isolating inactive promoters is greater, thus favouring the use of a low copy number gene in this project.

The overall approach for this project involved the use of two non-sugarcane grass species, maize and sorghum, as sources of the corresponding promoters of the SS gene sequence. A maize Lambda genomic library and a sorghum Bacterial Artificial Chromosome (BAC) library were screened with the SS gene sequence as the first step in isolating the upstream promoter regions.

The maize genomic library purchased from Stratagene uses the Lambda FIX II vector and the host strains XL1-Blue MRA and XL1-Blue MRA (P2). A total of 280

000 clones were screened from this library. The Sorghum (Btx623 cultivar) BAC library was purchased from the Clemson University Genomics Institute (CUGI). The BAC library uses the pIndigoBac536 vector and contains a total of 110 592 clones which are arrayed over a set of six filters. The two genomic libraries differ from each other in various ways (Table 2).

Table 2: Differences between the maize Lambda and sorghum BAC genomic libraries

Lambda Library (Maize)	Bacterial Artificial Chromosome Library (Sorghum)
The insert sizes in this library are between 9 and 23kb (Lambda Library User Manual, Stratagene).	The average insert size in this library is 130 kb with a range of 40 to 280 kb (Tomkins *et al.*, 1999).
The library is a random clone suspension having an estimated titre of 1.0×10^6 pfu/ml (Lambda Library User Manual, Stratagene).	The library is an ordered membrane array containing 110 592 clones gridded robotically onto filters. Each filter contains 18 432 clones and each set of six filters provides a genome coverage of 4x (Tomkins *et al.*, 1999).
The clone suspension must be plated out followed by plaque lifts prior to screening the clones with the radioactively labelled SS gene sequence.	The arrays can be analysed by screening with the radioactively labelled SS gene sequence directly.
Three screenings are needed to obtain individual clones.	Individual clones are identified after a single screening.

The maize Lambda and sorghum BAC library were screened using the SS gene sequence. The clones identified were large DNA fragments that could not be sub-cloned into a plasmid vector hence restriction analysis was implemented to obtain smaller fragments. Restricted fragments containing the SS gene sequence were identified using Southern blot analysis and cloned into pBluescript. Sequence analysis was carried out on the cloned fragments and these sequences were aligned to the SS gene sequence to determine if the upstream regulatory region was contained within the fragment.

Physical maps of the cloned DNA sequences were constructed to facilitate sequencing and the location of the promoter and intron regions. Primers were designed to amplify the promoter and promoter and intron regions respectively using polymerase chain reaction (PCR). The amplified fragments were fused transciptionally inframe to a β-glucuronidase (GUS) reporter gene in a test vector (pBScr-GUS000) for direct delivery into the plant. It was envisaged that the plasmid constructs would be used in the future to generate stable transformants in which the isolated promoter activity could be evaluated in sugarcane using both quantitative and qualitative analysis.

4.2 Tissue-specific expression of the SS gene sequence in sugarcane, maize and sorghum

4.2.1 Quality of isolated RNA

The isolation of clean intact RNA from plant material is essential for experiments involving Northern blot analysis. Total RNA was isolated from young and mature leaves, immature internodes (internodes 1-4), maturing internodes (internodes 5-9), mature internodes (internodes 10-12) and roots of maize, sorghum and four sugarcane cultivars, Black Cheribon, Coimbatore, N19 and 88H0019 as described in chapter 3.

The difficulty in RNA isolation is its easily degradable nature by RNases (Strommer *et al.,* 1993). Figure 4 shows intact total RNA run on a 1.2% agarose gel with clear

separation of the two abundant species of eukaryotic RNA, the 28S rRNA (approximately 5kb) and 18S rRNA (approximately 2kb) (Hillis and Davis, 1986).

(A)

Sugarcane

Zm   Sb   N19   88H   Coim   Cher

→ 28S rRNA

→ 18S rRNA

Chloroplast RNA

(B)
Top of sugarcane stem

Internodes 1-4   Internodes 5 -9   Internodes 10 - 12

Ly   Lm   S1-4   S5-9   S10-12   R

→ 28S rRNA

→ 18S rRNA

Chloroplast RNA
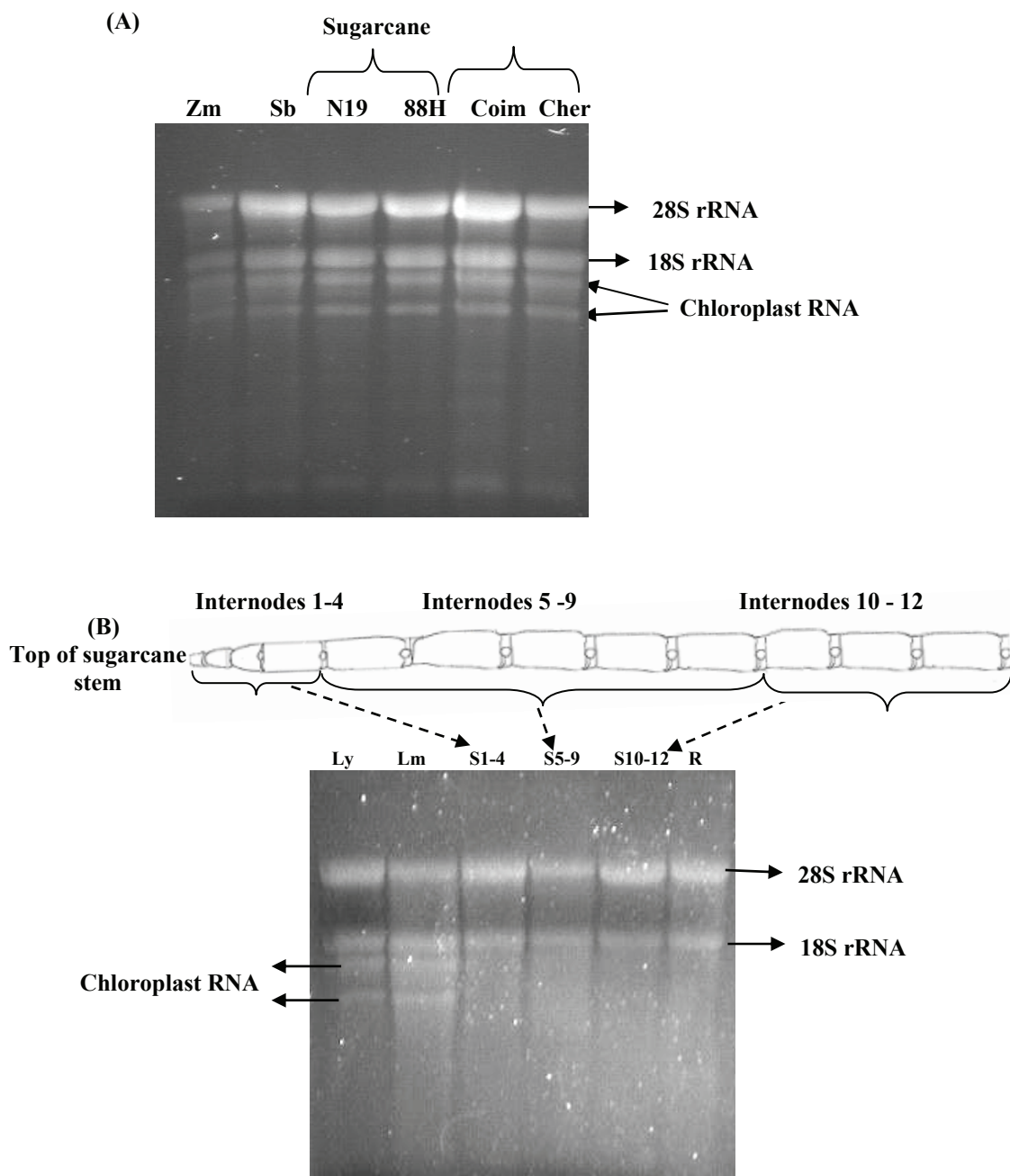
Figure 4: Agarose gel electrophoresis separation of typical total RNA (10 μg per lane). (A) Total RNA isolated from young leaves of maize (Zm), sorghum (Sb), N19, 88H0019 (88H), Coimbatore (Coim) and Black Cheribon (Cher). (B) Total RNA isolated from young (Ly) and mature (Lm) leaves, immature (S1-4), maturing (S5-9) and mature (S10-12) stems and roots (R) of sugarcane. The total RNA was separated electrophoretically on a 1.2% agarose gel and visualized using ethidium bromide staining.

The respective ribosomal bands were sharp with no smearing observed on the stained gel. This was indicative of high quality RNA undergoing no degradation during preparation. The banding pattern of the ribosomal bands showed a typical profile for RNA isolated from stems and roots with the 28S rRNA and 18S rRNA subunit. Leaf RNA showed a typical banding pattern which included the 28S rRNA and 18S rRNA subunit and the additional chloroplast RNA bands.

4.2.2 Northern analysis

In order to confirm the expression pattern of the SS gene sequence, Northern blot analysis was carried out. Figure 5 shows results of Northern analysis demonstrating the hybridisation of the sorghum SS gene sequence to total RNA isolated from immature, maturing and mature stems of maize, sorghum, N19, 88H0019, Black Cheribon and Coimbatore.

The results show that the SS gene sequence was not expressed in the leaves of maize, sorghum or the sugarcane cultivars. The prominent expression of the SS gene is observed only in the stems of the sugarcane hybrids N19 and 88H0019. Sorghum also shows high expression levels of the SS gene in the stems only. This therefore confirms the tissue specific expression pattern of this gene. The patterns of expression in the two ancestral *Saccharum* species vary greatly from each other, even though they are largely stem specific. Black Cheribon shows SS gene expression only in the young stems while Coimbatore shows expression in all the stem tissues as well as in the roots.

The expression of this gene other than in the stems was only observed in the roots of maize and Coimbatore but to a lesser degree in maize. The results from this Northern analysis provided a foundation substantiating the use of this sorghum gene sequence showing stem-specific expression for the isolation of corresponding stem-specific promoters from maize and sorghum. Furthermore, is shows the presence of homologous sequences in the tested plants.
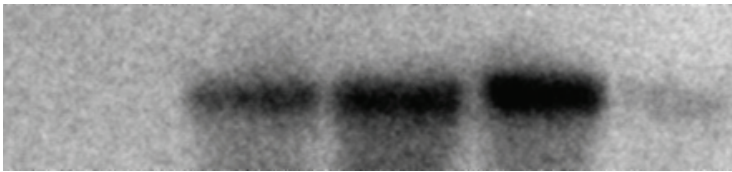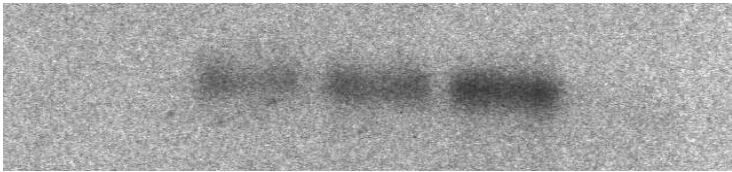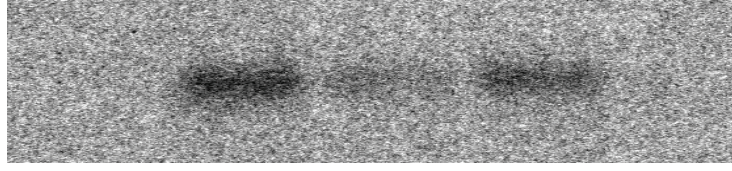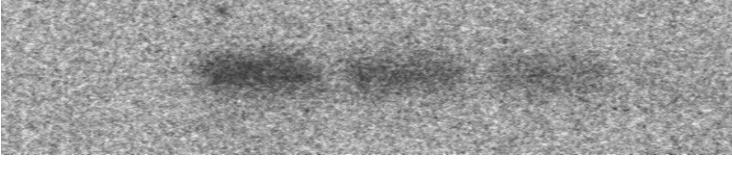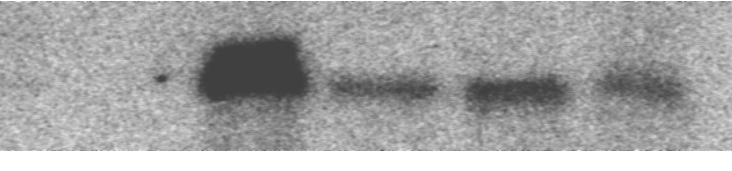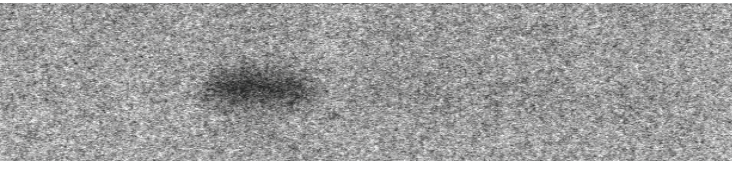
LY	LM	S1-4	S5-9	S10-12	R	Plant Source

Figure 5: Northern blots of total RNA illustrating the tissue-specific expression pattern of the SS gene sequence in maize, sorghum, N19, 88H0019, Coimbatore and Black Cheribon. Lanes contained total RNA (10μg) from young (Ly) and mature (Lm) leaves, immature (S1-4), maturing (S5-9) and mature (S10-12) stems and roots (R) of maize, sorghum and four sugarcane cultivars, N19, 88H0019, Coimbatore and Black Cheribon. The isolated RNA was denatured and electrophoretically separated on a 1.2% agarose gel followed by Northern blotting using the radioactively labeled SS gene sequence as the probe.

4.3 SS gene copy number in sugarcane, maize and sorghum

4.3.1 Quality of isolated DNA

The isolation of high quality DNA from the plant material was essential for the preparation of Southern blots. Figure 6 shows the quality and purity of genomic DNA isolated from maize, sorghum, N19, 88H0019, Coimbatore and Black Cheribon. The genomic DNA was observed as a sharp distinct band of high molecular weight with no smearing. This was indicative of minimal degradation. Quantities of isolated genomic DNA were measured by spectrophotometric analysis. Total yield of DNA from 5 g plant material was in the range of 450 and 750 µg.
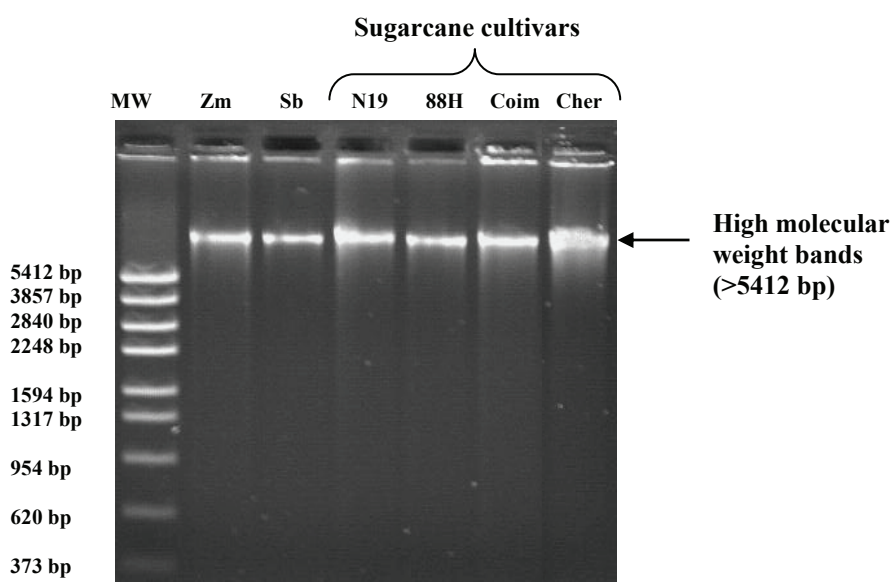
Figure 6: **Agarose gel electrophoretic analysis of genomic DNA**. Genomic DNA from *Zea mays (Zm), Sorghum bicolor* (Sb), N19, 88H0019, Coimbatore (Coim) and Black Cheribon (Cher) were analysed on a 1% agarose gel followed by visualisation by ethidium bromide staining. The size range of the molecular weight marker used (MW) is shown on the left.

4.3.2 Genomic Southern blot analysis

Genomic Southern blot analysis was performed to investigate the copy number of the SS gene sequence in maize, sorghum and the four sugarcane cultivars (N 19, 88H0019, Coimbatore and Black Cheribon). This was significant in assessing the SS genes suitability for isolating corresponding promoters (Section 4.1).

Four six-base cutting enzymes were used to restrict genomic DNA to produce a number of fragments. The choice of enzymes was based on availability and their moderate cutting nature. The enzymes selected were *Eco*RI, *Hin*dIII, *Pst*I and *Xba*I. The use of different enzymes in this study would provide different banding profiles for each of the DNA samples. It was argued that small fragments would be more likely to represent single gene copies as compared to large fragments which could represent multiple gene copies.

Figure 7 shows a Southern blot prepared using restricted genomic DNA from maize, sorghum, N19, 88H0019, Coimbatore and Black Cheribon. Analysis of the Southern blot was carried out by visually counting the number of restricted genomic fragments hybridising to the SS gene sequence to determine the number of hybridising bands (Table 3). The signal intensities of some of these bands were low; these may therefore contain only a small stretch of the SS gene sequence. In such cases the bands were disregarded.

The results obtained from the genomic Southern blot show the successful radioactive labelling of the SS gene sequence. There are distinct hybridising bands of both high and low molecular weights observed for sorghum and the four sugarcane cultivars thus enabling profile comparisons. As expected, each enzyme used in this study gave a different banding profile but some almost identical profiles can be seen across the different genomes for a particular enzyme. Good examples are shown by N19, 88H0019 and Black Cheribon restricted with *Pst*I and *EcoR*I respectively (Figure 7).

The restriction enzyme *Hin*dIII produced ambiguous results for the different plant samples and the profiles obtained were not informative to this study. The results from the *Hin*dIII restrictions were therefore excluded from the analysis.

The banding profile obtained for maize shows distinct bands of low molecular weight; however the high molecular weight bands are smeared. Due to this smearing the precise number of bands cannot be interpreted. The banding profiles for the ancestral sugarcane cultivars are very different from each other. Coimbatore has distinct hybridising bands both of high and low molecular weight whereas Black Cheribon has mainly high molecular weight bands. This result was in keeping with the fact that they are different species of *Saccharum.*

Bearing in mind the polyploid nature of the sugarcane genome, the number of hybridising bands observed from the genomic Southern blot is very low. This provides strong evidence that the SS gene has a low copy number in the four sugarcane cultivars and sorghum.
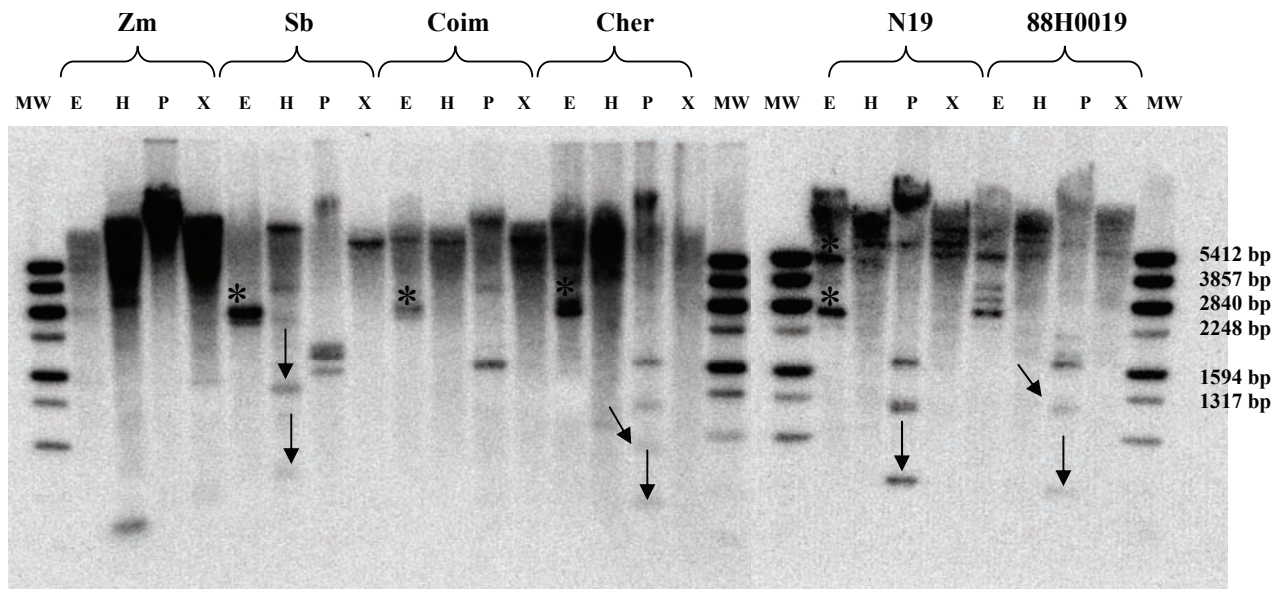
Figure 7: Southern blot analysis of *Zea mays* (Zm), *Sorghum bicolor* (Sb), Coimbatore (Coim), Black Cheribon (Cher), N19 and 88H0019 (88H) genomic DNA to determine copy number of the SS gene. Ten micrograms of genomic DNA from young leaves were digested with *Eco*RI (E), *Hin*dIII *(*H*)*, *Pst*I (P) and *Xba*I (X) and separated electrophoretically on a 1% agarose gel; thereafter the DNA was blotted onto a membrane for hybridisation analysis using the radioactively labeled SS gene sequence as the probe. MW = known molecular weight marker EL. Prominent bands are marked on the autoradiograph by asterisks and less prominent bands by arrows.

Table 3: The number of hybridising bands following Southern blot analysis using visual assessment for *Zea mays*, *Sorghum bicolor*, N19, 88H0019, Coimbatore and Black Cheribon after restriction with *Eco*RI (E), *Hin*dIII (H), *Pst*I (P) and *Xba*I (X).

		Zea mays	*Sorghum bicolor*	Coimbatore	Black Cheribon	N19	88H0019
E	*Eco*RI	-	2	3	5	4	4
P	*Pst*I	-	3	3	6	5	6
X	*Xba*I	-	1	-	-	3	4

(-) – Results were not included due to smeared bands

The number of individual hybridising bands obtained for the different restriction enzymes on the Southern blot does not necessarily correspond to the number of gene copies. Prominent or high molecular weight hybridising bands on the Southern blot may contain more than one copy of the SS gene sequence. Such examples include Sorghum, Coimbatore, Black Cheribon and N19 restricted with *EcoR*I (marked with asterisks) (Figure 7). Less prominent bands or low molecular weight bands on the Southern blot may be considered as less than one gene copy. Such examples are observed for Sorghum restricted with *Hind*III, N19 restricted with EcoRI, 88H0019 and Black Cheribon restricted with *Pst*I (marked with arrows) (Figure 7).

Overall the indications from this analysis show that the SS gene copy number is low in sorghum and in the four sugarcane cultivars. The comparison between sorghum and sugarcane shows that sorghum displayed a fewer number of hybridising bands on the Southern blots than across the four sugarcane cultivars. This makes sense since sugarcane is genetically more complex than sorghum.

4.4 Isolation of SS clones from the Lambda genomic library (maize)

A Lambda genomic library was used in this study to identify candidate clones encoding the SS gene sequence from maize. Insert DNA containing the SS gene sequence would be sub-cloned into a plasmid vector for restriction analysis and sequencing, thus enabling the localisation and characterisation of the corresponding upstream promoter region of this gene.

At the outset, the titre of the library was estimated since the number of clones to be screened needed to be at least 280 000 to ensure a good representation of the maize genome (calculation not shown). There maybe different copies of the SS gene sequence in maize (Results from Figure 7 and Table 3 are inconclusive) and it would be desirable to isolate these. An added consideration is that the maize genomic library is amplified and the same copy of the SS gene sequence may be isolated more than once. Therefore isolating a number of clones increase the chances of finding different copies of the SS gene sequence.

The Lambda library constructed in the Lambda FIX® II vector was screened using the 32 P- labeled SS gene sequence. Selected positive plaques were subjected to successive rounds of purification, re-infection and re-plating to ensure well separated plaques of single plaque purity.

The primary screening investigated a total of 280 000 plaque forming units (pfu) which were plated to produce a lawn of infected bacterial cells. Lysis was visible following 4-5 hours of incubation. Following lifts onto membranes for hybridisation analysis, a total of six signals was identified. Secondary and tertiary screenings were conducted using dilutions of phage to better obtain single plaque purity. The number of phages plated out for secondary and tertiary screenings was between 20 and 50 pfu thus ensuring well separated plaques on the agar plate for the isolation of individual plaques.

The six clones identified after screening the maize genomic library following the tertiary selection were named according to the membrane number they were located on i.e. 4, 5, 6b, 6d, 9a and 9b (Figure 8). These six clones were used for further analysis in this study.
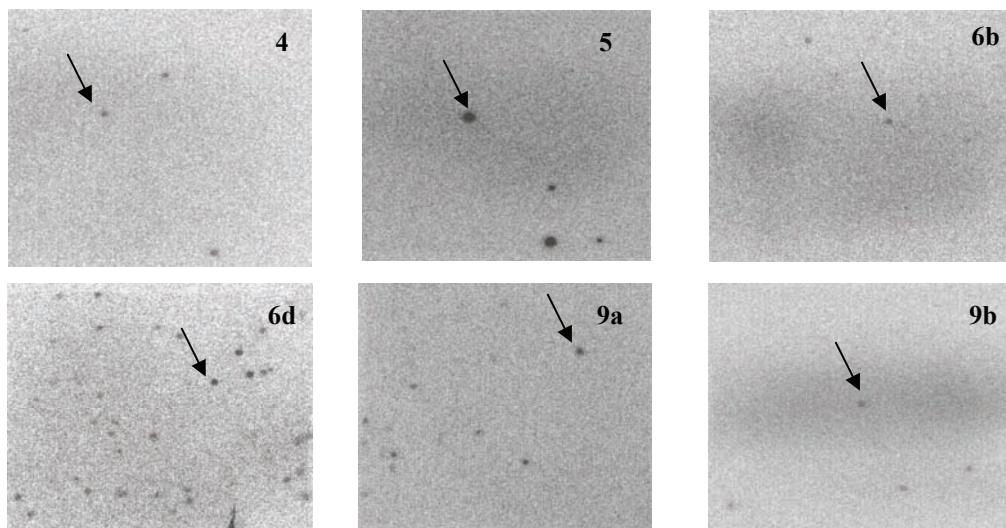
Figure 8: Autoradiograph of the tertiary selection of individual plaque forming units obtained from secondary screening using hybridisation analysis. The panels show the six stem-specific clones (4, 5, 6b, 6d, 9a and 9b) chosen from the maize Lambda library for further analysis. The arrows on the autoradiograph show the hybridising plaque forming units selected.

4.5 <u>Identification of SS clones from the Bacterial Artificial Chromosome genomic library (sorghum)</u>

The screening of a Bacterial Artificial Chromosome library was used to identify clones containing the SS gene sequence from the genome of sorghum. The isolation of candidate clones containing the SS gene sequence from the sorghum genome would allow for DNA fragments to be sub-cloned into a plasmid vector for sequencing and mapping thus enabling the localisation and characterisation of the corresponding promoter region of this gene. The BAC library purchased from the Clemson University contained 110 592 clones arrayed over 6 filters.

From the total 110 592 BAC clones on the membrane set, four were found to contain the SS gene sequence. An example is shown in Figure 9. These clones were allocated addresses and named with respect to their microtitre well location and position i.e. K1, E18, K24 and A11 (Table 4). These clones were ordered from Clemson University for further analysis in this study.
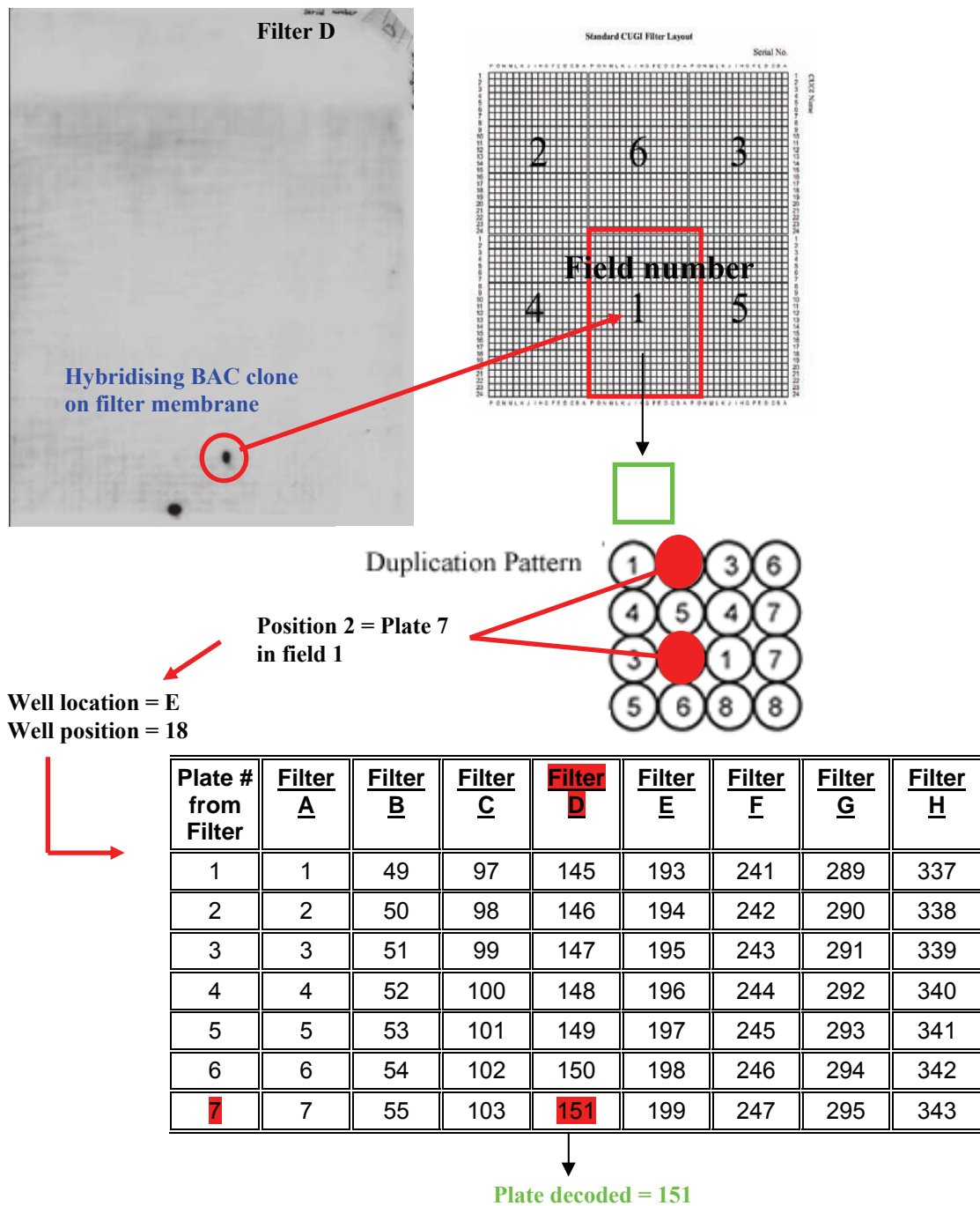
Figure 9: A hybridising BAC clone designated E18 obtained from screening filter D with the SS gene sequence. Clone E18 showed hybridisation on filter D and the layout of the grid was used to identify the field number 1. Within the respective fields, the well location and position were established to be E and 18 respectively. The plate number was identified as 7 by determining the orientation of the duplicate spots and then referring to the table for each field. This library has more than one filter therefore the plate number of clone E18 was decoded by using the plate number (7), well location (E18) and filter letter (D) from the conversion table and was found to be 151.

Table 4: The clones hybridising to the SS gene sequence obtained from the sorghum BAC library showing their filter letter, field number, well location and position, plate number and plate decoder number.

Filter Letter	Number of SS clones identified	Field number	Microtitre well location and position (Clone name)	Plate decoder number
A	0	-	-	-
B	1	1	K1	61
C	0	-	-	-
D	2	1	E18 and K24	151 and 163
E	0	-	-	-
F	1	4	A11	286

4.6 Analysis of library clones for SS fragments

4.6.1 Isolation of maize insert DNA from Lambda Library

The Lambda maize library clones contain inserts with sizes between 9 and 23 kb (Table 2). Isolated DNA from the six maize clones 4, 5, 6b, 6d, 9a and 9b (Figure 9) was restricted with various enzymes (*Hin*dIII, *Eco*RI, *Pst*I and *Xba*I). The restrictions produced numerous fragments for the maize clones (Figure 10). The purpose was to undertake Southern blotting to identify specific fragments containing the SS gene sequence in order to analyse and map the SS gene and its upstream promoter region.

4.6.2 Plasmid DNA isolation from sorghum clones

Plasmid DNA was isolated from the four sorghum clones obtained after screening the BAC library (A11, E18, K1 and K24) (Table 4) and as for the DNA from the maize clones, they were restricted with a range of enzymes to produce numerous small fragments (Figure 10)
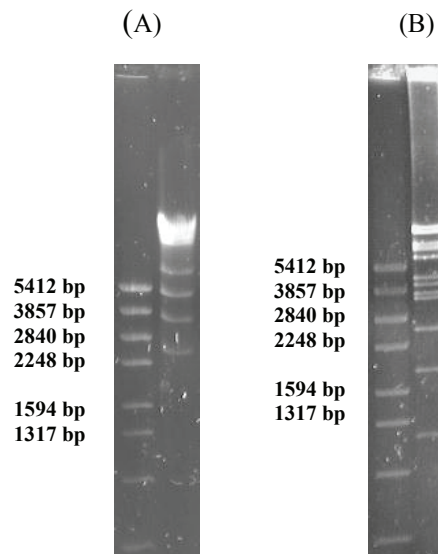
Figure 10: Agarose gel electrophoresis separation of isolated DNA from maize clone 4 and plasmid DNA from sorghum clone K24 restricted with *Xba*I (A) and *Eco*RV (B) respectively.

4.6.3 Southern analysis of maize and sorghum clones

The restricted DNA samples from the maize and sorghum clones were used in the preparation of Southern blots and screened using the SS gene sequence. The Southern blot of the restricted maize and sorghum clones show successful radioactive labelling of the SS gene sequence and restriction patterns for each clone (Figures 11 and 12). High signal fragments that were also considered to be of a suitable size for cloning were identified. A total of seven candidate maize fragments (a-g) and twelve sorghum candidate fragments (h-s) were chosen based on their hybridisation intensity and size. The selected fragments are marked on the autoradiographs with lowercase letters and summarised in Table 5.

The Southern blot of restricted maize DNA shows that the six clones display different hybridisation intensities from each other. For example restricted fragments from clone 5 show high signal intensity as compared to 6b and 6d. Restrictions of clone 6b with

*Xba*I, *Hind*III and *Pst*I produced either low or no signals on the blot. This suggests the possibility that the original isolates from the maize library were false positives. A similar hybridisation signal was observed with restricted fragments of clone 6d and 9a. By contrast with the maize clones, all four of the restricted sorghum clones give a strong hybridisation signal on the blots. This shows that correct addresses were allocated to the identified clones in the sorghum BAC library.
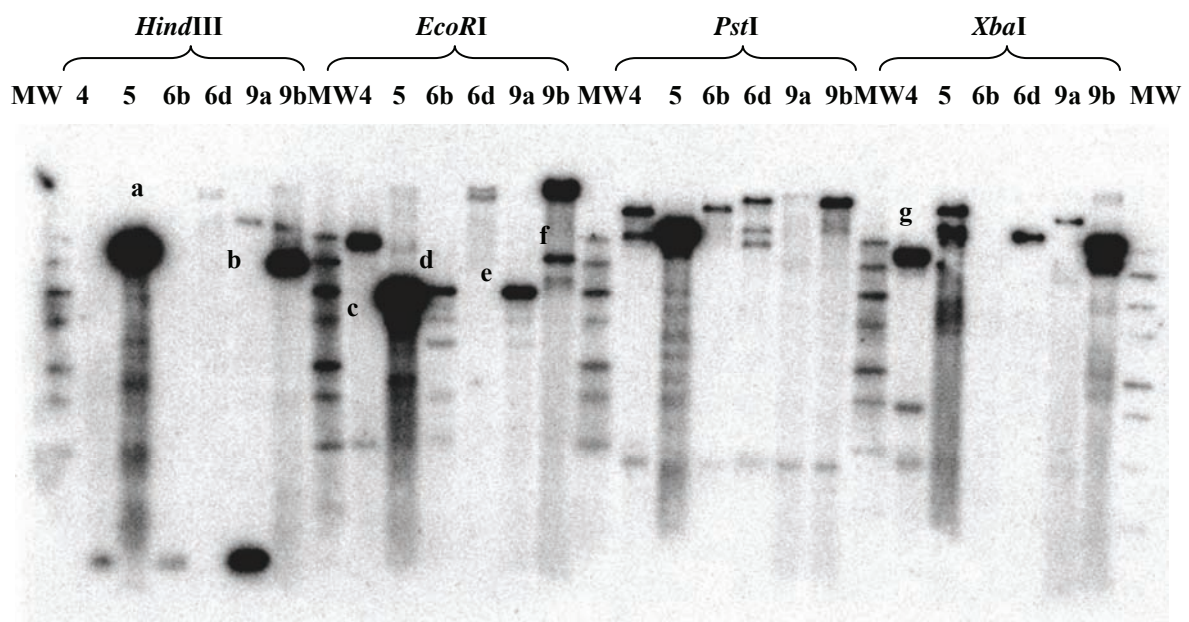
Figure 11: Southern blot analysis of restricted lambda genomic DNA (1 μg per lane) probed with the SS gene sequence. One microgram of Lambda genomic DNA from the maize clones 4, 5, 6b, 6d, 9a and 9b was restricted with *Hind*III, *Eco*RI, *Pst*I and *Xba*I respectively and electrophoresed on a 1% agarose gel. The DNA was denatured and blotted onto a nylon membrane for hybridisation analysis. The fragments containing the SS gene sequence and of a cloning size range are marked by letters on the autoradiograph. These included fragments 5 *Hin*dIII (a), 9b *Hin*dIII (b), 5 *Eco*RI *(c),* 6b *Eco*RI *(d),* 9a *Eco*RI *(e),* 9b *Eco*RI (f) and 4 *Xba*I *(g).*
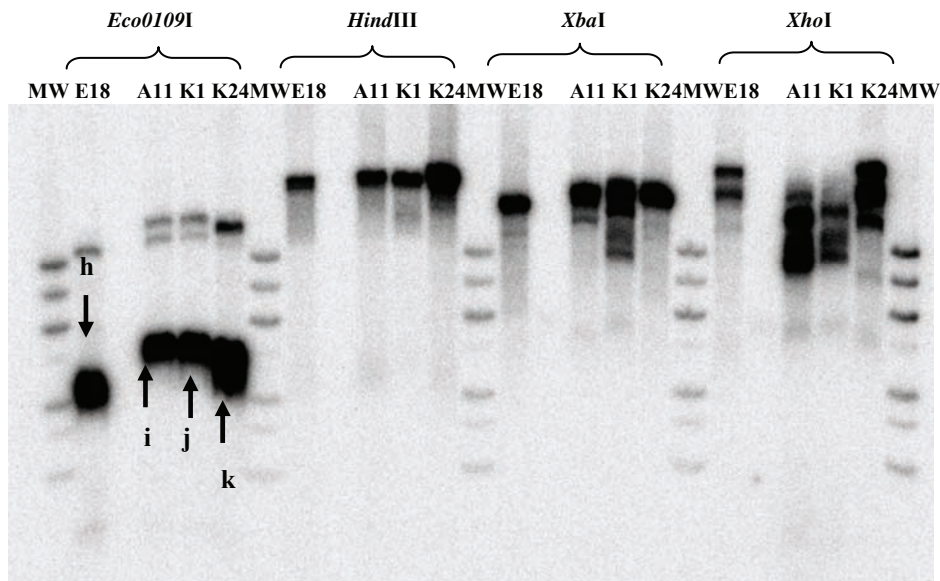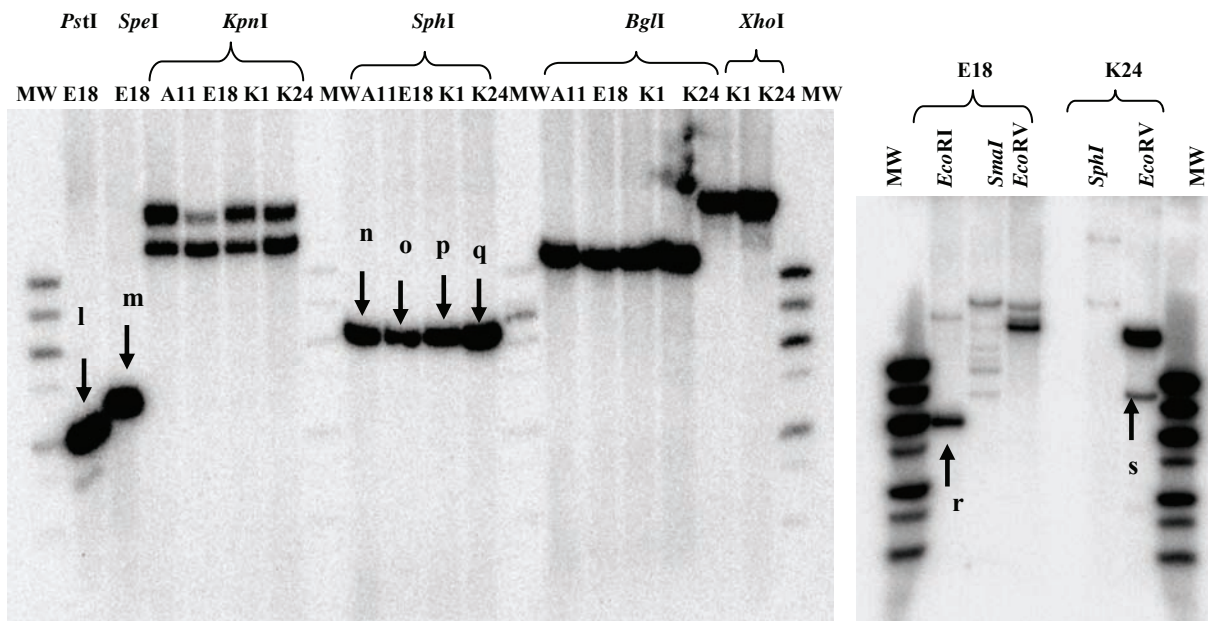
Figure 12: Southern blot analysis prepared with plasmid DNA from the sorghum clones restricted with a range of restriction enzymes. One microgram of plasmid DNA was restricted with a specific enzyme and electrophoresed on a 0.8% agarose gel. The DNA was denatured and blotted onto membranes for hybridisation analysis using the ^{32}P-labeled SS gene sequence as the probe. The blots were labelled A, B and C. Candidate DNA fragments h, i, j, k, l, m, n, o, p, q, r and s marked on the autoradiographs were selected for cloning and sequence analysis.

Table 5: Selected candidate DNA fragments from the maize and sorghum clones following Southern blot analysis (Figures 11 and 12). These clones were selected for sub-cloning into the pBluescript vector.

Maize												
Fragment	**a**	**b**	**c**	**d**	**e**	**f**	**g**					
Clone	5	9a	5	6b	9a	9b	4					
Restriction enzyme	*HindIII*	*HindIII*	*EcoRI*	*EcoRI*	*EcoRI*	*EcoRI*	*XbaI*					
Sorghum												
Fragment	**h**	**i**	**j**	**k**	**l**	**m**	**n**	**o**	**p**	**q**	**r**	**s**
Clone	E18	A11	K1	K24	E18	E18	A11	E18	K1	K24	E18	K24
Restriction enzyme	*Eco0109I*	*Eco0109I*	*Eco0109I*	*Eco0109I*	*PstI*	*SpeI*	*SphI*	*SphI*	*SphI*	*SphI*	*EcoRI*	*EcoRV*

4.7 Sub-cloning and physical mapping of SS fragments

4.7.1 Direct cloning of hybridising fragments into pBluescript

The principle of direct sub-cloning involves the cloning of a DNA fragment produced by restriction with one enzyme into a dephosphorylated vector restricted with the same enzyme. This strategy was used to clone the seven maize and twelve sorghum candidate fragments into pBluescript.

Candidate fragments were located on the gel, excised and the DNA purified. Direct cloning of these fragments into pBluescript was carried out. To amplify the plasmid DNA transformation of *E. coli* was carried out. Blue-white screening was implemented to identify colonies with plasmid and insert. Both blue and white colonies were obtained. The white colonies were investigated using restriction analysis of the isolated plasmid DNA but no inserts were found. No restricted DNA fragments from maize or sorghum were cloned using this strategy.

4.7.2 Forced cloning of hybridising fragments into pBluescript

Since the direct cloning of large fragments into the pBluescript vector proved to be difficult, the strategy of forced cloning was implemented. The principle involves restriction of fragments originally produced by one enzyme with a second enzyme in order to obtain fragments with different terminal ends. These fragments would be sub-cloned into a vector restricted with the same enzymes, where self-ligation was then impossible. The added advantage of using this approach was directional cloning.

An example of this strategy is shown in Figure 14 using fragment (a) originally obtained from restriction of maize clone 5 with *Hind*III (4400 bp) (Figure 11 and Table 5). Following trials with several restriction enzymes, *Eco*RI was found to produce a suitable number of fragments and molecular sizes. The electrophoretic separation of this digest on an agarose gel showed three fragments with molecular weights of 2200 bp (C), 1400 bp (B) and 750 bp (A). The two terminal fragments with *Hind*III and *Eco*RI sites on flanking ends were cloned into a plasmid vector restricted with the same restriction enzymes.

The maize clone 5 was the only clone sub-cloned using the forced cloning strategy. Figure 13 shows the sub-cloning strategy carried out for this clone.

4.7.3 Physical mapping of maize clone 5

The construction of a physical map for the maize clone 5 fragment (a) was based on the identification of restriction sites within the fragment. The map allows for the use of these restriction sites to obtain the complete sequence of the fragment (4.9).
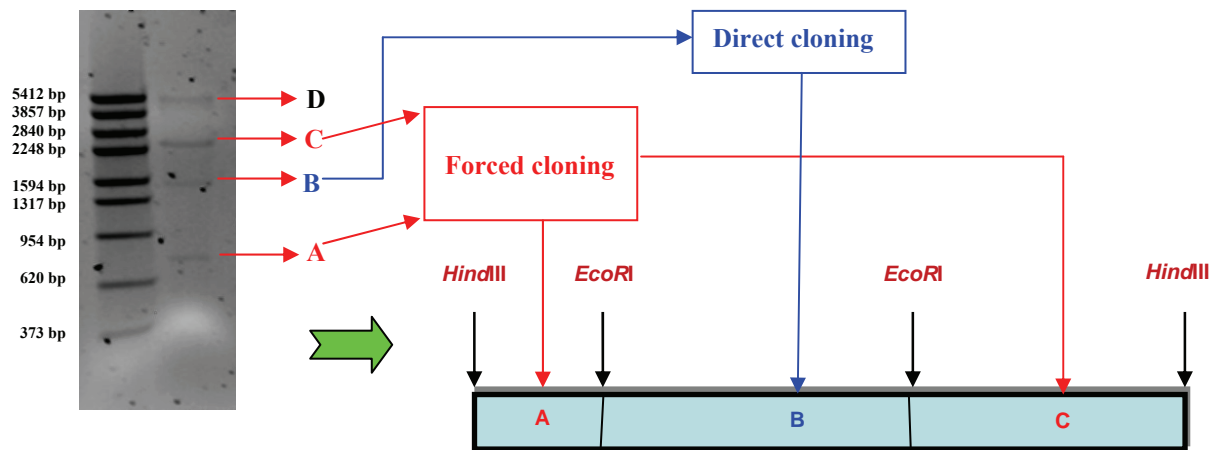
Electrophoretic separation of the 4400 bp maize genomic fragment restricted with *EcoR*I showed three fragments with molecular weights 2200 bp(C), 1400 bp (B) and 800 bp (A). The terminal fragments A and C have *Hind*III and *EcoR*I ends and B only *EcoR*I ends. Band D is undigested DNA.

Schematic representation showing the fragments produced following restriction with *EcoR*I. Terminal fragments A and C were cloned into a plasmid vector (pBS) restricted with the same restriction enzymes. To amplify the plasmid DNA transformation of *E.coli* was carried out.
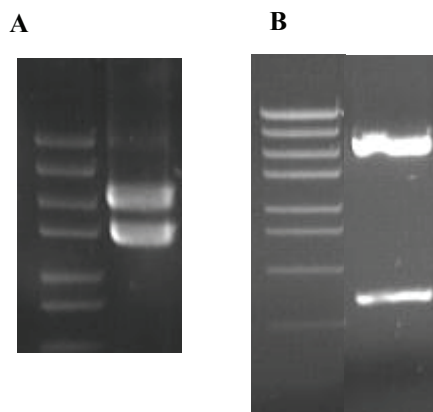
The isolated plasmid DNA was restricted with *Hind*III and *EcoR*I and electrophoretically separated on an agarose gel. The insert sizes of 2400 bp (A) and 800 bp (B) were confirmed.
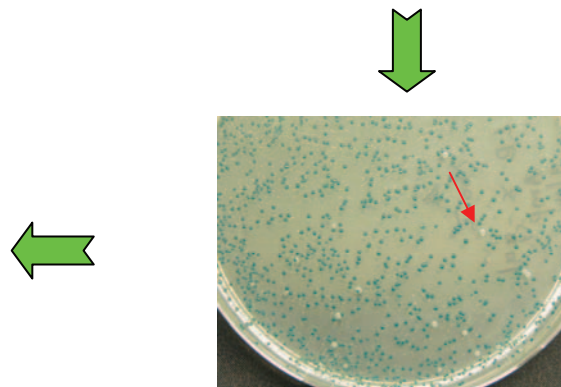
The blue-white screening technique was implemented as a preliminary selection to identify and isolate *E.coli* bacterial colonies successfully transformed with recombinant plasmid.

Construction of a physical map

Figure 13: Flow diagram showing the cloning strategy implemented for the maize clone 5 fragment to allow for the construction of a physical map.

The physical map of the clone was constructed by restricting the DNA fragments A, B and C with the enzymes *EcoR*I, *EcoR*V, *Eco0109*I, *Nhe*I, *Pvu*I, *Cla*I, *BamH*I, , *Sph*I and *Sma*I respectively. Of the ten enzymes tested only *Sph*I and *Sma*I proved to be useful since they cut only once within fragment C and again within the multiple cloning site of pBluescript. These restriction sites were identified and assembled on the map to produce a physical map (Figure 14). However, no useful restriction sites were found for fragments A and B. The restriction sites were assembled on the map using distances based on the sizes of the restriction fragments obtained from electrophoretic separation on an agarose gel.

Fragment B has been cloned using the direct cloning strategy; therefore the orientation of the fragment within the vector was unknown. Two possible models for assembly of the three fragments were proposed (Figure 14). Sequencing had to be carried out to determine the correct orientation of the fragment B and also to obtain the complete sequence in one piece.
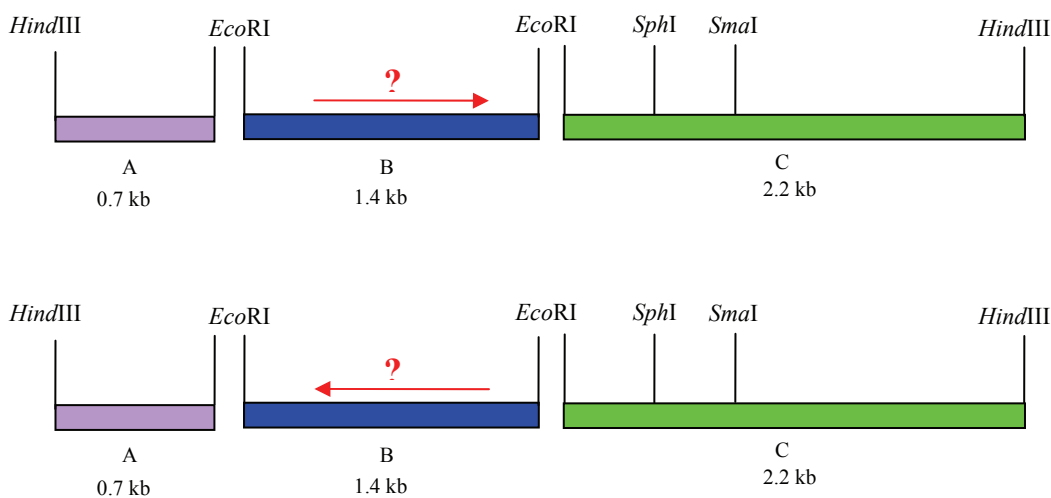
Figure 14: Physical map of the maize clone 5 divided into the three fragments A, B and C showing their assembly with respect to the orientation of the fragment B. The restriction sites *Sph*I and *Sma*I marked on the map cut once within the fragment C and cleave within the multi-cloning site of pBluescript.

4.8 Sequence of maize clone 5

The complete sequencing of the maize clone 5 fragment involved three approaches. The first strategy involved the use of the M13Forward (M13F) and M13Reverse (M13R) primers flanking the multiple cloning site in pBluescript. Sequencing with M13F and M13R primers gives 400-450 bp of reliable sequences. The second strategy was used for sequencing fragments greater than 800 bp by using restriction sites identified in the physical map to carry out re-ligations i.e. cleavage of sequenced stretches of DNA to sequence inaccessible regions in the truncated plasmid. The third strategy was used if no useful restriction sites were found within these sequences and involved the designing of primers at strategic positions by using known sequences of the fragment thus allowing for further stretches of DNA to be sequenced.

The flanking fragment A cloned in pBluescript was sequenced with M13F and M13R primers contained in the vector. Since fragment A was 0.7 kb in length, the entire region was sequenced.

The flanking fragment C was a large fragment (2.2 kb) and the use of the physical map was essential. The M13F and M13R primers were used to sequence the terminal ends of this fragment. The cleavage site *Sph*I within the fragment and *Spe*I from the multiple cloning site were used to cleave a 0.42 kb stretch of sequenced DNA to sequence the inaccessible region. The cleavage site *Sma*I within fragment C and in the multiple cloning site of pBluescript was used to cleave a 0.85 kb stretch to obtain the sequence for a central stretch of 0.78 kb.

Fragment B was sequenced firstly using the M13F and M13R primers. Since this fragment was 1.4 kb a central stretch of 0.4 kb could not be sequenced directly and suitable restriction sites were not available. Therefore primers R3EF and R3ER were designed from the known seqences in order to obtain the complete sequence. The sequencing strategy is summarised in Figure 15. The complete sequences for the three fragments were obtained (Appendix A).
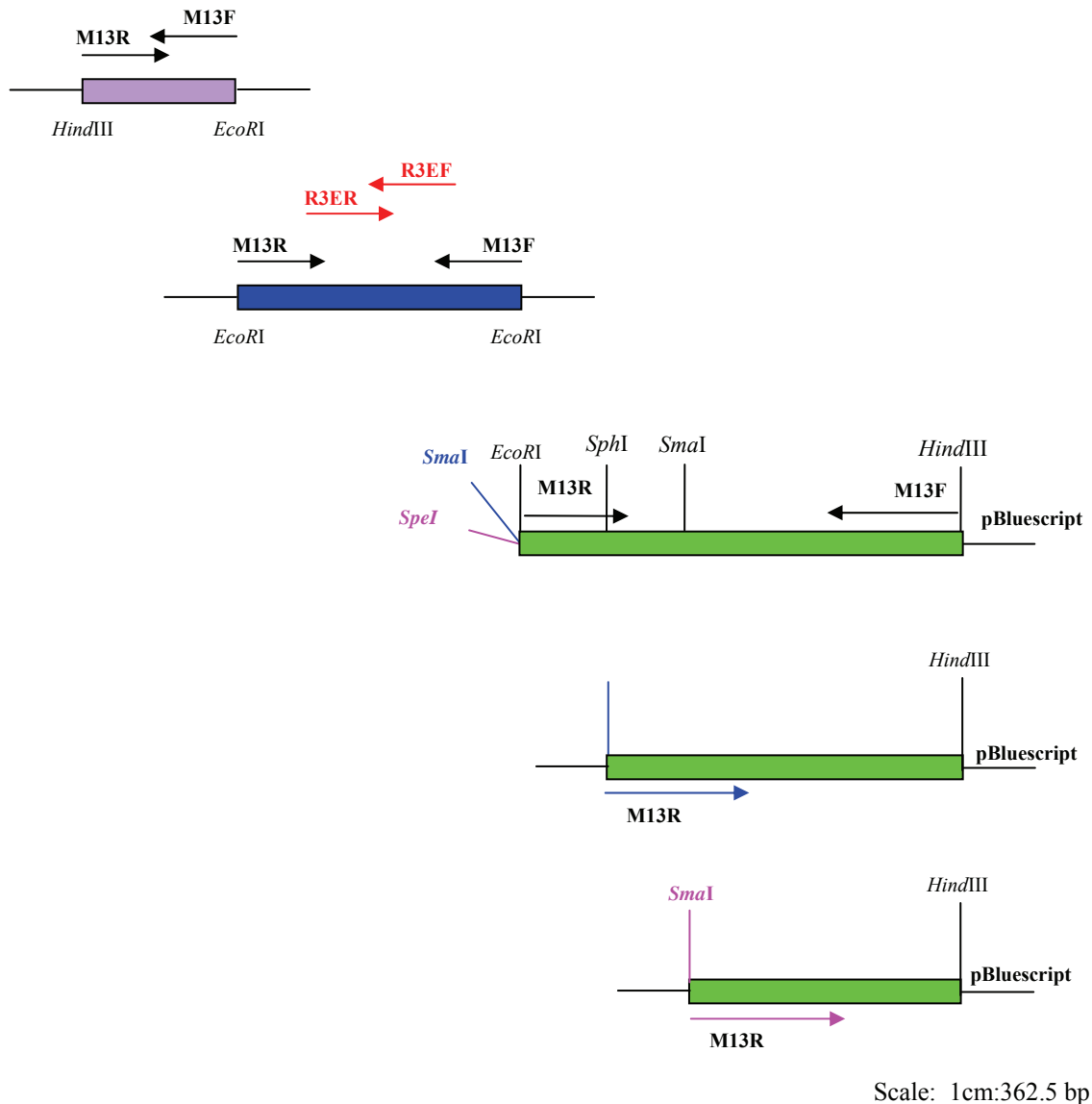
Figure 15: Schematic representation of the sequencing strategy implemented to obtain the complete sequences for fragment A, B and C in pBluescript for the maize clone 5. The direction of the arrows represents the direction and the sequence obtained following re-ligation using a particular enzyme.

The sequences obtained from each fragment were assembled into one piece for future analysis. BLAST searches were carried out with fragments A, B and C to determine their possible homology to known sequences on the NCBI database. Results for fragment C show 95% sequence similarity with the *Saccharum* hybrid mRNA (EST clone, Accession number AW746904). Fragment B revealed significant sequence similarity (87%) to *Saccharum* hybrid cultivar isolate c51 promoter region (Accession no. AY78 1895) while fragment A showed 91% sequence similarity to the *Zea mays* retrotransposon *cinful* (Accession no. AF0491 10). All three fragments showed

high sequence similarity to maize sequences thus confirming that all three fragments were from maize.

Based on the results from the BLAST search, fragment B was orientated to fragments A and C. The sequences from all three fragments were assembled in the order of retrotransposon, promoter region and coding region (Figure 16). The intron region was deduced by the stretch of missing DNA in the coding region. The lengths of the possible retrotransposon, promoter, intron and SS gene regions within the maize clone 5 fragment were found to be 325 bp, 2378 bp, 264 bp and 129 bp respectively (Appendix A).

Scale: 1cm:362.5 bp
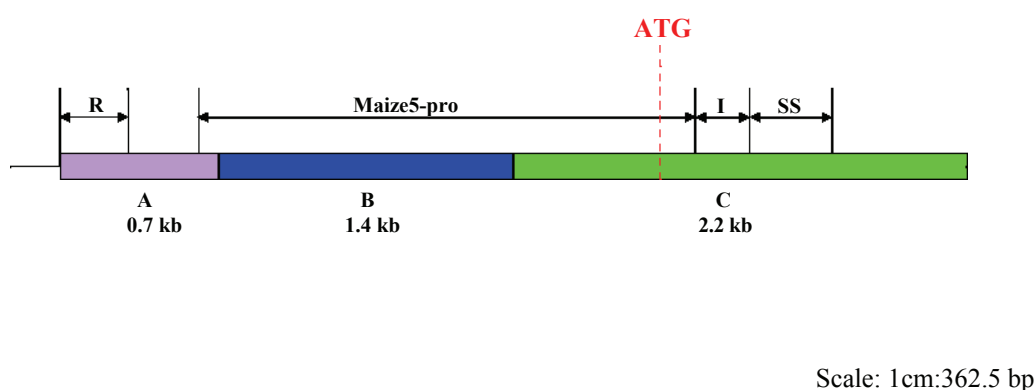
Figure 16: Schematic representation of the maize clone 5 showing the retrotransposon *cinful* (R), promoter region, intron (I) and the SS coding sequence (SS) in relation to fragments A, B and C.

4.9 Analysis of promoter region

For the purpose of promoter analysis, a stretch of DNA sequence from nucleotides 664- 3194 from the maize clone 5 sequence (Appendix A) was selected and designated the maize5-pro. A BLAST search of the deduced SS promoter sequence from maize clone 5 revealed significant homology to *Saccharum* hybrid cultivar isolate c5 1 promoter region (Accession no. AY78 1895). A sequence comparison was made between the SS gene promoter region from sugarcane (2947 bp in length) obtained from the NCBI database and the maize5-pro (2531 bp in length) (Figure 17). Implementing this comparison would help to understand the level of genetic conservation between the two promoters thus helping to provide more information on the usefulness of the maize promoter for driving transgene expression in sugarcane and the chances of promoter silencing.

```
Saccharum 1506 AAAAAAGATGCCATAAAATTGCTTGAAGCTCCTGGTAATAAGCAGCTGGTAAATAATTCCTTAAAACGAGAAAAGAAGAACCTCACTAATATCT 1599
Maize     1675 AAAAAAAT....TACAGTAGCAGCCCCCTCCTGATT.TCCAAAGAAAG.ACATAATTG.TTAAAACGAGAAAAGAAAAAAACTCGCGAATA.CT 1759

Saccharum 1600 CC.......TTTGCTTACCTTCA...GTTCA..TCAGCCAACGACGAGG...TAGGGTTCAGTATCATGATCCAATTAT...CCCATCGTGACA 1675
Maize     1760 CCATAGTATTTTGCGTACGTACGTTCGTCCAGTTCAGCCAACGACGAGGAGGTAGGGTTCAATATTATGACCCGATTATTATCCCATCGTGGCA 1853

Saccharum 1676 ....TAGCCTTGTCCTTGATGA....TTCGAGATGCAATTCTAATTCTCATC....ATATCATCGACTAGGTAACACAGAAAACAAAC.TTTTT 1756
Maize     1854 CATATAGCCTTGTCCTTGATGACTAATTCGAGATGCAATTCTAATTCTCACCGTGTATATAATCGACTAGGTAACACAGAAAACAAGCCTTTTT 1947

Saccharum 1757 TCTTCGTCAATT....GCACTGCA...........ACCGTTGCTTTTTG.TGATGTGCAGTTGTGCACCCACATCACAACAGCAC.TCAACACA 1833
Maize     1948 TCTTTGTCGATTAATCGCACTGGTCTGGACTCTGGACCGTTGCTTTTTGGTGACGTGCAGTTGTGCACCCATATCACAAAACAGCGTGTAAATA 2041

Saccharum 1834 TGCCACTTCAAAGTTCGAATCGACACC..AGAACTGACGGGAGAAAAGA...AAACAAATTAACAAAACTGTAGAATAGATCATCCAGTCATCC 1922
Maize     2042 GGCAACCTCGAAGTTCGAATCGACACCTCACACGTCACACCAGAAGGGACGCAAGAAAAGAAACAAAATTATACTATTATT..GCAATAGACC 2133

Saccharum 1923 AGCGTCCAAAAA....GTCCTGCTAGCTATAGATGCAACCTAATAACTTTGCTGACCTAGTCACTCCG...AATTCCAACATCACATCATCGTA 2009
Maize     2134 AGCGTCCAAAAAAAGGAGAGGTTAGCTATAGACTTAGC-TAATAACTTTGCTGATCTAGCCCCTCCGCCGAATTCCAAAACCACATGATAGTA 2226

Saccharum 2010 GTAGGCTCATTGTCATAGCATTCCTCAACAGCACTGTTAACAAGCAGATGCAAACAAGCAGATGCACATTCACCGGTCCCAATTGCACTGTTAA 2103
Maize     2227 GTAGGCTCAATGTCATA...........................CAGATACAGTGGAAAGGAGGCACATTCACCGGCCACAGCTAACCCGACAA 2293

Saccharum 2104 CCCGACAACCGCCTCTATTCCTGT......CACCTCTAATCA.......CGTACGAGCAAAGCA.....CTAATCAATC.TCTCTCCCCTGCCC 2178
Maize     2294 CCGGGCCCCCTGCTGTCTTCCTTTGCCGCGCGCGCCTCTAATAATCTCGTACGTACGACCAAATTAAAACGCTAATCAACAATCTCTTCCC.CGTA 2386

Saccharum 2179 TGTAACCAAAGTGCCGAA...TCTCTTCGCTGATCTGGCTGCTGCACCGCTGCAGCCTGCGAGGAGAGGTGGGGGTCGCTCATCAGTTAACTA 2269
Maize     2387 CGTAACCAAAGTGCCGAAATGTCCCCTCGCTGATTTTTAATTTG.......GC..........................TCA...ATTTAACTA 2445

Saccharum 2270 GTGACG...CCAAGGACGGCGTGCAACGTGCAGCAACAATGAGGCGCAGGAT.GCACACCTCACAA...TGCCCTGCACC.GCAATGAAGCCT. 2354
Maize     2446 CGGACGACGCGCAAGGACGGCGTGTACAGCGTGCAGCAACAATGAGGCGCAGCCCAGCGCAGCGAGCCAGCGTGCGACGCACATGCAATGAAGCCTC 2539

Saccharum 2355 .ACATCTCCCG......AAGGTACAC.......TTGTCTCCATGGACACACATGCTGGAACCTGGA.....GAGATGCATGCAAGCAAGCAGAC 2429
Maize     2540 TACAGCTCCGCTCCCAAAAGGTACGCGCGCTTGTTGTCTCCACGGACACACATGCTGGAGCCTGGAACCTGGAGAGAGAGATGCATGCAAAAA.AC 2632

Saccharum 2430 CACACATCA.TCGATCGGTCTGTGAACCTGTGATCACTTGGTGTTGGTGATCTATCGATCTCTCCCACAGATTCACGCACAGGGGCCTGCTGGG 2522
Maize     2633 CACACAGCAATCCATCGGTCTGTAATC....GACC.....GTCTCTCCCACAGATTCACCTCACCGCGCGCTGGTGCTGCGTGAAAGGACCAGG 2717

Saccharum 2523 TGAAAGAACCAAGACACCGTGCACAC....CGCCCCTTTTGGACCCTTTCCATGTGTCATGAC..ACGGT....CATGGGCACCTTTTGGTTGC 2606
Maize     2718 ACCAAGA.CCAAGACGCCGTGCACACACACCGCCCGCTTTGGACCCTTCCCATGTGTCACGCATGAGGGTGACTCATGGCCACCTTTTGGTTGC 2810

Saccharum 2607 ATTGCATGACATGTTCATGTGTGCTC..GTACAGCTCCTCAAGATTCCC......GATCATGATACCAGCGAC..........GACAGCTCTTT 2682
Maize     2811 ACTGCGTGGCATGTTCATGTGTGTACCTGTACAGCTCCTCAAGATTCCCAGTCATGATCATGATACGAGCGACAGATCAAGGGGCCGGGGATTT 2904

Saccharum 2683 ATTAGATCA.AGGGGGATTTTTAAAAAAAAAATC.....GGCAGAGTGCTAAGAAGCCCTTTTGTGTTCTCCCCATTCTGGCCTCGCCGC...C 2767
Maize     2905 AACAAAGAGAGAGAGAGAGAGAAAAAAACCCACCCGGCAGACATGCGCTTA.GCCCT..TGCGTTCTCCCCGTTCTGGCCTCGCCGCGCGCCC 2995

Saccharum 2768 GGGCCTC........TATATATAGCGCTCCCA.TCTCACCACCTT.GCTTCACACAAGCTCGAATGCTCGATCGATCCCACTCTCAGCTGATCG 2851
Maize     2996 GGTCTCTCCTCTTCTATATATAGCGTCCCGGGTCTCACGGCATTTGCCTCACACAACC....AAGCTCGATCGATCTCACTCTCAGCTGGTCG 3085

Saccharum 2852 CTCACTCTTGCAGCTCGA........TCAGTCTTAG........CTCTAGCCTCTAGCTAGCCAACTAGCCACTCCTTCGTGTAGCCATCAGCC 2929
Maize     3086 CTCACTCTTGCAGCTCCACCCTTCACTCAGTCTTAGGCTCTTAGCTCTAGGCTCTAGCTAGCCG...CGCCACTCCTTCGTGTAGCCATCAGCC 3176

Saccharum 2930 TTCTGATCGTCACCAATG 2947
Maize     3177 TTCTCATCGCCACCAATG 3194
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Figure 17: Alignment of nucleotide sequences of the 5' regulatory regions of the SS gene promoter from *Saccharum* (2947 bp) and maize (2947 bp). Significant sequence homology was observed between nucleotide 1675-3194 in maize (Appendix A) and nucleotide 1506-2947 in sugarcane. Homologous nucleotides are represented by shading. The transcription start sites (TSS) are in bold type and highlighted in bright green, the start codon (ATG) is in bold type and underlined and the TATA boxes are highlighted in yellow. The CAAT-like sequences similar in maize and sugarcane are highlighted in red, turquoise and grey. The sugarcane and maize5-pro promoter sequences were aligned and it was found that they share a 68% sequence similarity as shown by global alignments (BioEdit software). There are also numerous indels and substitutions between these promoters.

From this alignment, significant sequence similarity (68%) was observed between nucleotide 1675–3 194 in maize and nucleotide 1506-2947 in sugarcane (Figure 17). The maize and sugarcane promoter sequences were found to have identical TATA boxes (TATATATA) at positions 3011 and 2775 respectively and both were located 30 nucleotides from the TSS. Seven CAAT-like sequences were also identified

different distances from the TATA box for maize and sorghum (Figure 17). The consensus sequences of the CAAT-like sequences identified in the maize and sugarcane promoters were found to be different. In maize5-pro the CAAT-like sequences were positioned at nucleotides 1889, 2124, 2234, 2433 and 2528 (CAAT) and 2368 and 2479 (CAACAAT) upstream of the putative TATA-box. In sugarcane, the CAAT-like sequences were located at nucleotides 1657, 1703, 1764, 2089, 2160 and 2343 (CAAT) and 2368 (CAACAAT) upstream of the putative TATA-box (Figure 17). For both these promoter sequences, the distance between the putative CAAT-like boxes and the TSS is greater than the average of 80 bp as reported for several plant promoters by An and Kim (1993).

A detailed analysis of this promoter sequence revealed several promoter elements that are common in other plant promoters. The Neural Network Promoter Prediction (NNP2.1, Rees and Eeckman, 1995) tool was used to identify putative TATA-box elements and transcriptional start sites (TSS) for the maize and sugarcane SS gene promoter (Accession no. AY78 1895) (Table 6). These core elements are known to function as binding sites for Polymerase II, transcription factors and other proteins that are involved in the transcription initiation process.

Table 6: Putative core promoter elements in the upstream sequence of the SS coding sequence as predicted by the NNP2.1 tool[a]

Promoter sequence	Position of core promoter	Score	Promoter sequence[b]
Saccharum (Accession no. AY781895)	2775 - 2805	1.00	GCCGGGCCTC<u>TATATATA</u>GCGCTCCCATCTCACCACCTTG**C**TTCACACAA
Maize5-pro	3011 - 3041	1.00	TCTCCTCTTC<u>TATATATA</u>GCGTCCCGGGTCTCACGGCATT**T**GCCTCACAC

[a] Neural network promoter prediction.
[b] Predicted transcriptional start sites are indicated in bold type and the predicted TATA box sequences are underlined.

The promoter region sequence for maize revealed several important *cis* elements. The score obtained from the promoter prediction tool for the core promoter elements were 1.00 for both maize and sugarcane (Table 6). This shows the high predication accuracy for the core promoter elements and their location. The position of the core promoter element for maize was identified between positions 3011 and

3041 in relation to the transcriptional start site (TSS). To further confirm that the putative core promoter element identified from the NNP software program was correct, a BLAST search was carried out. The BLAST search used the region upstream from the ATG start codon to the predicted TSS to be compared with maize EST clones from the NCBI data base. The results obtained showed that no RNA sequence was found upstream of the deduced TSS site. This therefore confirmed the location of the TSS within the core promoter region.

The putative promoter, retrotransposon and intron regions were found to be AT-rich (56%, 55% and 55% respectively) as compared to the SS gene sequence (26%). Therefore, the AT-content is similar for the promoter, retrotransposon and intron regions while the SS coding region is much lower. The TATA box (TATATATA) for maize5- pro is located 30 bp upstream of the deduced transcriptional start site.

4.10 Transcription factors and regulatory functions of the putative promoter

The promoter region was analysed for the possible presence of *cis*-acting regulatory elements by comparison with known sequences using the Signal Scan Program, PLACE (Higo *et al.,* 1999). This analysis revealed a number of conserved DNA motifs upstream of the SS gene (Table 7).

Searches based on the use of PLACE software for the investigation of regulatory elements in the promoter region revealed a MYB-like recognition site (position 2005). Various other putative *cis*-acting elements were also identified in the maize5-pro involved in driving the expression of the SS gene (Table 7). These include various signal-responsive elements such as light- and phytohormone-responsive elements. The light responsive element identified was the GATA-box (positions 2220, 2246 and 2872) which is a characteristic cis-regulatory element of light-regulated promoters (Guilfoyle, 1997). The phytohormone-responsive elements identified include auxin (ABRE) (position 2000) and abscissic acid (ARFAT) (position 2574) responsive elements.

The AAAG-motif is a potential target site of the Dof class of DNA binding proteins. Dof proteins are transcription factors that are unique to plants and have been found to interact with a variety of promoters that drive photosynthetic genes, seed storage protein genes and genes responsive to plant hormones and/or stress signals (Yanagisawa and Schmidt, 1999). Thirteen AAAG-motifs were identified in the putative promoter region using the PLACE based searches.

The transcription factors between the maize5-pro and the sugarcane promoter were compared to determine the number of unique and similar regulatory elements (Figure 18). This would allow for the determination of the degree of similarity between two promoters driving the same gene in different plants.

The comparisons made between the putative transcription factors in maizepro-5 and the sugarcane promoter show that both promoter sequences are very similar as they share ten of the same transcription factors (Figure 18). These include the DOFCOREZM, GATAbox, ABRE, CACTFTPPCA1, ANAERO2CONSENSUS, ANAERO1CONSENSUS, CGACGOSAMY3, BOXCPSAS1, MYBCORE and ARFAT transcription factors. However, the transcriptional factors WBOX, SRE and SP8BFIBSP8BIB are unique to the maize5-pro and the TAAG motif to the sugarcane promoter.

The CACTFTPPCA1 and DOFCOREZM elements were the most numerous than any of the other elements in both maize5-pro and sugarcane. Seven DOFCOREZM elements were found in maize and four in sugarcane. This is not uncommon since this recognition site is unique to plants. Eight CACTFTPPCA1 recognition sites are found in sugarcane and four in maize; these are also found in the promoter driving the C4 phosphoenolpyruvate carboxylase gene (Gowik *et al.,* 2004).

Table 7: Putative regulatory elements present in the maize5-pro and their positions

Regulatory element[a]	Consensus sequence	Position[b]	Proposed function	Reference
GATA-box	GATAA	2220, 2246 and 2872	Cis-element for light and tissue specific responsiveness	Teakle *et al*., 2002
ABRE	ACGTG	2000	Abscisic acid responsive element	Simpson *et al*., 2003
ANAERO1CONSENSUS	AAACAA	1934	Found in promoters of anaerobic genes involved in the fermentative pathway	Mohanty *et al*., 2005
ANAERO2CONSENSUS	AGCAGC	1689	Found in promoters of anaerobic genes involved in the fermentative pathway	Mohanty *et al*., 2005
ARFAT	TGTCTC	2574	Binding site found in the promoters of auxin responsive genes of *Arabidopsis thaliana*	Ulmasov *et al*., 1997
BOXCPSAS1	CTCCCAC	2668	Box C found in pea asparagines synthetase (AS1) gene and binds with nuclear proteins	Ngai *et al*., 1997
CACTFTPPCA1	CACT	1965, 3070, 3088, 3109 and 3153	Cis-regulatory element for mesophyll-specific gene expression in the C4 plant *Flaveria trinervia*, the promoter of the C4 phosphoenolpyruvate carboxylase gene	Gowik *et al*., 2004
CCAATBOX1	CCAAT	1889, 2528 and 2479	Found in the promoter of heat shock protein genes and is located immediately upstream from the most distal HSE of the promoter. This element acts in cooperatively with HSEs to increase heat shock promoter activity	Rieping and Schoffl, 1992
CGACGOSAMY3	CGACG	2505 and 2876	May function as a coupling element for the G box element	Hwang *et al*., 1998
DOFCOREZM	AAAG	1710, 1714, 1738, 2098, 2256, 2556, 2707 and 2909	Dof core recognition sequence that are unique to plants	Yanagisawa and Schmidt, 1999
MYBCORE	CAGTTG	2005	Binding site for all animal MYB and at least two plant MYB proteins ATMYB1 and ATMYB2. ATMYB2 is involved in regulation of genes that are responsive to water stress in Arabidopsis	Urao *et al*., 1993
SP8BFIBSP8BIB	TACTATT	2113	A nuclear factor that binds to regions of three different genes encoding for major proteins of sweet potato tuberous roots	Ishiguro and Nakamura, 1992
SRE	TTATCC	1838	A sugar-repressive element found in down-regulated genes after main stem decapitation in Arabidopsis	Tatematsu *et al*., 2005
WBOX	TGACT	1873 and 2786	The SUSIBA2 binds to the WBOX element in barley isoamylase1 promoter and participates in sugar signalling	Sun *et al*., 2003

[a]Motifs were obtained from the PLACE database.
[b]Positions are relative to the TSS in the maize promoter sequence and the 5′ most nucleotide of the sequence is numbered.

The three regulatory elements unique to maize5-pro, the WBOX, SRE and SP8BFIBSP8BIB function in binding proteins involved in sugar signalling, binding of major proteins of sweet potato and as a sugar repressive element respectively. The function of the SS gene is unknown at this point; therefore a promoter with sugar

signalling elements may help to give an idea of the function this gene plays in the stem. The unique element in sugarcane (TAAG motif) is also found in *Solanum tuberosum* and is a target site for trans-acting StDof1 protein controlling guard cell-specific gene expression (Plesch *et al.,* 2001).

Figure 18: Alignment of nucleotide sequences of the 5' regulatory regions of the SS gene promoter from *Saccharum (*2947 bp) and maize (3194 bp) and their putative transcription factors identified using the Signal Scan Program, PLACE. Homologous nucleotides are represented by shading and indels by a dot (.). The transcription start sites (TSS) are underlined and highlighted in green, the start codon (ATG) is in bold type and underlined and the TATA boxes are in bold script and underlined.

4.11 <u>Construction of *GUS* transformation vector</u>

The known sequences from the flanking fragments (fragments A and C) (Section 4.8) were used to design primers (forward and reverse) for the amplification of the promoter with intron region (2.8 kb) (Appendix A). The primers had incorporated restriction sites, *SacII* and *Spe*I for their addition to the 5' and 3' ends of the promoter. The forward and reverse primers were designated ZM51F2 and ZM51R2 respectively (Figure 19). The forward primer was designed downstream from the retrotransposon. The amplified fragment was fused transcriptionally inframe to a β-glucuronidase (GUS) reporter gene in a transformation vector (pBScr-GUS000) for direct delivery into the plant. Figure 23 shows a schematic representation of the cloning strategy employed together with the respective primers designed.

The promoter with intron region was successfully amplified using the designed primers. This was verified by restriction and sequence analysis including BLASTN searches of the amplified product. The amplified fragment was fused transciptionally inframe to a β-glucuronidase (GUS) reporter gene in a transforamtion test vector (pBScr-GUS000) for direct delivery into the plant. The construct was designated GUS-maize5-pro. It was envisaged that this test construct would be used in the future to generate stable transformants in which the isolated promoter activity could be evaluated in sugarcane using both quantitative and qualitative analysis.
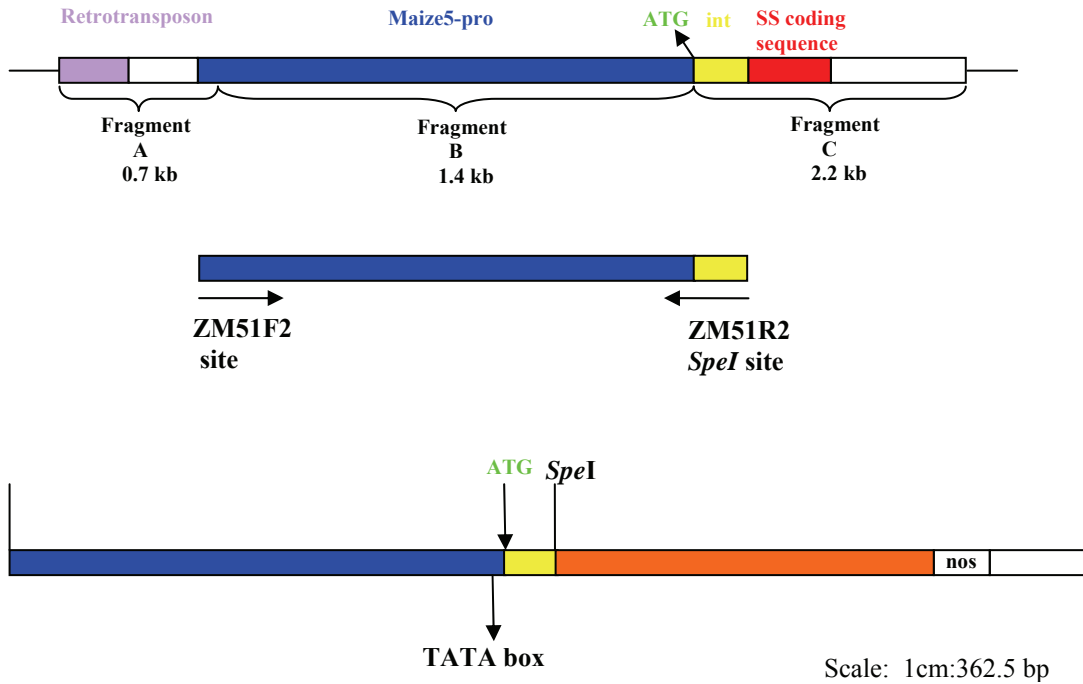
Figure 19: **Schematic representation showing the cloning strategy implemented for the maize5-pro with intron (int) region into the GUS test vector.** Restriction sites are marked on the complete maize clone 5 DNA fragment and the primers (ZM51F2 and ZM51R2) designed for the amplification of the promoter with intron region for the construction of the GUS test construct. The primers are shown below the arrows with their respective incorporated restriction site. The GUS test plasmid is represented as a linearised vector by restriction at the unique site showing the maize5-pro, *nos* terminator and GUS gene.

Chapter 5
Discussion and Conclusion

This project was aimed at isolating a stem-specific promoter; therefore it was essential to first identify a stem-specific gene. A partial gene sequence (EST clone, Accession number AW746904), named SS (stem-specific), was known from previous work to display a stem-specific expression pattern in sugarcane. This gene was selected for further analysis to determine if it could be a candidate for promoter recovery from sorghum or maize. To date the SS source gene has an undescribed function. This approach has been used by various researchers in the field as described in section 2.6.

The first phase of a project of this nature involves determining the level of expression and the expression pattern of a specific gene. For this purpose in the present study, RNA was isolated from leaves, immature, maturing and mature stems and root and used for Northern blot analyses. Implementing Northern analyses enabled the clear identification of the expression pattern of the SS gene sequence in maize, sorghum and sugarcane genotypes N19, 88H0019, Coimbatore and Black Cheribon. The results obtained confirmed the tissue-specific expression of the gene across the species under study and its suitability as candidate for the isolation of a stem-specific promoter. Although the function of this gene is still undescribed, it is interesting to speculate on its role in the stem, perhaps involvement with sucrose accumulation or other aspect of maturation.

In studies to determine the tissue-specific expression pattern of a gene, Northern blot analysis is not the only suitable method. More sensitive methods such as real-time PCR, *in situ* RNA hybridisation and microarrays can also be used. Work carried out by Jain *et al.* (2007) investigated the tissue-specific expression pattern of two phototropin genes *OsPHOT1* and *OsPHOT1* at the transcriptional level by performing real-time PCR with total RNA extracted from 6-day-old dark-grown and light-grown seedlings, shoots, roots, mature leaves, and flowers, using gene-specific primers. *In situ* hybridization was also used by Li *et al.* (1996) to investigate cell-specific expression of mitochondrial transcripts in maize seedlings. However, for this study Northern blot analysis was suitable to determine the expression pattern of the SS gene since it was

sensitive enough and the technology was available in the laboratory.

From the results obtained in the Northern analysis, the SS gene is expressed in the stem of the ancestral sugarcane cultivars; however, their expression patterns differ from those of the hybrid cultivars. Research carried out by Harvey and Botha (1996) indicated that there had been a gradual decline in DNA diversity (84% reduction) from the early inter-specific crosses to the commercial hybrids, probably as a result of backcrossing and inbreeding strategies used in the previous 5 to 6 generations of sugarcane breeding. Although there has been a gradual decline in DNA diversity, the ancestral and hybrid cultivars are still to some extent genetically diverse thus explaining the differences in expression of the SS gene. This information could be useful for future thought in isolating this promoter from ancestral sugarcane cultivars for driving transgene expression in the hybrid cultivars.

In the case of the two ancestral cultivars, Coimbatore and Black Cheribon, the SS gene was expressed down the length of the sugarcane stem and the roots for Coimbatore while Black Cheribon showed expression only in the young stems (internode 1-4). These ancestral cultivars are different species of *Saccharum* which could explain the differences obtained in their expression patterns. The expression of this gene in the roots of Coimbatore could suggest its function in nutrient uptake in the root cells, especially in root epidermis cells. This gene might therefore be involved with the phosphate or other category of transporter family, which takes up phosphate or other ions at the root-soil interface.

The second phase of this project involved the assessment of the gene copy number of the SS gene in maize, sorghum, N19, 88H0019, Coimbatore and Black Cheribon. The procedure for the isolation of a genomic clone can be complicated if there is more than one copy of the gene in the plant species. Therefore, it was necessary to determine the copy number of the gene by Southern blot analysis (An and Kim, 1993). Where a gene is present in multiple copies there are chances that most cloned copies of the genes may have nonfunctional promoters. In addition each functional copy of the gene may be assigned different levels of expression and how much of the total expression is assigned to each copy is unknown. Therefore there is

great uncertainty surrounding the levels of expression of the functional copies of such a promoter. To obtain the best estimate copy number, more than one restriction endonuclease was used to digest the plant DNA (An and Kim, 1993). The Southern blot data of the SS gene suggests that this gene is present at a low copy number in the genomes of these plants.

Confirmation of low copy number was also obtained following the screening of the sorghum BAC library with the SS gene. This particular sorghum BAC library has a 4x genome coverage and four clones were identified following the screening. This outcome could therefore suggest that the four clones are one and the same.

The approach to promoter isolation implemented in this project was to screen two large insert libraries i.e. the maize lambda and sorghum BAC library to identify clones containing the SS gene sequence. In identifying this gene sequence the corresponding promoter region can be located by sequence analysis. The availability of large-insert genomic libraries is crucial for genome analysis, physical mapping, genome sequencing, positional and map-based gene cloning, screening for specific genomic sequences in organisms with very large genomes and isolation of promoter regions (Woo *et al*., 1994, Budiman *et al.,* 2007; Saisho *et al.,* 2007). The BAC libraries have been constructed and used for genome analysis of many organisms, including crop plants. The main advantage of using BACs for genomic library construction is the stability of the large, very low or single-copy clones and this system has also proven to be an invaluable tool in stably maintaining large DNA fragments (Wild *et al.,* 2007).

The sorghum BAC library used for the purpose of this project was screened using the radioactively labelled SS gene sequence as the probe and four clones were identified. The same probe was used to probe the Lambda-based maize genomic library. A total of six clones were identified from this library. From this study the screening of the BAC library proved to be much simpler and less time consuming than that of the Lambda genomic library. The BAC library required a single screening with the radioactively labelled SS gene sequence for a pure clone to be identified for further analysis. The maize Lambda genomic library on the other hand required primary, secondary and tertiary screening procedures to be implemented in order to

obtain a pure clone thus proving to be more tedious and time consuming. This also suggested that this form of screening is more costly in terms of the amount of solutions prepared (including radioactivity), nylon filters used and the large number of false positives obtained.

The screening of recombinant Lambda phages by the traditional method yielded a large number of false positives. de Andrade *et al.* (2005) devised an approach to modify the original technique. This approach allowed for distinguishing, with great accuracy, a true positive spot from a false one using a single agar-plate and nylon filter. This modified technique was also applied to this project as explained in Chapter 3. It was agreed that this modification proved to be less tedious than the plating of several dilutions and had the obvious advantage of preventing the wastage of several expensive nylon filters. However, false positives were still obtained in this project following screening. This could be as a result of a short stretch of sequence from the SS gene hybridising to the clone sequence.

Maize became the main focus genomic resource for this project since more success was obtained with cloning the smaller fragments from the Lambda genomic library into a pBluescript vector as compared to the BAC library clones. The isolation of plasmid DNA for the BAC clones proved to be a challenge and due to the low yields of isolated plasmid DNA, cloning of these fragments into the pBluescript vector proved to be difficult. Hence, focus was placed on the maize genomic clones with respect to cloning and further characterisation of the SS promoter region in maize.

While the BAC library has many advantages over the Lambda genomic library the clone inserts obtained from the BAC library were large fragments and hence plasmid DNA recovery levels were low. This limited the amount of available plasmid DNA for use in the cloning procedure. Scaling up the experiment could have been a solution to extracting more plasmid DNA but it was decided that greater focus be placed on the Lambda library since it was not problematic. A similar problem was faced by Wild *et al.* (2007); however, in their study they solved the problem by constructing vectors that retain the advantages of single-copy stability of the BAC clones.

The clones obtained from the Lambda genomic library on the other hand proved to

yield high quantities of Lambda genomic DNA to carry out numerous cloning procedures. The fragments from this library were also within a reasonable size range and forced cloning was implemented for sequence analysis of the clones.

The next step in this study involved the characterisation of the isolated regions by DNA sequence analysis. A sequence comparison was made between the SS gene promoter region from sugarcane (2947 bp in length) obtained from the NCBI database and the maize5-pro (2531 bp in length). This was important in determining the level of sequence homology that existed between the maize and sugarcane promoter. This had a two-fold benefit, one involved the determination of the usefulness of this promoter for driving transgene expression in sugarcane and secondly the chances of promoter silencing when introduced into the sugarcane genome.

A sequence homology of 68% was observed between the two promoters following sequence analysis. This proved to be a valuable finding since it shows the close genetic relationship that is shared between maize and sugarcane. Although they are close monocotyledonous relatives they still show differences in their promoter sequences. The differences between the sequences involved numerous substitutions, deletions and differences in the types of transcription factors identified. This is significant to this project since gene silencing as a result of DNA-DNA pairing could pose a great problem in the use of this promoter for driving transgene expression in sugarcane.

Having this degree of sequence divergence could help to lower the chances of gene silencing when the maize5-pro is introduced into the sugarcane genome. In the nopaline synthase promoter-based system, the extent of promoter homology was about 300bp (Matzke and Matzke, 1993) and no gene silencing was observed. However, when Vaucheret (1993) investigated the 35Spro-based system silencing was observed even when promoter homology comprised only 90bp. Therefore 90bp is probably not enough for direct DNA-DNA pairing; it still does not eliminate the possibility of recognition of DNA sequence homology. Rossignol and Faugeron (1994) showed for direct DNA-DNA interaction, the homology between promoter sequences should exceed a minimum length of about 300 bp.

Following detailed sequence analysis of the promoter region obtained, several

promoter elements that are common in other plant promoters were identified. The TATA box sequences identified were identical and both were located 30 nucleotides from the TSS. For both the promoter sequences the distance between the putative CAAT-like boxes and the TSS was greater than the average of 80 bp reported for several plant promoters. The presence of a TATA box sequence categorises the SS gene promoter from maize as a TATA box- containing promoter as classified by Joshi (1987). The localisation of the TATA-box and TSS in the maize promoter is compatible with the results previously obtained by Joshi (1987) for 79 higher plants. It must be noted that the distance between the putative TATA-box and the TSS for this promoter (30 bp) falls in the range of 32± 7 bp as determined by Joshi (1987). Having identified the ATG, the length of the 5'UTR leader sequence was calculated using the distance between the transcriptional start site obtained from the NNP software program and the ATG start codon. According to Joshi (1987) the length of the leader sequence varies from 9 to 193 bp, which agrees with the 150 bp distance obtained for the maize promoter.

The promoter region was analysed for *cis*-regulatory elements, a number of conserved DNA motifs were observed. However, it must be noted that since transcription factor genes encoding proteins are short stretches of DNA sequences, there is a high probability of finding random sequences in the promoter which have no significance. The analysis of the promoter regions for both maize and sorghum showed a sharing of ten similar transcription factors (DOFCOREZM, GATA-box, ABRE, CACTFTPPCA1, ANAERO2CONSENSUS, ANAERO1CONSENSUS, CGACGOSAMY3, BOXCPSAS1, MYBCORE and ARFAT transcription factors). This further confirms that maize and sorghum share a degree of sequence homology due their close genetic relation.

Specific transcription factors, Dof proteins, are plant-specific transcription factors which interact with a wide variety of promoters of plant-specific genes in both monocotyledonous and dicotyledonous (maize, barley, *Arabidopsis,* tobacco and pumpkin) (Yanagisawa, 2000). Maize Dof1 was found to activate transcription from synthetic promoters to varying extents in greening and etiolated protoplasts. But of greater significance to this project is the findings of Yanagisawa (2000) in which Dof 1 regulates the activities of the cyppdk and dpepcZm2A promoters as well as the C4pepc

promoter in maize mesophyll protoplasts. Therefore, it is likely that Dof proteins may play regulatory roles in the co-ordinated expression of genes related to the plant-specific pathway for carbon metabolism. This could therefore provide insight to the function of the SS gene in the stems of maize and sugarcane since it is in the stems of sugarcane that sucrose accumulation takes place.

The MYB gene family represents one of the largest regulatory factor families in plants. One of the most important functions for MYB factor is to control development and determine cell fate and identity (Stracke *et al.,* 2001). Studies carried out by Gavnholt *et al.* (2002) showed that MYB recognition sites were also identified in the promoter region of LpLAC5-4 laccase from ryegrass which is involved in lignin polymerisation. Furthermore, MYB transcription factors are involved in regulation of signal transduction pathways responding to plant growth regulators as well as in the regulation of the branch of phenylpropanoid metabolism involved in lignin formation (Martin and Paz-Ares, 1997). MYB genes are thought to play a role in the lignification process since they are highly expressed in xylem tissues thus supporting the idea that this promoter drives a stem-specific gene. Although the function of this gene is unknown, it could play a role in the lignification process in the stem.

The presence of the MYB recognition sites, the plant-specific Dof motif, light responsive elements and phytohormone-responsive elements in the promoter sequence might play a role in the processes that occur in the stem tissues, thus providing a broad but inconclusive role for the SS gene in the sugarcane stem.

The final step in this project was the incorporation of the promoter region into a test construct by fusing the candidate promoter to the reporter gene GUS. This project set out to obtain GUS constructs, one with and one without the intron and from there make comparisons with respect to their influence on the level of gene expression in sugarcane. Introns are intervening sequences present in the pre-mRNA but absent in the mature RNA. Introns inserted inside the coding region of the reporter gene have been shown to enhance gene expression (Tanaka *et al.,* 1990). Further research by Rose (2004) showed that mRNA accumulation in transgenic plants declined with distance from

the promoter. In that work, measurements of GUS enzyme activity revealed that the intron elevated GUS activity. The findings supported the model in which introns increase transcription and promote translation. A GUS construct with the intron was obtained from this study.

The construct (GUS-maize5-pro), the main product of this study, is currently being used to generate stable transformants in which the isolated promoter activity will be evaluated in sugarcane. Transformation will be carried out by microprojectile bombardment of sugarcane embryogenic callus with the GUS-maize5-pro construct and subsequent selection and growth of transformants. Future analytical work will involve GUS assay analysis of various tissues of these transgenic sugarcane plants. Histochemical localisation of GUS-activity and GUS activity assays would determine the functionality and strength of this promoter (Nitz *et al.,* 2001, Rose, 2004). The results envisaged would be the expression of the GUS gene specifically in the stem tissues of sugarcane.

If the maize5-pro proves to be functional, tissue specific and stable in sugarcane, it will be of great significance to the transgenic programme at SASRI. It will help to target transgene expression in the stem tissue thus reducing the impact on non-target tissue. The isolated promoter may be used to manipulate certain aspects of metabolism and increase pathogen resistance in the stem of sugarcane. For instance, the manipulation of sucrose accumulation in the stem or successful control of stem borer will largely depend on the availability of promoters that are specific to or highly active in stem tissues. Therefore strong stem-specific promoters would be very valuable for crop improvement research. Manipulation of these crucial factors will be invaluable in enhancing the productivity and economic well-being of the South African sugar industry.

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Appendix A

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   1 AAGCTTAAGA AACTTTTTTC ACGGATCAAG CTCGTTACGA AAAACGATCT
  51 AGCACCGCGA AAGGGGCTAC TGTTGGGTCT ATGCTTCGTC GCCGAAGGTC
 101 TTATAGAAAG AAGTGATCCT CGGATGAAGC TGTTCGCACA AGATAGCCGA
 151 AGGTGCCTTT TCGCAGAGCT TCGGCATTAC AAACCGACTT AAAGATAGAA
 201 TGACCTTTTA GTCCATAAAG GTCTGAGTCA AACGTTGTAA GTTCTTATAA
 251 GGGGCATACT TGTAATTCCT CACAGGCTGC GTCCTGTGCC TATAAATAGT
 301 GAACAGTATT CCGTTACTGT TCACGCATTT TCTGACATTT TGCAATCGCA
 351 TTTCTCGGAA TACAACCTTT GTCAAGGCAT AGGTATCATT GTATTTTATG
 401 ATTCAATATA TTAAGTGAAT ATTATATAAT GCATCTGTGG ATCATTTATT
 451 CATTCTTACC TTTTACTTTG CATTATTCTA CAACGTTTAT TGAAAATTTA
 501 TTACGAAGGT TCAACTTCGT AATAAGACGC TTATCAACCT TCGTCTAAGA
 551 TCCATTATCC TCAAAGGTAT AATGCTTCGC GGACGAAGGA CAGTATCATT
 601 TAACATTCTA TGTTGCCTTG TTCTTAATTC ATAGCATTTG AGAACAAGTC
 651 CCCAACAGAG ATAGCCTGAT ATCTGAACTT TGGAAAGTGG TGGAATGTAC
 701 AATTCCAAAT TAAATAAGTT ACTTTATTGA GTGAATTCGC CCCGATAATT
 751 CCTTCAACAG TCTTGCTCCC AAACACGTCC TACAAGTTTC TTCATCCTTT
 801 GTGTCTTCTT TATGTCTTAC AACTGTTAGA TATGTATTAG ACGAAATCAA
 851 CCTCTCATGT TCTTATACTC ATATGAGTTC GATAAATACT TTATCCGTCT
 901 TAGTTTTTAA CACGTAACCA CTTTTAGTTC GAAGACCAAA AATTGTATGT
 951 AATTCTTTAT ATACTATTGC ATCGACATAT GCATGTCTAA AAAGAGAATA
1001 CACCTTATAT TATGACATTA GAATAAATAT ATAGTGGTTA TTCCTTATAT
1051 TAGGAGGACA GAGGGAGAAC ATATCCAAGG CACACTATGG AGTTCATGAT
1101 CGAAACACAT GATGACTGGG TGTGTTTGTT GGTTAATTAA ATAAAAGAAG
1151 AAATTTCCTT TCTTCAAATT TTGATCTGTC CCTTTCACTC CTGTCCATGT
1201 AACTGTCTGC CACTCCACTG AGACACAAGG ATAAGGATAA GAGGTATTAC
1251 CGTTTTAAAT GATGGATTAA ATACTTTCTC TGTTCTTTTT TATTTGTCGC
1301 GGTTTAGTTA AAAATGAACT AGCTTACGAT AAATATTGGA GAACGGAAGT
1351 AGTATAATTT ATGGCTTTGT CATTGTCGAA CTCAAGGCCG CACCAATATG
1401 ATTGAACTAC GGTAAAGTGT TCAATTTCAA GATATATTAG AGCCAATGAC
1451 AGAAATTATA TATTCTCAAG GAAAATATTT GCTAATAACA ACAACGACGA
1501 CAACAACAAC AACAATAATA ATAATAAAAG GATGTCATAA AATTACTTGT
1551 TGAAGCTCCT CCTATTAAGC AGCTAGTGCA TTTGCGTAGA TTTACTCTCC
1601 CTATTCATCA TCTATACTCT CTCATCAAGC TGTAACATAA TTCTCGCTAT
1651 ATCTATGTTC GAGCACCTTA ATATAAAAAA ATTACAGTAG CAGCCCCCTC
1701 CTGATTTCCA AAGAAAGACA TAATTGTTAA AACGAGAAAA GAAAAAACTC
1751 GCGAATACTC CATAGTATTT TGCGTACGTA CGTTCGTCCA GTTCAGCCAA
1801 CGACGAGGAG GTAGGGTTCA ATATTATGAC CCGATTATTA TCCCATCGTG
1851 GCACATATAG CCTTGTCCTT GATGACTAAT TCGAGATGCA ATTCTAATTC
1901 TCACCGTGTA TATAATCGAC TAGGTAACAC AGAAAACAAG CCTTTTTTCT
1951 TTGTCGATTA ATCGCACTGG TCTGGACTCT GGACCGTTGC TTTTTGGTGA
2001 CGTGCAGTTG TGCACCCATA TCACAAAACA GCGTGTAAAT AGGCAACCTC
2051 GAAGTTCGAA TCGACACCTC ACACGTCACA CCAGAAGGGA CGCAAGAAAA
2101 GAAACAAAAT TATACTATTA TTGCAATAGA TCCAGCGTCC AAAAAAAAGG
2151 AGAGGTTAGC TATAGACTTA GCTAATAACT TTGCTGATCT AGCCCCTCCG
2201 CCGAATTCCA AAACCACATG ATAGTAGTAG GCTCAATGTC ATACAGATAC
2251 AGTGGAAAGG AGGCACATTC ACCGGCCACA GCTAACCCGA CAACCGGGCC
2301 CCCTGCTGTC TTCCTTTGCC GCGCGCCTCT AATAATCTCG TACGTACGAC
2351 CAAATTAAAA CGCTAATCAA CAATCTCTTC CCCGTACGTA ACCAAAGCTG
2401 CCGAAATGTC CCCTCGCTGA TTTTTAATTT GGCTCAATTT AACTACGGAC
2451 GACGCCAAGG ACGGCGTACA GCGTGCAGCA ACAATGAGGC GCAGCCCAGC
2501 GCAGCGAGCC AGCGTGCGAC GCACATGCAA TGAAGCCTCT ACAGCTCCGC
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2551  TCCCAAAAGG  TACGCGCGCT  TGTTGTCTCC  ACGGACACAC  ATGCTGGAGC
2601  CTGGAACCTG  GAGAGAGATG  CATGCAAAAA  ACCACACAGC  AATCCATCGG
2651  TCTGTAATCG  ACCGTCTCTC  CCACAGATTC  ACCTCACGCG  CCGCTGGTGC
2701  TGCGTGAAAG  GACCAGGACC  AAGACCAAGA  CGCCGTGCAC  ACACACCGCC
2751  CGCTTTGGAC  CCTTCCCATG  TGTCACGCAT  GAGGGTGACT  CATGGCCACC
2801  TTTTGGTTGC  ACTGCGTGGC  ATGTTCATGT  GTGTACCTGT  ACAGCTCCTC
2851  AAGATTCCCA  GTCATGATCA  TGATACGAGC  GACAGATCAA  GGGGCCGGGG
2901  ATTTAACAAA  AGAGAGAGAG  AGAGAGAGAA  AAAAACCCA  CCCGGCAGAC
2951  TGCGCTTAGC  CCTTGCGTTC  TCCCCGTTCT  GGCCTCGCCG  CGCCCGGTCC
3001  TCTCCTCTTC  <mark>TATATATA</mark>GC  GTCCCGGGTC  TCACGGCATT  <mark>T</mark>GCCTCACAC
3051  AACCAAGCTC  GATCGATCTC  ACTCTCAGCT  GGTCGCTCAC  TCTTGCAGCT
3101  CCACCCTTCA  CTCAGTCTTA  GGCTCTTAGC  TCTAGGCTCT  AGCTAGCCGC
3151  GCCACTCCTT  CGTGTAGCCA  TCAGCCTTCT  CATCGCCACC  A**ATG**GCCACT
3201  GCCGAG*GTAG  CTCGATCGAT  CGACCCTAGT  CTTCGATTCA  TATATACATA*
3251  *CATACATGTG  CACGGATCGT  TTGGATCCGA  ATAATACCTA  TATATGAATA*
3301  *ATGATCTCTG  TCTCATCGAT  GATCGGTCGG  GTCAATCGCC  CATCTTCGAT*
3351  *GAGACGTACA  GCGATGGACG  TGCGGATCCT  ATGTTAACAA  ATGGAGGACG*
3401  *CTAAGATTAA  GTGTGGCGCT  AAGATGTAGG  AGTATATGCT  TTGATTTTTA*
3451  *CGCGCCGTTC  ACGTGTGCAG  GTCCAGACCC  CGACCGTCGT  GGCGGCCGAA*
3501  *GAAGCGCCCG  TGGTGGAGAC  CCCGCCGCCG  GCCGTCGTGC  CCGAGGAGTC*
3551  *TGCCCCCGCC  GAG*GCTGAGC  TGAGCTGAGC  TGCTGCTCCT  GCTGCTCTGC
3601  TGCTCCCGAG  ACTTCGGCGG  CCGAGCAGCA  GCACCGCCGA  CCGGAGGCGC
3651  TGCTCTGCTG  GTGCAGGACC  GCGCGGCGAC  GCCAGCGAGG  CTGTGCTCTG
3701  CTGTCCAGAA  CTCCGCGCCG  TCCAGACCCC  GACCGTGGTG  GCGACCGAGG
3751  AAGCGCCGGT  GGTGGAGACC  CCGGCGCCGG  CCGTCGTGCC  CGAGGAGGCT
3801  GCCCCCGCCA  GAGGTTGGTT  GCTCCCGAAG  GAAGCTCGCC  GCGGACGAGC
3851  CCGGAGGCTC  CTGCTTCCTG  GCTGGTTCCC  CAAGAAGCCG  GCCGCCCGAG
3901  GCGGAGGCTG  CTGCTCCTGA  GGAGCCCGAG  AAGGCCAGCG  AGTGAGGCCG
3951  TGCGCACGCA  GCGGCGGCGG  CCAGGGCCCA  GGGGATCGGC  GTGCGCGCGT
4001  GGCGGGGTAG  TCGCTCGCGC  GCGTACATGG  CAGTATGACA  CGGGATTTTG
4051  CTGTTCAGTA  CGCTACGGTT  ACTGGCGTCT  CTCTCTGTGT  GTGTGTGTCG
4101  TACGTAGCCC  GGTACGTAGC  TAGCAGTGAC  TGTCGCGTGG  ACTTGTCCGG
4151  TGCGGTCTGC  GAGTGACGTG  GGCTAAATAA  AGTGTGCGTC  GGGTGGTAAT
4201  GGTGGTGGGG  CCCGGTGTCA  GTGGCGCGGT  GCCCCATGCC  TGCCGCGGCT
4251  GCAGCTTGTT  GTGTCTTGTG  TTGGTTGGTT  CCTGTTCTTT  CTGTCTATCT
4301  ATTCTCACGC  ATGGCAGTTT  GGGTATGGTA  TGGGTCGTCT  TCTTTGGTTA
4351  CGTTCGCTGC  ATTCTGTTTG  TGATGATATG  ATCTTCTTTA  TATGATAATA
4401  GAGCCAGTCG  TTCATCGGCA  AACCTGCAAA  GCTT
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Figure 20: Complete DNA sequence of the maize clone 5 showing the fragments A (between red arrows), B (between blue arrows) and C (between green arrows). The maize retrotransposon cinful,intron region and SS gene coding sequences are shown in italics, red text and green text respectively. The six CAAT-like sequences identified are highlighted in red, the TATA box in yellow and the transcriptional start site in green.