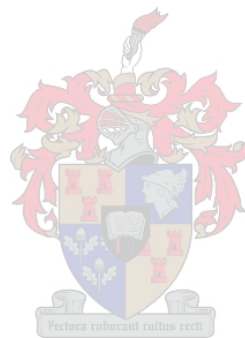# The characterization of Vacuolar Pyrophosphatase expression in sugarcane

by

Johannes Cornelius Swart

Thesis presented in fulfilment of the requirements for the degree of

Masters of Science at Stellenbosch University

April 2005

Supervisor: JH Groenewald

Co-Supervisor: FC Botha

DECLARATION

I, the undersigned, herby declare that the work contained in the thesis is my own original work, and that I have not previously, in its entirety or in part, submitted it at any university for a degree.

1 December 2004

ABSTRACT

Vacuolar Pyrophosphatase (V-PPase) has never been studied in sugarcane before and to date nothing is known about V-PPase in sugarcane, except for the sequences of a few expressed sequence tags (ESTs). The aim of this project was to characterize V-PPase expression in several hybrid sugarcane varieties that differ significantly in sucrose content, with the main objective of the study to assess whether V-PPase is correlated in any way to the sucrose storage phenotype. Therefore, the goals of this project were to (i) develop molecular tools for the detection and quantification of V-PPase on a DNA, RNA, protein and enzyme level and (ii) to use these tools to characterize the expression of V-PPase within the culm of the three hybrid varieties.

The cDNA sequence of the catalytic subunit of the sugarcane V-PPase gene was cloned, expressed in a bacterial system and the V-PPase peptide was purified. This peptide was used for the immunization of mice and the production of polyclonal anti-VPPase antiserum. Anti-VPPase antiserum reacted specifically with a single polypeptide among vacuolar membrane proteins. Moreover, anti-VPPase antiserum recognized V-PPase from various monocotyledons and dicotyledons. The anti-VPPase antiserum was used for the establishment of an ELISA system to determine V-PPase protein content in vacuolar membrane preparations. This system proved to have several advantages over the protein blotting technique and shared a strong linear relation with V-PPase specific activity, showing that these two tests are compatible and reliable. The optimisation of sugarcane V-PPase zero-order kinetics was fundamental in order to measure V-PPase specific activity accurately. It had a relative broad pH optimum, retaining more than 90% of its maximum activity between pH 6.50 and 7.25. V-PPase required both Mg^{2+} and K^+, in addition to PPi, for maximum activity *in vitro*. The reported kinetic variables are within range of previous data determined for other species, including mung bean, red beet and sugar beet.

V-PPase protein level and specific activity within the sugarcane culm followed a similar trend , withoiofofoenaobserved for sucrose accumulation rates observed in sugarcane. Moreover, V-PPase protein contents and specific activity share the same general trend as total sucrose content in a specific tissue compared among the three varieties. No significant differences were observed in V-ATPase activity among the three varieties. Our findings suggest that V-PPase may play a role in sucrose accumulation in sugarcane.

OPSOMMING

Vakuolêre Pirofosfatase (V-PPase) is nog nooit in suikerriet gekarakteriseer nie en tot op hede is daar geen literatuur oor V-PPase in suikerriet beskikbaar nie, behalwe vir die volgordes van enkele ESTs. Die doelwit van hierdie projek was om die uitdrukking van V-PPase te karakteriseer in verskeie hibried variëteite wat beduidend in sukrose inhoud verskil met die hoof doelwit van die studie, om vas te stel of V-PPase op enige manier gekorrileer kan word met die sukrose storingsfenotipe. Dus, die onderskeie mylpale van die projek om hierdie doelwit te bereik was (i) die ontwikkeling van molekulêre instrumente om V-PPase te karakteriseer en kwantifiseer op DNA-, RNA-, protein- en ensiem vlak en (ii) die gebruik van hierdie instrumente om V-PPase uitdrukking in die stingel van die drie variëteite te karakteriseer.

Die cDNA-volgorde van die katalitiese sub-eenheid van die suikerriet V-PPase ensiem is gekloneer, in 'n bakteriële uitdrukkingsisteem uitgedruk en daarna is die V-PPase peptied gesuiwer. Die peptied is daarna vir die immunisering van muise en die produksie van poliklonale anti-VPPase antiserum gebruik. Die anti-VPPase antiserum het spesifiek met 'n enkele polipeptied van die vakuolêre membraan proteine gereageer. Die anti-VPPase antiserum het ook die V-PPase proteine van verskeie mono- en dikotiele spesies herken. Die anti-VPPase anti-serum is verder vir die daarstelling van 'n ELISA sisteem gebruik vir die bepaling van die hoeveelheid V-PPase protein in vakuolêre membraanpreparate. Hierdie sisteem het verskeie voordele bo die algemene proteien oordrag tegniek getoon. Die ELISA sisteem het 'n lineêre verwantskap met V-PPase spesifieke-aktiwiteit gehad, wat bewys dat hierdie twee toetse vergelykbaar en akkuraat is.

Die bepaling van die suikerriet V-PPase se nul-orde kinetika was fundamenteel om te verseker dat V-PPase se spesifieke-aktiwiteit, akkuraat gemeet sal word. Dit toon 'n breë pH optimum en behou meer as 90% van die maksimum aktiwiteit tussen pH 6.50 en 7.25. V-PPase benodig beide Mg^{2+} en K^+, addisioneel tot PPi, vir maksimale *in vitro* aktiwiteit. Die vermelde kinetiese verandelikes van suikerriet V-PPase in hierdie studie is min of meer dieselfde as wat deur vorige navorsers vir ander spesies, insluitend boontjies, rooibeet en suikerbeet gerapporteer is.

Die suikerriet V-PPase nul-orde kinetika parameters en die ELISA sisteem is verder gebruik om V-PPase uitdrukking in die kolom van die drie hibried variëteite te karakteriseer. 'n Soortgelyke patroon is waargeneem vir V-PPase proteienvlakke en spesifieke-aktiwiteit in die suikerriet kolom aan die patroon wat waargeneem word vir sukrose akkumuleringstempo in die suikerriet stingel. Die hoeveelheid V-PPase protein en spesifieke-aktiwiteit toon ook dieselfde algemene patroon as sukrosevlakke tussen die hibried variëteite in 'n spesifieke weefsel. Geen beduidende verkil in V-ATPase spesifieke-aktiwiteit is tussen die drie variëteite waargeneem nie. Ons bevindinge dui daarop dat V-PPase moontlik 'n rol mag speel in die akkumulering van sukrose in suikerriet.
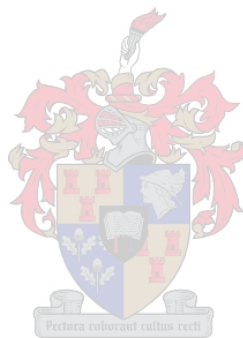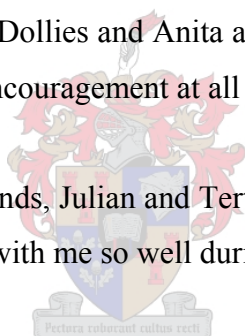
ACKNOWLEDGEMENTS

Thank you seems to be inadequate to use when I think about Hennie Groenewald. Without his support, encouragement and advice every day for the past two years, I would not have been able to complete this project. He is the most-dedicated supervisor that any student can ask for. I look forward to working with him during my doctorate.

I would like to thank Prof. Frikkie Botha for all his invaluable input in this project. His passion for science has encouraged me and strengthened my mind to something I will be grateful for throughout my career.

I appreciate the support of the staff and students of the Institute for Plant Biotechnology. Fletcher Hilten and Sue Bosch, thanks for all your advice whenever I burst in on you.

I would like to thank my parents, Dollies and Anita and my two brothers, Diederik and Petrus for their unconditional love and encouragement at all times.

Special thanks to my two best friends, Julian and Tertius Boshoff. I appreciate everything that you did for me and that you bore with me so well during the last two years.
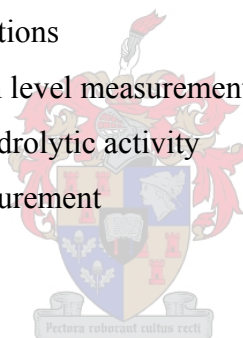
TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

FIGURES

6.1 Simplified illustration of the possible role that V-PPase may play in sucrose accumulation.

TABLES

LIST OF ABBREVIATIONS

°C	degrees centigrade
APS	ammonium persulfate
ATP	adenosine 5'-triphosphate
bp	Base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Da	Dalton
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
DNA	deoxyribo nucleic acid
DTT	1,4-dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
e.g.	for example
EST	expressed sequence tag
FW	fresh weight
g	gram
x g	gravitational force
G6PDH	glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
gDNA	genomic DNA
GST	glutathione S-transferase
H	hour
IgG	Immunoglobulin G
IPTG	isopropyl-beta-D-thiogalactopyranoside
J	Joule
K_m	substrate concentration producing half maximal velocity
L	Litre
M	molar
min	minute
PAGE	polyacrylamide gel electrophoresis
PPase	pyrophosphatase
PFK	6-phosphofructokinase (EC 2.7.1.11)
PFP	pyrophosphate-dependant phosphofructokinase (pyrophosphate:D-

	fructose-6-phosphate 1-phosphotransferase, (EC 2.7.1.90)
PPi	inorganic pyrophosphate
Pi	inorganic phosphate
PVPP	polyvinylpolypyrrolidine
RNA	ribonucleic acid
rpm	revolutions per minute
SASRI	South African Sugarcane Research Institute
SDS	sodium dodecyl sulphate
SE	standard error
SPS	sucrose phosphate synthase (UDP-glucose:D-fructose-6-P 2-α-D-glucotransferase, EC 2.4.1.14)
SuSy	sucrose synthase (UDP-glucose:D-fructose-2-α-D-glucosyl-transferase, EC 2.4.1.13)
TEMED	N,N,N',N'-Tetramethylethylenediamine
TBE	tris-borate/EDTA electrophoresis buffer
TBST	tris-buffered saline containing Tween
TE	tris/EDTA
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	uridine 5'-diphosphate
UDPGlc	uridine 5'-diphosphoglucose
UGPase	uridine 5'-diphosphoglucose pyrophosphorylase
UTP	uridine 5'-triphosphate
UV	ultra violet
V-ATPase	vacuolar H^+-translocating ATP phosphatase, EC 3.6.1.3
V-PPase	vacuolar H^+-translocating inorganic pyrophosphatase , EC 3.6.1.1

CHAPTER 1

GENERAL INTRODUCTION

Sugarcane (*Saccharum spp.*) is a C_4 grass cultivated in tropical and subtropical regions around the world. It is a crop plant that accumulates carbohydrate in the form of sucrose and under optimal conditions, commercial varieties have the capacity to store up to 25% of their fresh weight as sucrose (Moore and Maretzki 1997). Moreover, cane sugar represents 75% of the sucrose consumed globally; the rest made up by sugar beet. South Africa is the sixth largest exporter of sucrose in the world and earned R1.7 milliard in foreign exchange during the 2002/3 season (sasa.org.za). Approximately 240 000 jobs are provided directly and indirectly by the sugarcane industry, which means that at least a million people are dependent on the sugar industry in South Africa (sasa.org.za).

Since the late 1800's, the increase in sucrose yield has been accomplished through conventional breeding programmes. In Australia, varietal improvement has been estimated to have increased sucrose yield by 1 - 1.5% per annum over the last 50 years, while maintaining disease resistance and sugar quality standards (Chapman 1996). This increase in sucrose yield is the result of carbon partitioning modification and the overcoming of productivity barriers in both the source and sink organs, through these conventional breeding programmes (Moore et al. 1997). Unfortunately this rate of yield increase is well below that achieved in other major field crops such as maize, rice and wheat (Moore 1989). One possible significant factor believed to have contributed to the lack of progress in improving stem sucrose content, is the narrow gene pool currently being used in these breeding programs (Grof and Campbell 2001).

The challenge currently facing the industry is to further increase productivity, although it is recognised that there is a reciprocal relationship between growth rate in plant tissue and sucrose recovery (Komor et al. 1987). The biophysical capability of the sugarcane stem to accommodate a significant increase in sucrose concentration has been assessed by Moore et al. (1997) and concurs with an extrapolation derived by Bull and Glasziou (1963) that the *Saccharum* complex is potentially capable of storing more than 25% sucrose on a fresh weight basis. This estimate is almost double the amount of sucrose stored by current commercial varieties (Grof and Campbell 2001). Within this context, there is considerable scope to exploit the modern sugarcane varieties for enhanced field performance.

Sucrose accumulation have been studied more in sugarcane than any other plant, because it accumulates very high concentrations of this metabolite (Hawker et al. 1987). The principal steps of the metabolic pathways of sucrose synthesis and hydrolysis have been known in plants for more than 20 years. However, the regulation of sucrose synthesis and storage is not yet completely understood and attempts to increase sucrose accumulation in sugarcane through genetic engineering has failed (Grof and Campbell 2001). A prodigious amount of work in groups spearheaded by prominent researchers such as Stitt and the Hubers has provided much detail on the modulation of the pathways of sucrose synthesis in particular (Huber and Akazawa 1986; Stitt et al. 1987). To achieve the ultimate aim of increasing the concentration of sucrose in the stem of the sugarcane plant, it is necessary to identify the principal rate limiting or co-limiting steps in the entire sucrose accumulation process. Current research to improve sugarcane productivity has taken the viewpoint that the product yield of photosynthate is limited at a sink rather than a source level (Gifford and Evans 1981; Krapp et al. 1993). Therefore, an understanding of the regulation of sucrose import and storage at the sink level becomes essential. From this perspective, the identification of key regulatory pathways in metabolism is a prerequisite for the ultimate genetic engineering, to direct and increase carbon allocation to one or more sinks. Grof and Campbell (2001) identified the rate of sucrose transport into the storage parenchyma and vacuoles of sugarcane as one of these rate-limiting steps of sucrose accumulation.
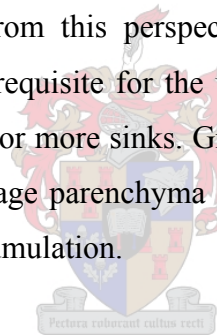
Another rate-limiting or controlling step of sucrose accumulation in plants may be pyrophosphate (PPi) metabolism and therefore PPi-linked enzymes. Several cytosolic enzymes are dependent on PPi. Plant cells contain a considerable pool of PPi in the cytosol (Chanson et al. 1985; Edwards and Rees 1986), whereas the cytosol of higher plants cells contains little or no soluble pyrophosphatase and alkaline pyrophosphatase is only located in the plastids (Gross and Stitt 1986; Weiner et al. 1987). One of these PPi-linked enzymes that has previously been shown to have an influence on sucrose synthesis and the PPi status of the plant cell is pyrophosphate:fructose-6-phosphate-1-phophotransferase (PFP), which plays a role in glycolysis analogous to that of phosphofructokinase (PFK) (Stitt 1990; Kruger 1997). Whittaker and Botha (1999) have shown that sugarcane PFP internodal specific activity varies significantly between varieties and is inversely correlated with sucrose contents. Supporting the theory that PPi plays an important role in sucrose metabolism, constitutive overexpression of soluble pyrophosphatase resulted in higher levels of sucrose and reducing sugars, increased uridine 5'-diphosphoglucose (UDPGlc), decreased levels of phosphates and other

phosphorylated intermediates, and less starch, compared to wild type potato tubers (Sonnewald 1992; Jellito et al. 1992). Apart from PFP several other metabolic reactions are dependent on PPi: cytosolic sucrose mobilization via sucrose synthase (SuSy), UDPGlc pyrophosphorylase (UGPase), fructokinase (Stitt 1990; Kruger 1997) and V-PPase (Maeshima 2000). These PPi-consuming reactions are ubiquitous in higher plants and are often present at high activities (Stitt 1990; Kruger 1997).

Thus, in context with the above statements that the rate of sucrose transport into the storage parenchyma and vacuoles as well as the PPi status of plant cells may be some of the rate-limiting steps of sucrose accumulation, the focus of this study was the characterization of an H^+-pumping inorganic pyrophosphatase situated in the vacuolar membrane (tonoplast). The vacuole of higher plants is a dynamic, acidic organelle that occupies more than 90% of the cell's volume (Maeshima 2001). The vacuole governs numerous cellular processes, including the regulation of cytosolic homeostasis, recycling of cellular components, space filling and the storage of inorganic ions, organic acids and sugars (Hedrich and Schroeder 1989; Maeshima et al. 1996; Taiz 1992). Many of these processes are directly or indirectly related to either the transmembrane electrochemical gradient across the vacuolar membrane or the acidic pH in the vacuole. The vacuolar membrane of plant cells contains two distinct H^+ pumps, i.e. vacuolar H^+-translocating ATPase (V-ATPase; EC 3.6.1.3) and H^+-translocating inorganic pyrophosphatase (V-PPase; EC 3.6.1.1) (Rea and Poole 1993; Rea and Sanders 1987; Sze 1985). Both these enzymes catalyse the electrogenic H^+-translocation from the cytosol to the vacuole lumen to generate an inside-acid pH difference and an inside-positive electrical potential difference (Rea et al. 1992). The H^+-gradient generated by these two proton pumps powers the secondary active transport of various metabolites and solutes, including sucrose across the vacuolar membrane (Hedrich and Schroeder 1989; Taiz 1992).

The V-PPase, however, has the unusual characteristic of exclusively using PPi as an energy source, whereas V-ATPases use ATP (Rea et al. 1992). PPi, the substrate of V-PPase, is one of the key regulatory substrates of several cytosolic abundant enzymes and sometimes under energy limited conditions an enigmatic alternative to ATP (Stitt 1998). From a theoretical point of view, it is feasible that V-PPase may influence sucrose and phosphate metabolism in two ways because of its specific properties (Refer to Chapter 2, section 2.5, The role of PPi and V-PPase in sucrose metabolism). Firstly, V-PPase may facilitate an important contribution to the disposal of cytosolic PPi, which could if accumulated, inhibit sucrose

synthesis and gluconeogenisis and favour the breakdown of sucrose through glycolysis. Secondly, V-PPase may use PPi as an energy donor to increase the vacuolar sink strength, by generating a proton motive force across the vacuolar membrane to drive the secondary transport of sucrose from the cytosol to the vacuole.

To date, nothing is known about V-PPase in sugarcane except for the sequences of a few ESTs. In this study, the expression of V-PPase was characterized in three hybrid varieties, US6656-15, NCo376 and N24, which differ significantly in sucrose content (Whittaker and Botha 1999). The main aim of this project was to assess whether V-PPase activity correlates to the sucrose storage phenotype in any way. Five different goals were identified to accomplish the main aim of this project: 1. The molecular cloning of the sugarcane V-PPase catalytic site and the introduction of this cDNA into a bacterial expression system for protein production and purification (Chapter 3). 2. The production of anti-VPPase polyclonal antiserum and the establishment of an ELISA system for V-PPase protein determinations (Chapter 3). 3. The determination of the zero-order conditions for sugarcane V-PPase substrate hydrolysis (Chapter 4). 4. The characterization of V-PPase expression within the sugarcane culm of three commercial varieties that differ significantly in their ability to accumulate sucrose (Chapter 5) and finally (5) to determine if the total extractable V-PPase activity is correlated to the sucrose storage phenotype in any way (Chapter 5).

This project forms the basis of future investigations to elucidate the potential role of V-PPase in PPi and sucrose metabolism with transgenic technology - a possible rationalization to an intricacy spanning over more than twenty years.

CHAPTER 2
Vacuolar H$^+$-Inorganic Pyrophosphatase: 1989-2004

The vacuoles of plant cells are multifunctional organelles that are central to cellular strategies of plant development (Marty 1999). In plant cells the vacuole is an acidic dominant organelle, which characteristically occupies more than 90% of the total intracellular volume of most mature cells (Maeshima 2001). Vacuoles in higher plants are functionally related to the vacuoles of algae, yeast and the lysosomes of animal cells, containing a variety of hydrolytic enzymes, although their functions are remarkably diverse (Boller and Weimken 1986; Taiz 1992). They are lytic compartments, function as reservoirs for ions, metabolites and proteins, including pigment, and moreover, are crucial to the processes of detoxification and general cell homeostasis (Nishimura and Beevers 1979; Wink 1997; Leigh 1997; Rea et al. 1998; Martinoia et al. 1993). Vacuoles are involved in cellular responses to environmental and biotic factors that provoke stress (Blumwald and Poole 1987; Apse et al. 1999). In the vegetative organs of plants they are the driving force for hydraulic stiffness and growth (Maeshima et al. 1996). In seeds and specialized storage tissues, they serve as sites for storing reserve proteins and soluble carbohydrates (Shewry et al. 1995). Correspondingly, the vacuole plays a prominent role in many important physiological processes, including: metabolite storage, pH, and ionic homeostasis (Boller and Weimken 1986; Taiz 1992; Maeshima et al. 1996). A large number of proteins in the vacuolar membrane (tonoplast) support the function of multifaceted vacuoles, including carriers, ion channels, receptors, structural proteins and active pumps (Maeshima 2001).

2.1 Vacuolar membrane H$^+$-pumps

Several major proteins of the tonoplast have been extensively investigated and information on their molecular properties has accumulated over the past decade. The two most abundant protein groups of the tonoplast are vacuolar H$^+$ pumps and water channels (aquaporins) (Maeshima 2001). The vacuolar membrane of plant cells contain two distinct H$^+$ pumps i.e., vacuolar H$^+$-translocating ATPase (V-ATPase; EC 3.6.1.3) and vacuolar H$^+$-translocating inorganic pyrophosphatase (V-PPase; EC 3.6.1.1) (Sze 1985; Rea and Sanders 1987; Rea and Poole 1993). Although each enzyme is specific in its use of its respective substrate, both catalyse the electrogenic H$^+$ translocation from the cytosol to the vacuolar lumen to generate an inside-acid pH and a cytosol-negative electrical potential difference (Ikeda et al. 1991; Rea et al. 1992). According to the chemiosmotic model for energy-dependent solute transport, the proton-motive force generated by either V-ATPases or V-PPase can be used to drive

secondary transport of various solutes into the vacuole, including ions, amino acids, xenobiotics and sugars (Sze 1985; Hedrich and Schroeder 1989; Hedrich et al. 1989). This proton-motive force can also be related to the control of cell volume and cell turgor and the regulation of cytoplasmic ions and pH (Boller and Weimken 1986; Taiz 1992; Maeshima et al. 1996; Maeshima 2000b). Unlike the complex V-ATPases involved in ATP synthesis (Boyer 1997), V-PPase is representative of simple energy-transducing enzymes. This proton pump consists of a single polypeptide and its substrate, inorganic pyrophosphate (PPi), is one of the simplest high-energy compounds in the cell (Baltscheffsky et al. 1999; Maeshima 2000b).

Among the membrane proteins in vacuoles, V-ATPase and V-PPase have been well characterized in terms of their molecular structures and enzymatic properties (Maeshima and Yoshida 1989; Gogarten et al. 1989; Rea et al. 1992; Sze et al. 1992; Matsuura Endo et al. 2003). Most of what is known of H^+ pumping PPases, was derived from studies of the two prototypes: the vacuolar H^+-PPase (V-PPase) from plants and the H^+-PPi synthase from *Rhodospirillum rubrum* (*R. rubrum*). Prototypical plant V-PPases are found primarily in vacuolar and Golgi membranes and have a near obligatory requirement for millimolar K^+ for activity. Moreover, they appear to operate predominantly in a hydrolytic mode (pumping H^+ at the expense of PPi) and contribute to the transmembrane H^+ gradient that drives the secondary transport of a broad range of solutes in and out of the vacuole (Rea et al. 1992; Rea and Poole 1993). The prototypical phototropic bacterial H^+-PPi synthase found in the chromatophores of some purple, nonsulfure α-proteobacteria is, by comparison, relatively insensitive to monovalent cations, freely reversible and considered to be responsible for the maintenance of H^+ gradients across photosynthetic membranes (when irradiance is insufficient to sustain direct H^+-coupled ATP synthesis) through the use of photosynthetically produced cellular PPi reserves (Baltscheffsky et al. 1999; Nyren and Strid 2004). H^+ pumping PPases are collectively termed 'V-PPase'. These pumps are composed of a single 75-81 kDa, 14-16-span intrinsic membrane protein, and belong to a fourth category of H^+-phosphohydrolase, distinct from F_1F_0 plasma-membrane and vacuolar H^+-ATPases (F-, P- and V-ATPases, respectively)(Rea et al. 1992; Zhen et al. 1997).

2.1.2 Pyrophosphatase families

Soluble PPases of both prokaryotes and eukaryotes have been shown to form a large family of homologous enzymes. Although the cytoplasmic PPase has not yet been purified from plants

(Maeshima 2000a), a putative soluble PPase gene has been cloned from *Arabidopsis* (Kieber and Signer 1991), potato (Rojans-Beltran et al. 1999) as well as barley (Visser et al. 1998). In addition to the well-known soluble PPases, membrane-bound PPases have recently been identified in the plant thylakoid membrane (Jiang et al. 1997) and plant mitochondria (Zancani et al. 1995). These membrane-bound PPases, however, did not show proton pump activity. For the interim, PPases in a wide variety of organisms can be divided into three categories, namely: membrane associated PPase, soluble PPase, and H^+-PPase (Table 2.1). Only H^+-PPases, which are found in the vacuolar membranes of plants and *Rhodospirullum rubrum* (chromophore), have the ability to transport protons across the tonoplast membrane (Maeshima 2000b).

2.2 V-PPase: Gene and Protein properties

2.2.1 Molecular cloning

To date, several V-PPase cDNA clones have been reported from various plants. The first cDNA clone for V-PPase was isolated from *Arabidopsis* (Genbank accession no. M81892). This was accomplished by immunological screening of an expression library with an anti-VPPase antibody to the mung bean enzyme. Only a single copy was isolated from barley (D13472) (Tanaka et al. 1993), mung bean (AB009077) (Nakanishi and Maeshima 1998) and pumpkin (D86306) (Maruyama et al. 1998). Two V-PPase cDNA species were isolated from red beet (L32791, L32792) (Kim et al. 1994) and rice (D45383, D45384) (Sakakibara et al. 1996), whereas three V-PPase cDNA species were isolated from *Nictotiana tabacum* (TVP5, TVP9 and TVP 31) (Lerchl et al. 1995). In these cases, the nucleotide sequence of the different clones is highly homologous within the coding region, but differs significantly in the untranslated regions (Maeshima 2000a). Therefore, these different genes for V-PPase may be differentially, individually regulated in plants. Kim et al. (1994) reported a difference in the transcript levels in leaves and roots of red beet between the two isoforms BVP1 and BVP2. Lerchl et al. (1995) isolated 24 cDNA clones for V-PPase from tobacco, and grouped them into three different classes. They found that the levels of mRNAs for these V-PPase isoforms were different in several tissues, such as leaf, stem, root, sepal, and petal. These findings indicate that the number of V-PPase isoforms differs between species and that they are differentially regulated.

Table 2.1. Classification of inorganic pyrophosphatases

Ppase	Subunit mass (kDa)	Amino acid number	Ref.
H$^+$-PPase (PPi synthase)			
Plant (vacuole)	80.[b]	761-771	(Rea and Poole 1993)
Rhodospirillum rubrum (chromophore)	67.5[b]	600	(Nyren et al. 1991; Baltscheffsky et al. 1998)
Soluble PPase			
Escherichia coli	20[b]	175	(Baykov et al. 1999)
Saccharomyces cerevisiae	32.5[b]	286	(Baykov et al. 1999)
Arabidopsis thaliana	30[b]	263	(Kieber and Signer 19910)
Potato	24, 25[b]	211, 217	(Rojans-Beltran et al. 1999)
Barley	29[b]	215	(Visser et al. 1998)
Membrane-associated PPase			
Spinach (chloroplast thylakoid)	55		(Jiang et al. 1997)
Saccharomyces cerevisiae (mitochondria)	32[b]	310	(Lundin et al. 1991)
Sulfolobus acidocaldarius (plasma membrane)	17-18		(Meyer and Schafer 1992)
Pea (mitochondria)	35		(Zancani et al. 1995)
Syntrophus gentanae	Unknown		(Schocke and Schink 1998)

[a] Number of amino acid residues of the polypeptide deduced from the cloned DNA
[b] Molecular mass calculated from the cDNA
From Maeshima (2000b)
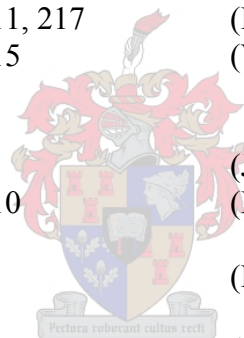
However, enzyme isoforms are generally different from each other in enzymatic function, regulatory mechanism, tissue- (or cell-) specificity of gene expression, or growth stage specificity. There is no report on the difference in the enzymatic activity of V-PPase isoforms, although the organ-specific expression of V-PPase has been reported in several plant species as described above. Nevertheless, it remains unclear whether a multigene family reminiscent of other primary ion translocases encodes V-PPase isoforms. Primary ion translocases usually have several isoforms and are encoded by a multigene family. For instance, there are more than 10 isoforms of the plasma membrane H^+-ATPase in *Arabidopsis (AHA1-10)* (Sussman 1994) and at least four cDNA clones encoding the 16 kDa proteolipid subunit of the V-ATPase in oats (Sze et al. 1992). In addition, each isoform of the plasma membrane H^+-ATPase is expressed in a tissue- and organ-specific manner and differ from others in its biochemical and regulatory characteristics (De Witt et al. 1991; Houlne and Boutry 1994; Palmgren and Christensen 1994).

2.2.2 Tertiary structure

V-PPases of several plant species have been reported to consist of 761-771 amino acids with a P*I* of approximately 5.0 (Maeshima 2000a). In addition, V-PPase exists as a dimer of two identical subunits with a molecular mass of approximately 80 kDa (Maeshima 2000a). Early radiation inactivation studies conducted by Chanson and Pilet (1989) yielded a functional mass of 160 kDa for PPi hydrolysis. However, Sarafian et al. (1992) reported that the radiation-inactivation size of PPi-dependent H^+ translocating was about 446 kDa. More recent studies conducted by Tzeng et al. (1996) confirmed the initial findings and reported similar values of 141 kDa and 158 kDa for PPi-dependent H^+ translocation. Alternative methods used to calculate the molecular mass of the V-PPase enzyme resulted in similar results as reported by Chanson and Pilet. The native molecular size for V-PPase, determined with gel permeation HPLC resulted in a mass of 135 kDa (Sato et al. 1991). SDS-PAGE after cross-linking of the purified V-PPase with dimethyl submerimidate showed a band of 158 kDa (Maeshima 1990). These studies made it clear that a single catalytic subunit is insufficient for H^+ transport activity; however, the exact degree of oligomerizaton of V-PPase in the vacuolar membrane remains to be determined directly. It is also evident from this work that the molecular mass of the V-PPase enzyme is approximately 150 kDa.

2.2.3 Conserved segments in the V-PPase primary structure

The V-PPase amino acid sequences among land plants are highly conserved with 86% to 91% homology (Maeshima 2000a). The least conserved region is the N-terminal part (the first 60

residues). Recently, the primary structures of V-PPase have been reported for *R. rubrum* (database accession no. AF044912) (Baltscheffsky et al. 1998; Baltscheffsky et al. 1999), a marine alga *Acetabularia acetabulum* (D88820) (Ikeda et al. 1999), and green alga *Chara corallina* (AB018529) (Nakanishi et al. 1999). The overall identities of amino acid sequences of V-PPase among these three phylogenically separated organisms were low (35-46%). Moreover, the identity of *R. rubrum* and *A. acetabulum* V-PPase is 36-39% and 47% respectively compared to land plant V-PPase.

However, multiple amino acid alignments of V-PPase from all land plants, *Chara, Acetubularia* and *Rhodospirillum,* revealed three highly conserved regions (CS1, CS2, CS3) (Nakanishi et al. 1999; Baltscheffsky et al. 1999; Maeshima 2000a). After comparing V-PPase and soluble PPase, Rea et al. (1992) proposed that two motifs, DxxxxDxKxxxxD and (E/D)xxxxxxxKxE, are putative catalytic sites of V-PPase (Fig. 2.1). Although the former motif is not common to V-PPase of mung bean, tobacco, beet and barley, the latter motif is conserved as the sequence, DVGADLVGKVE, among all available V-PPases except for those of *Arabidopsis* (DVGADLVGK<u>I</u>E). This sequence, DVGADLVGKVE (CS1) was assumed to include the catalytic domain for substrate hydrolysis (Rea et al. 1992; Rea and Poole 1993). Takasu et al. (1997) supported these results and confirmed that this sequence, which contains the V-PPase catalytic site, is exposed to the cytosol as illustrated in cytoplasmic loop *e* (Fig. 2.1). The comparison of the primary structures of V-PPase of various organisms also revealed that there are two consensus acidic regions, DNVGDNVGD (acidic region 1) (CS2) and DT*X*GDP*X*KD (acidic region 2) (CS3). The former segment is near the DVGADLVGKVE motif in cytoplasmic loop *e,* and the latter is in loop *m* between the 13th and 14th transmembrane domains (Fig. 2.1). In a preliminary observation, the individual replacement of three Glu residues in CS3 of mung bean V-PPase resulted in the loss of the enzymatic activity (Nakanishi and Maeshima 1998). Therefore, CS3 may also be exposed to the cytosol and play a critical role in the catalytic function together with CS1 and CS2. It has been established that the putative substrate binding-site is in cytoplasmic loop *e* and it contains the sequence DVGADLGKVE. There are ten negatively charged residues and five positively charged residues in loop *e*, and the net charge of the loop is negative, thus it is suitable for binding the Mg_2PPi complex (Maeshima 1991; Baykov et al. 1993). The catalytic domain probably comprises of several cytosolic loops, including loops *e* and *k*. The C-terminal of the enzyme seems to be more important for the function than the N-terminal part, because the

sequence homology of the C-terminal part (90%) among various V-PPases is higher than that of the N-terminal part (less than 40%) (Maeshima 2001).
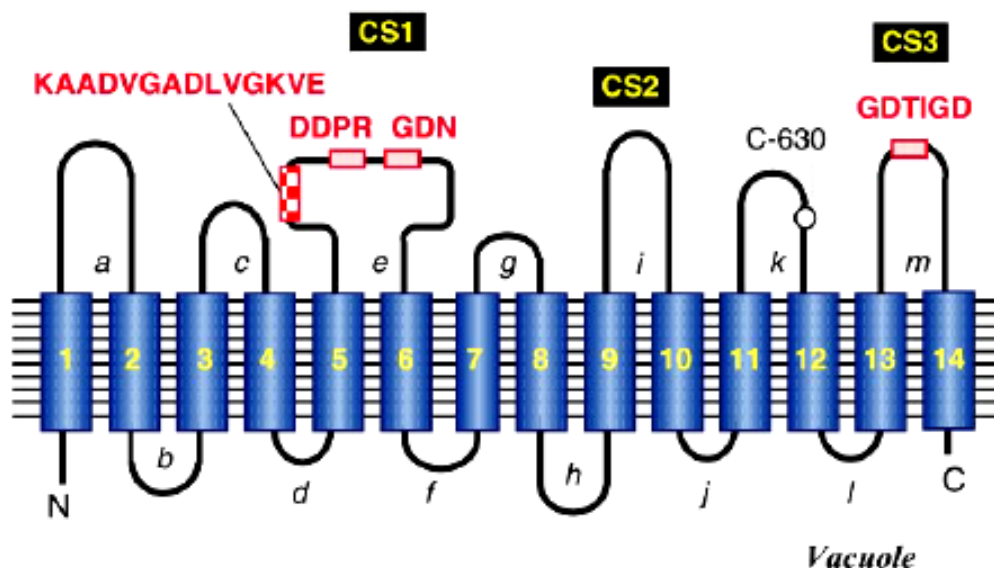
Fig 2.1. Transmembrane model of mung bean V-PPase. A structural model of V-PPase. (A) The 14 putative transmembrane helices are depicted as cylinders and conserved motifs are shown in boxes. The NEM-binding cysteine residue (Cys630 of Vigna V-PPase, Cys634 of Arabidopsis enzyme) (161) is shown as a circle. The hydrophilic loops are numbered from a to m. The conserved segments in the cytosolic loops are indicated as CS1, CS2, and CS3 (Maeshima 2001).

2.2.4 Catalytic properties of V-PPase

PPi hydrolysis supplies a free energy change in the cytosol of 27.4 kJ/mol at a pH of 7.3 (Davies et al. 1993). Maeshima et al. (1994a) determined that the H^+/PPi stoichiometry is one and the steady state pH generated by V-PPase is 3.2. The specific activities of V-PPase in the vacuolar membrane fluctuate and are dependent on the specific plant species, tissue and the assay conditions used. Typical values are 1.10, 0.30, 0.52, 0.35, 1.56 and 0.22-0.71 μmol PPi mg^{-1} of membrane protein for the seedling hypocotyl of mung bean (Maeshima and Yoshida 1989), storage tissue of red beet (Britten et al. 1989; Sarafian and Poole 1989), *Arabidopsis* leaf (Schmidt and Brisken 1993), cotyledon of pumpkin seedling (Maeshima et al. 1994b), *Acetabularia* (Ikeda et al. 1991) and CAM plants (Becker et al. 1995), respectively.

The substrate of V-PPase is commonly known as PPi but the actual substrate is a Mg^{2+}-PPi complex (Mg_2PPi) (Leigh et al. 1992; Rea and Poole 1993). The purified enzyme requires phospholipid for catalysis (Britten et al. 1989; Maeshima and Yoshida 1989; Sarafian and Poole 1989). The specific activities of purified V-PPase from mung bean and red beet were 8.5 and 3.0 μmol PPi mg^{-1} protein respectively and CAM plants V-PPases have similar

specific activities (Becker et al. 1995). V-PPase assays using purified vacuolar membranes reach their maximal velocity at more than 200 μM PPi in the presence of 1 mM $MgSO_4$. The K_m values for PPi have been reported to be 130 μM (Maeshima 1991), 2-5 μM (Baykov et al. 1993) and 2 μM (Gordon-Weeks et al. 1996).

Similar to V-ATPase, V-PPase requires free Mg^{2+} as an essential cofactor. Binding of Mg^{2+} stabilizes and activates the enzyme. Maeshima (1991) reported that the K_m value for Mg^{2+} is 42 μM, whereas Gordon-Weeks et al. (1996) reported Mg^{2+} K_m values of 20-23 μM. The exact number of Mg^{2+} binding sites on the V-PPase enzyme is not yet known. Baykov et al. (1993) have reported the presence of low-affinity (K_m = 23-31 μM) and high affinity (K_m = 0.25-0.46 μM) Mg^{2+} binding sites for mung bean V-PPase. V-PPase, like yeast cytosolic PPase, has been proposed to have two different Mg^{2+} binding sites in a single enzyme molecule. Free $[Mg^{2+}]$ concentration has been determined to be approximately 0.4 mM in the cytosol of mung bean root tips (Yazaki et al. 1988). Maeshima (2000b) speculates that under these conditions V-PPase could express more than 90% of its full activity. Binding of Mg^{2+} to V-PPase not only activates the enzyme, but also protects it from heat inactivation (Gordon-Weeks et al. 1996).

Furthermore, potassium is also regarded as an essential co-factor of V-PPase. K^+ stimulates V-PPase more than 3-fold in most cases (Maeshima 2000b). The K_m value for K^+ stimulation is 1.27 mM (Gordon-Weeks et al. 1997). Maximal in *vitro* activity could be obtained in the presence of more than 30 mM KCl in most cases. Gordon-Weeks et al. (1997) also reported that Tris at more than 25 mM inhibited this activation of V-PPase by K^+, and the inhibitory effect of Tris, and Bis-Tris-propane, was marked at KCl concentrations less than 10 mM. At present, the biochemical mechanism of competitive inhibition of K^+ activation by Tris and other pH buffers remains to be resolved. There has been a dispute whether or not V-PPase transports K^+ into the vacuole. Davies et al. (1992) have proposed from patch clamp studies of red beet vacuoles that the V-PPase functions as a H^+/K^+ symporter with a coupling ration of 1.3 H^+: 1.7 K^+: 1 PPi. (Obermeyer et al. 1996). Obermeyer et al. (1996) also analysed vacuoles of *Chenopodium rubrum* by the patch clamp technique, and obtained evidence for the possible role of V-PPase in K^+ transport. However, reconstitution of V-PPase into proteoliposomes and $^{42}K^+$ failed to confirm the ability of V-PPase to transport K^+ (Sato et al. 1994). Supporting theses findings, Ros et al. (1995) also found that no active transport of K^+ by V-PPase was detectable by fluorescent probe measurements. Gordon-Weeks et al. (1997)

suggested the need to re-examine the K^+ transport assay using a pH buffer that does not affect K^+ stimulation.

2.3 Inorganic Pyrophosphate as a cellular energy source

PPi is produced as a by-product in a horde of anabolic reactions including: the activation of amino acids by amino acyl-tRNA synthases, activation of fatty acids by thiokinases to form CoA esters, activation of carbohydrates by uridyl and adenyl transferases, and during the elongation reactions involved in the synthesis of proteins, nucleic acids and polysaccharides (Wood 1985; Stitt 1998). In the mid-1960s Calvin (1960) and Lipman (1965) suggested a role for pyrophosphate as a high-energy bond donor in the primeval earth. They proposed that the reactions found in primitive life forms evolved from prebiotic systems and that contemporary organisms could have retained the ability to employ PPi as a high-energy compound.

One doctrine of cellular bioenergetics (stemming from animal biochemistry) is that plants never only utilize the anhydride bond of PPi as in the case of animal cells, but rather always hydrolyse PPi by an H^+ inorganic pyrophosphatase that couples PPi hydrolysis with H^+ transport across the vacuolar membrane (Maeshima 2001). The use of the anhydride bond energy to transport H^+ across the vacuolar membrane makes the above listed synthetic reactions energetically favourable. The large amount of PPi produced during biosynthesis is therefore not wasted, but is employed in the cytosol to enhance the energetic efficiency of several cellular processes (Davies et al. 1993; Stitt 1998). The abundance and ubiquity of V-PPase in plants suggests a steady supply of cytosolic PPi and a PPi:Pi mass action ratio poised in favour of transtonoplast H^+-translocation (Stitt 1998). In contrast to animal cells, Sonnewald (1992) and Geigenberger et al. (1998) reported that the plant cytosol lacks soluble inorganic pyrophosphatase and therefore must liberate PPi by an alternative pyrophosphatase, in which V-PPase may play an important role. Although, soluble pyrophosphatase genes have been identified in potato (Rojans-Beltran et al. 1999) barley (Visser et al. 1998) and *Arabidopsis* (Kieber and Signer 1991), no *in vitro* catalytic activity estimates for these proteins exists. Therefore, the contribution of soluble pyrophosphatases to the regulation of cytosolic PPi levels cannot be estimated The earliest estimates of PPi levels in plant tissues fell in the range of 5-39 nmol g^{-1} fresh weight (Edwards et al. 1984; Smyth and Black 1984; Dancer and ap Rees 1989). Non-aqueous density fractionation of extracts from spinach leaves and membrane filtration of wheat mesophyll protoplasts indicate cytosolic PPi concentrations of between 200 and 300 μM (Weiner et al. 1987). Analysis of the charophyte alga, *Chara*, in

which the cytoplasm, chloroplasts, and vacuoles can be assayed in isolation, yields cytoplasmic, chloroplastic, and vacuolar PPi concentrations of 193, <1 and 2-3 μM, respectively (Takeshige and Tazawa 1989).

Assuming a mean cytoplasmic Pi concentration of 5 mM (Rebeille et al. 1984), and an equilibrium constant for PPi hydrolysis of 2320 (Guynn et al. 1974), an overall free energy yield of about 25 kJ/mol would be predicted under physiological conditions if cytosolic pyrophosphate concentration is 200-300 μM (Weiner et al. 1987). Given an average transtonoplast electrical potential energy of 18 kJ/mol (Rea and Sanders 1987; Takeshige and Tazawa 1989) and a H^+:PPi stoichiometric ratio of one (Johannes and Felle 1990; Schmidt and Brisken 1991), the free energy liberated by the hydrolysis of cytosolic PPi would exceed the theoretical minimum required for vacuolar energization by approximately 7 kJ/mol (Rea and Poole 1993).

2.3.1 V-PPase and PPi metabolism

An elementary question that still arises concerning V-PPase and V-ATPase, is why should there be two transport systems simultaneously pumping the same ion into the same intracellular compartment? A simple answer to this might be that V-PPase salvages the free energy of the PPi, which is a by-product of essentially all major biosynthetic pathways, while at the same time ensuring the recycling of phosphate. Although a soluble PPase would merely thermally dissipate the free energy of PPi hydrolysis, a biological useful output could be retrieved if some of this energy was conserved as a transmembrane pH gradient. Such energy conservation may not be critical under optimal metabolic conditions, but could be vital under conditions of stress, such as anoxia due to flooding, when normal ATP supply is drastically reduced, or in chilling, where a reduction in ATP supply may be augmented by the temporary inactivation of the V-ATPase through cold-induced dissociation of this enzyme (Kasamo 1988; Yoshida et al. 1989; Johannes and Felle 1990; Yoshida 1991; Moriyama and Nelson 1989; Parry et al. 1989; Ward et al. 1992).

A remarkable characteristic of cellular PPi levels is its consistency. PPi levels in the cytosol are remarkably insensitive to abiotic stresses such as anoxia or Pi starvation, or following the addition of respiratory poisons, which elicit a significant reductions in cellular ATP pools (Plaxton 1996; Stitt 1998). Cellular ATP levels on the other hand change dramatically under these conditions. Other PPi-linked enzymes, such as pyrophosphate:fructose-6-phosphate-1-

phophotransferase (PFP), which plays a role in glycolysis analogous to that of phosphofructokinase (PFK), are found in a number of anaerobic micro organisms (Mertens 1991), and these are also induced by anoxia in some plants (Mertens et al. 1990). Coordinated stabilization of PPi levels and utilization of such enzymes could provide a back-up system for metabolism under stress conditions. Retention of a functional V-PPase in plants, along with other enzymes utilizing the free energy of PPi, may therefore be related to the wider range of environmental conditions experienced by plants compared to animals.

2.4 Regulation of V-PPase gene expression and activity

2.4.1 Proton pump miscellany during cell growth

The hypocotyls of seedlings are often used as typical examples of young growing tissues because the shoots of etiolated seedlings grow so rapidly. Maeshima (1990) reported that etiolated hypocotyls of mung bean grow at a rate of about 4 cm per day at 26°C. The vacuolar membranes of these growing hypocotyls contain both V-PPase and V-ATPase activity, acting in concert with each other to generate a cytosol-negative electrical potential difference across the tonoplast. The hypocotyls of mung bean can be separated into the dividing, elongating and mature regions. The rapid elongation of cells occurs in the middle part of the hypocotyls whereas cell division only occurs in the apical meristem at the top part of the hypocotyl. The apical meristem is composed of relatively small cells with small central vacuoles, whereas the mature region of the hypocotyl consists of large mature cells. Cell volume can increase more than 20-fold during elongation of the hypocotyl, as judged from the size of protoplasts derived from these tissues (Maeshima et al. 1996).

The levels of V-PPase and V-ATPase are higher in the dividing region than those in the elongating and mature regions of mung bean hypocotyl. In addition, the substrate levels for these enzymes, on a fresh weight basis, are also higher in the dividing region than those in the elongating region. The vacuoles of the cells in the dividing region of mung bean hypocotyls, occupy more than 50% of the volume of each cell. Therefore, Maeshima et al. (1996) estimated the cytoplasmic concentrations of ATP and PPi to be in the mM and μM range, respectively. Moreover, they state that these concentrations are sufficient to support the maximal activities of V-PPase and V-ATPase. The comparison of V-PPase and V-ATPase catalytic activities in mung bean hypocotyls revealed that *in vitro* V-PPase activity is four times higher than that of V-ATPase (Maeshima et al. 1996). It has been confirmed to be due to active transcription of the V-PPase gene, i.e. coarse regulation (Nakanishi and Maeshima

1998). It has also been concluded that the relative distribution density of V-PPase with respect to the surface area of vacuolar membrane did not change during tissue elongation, although the size of vacuoles in young cells is less than 1% of that in mature cells. V-PPase is therefore assumed to be the main proton pump in the vacuolar membrane of mung bean hypocotyls.

Smart et al. (1998) investigated the changes in the key enzymes involved in the development of cotton fibres after anthesis. Fibre cells are single-celled trichomes that elongate at peak rates in excess of 2mm/day. It was found that the V-PPase was constantly transcribed during cell elongation and reached a peak a few days after the peak rate of fibre elongation. The increased level of V-PPase has been thought to support the acidification of expanding vacuoles by utilizing a proton motive force to activate the secondary transport of various solutes into the vacuole to lower the osmotic potential which would in turn lead to an increase in the osmotic pressure (Rea and Sanders 1987). Lerchl et al. (1995) reported the occurrence of changes in the mRNA levels of V-PPase during leaf development of tobacco, with respect to the conversion from a sink to a source organ. All steady-state mRNA levels of three isoforms of V-PPase were high in young sink leaves, but they decreased during leaf maturation. Furthermore, they reported daily rhythms of V-PPase mRNA accumulation with a minimum at high noon and a several fold increase in signal at night (Lerchl et al. 1995). The comparison of V-PPase and V-ATPase activities in mung bean hypocotyls (Maeshima 1990) and pear fruit (Shiratake et al. 1997) also revealed that V-PPase activity is several times higher than V-ATPase activity. II In contrast to the V-PPase activity that decreases during tissue development, V-ATPase level is relatively constant during growth and maturation. As a result V-ATPase becomes the major proton pump of vacuolar membranes in mature tissues, whereas V-PPase is the main proton pump of vacuolar membranes in most young tissues (Maeshima 2000b).

2.4.2 Physiological significance of V-PPase in cell growth

Plant growth is accompanied by the expansion of cells, and both hydrostatic and osmotic forces support cell expansion (Maeshima et al. 1996). In most cases, the expansion of a cell is due to an increase in vacuolar volume, rather than an increase in cytoplasmic volume. It is quite rational that vacuolar enlargement cannot occur without an increase in vacuolar contents and the active biogenesis of the vacuolar membrane. In order to maintain the high osmotic pressure of the contents of the expanding vacuole, the vacuole must actively import solutes since the osmotic pressure depends on concentrations of the solutes in the vacuole. Both V-

PPase and V-ATPase are primary active transporters that provide the transmembrane H^+ gradient for secondary active transporters (Maeshima et al. 1996) (Fig. 2.2). Measurement of enzymatic activities and immunohistochemical quantification of the vacuolar proton pumps, showed that V-PPase is present at higher levels than that of V-ATPase in young tissue (Maeshima 1990; Shiratake et al. 1997).

Maeshima et al. (1996) claimed that this disparity between V-PPase and V-ATPase is equitable from the perspective of cell energetics. In growing tissues, RNAs, proteins, and polysaccharides are actively synthesized for construction of cells and as a result, a significant pool of PPi is produced as a by-product of these metabolic processes (Fig. 2.2). The β-oxidation of fatty acids also generates PPi. Takeshige and Tazawa (1989) reported that PPi is predominantly present in the cytosol of plant cells at a concentration of 0.2 mM. If PPi accumulates at high concentrations in the cytosol, it will inhibit these reactions (Maeshima 1990; Maeshima et al. 1996). From an energetic perspective it seems reasonable that V-PPase may aid to scavenge the accumulated PPi and maintain the PPi/Pi equilibrium in the cytosol. V-PPase has the advantage that it utilizes this low-cost substrate as an energy source for the active transport of protons and the acidification of the expanding vacuole as well as the activation of the secondary transport system of solutes into the vacuole. In mature cells, metabolic activity decreases and therefore also the amount of PPi produced. In addition, the rate of solute transport into the vacuole decreases and expansion of the vacuole ceases. Accordingly, V-PPase activity has been reported to be lower than that of V-ATPase in mature tissues (Maeshima et al. 1996; Shiratake et al. 1997). The existence of V-PPase in the plant vacuolar membrane seems to be a backup system to conserve energy or ATP, which is a universal energy source of many cellular activities, such as the synthesis and transport of cellular components.
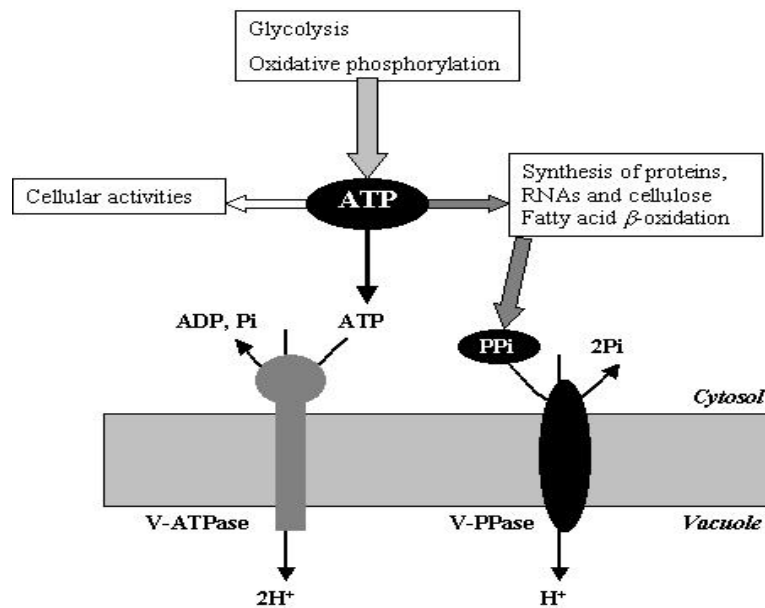
Fig. 2.2 V-PPase, V-ATPase and their substrates. PPi is supplied as a by-product of biosyntheses of macromolecules such as RNAs, cellulose and β-oxidation of fatty acids. From Maeshima (2000b).

2.4.3 V-PPase and stress conditions

V-PPase could be important in plant cells under anoxia and cold stress (Rea and Poole 1993). Carystinos et al. (1995) reported that the relative levels of transcript and enzyme activity of V-PPase increased notably under anoxia and chilling (10°C) in seedlings of anoxia-tolerant rice species. There was a 75-fold increase in the enzyme activity from 0.0133 to 1.0 μmol PPi mg^{-1} of vacuolar membrane protein after six days of anoxia. V-PPase protein amount and activity decreased to the original level after it was returned to air. They proposed that the induced V-PPase might replace V-ATPase under energy stress to maintain the vacuole acidity. Similarly, V-PPase activity, but not V-ATPase, increased approximately 1.5- to 2-fold in mung bean hypocotyls under low-temperature stress at 4°C (Darley et al. 1995). This increment was proposed to be due to a shift toward fermentative metabolism in hypocotyl cells, because the rate of ATP generation decreased.

This phenomenon of vacuolar proton pump differentiation during stress is very compelling from a metabolic perspective (Maeshima 2000a) (Fig. 2.4). Provided that adequate PPi is being generated in the biosynthetic reactions, substituting SuSy, PFP and V-PPase for invertase, PFK and V-ATPase respectively would allow ATP to be conserved, and might improve plant cell performance in hypoxic conditions. For example, when sucrose is

18

mobilised via SuSy and UGPase, the UTP formed in the reverse reaction of UGPase can be converted to ATP by a cytoplasmic nucleoside-5-diphosphate kinase (Dancer et al. 1990), and used to phosphorylate fructose. In this way, the energy in the (1-2) glycosidic bond of sucrose is largely conserved as two phosphoester bonds. If the hexose phosphates are then further metabolised to triose phosphate via PFP, the conversion of one molecule of sucrose to four molecules of triose phosphates costs just two molecules of PPi, which can be recycled from biosynthesis reactions. This contrasts with an energy requirement of three molecules of ATP when a molecule of sucrose is converted to four molecules of triose phosphate via invertase, hexo- and fructokinase, and PFK. The PPi dependent route will double the net ATP yield during fermentation, and will also greatly decrease the ATP requirement when sucrose is converted to starch, cell wall components, protein, lipid or other components in the cell.

There is correlative evidence supporting a role for PPi metabolism and V-PPase under anaerobic conditions. Anaerobiosis leads to an increase of V-PPase (Carystinos et al. 1995), PFP activity (Mertens et al. 1990; Mohanty et al. 1993) and sometimes sucrose synthase activity (Salonoubat and Belliard 1989; Chourey et al. 1991; Ricard et al. 1991; Guglielminetti et al. 1995). Further metabolic cycling around PFP increases massively in hypoxic banana fruit (Hill and ap Rees 1995), and inhibitors of V-PPase resulted in vacuolar acidification in air but not in anaerobic conditions (Brauer et al. 1997), implying that PFP and V-PPase becomes more important in anaerobic conditions. Furthermore, PPi concentrations often remain stable in anaerobic tissues, whereas ATP levels rapidly decrease (Dancer and ap Rees 1989; Dancer et al. 1990; Mohanty et al. 1993).

V-PPase indeed confers a double advantage during stress: not only will it serve to diminish ATP consumption, but it will also contribute to the stabilization of cytoplasmic pH. It has been suggested that pH regulation was originally the principle role of H^+ pumps in evolution (Raven and Smith 1976). To consider the relative importance of PPi versus ATP as an energy donor in the plant cytosol, Davies et al. (1993) computed the standard free energy changes for PPi and ATP hydrolysis under a variety of cytosolic conditions. The results indicated that PPi would be particularly favoured as a phosphoryl donor, relative to ATP, under cytosolic conditions known to accompany stresses such as anoxia or nutritional Pi deprivation. This underscores the importance of PPi as an autonomous energy donor of the plant cytosol.

V-PPase expression is also regulated under mineral deficiency stress. Kasai et al. (1998) have examined the effect of mineral nutrients, such as K^+, NO_3, and Ca^{2+}, on V-PPase in rye roots. Both PPi hydrolysis and PPi-dependent proton pumping activities in the plants grown under mineral-deficient conditions were three times greater than those in plants grown under normal conditions. The increased PPi-hydrolysis activity in the vacuolar membrane was 0.14 µmole PPi min^{-1} mg^{-1} vacuolar membrane protein. Since there was no difference in the amount of V-PPase protein, it was suggested that there is an activation of V-PPase in the rye grown under nutrient stress conditions, and that a high activity of V-PPase resulted in the marked reduction of PPi level in roots grown in mineral-deficient medium. They suggested that Ca^{2+} or cytokinin might modulate the V-PPase activity.

2.5 The role of PPi and V-PPase in sucrose metabolism

2.5.1 General overview of sucrose transport in plants

Plants are autotrophic organisms that are able to synthesize complex molecules by reducing C, N and S from simple molecules. As a major translocatable product of photosynthesis, sucrose (glucose + fructose) is the main soluble component of the phloem sap (Zimmermann and Ziegler 1975). Even in species translocating derivatives of sucrose, e.g. raffinose, stachynose and verbascose, or polyols, e.g. mannitol and sorbitol, sucrose is still present in significant amounts in the phloem sap (Lemoine 2000). The selection of sucrose as the major transport sugar in plants has been related to its non-reducing nature and relative insensitivity to metabolism (Arnold 1968). This represents an advantage for a substrate translocated over long distance in the plant (Giaquinta 1980).

In plants, sucrose has to cross several membranes after being synthesised in source organs until it is stored or metabolised in sink organs (as sucrose or products thereof). Sucrose transport from source to sink tissue is illustrated in a simplified diagram in Fig. 2.3. Following the synthesis of sucrose in the cytoplasm of source organs, the first transmembrane event according to Lemoine (2000) is the transport of sucrose into the vacuole across the tonoplast. The amount of sucrose temporarily stored in the vacuole will determine the pool of sucrose available for export (Lemoine 2000) (Fig. 2.3, step1). Following the transport of sucrose out of the vacuole into the cytosol, sucrose has to exit the mesophyll cell to the apoplasm (Fig. 2.3 step 2), and from the apoplasm enter the phloem (Fig. 2.3 step 3). Several modes of transport are possible to exit this long distance pathway, as different situations are encountered among species, i.e. apoplastic vs. symplastic unloading. When sucrose is unloaded into the apoplastic

space (Fig. 2.3 step 4), it can either be taken up as sucrose into the sink cell (Fig. 2.3 step 5), or cleaved by cell wall invertase to hexoses that are transported by specific carriers into the cytosol (BÜttner and Sauer 2000). After the sucrose arrives in the sink tissue, it can be used for sink growth or development (metabolic sink) or can be stored as sucrose in the vacuoles of the storage cells, e.g. sugar beet and sugarcane (Fig. 2.3 step 6). There might be some additional steps, such as retrieval along the translocation path; however, the corresponding carriers are responsible for the same type of transport as the one described in step 3, Fig. 2.3.

Sucrose transporters in plants can be divided into three groups according to the different steps illustrated in Fig. 2.3 (Brisken et al. 1985). Firstly, plasma membrane influx carriers that are responsible for the entry of sucrose into cells that are of the proton/sucrose symporter type. Secondly, tonoplast carriers that operate as sucrose/proton antiporters as the vacuole is the acidic compared to the cytoplasm. Thirdly, plasma membrane efflux carriers responsible, for example for the unloading of sucrose in sink organs or for sucrose exudation from the mesophyll cells in close vicinity to the phloem (Fig. 3 steps 2 and 4). Efflux carriers could be in theory either facilitators or antiporters.
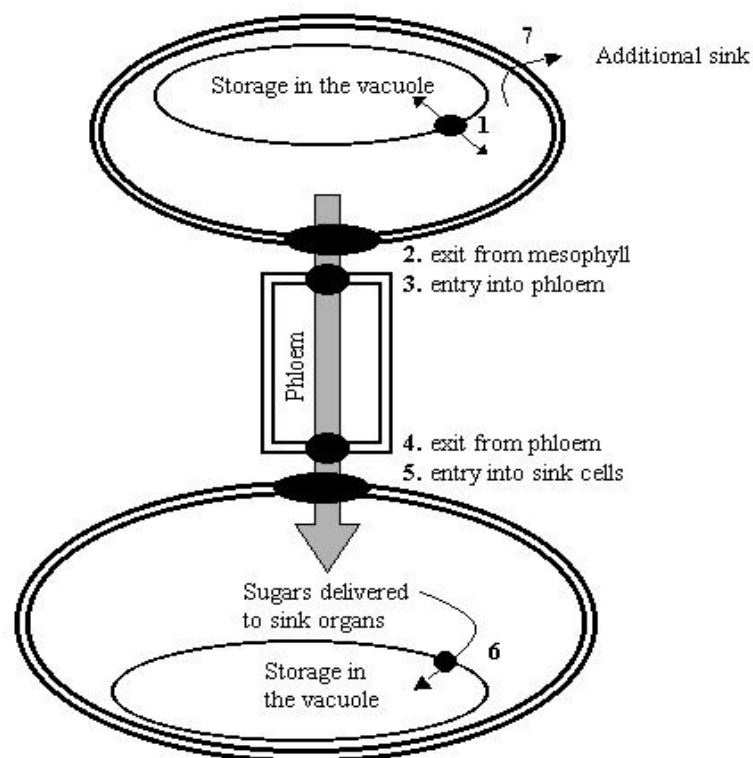
Fig. 2.3 Transmembrane steps mediated by a sucrose transporter. The flow of sucrose from the source organ (upper part) to the sink organs (lower part) through the phloem is represented as a large arrow, and the numbers refer to the different events of membrane transport. From Lemoine (2000).

2.5.2 PPi and its role in sucrose metabolism

As mentioned earlier, PPi is produced as a by-product of a host of reactions involved in macromolecule biosynthesis. One dogma of cellular bioenergetics is that the anhydride bond of PPi is never utilized and that PPi produced in anabolism is always removed by the hydrolytic action of an inorganic alkaline pyrophosphatase, thereby providing a thermodynamic "pull" for biosynthetic processes. Macromolecule biosynthesis, however, remains thermodynamically favourable no matter how the low concentration of PPi is maintained, whether by hydrolysis or by some other means, including the utilization of the high energy of the PPi bond (Wood 1985).

Several cytosolic enzymes are dependent on PPi. The discovery in 1979 of the strictly cytosolic pyrophosphate: fructose-6-phosphate phosphotransferase (PFP) in plants (Carnal and Black 1979) and the subsequent observation of its potent activation by μM levels of the regulatory metabolite fructose-2,6-bisphosphate (Fru-2,6-P_2) (Sabularse and Anderson 1981) led to a surge of research on the role of PPi in plant sugar and phosphate metabolism. During gluconeogenesis, PFP is thought to operate in the direction of PPi synthesis in order to maintain the level of PPi (Hatzfeld et al. 1990; Sung et al. 1988). This reaction, which is near thermodynamic equilibrium (Weiner et al. 1987), may however work in the opposite direction when other substrates such as starch are metabolised, or when the ATP status of the cell is low (Hatzfeld et al. 1989; Mertens 1991). Apart from PFP several other metabolic reactions are dependent on PPi. Cytosolic sucrose mobilization via sucrose synthase (SuSy), uridine 5'-diphosphoglucose (UDPGlc) pyrophosphorylase (UGPase), fructokinase (Stitt 1990; Kruger 1997) and V-PPase (fig. 2.4). Characterized by their high extractable activity, these PPi-consuming reactions are ubiquitous in higher plants and are often present at high activities (Stitt 1990; Kruger 1997). Key features of glycolysis via the sucrose synthase pathway are the cycling of uridylates (UDP/UTP) and PPi as well as the conservation of the glycosidic bond energy of sucrose. During gluconeogenisis, PPi is produced by both PFP and UGPase and UTP is reduced by UGPase. The reaction catalysed by UGPase is readily reversible, and the equilibrium in vivo depends on the concentration of PPi (Stitt 1998), which is kept constant in plant cells as discussed earlier.

As discussed earlier, plant cells contain a considerable pool of PPi in the cytosol (Chanson et al. 1985; Edwards and ap Rees 1986) and PPi has an estimated *in vivo* free energy of hydrolysis of about half that of ATP (Weiner et al. 1987; Davies et al. 1993). Considering this

it can be appreciated that this phosphoanhydride is a major energy source and that cytosolic PPi can act as an energy donor to various metabolic systems. During sucrose synthesis, there is a need for the disposal of the PPi produced by UGPase, which would otherwise hinder sucrose synthesis and favour the formation of fructose 1,6-bisphophate and glycolytic flux (Fig. 2.4). The accumulation of PPi in the cytosol to abnormal levels could therefore inhibit sucrose synthesis. However, the cytosol of higher plants cells contains little or no soluble pyrophosphatase and alkaline pyrophosphatase is only located in the plastids (Gross and Stitt 1986; Weiner et al. 1987). V-PPase may have a dual responsibility during sucrose synthesis, based on its enzymatic properties. Firstly, the disposal of cytosolic PPi, which could otherwise inhibit these sucrose synthesising enzymes and secondly the use of this phosphoanhydride energy bond to pump H^+ into the vacuole and activate the secondary transport of various solutes, including sucrose into the vacuole.
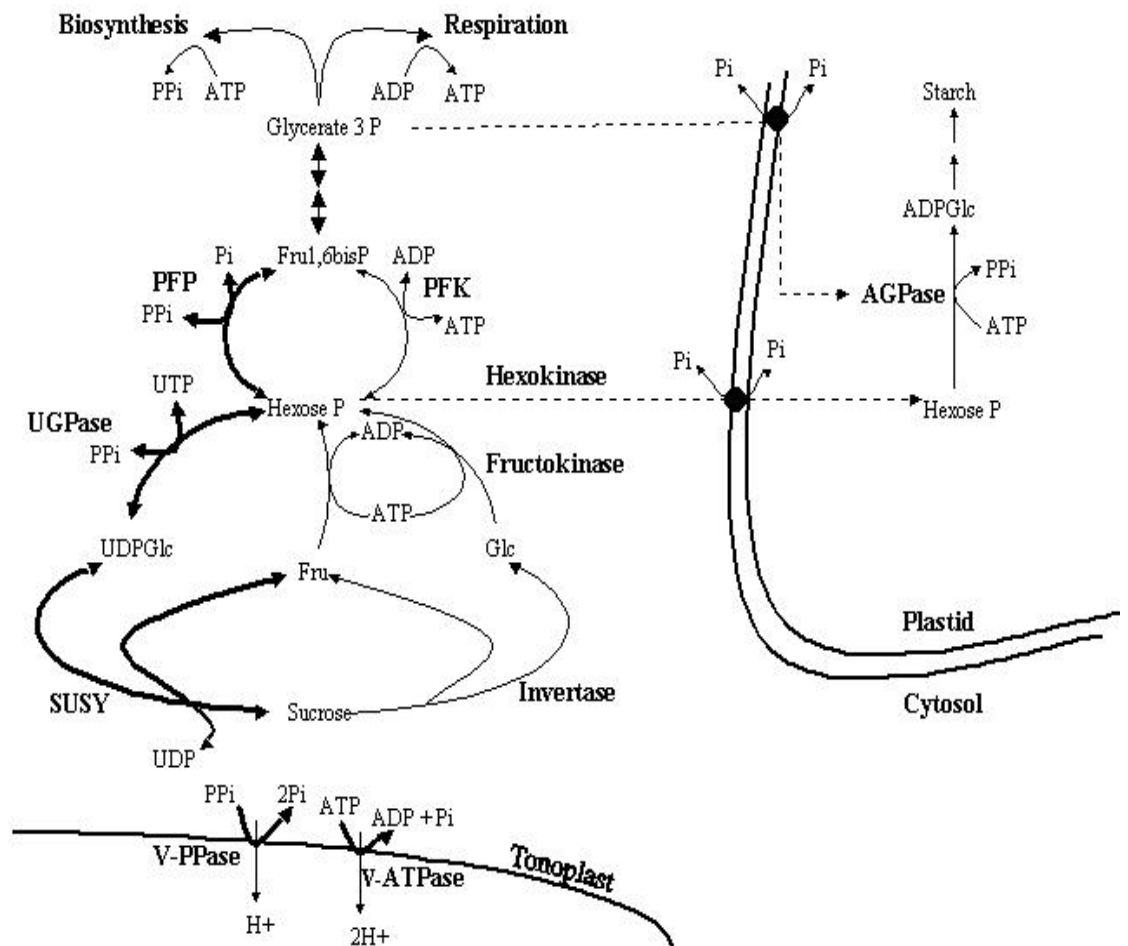
Fig. 2.4 Pyrophosphate-utilising reactions in plant metabolism in a heterotrophic cell. The PPi-utilising reactions are shown on the left hand side in bold script. The regulatory effect of 3 PGA on AGPase is shown by a dotted line. The entry of hexose phosphate and 3 PGA into the plastid occurs via the TPT and is shown as an exchange with orthophosphate (Pi). From Stitt (1998).

Specific inhibition of V-PPase and sucrose synthesis has been obtained by inhibitor studies with fluoride (Quick et al. 1989) or the nonhydrolyzable PPi analog imidodiphosphate (Neuhaus and Stitt 1991), which are potent inhibitors of V-PPase (Wang et al. 1986; Chanson and Pilet 1987; Chanson and Pilet 1988) but not PFP (Neuhaus and Stitt 1991; Quick et al. 1989; Van Schaftingen et al. 1982). Administering these inhibitors to detached leaves via the transpiration stream strongly inhibits sucrose synthesis concomitant with the depletion of cellular UDPGlc and accumulation of PPi, hexose monophosphates, and fructose 1,6 bisphosphate. These results strongly indicated a pivotal role for V-PPase in ensuring PPi removal during photosynthetic sucrose synthesis. However, Zhen et al. (1997) argued that the use of inhibitors *in vivo* is burdened with assumptions. The interference perceived when imidodiphosphate is used as an inhibitor of V-PPase but not PFP, is not conclusive, because it depends on the assumption that it does not affect other reactions that might modify PPi levels in the cytosol. Whether or not the latter argument is true, has not yet been established.

2.5.3 Overexpression of soluble pyrophosphatase in the cytosol

Sonnewald (1992) transformed tobacco and potato with soluble alkaline pyrophosphatase from *E. coli*, expressing the pyrophosphatase under the control of the constitutive 35S promotor, and targeting the heterologous protein to the cytosol. The resulting plants contained significantly less PPi and showed a dramatic phenotype with altered levels of metabolites in primary metabolism, and major changes in their carbohydrate levels, sink-source relations, phenotype and growth rate (Sonnewald 1992; Jellito et al. 1992). These studies established that PPi plays an essential role in plant metabolism, growth and development.

In source leaves of tobacco and potato, overexpression of alkaline pyrophosphatase led to a large accumulation of sugars and less starch. UDPGlc accumulated and the hexose phosphates as well as other phosphorylated metabolites decreased (Jellito et al. 1992), showing that the removal of PPi has altered the balance of the reactants in the equilibrium reaction catalysed by UGPase (Jellito et al. 1992). This changed balance between UDPGlc and sugar phosphate intermediates was proposed to alter the balance between sucrose and starch synthesis in favour of the former (Sonnewald 1992; Jellito et al. 1992).

Analysis of the tubers from potato transformants over expressing pyrophosphatase has shown that PPi is also involved in the metabolism and growth of sink organs. Constitutive overexpression of pyrophosphatase resulted in higher levels of sucrose and reducing sugars,

increased UDPGlc, decreased levels of phosphates and other phosphorylated intermediates, and less starch, compared to wild type tubers (Sonnewald 1992; Jellito et al. 1992). Since sucrose degradation in growing tubers occurs via sucrose synthase, a simple explanation for this inhibition of sucrose mobilisation would be that low PPi restricts the conversion of UDPglucose to hexose phosphates by UGPase. When PPase was overexpressed under the control of the patatin promotor to restrict expression to storage parenchyma cells, metabolites, sucrose and starch changed as expected from a block at UGPase (Trethewey et al. 1998). Intriguingly, moderate constitutive overexpression of PPase led to a general increase of nucleotides and tuber proteins involved in sucrose-starch interconversion and a slight stimulation of starch synthesis (Geigenberger et al. 1998), indicating that signals related to PPi or PPi metabolism in cells where the patatin promotor is inactive may stimulate sink function.

Sikora et al. (1998) studied the effect of sucrose starvation on logarithmically growing suspension-cultured tobacco cells using immunogold election microscopy with antisera against V-PPase and V-ATPase. After a period of 32 hours, V-PPase and V-ATPase polypeptides were no longer detectable, following growth in the absence of exogenous supplied sucrose. Sucrose is taken up at the plasma membrane of plant cells by a H^+-symporter (Fig 3. step 5) (Bush 1993) and sucrose can be transported across the tonoplast (Fig 3. step 1 and 6), against a concentration gradient, by a H^+-antiporter (Getz and Klein 1995). Uptake into the vacuole is therefore dependent upon an H^+-gradient across the tonoplast for which V-PPase and V-ATPase are responsible (Niland and Schmitz 1995). Under conditions when sucrose cannot be expected to accumulate in the vacuole, e.g. during starvation, the necessity to continue to pump protons into the vacuole is obviously no longer as great as it was. Moreover, since during active sucrose synthesis and storage the cell synthesizes PPi (Rea and Poole 1993) it can be assumed that under conditions of sucrose deficiency, not only ATP, but also PPi will be in limited supply. Niland and Schmitz (1995) concluded that it is therefore not unreasonable for the cell to adapt to sucrose starvation by down-regulating the expression of its two tonoplast-located electrogenic transporters.

2.5.4 Where to go now?

Various reports have indicated the possibility that V-PPase may play an important role during sucrose synthesis and accumulation (Quick et al. 1989; Neuhaus and Stitt 1991; Sonnewald 1992; Jellito et al. 1992; Niland and Schmitz 1995; Sikora et al. 1998; Geigenberger et al.

1998). As explained earlier the cytosol of higher plants cells contains little or no soluble pyrophosphatase, and a significant pool of pyrophosphate (Weiner et al. 1987). V-PPase may play a role in sucrose and phosphate metabolism in two ways, based on its enzymatic properties. Firstly, V-PPase may aid in the disposal of cytosolic PPi during photosynthetic sucrose synthesis. Secondly, V-PPase may play a pivotal role in the supply of energy for tonoplast energisation via the pyrophosphate-dependent proton pump (Davies et al. 1993; Zhen et al. 1997) to activate the secondary transport of various solutes including sucrose (Grof and Campbell 2001), into the vacuole. Direct and indirect evidence to this effect as been obtained with the over expression of soluble pyrophosphatase in the cytosol of potato plants (Sonnewald 1992; Jellito et al. 1992; Geigenberger et al. 1998) the in vivo specific inhibition of V-PPase (Quick et al. 1989; Neuhaus and Stitt 1991) and immuno-histochemical studies on suspension cultures (Sikora et al. 1998; Niland and Schmitz 1995). These reports suggest that V-PPase may indeed play an important role in sucrose and phosphate metabolism but this issue has to be further addressed to elucidate the possible role of V-PPase during sucrose accumulation in a crop such as sugarcane.

V-PPase has been well characterized in various species, but no work has been done on sugarcane. Sugarcane, as one of the world's most efficient accumulators of sucrose, is a suitable model to establish the possible role that V-PPase may play in sucrose metabolism. The characterization of V-PPase in sugarcane varieties that differ significantly in sucrose contents could therefore direct us to an answer. Our hypothesis is that V-PPase may contribute in the disposal of cytosolic PPi, which if accumulated will inhibit sucrose synthesis and stimulate the breakdown of sucrose through glycolysis. In addition, by doing so V-PPase may use the phosphoanhydride energy from PPi to establish a trans-vacuolar H^+ gradient and therefore increase the sink strength, which is important for the storage of sucrose in the vacuole. A correlation between V-PPase activity and the sucrose storage phenotype should therefore give an indication whether or not V-PPase plays a role in sucrose accumulation in sugarcane. In this thesis we report the first work done on V-PPase in sugarcane. This project forms the platform for future investigation using transgenic technology to determine the potential role of V-PPase in sucrose and phosphate metabolism.

CHAPTER 3

Production of sugarcane V-PPase polyclonal antiserum and development of an ELISA system for V-PPase protein content determination

3.1 INTRODUCTION

The vacuoles of higher plants cells are acidic organelles and have previously been identified as sites for depositing of various inorganic ions, organic acids and sugars, as well as the lysis of intracellular components (Nishimura and Beevers 1979; Wagner 2004). The secondary active transport of these metabolites and solutes, including sucrose across the vacuolar membrane are dependent on the H^+-gradient generated by vacuolar membrane H^+ pumps (Hedrich and Schroeder 1989; Taiz 1992). The vacuolar membrane of plant cells contain two distinct H^+ pumps i.e., vacuolar H^+-translocating ATPase (V-ATPase; EC 3.6.1.3) and H^+-translocating inorganic pyrophosphatase (V-PPase; EC 3.6.1.1) (Sze 1985; Rea and Sanders 1987; Rea and Poole 1993). V-PPase is widely distributed in all plant species, including mosses, ferns and algae, but unlike V-ATPases does not occur in mammalian cells (Takeshige et al. 1988; Ikeda et al. 1991; Maeshima et al. 1994). Both these vacuolar pumps catalyse the electrogenic H^+ translocation from the cytosol to the vacuolar lumen to generate an inside-acid pH and a cytosol-negative electrical potential difference (Ikeda et al. 1991; Rea et al. 1992). Unlike the complex V-ATPases involved in ATP synthesis (Boyer 1997), V-PPase is representative of simple energy-transducing enzymes and its substrate, inorganic pyrophosphate (PPi), is one of the simplest high-energy compounds in the cell (Baltscheffsky et al. 1999; Maeshima 2000b).

V-PPase cDNAs have been cloned from various land plants such as barley (D13472) (Tanaka et al. 1993), mung bean (AB009077) (Nakanishi and Maeshima 1998), red beet (Kim et al. 1994) and tobacco (X77915, X83730, X83729) (Lerchl, Konig, et al. 1995), but have not yet been cloned from sugarcane, except for a few ESTs. Therefore, the V-PPase protein has also not been characterized in sugarcane before. Multiple amino acid sequence alignment of V-PPases from organisms revealed various highly conserved regions (Baltscheffsky et al. 1999; Nakanishi et al. 1999; Maeshima 2000a). One of the cytoplasmic loops (*e*) contains a putative substrate-binding motif, DVGADLVGKVE, and an acidic region, DNVGDNVGD, which is believed to together, form the domain for substrate hydrolysis (Rea and Poole 1993; Cooperman et al. 1992). This work was supported by an immunochemical study where

antibodies were raised specifically against the DVGADLVGKVE motif and inhibited the protein's activity (Takasu et al. 1997).

V-PPase is a viable enzyme to study for mainly three reasons. Firstly, it is a proton-pump model with which to study how the hydrolysis of phosphate esters is coupled with proton translocation across a membrane because the enzyme exists as a dimer of two identical subunits with a molecular mass of approximately 80 kDa (Kim et al. 1994; Maeshima 2000a). Secondly, the enzyme utilizes a simple substrate PPi that has a high energy-phosphoanhydride bond. PPi is generated as a by-product of anabolic reactions, including the activation of amino acids by amino acyl-tRNA synthesis, activation of fatty acids by thiokinases from CoA esters, activation of carbohydrates by uridyl and adenyl transferases, and during the elongation reactions involved in the synthesis of proteins, nucleic acids and polysaccharides (Sung et al. 1988; Plaxton 1996). Moreover, PPi is an important regulator of glycolytic enzymes both within the cytosol and plastid of plant cells, including: ADP-Glucose pyrophosphorylase (AGPase), ATP-dependent phosphofructokinase (PFK), pyruvate kinase (PK), PEP carboxylase (PEPCase), Sucrose synthase (Susy) and PPi-dependent phosphofructokinase (PFP) (Geigenberger *et al.,* 1996). Thirdly, this efficient proton pump coexists with H^+-ATPase in a single vacuolar membrane in plant cells. These two proton pumps are responsible for several cellular processes that are directly or indirectly related to either the trans-membrane electrochemical gradient across the vacuolar membrane or the intra-vacuolar acidic pH (Maeshima et al. 1996). These include: storage of metabolites and ions; regulation of cytosolic homeostasis; degradation and recycling of cellular components and vacuolar expansion.

Previously it has been established that V-PPase plays an important role in a number of physiological and metabolic processes. Several studies have indicated that V-PPase may be the main proton pump in vacuolar membranes in most young tissues and have indicated the importance of V-PPase during cell growth and expansion (Maeshima 1990; Shiratake et al. 1997; Smart et al. 1998). A possible explanation from these findings is that V-PPase supports the acidification of the expanding vacuole and stimulates the secondary transport system by utilizing a proton motive force across the tonoplast. Moreover, the significance of V-PPase has been well established in stress conditions, including mineral nutrient stress (Kasai et al. 1998) and toxic NaCl levels (Colombo and Cerana 1993), as well as anoxia and cold stress (Rea and Poole 1993; Darley et al. 1995; Carystinos et al. 1995). Carystinos et al. (1995)

proposed that anoxia and cold stress induce V-PPase and might replace V-ATPase under energy stress to maintain the vacuole acidity.

Moreover, several studies have indicated that V-PPase and PPi metabolism may play a pivotal role during photosynthetic sucrose synthesis and accumulation. Strong indirect evidence to this effect has been obtained by inhibitor studies with fluoride (Quick et al. 1989) and the nonhydrolyzable PPi analog imidodiphosphate (Neuhaus and Stitt 1991), which are potent inhibitors of V-PPase (Wang et al. 1986; Chanson and Pilet 1987; Chanson and Pilet 1988) but not PFP (Neuhaus and Stitt 1991; Quick et al. 1989; Van Schaftingen et al. 1982). Administering these inhibitors to detached leaves via the transpiration stream leads to the strong inhibition of sucrose synthesis concomitant with the depletion of cellular UDP-glucose and accumulation of PPi, hexose monophosphates, and fructose 1, 6 bisphosphate. Concurrently, analysis of potato tuber transformants with overexpressed soluble pyrophosphatase resulted in higher levels of sucrose and reducing sugars, increased UDP-glucose, decreased levels of phosphates and other phosphorylated intermediates, and less starch, compared to wild type tubers (Sonnewald 1992; Jellito et al. 1992). Moreover, Sikora et al. (1998) studied the effect of sucrose starvation on logarithmically growing suspension-cultured tobacco cells using immunogold election microscopy with antisera against V-PPase and V-ATPase. After 32 hours of growth in the absence of exogenous sucrose, V-PPase and V-ATPase polypeptides were no longer detectable.

V-PPase is a unique enzyme with a suggested diverse range of physiological impacts on plant metabolism (Maeshima et al. 1996; Baltscheffsky et al. 1999; Maeshima 2000a). The potential role of V-PPase in sucrose accumulation has not been studied yet. Moreover, nothing whatsoever is known about V-PPase in sugarcane. The first step in studying V-PPase in sugarcane is the development of molecular tools to do so. The quantification of V-PPase protein in other plant species was done by protein blotting of tonoplast membrane proteins (Maeshima 2000a). This well established method requires a large amount of protein in comparison to ELISAs, is time consuming and difficult to use when comparing a large number of samples. In addition, V-PPase is only expressed in the vacuolar membranes of plant cells and merely contributes to an estimated 10% of the total vacuolar membrane protein (Maeshima et al. 1996). The establishment of a sugarcane V-PPase ELISA system will provide an easy, quick and sensitive method to analyse V-PPase protein levels. The aim of this study was therefore to firstly produce and characterize polyclonal antiserum against

sugarcane V-PPase and secondly, to optimise and validate an ELISA system for V-PPase protein quantification.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals
All auxiliary enzymes, cofactors and substrates used for sample preparations were from either Sigma Chemical Company (St Louis, MO, USA) or Roche (South Africa). Anti-mouse IgG (whole molecule) horseradish peroxidase conjugate and enhanced chemiluminescence (ECL) protein blotting detection reagents were supplied by Amersham International. The standard protein molecular mass markers were from Sigma Chemical Company. All other solvents and biochemicals were of analytical grade.

3.2.2 Plant material
The commercial sugarcane variety NCo310 was used for all extractions. Mature, non-flowering, field grown plants were sampled. Plants grew in the Stellenbosch area, South Africa. Stalks from separate plants with approximately 20 aboveground internodes were randomly selected and harvested in the morning. The first leaf with the uppermost visible dewlap was defined as number one. The internode immediately below the point of leaf attachment was designated by the same number, according to the system of Kuijper (van Dillewijn C 1952).

3.2.3 Sample preparation
Internodes selected for analysis were excised form the culm and the rind carefully removed. The underlying, spanning core of the periphery was rapidly sliced and frozen in liquid nitrogen. Samples were stored at -80°C until further use.

3.2.4 RNA preparation
Total RNA was extracted using a modified phenol-based method described by Bugos et al. (1995). Tissue was ground to a fine powder in liquid nitrogen. Total nucleic acids were extracted in a solution containing 1:2 (w/v) of 25:24:1 phenol/chloroform/isoamyl alcohol solution and the same volume of homogenisation buffer containing 0.1 M Tris-HCl (pH 7.5), 1 mM ethylene diamine tetra-acetic acid (EDTA), 1% (w/v) sodium dodecyl sulphate (SDS), 0.1 M NaCl and 100 mM β-mecaptoethanol. Sodium acetate (pH, 5.2) was added to a final

concentration of 0.1 M, the emulsion was mixed and incubated on ice for 15 min followed by a 15 min centrifugation at 12 000 × g at 4°C. The aqueous phase was transferred to a new tube and one volume isopropanol added, followed by incubation at -20°C overnight to precipitate the nucleic acids. Nucleic acids were collected by centrifugation at 10 000 × g for 10 min. The pellet was washed with 70% (v/v) ethanol, centrifuged for 5 min at 10 000 × g, and air-dried. Nucleic acids were resuspended in 750 µl diethyl pyrocarbonate (DEPC) treated water and the insoluble material was removed by centrifugation at 10 000 × g for 5 min. LiCl was added to the supernatant to a final concentration of 2 M to selectively precipitate the RNA overnight at 4°C. RNA was recovered by centrifugation at 12 000 × g for 15 min at 4°C. The pellet was washed as before with 70% (v/v) ethanol and air-dried. The pellet was resuspended in DEPC-treated water and all remaining insolubles removed by centrifugation at 10 000 × g for 5 min at 4°C. The isolated RNA was quantified by UV spectrophotometry and the quality was assessed by electrophoresis through a 1.2% (w/v) agarose gel. The RNA preparations were stored at -80°C until further use.

3.2.5 cDNA synthesis and amplification of V-PPase

First strand cDNA synthesis was prepared from total RNA with Promega's Smart cDNA kit according to the manufacturer's instruction manual. Primers were designed to correspond to the consensus nucleotide sequences, which code for the catalytic domain for substrate hydrolysis (CS1) of V-PPase (Rea et al. 1992; Rea and Poole 1993). The amplification protocol was 5 min at 95°C (once), 1 min at 95°C, 1 min at 55°C and 2 min at 72°C for 20 cycles. Another 20 cycles was done at an annealing temperature of 50°C. Amplified DNA fragments (600bp) were purified from the gel and cloned into the vector pGEMTeasy (Promega). The V-PPase fragment was restricted from pGEMTeasy and ligated into the BamHI site of the pGEX vector (Pharmacia Biotech, Glutathione S-transferase (GST) Gene Fusion System) to give rise to the vector, pGEXVP. *Escherichia coli* (*E.coli*) MCI061 was transformed with the pGEXVP vector and a plasmid miniprep (GenElute™ plasmid miniprep kit) supplied by Sigma, was done according to the manufacturers instructions.

3.2.6 Sequence analysis

Automated DNA sequencing was performed with an Applied Biosystem ABI Prism 373 Genetic Analyzer using an ABI BigDye™ terminator cycle sequencing ready reaction kit according to the manufacturer's recommendations (Perkin-Elmer, Boston, Massachusetts, USA). The dye terminator cycle sequencing was done with the above-described reverse

31

primer and the 5` pGEX primer (5`- GGGCTGGCAAGCCACGTTTGGTG - 3`) that binds to the pGEX vector.

3.2.7 Expression and purification of the V-PPase peptide

The GST integrated Gene Fusion System, supplied by Pharmacia Biotech was used for purification and detection of V-PPase expressed fusion proteins. All buffers and mediums used were prepared, according to the instruction manual unless otherwise stated.

BL21 strain *E.coli* cells were transformed with the characterized pGEXVP vector and plated out on 2 × YTA medium. Colony PCR was done with supplied forward primers and the above described reverse primers to screen for positive colonies containing the V-PPase cDNA insert. Three litres of 2 × YTA medium was inoculated with a single positive colony of BL21 *E.coli* cells and grown at 37°C, slowly shaking, until an OD of 1.2 (A_{600}) was reached. V-PPase protein expression was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) (Roche) to a final concentration of 0.1 mM and allowed to incubate for an additional 2 hours at 22°C vigorously shaking. Cultures were sedimented at 4°C with centrifugation at 7 700 × g for 10 minutes and completely suspended in 50 μl of ice-cold 1 × PBS (140 mM NaCl, 2.7 mM KCL, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) per ml of culture. One tablet of Complete Protease inhibitor cocktail (Roche) was added to 50 ml of cell suspension. Lysozyme was added to a final concentration of 0.1 volumes of a 10 mg/ml stock solution containing 25 mM Tris-HCl (pH 8.0) and incubated on ice for 30 minutes. The suspension was frozen overnight at –80°C in 20 ml aliquots, where after it was sonicated at 14 watts (Virsonic-60) with 6 bursts of 10 sec each with 1-minute intervals on ice. Triton X-100 was added to a final concentration of 1% (v/v) to the bacterial sonicate and incubated for 30 min at 4°C with gentle agitation. Cell rests were removed with centrifugation at 12 000 × g for 10 min at 4°C after which the supernatant was filtered (0.45 μm).

The filtered bacterial sonicate was eluted through a disposable affinity column, packed with Glutathione Sepharose 4B (Pharmacia Biotech), as indicated in the GST Gene Fusion System technical manual. The semi-purified GST-VPPase fusion protein preparations were then run on a discontinuous 10% vertical SDS-PAGE slab protein gel and the GST-VPPase protein band excised and mashed in an equal volume of PBS.

3.2.8 Protein determinations

Protein was measured according to the method of Bradford (1976) using Biovine Serum Albumine (BSA) as a standard protein (Sigma).

3.2.9 The production of mouse polyclonal ascitic fluid antibodies

Mice were immunized according to the method of Tung et al. (1976). One volume of 1 mg/ml antigen in PBS was emulsified with 9 volumes of complete Freud's adjuvant. Each mouse was immunized intraperitoneally with 0.2 ml of this emulsion at weekly intervals for 3 weeks after which immunizations were given at 2 weeks intervals. Immunization was boosted with another 0.2 ml emulsion, containing 1 volume of 1 mg/ml antigen in PBS and 9 volumes of Freud's incomplete adjuvant. Mice were tapped using sterile 1.8 mm diameter needles and pasteur pipettes as soon as noticeable volumes of ascitic fluid accumulated in the abdominal cavity (usually 4-6 weeks after the 1st immunization). Cells were removed form the ascitic fluid by centrifugation at 200 × g for 10 min. The clarified fluid was stored at -20°C until required. The day before use, the ascitic fluid was removed from the freezer and allowed to thaw at 4°C. The fibrin clot was removed by centrifugation at 12 100 × g for 10 min at 4°C. An equal volume of glycerol was added to the supernatant and stored at -20°C without any apparent loss of immuno-reactivity.

3.2.10 Vacuolar membrane preparations

Vacuolar membranes were prepared as first described by Maeshima and Yoshida (1989) and Rea and Poole (1985). Tissue were ground into a fine powder with liquid nitrogen and homogenized in 1:2 (w/v) homogenisation buffer containing 0.25 M sorbitol, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1% (w/v) polyvinylpyrolidone-40, 1% ascorbic acid and 50 mM Mops/KOH buffer, pH 7.6. The homogenate was filtered and centrifuged at 3 600 × g for 10 min. The supernatant was transferred to new tubes and centrifuged at 120 000 × g for 30 min using a SW 28 Beckman rotor. The microsomal pellet was dissolved in 9 ml of 10 mM potassium phosphate (pH 7.8), 0.3 M Sucrose, 1 mM EGTA and 2 mM DTT. The suspension was overlaid with the same volume of overlaying buffer consisting of 5 mM Mops/KOH (pH 7.3), 0.25 M sorbitol, 1 mM EGTA and 2 mM DTT. After centrifugation at 120 000 × g for 30 minutes, the interface portion was collected and diluted in half a volume of 5 mM Mops/KOH (pH 7.3), 0.25 M Sorbitol, 1 mM EGTA and 2 mM DTT and centrifuged at 130 000 × g for 30 min. The resulting white pellet (vacuolar membranes) was suspended in 20 mM Tris-acetate, pH 7.5, 20 % (w/v) glycerol, 2 mM DTT, 1 mM EGTA and 2 mM MgCl$_2$.

The protein content of the preparations was determined by the method of Bradford (1976) and the membrane suspensions were flash frozen in liquid nitrogen and stored at -80°C.

3.2.11 SDS PAGE and protein blotting

Proteins were resolved in a 10% (m/v) polyacrylamide gel with a 6% (m/v) polyacrylamide stacking gel. The separation gel contained 200 mM Tris-HCl (pH 8.8), 100 mM glycine, 12% (m/v) polyacrylamide, 0.12% (m/v) N'N'-methylene bisacrylamide, 5% (v/v) glycerol, 0.4% (m/v) SDS, 0.1% (m/v) ammonium persulfate and 0.1% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED), whilst the stacking gel contained 70 mM Tris-HCl (pH 6.8), 6% (m/v) polyacrylamide, 0.06% (m/v) N'N'-methylene bisacrylamide, 5% (v/v) glycerol, 0.1% (m/v) SDS, 4 mM EDTA, 0.1% (m/v) APS and 0.1% (v/v) TEMED. Polyacrylamide gels were run at 120 V in a slab electrophoresis unit (SE 250 Mighty Small II) containing 100 mM Tris-HCl, 150 mM glycine and 0.1% (m/v) SDS in the electrophoresis tank.

Separated polypeptides were transblotted onto nitrocellulose in 25 mM Tris-HCl, containing 192 mM glycine and 10% (v/v) methanol at 15 Volts for 1 hour. After blotting the proteins onto nitrocellulose, the nitrocellulose was first dried between filter paper before any further manipulations. Following transfer, the blots were stained in Ponceau-S (0.2% [m/v] Ponceau-S, 3% [m/v] trichloroacetic acid), to be able to mark the standard molecular weight bands. The blots were then thoroughly rinsed in saline and non-specific binding blocked for 1 hour in blocking solution containing 4% (m/v) BSA in saline at room temperature. All subsequent steps in the procedure were carried out at room temperature. Blots were incubated in saline containing 4% (m/v) BSA, 0.1% SDS and the anti-VPPase anti-body (1/100) for 16 hours. Unbound antibody was removed by washing the nitrocellulose 4 times for 5 minutes in wash solution containing saline and 0.1% tween. The nitrocellulose was then incubated for 1 hour in wash solution containing avidin-horseradish peroxidase conjugate (1/2500), 0.1% BSA and saline. The nitrocellulose was washed as before and finally it was stained for horseradish peroxidase binding using the substrate 4-chloro-1-naphthol. After staining the nitrocellulose it was washed with distilled water, dried and stored in an aluminium foil envelope.

3.2.12 V-PPase catalytic measurement

For analytic measurements 0.5 μg of tonoplast membrane proteins were used. Assays were done as previously described by Rea and Poole (1985), Maeshima and Yoshida (1989) Leigh

et al. (1992), Baykov et al. (1993), Kasai et al. (1998), Nakanishi and Maeshima (1998) Baltscheffsky et al. (1999) and Wang et al. (2000), with some changes in order to work at zero order kinetics (Chapter 4). The reaction solution contained 0.25 mM sodium pyrophosphate, 50 mM KCl, 1 mM sodium molybdate, 0.03% (v/v) Triton X-100 and 30 mM Tris/Mes (pH 7.0) and were done in a final volume of 0.05 ml. The reaction was started with the addition of $MgSO_4$ to a final concentration of 1 mM. The reaction was allowed to continue at 30°C for 30 min, where after it was terminated with stopping solution. Iced cold stopping solution was added to make up a final volume of 0.2 ml reaction mix consisting of 6 parts 0.42% ammonium molybdate in 1 N H_2SO_4 and 1 part 10 % (w/v) ascorbic acid. After incubation for 10 min at 30°C the amount of Pi released was determined colorimetrically as previously described by Hodges and Leonard (1974). Specific activity was expressed as µmol PPi mg^{-1} of tonoplast membrane protein.

3.2.13 Establishment of an ELISA for V-PPase protein measurement

Nunc-Immuno Maxisorp® plates (Nalge Nunc, Denmark) were used for all ELISA assays. Plates were coated with tonoplast membrane protein (0.5 µg/ml) in Saline at 50 µl/well for 16 hours at 22°C. At the end of the incubation period the coating solution was decanted after which 0.2 ml BSA (1% w/v in Saline) was dispensed into each well to block the remaining adsorption sites. The plates were then incubated for 30 min at 22°C, gently shaking after which it was washed 4 times with saline. This was followed by the addition of anti-VPPase antiserum (1/200) and 0.01% (w/v) SDS in blocking solution at a volume of 200 µl/well, gently shaking for 2 hours at 22°C. Wells were again decanted and plates were washed as before. Anti-mouse peroxidase conjugate (AEC-Amersham, South-Africa) was diluted to 1/5000 and dispensed at a volume of 50 µl/well and incubated for 1 hour at 22°C. The well contents were again decanted and washed 7 times with saline. Plates were developed for 15 min using BM Blue™ soluble substrate (Roche, South Africa) at 50 µl/well at 22°C. The reaction was stopped with the addition of 25 µl stopping solution containing 0.2 M H_2SO_4. The absorbance values of the wells were then read at 450 nm.

3.3 RESULTS AND DISCUSSION

3.3.1 Cloning and sequencing of the sugarcane VPPase catalytic domain

V-PPase cDNA was amplified by PCR using these gene-specific primers (forward, 5′-ATA**GGATCC**ACTGGTTATGGTCTTGGTGGT-3′; reverse, 5′-ATA**GGATCC**TAGICCA GCCCAIAGACCAAC-3′). Primers were designed based on the V-PPase cDNA sequences of mung bean (Nakanishi et al. 2001) *Oryza sativa* (Sakakibara et al. 1996) and *Hordeum vulgare* (Tanaka et al. 1993). These primers amplify cytosolic loop *e* and contain both the conserved putative substrate binding motif and acidic region. The sugarcane sequence was amplified using cDNA prepared from young leaf-roll and internodal tissue as template.

Nucleotide sequence analysis of the 585 bp fragment amplified from Sugarcane cDNA (NCBI accession number: caf 22023) was conserved among various land plants and amino acid sequence analysis, based on the cDNA, showed the amino acid motif – DVGADLVGKVE - for the putative substrate-binding site. In the acidic region, sugarcane V-PPase has the sequence – DSVGDNVGD – where the second residue S replaces N compared to other organisms. In the motif DVGADLVGKVE of V-PPase cytosolic loop *e,* the third residue (Gly) is substituted by Ala in *Acetabularia* (Ikeda et al. 1999), and the tenth residue (Val) is substituted by Ile in the *Arabidopsis* enzyme (Sarafian et al. 1992). Amino acid differences in the substrate-binding motif of V-PPase are common between land plants. This cloned cytosolic loop *e* of sugarcane V-PPase was introduced into a bacterial system to express and purify this peptide for the immunization of mice.

3.3.2 Expression and purification of the GST-VPPase fusion peptide in a bacterial system

After cytosolic loop *e* was amplified, fragments were cloned into the pGEX bacterial expression vector (pGEX-VPPase) as outlined in the *Methods and Material* section. *E.coli* cells, strain BL21, were used to express the cDNA and purify the V-PPase peptide. The V-PPase fragment codes for a calculated 20.5 kDa peptide. The GST peptide (26 kDa) plus the V-PPase peptide will therefore produce a fusion protein of 46 kDA. When crude bacterial sonicates of both the pGEX control and pGEX-VPPase was compared on a SDS-Page before induction (Fig. 3.1, BI), no differentially expressed bands could be identified in the 45 kDa range. After IPTG induction though (Fig. 3.1, AI), a differential expressed protein in the 45-kDa range could be observed for the pGEX-VPPase construct if compared to the pGEX sonicate.
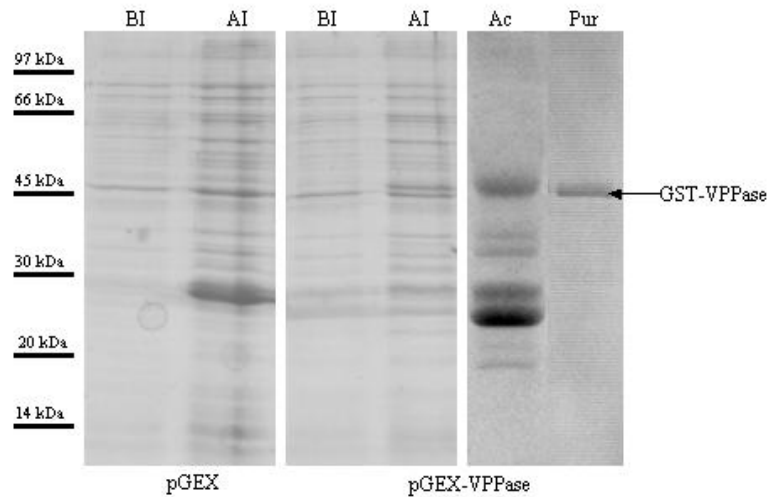
Fig. 3.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant sugarcane V-PPase peptide-e. Crude protein sonicates of bacterial lines that contain the pGEX and the pGEX-VPPase expression vector were analysed before IPTG induction (BI) [10 μl] and after IPTG induction (AI) [10 μl]. The crude pGEX-VPPase bacterial line protein preparations were subjected to SDS-PAGE after GST affinity column purification (Ac) [10 μl] and after excision from the prepative SDS-PAGE gel to purify GST-VPPase fusion proteins (Pur) [5μg].

Following IPTG induction, crude protein extracts were subjected to affinity chromatography (Fig. 3.1, Ac) for purification of the GST-VPPase fusion protein. Affinity chromatography was not very successful, although a single band in the 45 kDa range with contamination of lower molecular weight proteins could be identified. Alternative methods had to be used to completely purify V-PPase for immunization. These semi-purified GST-VPPase fusion protein preparations were consequently subjected to preparative vertical SDS-PAGE slab protein gels and the putative GST-VPPase protein bands were excised based on molecular weight. The excised band was again run on a SDS-PAGE gel to verify the molecular weight and purity of the peptide (Fig. 3.1, Pur). This purified GST-VPPase peptide was used to immunize mice and produce polyclonal anti-VPPase antibodies.

3.3.3 Inhibition of substrate hydrolysis of V-PPase by the peptide antiserum

The polyclonal VPPase antiserum produced in mice was used as described in the methods and materials section to immuno precipitate V-PPase catalytic activity. This was done to firstly establish that the purified antigen (Fig. 1, Pur) used for immunisation was indeed GST-VPPase and secondly, if the Ab recognizes native sugarcane V-PPase proteins, because the mice were immunized with denatured proteins. Activity was measured at zero order kinetics (Chapter 4). The anti-VPPase antibody was clearly effective at removing V-PPase activity from the vacuolar membrane enriched preparations (Fig 3.2).
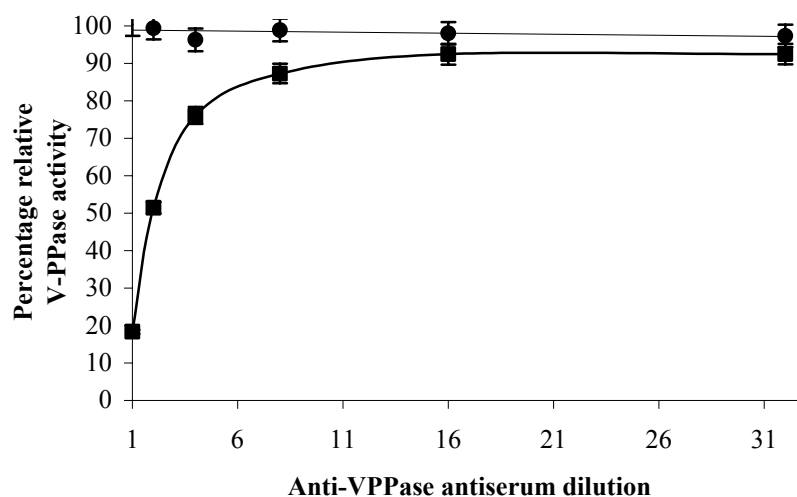
Fig. 3. 2. Effect of anti-VPPase IgG on the activity of V-PPase in tonoplast membranes. 1μg of tonoplast membrane was used in all the assays with the same volume but different dilutions of anti-VPPase antiserum (■) and day 0 serum (serum taken at day 0, before immunization) (●). Tonoplast membranes were incubated for 3 hours at 22°C where after pyrophosphatase activity was measured. Each point represents the mean value of one experiment using three wells. Vertical bars represent standard deviational errors. Data points were fitted with second order polynomial regression.

The initial activity (100%) was 0.18 μmol PPi mg^{-1} of tonoplast membrane protein from young internodal tissue. V-PPase activity was inhibited with increasing amounts of antiserum while the control serum showed no inhibition (Fig 3.2). Over 80 % of the V-PPase activity was immuno inhibited with 1 time diluted V-PPase antiserum. This result concurrent with the immunoblot (Fig. 3.3) gives us a strong indication that the purified GST-VPPase antigen was indeed sugarcane V-PPase. The antiserum is also very specific for V-PPase in sugarcane. It is quite clear that although a denatured antigen was used for immunization, the antibody was still able to recognize the antigen under native conditions, which is a prerequisite for an ELISA system. The inhibitory effect of the antiserum raised against CS1 also confirms the proposal of Cooperman et al. (1992), Rea and Poole (1993) and Baykov et al. (1999) that the conserved amino acid motif: DVGADLVGKVE and DSVGDNVGD of V-PPase participates directly in PPi and Mg^{2+} binding. In these previous studies similar polyclonal anti-VPPase antiserum were also raised against CS1 and inhibited V-PPase catalytic activity.

3.3.4 Immunoblotting with the peptide antibodies

Previously it has been estimated that V-PPase represents about 10% of the total vacuolar proteins (Maeshima et al. 1996). When the vacuolar proteins are separated on a SDS-PAGE gel, the majority of proteins cluster between 45 kDa and 97 kDa (Fig 3.3).
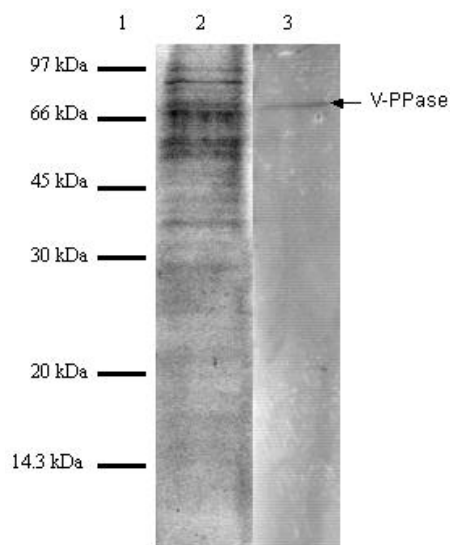
Fig. 3.3 SDS-polyacrylamide gel electrophoresis of tonoplast membrane proteins and immunoblot analysis with anti-VPPase. *Lane 1*, Molecular weight marker; *Lane 2*, gel stained with Coomassie Brilliant Blue; *Lane 3*, immunoblot with anti-VPPase IgG and horseradish peroxidase-linked secondary antibodies.

Antibodies against the GST-VPPase fusion protein reacted specifically with a single polypeptide among this cluster of the tonoplast proteins (Fig 3.3). V-PPase of land plants have been reported to consist of 761-771 amino acids with their calculated molecular masses ranging from 79 841 to 80 800 Da. SDS-PAGE profile analysis of these plants, on the other hand has proposed that V-PPase consists of a single polypeptide of 67 to 73 kDa (Maeshima 2000b). Immuno Blot analysis (Fig. 3.3) shows that the sugarcane V-PPase has a molecular weight of about 71 kDa, which is in the proposed molecular weight range of the known V-PPases in land plants. Based on the transcript size of V-PPase in sugarcane (Chapter 5, Fig. 5.3) one would expect a polypeptide of approximately 93 kD. This phenomenon has been observed in other species as well (Maeshima and Yoshida, 1989; Kim et al., 1994b). Rapid migration of the enzyme on the SDS gel may be due to the high hydrophobicity of the V-PPase. Hydrophobic amino acids account for about 63% of the total 766 residues in known land plants (Nakanishi and Maeshima, 1998).

3.3.5 Anti-VPPase antiserum titration for ELISA

Antibody titer was determined using a direct ELISA with 1μg/ml tonoplast prototein and a two fold dilution series of anti-VPPase antiserum (Fig. 3.4). The mouse anti-VPPase antiserum showed activity ($OD_{450nm} > 0.2$) up to dilutions of 1:6400. Low background readings were observed with the control serum (serum taken directly before immunization) at

this antiserum dilution (OD_{450nm} = 0.122). The anti-VPPase antibody dilution selected for ELISA's was 1:200, as this antibody concentration gave an OD reading of approximately 1.2, which is at a level of very high sensitivity and specificity, midway between the linear and plato area of the graph (Fig 3.4). Interassay variability is less than 10 % at this dilution. This titer is not very high, but a highly specific (Fig. 3.3) immune response reading (OD_{450nm} = 1.2) is observed here with a relative low background (OD_{450nm} = 0.164).
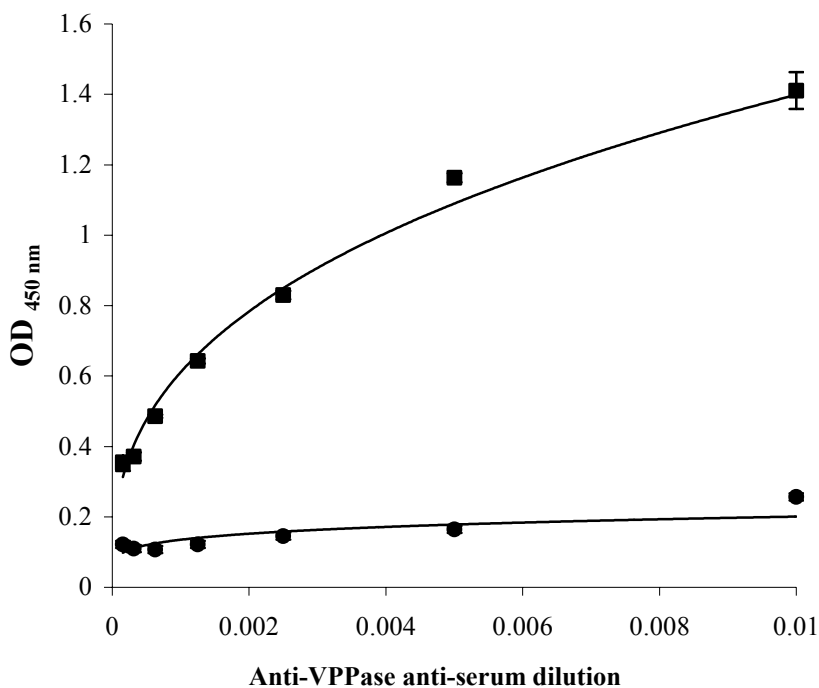
Fig. 3.4. The titration curve obtained for polyclonal anti-VPPase ascitic fluid antibodies. Plates were coated with 1μg/ml tonoplast protein. Anti-VPPase antiserum (■) and control serum (◆) were added in a twofold dilution series. Each point represents the mean value of 1 experiment using three wells each. Vertical bars represent standard errors. Data points were fitted with second order polynomial regression.

3.3.6 Tonoplast membrane protein titration and the use of detergents for ELISA

The effect of different detergents on the sensitivity of the ELISAs was determined for two reasons. (i) Plates were coated with isolated vacuolar membranes, the use of a detergent will therefore help to denature the membranes, solubilize the proteins and thereby improve the ability of the antibodies to recognise the V-PPase in the membrane fractions. (ii) The use of a detergent during the first coating stage of the ELISA will prevent the adsorption of proteins to the immunoplate and therefore it is reasonable to use it during the second stage of the ELISA with application of the primary antibody, after proteins have already been adsorbed to the immunoplate. Several detergents were evaluated (data not shown) and SDS gave the best results (Fig 3.5).

The standard curve for this assay gave a very good 2nd order polynomial fit (R^2=0.99). The direct ELISA developed for native sugarcane V-PPase protein level detection has a linear range between 0.3 - 1.3 μg/ml tonoplast membrane proteins whilst without SDS, sensitivity is much lower. Interassay variability is less than 10 % within this dilution range.
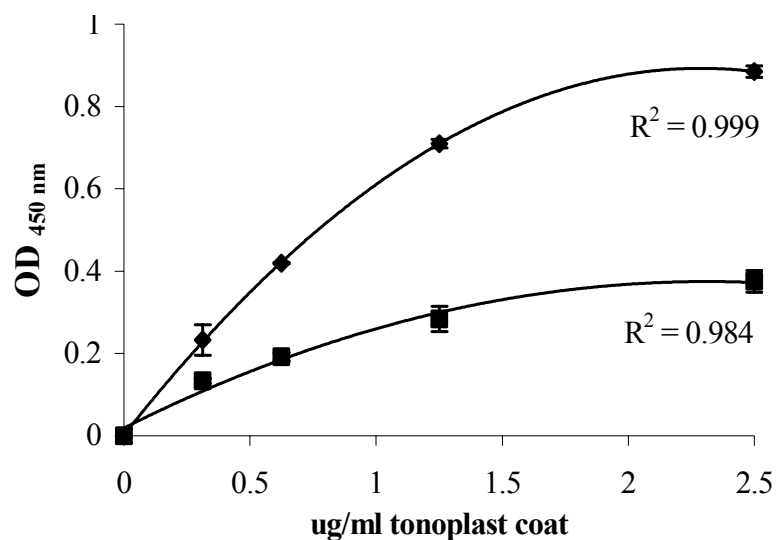
Fig. 3.5. A typical standard curve for the established direct competitive V-PPase ELISA. Plates were coated with a two fold dilution series of tonoplast membrane proteins. Anti-VPPase antiserum were added at a 1/200 dilution with no SDS (■) or 0.5% SDS (w/v) (◆). Data represent the mean of two experiments using three wells each. Vertical bars represent standard deviational errors. Each data point is the result of OD $_{450 nm}$ minus day o serum background. Data points were fitted with second order polynomial regression.

This linear detection range translates to 15 ng – 65 ng protein per assay, whereas protein blotting requires 10 – 20 μg vacuolar membrane protein to detect V-PPase proteins. This proves the ELISA system to be between 300 and 600 times more sensitive for the detection of V-PPase proteins in vacuolar membrane preparations than the protein blotting technique.

3.3.7 Correlation of V-PPase ELISA determined protein levels and catalytic activity

V-PPase activity is believed to be coarsely regulated in plant cells (Maeshima 2000b). This means that the amount of V-PPase protein should correlate with the total extractable activity and therefore, the use of an ELISA system to quantify V-PPase protein levels should give a good indication of the specific activity. In order to assess the reliability of the present direct sandwich-ELISA to quantify the specific activity of V-PPase in tonoplast membranes of sugarcane, the relative amounts of V-PPase protein were determined and compared to the catalytic activity (as outlined in the *methods and materials* section). The V-PPase catalytic activity determined with the ELISA system at a known vacuolar membrane protein amount will make it possible to calculate the specific activity. The relative V-PPase protein levels

(OD 450) determined by this sandwich-ELISA showed a strong linear correlation ($R^2 = 0.990$) with V-PPase catalytic activity (Fig. 3.6).
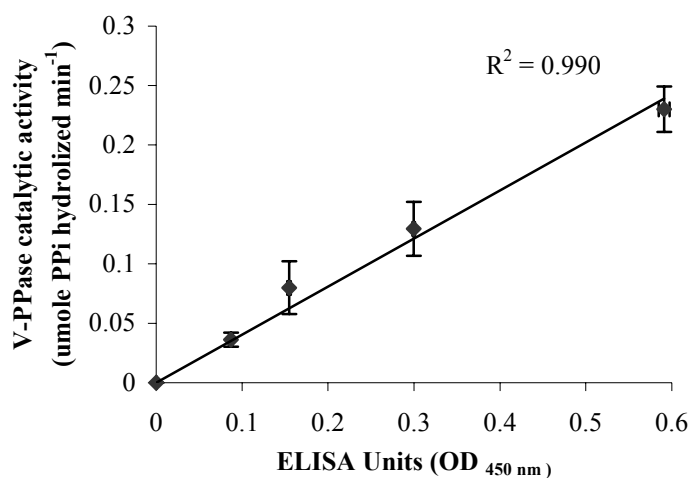
Fig. 3.6. Correlation between V-PPase protein level and catalytic activity in tonoplast vesicles from young sugarcane tissue in the variety NCo310. Immuno plates were coated with a twofold dilution series of vacuolar membranes and a 1/200 dilution of antiserum was used for ELISA The same dilution series was used to measure V-PPase catalytic activity. ELISA and V-PPase catalytic activity was done under the exact same conditions described in the methods and materials section. Each point represents the mean value of 2 experiments using three wells each. Vertical and horizontal bars represent standard errors.

These results indicate that the relative V-PPase protein levels determined with this ELISA system can be used to determine V-PPase activity in these specific tissues. This correlation was only done with tonoplast membrane from young internodal tissue from the NCo310 variety. The general applicability of this correlation will be confirmed in other tissues (Chapter 5).

3.3.8 Interspecies immuno activity

As earlier stated, cytosolic loop e has been identified as a highly conserved segment in the V-PPase genes of land plants. The first conserved segment includes the catalytic domain for substrate hydrolysis (Rea et al. 1992; Rea and Poole 1993) to which the antiserum was raised. Although this segment is highly conserved among species, peptide comparison of the sugarcane V-PPase fragment showed between 70% - 90% homology with the species in table 3.1. As a result of this homology among various species, it is expected that the anti-VPPase antiserum will show interspecies immuno activity in both mono- and dicotyledons. In this optimised ELISA system, anti-VPPase antiserum shows immuno activity with various monocotyledons and dicotyledons tested (table 3.1).

Table 3.1. Cross reactivity of polyclonal sugarcane VPPase antiserum with other monocots and dicots

Species	ELISA UNITS (% of Sugarcane)	SE (n =3)
Sugarcane	100	1.0
Sorghum	58.5	5.4
Zea mays	70.5	1.3
Hordeum vulgares	61.9	1.8
Tomato	67.7	4.0
Mung Bean	61.5	6.9

Data for ELISA UNITS were from direct competitive ELISA as described in the section 'Methods and Materials' on tonoplast proteins. The amount of cross-reactant giving the same degree of response (absorbance at 450 nm) in the assay was expressed as a % of the reaction given by the relevant amount of V-PPase in sugarcane. The % cross-reactivity is the mean of three replicates and the standard error (SE) was calculated.

Sugarcane vacuolar membrane proteins showed the highest immuno activity with the anti-VPPase antiserum as expected, because the antiserum was raised against a sugarcane V-PPase peptide. The differences observed in immuno activity between the species cannot be interpreted as the degree of cross reactivity because the specific concentration of V-PPase might differ significantly between the various samples. This experiment was only performed to illustrate that the sugarcane V-PPase anti-body can be used in various species for the immuno detection of V-PPase.

To conclude, the catalytic subunit of sugarcane V-PPase was successfully cloned, expressed in a bacterial system and V-PPase peptides purified. These peptides produced highly specific antiserum in mouse and were used for the establishment of an ELISA system for V-PPase protein determinations in sugarcane vacuolar membrane preparations. The establishment of this ELISA system was a prerequisite for the characterization of V-PPase expression in sugarcane and also proved to have several advantages over the protein blotting technique to quantify V-PPase protein levels. In this study we established the technology platform to investigate the role of V-PPase in sucrose accumulation.

CHAPTER 4

Kinetic properties of sugarcane Vacuolar H$^+$-Pyrophosphatase

4.1 INTRODUCTION

Plants, some bacteria and animal cells contain alternative metabolic pathways, which either utilize nucleotides or inorganic pyrophosphate (PPi) as energy sources (Black et al. 1987; Stitt 1998). Although the full significance of this phenomenon remains to be determined, this pattern of alternate energy source utilisation is exemplified by the presence of two parallel H$^+$-pumps in the vacuolar membrane (tonoplast) of plants. These are the vacuolar H$^+$-ATPase (V-ATPase, EC 3.6.1.3), an enzyme common to the endomembranes of all characterized eukaryotes (Nelson and Taiz 1989; Sze et al. 1992), and vacuolar H$^+$-pyrophosphatase (V-PPase EC 3.6.1.1), which apparently is ubiquitous in plants, but otherwise present in only a few phototrophic bacteria (Rea et al. 1992; Rea and Poole 1993). Both enzymes catalyse electrogenic H$^+$-translocation from the cytosol to the vacuole lumen to establish an inside-acid pH difference and inside-positive electrical potential difference, which are employed to energize the H$^+$-coupled, secondary transport of solutes across the tonoplast (Sze 1985; Blumwald 1987).

The potential bioenergetic impact of V-PPase is illustrated by its intrinsic abundant catalytic activity. Based on maximum catalytic activity the V-PPase is capable of generating a steady-state transtonoplast H$^+$-electrochemical potential difference of similar or greater magnitude than the V-ATPase on the same membrane (Maeshima and Yoshida 1989; Johannes and Felle 1989). Abundance estimates indicate that V-PPase constitutes approximately 1% of *Beta vulgaris* storage root (Britten et al. 1989; Rea et al. 1992) and 5-10% of *Vigna radiata* hypocotyl (Maeshima and Yoshida 1989; Rea et al. 1992) total vacuolar membrane protein. When taking into account that the vacuole of a typical mature plant cell represents 50-90% of total intracellular volume (Raven and Smith 1976) and hydrolysis by V-PPase may significantly contribute to the disposal of cytosolic PPi (Weiner et al. 1987; Takeshige and Tazawa 1989), it is clear that the V-PPase has the capacity to influence overall cellular PPi status substantially.

In vitro, V-PPase requires both Mg^{2+} and K$^+$, in addition to PPi for optimal activity (Walker and Leigh 1981; Rea and Poole 1985; Wang et al. 1986; White et al. 1990; Davies et al. 1991). However, the response of the enzyme to changes in both [PPi]$_{tot}$ and [Mg^{2+}]$_{tot}$ can be complex (Wang et al. 1986; Leigh and Pope 1987; Johannes and Felle 1989; White et al. 1990). In a

reaction medium containing PPi, Mg^{2+} and K^+, the complexes and ions present include free Mg^{2+}, free PPi, MgPPi, Mg_2PPi, K^+ and KPPi as well as various protonated forms of the complexes (Johannes and Felle 1989; White et al. 1990). This makes it very difficult to test the effect of individual complexes on the activity of V-PPase, because the concentration of any single complex cannot easily be changed without altering the concentration of some of the others. Nonetheless, previous kinetic studies have suggested that binding of Mg^{2+} stabilizes and activates the enzyme, K^+ stimulates V-PPase activity more than three times in most cases (Maeshima 2000a) and that the actual substrate is a Mg^{2+}-PPi complex (Mg_2PPi) (Leigh et al. 1992; Rea and Poole 1993; Baykov et al. 1993; Gordon-Weeks et al. 1996). In addition, both free PPi and Mg_2PPi might inhibit the enzyme, although this appears to depend on the tissue from which the tonoplast membranes are prepared (Wang et al. 1986; Johannes and Felle 1989; White et al. 1990).

Reported K_m values for PPi vary between 130 µM (Maeshima 1991), 2-5 µM (Baykov et al. 1993), and 2 µM (Gordon-Weeks et al. 1996). The apparent K_m values for Mg^{2+} have been reported to be 42 µM (Maeshima 1991) and 20-23 µM (Gordon-Weeks et al. 1996). Baykov et al. (1993) have reported the presence of high-affinity ($K_m = 20$-31 µM) and low-affinity ($K_m = 250$-460 µM) Mg^{2+}-binding sites in mung bean V-PPase. However, the exact number of Mg^{2+}-binding sites in V-PPase is still unclear. The K_m value for K^+ stimulation has been reported to be 1.27 mM (Gordon-Weeks et al. 1997). Cytosolic free Mg^{2+} concentration has been determined to be approximately 0.4 mM (Yazaki et al. 1988) whereas cytosolic free K^+ is thought to be maintained homeostatically around 80-100 mM (Leigh and Wyn Jones 1984; Malone et al. 1991). The reported cytosolic PPi concentration of the sugarcane variety NCo376 decreases from 270 µM in immature internode three to 166 µM in mature internode nine (Whittaker and Botha 1997). These physiological metabolite levels could allow V-PPase an estimated 90% of its maximum activity *in vivo* (Maeshima 2000a).

Specific activities of V-PPase in vacuolar membranes have been shown to vary depending on the plant species, tissue type and assay conditions. Reported catalytic values include 1.1, 0.3, 0.5, 0.4, 1.6 and 0.2-0.7 µmol PPi mg^{-1} of tonoplast protein for mung bean hypocotyls (Maeshima and Yoshida 1989), storage tissue of red beet (Britten et al. 1989; Sarafian and Poole 1989), *Arabidopsis* leaf (Schmidt and Brisken 1993), cotyledon of pumpkin seedling (Maeshima et al. 1994), *Acetabularia* (Ikeda et al. 1991), and CAM plants (Becker et al. 1995), respectively.

Previous investigations have proposed that V-PPase may play an important role in sucrose and phosphate metabolism because the cytosol of higher plants contains little or no soluble PPase and is almost exclusively located in the chloroplast stroma (Gross and Stitt 1986; Weiner et al. 1987). V-PPase may have a dual responsibility during sucrose synthesis and storage. V-PPase may firstly be accountable for the disposal of cytosolic PPi, which could otherwise inhibit sucrose synthesis and secondly use this energy to increase the sucrose sink pool by activating a proton motive force across the vacuolar membrane to drive the secondary transport of sucrose from the cytosol to the vacuole (Chapter 2). The zero-order kinetics for sugarcane V-PPase substrate hydrolysis have not been determined before. In order to elucidate the role of V-PPase in sucrose and phosphate metabolism and in particular the sucrose storage phenotype, molecular tools have to be developed and optimised in order to do so. The measurement of V-PPase hydrolytic activity will be a fundamental modus operandi during future work to elucidate the role of V-PPase in sucrose phosphate metabolism in sugarcane. The aim of this study was therefore to determine the zero-order conditions for sugarcane V-PPase hydrolytic activity.

4.2 METHODS AND MATERIALS

4.2.1 Chemicals

All cofactors and substrates used for tonoplast preparations and enzyme catalytic activity measurement were from either Sigma Chemical Company (St Louis, MO, USA) or Roche (Mannheim, Germany).
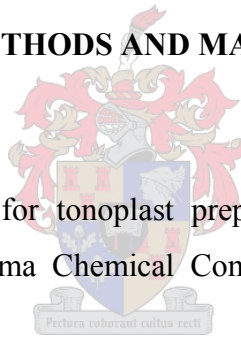
4.2.2 Plant material

The commercial sugarcane cultivar NCo310 was used for all the tonoplast preparations. Mature, non-flowering, field grown plants were sampled. Plants grew in the Stellenbosch area, South Africa. Stalks from plants with approximately 20 aboveground internodes were randomly selected and harvested in the morning. The first leaf with the uppermost visible dewlap was defined as number one. The internode immediately below the point of leaf attachment was designated by the same number, according to the system of Kuijper (van Dillewijn C 1952).

4.2.3 Sample preparation

Young internodes, three and four, were used for all the experiments. Internodes were excised from the stalk, and the rind carefully removed. The underlying tissue, spanning the core and the periphery, was rapidly sliced and frozen in liquid nitrogen. Samples were stored at -80°C until use.

4.2.4 Vacuolar membrane preparations

Vacuolar membranes were prepared as described previously by Maeshima and Yoshida (1989) and Rea and Poole (1985). Samples were removed from -80°C and ground into a fine powder with liquid nitrogen. For each membrane preparation, 10 to 12 g (fresh weight) of tissue was used and homogenized in 1:2 (w/v) homogenisation buffer containing 0.25 M sorbitol, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1% (w/v) polyvinylpyrolidone-40, 1% (w/v) ascorbic acid and 50 mM Mops/KOH buffer, pH 7.6.

The homogenate was filtered through a double layer of nylon cloth and centrifuged at 3 600 × g (Sorvall SLA-600 TC rotor) for 10 min. The supernatant was transferred to new tubes and centrifuged at 120 000 × g for 30 min using a SW 28 Beckman rotor. The microsomal pellet was suspended in 9 ml of suspension buffer (10 mM potassium phosphate (pH 7.8), 0.3 M Sucrose, 1 mM EGTA and 2 mM DTT). The suspension was overlaid with the same volume of overlaying buffer consisting of 5 mM Mops/KOH (pH 7.3), 0.25 M sorbitol, 1 mM EGTA and 2 mM DTT. After centrifugation at 120 000 × g for 30 minutes, the interface portion was collected and diluted in half a volume of 5 mM Mops/KOH (pH 7.3), 0.25 M sorbitol, 1 mM EGTA and 2 mM DTT and centrifuged at 130 000 × g for 30 min. The resulting white pellet (vacuolar membranes) was suspended in 20 mM Tris-acetate, pH 7.5, 20 % (w/v) glycerol, 2 mM DTT, 1 mM EGTA and 2 mM $MgCl_2$. The vacuolar membrane protein content of the preparations was determined by the method of Bradford (1976) and the membrane suspensions were rapidly frozen in liquid nitrogen and stored at -80°C until further analysis.

4.2.5 V-PPase hydrolytic activity

V-PPase hydrolytic activities were measured as the liberation of free Pi from PPi, estimated by the method of Ames (1966). Standard assays were done as previously described by Rea and Poole (1985).

The standard reaction mixture contained 3 mM sodium pyrophosphate, 50 mM KCl, 1mM sodium molybdate, 0.03% Triton X-100, 30 mM Tris/Mes (pH 8) and 3 mM $MgSO_4$. The reaction was started with the addition of $MgSO_4$. Membrane protein (1 μg) was made up to a final volume of 10 μl with water, added to 40 μl aliquots of reaction mixture, and incubated for 30 min at 30°C. The reaction was stopped with the addition of 150 μl of Ames reagent (6 parts 0.42% [w/v] ammonium molybdate in 1 N H_2SO_4 to 1 part 10 % [w/v] ascorbic acid) and allowed to stand for 10 min at 30°C. The amount of Pi released was colorimetrically determined

as previously described by Ames (1966). A_{820} was measured in a microplate against a reagent blank (tonoplast and reaction mix, without $MgSO_4$). V-PPase activity was calculated as one-half the rate of Pi liberation (= nmol PPi consumed per unit time). Variations in the assay pH, salt, or effector concentration are indicated in the text and legends.

The contamination of the tonoplast fraction by other organelle membranes was determined on pools of tonoplast fractions from the different varieties and tissues respectively. Vanadate (100μm), and Oligomycin (2 mg/l) sensitive ATPase activities were used as markers for the presence of plasma membrane (Rea and Turner 1990; Suwastika and Gehring 1999) and mitochondria or chloroplast membrane respectively (Penefsky 1985; Zheng and Ramirez 2000) and to determine the degree of contamination of non-vacuolar contamination.

4.3 RESULTS

In each of the following results V-PPase hydrolytic activity was measured according to the standard assay conditions as described in the methods and materials section unless otherwise stated.

4.3.1 pH dependence

Previous investigations have shown that V-PPase hydrolytic activity is pH sensitive and maximum activity is around pH 7 (Maeshima and Yoshida 1989). Sugarcane V-PPase displayed similar characteristics with a relatively broad pH optimum between pH 6.5 and pH 7.25 (Fig. 4.1).
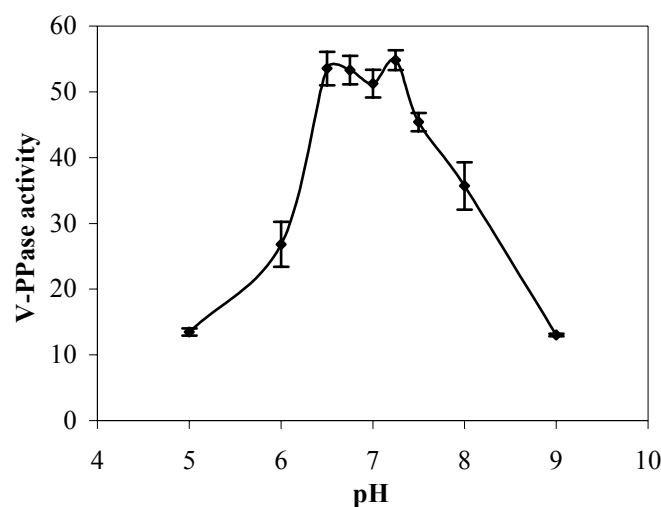
Fig. 4.1. pH dependence of sugarcane V-PPase hydrolytic activity. Activity was measured according to the standard assay with 30 mM Tris/Mes buffer, increasing in pH from 5 to 9. V-PPase activity is expressed as nmole PPi min^{-1} mg^{-1} membrane protein. Results are means ± SE of three independent assays.

4.3.2 Pyrophosphate dependence of hydrolytic activity

The PPi substrate dependency of sugarcane V-PPase activity is presented in Fig. 4.2. At a fixed total Mg^{2+} concentration $[(Mg)_{tot}]$ of 3 mM (Rea and Poole 1985), the maximum measured total V-PPase activity (63 ± 2.4 nmole PPi min^{-1} mg^{-1} protein, $n = 3$) occurred between 0.2 and 0.3 mM total PPi $[(PPi)_{tot}]$. Variation of PPi-dependent activity with $(PPi)_{tot}$ exhibited apparent substrate inhibition when $(PPi)_{tot}$ exceeded 0.3 mM, an observation which qualitatively agrees with that of White et al. (1990) and Leigh et al. (1992). The calculated apparent K_m for PPi is 0.178 ± 0.1 mM.
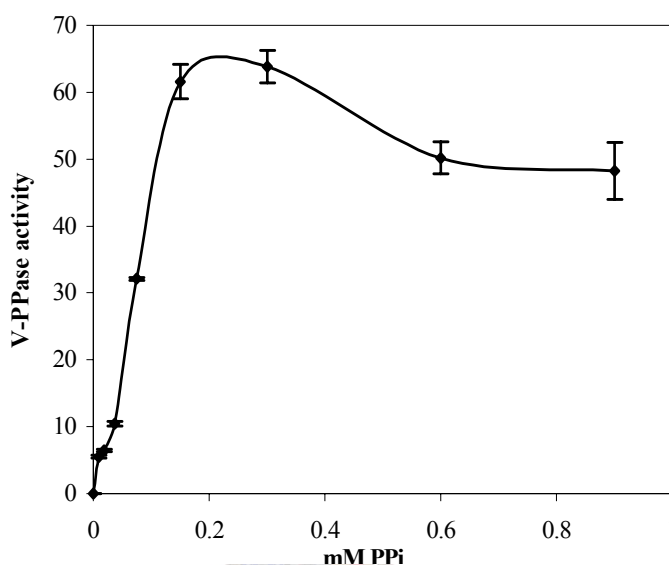
Fig. 4.2 Sugarcane V-PPase hydrolytic activity as a function of $(PPi)_{tot}$. Activities were determined using the standard assay conditions as explained in the methods and material section; 3 mM $MgSO_4$ buffered at pH 7 with 30 mM Tris/Mes was used throughout. V-PPase activity is expressed as nmole PPi min^{-1} mg^{-1} protein. Results are means $\pm$ SE of three independent assays .

4.3.3 Activation by potassium

V-PPase has the characteristic that it is completely dependent on K^+ (Wang et al. 1986). In vacuolar membranes, hydrolytic activity elicited by 0.3 mM $(PPi)_{tot}$ in the presence of 3 mM $(Mg^{2+})_{tot}$ increased with K^+ concentration, saturating at approximately 30 mM with maximal activity of 97.16 nmole PPi min^{-1} mg^{-1} protein (Fig. 4.3). V-PPase activity increased as a simple hyperbolic function of K^+ and displayed classic Michaelis Menten kinetics (R^2=0.976). The apparent K_m for K^+ stimulation of V-PPase activity is 2.28 ± 0.25 mM.

Fig. 4.3. Rate of PPi hydrolysis by V-PPase as a function of KCl concentration. The standard assay was used with 0.3 mM PPi and the reaction mix buffered at pH 7 with 30 mM Tris/Mes. V-PPase activity is expressed as nmole PPi min^{-1} mg^{-1} protein. Results are means ± SE of three independent assays.

4.3.4 Stimulation of V-PPase hydrolytic activity with Mg^{2+}

V-PPase requires free Mg^{2+} as an essential cofactor (Wang et al. 1986). Binding of Mg^{2+} stabilizes and activates the enzyme. V-PPase activity increased with Mg^{2+} concentration, saturating at approximately 1.3 mM MgSO$_4$ (Fig.4.4). Mg^{2+} stimulated V-PPase activity as a simple hyperbolic function similar to that of K$^+$ and also displays classic Michaelis Menten kinetics (R^2=0.976). The apparent K_m for Mg^{2+} stimulation of sugarcane V-PPase activity is 0.44 ± 0.1 mM.
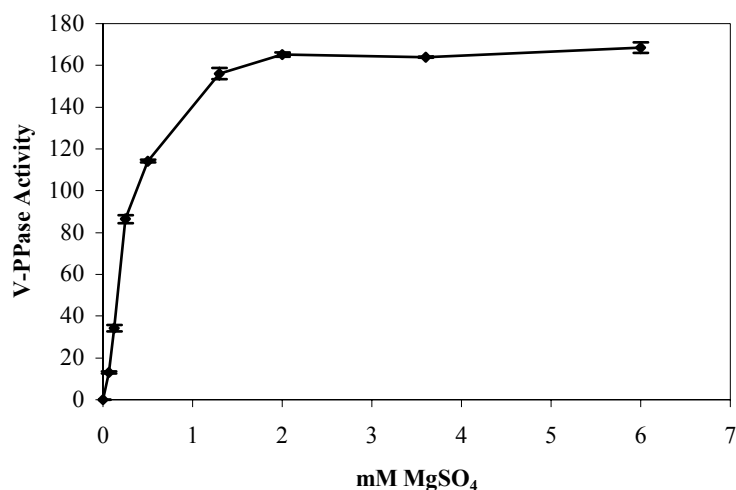
Fig. 4.4. Rate of PPi hydrolysis by V-PPase as a function of MgCl$_2$. The standard assay was used with 50 mM KCl; 0.3 mM PPi and the reaction mix buffered at pH 7 with 30 mM Tris/Mes. V-PPase activity is expressed as nmole PPi min^{-1} mg^{-1} protein. Results are means ± SE of three independent assays.

50

4.3.5 Tonoplast titration

V-PPase hydrolytic activity was measured using the optimised zero-order conditions as displayed in figures 4.1 to 4.4. Activity showed a linear increase ($R^2 = 0.9664$) between 0.1 μg and 1μg of tonoplast protein (Fig. 4.5). No activity was detectable without PPi and $MgSO_4$.
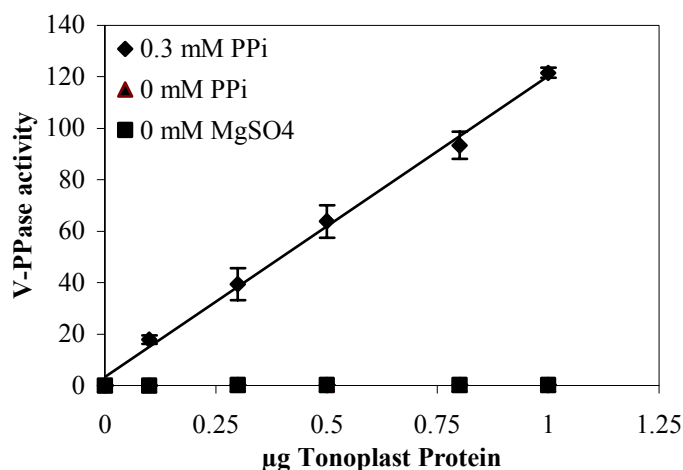
Fig. 5. Tonoplast titration for V-PPase hydrolytic activity. The assay medium contained 50 mM KCl, 0.3 mM PPi, 1.3 mM $MgSO_4$. Activity w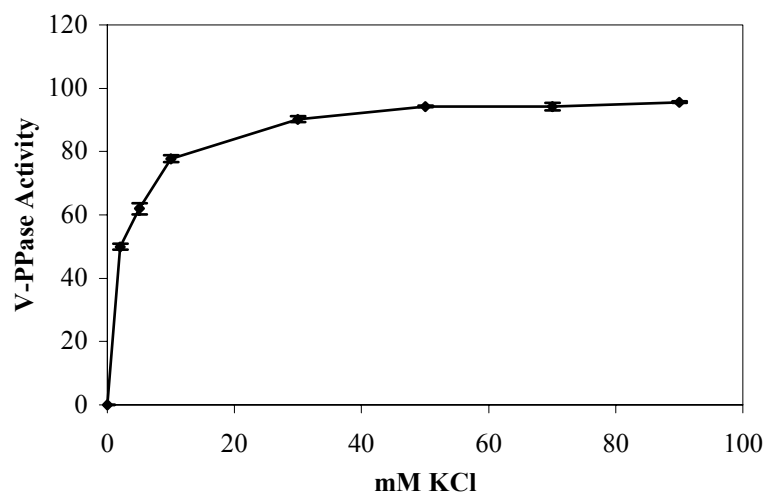as measured at pH 7 throughout. V-PPase activity is expressed as nmole PPi mg^{-1} protein. Results are means ± SE of three independent assays.

4.4 DISCUSSION

V-PPase requires both Mg^{2+} and K^+, in addition to PPi, for maximum activity *in vitro* (Davies et al. 1991). Moreover, the response of the enzyme to changes in the concentration of these substrates can be complex (Wang et al. 1986; Leigh and Pope 1987) and shows substantial differences between species (Maeshima 2000b). The optimisation of zero-order kinetics for sugarcane V-PPase hydrolytic activity is therefore a prerequisite to guarantee accurate hydrolytic activity measurements. Appreciable PPi hydrolysis by sugarcane tonoplast proteins with variation in each of the substrates was observed in this study.

Sugarcane V-PPase showed a broad pH optimum between 6.5 and 7.25 (Fig. 4.1). Similar pH activity profiles have been obtained with the V-PPases of isolated red beet vacuoles (Wagner and Mulready 1983) and the microsomal pellet of sugar beet (Karlsson 1974). Kinetic analysis of V-PPase hydrolytic activity as a function of [PPi], showed maximum activity between 0.2 and 0.3 mM with an apparent K_m of 0.178 mM ± 0.1 (Fig. 4.2.). Comparable K_m values for PPi has previously been reported in mung bean 0.130 mM (Maeshima 1991), whereas much lower values have previously been reported, 2-5 μM (Baykov et al. 1993) and 2 μM (Gordon-Weeks et al. 1996). The reported cytosolic PPi concentration of the sugarcane variety NCo376 is 270 μM in immature internode three (Whittaker and Botha 1997). This cytosolic PPi concentration could in

theory mean that the Sugarcane V-PPase would function at approximately 76% of its maximum catalytic activity in immature internode three if presumed that the cytosolic PPi concentrations were the same for the varieties NCo376 and NCo310.

In addition to PPi as the substrate for hydrolysis, V-PPase requires both free Mg^{2+} and K^+ ions as essential co-factors for activity. The binding of Mg^{2+} stabilizes and activates the enzyme, whereas K^+ stimulates V-PPase activity (Maeshima 2000b). Our results showed that sugarcane V-PPase activity increased as a simple hyperbolic function of KCl, saturating at approximately 30 mM (Fig 4.3). Similarly, $MgSO_4$ also stimulated V-PPase activity as a simple hyperbolic function and saturated at approximately 1.3 mM (Fig. 4.4). The calculated apparent K_m for K^+ stimulation of sugarcane V-PPase activity is 2.28 ± 0.25 mM. This value compares well with the reported K_m value of 1.27 mM for mung bean V-PPase (Gordon-Weeks et al. 1997; Maeshima 2000b). The K_m for Mg^{2+} stimulation of sugarcane V-PPase activity is 0.44 ± 0.1 and is in the same range as the reported low-affinity Mg^{2+} -binding sites ($K_m = 0.25 - 0.46$ mM) of mung bean V-PPase (Baykov et al. 1993).

Under the above determined zero-order conditions total V-PPase activity showed a linear response ($R^2 = 0.9664$) between 0.1 μg and 1μg of tonoplast protein (Fig. 4.5). The specific activity of sugarcane V-PPase from young internodes, i.e. internodes three and four, was 121.6 nmole PPi min^{-1} mg^{-1} protein. This value is lower than the reported V-PPase specific activities of mung bean 300 nmole PPi min^{-1} mg^{-1} protein (Maeshima and Yoshida 1989), storage tissue of red beet 520 nmole PPi min^{-1} mg^{-1} protein (Britten et al. 1989; Sarafian and Poole 1989) and *Arabidopsis* leaf 350 nmole PPi min^{-1} mg^{-1} protein (Schmidt and Brisken 1993). Maximum activity for V-PPase is generally present in young immature tissue in most species.

In this study the zero-order substrate concentrations and kinetics of sugarcane V-PPase were determined and compared with that of other species. This work will be fundamental to the characterization of V-PPase expression in sugarcane during future work.

CHAPTER 5

Characterization of V-PPase expression in the sugarcane culm

5.1 INTRODUCTION

The vacuole of higher plants is a dynamic, acidic organelle that occupies more than 90% of the cell's volume (Maeshima 2001). Typical plant vacuoles are 50 to 100 μm in diameter (Maeshima and Nakanishi 1998). The vacuole governs numerous cellular processes, including: the regulation of cytosolic homeostasis, recycling of cellular components, space filling and the storage of inorganic ions, organic acids and sugars (Hedrich and Schroeder 1989; Taiz 1992; Maeshima et al. 1996). Many of these processes are directly or indirectly related to either the transmembrane electrochemical gradient across the vacuolar membrane or the acidic pH in the vacuole. Two distinct primary proton translocating enzymes, vacuolar H^+-ATPase (V-ATPase, EC 3.6.1.3) and vacuolar H^+-pyrophosphatase (V-PPase, EC 3.6.1.1), are located in the vacuolar membrane (Sze 1985; Hedrich and Schroeder 1989; Taiz 1992; Rea and Poole 1993). Although both these enzymes catalyse the electrogenic H^+ translocation from the cytosol to the vacuolar lumen to maintain an inside-acid pH and a cytosol-negative electrical potential difference, both enzymes are specific for the use of its substrate (Rea et al. 1992). The H^+-gradient generated by these two proton pumps, powers the secondary active transport of various metabolites and solutes across the vacuolar membrane (Hedrich and Schroeder 1989; Taiz 1992).

V-PPase is generally studied from two perspectives. Firstly, the V-PPase enzyme molecule is a great proton-pump model with which to study how the hydrolysis of phosphate ester is coupled with proton translocation across the vacuolar membrane, because the enzyme consists as a dimer of two identical subunits with a molecular mass of approximately 80 kDa (Maeshima and Yoshida 1989; Kim et al. 1994). Secondly, its substrate inorganic pyrophosphate (PPi), is one of the simplest high-energy compounds (Baltscheffsky et al. 1999; Maeshima 2000) and is produced as a by-product in the activation or polymerisation steps of a wide range of biosynthetic pathways. Therefore, the biochemical properties of V-PPase and the detailed mechanisms of the enzyme reaction have been extensively analysed (Rea and Poole 1993; Baykov et al. 1993). To date, several V-PPase cDNA clones from various plants have been characterized . The first cDNA clone for V-PPase was isolated from

Arabidopsis (genbank accession no. M81892). Only one V-PPase cDNA clone was cloned from Barley (D13472) (Tanaka et al. 1993), mung bean (AB009077) (Nakanishi and Maeshima 1998) and pumpkin (D86306) (Maruyama et al. 1998). Two V-PPase cDNA species were isolated from red beet (L32791, L32792) (Kim et al. 1994) and rice (D45383, D45384) (Sakakibara et al. 1996), whereas three V-PPase cDNA species of tobacco (*TVP5, TVP9* and *TVP* 31) (Lerchl et al. 1995) were isolated.

The expression of V-PPase has been well documented, but from a physiological and metabolic point of view, the same question is still pending: Why do V-PPase and V-ATPase co-exist in the vacuolar membranes of plants but not in those of yeast or animal acidic compartments? The vacuolar membranes prepared from all of the land plants species, including mosses, ferns and algae show V-PPase activity in addition to V-ATPase activity, which coincides with proton pump activity (Takeshige et al. 1988; Ikeda et al. 1991; Maeshima et al. 1994b). Numerous studies have shown that V-PPase is the main proton pump of most young tissues and V-ATPase becomes the major proton pump in mature tissues (Maeshima 1990; Maeshima et al. 1994a; Lerchl et al. 1995; Shiratake et al. 1997; Smart et al. 1998). In addition, the level of V-PPase is regulated under various stress conditions, including: mineral deficiencies (Colombo and Cerana 1993; Kasai et al. 1998), anoxia and cold stress (Yoshida et al. 1989; Rea and Poole 1993; Darley et al. 1995; Carystinos et al. 1995) and drought accompanied with toxic salt levels (Gaxiola et al. 2001).

Various reports have indicated the possibility that V-PPase may play an important role during sucrose synthesis and accumulation (Quick et al. 1989; Neuhaus and Stitt 1991; Sonnewald 1992; Jellito et al. 1992; Niland and Schmitz 1995; Sikora et al. 1998). A simple explanation for this may be that V-PPase firstly makes a significant contribution to PPi removal concurrent with other PPi phosphatase enzymes in the cytosol during sucrose synthesis. Secondly, V-PPase may play a pivotal role in the supply of energy for tonoplast energisation via the pyrophosphate-dependent proton pump (Davies et al. 1993; Zhen et al. 1997) to activate the secondary transport of various solutes including sucrose, into the vacuole. During sucrose synthesis, there is a need for the removal of the PPi produced by uridine 5'-diphosphoglucose (UDPGlc) pyrophosphorylase (UGPase), which would otherwise inhibit the production of UDPGlc. Inhibition of UDPGlc production will concurrently inhibit sucrose phosphate synthase (SPS) and ultimately inhibit sucrose synthesis and favour the formation of fructose 1,6-bisphophate and glycolytic carbon flow. One molecule of PPi is produced in the

reaction catalysed by UGPase per molecule of sucrose produced (Neuhaus and Stitt 1991). The accumulation of PPi in the cytosol to abnormal levels could therefore inhibit sucrose synthesis. Strong indirect evidence to this effect has been obtained by inhibitor studies with fluoride (Quick et al. 1989) and the nonhydrolyzable PPi analog imidodiphosphate (Neuhaus and Stitt 1991), which are potent inhibitors of V-PPase (Wang et al. 1986; Chanson and Pilet 1987; Chanson and Pilet 1988) but not PFP (Neuhaus and Stitt 1991; Quick et al. 1989; Van Schaftingen et al. 1982). Administering these inhibitors to detached leaves via the transpiration stream leads to the strong inhibition of sucrose synthesis concomitant with the depletion of cellular UDPGlc and the accumulation of PPi, hexose monophosphates, and fructose 1, 6 bisphosphate. Concurrently, analysis of potato tuber transformants with overexpressed soluble pyrophosphatase resulted in higher levels of sucrose and reducing sugars, increased UDPGlc, decreased levels of phosphates and other phosphorylated intermediates, and less starch, compared to wild type tubers (Sonnewald 1992; Jellito et al. 1992). Since sucrose degradation in growing tubers occurs via sucrose synthase, a simple explanation for this inhibition of sucrose mobilisation was that low PPi restricts the conversion of UDPglucose to hexose phosphates by UGPase. When PPase was overexpressed under the control of the patatin promotor to restrict expression to parenchyma cells, metabolites, sucrose and starch changed as expected from a block at UGPase (Trethewey 1998). Intriguingly, moderate constitutive overexpression of PPase led to a general increase of nucleotides and tuber proteins involved in sucrose-starch interconversion and a slight stimulation of starch synthesis indicating that signals related to PPi or PPi metabolism in cells where the patatin promotor is inactive may stimulate sink function (Geigenberger et al. 1998). Sikora et al. (1998) studied the effect of sucrose starvation on logarithmically growing suspension-cultured tobacco cells using immunogold election microscopy with antisera against V-PPase and V-ATPase. After 32 hours of growth in the absence of exogenous sucrose, V-PPase and V-ATPase polypeptides were no longer detectable.

Plants contain a considerable pool of PPi in the cytosol, which is produced as a relatively inexpensive source of energy in the activation or polymerisation steps of a wide range of biosynthetic pathways (Chanson et al. 1985; Edwards and Rees 1986). The reported cytosolic PPi concentration of the sugarcane variety NCo376 decreases from 270 μM in immature internode three to 166 μM in mature internode nine (Whittaker and Botha 1997). Furthermore, PPi has an estimated *in vivo* free energy of hydrolysis about half of that of ATP (Weiner et al. 1987; Davies et al. 1993). However, the cytosol of higher plant cells contains

little or no soluble pyrophosphatase and alkaline pyrophosphatase is only located in the plastid (Gross and Stitt 1986; Weiner et al. 1987). Because soluble PPase activity is largely absent from the cytosol and almost exclusively located in the chloroplast stroma (Weiner et al. 1987), V-PPase may have a dual responsibility during sucrose synthesis and storage. V-PPase may firstly be accountable for the disposal of cytosolic PPi, which could otherwise inhibit sucrose synthesis and secondly use this energy to increase the metabolic sink pool by activating a proton motive force across the vacuolar membrane to drive the secondary transport of various metabolites, including sucrose from the cytosol to the vacuole.

Sugarcane as the world's largest producer of sucrose is a good model in which to study the metabolic role of V-PPase and its possible link to sucrose and phosphate metabolism. To date, nothing is known about V-PPase in sugarcane except for the sequences of a few ESTs. The aim of the present study was, therefore, to compare V-PPase at genomic-, transcript-, protein- and enzymatic level between hybrid sugarcane varieties that differ significantly in sucrose content. The main objective of this study was to assess whether the expression and/or activity of V-PPase is correlated in any way to the sucrose storage phenotype.

5.2 METHODS AND MATERIALS

5.2.1 Chemicals

All auxiliary enzymes, cofactors and substrates used for sample preparations were from either Sigma Chemical Company (St Louis, MO, USA) or Roche (South Africa)..

5.2.2 Plant materials

Eighteen month-old sugarcane with approximately twenty aboveground internodes was randomly selected and harvested. Hybrid varieties: US6656-15, NCo376 and N24 were used. The plants grew at the South African Sugarcane Research Institute (SASRI), north of Durban, Kwazulu-Natal, South Africa. Mature, non-flowering, field grown plants were sampled at 09:00 hours during early January, which is mid-summer. Six internodes were harvested to represent immature (internodes three and four), maturing (internodes seven and eight) and mature (internodes eleven and twelve) culm tissue. The first leaf with the uppermost visible dewlap was defined as number one. The internode immediately below the point of leaf attachment was designated by the same number, according to the system of Kuijper (van Dillewijn C 1952).

5.2.3 Sample preparation

Internodes selected for analysis were excised from the stalk and washed with water and 70% [v/v] ethanol, and the rind carefully removed. The underlying, spanning core of the periphery was rapidly sliced into small pieces and frozen in liquid nitrogen. Samples were stored at -80°C until further use.

5.2.4 DNA Extraction

Genomic DNA was isolated from sugarcane tissue according to the method of Dellaporta et al. (1983).

5.2.5 Southern Blot Analysis

Ten microgram of genomic DNA was fully digested with Hind III overnight at 37°C according to the recommended protocol of the restriction enzyme company (Promega). After a 24:1 (v/v) chloroform/isoamylalcohol extraction, the DNA was precipitated with $MgCl_2$ and 70% ethanol (v/v). The samples were air dried, resuspended in distilled water and electrophoresed through a 0.8 % (w/v) agarose gel at 100 V. DNA was then denatured with 1.5 M NaCl and 0.5 M NaOH for 15 min and transferred to neutralisation solution containing 0.5 M Tris-Cl (pH 7.4) and 1.5 M NaCl, for another 15 min. The gel was equilibrated in 10x SSC (1.5 M NaCl and 0.3 M $Na_3C_6H_5O_7$; pH, 6.8) at room temperature for 20 min. The DNA was then transferred overnight onto positively charged nylon membranes (Boehringher Manheim) by the downward blot method. The DNA was then cross-linked to the membrane under UV light (1200 mJ cm^{-2}, 2 min).

5.2.6 RNA Extraction

RNA was extracted according to a modified method of Bugos et al. (1995). Tissue was ground to a fine powder in liquid nitrogen and added 1:2 (w/v) to 25:24:1 phenol:chloroform:isoamyl alchohol and vortexed. There after homogenisation buffer (0.1 M Tris-HCl (pH 7.5), 1 mM ethylene diamine tetra-acetic acid (EDTA), 1% (w/v) sodium dodecyl sulphate (SDS), 0.1 M NaCl and 100 mM β-mecaptoethanol) was added at the same volume as the 25:24:1 phenol:chloroform:isoamyl alchohol. Sodium acetate (pH 5.2) was added to a final concentration of 0.1 M, the solution was mixed and incubated on ice for 15 min followed by a 15 min centrifugation at 12 000 x g, 4°C. The aquas phase was transferred to a new tube and one volume isopropanol added, followed by incubation at -70°C for at least 30 min. Precipitated RNA was pelleted by centrifugation at 10 000 x g for 10 min at 4°C. The

pellet was washed with 70% (v/v) ethanol, centrifuged for 5 min at 10 000 x g, and air-dried. RNA was resuspended in 750 µl diethyl pyrocarbonate (DEPC) treated water, and insolubles removed by centrifugation at 10 000 x g for 5 min at 4°C. The supernatant was transferred to a microcentrifuge tube, and RNA precipitated using LiCl (final concentration of 2 M) and incubated overnight at 4°C. RNA was recovered by centrifugation at 12 000 x g for 15 min at 4°C. The pellet was washed with 70%(v/v) ethanol, centrifuged at 10 000 x g for 5 min and air-dried. The pellet was resuspended in DEPC treated water and all remaining insolubles removed by centrifugation at 10 000 x g for 5 min at 4°C. RNA was quantified spectophotometrically.

5.2.7 Preparation of the cDNA radiolabeled probe and hybridisation

The same V-PPase cDNA clone (genbank accession no. CAF22023), which was earlier reported (Chapter 3), was used in this study for the preparation of V-PPase cDNA radioactive labelled probes for southern and northern blot analysis.

Total RNA (5µg) was used for first strand cDNA synthesis (Superscript^{TM}II, GibcoBRL Life Technologies Inc., Gaithersberg, MD USA) according to the manufacturer's instructions. The catalytic region of V-PPase was amplified by PCR using gene-specific primers (forward, 5′-ATAGGATCCACTGGTTATGGTCTTGGTGGT-3′; reverse, 5′-ATAGGATCCTAGICCAG CCCAIAGACCAAC-3′). The amplification protocol was 5 min at 95°C (once), 1 min at 95°C, 1 min at 55°C and 2 min at 72°C for 20 cycles. Another 20 cycles were done at an annealing temperature of 50°C. The amplified product was visualized in ethidium bromide-stained 1.2 % (m/v) agarose gel with an expected size of 600 bp. The amplified product was purified from the gel using QIAGENs QIAquick gel extraction Kit and ligated into the pGEMTeasy vector. For the preparation of radiolabeled probes, the fragment of interest was radioactively labelled using 25 µCi [$\propto$-^{32}P] dCTP (Amersham). Re-amplification using PCR was done under the same conditions as used in the initial amplification procedure using 1 ng of the pGEMTeasy vector with V-PPase insert for only 10 cycles.

Membranes were prehybridised in 15 ml of RAPIDhyb^{TM} rapid hybridization buffer at 65°C for 30 min. The probe was boiled for 5 min, added to the hybridisation bottle and incubated overnight at 65°C. The hybridised blots were washed twice for 15 min each at 50°C and 55°C in wash solution 1 (1 x SSC, 0.1% (w/v) SDS) and twice for 15 min each at 60°C and 65°C in wash solution 2 (0.5 x SSC, 0.1% (w/v) SDS). The blots were exposed to a supersensitive

Cyclone Phosphor screen (Packard) for 16 to 18 hours. Hybridisation was visualised using the Cyclone[TM] Storage Phosphor System (Packard Instrument Co., Inc., Meriden, USA).

5.2.8 Northern Blot Analysis

Fifteen µg of total RNA per sample were loaded on a 1.2% (w/v) agarose gel (made up with 1 time diluted Tris-Borate/EDTA electrophoresis buffer (TBE)). RNA was separated at 100 V until the dye front had moved 8 cm. The gel was trimmed and stained in ethidium bromide solution (0.4 µg ml^{-1}) for 15 min. RNA was visualized under UV light. The gel was then equilibrated in 10x SSC for 20 min, and the membrane wetted in water and equilibrated in 10 X SSC for 10 min. RNA was transferred to a positively charged nylon membrane by downward capillary blotting using 10 X SSC overnight at room temperature. After transfer the membrane was removed and RNA was UV cross-linked to the membrane on both sides for 1.5 min at 1200 mJ cm^{-2}.

Probe preparation, prehybridisation and hybridisation of membranes, washing steps, exposure and developing of films were carried out as for the Southern blot analysis method above. Total RNA and V-PPase mRNA signal intensities were analyzed using the AlphaImager[TM]2000 documentation and analysis system (Alpha Innotech Corporation, San Leandro, USA). The V-PPase mRNA band size was calculated with the above-mentioned software and the use of standard RNA marker supplied by Promega.

5.2.9 Vacuolar membrane preparations

Because of the low ratio of protein to lipid in the tonoplast membrane, tonoplast vesicles usually have a relatively low density, and therefore can be separated from other cellular membranes by density-gradient centrifugation (Kawamura et al. 2000; http://www.plantphys.net). Vacuolar membranes were prepared as first described by Maeshima and Yoshida (1989) and Rea and Poole (1985). Samples were removed from -80°C and ground into a fine powder with liquid nitrogen. For each membrane preparation 10 to 12 g fresh weight of tissue was used and homogenized in 1:2 (w/v) homogenisation buffer containg 0.25 M sorbitol, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1% (w/v) polyvinylpyrolidone-40, 1% (w/v) ascorbic acid and 50 mM Mops/KOH buffer (pH 7.6).

The homogenate was filtered through a double layer of nylon cloth and centrifuged at 3 600 × g in a Sorvall SLA-600 TC rotor for 10 min. The supernatant was transferred to new tubes

and centrifuged at 120 000 × g for 30 min using a SW 28 Beckman rotor. The microsomal pellet was suspended in 9 ml of suspension buffer (10 mM potassium phosphate (pH 7.8), 0.3 M Sucrose, 1 mM EGTA and 2 mM DTT). The suspension was overlaid with the same volume of overlaying buffer consisting of 5 mM Mops/KOH (pH 7.3), 0.25 M Sorbitol, 1 mM EGTA and 2 mM DTT. After centrifugation at 120 000 × g for 30 minutes, the interface portion was collected and diluted in half a volume of 5 mM Mops/KOH (pH 7.3), 0.25 M Sorbitol, 1 mM EGTA and 2 mM DTT and centrifuged at 130 000 × g for 30 min. The resulting white pellet (vacuolar membranes) was suspended in 20 mM Tris-acetate, pH 7.5, 20 % (w/v) glyserol, 2 mM DTT, 1 mM EGTA and 2 mM $MgCl_2$. The vacuolar membrane protein content of the preparations was determined by the method of Bradford (1976) and the membrane suspensions were flash frozen in liquid nitrogen and stored at -80°C until further analysis.

5.2.10 ELISA for V-PPase protein level measurement

The production and characterization of a sugarcane anti-VPPase polyclonal anti-body and the optimization of an ELISA system for V-PPase protein level measurement in sugarcane was earlier reported in detail (Chapter 3). Nunc-Immuno Maxisorp® plates (Nalge Nunc, Denmark) were used for ELISA assays. Plates were coated with tonoplast vesicles (1 μg/ml) in Saline at 50 μl/well for 16 hours at 22°C. At the end of the incubation period the coating solution was decanted after which 0.2 ml BSA (1% w/v in Saline) was dispensed into each well to block the remaining adsorption sites. The plates were then incubated for 30 min at 22°C, gently shaking after which it was washed 4 times with saline. This was followed by the addition of anti-VPPase anti-serum (1/200) and 0.01% (w/v) SDS in blocking solution at a volume of 200 μl/well, gently shaking for 2 hours at 22°C. Wells were again decanted and plates were washed as before. Anti-mouse peroxidase conjugate (AEC-Amersham, South-Africa) was diluted to 1/5000 and dispensed at a volume of 50 μl/well and incubated for 1 hour at 22°C. The well contents were again decanted and washed 7 times with saline. Plates were developed for 15 min using BM Blue™ soluble substrate (Roche, South Africa) at 50 μl/well at 22°C. The reaction was stopped with the addition of 25 μl stopping solution containing 0.2 M H_2SO_4. The absorbance values were then read at 450 nm.

5.2.11 V-PPase and V-ATPase hydrolytic activity measurement

V-PPase and V-ATPase hydrolytic activities were measured as the liberation of free Pi from ATP or PPi and estimated by the method of Ames (1966). Assays were done as previously described by Maeshima and Yoshida (1989) with some changes.

V-PPase hydrolytic activity was measured at zero order kinetic (Chapter 4). For V-PPase hydrolysis, the reaction mixture contained 0.25mM sodium pyrophosphate, 50 mM KCl, 1mM sodium molybdate, 0.03% Triton X-100 and 30 mM Tris/Mes (pH 7) and 1 mM $MgSO_4$. The reaction was started with the addition of $MgSO_4$. Tonoplast protein (0.5-1 µg) was made up to a final volume of 10 µl with water, added to 40 µl aliquots of reaction mixture, and incubated for 30 min at 30°C. The reaction was stopped by with the addition of 150 µl of Ames reagent (6 parts 0.42% [w/v] ammonium molybdate in 1 N H_2SO_4 to 1 part 10 % [w/v] ascorbic acid) and incubated for 10 min at 30°C. The amount of Pi released was calorimetrically determined as previously described by Ames (1966). A_{820} was measured in a microplate against a reagent blank (tonoplast and reaction mix, without $MgSO_4$). To compare V-PPase and V-ATPase activities on a mole/mole basis, V-PPase activity was calculated as one-half the rate of Pi liberation (= nmol PPi consumed per unit time).

V-ATPase hydrolytic activity was estimated as described above but the reaction mixture contained 40 mM Tris/Mes (pH 6.5), 1mM sodium molybdate, 0.03% Triton X-100, 3 mM $MgSO_4$, 3 mM ATP and 100 mM KCl. Substrate amounts were used according to Williams et al. (1990) at zero order kinetics for sugarcane. The contamination of the tonoplast fraction by other organelle membranes was determined on pools of tonoplast fractions from the different cultivars and tissues respectively. Vanadate (100µm), and Oligomycin (2 mg/l) sensitive ATPase activities were used as markers for the presence of plasma membrane (Rea and Turner 1990; Suwastika and Gehring 1999) and mitochondria or chloroplast membrane respectively (Penefsky 1985; Zheng and Ramirez 2000) and to determine the degree of contamination of non-vacuolar contamination.

5.2.12 Sugar Extraction and Measurement

Soluble sugars were extracted in 70% (v/v) ethanol and 30% HM-buffer (100 mM HEPES [pH 7.8], 20 mM MgCl2), (1/10, w/v) with incubation at 65ºC overnight. The samples were then centrifuged at 10 000 x g, for 10 minutes at room temperature. The supernatant was dried under vacuum and resuspended in 1 ml 10% (v/v) isopropanol. For sucrose determinations all

samples were diluted 10 times. Sucrose, glucose and fructose concentrations were determined spectophotometrically using the enzymatic method described by Bergmeyer and Bernt (1974). Purity was expressed as the percentage molar ratio between sucrose and the total sugar pool including glucose, fructose and sucrose.

5.2.13 Statistical methods

Analysis of the ELISA and V-PPase catalytic activity date were performed in 4 independent replicate samples from 4 individual plants. Data were analysed for variance and the least significant difference using the Fischer Post Hoc test. Significant differences between treatments are indicated at $P<0.05$ (a,b,c).
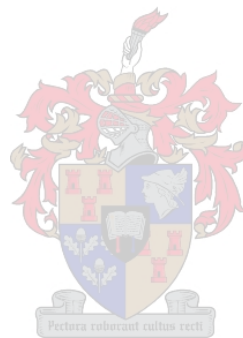
5.3 RESULTS

In this study three hybrid varieties, US6656-15, NCo376 and N24 that had previously been shown to differ significantly in sucrose content (Whittaker and Botha 1999), were used to characterize V-PPase on transcriptional level, protein abundance and specific activity. In addition, two ancestral varieties, *S. robustum* and *S. spontaneum* (Coimbatore), were compared to these three varieties in terms of the genomic organisation of the V-PPase genes. *S. robustum* is a high sucrose accumulating variety, whereas *S. spontaneum* accumulates significantly lower sucrose levels (Moore and Maretzki 1996; Whittaker and Botha 1999).

5.3.1 Soluble sugars

Previous investigations have shown that within the developing stalk of NCo376 there is a sharp increase in sucrose content between internodes 2 and 7 (Botha et al. 1996; Whittaker and Botha 1997). A similar relationship exists in US6656-15, NCo376 and N24, analysed in the present study. There was a sharp gradient in sucrose content between immature, maturing and mature internodes in each of the three varieties (Fig. 5.1A.). The sucrose content in the
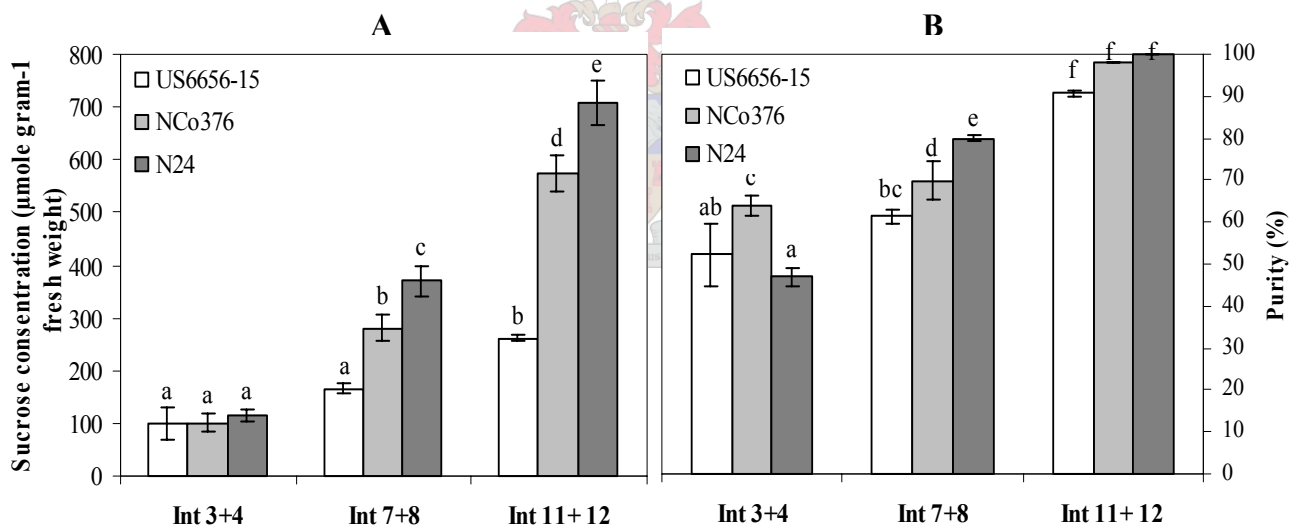
Fig. 5.1 Sucrose content and purity of the hybrid varieties used. Soluble sugars were extracted from the same tissue samples as DNA, RNA and tonoplast extracts. Soluble sugar contents were determined from three sets of internodes to represent immature (internodes 3+4), maturing (internodes 7+8) and mature (internodes 11+12) culm tissue from three varieties: US6656-15, NCo376 and N24. Only sucrose contents (**A**) and percentage purity (**B**) are displayed. Data points are the mean of four plants each and vertical error bars represent standard errors.

immature internodes is significantly lower than in those of the maturing and mature internodes respectively, except for US6656-15 that showed no significant differences between the immature and maturing internodes.

Results obtained for the comparison of the sucrose content in immature, maturing and mature internodes between the three varieties supports the findings of (Whittaker and Botha 1999). There were no significant differences in the immature internodes between the three varieties, whereas significant differences in sucrose content were observed in the maturing and mature internodes between US6656-15, NCo376 and N24. N24 has the highest sucrose contents, NCo376 intermediate and US6656-15 the lowest.

The percentage purity sharply increases from the immature internodes to the mature internodes in all three of the hybrid varieties (Fig. 5.1B.). In all three of the varieties, the immature internodes have the lowest percentage purity, maturing internodes intermediate, whereas mature internodes have the highest percentage purity. Although no significant differences were observed in the percentage purity between the immature and maturing internodes of US6656-15, NCo376 and N24 showed significant differences. Comparison of the percentage purity in specific internodes between the three varieties showed that US6656-15 has the lowest purity, NCo376 intermediate and N24 the highest in maturing internodes. No significant differences in the percentage purity were observed between the three varieties in both immature- and mature internodes except for NCo376 that showed a significant higher percentage purity than US6656-15 and N24 in immature internodes.

5.3.2 Genomic organisation of the V-PPase gene

The genomic organisation of the V-PPase gene was compared between two ancestral species (*S. robustum* and *S. spontaneum* (Coimbatore)) and three different hybrid varieties (US6656-15, NCo376, N24) (Fig.5.2).

The V-PPase cDNA used to probe the southern blot contained no *Hind III* restriction sites. The banding pattern exhibited by the southern blot analysis across the three hybrid- and two ancestral varieties was very simple and almost identical. Two major bands as well as two minor bands appear in each of these lines or varieties. Coimbatore has an additional faint band, which does not appear in any of the hybrid varieties.
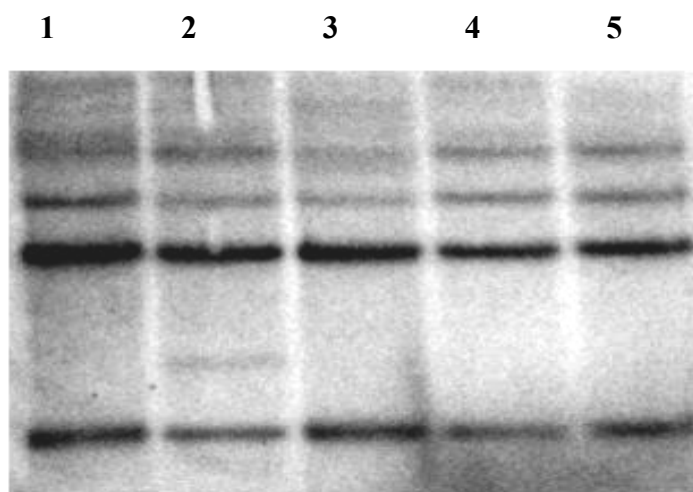
Fig. 5.2. Genomic southern analysis of V-PPase. A cDNA clone containing the catalytic site for V-PPase, which is highly conserved among all land plants was used to probe completely digested gDNA, using *Hind III*: (1) *S. robustum*; (2) *S. spontaneum* (Coimbatore); (3) US6656-15; (4) NCo376; (5) N24.

5.3.3 Transcript expression patterns

The V-PPase transcript expression levels were analysed in immature-, maturing- and mature internodes of US6656-15, NCo376 and N24 with northern blot analysis (Fig. 5.3).
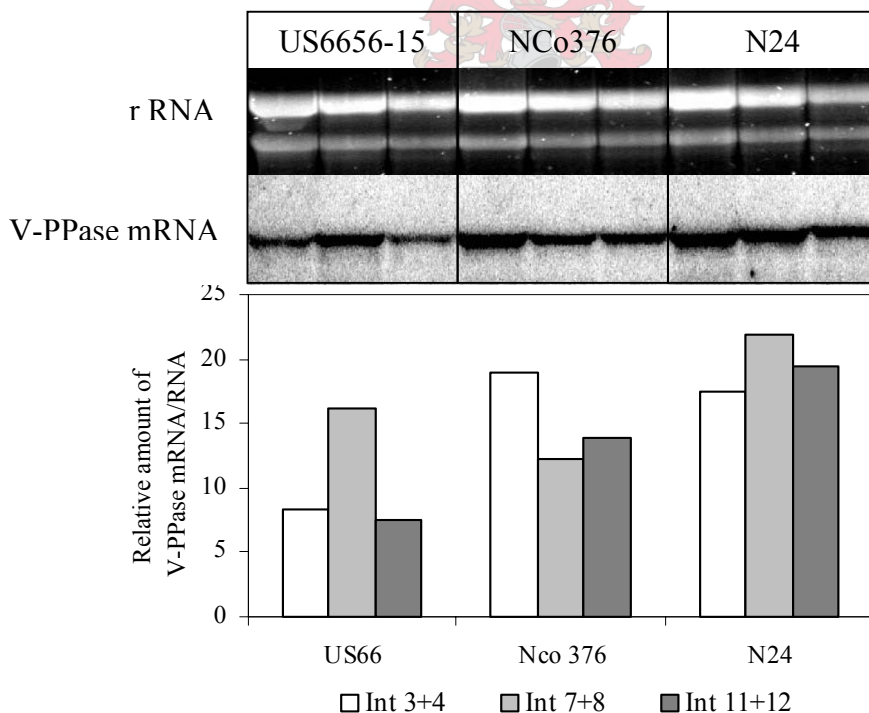
Fig. 5.3. Northern blot analysis of V-PPase gene expression. RNA was extracted from three sets of tissue: internodes 3+4, Int 7+8 and Int 11+12, from three hybrid varieties: US6656-15, NCo376 and N24. The relative amount of V-PPase mRNA is expressed as the V-PPase mRNA band intensity/rRNA band (top) intensity.

The V-PPase cDNA probe hybridised to a single mRNA species (or molecule) of 2.3 kb. V-PPase transcript levels (V-PPase mRNA band intensity/rRNA top band intensity) showed that

maturing internodes have relatively higher transcript level than immature and mature internodes. This trend is however, only noticeable in US6656-15 and N24, but not reiterated in NCo376. This may be due to an unknown experimental error and makes transcript levels in NCo376 contemptuous. V-PPase transcript levels of N24 in immature, maturing and mature internodes seem to be relatively higher than in comparison with US6656-15. Overall, there seems to be a trend of an increase in V-PPase transcript level from US6656-15 to NCo376 and N24. This experiment was however not repeated and therefore no significant conclusions can be drawn from these results without supporting evidence. Previously it has been shown that V-PPase is coarsely regulated (Yoshida 1991; Carystinos et al. 1995; Ballesteros et al. 1996; Nakanishi and Maeshima 1998; Nakanishi and Maeshima 1998) and therefore these results and conclusions need to be supported with V-PPase protein levels and catalytic activity (section 5.3.4).

5.3.4 V-PPase and V-ATPase specific activity within the sugarcane culm

Tonoplast extracts from the same tissue as soluble sugar-, DNA- and RNA extractions were used to determine and compare V-PPase and V-ATPase specific activity in immature, maturing and mature internodes of US6656-15, NCo376 and N24 (Table 5.1). Non vacuolar membrane contamination was less than 5% in all preparations, with no differentiation between varieties and the developmental stage of the internodes as determined with Vanadate ($100\mu M$), and Oligomycin (2 mg/l) sensitive ATPase activities as markers for the presence of plasma membrane and mitochondria or chloroplast membranes respectively (data not shown).

Table 5.1. Specific activity of V-PPase and V-ATPase. Activity was measured in internodes, 3+4, 7+8 and 11+12 of US6656-15, NCo376 and N24. Each value is the mean ± SE from four separate plants.

	V-PPase activity (nmole PPi min^{-1} mg^{-1} protein)			V-ATPase activity (nmole Pi min^{-1} mg^{-1} protein)		
Variety	Internode 3+4	Internode 7+8	Internode 11+12	Internode 3+4	Internode 7+8	Internode 11+12
US6656-15	65.5 ± 4.0	92.9 ± 15.1	55.4 ± 1.4	146.9 ± 9.9	86.8 ± 3.8	73.3 ± 1.3
NCo376	136.6 ± 22.6	172.9 ± 26.4	89.6 ± 6.5	161.1 ± 2.3	79.7 ± 7.5	53.3 ± 2.3
N24	174.4 ± 27.1	243.5 ± 32.4	153.3 ± 22.6	113.0 ± 5.6	88.7 ± 3.6	37.7 ± 3.8

V-PPase specific activity was intermediate in the immature internodes, peaks in the maturing internodes and is the lowest in the mature internodes in all three of the hybrid varieties (Table 5.1.), although this increase is not significant in the immature and maturing internodes of N24. In contrast to V-PPase-, V-ATPase specific activity is the highest in the immature

internodes and decreases in both the maturing and mature internodes in all three of the varieties.

5.3.5 V-PPase specific activity and protein content across varieties

The use of an ELISA system to determine and compare V-PPase protein levels is more advantages than the protein blotting technique (Chapter 3). To determine whether the measured V-PPase protein levels truly reflect the amount of V-PPase specific activity, the same vacuolar membrane preparations, which were used for specific activity determinations, were analysed using the V-PPase ELISA system and correlated with the V-PPase specific activity (Fig. 5.4, 5.5, 5.6; all results are the average of four independent experiments and vertical bars represent standard errors).

V-PPase specific activity measured in immature internodes 3+4 revealed major differences between the three genotypes whereas no significant differences in V-ATPase specific activity were detectable (Fig. 5.4A). V-PPase specific activity was significantly lower in US6656-15 than in NCo376 and N24, whereas no significant difference in V-PPase specific activity was detected between NCo376 and N24. There was a significant difference in V-PPase protein content between US6656-15 and N24, with no significant differences between US6656-15 and NCo376, and NCo376 and N24 respectively (Fig. 5.4B). A significant linear correlation ($r^2 = 0.959$, $P = 0.05$) between V-PPase activity and protein content was found (Fig. 5.4C).

Fig 5.4. Comparison of V-PPase, V-ATPase activity and protein content in immature internodes 3+4. V-PPase activity and protein content were measured in internodes 3+4 of US6656-15, NCo376 and N24. V-PPase activity (**A**) is expressed as nmole PPi min^{-1} mg^{-1} protein and V-ATPase activity is expressed as nmole Pi min^{-1} mg^{-1} protein to compare V-PPase and V-ATPase activity on a mole-to-mole basis. V-PPase protein content was measured with an ELISA system (**B**) and is expressed as V-PPase ELISA units (OD 450 min day 0 serum background). Correlation of V-PPase activity and protein content (**C**).

Comparison of V-ATPase specific activity in maturing internodes 7+8 revealed no significant differences between the three varieties, similar to the results obtained for immature internodes 3+4 above. V-PPase activity, however differed significantly with the highest activity in N24, intermediate in NCo376 and lowest in US6656-15 (Fig 5.5A). V-PPase protein content was the lowest in US6656-15 and significantly higher in NCo376 (Fig.5.5B). N24 showed no significant difference in protein content with NCo376. There was again a significant linear correlation ($r^2 = 0.999$, $P = 0.05$) between V-PPase activity and protein content (Fig. 5.5C).

Fig 5.5. Comparison of V-PPase, V-ATPase activity and protein content in maturing internodes 7+8. V-PPase activity and protein content were measured in internodes 7+8 of US6656-15, NCo376 and N24. V-PPase activity (**A**) is expressed as nmole PPi min^{-1} mg^{-1} protein and V-ATPase activity is expressed as nmole Pi min^{-1} mg^{-1} protein to compare V-PPase and V-ATPase activity on a mole-to-mole basis. V-PPase protein content was measured with an ELISA system (**B**) and is expressed as V-PPase ELISA units (OD 450 min day 0 serum background). V-PPase activity and protein content are correlated (**C**).

Results obtained in mature internodes 11+12 were very similar to the results obtained for maturing internodes 7+8 and immature internodes 3+4 (Fig. 5.6). Again, there was no significant difference in V-ATPase activity between NCo376 and N24 (Fig. 5.6A). US6656-15 showed a significant higher level of V-ATPase activity than N24, but no significant difference could be detected in comparison with NCo376. V-PPase catalytic activity showed significant differences between the three hybrid varieties (Fig 5.6A). V-PPase activity was the lowest in US6656-15, intermediate in NCo376 and the highest in N24. V-PPase protein content showed the same trend as the V-PPase activity, with significant differences between the three cultivars (Fig. 5.6B). V-PPase protein was the lowest in US6656-15, intermediate in NCo376 and the highest in N24. There was again a significant linear correlation ($P = 0.05$) between V-PPase activity and protein content (Fig. 5.6C).
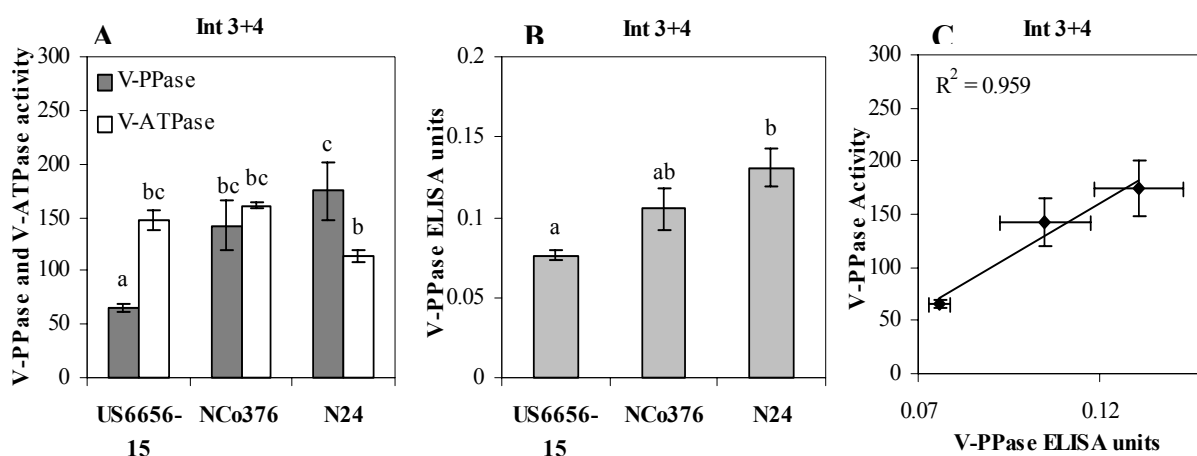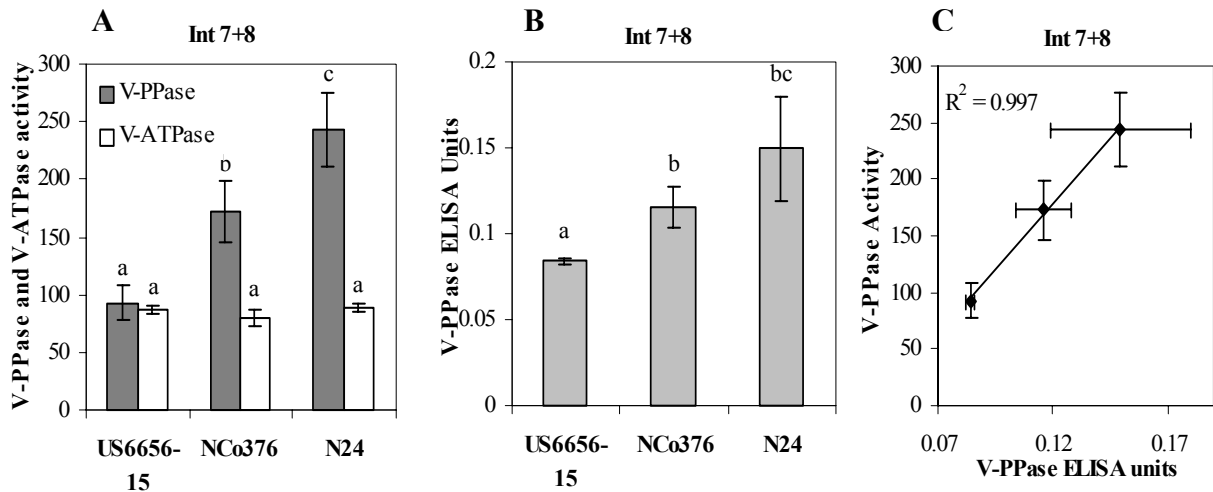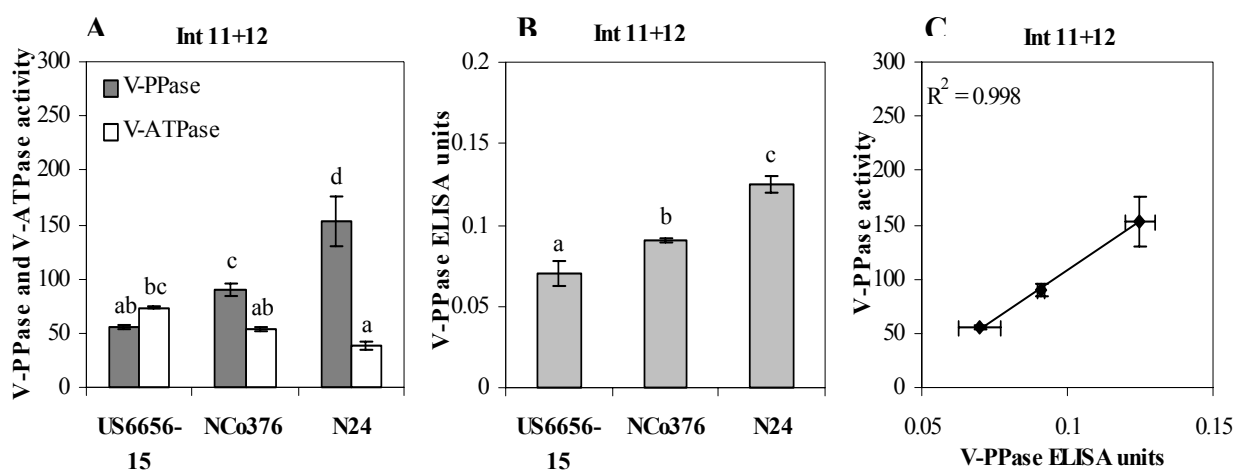
Fig 5.6. Comparison of V-PPase, V-ATPase activity and protein content in mature internodes 11+12. V-PPase activity and protein content was measured in internodes 11+12 of US6656-15, NCo376 and N24. V-PPase activity (**A**) is expressed as nmole PPi min^{-1} mg^{-1} protein and V-ATPase activity is expressed as nmole Pi min^{-1} mg^{-1} protein to compare V-PPase and V-ATPase activity on a mole-to-mole basis. V-PPase protein content was measured with an ELISA system (**B**) and is expressed as V-PPase ELISA units (OD 450 min day 0 serum background). V-PPase activity and Protein content are correlated (**C**).

5.3.6 Total Vacuolar proton pumping hydrolytic activity

There are definite patterns, both within the culm (int. 3+4, 7+8 and 11+12) of the three varieties respectively and between varieties when V-PPase activity is expressed as a percentage of the total vacuolar proton pumping catalytic activity (Table 5.2).

Table 5.2. Percentage V-PPase activity. V-PPase as a percentage of the total vacuolar catalytic activity (sum of the average V-PPase and V-ATPase specific activity).

Varietiy	Percentage V-PPase specific activity		
	Int 3+4	Int 7+8	Int 11+12
US6656-15	30.8	51.7	43.0
NCo376	45.9	68.4	62.7
N24	60.7	73.3	80.3

The percentage V-PPase activity is the lowest in the immature internodes, the highest in the maturing internodes and intermediate in the mature internodes in all three of the cultivars, with the exception of N24 where the highest percentage V-PPase activity is in the mature internodes. Comparison of the percentage V-PPase activity between the three varieties within the different tissue types show that the percentage V-PPase activity is the highest in N24, intermediate in NCo376 and the lowest in US6656-15 within immature-, maturing and mature internodes.

5.4 DISCUSSION

The aim of the present study was to compare V-PPase at genomic-, transcript-, protein- and enzymatic level between hybrid sugarcane varieties that differs significantly in sucrose content. The main objective of this study was to assess whether a relationship between V-PPase expression and sucrose content exists.

The developmental characterization of V-PPase in various land plant species has shown that V-PPase is the main proton pump of vacuolar membranes in young tissues and usually decreases during tissue development. V-ATPase on the other hand, is expressed relatively constantly during growth and maturation. The comparison of V-PPase and V-ATPase activities in mung bean hypocotyls (Maeshima 1990) and pear fruit (Shiratake et al. 1997) revealed that V-PPase activity is several times higher than V-ATPase activity. In contrast to the V-PPase activity that decreased during tissue development, V-ATPase levels were relatively constant during growth and maturation. As a result V-ATPase becomes the major proton pump of vacuolar membranes in mature tissues, whereas V-PPase is the main proton pump of vacuolar membranes in most young tissues (Maeshima 2000). V-PPase has never been investigated in sugarcane before, nor has its expression been characterized in other species that differ significantly in their ability to accumulate sucrose.

Sucrose content determinations between the sugarcane varieties US6656-15, NCo376 and N24 showed a sharp gradient between the immature, maturing and mature internodes in each of the three varieties (Fig. 5.1*A)*. Comparison of the sucrose content of these internodes between the three varieties showed significant differences in the maturing and mature internodes of US6656-15, NCo376 and N24. N24 contained the highest sucrose levels, NCo376 was intermediate and US6656-15 contained the lowest levels.

Comparison of the genomic organisation of V-PPase between the two ancestral- and three hybrid varieties showed no major differences and they share the same banding pattern, except for *S. spontaneum* that contains an extra fragment. V-PPase transcript levels showed that maturing internodes have relatively higher transcript level than immature and mature internodes. This trend is however, only noticeable in US6656-15 and N24. Transcript analysis of V-PPase in the three hybrid varieties seemed to have an overall trend of an increase in V-PPase transcript level from US6656-15 to NCo376 and N24. These results are supported with the V-PPase protein and catalytic activity data (Fig. 5.4A and B; Fig. 5.5 A and B; Fig 5.6 A

and B). V-PPase has previously been shown to be coarsely regulated, as earlier stated and therefore rectifies the observed trend in V-PPase mRNA transcript levels (Fig. 5.3).

The characterization of V-PPase expression on protein and enzyme level revealed exciting differences in all three of the hybrid varieties in comparison with results obtained for other land plants that do not accumulate significant levels of sucrose in comparison with sugarcane. We found that sugarcane V-PPase does not follow the conventional expression pattern found in various other species. Sugarcane V-PPase expression and specific activity was intermediate in immature internodes, the highest in the maturing internodes and the lowest in the mature internodes in all three of the hybrid varieties although this increase is not significant in N24 between the immature and maturing internodes (Table 5.1). V-ATPase activity on the other hand was the highest in immature internodes and the lowest in mature internodes in all three of the hybrid varieties (Table 5.1). This pattern of V-PPase specific catalytic activity within the culm is reflected by corresponding patterns of protein content (Fig. 5.4B, 5.5B, 5.6B).

Sugarcane V-PPase protein content and specific activity shared a significant linear relation within a certain tissue type, across the three hybrid varieties ((immature internodes, Fig. 5.4 C), (maturing internodes, Fig. 5.5 C), (mature internodes, Fig 5.6 C)). This correlation proves that there is a significant direct linear correlation between the measured V-PPase catalytic activity and V-PPase protein content. The anti-VPPase antiserum is highly specific for sugarcane V-PPase (Chapter 3) and therefore minimizes the possibility that our tonoplast fractions are contaminated with cytosolic soluble pyrophosphatases. Our results suggest that V-PPase activity in sugarcane, as in other species (Yoshida 1991; Carystinos et al. 1995; Ballesteros et al. 1996; Nakanishi and Maeshima 1998) is coarsely regulated, although V-PPase does not follow the same expression pattern. This pattern of V-PPase expression and catalytic activity shares the same trend as sucrose accumulation rates within the sugarcane culm, where sucrose accumulation rate is the highest in maturing internodes (Botha, Whittaker, et al. 1996).

Previous investigations in some land plant species has postulated that V-PPase may play a role in sucrose metabolism during sucrose synthesis, in ensuring PPi removal during photosynthetic sucrose synthesis (Quick et al. 1989; Neuhaus and Stitt 1991), as well as sucrose storage (Sikora et al. 1998). Our results showed that V-PPase protein content and specific activity differed significantly in the immature, maturing and mature internodes

between the three hybrid varieties. The lowest V-PPase activity (Fig. 5.4A, 5.5A, 5.6A) was found in US6656-15 (low sucrose accumulating tissue (Fig. 5.1A)) and the highest V-PPase activity (Fig. 5.4A, 5.5A, 5.6A) in N24 (high sucrose accumulating tissue (Fig. 1A)). This pattern of V-PPase protein content and activity is evident in the immature, maturing and mature internodes. The differences in V-PPase activity between the sugarcane varieties are reflected by corresponding differences in the V-PPase protein content (Fig. 5.4B, 5.5B, 5.6B), which share significant linear relations (Fig 5.4C, 5.5C, 5.6C). Transcriptional analysis of V-PPase (Fig. 5.3) seems to follow the same trend as V-PPase protein content and enzyme activity, where the lowest transcript levels is in US6656-15 and the highest in N24. V-ATPase activity on the other hand, shows no significant difference in specific activity in immature, maturing internodes between the three hybrid varieties (Fig. 5.4A, 5.5A, 5.6A). US6656-15 on the other hand shows significant higher levels of V-ATPase activity in mature internodes between the three hybrid varieties. Therefore, this variation in V-PPase activity between the low sucrose accumulating varieties and the high sucrose accumulating varieties shares the same trend as sucrose contents between these three varieties, whereas V-ATPase shows no such differentiation.

Analysis of the percentage V-PPase specific activity (Table 5.2) showed that V-PPase is the main proton pump in the maturing internodes, whereas V-ATPase seems to be more prevalent in the immature and mature internodes. Moreover, the percentage V-PPase specific activity seems to differentiate between US6656-15, NCo376 and N24 and is not the same within a specific tissue type between the three varieties. V-PPase appears to be the more dominating proton pump in relation to V-ATPase in N24 in comparison with NCo376 and US6656-15. The differentiation between V-PPase and V-ATPase specific activity appears to accompany the differences observed in sucrose content between the three varieties, where N24 has higher sucrose content and V-PPase specific activity in comparison with NCo376 and N24.

Growth of any plant species is impossible without vacuole expansion, since the vacuole occupies an estimated 90% of the cell's volume (Maeshima 2001). To maintain the high osmotic pressure of the expanding vacuole, the vacuole must actively incorporate solutes such as sugars and inorganic ions. Previous investigations proposed that V-PPase and V-ATPase provide the power for the secondary active transporters of these solutes into the vacuole (Maeshima 2000). Our results show that V-PPase seems to be the more dominating vacuolar proton pump within the sugarcane culm of varieties that contains significant higher levels of

sucrose. Moreover, it is evident from this project that there is a definite differentiation between V-PPase and V-ATPase specific activity within specific tissues between the hybrid varieties that differs significantly in sucrose content. Sugarcane V-PPase also does not follow the conventional expression pattern found in other characterized species.

V-PPase transcript levels, protein content and specific activity shares the same trend as sucrose content within the same tissue type between the three hybrid varieties. Therefore, it can be concluded from this study that V-PPase does seem to play a role in sucrose synthesis and accumulation. The use of transgenic technology during future studies will provide us novel insight in the specific role that V-PPase may play in sucrose synthesis, storage as well as phosphate metabolism.
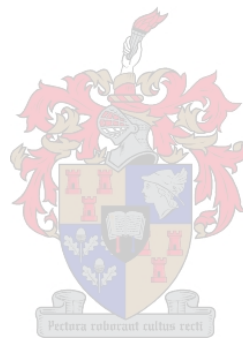
CHAPTER 6

GENERAL DISCUSSION

Sucrose accumulation has been studied more in sugarcane than in any other plant, because it accumulates very high concentrations of this metabolite (Hawker, Smith, et al. 1987). The principal steps of the metabolic pathways of sucrose synthesis and hydrolysis have been known for more than 20 years but the regulation of sucrose synthesis and storage is not yet completely understood. As a result most attempts to increase sucrose accumulation in sugarcane through genetic engineering have failed (Grof & Campbell, 2001). Current research to improve sugarcane productivity has taken the viewpoint that the product yield of photosynthate is limited at a sink rather than a source level (Gifford & Evans, 1981; Krapp *et al.*, 1993). Therefore, an understanding of the regulation of sucrose import and storage at the sink level becomes essential. Grof & Campbell (2001) identified the rate of sucrose transport into the storage parenchyma and vacuoles of sugarcane as one of these rate-limiting steps of sucrose accumulation. The vacuolar membrane of plant cells contains two distinct H^+ pumps, i.e. V-ATPase and V-PPase (Rea & Poole, 1993; Rea & Sanders, 1987; Sze, 1985). Both these enzymes catalyse the electrogenic H^+-translocation from the cytosol to the vacuole lumen to generate an inside-acid pH difference and an inside-positive electrical potential difference. The H^+-gradient generated by these two proton pumps, powers the secondary active transport of various metabolites and solutes, including sucrose, across the vacuolar membrane (Hedrich & Schroeder, 1989; Taiz, 1992).

Although previous findings indicated that V-PPase may play an important role in sucrose and phosphate metabolism its precise role has not yet been clarified and needs further investigation (Jellito *et al.*, 1992; Sonnewald, 1992; Davies *et al.*, 1993; Zhen *et al.*, 1997; Sikora *et al.*, 1998). V-PPase has never been investigated in sugarcane before. To date nothing, except for the sequences of a few ESTs, is known about sugarcane V-PPase. In view of these facts, sugarcane should be a great model to elucidate the role of V-PPase in sucrose and phosphate metabolism.

The primary aim of this project was to assess whether V-PPase activity correlates to the sucrose storage phenotype of sugarcane in any way. Therefore, the expression of V-PPase was characterized in three hybrid varieties, US6656-15, NCo376 and N24, which differ significantly in sucrose content (Whittaker & Botha, 1999). Due to the fact that V-PPase has

never been investigated in sugarcane before, molecular tools had to be developed and optimised to characterize V-PPase expression in sugarcane. Four different goals were identified to achieve the primary aim of this project. 1. The molecular cloning of the sugarcane V-PPase catalytic site and the introduction of the cDNA into a bacterial expression system for protein production and purification (Chapter 3). 2. Production of anti-VPPase polyclonal antiserum and the establishment of an ELISA system for V-PPase protein determinations (Chapter 3). 3. The determination of zero order kinetics for sugarcane V-PPase substrate hydrolysis (Chapter 4). 4. The characterization of V-PPase expression within the sugarcane culm of three hybrid varieties that differ significantly in their ability to accumulate sucrose (Chapter 5).

The first step in the development of molecular tools to characterize V-PPase expression in sugarcane was the cloning of the V-PPase catalytic region. The sugarcane V-PPase catalytic region was successfully cloned, expressed and purified from a bacterial system. Amino acid sequence analysis, based on the sugarcane V-PPase cDNA sequence (caf 22023) revealed exactly the same conserved amino acid motif for the putative substrate-binding site, as in other land plant species. The only difference noticed, was that the acidic region of the sugarcane V-PPase had a single amino acid substitution, S for N, in the second amino acid position if compared to other land plants. This purified peptide was used for the immunization of mice and the production of anti-VPPase polyclonal antiserum and the establishment of an ELISA system for V-PPase protein determinations.

Results obtained with protein blot analyses showed that the polyclonal antiserum reacted specifically with a single polypeptide with a molecular weight of approximately 71 kDa, which falls within the expected molecular weight range of the V-PPases of land plants. The establishment of an ELISA system for V-PPase protein quantification was therefore undertaken as the second goal. This system proofed to have several advantages over the well-known protein blotting technique. Titration analyses indicated that a 1:200 dilution of the antisera was optimal for the ELISAs, whereas a 1:100 dilution was required for the protein blot analyses for the detection of V-PPase. In addition, this direct ELISA system developed for native sugarcane V-PPase protein quantification has a linear detection range between 0.3 and 1.3 μg/ml vacuolar membrane proteins. This translates into 15 – 65 ng protein / ELISA, whereas protein blotting requires 10 – 20 μg vacuolar membrane protein to detect the V-PPase protein. The ELISA system also has the advantage that up to 96 samples can easily be

analysed and compared at once, while with protein blotting this is only possible with 20 samples. With the ELISA system it is also possible to statistically analyse and compare V-PPase protein levels between samples. Moreover, the relative V-PPase protein levels (OD_{450}) as determined by this ELISA showed a strong correlation ($R^2 = 0.99$) with V-PPase catalytic activity. This proved that the V-PPase ELISA system could be used as a reliable system to indirectly determine V-PPase specific activity in the young tissues of the variety NCo310.

The zero-order kinetics for substrate hydrolysis has not been determined for sugarcane V-PPase before. Previous investigations have shown that V-PPase requires, in addition to PPi, both Mg^{2+} and K^+ in a broad pH range for optimal activity *in vitro* (Davies *et al.*, 1991). Moreover, the response of the enzyme to changes in the concentration of these substrates, can be complex (Leigh & Pope, 1987; Wang *et al.*, 1986) and shows substantial differences between species (Maeshima, 2000b). The third goal of this study was therefore to establish the conditions for zero order kinetics for substrate hydrolysis by sugarcane V-PPase.

Sugarcane V-PPase had a broad pH optimum between 6.5 and 7.25, which is similar to the pH activity profiles obtained for V-PPases of isolated red beet vacuoles (Wagner & Mulready, 1983) and the microsomal pellet of sugar beet (Karlsson, 1974). Kinetic analysis of V-PPase hydrolytic activity as a function of PPi concentration, showed maximum activity at 0.3 mM with an apparent K_m of 0.178 mM $\pm$ 0.1. The reported cytosolic PPi concentration of the sugarcane variety NCo376 is 270 µM in immature internode three (Whittaker & Botha 1997). Based on this concentration V-PPase could function at approximately 76% of its maximum activity in immature sugarcane internodal tissues.

As mentioned earlier V-PPase requires both free Mg^{2+} that stabilizes and activates the enzyme and K^+ for stimulation of activity (Maeshima, 2000b). Here we showed that sugarcane V-PPase activity increased as a simple hyperbolic function of KCl concentration, saturating at approximately 30 mM. $MgSO_4$ also stimulated V-PPase activity as a simple hyperbolic function similar to that of K^+ and saturated at approximately 1.3 mM. The calculated K_m for K^+ stimulation of sugarcane V-PPase activity is 2.28 mM $\pm$ 0.25 whereas the K_m for Mg^{2+} stimulation is 0.44 mM $\pm$ 0.1. These values are in the same range as the reported K_m values for K^+ stimulation (Gordon-Weeks *et al.*, 1997;Maeshima, 2000b) and reported low-affinity Mg^{2+} -binding sites ($K_m = 0.25 - 0.46$ mM) of mung bean V-PPase (Baykov *et al.*, 1993).

The fourth and primary goal of this study was to use these molecular tools to characterize and compare the expression of V-PPase within the sugarcane culm of three hybrid varieties that differ significantly in sucrose content. The characterization of V-PPase activity in the developing tissues of several species has shown that V-PPase is the main proton pump in vacuolar membranes in young, immature tissues and usually decreases with tissue maturation (Maeshima 1990; Shiratake et al. 1997; Smart et al. 1998). In contrast, V-ATPase is expressed relative constantly during growth and maturation and as a result becomes the major proton pump of vacuolar membranes in mature tissues (Maeshima, 1990; Shiratake *et al.*, 1997; Maeshima, 2000b). A highlight in the current study was therefore the finding that V-PPase and V-ATPase activity does not follow these expression patterns in sugarcane. V-PPase expression and specific activity was intermediate in immature internodes, the highest in the maturing internodes and the lowest in the mature internodes in all three of the varieties, except for N24 where this difference between immature and maturing internodes was not significant. V-ATPase activity on the other hand was the highest in immature internodes and the lowest in mature internodes of the three hybrid varieties. This pattern of V-PPase expression is similar to the pattern observed for sucrose accumulation rates observed in sugarcane. Although sucrose content is the highest in mature internodes, sucrose accumulation rates are the highest in the maturing internodes, (internodes 3-7) (Botha, Whittaker, et al. 1996).
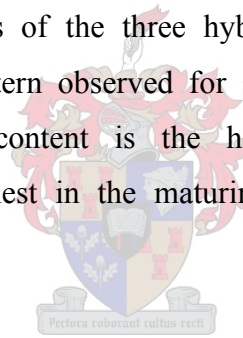
The comparison of V-PPase and V-ATPase activity between the three varieties that differ significantly in sucrose content, revealed exciting differences. V-PPase protein levels and activities differed significantly within immature, maturing and mature internodes between the varieties. In contrast, no significant differences were observed for V-ATPase activity, except in the case of US6656-15, which contained significant higher levels of V-ATPase activity in mature internodes in comparison with maturing and immature internodes. Moreover, these differences observed in V-PPase protein content and activity shared the same trend as sucrose content within the maturing- and mature tissue in the three varieties. V-PPase specific activity and protein content (ELISA units) shared a linear correlation ($R^2 = 0.99$) in both maturing and mature internodes in the three hybrid varieties. These results therefore support the use of V-PPase protein content as an indication of specific activity in various sugarcane tissues and varieties.

This differentiation in V-PPase specific activity and protein content between the hybrid varieties that differ significantly in sucrose content suggests a possible role for V-PPase in sucrose metabolism. Sucrose accumulation is the result of sucrose synthesis, transport and storage against a concentration gradient into the vacuoles of sink organs, where it is stored at very high concentrations (Gifford & Evans, 1981; Krapp, Hoffmann, et al., 1993; Grof & Campbell 2001). Sucrose synthesis is dependent on the disposal of PPi produced by UGPase, which would otherwise inhibit the production of UDPGlc. Inhibition of UDPGlc production will concurrently inhibit SPS and ultimately inhibit sucrose synthesis and favour the formation of fructose 1,6-bisphophate and glycolytic carbon flow. Plants contain a considerable pool of PPi in the cytosol, which is produced as a relative inexpensive source of energy in the activation or polymerisation steps of a wide range of biosynthetic pathways (Chanson et al. 1985; Edwards & Rees 1986). However, the cytosol of higher plants cells contains little or no soluble pyrophosphatase and alkaline pyrophosphatase is only located in the plastids (Gross & Stitt 1986; Weiner et al. 1987). Because soluble PPase activity is largely absent from the cytosol and almost exclusively located in the chloroplast stroma (Weiner et al. 1987), V-PPase may have a duel responsibility during sucrose synthesis and storage in sink tissues (Fig. 6.1).
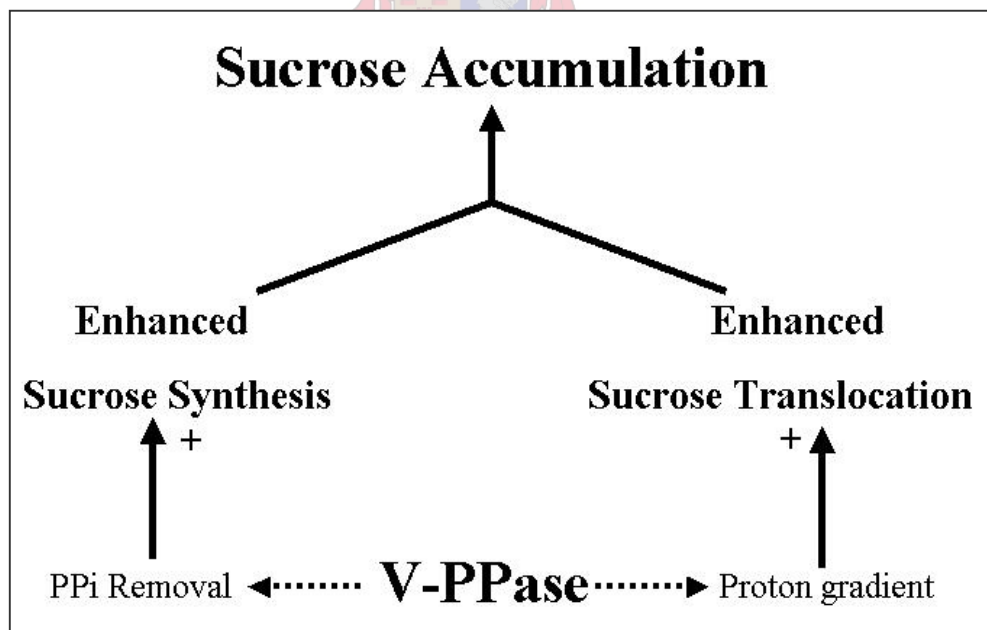

Fig. 6.1 Simplified illustration of the two possible roles V-PPase may play in sucrose accumulation.

Firstly it may contribute in the disposal of cytosolic PPi, which could otherwise inhibit sucrose synthesis (Jellito *et al.*, 1992;Sonnewald, 1992). Secondly, V-PPase may use the

energy from the hydrolysis of PPi to increase the sucrose sink pool by activating a proton motive force across the vacuolar membrane to drive the secondary transport of sucrose from the cytosol to the vacuole (Davies *et al.*, 1993; Zhen *et al.*, 1997; Sikora *et al.*, 1998). Our results showed that there is a definite differentiation between V-PPase and V-ATPase activity between hybrid sugarcane varieties that differ significantly in sucrose contents. Moreover, V-PPase seems to be the more important vacuolar H^+ pump in high sucrose accumulating hybrid sugarcane varieties. V-PPase shares the same trend as sucrose content in both maturing and mature tissues between the three varieties and follows the same trend as sucrose accumulation rates within the sugarcane culm.

The data suggest that V-PPase may possibly play a role in sucrose and phosphate metabolism in sugarcane. This is the first reported work on V-PPase where its protein expression and catalytic activity has been correlated with the sucrose storage phenotype in any species. This is also the first report on V-PPase in sugarcane. These results suggest an intriguing correlation between sucrose storage and V-PPase expression, which needs to be further investigated. The potential roles of V-PPase as a PPi scavenger to maintain sucrose synthesis and/or an activator of sucrose transport across the tonoplast for storage need to be elucidated. Work is currently underway to analyse the potential changes in sucrose and PPi metabolism and in particular the sucrose accumulation phenotype in the sink tissues of transgenic sugarcane with reduced or increased V-PPase activity.
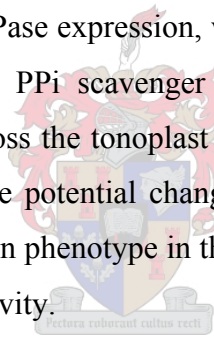
LITERATURE CITED

1. Ames, B. N. (1966). Assay of inorganic phosphate, total phosphate and phosphatase. *Methods Enzymology* **8**, 115-118.

2. Apse, M. P., Aharon, G. S., Snedden, W. A., & Blumwald, E. (1999). Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. *Science* **285**, 1256-1258.

3. Arnold, WN. (1968). The selection of sucrose as the translocate of higher plants. *Journal of Theoretical Biology* **21**, 13-20.

4. Ballesteros, E., Donaire, J. P., & Belver, A. (1996). Effects of salt stress on H^+-ATPase and H^+-PPase activities of tonoplast-enriched vesicles isolated from sunflower roots. *Physiologia Plantarum* **97**, 259-268.

5. Baltscheffsky, M., Nadanacive, S., & Schultz, A. A pyrophosphate synthase gene: molecular cloning and sequencing of the cDNA encoding the inorganic pyrophosphate synthase form *Rhodospirillum rubrum. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression*, 301-306. 1998.

6. Baltscheffsky, M., Schultz, A., & Baltscheffsky, H. (1999). H+-PPases: a tightly membrane-bound family. *FEBS Letters* **457**, 527-533.

7. Baykov, A. A., Cooperman, B. S., Goldman, A., & Lahti, R. (1999). Cytoplasmic inorganic pyrophosphatase. *Progress in Molecular and Subcellular Biology* **23**, 127-150.

8. Baykov, AA., Bakuleva, NP., & Rea, PA. (1993). Steady-state kinetics of substrate hydrolysis by vacuolar H^+ -pyrophosphatase: A simple three-state model. *European Journal of Biochemistry* **217**, 755-762.

9. Becker, A., Canut, H., Lüttge, U., Maeshima, M., Marigo, G., & Ratajczak, R. (1995). Purification and Immunological Comparison of the Tonoplast H^+-Pyrophosphatase from Cells of *Catharanthus roseus* and Leaves from *Mesembryanthemum crystallinum*

performing C-3-Photosynthesis and the Obligate CAM-Plant *Kalanchoe daigremontiana*. *Journal of Plant Physiology* **146**, 88-94.

10. Bergmeyer, H. U. & Bernt, E. (1974). Sucrose, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis. *Verlag, Weiheim.* 1176-1179.

11. Black, C. C., Mustardy, L., Sung, S. S., Kormanik, P. P., Xu, D.-P., & Paz, N. (1987). Regulation and roles of alternative pathways of hexose metabolism in plants. P*hysiologia Plantarum* **69**, 387-394.

12. Blumwald, E. (1987). Tonoplast vesicles as a tool in the study of ion-transport at the plant vacuole. *Plant Physiology* **69**, 731-734.

13. Blumwald, E. & Poole, R. J. (1987). Salt tolerance in suspension cultures of sugar beet: induction of Na^+/H^+ antiport activity at tonoplast by growth in salt. *Plant Physiology* **83**, 884-887.

14. Boller, T. & Weimken, A. (1986). Dynamics of vacuolar compartmentation. *Annual Review of Plant Physiology* **37**, 137-164.

15. Botha, F. C., Whittaker, A., Vorster, D. J., & Black, K. G. (1996). Sucrose accumulation rate carbon partitioning and expression of key enzyme activities in sugarcane stem tissues. *In: Wilson JR, Hogarth DM, Campbell JA, Garside AL (eds) Sugarcane: Research Towards Efficient and Sustainable Production.CSIRO Division of Tropical Crops and Pastures, Brisbane* 98-101.

16. Boyer, P. (1997). The ATP synthase: a splendid molecular machine. *Annual Review of Biochemistry* **66**, 717-749.

17. Bradford, M. (1976). A rapid and sensitive method for the quantitaton of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248-254.

18. Brauer, D., Uknalis, J., Triana, R., Schacharhill, Y., & Tu, S. I. (1997). Effects of bafilomycin A(1) and metabolic inhibitors on the maintenance of vacuolar acidity in maize root hair cells. *Plant Physiology* **113**, 809-816.

19. Brisken, DP., Thornley, WR., & Wyse, RE. (1985). Membrane transport in isolated vesicles from sugar beet taproot: evidence for a sucrose/H^+-antiport. *Plant Physiology* **78**, 871-875.

20. Britten, CJ., Turner, JC., & Rea, PA. (1989). Identification and purification of substrate-binding subunit of higher plant H+-translocating inorganic pyrophosphatase. *FEBS Letters* **256**, 200-206.

21. Bugos, R. C., Chiang, V. L., Zhang, E.-H., Campbell, E. R., Podila, G. K., & Campbell, W. H. (1995). RNA isolation from plant tissues recalcitrant to extraction in guanidine. *Biotechniques* **19**, 734-737.

22. Bull, T. A. & Glasziou, K. T. (1963). The evolutionary significance of sugar accumulation in *Saccharum. Australian Journal of Biological Sciences* **16**, 737-742.

23. Bush, D. R. (1993). Proton-coupled sugar and amino acid transporters in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**, 513-542.

24. Büttner, N. & Sauer, N. (2000). Monosaccharide transporters in plants: structure, function and physiology. *Biochimica et Biophysica Acta* **1465**, 263-274.

25. Calvin, M. (1960). *Journal of Graduate* **5**, 319-331.

26. Carnal, N. W. & Black, C. C. (1979). Pyrophosphate-dependent 6-phosphofructokinase, a new glycolytic enzyme in pineapple leaves. *Biochemical and Biophysical Research Communications* **86**, 20-26.

27. Carystinos, G. D., MacDonald, H. R., Monroy, A. F., Dhindsa, R. S., & Poole, R. J. (1995). Vacuolar H+-translocating pyrophosphatase is induced by anoxia or chilling in seedlings of rice. *Plant Physiology* **108**, 641-649.

28. Chanson, A., Fichman, J., Spear, D., & Taiz, L. (1985). Pyrophosphate-driven proton transport by microsomal membranes of corn coleoptiles. *Plant Physiology* **79**, 159-164.

29. Chanson, A. & Pilet, P. E. (1987). Localization in sucrose gradients of the pyrophosphate-dependent proton transport of maize root membranes. *Plant Physiology* **84**, 1431-1436.

30. Chanson, A. & Pilet, P. E. (1988). Characterizaton of the pyrophosphate dependent proton transport in microsomal membranes from maize roots. *Physiologia Plantarum* **74**, 643-650.

31. Chanson, A. & Pilet, P. E. (1989). Target molecular size and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the ATP-dependent and pyrophosphate-dependent proton pumps from maize root tonoplast. *Plant Physiology* **90**, 934-938.

32. Chapman, L. S. (1996). Increase in sugar yield from plant breeding from 1946 to 1994. *In 'Sugarcane: research towards efficient and sustainable production.(Eds Jr Wilson, DM Hogarth, JA Campbell and AL Garside)* 37-38.

33. Chourey, P., Talierco, E. W., & Kane, E. J. (1991). Tissue specific expression and anaerobically induced post-translational modification of sucrose synthase genes in *Sorghum bicolor*. *Plant Physiology* **96**, 485-490.

34. Colombo, R. & Cerana, R. (1993). Enhanced activity of tonoplast pyrophosphatase in NaCl grown cells of *Daucus-Carota*. *Plant Physiology* **142**, 226-229.

35. Cooperman, BS., Baykov, AA., & Lahti, R. (1992). Evolutionary conservation of the active site of soluble inorganic pyrophosphatase. *Trends in Biochemical Sciences* **17**, 262-266.

36. Dancer, J. E. & ap Rees, T. (1989). Phosphoribosyl pyrophosphate and the measurement of inorganic pyrophosphate in plant tissue. *Planta* **177**, 261-264.

37. Dancer, J. E., David, M., & Stitt, M. (1990). The subcellular compartmentation of uridine nucleotides and nucleoside-5-diphosphate kinase in leaves. *Plant Physiology* **92**, 637-640.

38. Darley, C. P., Davies, J. M., & Sanders, D. (1995). Chill-induced changes in the activity and abundance of the vacuolar proton-pumping pyrophosphatase from mung bean hypocotyls. *Plant Physiology* **109**, 659-665.

39. Davies, J.M., Darley, C.P., & Sanders, D. (1993). Energetics of the plasma membrane pyrophosphatase. *Trends in Plant Science* **2**, 9-10.

40. Davies, JM., Poole, RJ., Rea, PA., & Sanders, D. (1992). Potassium-transport into plant vacuoles energized directly by a proton-pumping inorganic pyrophosphatase. *Proceedings of the National Academy of Science of the United States of America* **89**, 11701-11705.

41. Davies, JM., Poole, RJ., & Sanders, D. (1993). The computed free energy change of hydrolysis of inorganic pyrophosphatase and ATP: apparent significance of inorganic pyrophosphate-driven reactions of intermediary metabolism. *Biochimica et Biophysica Acta* **1141**, 29-36.

42. Davies, JM., Rea, PA., & Sanders, D. (1991). Vacuolar proton-pumping pyrophosphatase in *Beta vulgaris* shows vectorial activation by potassium. *FEBS Letters* **278**, 66-68.

43. De Witt, N., Harper, J., & Sussman, M. (1991). Evidence for a plasma membrane proton pump in phloem cells of higher plants. *Plant Journal* **1**, 121-128.

44. Dellaporta, S. L., Wood, J., & Hicks, J. B. (1983). A plant DNA minipreparation. *Plant Molecular Biology Reporter* **1**, 19-21.

45. Edwards, J., ap Rees, T., Wilson, P. M., & Morell, S. (1984). Measurement of inorganic pyrophosphate in tissues of *Pisum sativum*. *Planta* **162**, 188-191.

46. Edwards, J. & ap Rees, T. a. (1986). Metabolism of UDP-glucose by developing embryos of round and wrinkled varieties of *Pisum sativum*. *Phytochemistry* **25**, 2033-2039.

47. Gaxiola, R. A., Li, J., Undurraga, S., Dang, L. M., Allen, G. J., & Alper, S. L. (2001). Drought- and salt-tolerant plants result from overexpression of the AVP1 H$^+$-pump. *Genetics* **98**, 11444-11449.

48. Geigenberger, P., Hajirezaei, M., Geiger, M., Deiting, U., Sonnewald, U., & Stitt, M. (1998). Overexpression of pyrophosphatase leads to increased sucrose degradation and starch synthesis, increased activities of enzymes for sucrose-starch interconversion, and increased levels of nucleotides in growing potato tubers. *Planta* **205**, 428-437.

49. Getz, H. P. & Klein, M. (1995). Characteristics of sucrose transport and sucrose-induced H+-transport on the tonoplast of red beet (Beta vulgaris L.) storage tissue. *Plant Physiology* **107**, 459-467.

50. Giaquinta, R. T. (1980). Phloem loading of sucrose-involvement of membrane ATPase and proton transport. *Plant Physiology* **63**,744-748.

51. Gifford, R. M. & Evans, L. T. (1981). Photosynthesis, carbon partitioning and yield. *Annual Review of Plant Physiology* **32**, 485-509.

52. Gogarten, J., Kibak, H., Dittrich, P., Taiz, L., Bowman, E., Bowman, B., Manolson, M., Poole, R. J., Date, T., Oshima, T., Konishi, J., Denda, K., & Yoshida, M. (1989). The evolution of vacuolar H$^+$-ATPase: implications for the origin of eukaryotes. *Proceedings of the National Academy of Science of the United States of America* **86**, 6661-6665.

53. Gordon-Weeks, R., Koren'kov, VD., Steele, SH., & Leigh, RA. (1997). Tris is a competitive inhibitor of K+ activation of the vacuolar H+-pumping pyrophosphatase *Plant Physiology* **114**, 901-905.

54. Gordon-Weeks, R., Steele, SH., & Leigh, R. A. (1996). The Role of Magnesium, Pyrophosphate, and Their Complexes as Substrates and Activators of the Vacuolar H+Pumping Inorganic Pyrophosphatase (Studies Using Ligand Protection from Covalent Inhibitors) *Plant Physiology* **111**, 195-202.

55. Grof, C. P. L. & Campbell, J. A. (2001). Sugarcane sucrose metabolism: scope for molecular manipulation. *Australian Journal of Plant Physiology* **28**, 1-12.

56. Gross, P. & Stitt, M. (1986). Alkaline inorganic pyrophosphatase and starch synthesis in plastids. *Planta* **167**, 140-145.

57. Guglielminetti, L., Perata, P., & Alpi, A. (1995). Effect of anoxia on carbohydrate metabolism in rice seedlings. *Plant Physiology* **108**, 735-741.

58. Guynn, R. W., Velosco, D., Lawson, R., & Veech, R. L. (1974). The concentration and control of cytoplasmic free inorganic pyrophosphate in rat liver in vivo. *Biochemical Journal* **140**, 369-375.

59. Hatzfeld, W.-D., Dancer, J., & Stitt, M. (1989). Direct evidence that pyrophosphate: fructo-6-phosphate phophotransferase can act as a glycolytic enzyme in plants. *FEBS Letters* **254**, 215-218.

60. Hatzfeld, W.-D., Dancer, J., & Stitt, M. (1990). Fructose-2,6-Bisphosphate, metabolites and "coarse" control of pyrophosphate:-fructose-6-phosphate phosphotransferase during triose-phosphate cycling in heterotrophic cell-suspension cultures of *Chenopodium rubrum. Planta* **180**, 205-211.

61. Hawker, J. S., Smith, G. M., Phillips, H., & Wiskich, J. T. (1987). Sucrose phophatase associated with vacuole preparations from red beet, sugar beet, and immature sugarcane stem. *Plant Physiology* **84**, 1281-1285.

62. Hedrich, R. & Schroeder, J. I. (1989). The physiology of ion channels and electrogenic pumps in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 539-569.

63. Hedrich, R., Kurkdjian, A., Guern, J., & Flugge, U. (1989). Comparitive studies of the electrical properties of the H$^+$-translocating ATPase and pyrophosphatase of the vacuolar-lysosomal compartment. *EMBO Journal* **8**, 2835-2841.

64. Hill, S. A. & ap Rees, T. (1995). The effect of hypoxia on the control of carbohydrate metabolism in ripening bananas. *Planta* **197**, 313-323.

65. Hodges, T. K. & Leonard, R. T. (1974). Purification of a Plasma Membrane-Bound Adenosine Triphosphatase form Plant Roots. *Methods Enzymology* **32**, 392-406.

66. Horwitz, H. (1958). *Plant Physiology* **33**, 81-93.

67. Houlne, G. & Boutry, M. (1994). Identification of an *Arabidopsis thaliana* gene encoding a plasma membrane H$^+$-ATPase whose expression is restricted to anther tissue. *Plant Journal* **5**, 311-317.

68. Huber, S. C. & Akazawa, T. (1986). A novel sucrose synthase pathway for sucrose degradation in cultured sycamore cells. *Plant Physiology* **81**, 1008-1013.

69. Ikeda, M., Satoh, S., Maeshima, M., Mukohata, Y., & Moritani C. A vacuolar ATPase and pyrophosphate in *Acetabularia acetabulum*. *Biochimica et Biophysica Acta-Biomembranes* **1070**, 77-82. 1991.

70. Ikeda, M., Tanabe, E., Rahman, M., Kadowaki, H., & Moritani C. A vacuolar inorganic H$^+$-pyrophosphatase in *Acetabularia acetabulum*: molecular cloning and comparison with higher plants and a bacterium. *Journal of Experimental Botany* **50**, 139-140. 1999.

71. Jellito, T., Sonnewald, U., Willmitzer, L., Hajirezaei, M., & Stitt, M. (1992). Inorganic pyrophosphate content an metabolite in leaves and tubers of potato and tobacco plants expressing *E.coli* pyrophosphatase in their cytosol. *Planta* **188**, 238-244.

72. Jiang, S. S., Fan, L. L., Yang, S. J., Kuo, S. Y., & Pan, R. L. (1997) Purification and characterization of thylakoid membrane-bound inorganic pyrophosphatase. *Archives of Biochemistry and Biophysics* **346**, 105-112.

73. Johannes, E. & Felle, H. (1990). Proton gradient across the tonoplast of *Riccia fluitans* as a result of the joint action of two electroenzymes. *Plant Physiology* **93**, 412-417.

74. Johannes, E. & Felle, H. H. (1989). The role of Mg^{2+} in proton transport by the tonoplast pyrophosphatase of *Riccia fluitans* vacuoles. *Physiologia Plantarum* **77**, 326-331.

75. Karlsson, J. (1974). Membrane-bound potassium and magnesium ion stimulated inorganic pyrophosphatase from roots and cotyledons of sugar beet (*Beta vulgaris* L.). *Biochimica et Biophysica Acta* **399**, 356-363.

76. Kasai, M., Nakamura, T., Kudo, N., Sato, H., Maeshima, M., & Sawada, S. (1998). The activity of the root vacuolar H+-pyrophosphatase in rye plants grown under conditions deficient in mineral nutrients. *Plant and Cell Physiology* **39**, 890-894.

77. Kasamo, K. (1988). Response of tonoplast and plasma membrane ATPases in chilling-sensitive and chilling-insensitive rice (*Oryza sativa L.*) culture cells to low temperature. *Plant Cell Physiology* **29**, 1085-1094.

78. Kawamura, Y., Arakawa, K., Maeshima, M., and Yoshida, S. (2000) Tissue specificity of E subunit isoforms of plant vacuolar H$^+$-ATPase and existence of isotype enzymes. *Journal of Biological Chemistry* **275**: 6515-6522.

79. Kieber, J. J. & Signer, E. R. (1991). Cloning and characterization of an inorganic pyrophosphatase gene from *Arabidopsis thaliana*. *Plant Molecular Biology* **16**, 345-348.

80. Kim, Y., Kim, E. J., & Rea, P. A. (1994). Isolation and characterization of cDNAs encoding the vacuolar H+-pyrophosphatase of *Beta vulgaris*. *Plant Physiology* **106**, 375-382.

81. Komor, E., Höfner, M., Wendler, R., Thom, M., & Dela Cruz, A. (1987). Nitrogen nutrition of sugarcane cells: regulation of amino acid uptake and the effect on cell growth and sucrose storage. *Plant Physiology and Biochemistry* **25**, 581-588.

82. Krapp, A., Hoffmann, B., Schäfer, C., & Stitt, M. (1993). Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism for the "sink regulation" of photosynthesis? *Plant Journal* **3**, 817-828.

83. Kruger, N. J. (1997). Carbohydrate synthesis and degradation. In: Dennis, D.T., Turpin, *D.M., Lefebvre, D.D., and Layrell, D.B., eds., Plant Physiology, Biochemistry and Molecular Biology* Longman. Harlow, U.K., 83-103.

84. Kulaev, IG. & Vagabov, V. (1983). Polyphosphate metabolism in micro-organisms. *Advanced Microbial Physiology* **24**, 83-171.

85. Leigh, R. A. (1997). Solute composition of vacuoles. *In Advances in Botanical Research, The Plant Vacuole, R.A.Leigh and D.Sanders, eds (London: Academic Press).* **25** , 171-194.

86. Leigh, R. A. & Pope, A. J. (1987). Understanding tonoplast function: some emerging problems. *In B Martin, ed, Plant Vacuoles: Their importance in Solute Compartmentation in Cells and Their Applications in Plant Biotechnology.Plenum Press, New York* 101-110.

87. Leigh, R. A. & Wyn Jones, R. G. (1984). An hypothesis relating critical potassium relations for growth to the distribution of this ion in the plant cell. *New Phytologist* **97**, 1-14.

88. Leigh, RA., Pope, AJ., Jennings, IR., & Sanders, D. (1992). Kinetics of vacuolar H^+-pyrophosphatase. The roles of magnesium, pyrophosphate, and their complexes as substrates, activators and inhibitors. *Plant Physiology* **100**, 1698-1705.

89. Lemoine, R. (2000). Sucrose transporters in plants: update on function and structure. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1465**, 246-262.

90. Lerchl, J., Konig, S., Zrenner, R., & Sonnewald, U. (1995). Molecular cloning, characterization and expression analysis of isoforms encoding tonoplast-bound proton-translocating inorganic pyrophosphatase in tobacco. *Plant Molecular Biology* **29**, 833-840.

91. Lipman, F. (1965). *The origin of Prebiological Sytems (Fox, S.W., ed), Acadamic Press, New York* 259-310.

92. Lundin, M., Baltscheffsky, H., & Ronne, H. (1991). Yeast PPA2 gene encodes a mitochondrial inorganic pyrophosphatase that is essential for mitochondrial function. *Journal of Biological Chemistry* **266**, 12168-12172.

93. Maeshima, M. (1990). Development of vacuoar membranes during elongation of cells in mung bean hypocotyls. *Plant Physiology* **31**, 311-317.

94. Maeshima, M. (1991). H^+-translocating inorganic pyrophosphatase of plant vacuoles: inhibition by Ca^{2+}, stabilization by Mg^{2+} and immunological comparison with other inorganic pyrophosphatases. *European Journal of Biochemistry* **196**, 11-17.

95. Maeshima, M. (2000a). Vacuolar H+-pyrophosphatase. *Biochimica et Biophysica Acta-Biomembranes* **1465**, 37-51.

96. Maeshima, M. (2000b). Vacuolar H+-pyrophosphatase. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1465**, 37-51.

97. Maeshima, M. (2001). Tonoplast transporters: Organization and function. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 469-497.

98. Maeshima, M., Hara-Nishimura, I., Takeuchi, Y., & Nishimura, M. (1994a). Accumulation of Vacuolar H+-Pyrophosphatase and H+-ATPase during Reformation of the Central Vacuole in Germinating Pumpkin Seeds. *Plant Physiology* **106**, 61-69.

99. Maeshima, M., Mimura, T., & Sato, H. (1994b). Distribution of vacuolar H$^+$-pyrophosphatase and a membrane integral protein in a variety of green plants. *Plant Cell Physiology* **35**, 323-328.

100. Maeshima, M. & Nakanishi, Y. (1998). Molecular Cloning of Vacuolar H$^+$-Pyrophosphatase and Its Developmental Expressionn in Growing Hypocotyl of Mung Bean. *Plant Physiology* **116**, 589-597.

101. Maeshima, M., Nakanishi, Y., MatsuuraEndo, C., & Tanaka, Y. (1996). Proton pumps of the vacuolar membrane in growing plant cells. *Journal of Plant Research* **109**, 119-125.

102. Maeshima, M. & Yoshida, S. (1989). Purification and properties of vacuolar membrane proton-translocating inorganic pyrophosphatase from mung bean. *Journal of Biological Chemistry* **264**, 20068-20073.

103. Magnuson, CE., Goeschl, JD., & Fares, Y. (1986). Experimental tests of the Munch-Horwitz theory of phloem transport: effect of loading rates. *Plant Cell Environment* **9**, 103-109.

104. Malone, M., Leigh, R. A., & Tomos, A. D. (1991). Concentrations of vacuolar inorganic ions in individual cells of intact wheat leaf epidermis. *Journal of Experimental Botany* **42**, 305-309.

105. Martinoia, E., Grill, E., Tommasini, R., Kreuz, K., & Amrhein, N. (1993). ATP-dependent glutathione S-conjugate 'export' pump in the vacuolar membrane of plants. *Nature* **364**, 247-249.

106. Marty, F. (1999). Plant Vacuoles. *The Plant Cell* **11**, 587-599.

107. Maruyama, C., Tanaka, Y., Mitsuda, N., Takeyasu, K., Yoshida, M., & Sato, H. (1998). Structural studies of the vacuolar H-pyrophosphatase: sequence analysis and identification of the residue modified by fluorescent cyclohexylcarbodiimide and maleimide. *Plant Cell Physiology* **39**, 1045-1053.

108. MatsuuraEndo, C., Maeshima, M., & Yoshida, S. (2003). Mechanisms of the decline in vacuolar H$^+$-ATPase activity in mung bean hypocotyls during chilling. *Plant Physiology* **100**, 718-722.

109. Mertens, E. (1991). Pyrophosphate-dependent phosphofructokinase: an anaerobic glycolytic enzyme? *FEBS Letters* **285**, 1-5.

110. Mertens, E., Larondelle, Y., & Hers, H. G. (1990). Induction of pyrophosphate:fructose-6-phosphate 1-phosphotransferase by anoxia in rice seedlings. *Plant Physiology* **93**, 584-587.

111. Meyer, W. & Schafer, G. (1992). Characterization and purification of a membrane-bound archaebacterial pyrophosphatase from *Sulfolobus acidocaldarius*. *European Journal of Biochemistry* **207**, 741-746.

112. Mohanty, B., Wilson, P. M., & ap Rees, T. (1993). Effects of anoxia on growth and carbohydrate metabolism in suspension cultures of soybean and rice. *Phytochemistry* **34**, 75-82.

113. Moore, P. H. (1989). Physiological basis for varietal improvement in sugarcane. *In 'Sugarcane varietal improvement'.(Eds KM Naidu, TV Screenivasan and MN Premachandran)* 19-55.

114. Moore, P. H., Botha, F. C., Furbank, R. T., & Grof, C. P. L. (1997). Potential for overcoming physico-biochemical limits to sucrose accumulation. *In 'Intensive sugarcane production: meeting the challenges beyond 2000'.(Eds BA Keating and JR Wilson)* 141-155.

115. Moore, P. H. & Maretzki, A. (1996). Sugarcane. *In: Zamski E, Schaffer AA (eds) Photoassimilate Distribution in Plants and Crops: Source sink Relationships.Marcel Dekker Inc, New York, NY* 643-669.

116. Moriyama, Y. & Nelson, N. (1989). Cold inactivation of vacuolar proton-ATPases. *Journal of Biological Chemistry* **264**, 3577-3582.

117. Nakanishi, Y., Matsuda, N., Aizawa, K., Kashiyama, T., Yamamoto, K., Mimura, T., Ikeda, M., & Maeshima, M. (1999). Molecular cloning and sequencing of the cDNA for vacuolar H+-pyrophosphatase from Chara corallina. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1418**, 245-250.

118. Nakanishi, Y. & Maeshima, M. (1998). Molecular cloning of vacuolar H+-pyrophosphatase and its developmental expression in growing hypocotyl of mung bean. *Plant Physiology* **116**, 589-597.

119. Nakanishi Y, Saijo T, Wada Y, Maeshima M (2001) Mutagenic analysis of functional residues in putative substrate-binding site and acidic domains of vacuolar H+-pyrophosphatase. *Journal of Biological Chemistry* **276**: 7654-7660

120. Nelson, N. & Taiz, L. The evolution of H$^+$-ATPases. *Trends in Biochemical Science* **14**, 113-116. 1989.

121. Neuhaus, H. E. & Stitt, M. (1991). Inhibition of photosynthetic sucrose synthesis by imidodiphosphate, an analog of inorganic pyrophosphate. *Plant Science* **76**, 49-55.

122. Niland, S. & Schmitz, K. (1995). Sugar transport into storage tubers of *Stachys sieboldii* Miq.: Evidence for symplastic unloading and stachyose uptake into storage vacuoles by an H$^+$-antiport mechanism. *Botanica Acta* **108**, 24-33.

123. Nishimura, M. & Beevers, H. (1979). Hydrolysis of protein in vacuoles isolated from higher plant tissue. *Nature* **277**, 412-413.

124. Nyren, P. & Strid, A. (2004). Hypothesis: the physiological role of the membrane-bound proton translocating pyrophosphatase in some phototrophic bacteria. *FEBS Letters* **77**, 265-270.

125. Nyren, P., Nore, B. F., & Strid, A. (1991). Proton-pumping N,N'-dicyclohexylcarbodiimide-sensitive inorganic pyrophosphate synthase from

Rhodospirillum rubrum: purification, characterization, and reconstitution *Biochemical Journal* **11**, 2883-2887.

126. Obermeyer, G., Sommer, A., & Bentrup, FW. (1996). Potassium and voltage dependence of the inorganic pyrophosphatase of intact vacuoles from *Chenopodium rubrum*. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1284**, 203-212.

127. Palmgren, M. & Christensen, C. (1994). Functional comparison between plant plasma membrane H$^+$-ATPase isoforms expressed in yeast. *Journal of Biochemistry* **269**, 3027-3033.

128. Parry, R. V., Turner, J. C., & Rea, P. A. (1989). High purity preparations of higher plant vacuolar H$^+$-ATPase reveal additional subunits: revised subunit composition. *Journal of Biological Chemistry* **264**, 20025-20032.

129. Penefsky, H. S. (1985). Mechanisms of inhibition of mitochondrial adenosine triphosphatase by dicyclohexylcarbodiimide and oligomycin: Relationship to ATP synthesis. *Proceedings of the National Academy of Science of the United States of America* **82**, 1589-1593.

130. Plaxton, WC. (1996). The organization and regulation of plant glycolysis. *Plant Molecular Biology* **47**, 185-214.

131. Quick, W. P., Neuhaus, H. E., Feil, R., & Stitt, M. (1989). Fluoride leads to an increase of inorganic pyrophosphate and an inhibition of photosynthetic sucrose synthesis in spinach leaves. *Biochimica et Biophysica Acta* **973**, 263-271.

132. Raven, J. A. & Smith, F. A. (1976). The evolution of chemiosmotic energy coupling. *Journal of Theoretical Biology* **57**, 301-312.

133. Rea, P. A., Li, Z.-S., Lu, Y.-P., Drodzdowics, Y. M., & Martinoia, E. (1998). From vacuolar GS-X pumps to multispecific ABC transporters. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 727-760.

134. Rea, P. A. & Poole, R. J. Proton -translocating inorganic pyrophosphatase in red beet (*Beta vulgaris* L.) tonoplast vesicles. *Plant Physiology* **77**, 46-52. 1985.

135. Rea, P. A. & Poole, R. J. (1993). Vacuolar H$^+$-translocating pyrophosphatase. *Annual Review of Plant Physiology and Plant Molecular Biology* **44**, 157-180.

136. Rea, P. A. & Sanders, D. (1987). Tonoplast energization: two H$^+$ pumps, one membrane. *Physiologia Plantarum* **71**, 131-141.

137. Rea, P. A. & Turner, J. C. (1990). *Methods in Plant Biochemistry* **3**, 385-405.

138. Rea, PA., Kim, Y., Sarafian, V., Poole, R. J., Davies, JM., & Sanders, D. (1992). Vacuolar H+-translocating pyrophosphatases: a new category of ion translocase. *Trends in Biochemical Sciences* **17**, 348-353.

139. Rebeille, F., Bligny, R., & Douce, R. (1984). Is the cytosolic Pi concentration a limiting factor for plant cell respiration. *Plant Physiology* **74**, 355-359.

140. Ricard, B., Rivoal, J., Spiteri, A., & Pradet, A. (1991). Anaerobic stress induced the transcriptional and translation of sucrose synthase in rice. *Plant Physiology* **95**, 669-674.

141. Rojans-Beltran, J. A., Dubios, F., Mortiaux, F., Portetelle, D., Gerbhardt, C., Sangwan, R. S., & Jardin, P. (1999). Identification of cytosolic Mg2+-dependent soluble inorganic pyrophosphatases in potato and phylogenetic analysis. *Plant Molecular Biology* **39**, 449-461.

142. Ros, R., Romieu, C., Gibrat, R., & Grignon, C. (1995). The Plant Inorganic Pyrophosphatase Does Not Transport K$^+$ in Vacuole Membrane Vesicles Multilabeled with Fluorescent Probes for H$^+$, K$^+$, and Membrane Potential. *Journal of Biological Chemistry* **270**, 4368-4374.

143. Sabularse, D. C. & Anderson, R. L. (1981). D-fructose-2,6-bisphosphate: a naturally occuring activator for inorganic pyrophosphatase: D-fructose-6phosphate 1-phosphotransferase. *Biochemical and Biophysical Research Communications* **103**, 848-855.

144. Sakakibara, Y., Kobayashi, H., & Kasamo, K. (1996). Isolation and characterization of cDNAs encoding vacuolar H$^+$- pyrophosphatase isoforms from rice (*Oryza sativa L.*). *Plant Molecular Biology* **31**, 1029-1038.

145. Salonoubat, M. & Belliard, G. (1989). The steady state level of potato sucrose synthase mRNA is dependent on wounding, anaerobiosis and sucrose concentration. *Gene* **84**, 181-185.

146. Sarafian, V. & Poole, RJ. (1989). Purification of H$^+$-translocating inorganic pyrophosphatase from vacuolar membranes of red beet. *Plant Physiology* **91**, 34-38.

147. Sarafian V, Kim Y, Poole RJ, Rea PA (1992) Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump (H$^+$-PPase) of Arabidopsis thaliana. *Proceedings of the National Academy of Science of the United States of America* **89**: 1775-1779.

148. Sarafian, V., Potier, M., & Poole, R. J. (1992). Radiation-inactivation analysis of vacuolar H(+)-ATPase and H(+)-pyrophosphatase from *Beta vulgaris L*. Functional sizes for substrate hydrolysis and for H+ transport. *Biochemical Journal* **283**, 493-497.

149. Sato, H., Maeshima, M., Ohsumi, Y., & Yoshida, M. (1991). Dimeric structure of H+-translocating pyrophosphatase from pumpkin vacuolar membranes. *FEBS Letters* **290**:177-180.

150. Sato, HM., Kasahara, M., Ishii, N., Homareda, H., Matsui, H., & Yoshida, M. (1994). Purified vacuolar inorganic pyrophosphatase consisting of a 75-kDa polypeptide can pump H+ into reconstituted proteoliposomes *Journal of Biological Chemistry* **269**, 6725-6758.

151. Schmidt, A. L. & Brisken, D. P. (1991). H$^+$/substrate stoiciometry of the tonoplast H$^+$-ATPase and H$^+$-PPase from red beet storage tissue. *Plant Physiology* **96**, 139.

152. Schmidt, AL. & Brisken, DP. (1993). Energy Transduction in Tonoplast Vesicles from Red Beet (*Beta vulgaris* L.) Storage Tissue: H^+/Substrate Stoichiometries for the H^+-ATPase and H^+-PPase. *Archives of Biochemistry and Biophysics* **301**, 165-173.

153. Schocke, L. & Schink, B. (1998). Membrane-bound proton-translocating pyrophosphatase of *Syntrophus gentianae*, a syntrophically benzoate-degrading fermenting bacterium. *European Journal of Biochemistry* **256**, 589-594.

154. Shewry, P. R., Napier, J. A., & Tatham, A. S. (1995). Seed storage proteins: Structures and biosynthesis. *The Plant Cell* **7**, 945-956.

155. Shiratake, K., Kanayama, Y., Maeshima, M., & Yamaki, S. (1997). Changes in H+-pumps and a tonoplast intrinsic protein of vacuolar membranes during the development of pear fruit. *Plant and Cell Physiology* **38**, 1039-1045.

156. Sikora, A., Hillmer, S., & Robinson, D. G. (1998). Sucrose starvation causes a loss of immunologically detectable pyrophosphatase and V-ATPase in the tonoplast of suspension-cultured tobacco cells. *Journal of Plant Physiology* **152**, 207-212.

157. Smart, L. B., Vojdani, F., Maeshima, M., & Wilkins, T. A. (1998). Genes involved in osmoregulation during turgor-driven cell expansion of developing cotton fibers are differentially regulated. *Plant Physiology* **116**, 1539-1549.

158. Smyth, D. A. & Black, C. C. (1984). Measurement of the pyrophosphate content of plant tissues. *Plant Physiology* **75**, 862-864.

159. Sonnewald, U. (1992). Expression of *E.coli* inorganic pyrophosphatase in transgenic plants alters photoassimilate partitioning in leaves. *Plant Journal* **2**, 571-581.

160. Stitt, M. (1998). Pyrophosphate as an energy donor in the cytosol of plant cells: an enigmatic alternative to ATP. *Botanica Acta* **111**, 167-175.

161. Stitt, M. (1990). Fructose-2,6-bisphosphate as a regulatory molecule in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 153-185.

162. Stitt, M., Huber, S. C., & Kerr, P. (1987). Control of photosynthetic sucrose formation. *In 'Photosynthesis'.(Eds MD Hatch and NK Boardman) The biochemistry of plants: a comprehensive treatise* **10**, 327-408.

163. Sung, S.-J. S., Xu, D.-P., Galloway, C. M., & Black, C. C. (1988). A reassessment of glycolysis and gluconeogenisis in higher plants. *Physiologia Plantarum* **72**, 650-654.

164. Sussman, M. (1994). Molecular analysis of proteins in the plant plasma membrane. *Annual Review of Plant Physiology and Plant Molecular Biology* **45**, 211-234.

165. Suwastika, I. N. & Gehring, C. A. (1999). The plasma membrane H^+-ATPase from *Tradescantia* stem and leaf tissue is modulated *in Vitro* by cGMP. *Archives of Biochemistry and Biophysics* **367**, 137-139.

166. Sze, H., Ward, J., Lai, S., & Perera, I. (1992). Vacuolar type H^+-translocating ATPases from plants: structure, function, and isoforms. *J Bioenerg Biomembr* **24**, 371-381.

167. Sze, H. (1985). H^+-translocating ATPases: Advances using membrane vesicles. *Annual Review of Plant Physiology and Plant Molecular Biology* **36**, 175-208..

168. Taiz, L. (1992). The Plant vacuole. *Journal of Experimental Botany* **172**, 113-122.

169. Takasu, A., Nakanishi, Y., Yamauchi, T., & Maeshima, M. (1997). Analysis of the substrate binding site and carboxyl terminal region of vacuolar H+-pyrophosphatase of mung bean with peptide antibodies. *Journal of Biochemistry* **122**, 883-889.

170. Takeshige, K., Tazawa, M., & Hager, A. (1988). Characterization of the H^+-translocating adenosine triphosphatase and pyrophosphatase of vacuolar membranes isolated by means of a perfusion technique from *Chara corallina. Plant Physiology* **86**, 1168-1173.

171. Takeshige, K. & Tazawa, M. (1989). Determination of the inorganic pyrophosphatase level and its subcellular localization in *Chara corallina. Journal of Biological Chemistry* **264**, 3262-3266.

172. Tanaka, Y., Chiba, K., Maeda, M., & Maeshima, M. (1993). Molecular Cloning of cDNA for Vacuolar Membrane Proton-Translocating Inorganic Pyrophosphatase in *Hordeum vulgare. Biochemical and Biophysical Research Communications* **190**, 1110-1114.

173. Trethewey, R.N., Geigenberger, P., Riedel, K., Hajiresaei, M.-R., Sonnewald, U., Stitt, M., Reismeier, J.W. and Willmitzer, L. (1998). Combined expression of glucokinase and invertase in potato tubers leads to a dramatic reduction in starch accumulation and a stimulation of glycolysis. *The Plant Journal* **15**, 109-118.

174. Tung A, Ju S, Sato S, Nisonoff A (1976). Production of large amounts of antibodies in individual mice. *Journal of Immunology* **116**, 676-681.

175. Tzeng, C., Yang, C., Yang, S. J., Jiang, S., Kuo, S., Hung, S., Ma, J., & Pan, R. (1996). Subunit structure of vacuolar proton-pyrophosphatase as determined by radiation inactivation. *Biochemical Journal*. **316**, 143-147.

176. van Dillewijn C (1952). Growth. General, grand period, growth formulae. *C van Dillewijn, ed.Botany of sugarcane, Veenen and Zonen, Netherlands* **1**, 97-162.

177. Van Schaftingen, E., Ledere, B., Bartrons, R., & Hers, H.-G. (1982). A kinetic study of pyrophosphate:fructose-6-phosphate phosphotransferase from potato tubers. *European Journal of Biochemistry* **129**, 191-195.

178. Visser, K., Heimovaara-Dijkstra, S., Kijne, J.W., & Wang, M. (1998). Molecular cloning and characterization of an inorganic pyrophosphatase from barley. *Plant Molecular Biology* **37**, 131-140.

179. Wagner GJ (2004) *In Recent Advances in Phytochemistry (Creasy, L , Hrazdina, G , eds), Plenum Publishing Corp , New York* 14: 1-45.

180. Wagner, G. J. & Mulready, P. (1983). Characterization and solubilization if nucleotide-specific Mg^{2+}-ATPase and Mg^{2+}-pyrophosphatase on tonoplast. *Biochimica et Biophysica Acta* **728**, 267-280.

181. Walker, R. R. & Leigh, R. A. (1981). Mg^{2+}-dependent, cation-stimulated inorganic pyrophosphatase associated with vacuoles isolated from storage roots of red beet (*Beta vulgaris* L.). *Planta* **153**, 150-155.

182. Wang, Y. Z., Leigh, R. A., Kaestner, K. H., & Sze, H. (1986). Electrogenic H^+-pumping pyrophosphatase in tonoplast vesicles of oat roots. *Plant Physiology* **81**, 497-502.

183. Wang BS, Ratajczak R, Zhang JH (2000). Activity, amount and subunit composition of vacuolar-type H+- ATPase and H+-PPase in wheat roots under severe NaCl stress. *Journal of Plant Physiology* **157**: 109-116

184. Ward, J. M., Reinders, A., Hsu, H.-T., & Sze, H. (1992). Dissociation and reassembly of the vacuolar H^+-ATPase complex from oat roots. *Plant Physiology* **99**, 161-169.

185. Weiner, H., Stitt, M., & Heldt, H. W. (1987). Subcellular compartmentation of pyrophosphate and alkaline pyrophosphate in leaves. *Biochimica et Biophysica Acta* **893**, 13-21.

186. White, P. J., Marshall, J., & Smith, J. A. C. (1990). Substrate kinetics of the tonoplast H^+-translocating inorganic pyrophosphatase and its activation by free Mg^{2+}. *Plant Physiology* **93**, 1063-1070.

187. Whittaker, A. & Botha, F. C. (1997). Carbon partitioning during sucrose accumulation in sugarcane internodal tissue. *Plant Physiology* **90**, 635-642.

188. Whittaker, A. & Botha, F. C. (1999). Pyrophosphate:D-fructose-6-phosphate 1-phosphotransferse activity patterns in relation to sucrose storage across sugarcane varieties. *Physiologia Plantarum* **107**, 379-386.

189. Williams, L., Thom, M., & Maretzki, A. (1990). Characteization of proton translocating ATPase and sucrose uptake in a tonoplast-enriched vesicle fraction from sugarcane. *Physiologia Plantarum* **80**, 169-176.

190. Wink, M. (1997). Compartmentation of secondary metabolites and xenobiotics in plant vacuoles. *In Advances in Botanical Research, The Plant Vacuole, R.A.Leigh and D.Sanders, eds (London: Academic Press)*. **25**, 141-169.

191. Wood, H. C. (1985). Inorganic pyrophosphate and polyphophates as sources of energy. *Curr.Top.Cell.Reg.* **26**, 355-369.

192. Yazaki, Y., Asukagawa, N., Ishikawa, Y., Ohta, E., & Sakata, M. (1988). Estimation of Cytoplasmic Free Mg^{2+} Levels and Phosphorylation Potentials in Mung Bean Root Tips by In vivo ^{31}P NMR Spectroscopy. *Plant and Cell Physiology* **29**, 919-924.

193. Yoshida, S. (1991). Chilling-induced inactivation and its recovery of tonoplast H^+-ATPase in mung bean cell-suspension cultures. *Plant Physiology* **95**, 456-460.

194. Yoshida, S., Matsuura, C., & Etani, S. (1989). Impaired of tonoplast H^+-ATPase as an initial physiological response of cells to chilling in mung bean [*Vigna radiata* (L.) Wilczek]. *Plant Physiology* **89**, 634-642.

195. Zancani, M., Macri, F., Perufflo, A. D. B., & Vianello, A. (1995). Isolation of the catalytic subunit of a membrane-bound H(+)- pyrophosphatase from pea stem mitochondria. *European Journal of Biochemistry* **228**, 138-143.

196. Zhen, R. G., Kin, E. J., & Rea, P. A. (1997). The molecular and biochemical basis of pyrophosphate-energised proton translocation at the vacuolar membrane. *In: Leigh, R.A., and Sanders, D., eds., Advances in Botanical Reserach, The Plant Vacuole* Academic Press, 297-337.

197. Zheng, J. & Ramirez, V. D. (2000). Inhibition of mitochondrial proton F0F1-ATPase/ATP synthase by polyphenolic phytochemicals. *British Journal of Pharmacology* **130**, 1115-1123.

198. Zimmermann, MH. & Ziegler, H. (1975). *M.H.Zimmermann, J.A.Milburn (Eds.), Transport in Plants, Encycl.Plant Physiology., Springer, Berlin* 245-271.