THE ANALYSIS AND REDUCTION OF STARCH IN SUGARCANE BY SILENCING ADP-GLUCOSE PYROPHOSPHORYLASE AND OVER-EXPRESSING β-AMYLASE

by

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Declaration

The experimental work in this thesis was supervised by Dr. J-H. Groenewald, and conducted in the Institute for Plant Biotechnology, at Stellenbosch University, South Africa. The results presented are original, and have not been submitted in any form to another university.

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in parts been submitted at any other university for a degree

Signed: …………………   Date: …………………

Abstract

Sugarcane is cultivated because of the high levels of sucrose it stores in its internodes. Starch metabolism has been a neglected aspect of sugarcane research despite the problems caused by it during sugarcane processing. Currently there is no information available on the starch content in different South African commercial sugarcane varieties. This project had two main aims of which the first was to determine the starch content in the internodal tissues of six commercial sugarcane varieties. The activities of ADP-Glucose Pyrophosphorylase (AGPase) and β-amylase were also determined. The second aim of the project was to manipulate starch metabolism in sugarcane using transgenesis. To achieve this, transformation vectors for the down-regulation of AGPase activity and over-expression of β-amylase activity were designed. These vectors were then used to transform sugarcane calli and the results were analysed in suspension cultures. Starch levels in sugarcane internodal tissue increased more than 4 times from young to mature internodes. There were also large differences between varieties. When mature tissues of different varieties were compared, their starch concentration varied between 0.18 and 0.51 mg g^{-1} FW, with the majority of the varieties having a starch concentration between 0.26 and 0.32 mg g^{-1} FW. NCo376's starch concentration was much lower than the rest at 0.18 mg g^{-1} FW and N19's was much higher at 0.51 mg. g^{-1} FW. There was also a very strong correlation between starch and sucrose concentration (R^2 = 0.53, p ≤ 0.01) which could be due to the fact that these metabolites are synthesized from the same hexose-phosphate pool. No correlation was evident between starch concentration and AGPase activity. This was true for correlations based on either tissue maturity or variety. β-amylase activity expressed on a protein basis was almost 5 times higher in the young internodes compared to mature

internodes, suggesting that carbon might be cycled through starch in these internodes. AGPase activity in the transgenic suspension cultures was reduced by between 0.14 and 0.54 of the activity of the wild type control. This reduction led to a reduction in starch concentration of between 0.38 and 0.47 times that of the wild type control. There was a significant correlation between the reduction in AGPase activity and the reduction in starch (R^2 = 0.58, p ≤ 0.05). β-amylase activity in the transgenic suspension cultures was increased to 1.5-2 times that of the wild type control. This led to a reduction in starch concentration of between 0.1 and 0.4 times that of the wild type control. Once again the increase in β-amylase activity could be correlated to the reduction in starch concentration of the transgenic suspension cultures (R^2 = 0.68, p ≤ 0.01). In both experiments there was no significant effect on sucrose concentration.
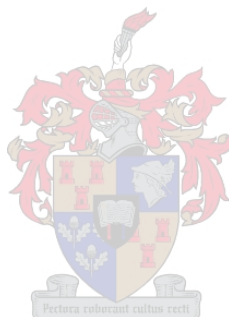
Opsomming

Suikerriet is 'n belangerike landbougewas omdat dit hoë vlakke van sukrose in die internodale weefsel kan berg. Navorsing op styselmetabolisme is 'n verwaarloosde aspek van suikerrietnavorsing ten spyte van die probleme wat dit veroorsaak in suikerriet prosesering. Daar is huidiglik geen inligiting beskikbaar oor die styselinhoud van kommersiële Suid-Afrikaanse suikerriet variëteite nie. Die projek het twee hoofdoelwitte gehad. Die eerste was om die styselinhoud van die internodale weefsel van ses kommersiële variëteite te meet. Die aktiwiteite van ADP-Glukose pirofosforilase (AGPase) en β-amilase is ook gemeet. Die tweede doelwit van die projek was om styselmetabolisme in suikerriet te verlaag deur middel van transgeniese tegnieke. Vir die doel is transformeringsvektore vir die afregulering van AGPase en die ooruitdrukking van β-amilase ontwerp. Die vektore is toe gebruik vir die transformering van suikerriet kalli en die resultate geanaliseer in suspensiekulture. Die styselvlakke in suikerriet internodes het meer as vier keer meer geword vanaf jong na volwasse internodes. Daar was ook groot variasie tussen die variëteite. Toe die volwasse weefsel van verskillende variëteite vergelyk was, het die styselvlakke tussen 0.18 en 0.51 mg g^{-1} vars massa gewissel met die meeste variëteite tussen 0.26 en 0.32 mg g^{-1} vars massa. Die styselkonsentrasie van NCo376 was veel laer op 0.18 mg g^{-1} vars massa en N19 was veel hoër op 0.51 mg g^{-1} vars massa. Daar was ook 'n sterk korrelasie tussen stysel en sukrose konsentrasie (R^2 = 0.53, p ≤ 0.01) wat moontlik verduidelik kan word deur die feit dat hierdie metaboliete gesintetiseer word uit dieselfde heksose-fosfaat poel. Daar was geen noemenswaardige korrelase tussen styselinhoud en AGPase aktiwiteit nie. Dit was die geval vir korrelasies gebaseer op beide die ouderdom van die weefsel en variëteit. β-amilase aktiwiteit uitgedruk in terme van die hoeveelheid protein was

bykans vyf keer hoër in die jong internodes as die volwasse internodes wat dui op moontlike sirkulering van stysel in die jong internodes. AGPase aktiwiteit in die transgeniese suspensiekulture was verlaag met tussen 0.14 en 0.54 keer die aktiwiteit van die wilde tipe kontrole. Hierdie verlaging het gelei tot die verlaging in styselkonsentrasie van tussen 0.38 en 0.47 keer die van die wilde tipe kontrole. Daar was 'n noemenswaardige korrelasie tussen die verlaging in AGPase aktiwiteit en die verlaging in stysel (R^2 = 0.58, p ≤ 0.05). β-amilase aktiwiteit was 1.5-2 keer verhoog in die transgeniese lyne in vergelyking met die wilde tipe kontrole. Dit het gelei tot 'n 0.1 tot 0.4 keer verlaging in styselvlakke. Daar was weereens 'n noemenswaardige korrelasie tussen die verhoging in β-amilase aktiwiteit en die velaging in styselkonsentrasie (R^2 = 0.68, p ≤ 0.01). Daar was in beide eksperimente geen noemenswaardige verandering in sukrose inhoud nie.
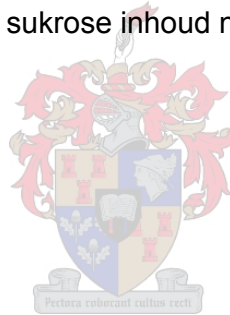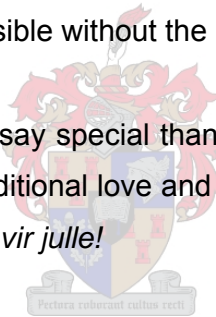
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Last but not least, I would like to say special thanks to my parents, Frans and Lucia Ferreira. Thanks for all the unconditional love and support over the years. This thesis is dedicated to you. *Ek is baie lief vir julle!*
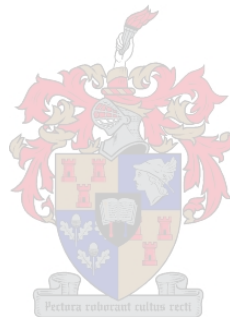
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List of abbreviations

2, 4 D	2,4-Dichlorophenoxyacetic acid
3-PGA	3-phosphoglycerate
ADP	Adenosine 5'-disphoshate
AGPase	Adenosine 5'-disphosphate - Glucose pyrophosphorylase
ATP	Adenosine 5'-triphosphate
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
DBE	Debranching enzyme
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E.C.	Enzyme Commission number
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
FW	Fresh wieght
g	Gravitational acceleration (9.806 m/s)
g	Gram
GBSSI	Granule bound starch synthase
gDNA	Genomic deoxyribonucleic acid
GWD	Glucan, water dikinase
h	Hour
IPB	Institute for Plant Biotechnology
ISA 3	Isoamylase 3
KOH	Potassium hydroxide
kPa	Kilopascal
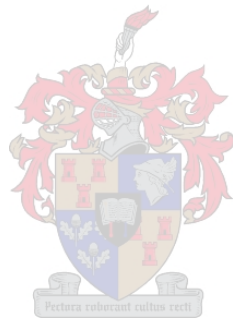
l	Liter
LiCl	Lithium chloride
M	Molar
mg	Milligram
$MgCl_2$	Magnesium Chloride
ml	Milliliter
mM	Millimolar
MOPS-KOH	3-(N-morpholino)propanesulfonic acid-potassium hydroxide
NA	Not available
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
ºC	Degrees celsius
OPP	Oxidative pentose phosphate
p	Phosphate
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Acidity
PPi	Pyrophosphate
PVP	Polyvinyl pyrrolidone
PVPP	Polyvinylpolypyrrolidone
PWD	Phospho, glucan dikinase
R^2	coefficient of determination
RNA	Ribonucleic acid
s	Second (time unit)
SADC	Southern African Development Community
SASA	South African Sugar Association

SASRI	South African Sugarcane Research Institute
SBEA	Starch branching enzyme A
SBEB	Starch branching enzyme B
SDS	Sodium dodecyl sulfate
SSI	Starch synthase I
SSIIa	Starch synthase IIa
SSIIb	Starch synthase Iib
SSIII	Starch synthase III
SuSy	Sucrose synthase
Tris-HCl	Tris(hydroxymethyl)-aminomethane
U	Units
UDP	Uridine disphosphate
USA	United States of America
v/v	Volume/ volume
w/v	Weight/ volume
Wx	Waxy
WI	Wisconsin

Chapter 1

General Introduction

General Introduction

Sugarcane is a C_4 grass cultivated for the production of sucrose in over 100 regions around the globe. It contributes 75% of the sucrose consumed annually and the Southern African Development Community (SADC), of which South African production accounts for approximately 50%, is the sixth largest exporter of sucrose in the world. Global sugar consumption has increased at about 2% per annum since 1960 and reached 150 million tons in 2005/06 (www.Illovo.co.za, 2006).

The South African sugarcane industry is one of the world's leading cost-effective producers of high quality sugar. The industry is diverse, including both agricultural activities and the production of raw sugar, refined sugar, syrups and a range of by-products. SADC produces an average of 2.5 million tons of sucrose per season. More than half of this is consumed within the SADC and the remainder is exported to markets in Africa, the Middle East, North America and Asia. The industry contributes an estimated average of R2 billion (2003 estimate) in South African foreign exchange earnings annually. The number of people directly and indirectly employed by the sugar industry is estimated at 350 000, which means that approximately one million people are dependent on the South African sugar industry (www.sasa.org.za, 2006).

For South Africa to remain internationally competitive, the continual development of new sugarcane varieties is essential. Traditional sugarcane breeding has been very successful in increasing the sucrose yield. Over the last half of the 20th century, traditional breeding techniques increased sucrose yield with 1 - 1.5% per year (Chapman, 1996). Since sugarcane is almost exclusively used for the production of

sucrose, research and selection and breeding programs have obviously focused on this aspect (Godshall et al., 1996).

Starch metabolism in sugarcane has largely been ignored and currently very little information is available on starch in commercially grown South African sugarcane. This is despite the fact that the problems caused by starch in sugar milling are well documented (Cuddihy et al., 1999; Godshall, 1996 and Schoonees, 2003). Minor polysaccharides in sugarcane, of which starch and dextran are the most important, lower the quality of sugarcane juice and raw sugar. During processing of sugarcane they increase viscosity and slow or inhibit crystallisation, which increases the loss of sucrose to molasses. This problem of starch in sugarcane is currently overcome by adding a bacterial α-amylase to the extraction process, which hydrolyse the starch before it can cause problems (Godshall et al., 1996).

Starch metabolism in general is of course divided into synthesis and degradation. As far as synthesis is concerned, the first three enzymes committed to starch synthesis have been widely researched and are ADP-Glucose Pyrophosphorylase (AGPase), Starch Synthase and the Starch Branching Enzymes (Kossmann and Lloyd, 2000). ADP-Glucose is the major substrate for starch biosynthesis in most plants (a small contribution is made by UDP-Glucose (Echeverria et al., 1988, Sasaki et al., 1980). Due to this, the enzyme responsible for producing ADP-Glucose from Glucose 6-phosphate, AGPase, is seen as a rate determining step (Tsai and Nelson, 1966; Hannah and Nelson, 1976; Lin et al., 1988a; 1988b). This heterotetrameric enzyme consists of two different subunits and the small subunit, seen as the catalytic subunit, has attracted most of the research.

The degradation of starch is a more complex process than synthesis and many enzymes seem to play a role. After the release of starch polymers from the starch granule, the α(1,6) "branch" linkages are degraded by die starch debranching enzymes (Manners, 1985). After this debranching, one of two enzymes is responsible for further degradation of the linear glucans. This is done by either glucan phosphorylase or β-amylase. It seems as though β-amylase, not glucan phoshorylase, is primarily responsible for the degradation of the linear glucans. The evidence for this is that while decreased activity of β-amylase does have an effect on the amount of starch degraded (Scheidig et al., 2002), mutants lacking glucan phosphorylase seems to have no effect (Zeeman et al., 2004b).

This project had two main aims. The first aim was to increase our knowledge of starch metabolism in sugarcane. This was done by determining the internodal starch content of commercial sugarcane varieties. The activities of two enzymes closely associated with starch metabolism, ADP-Glucose pyrophosphorylase and β-amylase were also determined. The second aim was to try and reduce the starch content of sugarcane. This was done by creating sugarcane transformation vectors for the reduction of AGPase activity and the over-expression of β-amylase activity. These vectors were then used for sugarcane transformation and the results analysed in suspension culture.

The layout of this thesis is as follows: Chapter two gives an overview of starch metabolism in plants and focus on starch in sugarcane. It also discuss the problems caused by starch during the sucrose extraction and refinement process and the possible negative effect it might have on carbon partitioning towards sucrose. The experimental work of this thesis is divided into two chapters. In chapter three the

starch and sucrose content of commercial South African sugarcane varieties were determined as well as the activities of AGPase and β-amylase. In chapter four the acitivities of AGPase and β-amylase were manipulated in an attempt to reduce starch. Two transformation constructs were designed, an AGPase silencing vector and a β-amylase over-expression vector. These vectors were then used for sugarcane transformation and the results analysed in suspension culture by measuring enzyme activities, starch content and soluble sugars content. Chapter five then gives a general discussion and conclusion of all the results.

Chapter 2

Literature Overview

Literature Overview

Sugarcane is one of only a few carbohydrate-storing plants that stores most of its carbohydrates in the form of sucrose rather than starch. For this reason, sugarcane is the world's major source of sucrose (Moore and Maretzki, 1997) and starch metabolism has been neglected in sugarcane agronomical research. The role of starch in sugarcane processing is, however, well researched. Polysaccharides, of which starch and dextran are the most important, cause various problems in sugar milling and thereby increase production costs and lower the quality of the raw sugar (for reviews on starch in sugarcane processing see Cuddihy et al., 1999; Godshall, 1996 and Schoonees, 2003). A penalty will usually be imposed on a sugarcane factory for producing raw sugar with a starch concentration of more than 140 parts per million (0.014 mg starch g^{-1} raw sugar). Since nine of the fourteen sugarcane mills in South Africa operate their own refinery, factories try to produce raw sugar with lower starch concentration for their own benefit. Starch is reduced in the sugarcane mill by adding a bacterial α-amylase to the extraction process which hydrolyse the starch before it can cause problems. Despite this, sugarcane with lower starch content still produces higher quality raw sugar with lower production costs of refined sugar (Personal communication B. Schoonees, Sugar Millers Research Institute, Durban, Kwazulu-Natal, RSA). This chapter will discuss starch synthesis and degradation in general to give a clear picture on what starch metabolism entails and the enzymes that play important roles. Two enzymes, ADP-Glucose pyrophosphorylase (AGPase) and β-amylase, will be discussed in detail since they will be further analysed in the experimental chapters due to the important role they play in starch metabolism. This chapter proceeds to discuss starch and

starch metabolism in sugarcane in terms of (a) the problems it causes in the sucrose extraction and refinement process and (b) its possible effect on carbon partitioning.

2.1. Starch

Starch is the major storage carbohydrate in most plants and is not only the primary source of energy food for most humans, but also has major industrial applications. It also plays an important physiological role in plants since transient starch synthesis serves to prevent periods of phosphate limitation of photosynthesis (Kossmann and Lloyd, 2000). Starch is a polymer of glucose and can be structurally divided into amylose and amylopectin (Mayer, 1895; 1896). These glucose units are linked to each other by either α(1,4) glycosidic or α(1,6) glycosidic bonds. Linear polymers are formed by α(1,4) glycosidic bonds, whilst branched linkages are formed by α(1,6) glycosidic bonds. It was initially thought that amylose is a linear molecule with the glucose units linked by α(1,4) glycosidic bonds, but it has been shown that amylose do contain 0.1% α(1,6) glycosidic branchpoints (Hizukuri and Takagi, 1984; Takeda et al., 1984; 1986). Amylopectin also consists mainly of α(1,4) glycosidic bonds, but has a far higher content of α(1,6) glycosidic branchpoints (4%) and thus have a much more branched structure (Banks and Greenwood, 1975). For a review on this, see Kossmann and Lloyd, 2000.

2.2. Starch synthesis

Starch synthesis is exclusively located in the plastids which mean that carbon substrate must be imported from the cytosol to the plastid for synthesis. The precise precursor for starch that is imported has led to some debate. The triose phosphate transporter plays an important role in the transport of carbon in photosynthetic tissue, but since virtually all non-photosynthetic tissue lacks the enzyme fructose 1,6 bisphosphatase (Entwislte and ap Rees, 1990), it means that this is most probably

not the case there, since the plastids will then not be able to convert triose-phosphates to hexoses. Labeling experiments has given further evidence that it is hexoses, and not triose phosphates, which is imported into the amyloplasts (Keeling et al., 1988; Viola et al., 1991). It is also not clear whether hexose phosphates (through the hexose phosphate transporter (transporter h in figure 2.1) or ADP-glucose (transporter g in figure 2.1) is transported to the cytosol and it seems to differ between species.

Although it was initially thought that AGPase activity is restricted to the plastid, a cytosolic isoform has been identified in the endosperm of at least two cereals (maize and barley) that constitutes the majority (85-95%) of the enzyme's activity (Thorbjornsen et al., 1996; Denyer et al., 1996). The reason for a cytosolic isoform in potentially all cereal endosperms is still not clear. Beckles et al. (2001) argued that it may facilitate the partitioning of large amounts of carbon from sucrose into starch when there is abundant supply of sucrose in the endosperm (storage organ). In tissues where AGPase is exclusively plastidial, the pathway from sucrose to starch involves the importation of hexose phosphates and ATP into the plastid. In the plastid these metabolites are not only used for starch synthesis, but also for fatty acid and amino acid synthesis, as well as for the oxidative pentose phosphate (OPP) pathway. If a plant or specific tissue possesses both cytosolic and plastidial AGPase activity, it allows the direct commitment of carbon from sucrose to starch synthesis without the involvement of the plastidial hexose phosphate and ATP pools. Starch synthesis may then only be dependent on the concentration of sucrose in the cytosol. When the sucrose concentration is high, the ADP-glucose concentration in the cytosol will also be high since the enzymes that convert sucrose to ADP-glucose are close to equilibrium. When sucrose concentration is low, most of the ADP-glucose for starch synthesis will be supplied via the importation of hexose phosphates into the plastid.

This mechanism therefore not only ensures that carbon is available for processes other than starch synthesis when the sucrose levels in the cytosol is low, but also allows carbon from sucrose to be committed directly to starch when sucrose is plentiful. In a model proposed by Beckles et al. (2001) it seems as though a cytosolic isoform of the enzyme is present when levels of ADP-Glucose and UDP-Glucose in the cytosol are similar. If ADP-Glucose levels are significantly lower than that of UDP-Glucose, most AGPase activity resides in the plastid (Beckles et al., 2001).
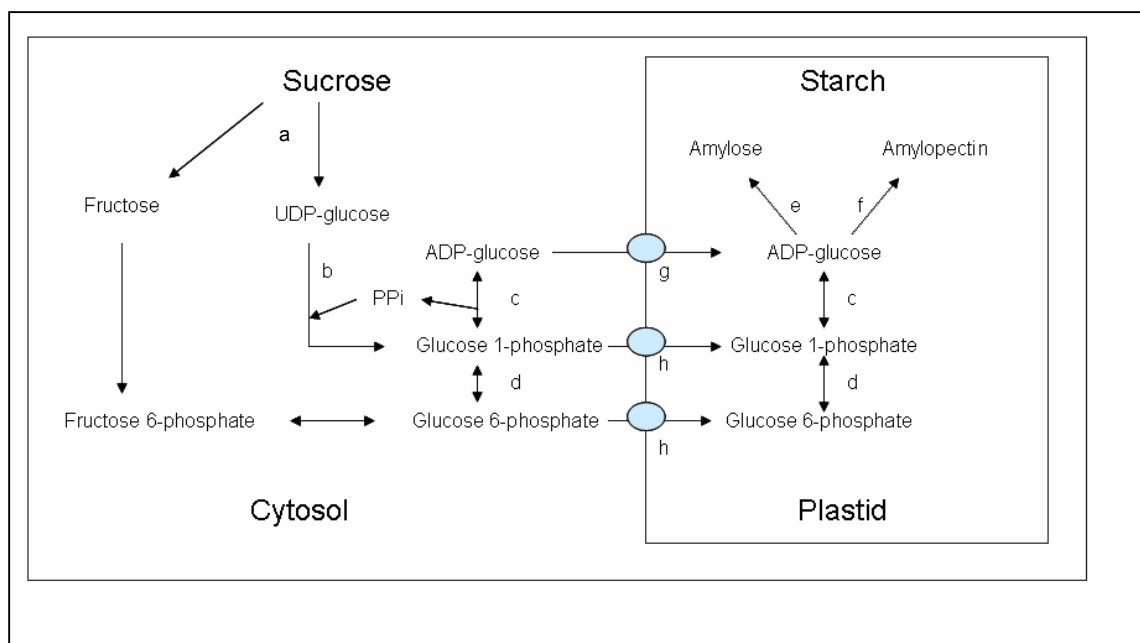
Figure 2.1. The major metabolites and enzymes involved in the conversion of sucrose to starch in storage organs. Carbon is shown entering the plastid either as a hexose-phosphate (Smith et al., 1995) or as ADP-glucose (Thorbjornsen et al., 1996; Denyer et al., 1996). The enzymes are: a, sucrose synthase; b, UDP-glucose pyrophosphorylase; c, ADP Glucose pyrophosphorylase; d, phosphoglucomutase; e, starch synthase (GBSSI); f, starch synthase and starch-branching enzyme; g, ADPglucose transporter; h, hexose phosphate transporter. PPi: inorganic pyrophosphate (Figure reproduced from Smith et al., 1997).

The advent of genetic engineering has given scientists the opportunity to study the role of specific enzymes in much more detail (Müller-Röber and Kossmann, 1994;

Oostergetel and van Bruggen, 1993). This has fuelled an enormous amount of research into starch synthesising enzymes, in particular ADP-Glucose pyrophosphorylase (AGPase, EC 2.7.7.27), starch synthase (EC 2.4.1.21), and starch branching enzyme (EC 2.4.1.18) (For a review see Smtih et al., 1997; Kossmann and Lloyd 2000). The synthesis of ADP-Glucose from Glucose 1-phosphate by the enzyme AGPase (enzyme c in figure 2.1) is seen as the first committed step to starch synthesis (Tsai and Nelson, 1966; Hannah and Nelson, 1976; Lin et al., 1988a; 1988b). As a result, a large amount of research has been done on AGPase and the role it plays in determining the rate of starch synthesis. It has been shown that both down-regulation (Müller-Röber et al., 1992) and over-expression of enzyme activity (Stark et al., 1992) have an effect on the amount of starch synthesised. This enzyme will later be discussed in more detail.

The next step in starch synthases is the transfer of the glucosyl moiety from ADP-glucose to the non-reducing end of an α(1,4) glucan and the reaction is catalysed by starch synthases (enzyme e and f in figure 2.1). The starch synthases are able to extend the α(1, 4) glucans to form both amylose and amylopectin (Kossmann and Lloyd, 2000). It is believed that there are four different isoforms: SSI, SSIIa, SSIIb and SSIII that play a role in amylopectin synthesis. An isoform that is encoded by the *Waxy (Wx)* locus, granule bound starch synthase (GBSSI) specifically elongates amylose. Even though there is a linear relationship between GBSSI activity and *waxy* gene dosage, amylose content is not linear to *waxy* gene dosage. This means that other factors, besides GBSSI, also determine the amylose content. The availability of ADP-Glucose as substrate and malto-oligosaccharide primers might be two such factors (Nelson and Rines, 1962; Shure et al., 1983).

All starch producing organisms probably contain at least one isoform of starch synthase other than GBSSI (Smith et al., 1995). Different isoforms of this enzyme each play a specific role in the synthesis of starch. The distribution of amylopectin chain lengths depends for example on the specific composition of starch synthase isoforms. According to a model for starch synthesis, SSI is primarily responsible for the synthesis of the shorter chains, i.e. those with 10 glycosyl units or less (French, 1984; Gidley, 1992; Giroux and Hannah, 1994). The extension of these molecules into longer chains is catalysed by SSII and/or SSIII. This process may involve branching before further extension of the molecule (Fontaine et al., 1993).

Starch branching enzymes (enzyme f in figure 2.1) are responsible for creating α(1, 6) linkages. The process of branching is not well understood, but it seems as though the α(1, 4) linkage chain is cleaved and reattached to form a α(1, 6) linkage (Borovsky et al., 1976; 1979). The variety of isoforms of starch branching enzymes in most plants allows for the possibility that each different isoform produces a different amylopectin structure, in terms of chain length and branch point frequency (Burton et al., 1995). All starch branching enzymes can be divided into two classes, i.e. Starch Branching Enzyme A (SBEA) and B (SBEB), based on their primary protein sequence (Burton et al., 1995). Branching of starch in maize is probably the result of both SBEA and SBEB. *In vitro*, studies suggest that SBEA essentially branches amylose and SBEB amylopectin (Guan and Preiss, 1993; Takeda et al., 1993). When amylose is the only substrate, SBEB creates longer branches than SBEA. When expressed in *E. coli*, results remain consistent in the sense that the B isoform creates longer chains than the A isoform (Guan et al., 1995). These results indicate that isoform B is involved in creating longer branches and isoform A shorter branches (Preiss and Sivak, 1996; Takeda et al., 1993). Mutants with no SBEA activity (Bhattacharyya et al., 1990; Burton et al., 1995; Mizuno et al., 1993; Stinard et al.,

1993) produced slightly longer chain lengths, but no real change in structure of amylopectin was observed (Baba and Arai, 1984; Colonna and Mercier, 1984; Lloyd, 1995).

2.3. ADP glucose pyrophosphorylase

2.3.1. Structure of AGPase

Based on their sensitivity to activation and inhibition, AGPases can be divided into nine distinct classes (Preiss, 1973; 1984; Iglesias et al., 1991; Preiss and Sivak, 1998a, 1998b). AGPases of higher plants fall into class VIII. The enzyme consists out of two distinctly different subunits that are products of two different genes. This is true for both photosynthetic and non-photosynthetic tissue (Morell et al., 1987; Preiss and Sivak, 1998a). In eukaryotes these two subunits form a heterotetrameric enzyme, consisting out of two small (α) and two large (β) subunits to form an $α_2β_2$ heterotetramer (Okita et al., 1990; Preiss and Sivak, 1998a; 1998b). The amino acid sequence of the α-subunit is highly conserved (85-95% identity) through different plant species, while that of the β-subunit is less conserved (50-60% identity) (Nakata et al., 1991). The α-subunit homotetramer of an AGPase isolated from potato tuber could be activated with 3-phosphoglycerate (3-PGA), but not the β-subunit (Ballicora et al., 1995). The data suggest that the α- and β-subunits are respectively the catalytic and regulatory subunits.

2.3.2. Catalytic properties of AGPase

AGPase catalyses the conversion of glucose 1-phosphate to ADP-glucose, using glucose-1-phosphate and ATP as substrates. The reaction, which was first described in soybean (Espada, 1962), has since been identified in various plant and bacterial extracts (Iglesias et al., 1991; Preiss, 1984). The reaction takes place in the presence of the divalent metal ion Mg^{2+} and is freely reversible *in vitro* with an equilibrium

constant close to one. However, the rapid hydrolysis of pyrophosphate by inorganic pyrophosphatase and the utilisation of the sugar nucleotide for starch or glycogen synthesis result essentially in the reaction being irreversible *in vivo* (Iglesias and Preiss, 1992). The enzyme is allosterically regulated by small effector molecules, activated by 3-PGA, an intermediate of the Calvin cycle, and inhibited by PPi (Iglesias et al., 1991; Preiss and Romeo, 1994; Preiss and Sivak, 1998b; Sivak and Preiss, 1998).

The characteristics of AGPase make it a prime target for manipulating starch biosynthesis. Because of its obvious appeal, it has been at the centre of various studies of starch metabolism. Various mutants have been constructed with the aim of lowering starch content, for example the *brittle-2* and *shrunken-2* maize mutants. The mentioned mutants contained only 25-30% of the starch content of wild type controls and retained only 5-10% of the AGPase activity (Tsai and Nelson, 1966; Dickenson and Preiss, 1969). Müller-Röber et al. (1992) made anti-sense constructs for the silencing of AGPase in potato. These anti-sense lines not only showed a significant decrease in starch, but also an increase in sucrose, proving that AGPase do play a crucial role in starch biosynthesis and carbon partitioning. These aspects of AGPase will be further investigated and discussed in chapter four.

2.4. The degradation of starch

2.4.1. Degradation in the plastids

Higher plants accumulate starch during the day as the end product of photosynthesis and degrade it as an energy source during the night. Since starch is found in the form of granules (Zeeman et al., 2002; 2004a), the first step in degradation of such a granule must be catalysed by an enzyme that can hydrolyze starch on the surface of the granule. Even though there are many enzymes that can do this *in vitro* (Scheidig

et al., 2002; Steup et al., 1983; Sun et al., 1995), it was initially thought that only endoamylase can do this *in planta*. Endoamylase produces soluble glucans that can be further degraded by other starch-degrading enzymes. However, this enzyme does not seem to be necessary for starch degradation. In mutation studies where all three isoforms were lacking, starch degradation was normal (Yu et al., 2005). This could indicate that the initial attack on the starch granule does not require endoamylase, or that the plant in which this study was done (Arabidopsis) has a unique endoamylase which could not be identified based on its primary amino acid sequence (Yu et al., 2005).

A newly discovered enzyme, glucan water dikinase (GWD), looks as though it might play an important role in the release of soluble glucans from the starch granule. Studies on potato show that the enzyme transfers phosphate from ATP to either the 6- or the 3-position of the glucosyl residues within the amylopectin fraction (Mikkelsen et al., 2004; Ritte et al., 2002). Although the frequency of phosphorylation in Arabidopsis leaf starch is very low, the presence of an active form of GWD appears to be very important in normal starch degradation *in vivo* (Nielsen et al., 1994; Ritte et al., 2004). In *sex1* mutants, where the GWD protein was eliminated or inactivated, there was a dramatic reduction in the amount of phosphate in the amylopectin and also in the rate of starch degradation. Mature leaves of these plants accumulated up to seven times more starch than those of the wild type plants (Caspar et al., 1991; Zeeman and ap Rees, 1999; Yu et al., 2001). The exact role of phosphorylation in starch degradation is still unclear, but it could make the polymer more susceptible to attack by enzymes (Blennow et al., 2000; 2002; Yu et al., 2001). However, the process of starch mobilisation from the granule is not that simple. A second GWD-like enzyme, phosphoglucan water dikinase (PWD), was discovered that is also required for normal starch degradation. Even though mutants lacking

GWD activity showed an increase in the amount of starch, they did not show any changes in the amount of phosphate in the starch (Baunsgaard et al., 2005; Kotting et al., 2005). It is speculated that these enzymes act together to release starch polymers from the granule, but the precise mechanism is not well understood and requires a great deal of further investigation (Smith et al., 2005).

Since starch in plants mostly consists out of amylopectin, which has a higher frequency of α(1,6) "branch" linkages, enzymes that specifically target these α(1,6) bonds play a very important role in starch degradation (Manners, 1985). They are called the starch debranching enzymes (DBE) and belong to the limit dextrinase (one enzyme) and isoamylase (three enzymes) classes (Nakamura, 1996). Limit dextrinase is thought to be responsible for the degradation of starch in the cereal endosperm, but knockout mutants of this enzyme show no difference in starch degradation, which suggests that one or more isoamylases are involved. Of the isoamylase class enzymes, knockout mutants have shown no clear patterns of starch degradation, although preliminary studies of Arabidopsis mutants lacking isoamylase three (*ISA3)* show a higher starch content in leaves (Delatte et al., 2006).

After the initial degradation of the starch branches mentioned above, only linear glucans are present in the plastids, which are further degraded via two distinct pathways. They can either be converted to glucose-1-phosphate, which is subsequently converted to triose phosphates and exported out of the plastid in a reaction catalysed by glucan phosphorylase, or to maltose in a reaction catalysed by β-amylase (figure 2.4) Scheidig et al., 2002).
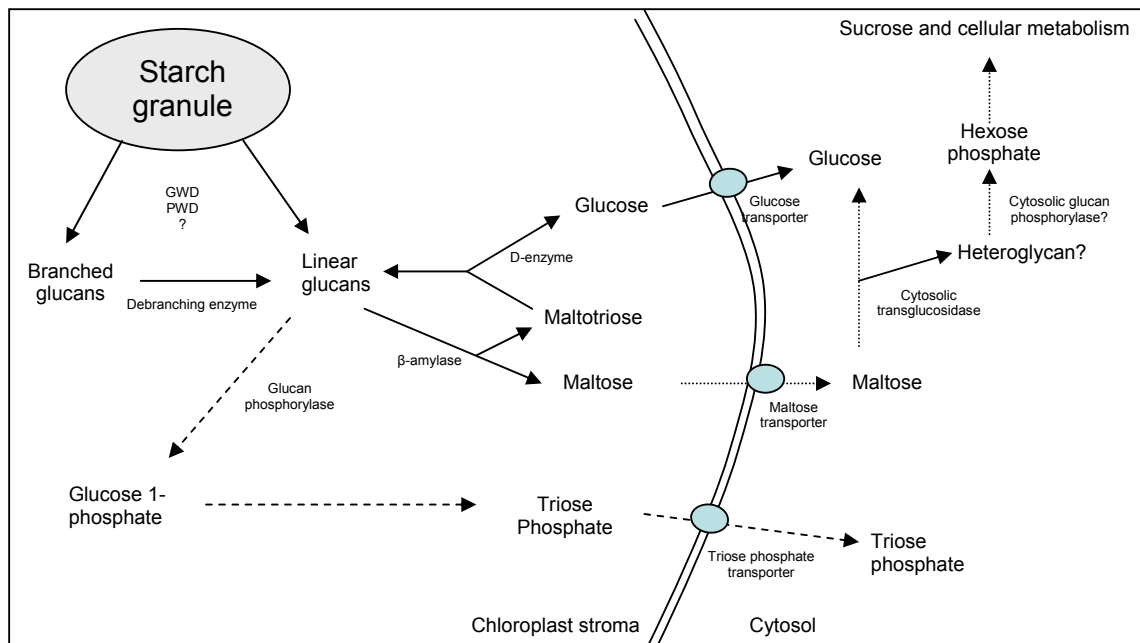
Figure 2.2. The proposed breakdown of starch in Arabidopsis leaves at night. Certain steps in the pathway remain unclear and these are marked by dotted lines and question marks. GWD is the abbreviation for glucan water dikinase and adds a β-phosphate group from ATP to either the 3- or 6-carbon of a glucosyl residue of amylopectin (Ritte et al., 2002). The abbreviation PWD refers to phosphoglucan dikinase, an enzyme that has the same function as GWD, but will only phosphorylate starch that already has phosphate groups (Smith et al., 2005). After this initial phosphorylation, the starch is attacked by the starch debranching enzymes which breakdown α(1,6) branches, leaving linear glucans in the plastid. These linear glucans are then further degraded by either glucan phosphorylase producing Glucose 1-phosphate, or β-amylase, producing maltose. (Figure reproduced from Smith et al, 2005)

2.4.2. The role of β-amylase

β-amylases are exoamylases that produce almost exclusively maltose as a product. These enzymes are abundant in both photosynthetic and storage organs of plants. Recent studies in Arabidopsis have shown that linear glucans are usually degraded by β-amylase rather than glucan phosphorylase, since plants lacking glucan pyrosphorylase have normal rates of starch degradation (Zeeman et al., 2004b) and plants lacking β-amylase activity have decreased rates of starch degradation (Fulton D, Dunstan H, Zeeman S and Smith S, unpublished data).

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In Arabidopsis there are nine genes that encode for β-amylases (Table 2.1). In this species, the majority of the β-amylase activity seems to be extra-plastidial and accounts for most of the starch degradation (80% in the rosette leaves) (Lin et al., 1988a). Since starch needs to be mobilised from the granule in the plastid, the extra-plastidial role of β-amylase seems to be to further degrade soluble starch. Scheidig et al. (2002) made an anti-sense construct of a potato β-amylase isoform that is very similar to the BAM3 gene in Arabidopsis (Table 1) and showed reduction in the mobilisation of starch from the granule proving that the enzyme does play a role in starch mobilisation.

Table 2.1. β-amylases present in Arabidopsis (Lloyd et al., 2005).

Protein	Gene name	Gene locus	Localisation to plastid (Predicted)[1]	Localisation to plastid (Experimental)
β-amylase 1	BAM1/ BMY7	At3g23920	Yes	NA[2]
β-amylase 2	BAM2/ BMY7	At4g00490	Yes	NA
β-amylase 3	BAM3/ BMY8/ ctBMY	At4g17090	Yes	Yes (Lao et al., 1999)
β-amylase 4	BAM4	At5g55700	No	NA
β-amylase 5	BAM5/BMY1/ RAM5	At4g15210	No	No (Wang et al., 1995; Laby et al., 2001)
β-amylase 6	BAM6	At2g32290	No	NA
β-amylase 7	BAM7	At2g45880	No	NA
β-amylase 8	BAM8	At5g45300	No	NA
β-amylase 9	BAM9	At5g18670	No	NA

[1]Transit peptide prediction was performed using two programs: TargetP and Predotar (Lloyd et al., 2005).

[2]Data not available

2.5. Starch in sugarcane

Starch levels in sugarcane stalks are very low, i.e. approximately 0.01% of fresh weight (Hawker, 1986). Due to this, and the fact that sugarcane stores such high levels of sucrose, sugarcane internodal cells are structurally different from starch storing plants. Sugarcane cells have a large central vacuole, which comprise over 90% of the cell volume and acts as a sucrose storage organelle (Ehwald et al., 1980). Suspension cultures display 40% lower vacuolisation (Komor, 1994), and it was shown that under unlimited growth conditions, there are also more amyloplasts than in culm tissue. Under unlimited growth condition there are also substantially more starch in sugarcane suspension cultures than in sugarcane internodal tissue, but when suspension cultures are under phosphate stress, starch is almost absent (Veith and Komor, 1993). The most important factor determining starch content of sugarcane seems to be varietal differences (Wood, 1962; Chen, 1968, Godshall et al., 1996), the time of season (Wood, 1962; Godshall et al., 1996), environmental factors (Wood, 1962) and the maturity of the plants / tissues (Wood, 1962; Bindon, 2000). Table 2.2 illustrates the difference in starch content between different sugarcane varieties in Lousiana, USA.

Table 2.2. Starch concentration in sugarcane juice of Louisiana sugarcane varieties (Adapted from Godshall et al., 1996)

Variety	Starch in mg g^{-1} FW			
	1990	1991	1992	Average
CP 720370	1.46	1.867	1.548	1.625
CP 79-318	0.986	1.092	0.986	1.021
LCP 82-89	0.566	0.701	0.538	0.602
CP 65-357	0.506	0.745	0.557	0.603
CP-74-383	0.611	0.59	0.636	0.612
CP 70-321	0.275	0.239	0.22	0.245

The starch content of commercial sugarcane varieties can be predicted by studying their percentage parentage from ancient wild sugarcane species (Wood, 1962). Current sugarcane varieties are interspecific hybrids originating from several species of the genus *Saccharum* from the *Poaceae* (grass) family (Stevenson, 1965). The wild type species that were originally used for sugarcane cultivation were *Saccharum spontaneum* and *S. robustum* and the cultivated species *S. officinarum* originated from *S. robustum* (Irvine, 1999). Two other cultivated species, *S. barberi* and *S. sinense*, are probably natural hybrids of *S. officinarum* and *S. spontaneum* (Daniels and Roach, 1987). Current commercial varieties, for all practical purposes, derived from *S. officinarium* and *S. spontaneum*. The starch content of these ancestral sugarcane species have been analysed. *S. officinarum* and *S. robustum* contained no starch at all, *S. sinense* contained very little, while the levels in *S. barberi* and *S. spontaneum* were quite high (Dutt and Narasimhan 1951). Moreover, the non-commercial variety, P.O.J. 2725, with no *S. barberi* and very little *S. spontaneum* parentage, had a very low starch content (Wood, 1962). On the other hand, NCo310, with a high percentage *S. officinarum and S. spontaneum* parentage, had very high levels of starch (Wood, 1962). This indicates that varieties with high *S. barberi* and *S. spontaneum* ancestry might have a higher starch content. Since most modern sugarcane varieties are essentially interspecific hybrids of *S. officinarum* and *S. spontaneum*, sugarcane breeders over the years have unintentionally selected for high starch containing sugarcane.

2.6. Why reduce the amount of starch in sugarcane?

2.6.1. Carbon partitioning

Current research strategies for improving the sucrose content of sugarcane are based on the idea that sucrose accumulation is regulated at the sink rather than the source (Gifford and Evans, 1981; Krapp et al., 1993). Starch as a competitive sink for

carbon in sugarcane stalk tissue has largely been ignored. This is due to the fact that starch contribute towards 2% of total metabolic products in immature tissues and the fraction in mature tissues is even smaller (Bindon, 2000). When starch is seen as a competitive sink for sucrose in terms of carbon allocated to storage carbohydrate, it contributes 10% in immature tissue (Bindon, 2000). There is also a substantial recovery of labelled maltose following the feeding of [U-^{14}C]-glucose, indicating significant cycling of carbon through starch. If there is so much cycling of carbon through starch, it might be a bigger competitor for carbon in immature tissue than the portioning data suggests (Bindon, 2000).

2.6.2. Minor polysaccharides in sugarcane and the problems they cause

Minor polysaccharides in sugarcane, of which starch and dextran are the most important, lower the quality of sugarcane juice and raw sugar in several ways. During processing of sugarcane they increase viscosity and slow down or inhibit crystallisation, which increases the loss of sucrose to molasses (Godshall et al., 1996). This is mostly due to starch gelatinisation. Starch gelatinisation is the disruption of molecular orders within the starch granule which leads to irreversible changes in properties such as granular swelling and native crystalline melting (Thomas and Atwell, 1999). This process is temperature and moisture dependent. The temperature at which it occurs differs with respect to plant species and the gelatinisation temperature of starch in sugarcane is estimated at 60ºC (Johnson, 1989).

Although early investigations into the minor polysaccharides have been covered in a broader scale, most of the recent research has been focused on starch and dextran. The polysaccharides found in sugarcane arise from two distinct sources, i.e. those that are produced via endogenous plant metabolic activity, for instance starch, and

those that are synthesised by micro-organisms after the cane is harvested, such as dextran (Imrie and Tilbury, 1972). The delay between cane harvesting and milling is the main cause of dextran formation. Dextran consists out of glucose subunits and is formed by micro-organisms in deteriorating cane. Polymers must contain at least 50% $\alpha(1,6)$ linkages to be defined as a dextran (Imrie and Tilbury, 1972).

As mentioned earlier, the starch content of any specific sugarcane stalk varies substantially in relation to various factors. Dextran levels are dependent on other factors such as transport time. The presence of starch and dextran reduce the efficiency of the extraction, and is especially problematic during the refinement process (Cuddihy et al., 1999). As mentioned earlier, such problems are currently overcome by adding enzymes that hydrolyse these polysaccharides while in the sugar mill. Even though these enzymes are relatively cheap and effective, sugarcane with lower starch content is still preferred, since higher quality sugar can be produced with lower production costs (Personal communication B. Schoonees, Sugar Millers Research Institute, Durban, Kwazulu-Natal, RSA).

Clearly more knowledge on starch in sugarcane is needed, especially in a South African context. Reducing starch in sugarcane might lead to higher sucrose yield and lower production costs.

Chapter 3

Determining the starch content and activities of the enzymes ADP-Glucose pyrophosphorylase and β-amylase in commercial South African sugarcane varieties

Abstract:

Currently there is very little information available on the starch content of South African commercial sugarcane varieties. In this study the internodal starch content of six commercial sugarcane varieties were determined as well as the activities of two important starch metabolising enzymes ADP-Glucose pyrophosphorylase (AGPase) and β-amylase. Starch concentration in internodal tissue increased more than 4 times from the young to mature internodes. There were also large differences between varieties. Comparative studies on the starch content of mature tissue showed that starch concentration varied between 0.18 mg g^{-1} FW and 0.51 mg g^{-1} FW. There was a very strong correlation between starch and sucrose concentration (R^2 = 0.53, p << 0.01) and no correlation between starch content and AGPase activity in the internodal tissue. This was true for correlations based on either tissue maturity or variety. β-amylase activity was much higher in the young internodes than in mature internodes, suggesting, in combination with the high AGPase activity, that there might be carbon cycling through starch in these internodes.

Determining the starch content and activities of the enzymes ADP-Glucose pyrophosphorylase and β-amylase in commercial South African sugarcane varieties

3.1. Introduction

Sugarcane is different from most carbohydrate storing plants, as it stores most of its carbon in the form of sucrose and not starch. Starch levels in sugarcane stalks are very low, i.e. approximately 0.01% of the fresh weight, and are affected by several factors (Hawker, 1985). Of these, the most important seem to be varietal differences (Wood,1962; Chen, 1968), the time of season (Wood, 1962; Godshall et al., 1996), environmental factors (Wood, 1962) and the maturity of the plants / tissues (Wood, 1962; Bindon, 2000). In this study we focused on starch in sugarcane in a South African context. Historically the starch content of South African sugarcane varieties is of the highest in the world (Alexander, 1954).
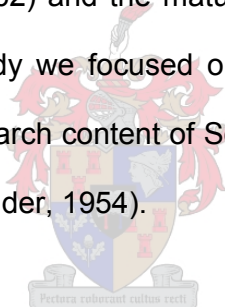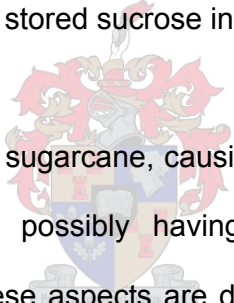
As mentioned above, varieties differ greatly in terms of their starch content. Of the modern sugarcane varieties NCo310, which is no longer commercially planted in South Africa, shows relatively high levels of starch while NCo376, a commercially planted variety in South Africa (Personal communication M. Butterfield, South African Sugarcane Research Institute, Mount Edgecombe, Kwazulu-Natal, RSA), has a lower starch content, i.e. approximately half that of NCo310 (Wood, 1962). Some ancestral sugarcane species contain more starch than others and the starch content of modern sugarcane varieties can be predicted by studying the parentage of the specific variety. Those with high *Saccharum barberi* and *S. spontaneum* ancestry tend to have higher starch content, while varieties with more *S. officinarum* ancestry contain less starch (Dutt and Narasimham, 1951). The correlation between starch content of

ancestral *Saccharum* species and those of commercial sugarcane varieties is discussed in more detail in Section 2.5.

In terms of changes in starch content during the growing season, starch levels increase with the onset of ripening and decrease as the cane matures (Chen, 1968; Batta and Singh, 1986). Fertilisers and environmental factors also play a role on starch content in sugarcane. Potassium treatment leads to an increase in growth and a decrease in starch accumulation, while nitrogen treatment also reduce the starch content in sugarcane, but the effect is not as great as it is with potassium. Wood (1962) argued that this slight decrease in the overall starch content might be due to the increase in the length of the internodes, since the starch content of nodes is up to four times higher than that of internodes. Phosphate, another major fertiliser used in sugarcane farming, plays an integral role in starch synthesis and its availability should therefore also play an important role in determining starch content. Although plenty of starch is produced (i.e. 100 mg (dry weight: FW)$^{-1}$) in sugarcane suspension cell cultures under optimal growing conditions, phosphate deficiency leads to an almost complete absence of starch in these cultures (Veith and Komor, 1993). Seemingly contradictory to this, studies on whole sugarcane plants, grown under supposedly different phosphate regimes, showed no variation in starch content. This apparent insensitivity to phosphate concentrations could, however, be explained by the already high levels of phosphate in all cultivated soils (Wood, 1962).

Another factor influencing starch content in sugarcane is the time lapse between harvesting and milling. Starch content initially declines slowly following harvesting, e.g. 13% after three days and 16% after five days, but after approximately five days it is rapidly degraded (69% decline after ten days) (Wood, 1962).

Studies on sugarcane's starch content in terms of tissue maturity show varying results. Bindon (2000) found that starch concentrations increase with tissue maturity, but an earlier study found the opposite result (Wood, 1962). This is probably due to different sampling techniques since Wood (1962) used whole stalk measurements, including nodal tissues which contain up to four times more starch than internodal tissues (Wood, 1962). Since younger internodes are shorter than mature internodes this part of the stalk would contain more nodes per unit fresh mass and therefore also more starch (Bindon, 2000). As a fraction of total carbohydrate content, starch decreases as the tissue matures. In immature tissue, starch can represent as much as 10% of the total carbohydrate pool, but this decreases to less than 1% as the tissue matures and the amount of stored sucrose increases (Bindon, 2000).

Starch is an unwanted product in sugarcane, causing problems during the extraction and refinement processes and possibly having a negative effect on carbon partitioning towards sucrose (These aspects are discussed in detail in section 2.6). Despite the fact that a large amount of research has been done on starch in the sucrose extraction and refinement processes, very little research has been done on starch in living sugarcane, especially in a South African context. The studies on the starch content of South African commercial varieties are outdated and therefore not relevant to current varieties. The aim was therefore to determine the starch contents of more modern sugarcane varieties at the onset of this project. The starch content of four different internodes, i.e. three, six, nine and twelve, of six different varieties, i.e. NCo310, NCo376, N12, N16, N19 and N27, were therefore determined. Excluding NCo310, these varieties contribute more than 70% of the total amount of sucrose produced in South Africa (Personal communication M Butterfield, SASRI). NCo310

was included in the study because of its role as a model variety in South African sugarcane research and, specifically, as a variety used in the production of transgenic plants. NCo310 and NCo376 also link this study to the work done by Wood in 1962. The activities of two enzymes important in starch metabolism, ADP-Glucose pyrophosphorylase and β-amylase, were also measured.

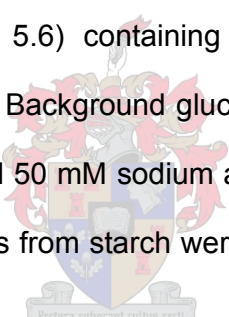## 3.2. Materials and methods

3.2.1. Biochemicals

All chemicals used for enzyme assays were purchased from Roche Biochemicals (Mannheim, Germany) unless stated otherwise. All other solvents and chemicals were of analytical grade.

3.2.2. Harvesting and sampling of tissue

Stalk tissue of six commercial South African sugarcane varieties were received from the South African Sugarcane Research Institute (SASRI), Durban, Kwazulu-Natal, South Africa. All varieties were 12 months old except for NCo310 which were ratoon sugarcane of 15 months old. Stalks were harvested and sent to Stellenbosch, Western Cape, South Africa *via* airfreight. Approximately 24h elapsed from the time of harvest until the material was processed in Stellenbosch. As mentioned earlier, although starch content of sugarcane starts to decline after harvesting, this is very slow for the first five days, after which there is a rapid decline (Wood, 1962). The nodes and rinds of the stalks were removed and the tissue of internodes 3, 6, 9 and 12 were ground separately in liquid nitrogen and stored at -80 ºC.

3.2.3. Starch determination

Starch content was determined using a method modified from Müller-Röber et al. (1992). Tissue samples of approximately 0.05 g were weighed and transferred to a micro-centrifuge tube containing 1 ml of 80% (v/v) ethanol. The tube was then incubated at 80 ºC for 30 minutes. Samples were centrifuged at 20 000 g and aliquots taken for determination of soluble sugars. The remaining ethanol was then discarded and the insoluble fractions were washed with 1ml 80% (v/v) ethanol before being dried under vacuum. This insoluble matter was re-suspended in 400 μl of 0.2 M KOH and heated at 95 ºC for 1 hour to dissolve the starch. After neutralisation with 70 μl of 1M Acetic acid, the sample was clarified by centrifugation at 20 000 g for 10 min. To digest the starch to glucose, a mixture of 20 μl of the supernatant and 20 μl of 50 mM sodium acetate (pH 5.6) containing 10 U/ml amyloglucosidase was incubated at 37 ºC for two hours. Background glucose amounts were determined by combining 20 μl sample and 20 μl 50 mM sodium acetate (pH 5.6) and incubating at 37 ºC for two hours. Glucose units from starch were determined using the method of Bergmeyer and Bernt (1974).

3.2.4. Sucrose determination

Sucrose was determined using the method of Bergmeyer and Bernt (1974).

3.2.5. Enzyme activity

0.05 g of ground samples were weighed out and proteins extracted using 100 μl volumes of protein extraction buffer containing 50 mM Mops-KOH (pH 7.5), 20 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM EDTA, 3% (w/v) PEG-3000, 2% (w/v) PVPP and 14.3 mM β-mecaptoethanol. After vigorous vortexing samples were incubated on ice for 20 minutes, centrifuged at 20 000 g for 10 min at 4ºC and the supernatant was used for

assaying enzyme activities. Protein contents of samples were determined using the method of Bradford (1976).

ADP-glucose pyrophosphorylase (AGPase) activity was measured using a method modified from Plaxton and Preiss (1987). The absorption of thirty microlitres of protein extract and 265 µl enzyme assay buffer (100 mM Tris-HCl [pH 7.0], 2 mM $MgCl_2$, 0.1 mM ADP-glucose, 0.4 mM NAD, 4U/ml glucose-6-phosphate dehydrogenase and 4U/ml phosphoglucomutase) was measured at 340 nm. The reaction was started by adding 5 µl of 50 mM sodium PPi and was analysed over 20 minutes. Mean V values were taken and used to calculate activity. All spectophotometric readings were performed in duplicate on a 96 well microtitre plate reader.

The activities of β-amylase were determined using an assay kit (Megazyme, Ireland), in which the release of p-nitrophenyl-glucoside from p-nitrophenyl-α-D-maltopentoase by the enzyme alpha-glucosidase is measured. The release of p-nitrophenyl-glucoside was spectrophotometrically determined at 410 nm, which is directly proportional to β-amylase activity. A unit of β-amylase activity is described as the amount of enzyme that is required to release one ụmol of p-nitrophenol from p-nitrophenyl-α-D-maltopentoase in one minute (Erdal, 1993; Santos and Riis, 1996). All spectrophotometric readings were performed in duplicate on a 96 well microtitre plate reader.

3.3. Results and Discussion

3.3.1. Starch and sucrose content in South African sugarcane varieties

The starch content of the six varieties analysed varied both in terms of variety and tissue maturity. All the lines showed the same overall pattern, where the starch content increases on a fresh weight basis up to internode 9, after which it plateau. The mean starch concentrations in the different internodes across all the varieties was 0.06 ± 0.01 mg g^{-1} FW for internode three, 0.11 ± 0.02 mg g^{-1} FW for internode six, 0.33 ± 0.03 mg g^{-1} FW for internode nine and 0.31 ± 0.04 mg g^{-1} FW for internode twelve (Figure 3.1a).

Based on the starch content of their mature tissues (internodes nine and twelve), the varieties were divided into three groups, i.e. a high starch variety (N19), medium starch varieties (NCo310, N12, N16 and N27) and a low starch variety (NCo376). The average starch concentration in the mature tissues of N19, i.e. 0.51 ± 0.05 mg g^{-1} FW, was almost 3X higher than that of NCo376 at 0.18 ± 0.06 mg g^{-1} FW, while the average starch concentration of the medium starch varieties, NCo310, N12, N16 and N27, was 0.31 ± 0.02 mg g^{-1} FW (Figure3.1a). Statistical analyses confirmed that the starch contents of the mature internodes of NCo376 and N19 were significantly different ($p \leq 0.01$). In addition, the medium starch varieties differed significantly from both N19 ($p \leq 0.01$) and NCo376 ($p \leq 0.05$) (Figure 3.1a). These differences in starch content between different varieties supports earlier studies (Godshall et al., 1996) while the low starch content of NCo376 compared to NCo310 observed in this study was also shown by Wood (1962) (figure 3.1a).
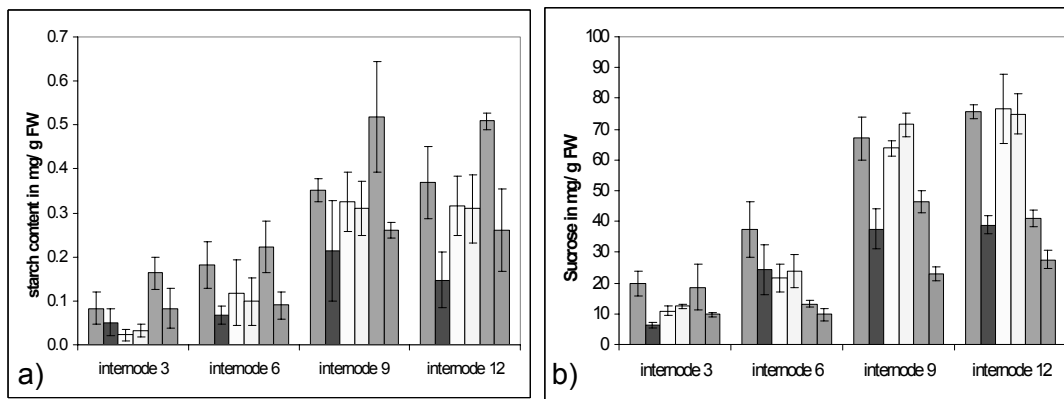
Figure 3.1. The starch (a) and sucrose (b) concentration in sugarcane internodal tissues in mg g^{-1} FW. In all cases the different coloured bars represent from left to right the different varieties NCo310, NCo376, N12, N16, N19, N27. The error bars represent the standard errors (n=4).

The sucrose content of all the internodal tissues was approximately 200X higher than that of starch at all levels of tissue maturity, but the two metabolites followed similar same patterns of accumulation. There was a significant correlation between starch and sucrose concentrations (R^2 = 0.53, p << 0.01) (Figure 3.2). This could be explained by the fact that they use the same hexose-phosphate pool as substrate for synthesis (Hill and ap Rees, 1994).
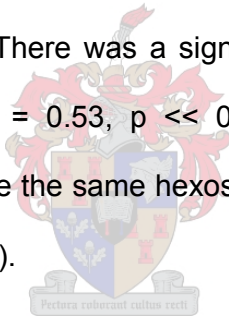
The varieties analysed could be divided into high sucrose and low sucrose varieties based on the sucrose content of their mature tissues. The high sucrose varieties, i.e. NCo310, N12 and N16, had an average sucrose concentration of 73.2 $\pm$ 3.0 mg g^{-1} FW, while the low sucrose varieties, i.e. NCo376, N19 and N27, had an average of 36.6 $\pm$ 2.2 mg g^{-1} FW. The sucrose content of these two groups was significantly different from each other (p << 0.01) (figure 3.1b).

Sucrose concentration should be closer for all varieties. Although all the sugarcane varieties were twelve months old, except for NCo310 which was eighteen months,

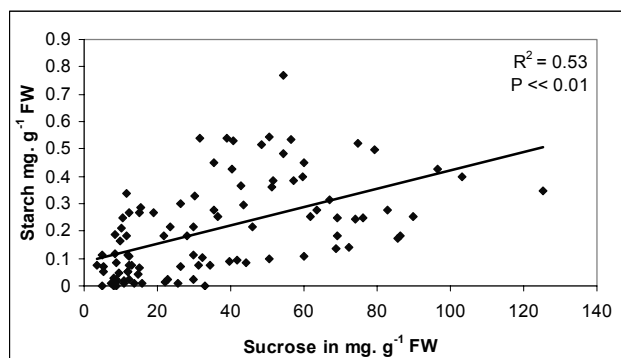environmental conditions possibly led to the "low sucrose" varieties being physiologically less mature.

Figure 3.2. Correlation between starch and sucrose concentrations across various sugarcane varieties and internodal tissue types.

3.3.3. Activities of enzymes directly associated with starch metabolism

AGPase activity was constantly the highest in N12 and N16 in all internodes, with the other varieties at similar, lower levels (figure 3.3a). No reproducible patterns in AGPase activity were observed across the different varieties. There was also no significant correlation between AGPase activity and starch content. This was true for correlations based on either tissue maturity or variety. It is possible that the measured AGPase activity could have been impacted upon by the twenty four hour delay between harvest and analysis. AGPase activity in maize has, for example, been shown to be fairly unstable and sensitive to high temperatures. Incubation at 57° C for ten minutes destroys 96% of the enzyme's activity (Hannah et al., 1980).

β-amylase activity was highest in internode three of all varieties, after which it decreased significantly in internode six and stayed low further down the stalk as the tissue matured. Based on the amount of β-amylase activity in internode three the varieties could be divided into three groups. These groups were N27 with the highest activity (2.82 units mg^{-1} protein $\pm$ 0.29), NCo310 with the lowest (0.36 units mg^{-1}

protein ± 0.12), and the varieties with medium activity, NCo376, N12, N16 and N19, with an average of 0.92 units mg^{-1} protein $\pm$ 0.13. There was a significant difference between NCo310 and N27 (p << 0.01). The medium activity varieties differed significantly from N27 (p << 0.01) and NCo310 (p ≤ 0.1) (figure 3.3b).

There is strong evidence that β-amylase is responsible for much of the *in planta* starch degradation in potato (Scheidig et al., 2002). The activity data presented here and the fact that large amounts of labelled maltose (the product of β-amylase) are recovered when immature sugarcane internodal tissues are fed with labelled glucose (Bindon 2000), suggests that there is cycling of carbon through starch at these internodes.
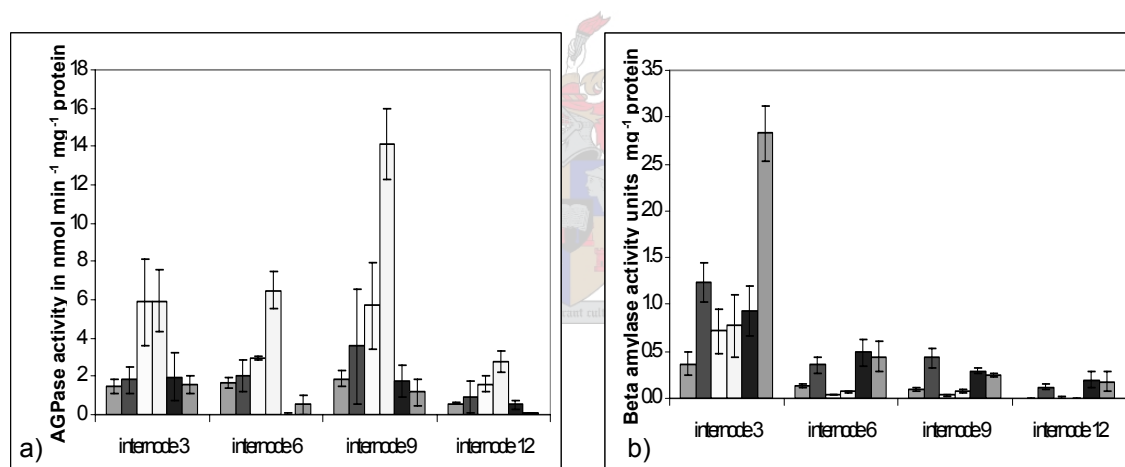
Figure 3.3. Activities of AGPase and β-amylase across various sugarcane varieties and internodal tissue types. (a) AGPase activities in sugarcane internodes in nmol minute^{-1} mg^{-1} protein.(b) β-amylase activity in sugarcane internodes tissue in units mg^{-1} protein. In all cases the different coloured bars represent from left to right the different varieties NCo310, NCo376, N12, N16, N19, N27. The error bars represent the standard errors (n=4).

3.4. Conclusion

Although starch makes up a very small percentage of the total metabolite pool in sugarcane, it is a significant minor metabolite which causes problems during sugar

milling. This is the first time that data on starch levels in most of the major sugarcane varieties in South Africa has been determined.

The starch content of sugarcane internodal tissue varies according to the age of the tissue. There is more than a 4X increase in starch concentrations between internode three, which had the lowest starch content, and internode nine, which had the highest starch content. Variety also plays an important role in determining starch content. N19, which the highest starch concentrations in the mature tissue, had almost 3X more starch than NCo376, which had the lowest concentration. There was a significant correlation between starch and sucrose concentrations, which could be due to the fact that the metabolites share the same substrate pool, i.e. the hexose-phoshates. The sucrose content of the varieties analysed varied a lot, indicating that some varieties were physiologically less mature than others.

AGPase activity was constantly the highest in N12 and N16. β-amylase activity was the highest in the youngest internodes (internode three) for all varieties after which activity sharply decreases as the tissue matures. There was high activity of both starch synthesising and degrading enzymes in the young tissues. This supports carbon partitioning data that there is cycling of carbon through starch in these internodes (Bindon, 2000).

Chapter 4

Reduction of the Starch Content of Sugarcane Suspension Cells by Silencing ADP-Glucose Pyrophosphorylase- or Over-expressing β-amylase Activity

Abstract

Starch is an unwanted product in sugarcane and reducing it could be of great value to the industry. In an attempt to reduce starch content of sugarcane, the activities of ADP-Glucose pyrophosphorylase (AGPase) and β-amylase were manipulated using transgenesis. Transformation vectors to reduce AGPase activity and increase β-amylase activity were constructed and used for the transformation of sugarcane calli. The results of the manipulations were analysed in suspension cultures. AGPase activity was reduced to between 0.14 and 0.54 times that of the wild type control. This led to a reduction in starch concentration of between 0.38 and 0.47 times that of the wild type control. β-amylase activity was increased in the transgenic lines by 1.5-2 times that of the wild type control. This increase in activity led to a reduction in starch concentration of between 0.1 and 0.4 times that of the wild type control. In both experiments the change in starch concentration could be correlated with the change in enzyme activity. There was also no significant effect on sucrose concentration in both experiments.

Reduction of the Starch Content of Sugarcane Suspension Cells by Silencing ADP-Glucose Pyrophosphorylase- or Over-expressing β-amylase Activity

4.1. Introduction

Starch is an unwanted product in sugarcane, causing problems during sucrose extraction and refinement and it could also possibly have a negative effect on carbon partitioning towards sucrose (See section 2.6). This study was focused on reducing starch in sugarcane by manipulating the activities of two enzymes, which were chosen because of the important role they play in starch metabolism. The enzymes were ADP-Glucose pyrophosphorylase (AGPase, E.C. 2.7.7.27), important for starch synthesis, and β-amylase (E.C. 3.2.1.2), important for starch degradation.

Although ADP-Glucose is preferentially used for starch synthesis and this substrate is produced by AGPase, this is not the only pathway through which starch is synthesised. UDP-Glucose can also serve as substrate for starch synthase, and glucan phoshorylase (E.C. 2.4.1.1) can both synthesise and degrade starch (Preiss, 1991). Despite this, knockout mutants or the genetic manipulation of AGPase activity has proven very successful over the years. As described in chapter two, AGPase is the rate limiting step in the biosynthesis of starch and glycogen (for review see Preiss and Sivak, 1996). Starchless mutants, such as *Brittle-2 (*Tsai and Nelson, 1966) and *Shrunken-2* (Dickenson and Preiss, 1969), which lack AGPase activity, as well as various experiments where starch content was affected by genetic manipulation (Müller-Röber et al., 1992; Stark et al., 1992; Sweetlove et al., 1996) demonstrate that AGPase plays a crucial role in starch synthesis.

AGPase is a heterotetrameric enzyme, encoded by two different genes. The two genes show similarity, indicating that they originated from an earlier gene duplication event (Greene and Hannah,, 1998). The small subunit seems to be the catalytic subunit of the enzyme and has been the focus of most of the genetic manipulation approaches. Many different strategies for manipulating this enzyme's activity have been reported. Other than simple over-expression and silencing of enzyme activity, the enzyme's sensitivity to 3-phosphoglycerate (3-PGA) activation and inorganic phosphate (Pi) inhibition has also been altered through mutagenesis (Greene et al., 1998). AGPase manipulation does not only reduce/increase the starch concentrations in plants, it also changes the structure of the starch. There is a strong correlation between the amount of AGPase activity and the amylose fraction in starch. Reduced AGPase activity seems to affect amylose synthesis more than that of amylopectin leading to a reduced ratio between amylose and amylopectin (Lloyd, 1995; Clarke et al., 1999).

Given that sugarcane is commercially planted for sucrose production, an added advantage for reducing starch could be to increase the partitioning of carbon towards sucrose. Müller-Röber et al. (1992) created an anti-sense construct of AGPase and silenced the enzyme in potato tubers. There was not only a significant reduction in starch (up to 96% less starch than in the control plants), but also a substantial increase in sucrose in the potato tubers (up to a ten fold increase).

As mentioned in chapter two, starch degradation is a much more complex process than synthesis. Bindon (2000) suggested that in sugarcane there might be substantial cycling of carbon through starch, especially in younger internodes, bringing the starch degrading enzymes into focus. It was believed that α-amylases

are responsible for the release of starch polymers from the starch granule, but studies on knockout mutants of all α-amylases, based on their primary sequence, in *Arabidopsis* show no effect on starch degradation, which indicates that α-amylase is either not necessary for normal starch degradation or that there is a α-amylase present that cannot be identified based on its primary protein sequence (Yu et al, 2005). The precise mechanism by which starch polymers are released from the starch granule is discussed in section 2.4.1. After the starch polymers are released from the granule, the α(1,6) "branch" linkages are degraded by starch debranching enzymes, yielding linear glucans. It seems as though β-amylase, and not glucan phosphorylase, is primarily responsible for the degradation of these linear glucans in the plastid. Evidence for this is that the reduction of β-amylase activity in potato led to a starch excess phenotype (Scheidig et al., 2002) whilst a reduction of glucan phosphorylase showed no effect on starch levels (Zeeman et al., 2004).

The aim of the experiments was to reduce starch content of sugarcane suspension cells. Starch metabolism was manipulated in sugarcane suspension cells through either the down-regulation of AGPase activity or the over-expression of β-amylase activity. Sugarcane suspension cultures exhibit similar growth and metabolic characteristics to the tissues of whole plants and can be used as a model system for research (Thom et al., 1982). Two sugarcane expression vectors were constructed using the sugarcane AGPase and the potato β-amylase sequences respectively. A partial cDNA sequence of sugarcane AGPase was cloned and used to create a RNAi construct. The β-amylase cDNA sequence used has a proven ability to degrade starch *in vivo* (Scheidig et al., 2002). The effect on enzyme activity and metabolite levels were analysed in sugarcane suspension cultures and plants were also regenerated for future work.

4.2. Materials and Methods

4.2.1. Biochemicals

All Oligonucleotides used in this study were ordered from Inqaba Biotec (Pretoria, South Africa). All enzymes and chemicals for gene isolation, molecular cloning and plasmid construction were purchased from Sigma-Aldrich (St. Louis, MO, USA), Roche Biochemicals (Mannheim, Germany), Promega (Madison, WI, USA) or Fermentas (Hanover, MD, USA). All other solvents and chemicals were of analytical grade.

4.2.2. Construction of an AGPase silencing vector

RNA was extracted from sugarcane leaf tissue using the CTAB method described by Doyle and Doyle, 1987. cDNA was synthesised from the total RNA template using SuperScript RNaseH Reverse Transcriptase[TM] (Invitrogen, Carlsbad, CA, USA).
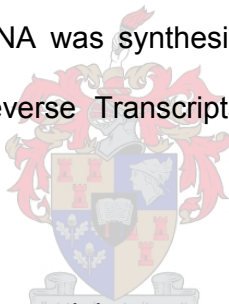
Primers for the amplification of a partial gene sequence of AGPase were designed from a sugarcane EST sequence available on the GenBank database (CA276527). These primers (5'-AGC ATT GGA AAG AGG GTT CAG GCT T-3' and 5'-TCT CCC TCG CAG CTT CTT GAA CAT-3') were then used to amplify the partial sequence of AGPase using the earlier synthesised cDNA as template. The PCR product was then separated by gel eletrophoresis and a fragment of the correct size was isolated. This fragment was then ligated, using T4 DNA ligase (Promega[TM]), into the pGEM-T-easy[TM] vector (Promega[TM]) to create pGEM-AGPase. A standard ligation protocol from Promega[TM] was used for all ligations.

After ligation and subsequent transformation of *Escherichia coli*, transformed colonies were selected using ampicillin selection. These colonies were then screened for the presence of pGEM-AGPase using PCR. The primers used for this PCR were the same pair used for the isolation of the partial AGPase sequence. After selecting a bacterial colony containing the plasmid, plasmid DNA was extracted using a plasmid DNA extraction kit from Sigma-Aldrich[TM]. The plasmid was then sequenced.

For the purpose of creating an intron region between two inverted repeats of AGPase in the final silencing vector, oligonucleotides were designed. These oligonucleotides were designed to contain splicing sites (Croy, 1993) and *Xba*I (T/CTAGA) and *Bam*HI (G/GATCC) cloning sites after they annealed with each other. The oligonucleotides were designated 5A (5'-CTA GAA TCT TGA AAT TAA TAT GCA TAT GTT ACC TGT G-3'), 5S (5'-GAT CCA CAG GTA ACA TAT GCA TAT TAA TTT CAA GAT T-3'), 3A (5'-GAT CCC ACC TGC ATC GAT CTG GCG TTT AAA AGA AGC T-3') and 3S (5'-CTA GAG CTT CTT TTA AAC GCC AGA TCG ATG CAG GTG G-3'),

For annealing purposes all four oligonucleotides were diluted to 100 ng µl^{-1}. 2 µl (200ng) of each of the oligonucleotides to be annealed (i.e. 5S and 5A, 3S and 3A) were added to 3 µl of 25 mM MgCl$_2$, and both solutions made up to 50 µl with sterile water. The solutions were then placed in a PCR machine for one cycle at 85°C (for 30 seconds) followed by a ramp of 0.05 ^{o}C down to 15°C.

The double stranded DNA was then ligated into the *Bam*HI and *Xba*I sites of the pBluescript SK (pSK) and pBluescript KS (pKS) vectors respectively (5A/5S in pSK

and 3A/3S in pKS). These intermediate RNAi vectors were named pSK 5'I and pKS 3'I and used for *E. coli* transformation. To confirm the successful hybridisation and ligation a PCR using the T7 (5'-AAT ACG ACT CAC TAT AGG-3') and T3 (5'-AAT TAA CCC TCA CTA AAG GG-3') primers were conducted on the *E. coli* colonies. Positive clones were selected for further experiments.

The AGPase fragment was cut from pGEM-AGPase with *Kpn*I (GGTAC/C) and *Pst*I (CTGCA/G). The digested product was separated by gel eletrophoresis and a 468 base pair fragment isolated. This fragment was then cloned into pSK 5'I and pKS 3'I using the *Kpn*I and *Pst*I sites. This created the RNAi intermediate vectors pSK 5'I-AGPase and pKS 3' I-AGPase. Both these vectors were then digested with *Kpn*I and *Xba*I. The digested product was then separated by gel eletrophoresis and a 502 base pair fragment from each vector was recovered. These two fragments were then cloned into the *Kpn*I site of the sugarcane expression vector, pU3Z to create the pHairpinAGPase-U3Z vector. This vector was then used for sugarcane transformation.

4.2.3. Creating β-amylase over-expression vector

β-amylase clone *pctBmyI* (GenBank accession number AF393847) was received and cloned into a sugarcane transformation vector. Cloning vector pSK-β-amylase containing *pctBmyI* was digested with *EcoR*I and subsequently blunt-ended using Klenow DNA polymerase (Fermentas™). *pctBmyI* was then cut from the vector by digesting with *Bam*HI. This fragment was ligated into the *Bam*HI and *Sma*I sites of sugarcane transformation vector pUBI510. The vector was named pUBI-β-amylase and used for sugarcane transformation.

4.2.4. Transformation of sugarcane

Two different sugarcane transformation experiments were carried out. In the first transformation the plasmid pHairpinAGPase was used, followed by the pUBI-β-amylase plasmid in the second transformation. Hereafter they will be referred to as the transformation vectors.

Sugarcane variety NCO310 Type 3 embryogenic calli (Ho and Vasil, 1983, Taylor et al., 1992) were transformed using a Particle Inflow gun (Finer et al., 1992). Tungsten particles (M17, Bio-Rad Laboratories, Hercules, CA, USA) (25 µl; 100 mg.ml^{-1}) were added to 5µg plasmid DNA (2.5 µg pUBIKM (selection plasmid) and 2.5 µg of the transformation vector). The former plasmid contains the *neomycin phosphotransferase II* (*nptII*) gene that encodes for geneticin resistance that was used for positive selection of the transformed calli. Calcium chloride (25 µl of 2.5 M CaCl$_2$ stock), spermidine (10 µl of 100 mM stock) and milliQ^{TM} water to a volume of 80 µl were added to the DNA suspension. The suspension was vortexed and incubated for 5 minutes on ice. The supernatant was discarded and the pellet resuspended in 15 µl of absolute ethanol. The calli were placed 12 cm from the point of outlet and bombarded with 3 µl DNA-coated tungsten particles. Helium, at a pressure of 1200 kPa, was used as the propellant. The chamber vacuum pressure was 90 kPa and the solenoid time setting was 50 x 10^{-6} s.

After bombardment, the calli were incubated in the dark at 28 °C on solid MS$_3$ media (4.43 g L^{-1} MS [Murashige and Skoog, 1962) basal salts and vitamins (Sigma-Aldrich^{TM}), 20 g L^{-1} sucrose, 0.5 g L^{-1} casein and 10 ml L^{-1} 2,4D; pH adjusted to 6.0 using 1 M KOH) containing 0.22 g L^{-1} gluconate. The calli were then

transferred to MS$_3$ medium containing geneticin (Roche^{TM}) after 7 days. The calli were subcultured every two weeks on this media. Once proliferated embryos were visible, the calli were transferred to the light on media in which 2,4 D was replaced with 1 mg L^{-1} kinetin (Sigma-Aldrich^{TM}). Germinated embryos were transferred onto media from which kinetin was omitted and which contained half the concentration of geneticin. Putative transgenic plantlets with well established roots were hardened off. Calli were kept in tissue culture for analysis. Suspension cultures were established from this calli along with NCo310 wild type as controls.

4.2.5. Analysis of transgenic plants

PCR analysis

Genomic DNA (gDNA) was extracted from transgenic sugarcane callus using a modified Tris-buffer method (Dellaporta et al., 1983). Plant material was ground to a fine powder in liquid nitrogen in a pre-cooled mortar and pestle. Ground samples were then transferred to 50 ml tubes containing 35 ml extraction buffer (100 mM Tris-HCl, 500 mM NaCl, 50 mM EDTA, 14.3 mM β-mercaptoethanol). After the tube was vigorously vortexed, 3.5 ml 20% SDS was added and the samples incubated at 70 °C for 60 minutes. Following incubation, 7 ml 5 M potassium acetate was added; the samples were incubated on ice for 20 minutes and then centrifuged for 10 minutes at 4 °C to pellet cellular debris. The supernatant liquid was filtered through a nylon filter. Ice-cold isopropanol (1 volume) was added onto the top of the supernatant liquid and precipitated gDNA was spooled out using a sterile Pasteur pipette. The DNA was resuspended in 1 ml of 1 M NaCl containing RNaseA (10 μg/ml). Samples were incubated at overnight at 37 °C. DNA was then extracted from the solution with one volume chloroform:isoamyl alcohol (24:1). The top layer containing the DNA was removed and the DNA was precipitated with one volume

isopropanol. DNA was then pelleted by centrifugation for 1 hour. The gDNA pellet was washed in 70% ethanol, dried and resuspended in 500 µl MilliQ^{TM} water.

Sugarcane callus transformed with pHairpinAGPase-U3Z

To test for the presence of the recombinant gene, a PCR analysis was conducted using only the forward primer of the primer set that was used for the isolation of the gene as described earlier (Section 4.2.2.). PCR conditions were set at 60 °C for annealing and two minutes extension time. Together with the transgenic clones, a positive control (pHairpinAGPase-U3Z plasmid) and a negative control (wild type NCO310 callus) were analysed. PCR results were assessed by gel electrophoresis.

Sugarcane callus transformed with pUBI-β-amylase

A PCR was conducted on transgenic clones to determine whether the recombinant gene was present. Primers (5'- ATG ACT TTA ACA CTT CAA TCA TC -3' and 5'- TTA CAC TAC TGC AAC CTC TGT AGC T -3'), designed from the *pct-Bmyl* gene, were used to amplify the whole gene fragment. PCR conditions were set at 60 °C annealing time and 2 minutes extension time. Together with the transgenic clones, a positive control (pUBI-β-amylase plasmid) and negative control (water and wild type NCO310 callus DNA) were analysed.

Enzyme activity measurement

Four duplicates of each transgenic line and control lines were grown in MS_3 suspension media (see section 4.2.4) for 14 days. After 14 days, cells were harvested and ground in liquid nitrogen in a mortar and pestle and frozen at -80 °C for later analysis. Protein extractions were performed using the same method as in Section 3.2.5.

AGPase activity was only determined on lines transformed with the AGPase silencing vector. AGPase activity was measured using a modified method of Plaxton and Preiss (1994, see Section 3.2.5).

β-amylase was only performed on lines transformed with the β-amylase over-expression vector. The β-amylase assay was conducted using a β-amylase assay kit as described in section 3.2.5 (Megazyme, Ireland).

Starch measurement

See Section 3.2.3

Soluble sugars measurement

Soluble sugars were measured using the method of Bergmeyer and Bernt (1974).

4.3. Results and discussion

4.3.1. Construction of AGPase silencing vector

After RNA was extracted from sugarcane leaf tissue (Figure 4.1a), cDNA was synthesised and used as template to amplify a partial sequence from the AGPase gene. A fragment of the expected size, i.e. 490 base pairs, was amplified and isolated after gel electrophoreses (Figure 4.1b). This fragment was then cloned into the pGEM-T-Easy vector system (Promega[TM]) and named pGEM-AGPase (Figure 4.1c).
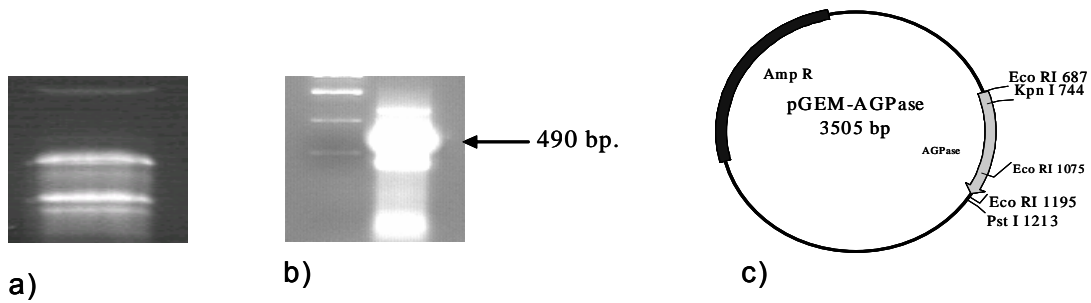
a)   b)   c)

Figure 4.1. Cloning of a partial AGPase sequence from sugarcane leaf tissue. (a) Total RNA extracted from sugarcane leaf tissue. (b) The 490 bp PCR product amplified from cDNA synthesised from sugarcane leaf RNA. (c) Cloning vector pGEM-AGPase containing the partial sequence of AGPase isolated from sugarcane leaf tissue.

pGEM-AGPase was sequenced and the sequence blasted on the GenBank database. Blast results confirmed that the gene fragment in pGEM-AGPase was in fact AGPase since it showed 99% homology to the EST sequence (CA276527) used for the design of the PCR primers. It also showed 95% homology to the maize AGPase gene *brittle-2* (GenBank accession number DQ118037) (figure 4.2).
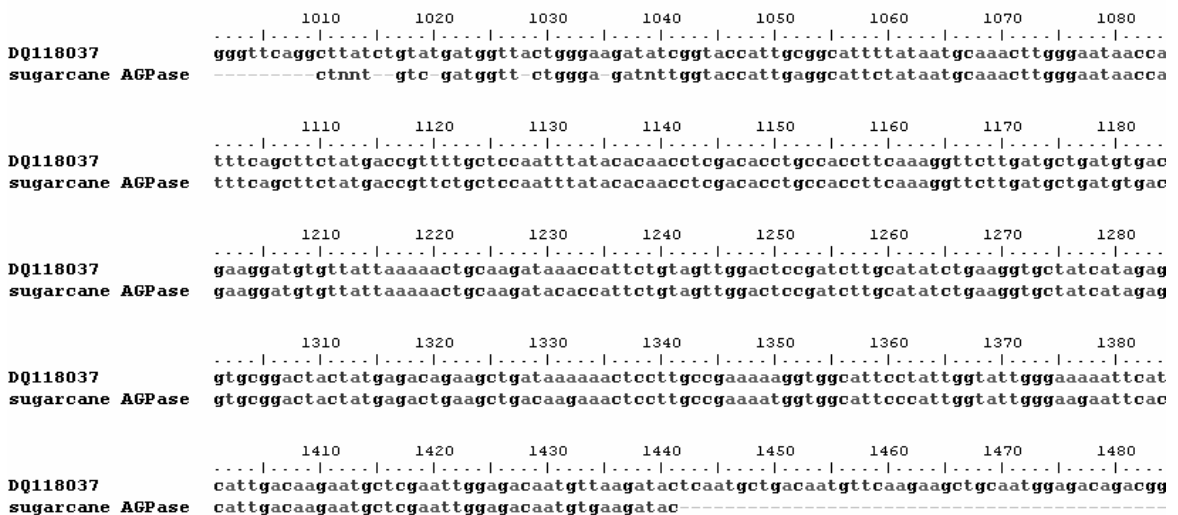

Figure 4.2. Partial gene sequence of AGPase isolated from sugarcane leaf tissue. The sequence showed 95% homology to the maize AGPase small subunit (*bt2*) (GenBank accession number DQ118037).

Oligonucleotides 5A and 5S and 3A and 3S were allowed to hybridise and ligated into pKS and pSK respectively. The success of the experiment was analysed by PCR. The slightly larger fragments (in comparison to the pBluescript SK control) confirmed that the ligation was indeed successful (Figure 4.3a). The intermediate RNAi vectors were named pSK 5'I and pKS 3'I respectively. Plasmid DNA was extracted from these colonies and sequenced to verify the results.
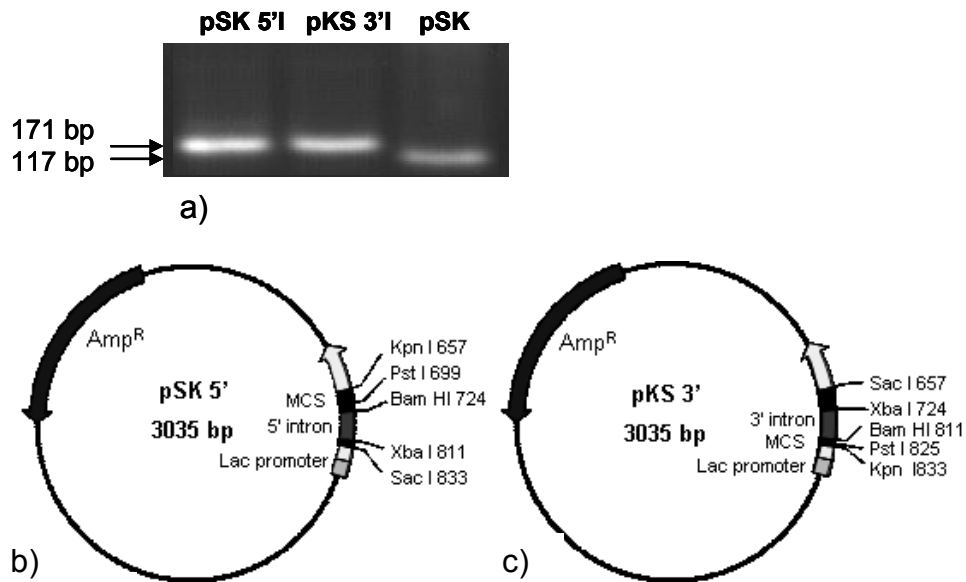

Figure 4.3. Construction of the two intermediate RNAi constructs. a) Gel electrophoresis of PCR products after the ligation of the intron. The 171 bp fragment contains the 54 bp intron regions. The 117 bp fragment is pBluescript vector with no intron region acting as control b) pBluescript KS with 54 bp. intron cloned into *Xba*I and *Bam*HI sites. c) pBluescript SK with 54 bp intron cloned in *Bam*HI and *Xba*I sites.

pSK 5'I and pKS 3'I was ligated with the partial AGPase sequence and designated pSK 5'I AGPase and pKS 3'I AGPase. *Kpn*I and *Xba*I digested fragments were then ligated into the *Kpn*I site of the sugarcane transformation vector pU3Z in a three-fragment ligation (see figure 4.4 for a schematic representation of the cloning process). The vector was then used for *E. coli* transformation and ampicillin was used to select colonies containing the transformation vector. This yielded only one

colony from which plasmid DNA was extracted and restriction analysis was conducted. This revealed the correct size fragments (figure 4.5) and sequencing proved that the vector was correct. The 1073 bp fragment observed in the *Kpn*I/*Xba*I digest is due to plasmid only digested by *Kpn*I and not both *Kpn*I and *Xba*I.
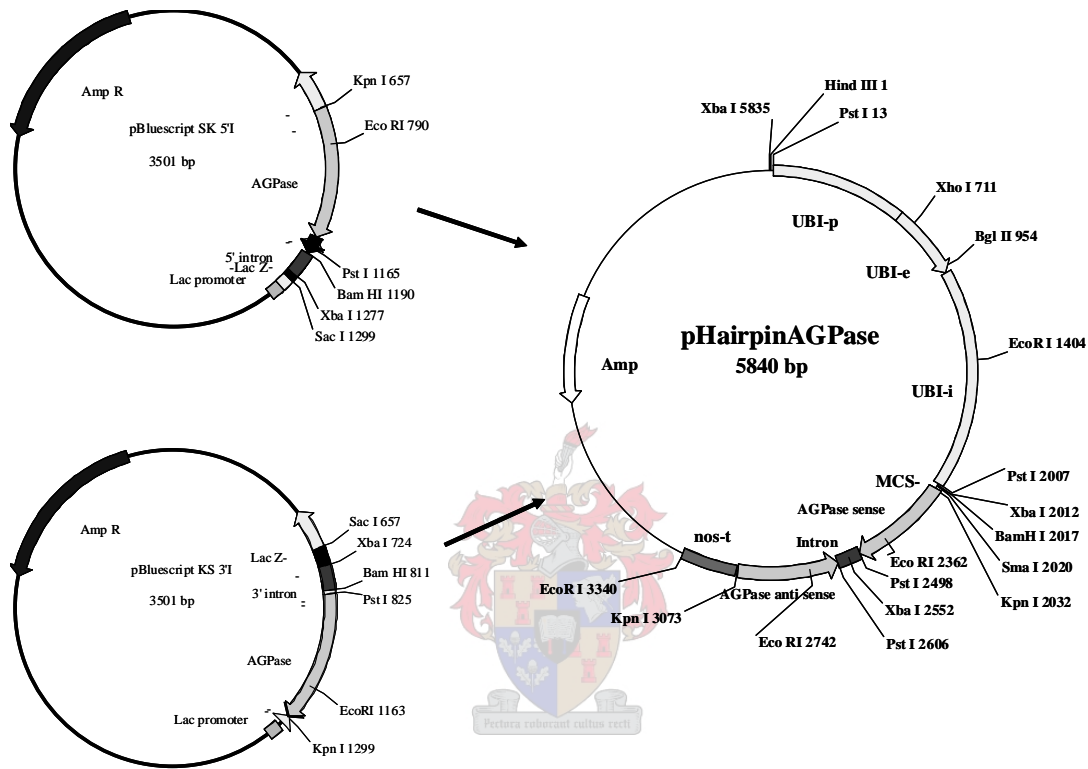

Figure 4.4. Schematic representation of the construction of pHairpin-AGPase. A 0.6 kb *Kpn*I-*Xba*I fragment from pSK 5'I-AGPase and an 0.6 kb *Kpn*I-*Xba*I fragment from pKS 5'I AGPase was cloned into the KpnI site of pU3Z in a three-fragment ligation.
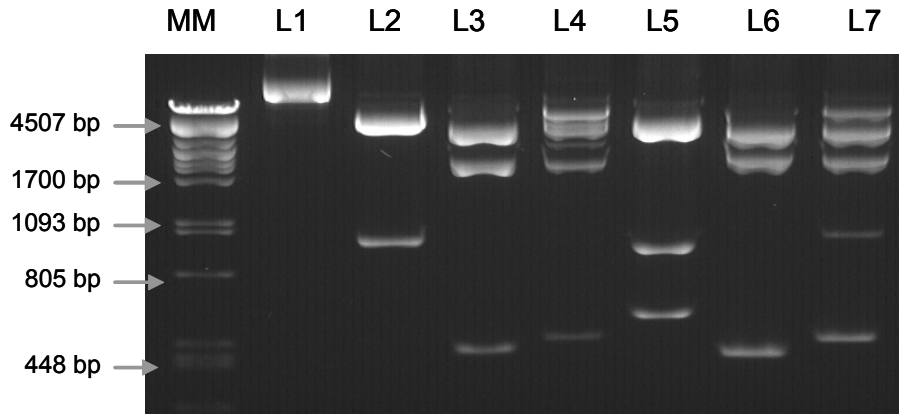
Figure 4.5. Characterisation of silencing construct pHairpinAGPase by restriction analysis: L1-uncut plasmid, L2-*Kpn*I, L3-*Pst*I, L4-*Xba*I, L5-*Eco*RI, L6-*Kpn*I/*Pst*I, L7-*Kpn*I/*Xba*I.

4.3.2. Construction of β-amylase over-expression vector pUBI-β-amylase

The *pctBmyI* β-amylase cDNA sequence (GenBank accession number AF393847) was cut from the pSK-β-amylase cloning vector using *Bam*HI and *Eco*RI. The *Eco*RI site was blunted using Klenow DNA polymerase (Promega™) and the fragment cloned into the *Bam*HI and *Sma*I sites of sugarcane transformation vector pUBI510. This vector was named pUBI-β-amylase (Figure 4.6). After transformation of *E. coli* using this vector, plasmid DNA was extracted from bacterial colonies. Putative clones were screened by restriction analysis. In one of the bacterial colonies analysed a 1032 base pair fragment was observed indicating the successful ligation of the β-amylase cDNA sequence into pUBI510 (figure 4.7). Sequencing confirmed the presence of the gene in the correct reading frame. pUBI-β-amylase was then used for sugarcane transformation.
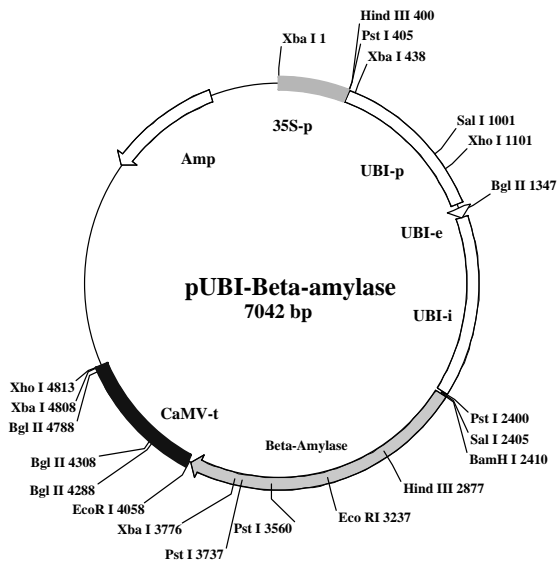
Figure 4.6. β-amylase over–expression vector. The β-amylase clone, *pct-Bmyl* (Scheidig et al, 2002), cloned into the pUBI-510 sugarcane vector using the *BamH*I and *Sma*I sites
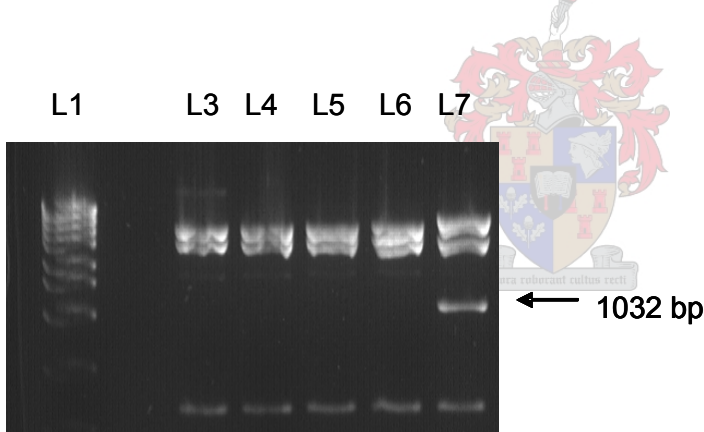
Figure 4.7. Characterisation of the construct pUBI-β-amylase. *Xba*I Restriction analysis of plasmid using *Xba*I. L1 contained the molecular marker and L7 the 1032 base pair fragment indicating a positive clone.

4.3.3. Characterisation of transgenic sugarcane suspension cell cultures

4.3.3.1. Molecular analysis of transgenic calli

After geneticin selection of transformed sugarcane callus, gDNA was extracted from resistant foci. The putative transgenic lines were tested for the presence of the transgenes using PCR. For the lines transformed with pHairpinAGPase-U3Z, the

expected 1073 bp fragment was observed. The positive control was pHairpinAGPase plasmid and negative control NCo310 callus gDNA. For the lines transformed with pUBI-β-amylase, the expected 1638 bp fragment was observed. Although there was unspecific binding of primers, the amplification of the correct band was seen as enough evidence to proceed with experiments. The positive control was pUBI-β-amylase plasmid and the negative control NCo310 callus gDNA. These results confirmed the presence of pHairpinAGPase-U3Z and pUBI-β-amylase in the transformed sugarcane callus (figure 4.8).
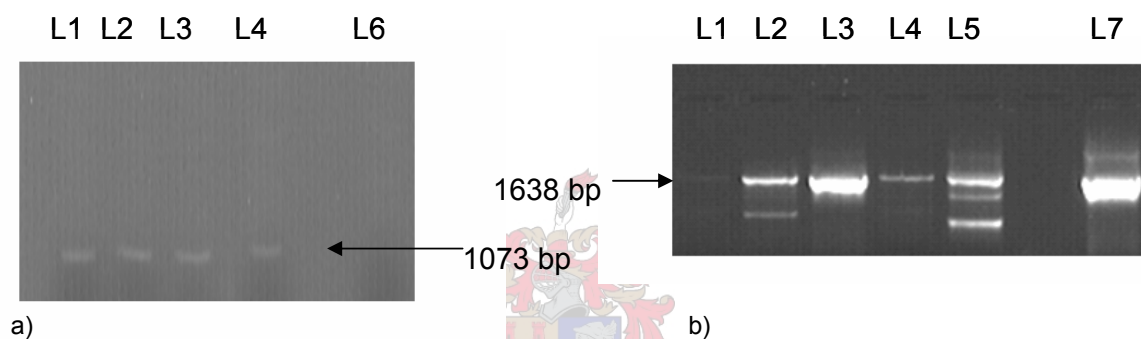

Figure 4.8. Characterisation of putative transgenic sugarcane calli lines. (a) Gel electrophoresis of PCR product after amplification of transgene using forward primer of the AGPase fragment. L1-L4 contains the transgenic lines 9, 10, 23 and the positive control pHairpinAGPase plasmid respectively. The fragment confirms the presence of the transgene. L6 contains the NCo310 wild type negative control. (b) Gel electrophoresis of PCR product after amplification of the transgene using gene specific primers of the transgenic β-amylase. L1 contains the negative control NCo310 callus and L2-L5 the transgenic lines 8, 9, 10 and 11. L7 contains the positive control pUBI-β-amylase plasmid. This fragment confirms the presence of the β-amylase transgene in sugarcane callus.

4.3.3.1. Analysis of transgenic lines with reduced AGPase activity

AGPase activity was reduced in three transgenic lines. The largest reduction in activity was in line ten with only 0.15 ($p \leq 0.01$) times the activity of the wild type control. This was followed by line nine with 0.22 ($p \leq 0.01$) times the activity of the

wild type control and line twenty-three with 0.54 (p ≤ 0.1) times the activity of the wild type control (figure 4.9a)

Starch and soluble sugars concentration

Starch concentration was reduced in the three transgenic lines analysed. Starch concentration in line nine was 0.38 (p ≤ 0.05), line ten was 0.46 (p ≤ 0.01) and line twenty three was 0.47 (p ≤ 0.01) times that of the wild type control (figure 4.9b). There was a significant correlation between the starch concentration and the AGPase activity in the transgenic and wild type control lines (R^2 = 0.58, p ≤ 0.05) (figure 4.9c). This could not be shown in sugarcane internodes (see chapter three), suggesting that in suspension cultures there is a stronger correlation between AGPase activity and starch synthesis than was the case in sugarcane internodes. The reduction in starch content due to a reduction in AGPase supports earlier studies showing that the change in activity of AGPase do have an effect on the starch content (Tsai and Nelson, 1966; Dickenson and Preiss, 1969 and Müller-Röber et al.,1992). There was not a significant effect on sucrose content as was the case in potato tubers (Müller Röber, 1992). There was no significant effect on the sucrose or glucose concentration in the transgenic lines, but there was a significant reduction in fructose concentration in line ten (p ≤ 0.01) (figure 4.9d).
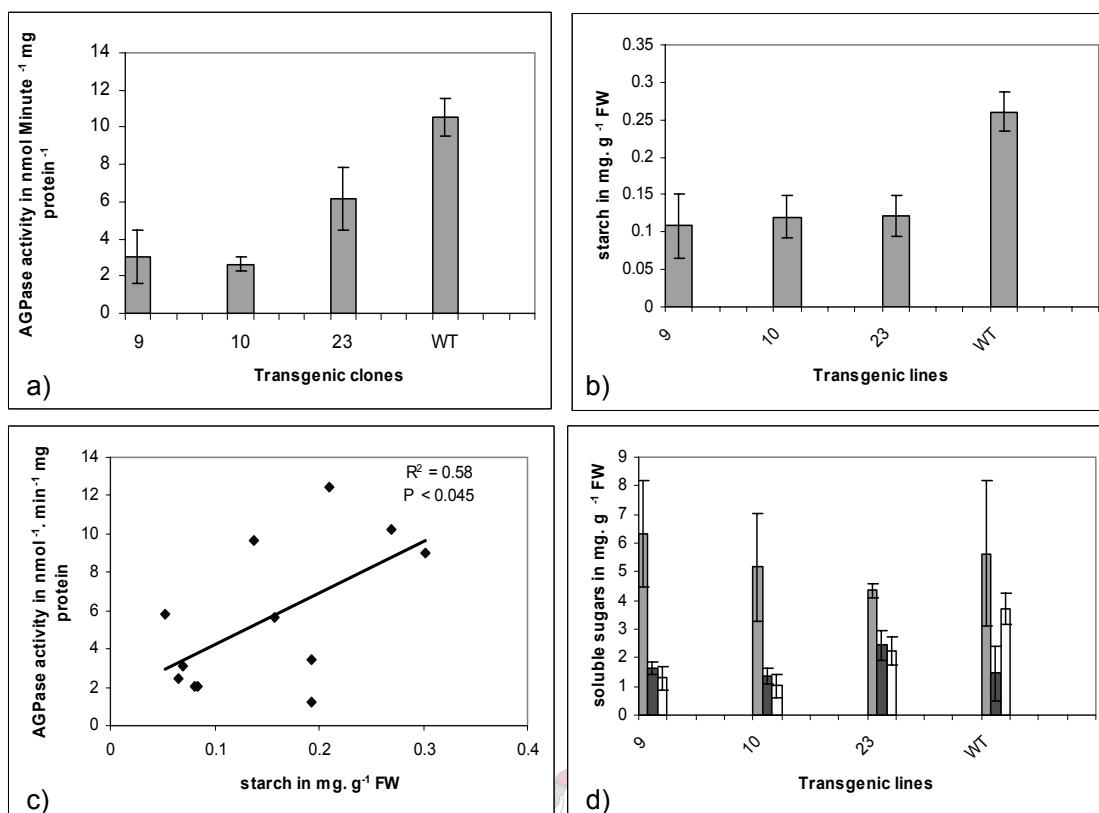
Figure 4.9. Analysis of transgenic clones with reduced AGPase activity. a) AGPase activity in transgenic and control lines. (b) Starch concentration in transgenic lines compared to the control. (c) The correlation between AGPase activity and starch concentration in the transgenic and control lines. (d) Soluble sugars concentration in transgenic and control lines. The columns represent from left to right sucrose, glucose and fructose. The error bars respresent standard error.

4.3.3.2. Transgenic lines with increased β-amylase activity

β-amylase activity was increased in four transgenic lines. The largest increase in enzyme activity was in line ten with a 2 times increase in activity ($p \leq 0.05$). This was followed by line eleven with a 1.6 times increase ($p \leq 0.05$), line nine with a 1.5 times increase ($p \leq 0.05$) and line eight with a 1.5 times increase ($p \leq 0.2$) (figure 4.10a).

Starch concentration and soluble sugars concentration

Starch concentration was reduced in the transgenic lines. The most significant reduction was in line ten, where the starch concentration was only 0.1 ($p \leq 0.01$) times that of the wild type control. This was followed by line eleven with 0.2 ($p \leq$ 0.01) times, line nine with 0.2 ($p \leq 0.01$) times and line eight with 0.4 times ($p \leq 0.2$) the starch concentration of the wild type control. There was a significant correlation between the starch concentration and β-amylase activity ($R^2 = 0.68$, $p \leq 0.01$). This correlation supports data that β-amylase activity plays a role in determining the starch content (Scheidig et al., 2002). No significant change in sucrose concentration of the transgenic lines could be observed. However, there was a significant increase in glucose levels in line eight and a significant increase in fructose in lines eight and nine. Glucose concentration in line eight was 2.9 ($p \leq$ 0.01) times that of the wild type control (Figure 4.10d). Fructose concentration in line eight was 1.8 ($p \leq 0.01$) times and in line nine 2 ($p \leq 0.01$) times that of the wild type control.
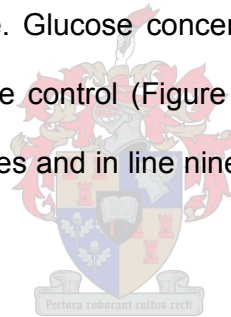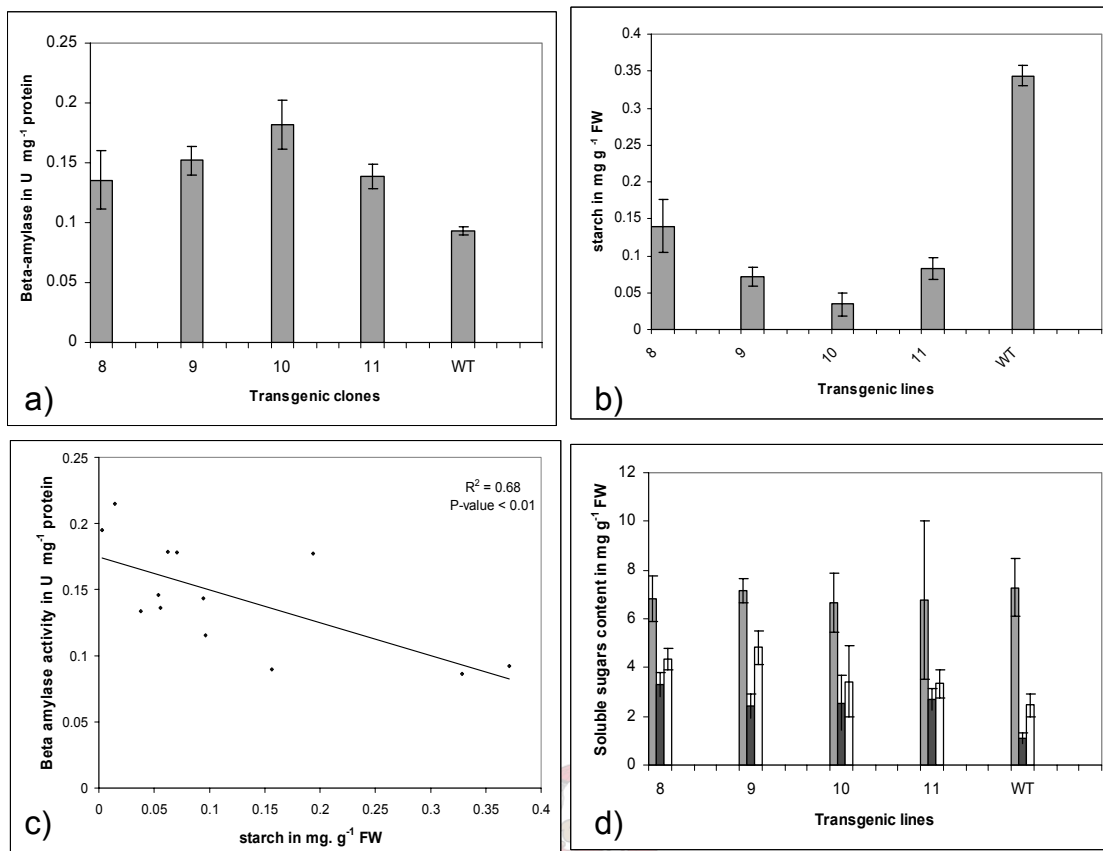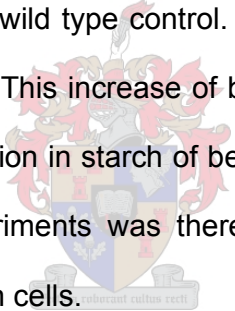
Figure 4.10. Analysis of transgenic clones with increased β-amylase activity (a) β-amylase activity in transgenic and wild type control lines. (b) Starch concentration in transgenic and wild type control lines. (c) The correlation between β-amylase activity and starch concentration in the transgenic and control lines. (d) Soluble sugars concentration in transgenic and control lines. The column represent from left to right sucrose, glucose and fructose. The error bars represent standard error.

4.4. Conclusion

This study was focused on reducing starch in sugarcane by manipulating the activity of two enzymes that are important in starch metabolism, i.e. reducing AGPase and increasing β-amylase activity. To achieve this, two transformation vectors were constructed and used for sugarcane calli transformation. The results were analysed in suspension cultures by measuring enzyme activity, starch and soluble sugars concentration

For AGPase silencing, a novel cloning system based on post transcriptional gene silencing (PTGS) by hairpin RNA (for review see Hammond et al., 2001) was established. The novelty of the cloning system is based on a 3-fragment ligation between the two relevant gene fragments, each containing one half of an intron sequence, and a sugarcane expression vector.

In both experiments, i.e. the reduction of AGPase activity and the over-expression of β-amylase activity, there was a reduction in starch concentration which could be correlated with either the reduction of AGPase activity or the increase of β-amylase activity. AGPase activity was reduced in three transgenic lines to between 0.14 and 0.54 times that of the wild type control. This led to a reduction in starch of between 0.38 and 0.47 times that of the wild type control. β-amylase activity was increased in four transgenic lines studied. This increase of between 1.5 to 2 times that of the wild type control, led to a reduction in starch of between 0.1 to 0.4 times that of the control. In neither of the experiments was there a significant effect on sucrose concentrations in the suspension cells.

Chapter 5

Conclusion

Conclusion

The two main aims of this project were firstly to increase our knowledge of starch metabolism in sugarcane and secondly to manipulate the enzyme activities of AGPase and β-amylase in an attempt to reduce the starch content of sugarcane. Although a lot of information is available on starch and the problems it causes during sucrose extraction and refinement (Cuddihy et al., 1999; Godshall, 1996 and Schoonees, 2003), this study provides the first information on the starch content of current commercially grown sugarcane varieties.

Starch metabolism in sugarcane was investigated by determining the starch content of different sugarcane varieties as well as the activities of certain enzymes involved in starch metabolism. The varieties that were analysed are responsible for over 70% of all sucrose harvested in South Africa (Mike Butterfield, SASRI, personal correspondence).
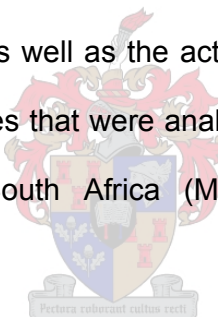
Results illustrated that the starch content of all varieties increased from young to mature internodes, with starch content being the highest at internode nine, after which it stabilises. This trend was true for all varieties, but the starch content varied greatly between varieties. Comparative analysis of the starch content in mature tissues (internodes nine and twelve) showed that the starch concentration of varieties varied between 0.18 mg g^{-1} FW and 0.58 mg. g^{-1} FW, with most varieties having a starch concentration of between 0.26 and 0.32 mg g^{-1} FW. NCo376's starch concentration was much lower at 0.18 mg g^{-1} FW and N19 much higher at 0.51 mg g^{-1} FW. Variation in the starch content of sugarcane varieties have previously been

observed (Godshall et al., 1996) and the low starch content of NCo376 compared to that of NCo310 corresponds to results published by Wood (1962) (figure 3.1a)

Based on the sucrose content in mature tissues, sugarcane varieties could be divided into two groups, i.e. low sucrose and high sucrose varieties. The low sucrose varieties included NCo376, N19 and N27 and had an average sucrose concentration of 36.63 mg g^{-1} FW. The high sucrose varieties included NCo310, N12 and N16 and had a sucrose concentration of 73.2 mg g^{-1} FW. These two groups did differ significantly ($p \leq 0.01$) (figure 3.1b). Results indicated that there is a strong linear correlation between starch and sucrose accumulation ($R^2 = 0.53$, $p \leq 0.01$) (figure 3.2). This could be explained by the fact that these metabolites are synthesised from similar hexose-phosphate pools (Hill and ap Rees, 1994). This does not, however, mean that varieties with high sucrose content had high starch content.

To further characterise starch metabolism, the activities of two enzymes that play an important role in starch metabolism, ADP-glucose pyrophosphorylase (AGPase) and β-amylase, were also determined. Although it has been shown that AGPase is a rate-determining step in starch synthesis in other plants (Tsai and Nelson, 1966; Dickenson and Preiss, 1969 and Müller-Röber et al., 1992) this could not be confirmed in sugarcane internodal tissue and needs further investigation. No correlation between AGPase activity and starch content could be observed (figure 3.3a). However, it has been shown that AGPase is very heat-labile in maize (Hannah et al., 1980), a close relative of sugarcane, and the 24 hour delay from sugarcane harvest to analysis might have led to the enzyme's degradation.

The highest level of β-amylase activity, which is responsible for most of the degradation of linear glucans in the plastid (Scheidig et al., 2002) was observed in the youngest tissue (internode three) after which activity sharply declined as the stalk matured (figure 3.3b). This apparent high breakdown of starch by β-amylase supports earlier studies which have shown that there is a lot of cycling of carbon through starch in younger internodes (Bindon, 2000).

The second aim of this project was to manipulate enzyme activities of AGPase and β-amylase in an attempt to reduce the starch content of sugarcane. This was done by reducing AGPase activity and increasing the activity of β-amylase, and analysing the results in suspension cultures. For AGPase silencing, a novel system for silencing genes in sugarcane was created. The system is based on post transcriptional gene silencing (PTGS) by hairpin RNA that leads to RNA interference (for review see Hammond et al., 2001) (figure 4.4). The novelty of the system is that the pBluescript vectors SK and KS, together with a sugarcane expression vector, is used to create a vector that leads to the formation of hairpin RNA.

Three transgenic lines and a control of NCo310 were analysed in suspension cultures and a significant reduction in AGPase enzyme activity was observed (figure 4.9a). This, in turn, led to a reduction in starch production (figure 4.9b) which could be correlated with AGPase activity (figure 4.9c). The reduction in starch due to reduction in AGPase was in line with earlier studies in potato where the reduction of AGPase by anti-sense led to a 94% reduction in starch in the tubers (Müller-Röber et al., 1992). Although Müller-Röber et al. (1992) also reported a ten fold increase in sucrose, no effect on sucrose levels were detected in sugarcane suspension cultures. The reason for this could be that since sugarcane is not a major starch

storing plant, even in suspension cultures where there is about seven times more starch than in mature sugarcane internodes, the effect of reducing starch will not have a huge effect on carbon flux. In potato tubers, on the other hand, there will be a big flux shift towards sucrose when the enzyme activity of AGPase is decreased.

To increase β-amylase activity, a β-amylase gene with proven ability to degrade starch (*pct-BmyI* (Scheidig et al., 2002)) was cloned into the sugarcane transformation vector pUBI510 to yield pUBI-β-amylase (figure 4.6). This vector was then used for sugarcane transformation. Four transgenic lines and a control line of NCo310 were analysed in suspension cultures and a significant increase in β-amylase activity was observed (figure 4.10a). This increase in activity led to a reduction of starch content in the transgenic lines that could be correlated with the reduced enzyme activity (figure 4.10b and 4.10c). Again there was no significant effect on sucrose content in the transgenic lines (figure 4.10d).

This study was conducted in non-photosynthetic tissue and the problems that could arise from reducing starch in photosynthetic tissue (leaves) were not investigated. This will only be investigated in regenerated sugarcane. The constitutive Ubiquitin promoter that was used for transgene expression will lead to transgene expression in leaves also which might have a negative effect on photosynthesis when whole plants are analysed. Starch is not only important as a storage carbohydrate for plants, but also serves an important physiological role as the end product of photosynthesis. Alterations in leaf starch levels might lead to altered rates in photosynthesis, because unlike sucrose it utilises triose-phosphates in the plastid (Sun et al., 1999). It also serves as a phosphate source during periods of phosphate limitation (Kossmann and Lloyd, 2000).

To conclude, this study showed that although there is very little starch in sugarcane internodes there were definite patterns of accumulation. The starch content was constantly almost two hundred times lower than the sucrose content. The apparent lack of patterns in the expression of AGPase will have to be further investigated. β-amylase activity was the highest in the youngest tissue,supporting partitioning data showing there is cycling of carbon through starch in these internodes (Bindon, 2000). Experiments to reduce the starch content of sugarcane in this study was successful and showed that, at least at suspension culture level, sugarcane does follow familiar patterns of starch metabolism. It will be of scientific as well as industrial interest to determine whether the same patterns could be observed in regenerated transgenic sugarcane plants. If this is indeed the case, the production of sugarcane with lower starch could reduce the industry dependency on the addition of α-amylase to reduce starch in sugar mills.

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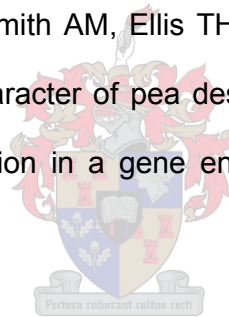
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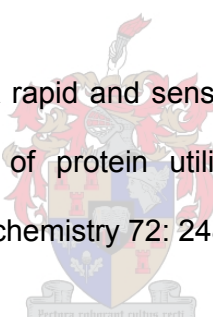
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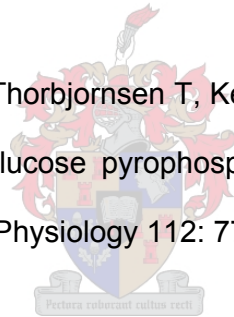
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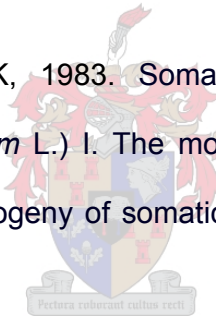
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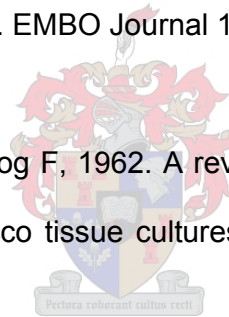
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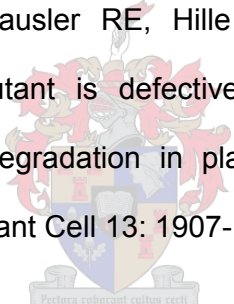
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