*XvVHA-c``1*- a novel stress responsive V-ATPase subunit c`` homologue isolated from the resurrection plant *Xerophyta viscosa* Baker

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A dissertation submitted in fulfilment of the requirements for the degree of Master of Science in the Department of Molecular and Cell Biology, Faculty of Science, University of Cape Town, South Africa

TABLE OF CONTENTS

In the name of God,
Most Gracious, Most Merciful!
He alone grants knowledge and He alone guides.
May He grant this work to be of benefit to all.
May He be pleased with us.

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Abbreviations

µg	microgram(s)
µl	microlitre(s)
^0C	degrees Celsius
ABA	abscisic acid
Ade	adenine
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
Bp	base pair(s)
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
Cl^-	chloride ion
Da	Dalton
DCCD	N,N` dicyclohexylcarbodiimide
dCTP	deoxy-cytosine triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	di-thiothritol
EDTA	ethylenediaminetetra-acetic acid
H^+	proton
His	histidine
Kbp	kilo-base pair
LB	Lauria-Bertani broth
Leu	leucine
M	molar
mg	milligram
min(s)	minute(s)
ml	millilitre
mM	millimolar

Na^+	sodium
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometre
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PMF	proton motive force
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RWC	relative water content
SDS	sodium dodecyl sulphate
SSC	sodium citrate
TBE	tris borate EDTA
Trp	tryptophan
Ura	uracil
V	volt
WT	wild type

List of figures and tables

List of figures

List of tables

Abstract

The resurrection plant *X. viscosa* is a model plant with the capacity to survive water deficit stress. *X. viscosa* can loose 95% of its relative water content, and remain within a quiescent state for long periods of time until favourable conditions re-immerge. The genes used by *X. viscosa* to tolerate extreme water loss could be used to transform crops to give them a degree of tolerance against environmental stresses. *XvVHA-c``1* was isolated from *X. viscosa* that underwent a dehydration stress. The aim of this project was to further characterise the molecular role of *XvVHA-c``1*in *X. viscosa*'s ability to tolerate water deficit stress. BLAST search results confirmed that *XvVHA-c``1* showed significant homology to plant subunit c`` proteolipids of the vacuolar H^+ATPase (V-ATPase). A Southern hybridisation analysis was conducted to confirm the presence of *XvVHA-c``1* in the *X. viscosa* genome and that *XvVHA-c``1* exists at a single gene locus. Northern hybridisation analyses were conducted on *X. viscosa* leaves subjected to stresses, to determine the expression of *XvVHA-c``1*. The stresses included a dehydration stress, a salinity stress and and freezing shock at -20^{0}C. An increase in the transcript was evident 72 and 96 hours after application (HAA) of 150 mM NaCl stress, at 18% and 10% RWC in the response to dehydration stress and an increase after 60 min exposure to -20^{0}C. A complementation study was conducted on *S. cerevisiae VMA3* knockout mutants to demonstrate *XvVHA-c``1*'s proposed functionality. The experimental strain was transformed with *XvVHA-c``1* cloned into a shuttle expression vector pYES2 and subjected to a 100mM $CaCl_2$ stress plates. *XvVHA-c``1* was able to rescue the mutant strain from 100 mM $CaCl_2$ stress and confirm its potential function. It was concluded that

XvVHA-c``1 displays stress-responsive gene expression and its potential role in *X. viscosa's* stress response is presented.

Chapter One

General introduction

1.1 Introduction

Water is an extremely important commodity for all life on earth and even though it covers most of the earth's surface area in the form of salt water and glaciers, access to fresh water for crop irrigation and consumption is an increasing problem (IFPRI, 2002). Water fulfils its ecological role in the biosphere by forming and completing the water cycle, but its accessibility as we require it, i.e. as fresh water, is decreasing per capita. The global problem in this regard is the rapid increase of the population. The world's population has doubled since 1950 from 3 billion to 6 billion and is projected to increase by the middle of the century from 6 billion to approximately 9 billion. If these projections are correct, the need for water will increase drastically.

Biotic and abiotic factors that affect water's availability per capita are pollution, meteorological disasters such as floods (in this case the water is available but not accessible as its flow rate is too high to capitalise) and climate change (IFPRI, 2002).

It is estimated that humans use more than 70% of the water that is available to them for agricultural practices. Currently 17% of global agriculture is irrigated (IFPRI, 2002). Forty percent of the world's food is produced using this 17% irrigation water. As an

increase of 3 billion people is expected by the middle of the century, we need to increase agricultural productivity. To do this, an increase in the amount of irrigation is required, but unlikely to materialise as the amount of available fresh water is decreasing. While the population will treble, water demand has increased by a factor of six, which simply put means that supply cannot meet demand.

Since the green revolution, agricultural productivity has increased due to the use of high-yielding crop varieties selected by traditional breeding methods, fertilizers and the unlimited use of irrigation water. The question then arises, how can we grow the food we need with the available source of irrigation water especially when traditional practices are failing us?

We could possibly solve the problem of diminishing fresh water by firstly, improving water and land resource management practices in agriculture, forestry and fisheries; secondly, increasing the understanding between agricultural and other water users, especially with regard to the environment; and thirdly, reducing water use or increasing the water productivity of agriculture. The latter option is not exclusively limited to, but may include implementing the efforts made to genetically enhance crops to tolerate intermittent periods of unfavourable meteorological events such as drought.

According to Grill and Ziegler (1998) plants have faced water shortage since they established themselves on land 450 million years ago. In response, plants have equipped

themselves with complex physiological and metabolic adaptations not to succumb to the threat of water deficit stress, especially since they are all sessile.

Rhodes (1987) defines stress as any physiochemical environmental factor capable of producing an injurious "strain" on a living organism. Furthermore, the difference between elastic and a plastic strain is defined by elastic strains being reversible, whereas plastic strains are irreversible but have the potential to be repaired.

Water's biological roles include acting as a solvent, as a transport medium, as an electron donor in the Hill reaction— a reaction that occurs in the chloroplast and is described by the light-induced transport of electrons from water to acceptors such as potassium ferricyanide, releasing oxygen as a by product— and to act as an evaporative coolant (Bohnert *et al.*, 1995). Additionally, water can serve to stabilize other molecules and organelles in both a hydrophobic and hydrophilic fashion. According to Bray (1997) when the rate of transpiration exceeds that of water uptake the plant experiences a water deficit, which is a component of different stresses that include drought, salinity and low and high temperature.

Water deficit could lead to the concentration of intracellular solutes, affect the cell volume and membrane shape, disrupt water potential gradients, cause the loss of turgor, disrupt membrane integrity and cause the denaturation of proteins (Bray, 1997). For a plant to be able to respond and survive cellular water deficit, it would depend on whole-plant mechanisms that are able to integrate the cellular responses (Bray, 1997).

How do scientists maintain or improve crop production in the light of a depleting water supply? One answer is to engineer a crop variety to withstand temporary periods of water deficit. One way to facilitate this is to study the physiological and metabolic mechanisms used by desiccation tolerant plants, such as resurrection plants.

1.2 Whole plant response to water deficit

For a whole plant to be able to respond to and survive water deficit it must be able to make use of mechanisms that orchestrate cellular responses (Figure 1.1) (Bohnert *et al.*, 1995). Resistance to water deficit may arise from either a tolerance toward the stress, or an adaptive mechanism that allows the avoidance of the stress (Bray, 1997).

The stress response is species and genotype dependent. The response is equally dependent on the length and severity of water loss, the age and stage of plant development, the organ, cell type, and the subcellular compartment in which the stress is experienced (Bray, 1997). It is thought, however, that the mechanisms that control stress perception and the genes consequently expressed are universal to all plants. This observation is based on the wide distribution of stress-adapted plants in many different families, the occurrence of many stress-tolerant relatives of many glycophytic (salt intolerant) species, and the genetic variability in stress tolerance of crop plants (Bohnert *et al.*, 1995).

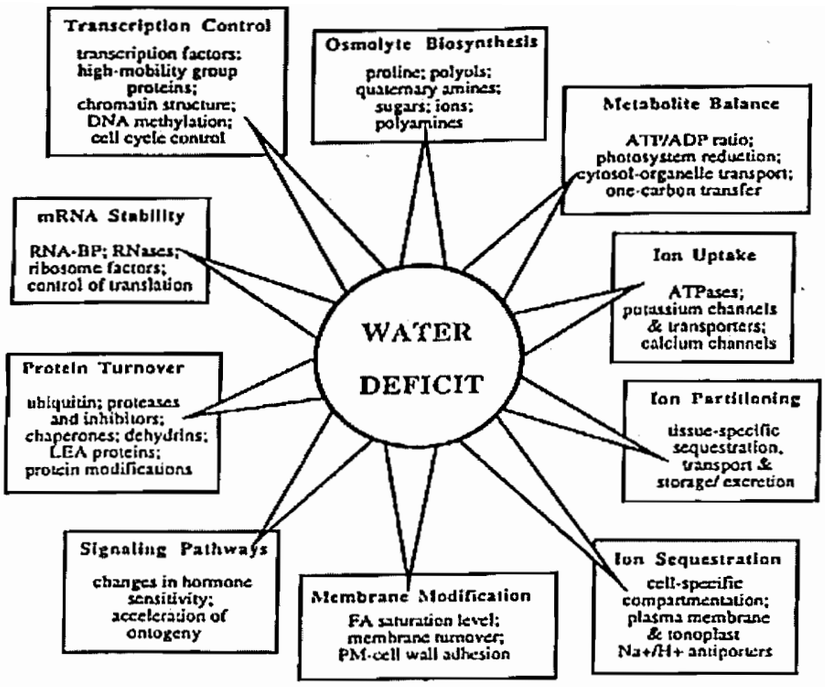

Figure 1.1 Whole plant responses to water deficit. Under stress from water many cellular processes change. These changes allow the plant to maintain metabolism and restore conditions that allow for continued growth under stress. (Bohnert *et al.*, 1995).

One of the cellular responses to water deficit stress includes *de novo* expression of proteins that assist the adaptation to a stress. Shinozaki and Yamaguchi-Shinozaki (1997) classify the proteins synthesised *de novo* in response to water deficit into two classes. The first class includes proteins that have a direct function in the stress tolerance, and the second class of proteins includes transcription factors that play roles in signal transduction and gene regulation in response to the stress. These include protein kinases, phospholipase C and 14-3-3 proteins, involved in cell cycle regulation (Yamaguchi-Shinozaki, 1997).

1.3 Some known adaptations to water deficit

In the following section some of the adaptations implemented by plants in response to water deficit stress are briefly introduced. The brief description is intended to give some background information into a still dynamic research discipline.

1.3.1 Metabolic adjustment

The adjustment of metabolism in response to water deficit is regarded as an inevitable and a general event, which is marked by the increase in expression of genes that code for metabolic enzymes. Ingram and Bartels (1996) report on the upregulated expression of glyceraldehyde-3-phosphate in response to drought stress and exogenous ABA treatment in *Craterostigma plantagineum*. It is also known that increased expression of this enzyme is triggered by other environmental stresses, indicating that the general requirement during stress is an increase in energy demand (Ingram and Bartels, 1996).

Protease activity is also known to increase in response to stress as an adjustment to metabolism (Ingram and Bartels, 1996). Proteases act to degrade non-native proteins and depolymerise vacuole storage peptides in order to increase the amino acid pool for *de novo* protein synthesis. Enzymes involved in sugar metabolism are critical for the adaptation to water deficit tolerance. For instance, the genes of sucrose-phosphate synthase and sucrose synthase are upregulated in expression in response to drought in *C. plantagineum*. Enzymes such as Δ1-pyrroline-5-carboxylate synthase and betaine aldehyde dehydrogenase are involved in the synthesis of proline and glycine betaine, respectively (Ingram and Bartels, 1996).

1.3.2 Osmotic adjustment

Osmotic adjustment entails the maintenance of water potential during mild water deficit by making use of sugars and other compatible solutes (Ingram and Bartels, 1996). Compatible solute accumulation lowers the intracellular osmotic potential, resulting in the reduction of intracellular water potential relative to that of external water potential and the flow of water into the cell (Bray, 1993). In *C. plantagineum* carbohydrate metabolism changes occurs on dehydration. The C8 sugar octulose has been recorded to accumulate to 90% of all soluble sugars in photosynthetically active leaves (Bartels and Salamini, 2001). Upon dehydration, the level of octulose decreases, whereas the levels of sucrose increase. The accumulation of sucrose in dehydrated tissue is a common characteristic of resurrection plants, but different metabolic routes may be used to synthesis sucrose (Bartels and Salamini, 2001).

The proton motive force (PMF) assists with the regulation of osmotic potential and ion compartmentation. Ion and water channels regulate water flux in response to water deficit (Ingram and Bartel, 1996).

1.3.3 Compatible solutes

Compatible solutes do not interfere with normal biochemical reactions and their accumulation in plants allows them to tolerate abiotic stress because they act as effective osmoprotectants (Bohnert, 1995). Compatible solutes are low molecular weight organic metabolites that act to stabilize folded protein structures, membranes (and/or membrane proteins) and have been implicated in cryoprotection. Examples of compatible solutes are

amino acids, quaternary ammonium compounds, polyols such as glycerol and sugars such as sucrose and trehalose (Rhodes, 1987).

1.4 Resurrection plants

There exists a group of higher plants, called "resurrection plants", that are capable of coping with drought stress by being desiccation tolerant. Pollen and seeds are capable of enduring extended periods of water loss in a quiescent state, which is lost once germination is initiated and when conditions are favourable to do so. Resurrection plants are different in this respect as they retain the water deficit survival capacity in their vegetative tissue. The resurrection capacity is not restricted to the main meristems of the plant as it is possible for mature leaf tissue to lose 95% of their water content. Upon re-introduction to water, dehydrated tissue can be restored to be photosynthetically active, with minimal, or without any apparent tissue damage throughout the dehydration-rehydration cycle (Gaff, 1971).

A common feature of these extraordinary plants is that they exist in mesic to arid climates in southern Africa, southern America and Western Australia, where they are exposed to waterless conditions in the micro niches that they inhabit. Their ability to survive drought confers on them additional endurance in conditions of high salt concentrations and fluctuations in temperature. Ultimately, resurrection plants are able to survive conditions that are uninhabitable by other higher plants, giving them an evolutionary advantage (Scott, 2000).

Craterostigma wilmsii has, like many other resurrection plants being studied, the ability to decrease the hydrated leaf area by 15% under dehydrating conditions (Sherwin and Farrant, 1996). This shrunken state correlates to the time when the leaf tissue loses most of its water. The reduction in size is evidently achieved by the plasmalemma and the cell wall forming a concertina, which is thought to minimize damage within and between cells. *Myrothamnus flabellifolia* on the other hand, does not display such drastic shrinkage, but it maintains the ability to desiccate to 5%-10% relative water content (RWC) (Farrant *et al.*, 2003).

Angiosperms with resurrection capacity are sub-divided into two groups, homoiochlorophyllous and poikilochlorophyllous (Gaff, 1971). Homoiochlorophyllous resurrection plants retain their chlorophyll during drying. Examples of homoiochlorophyllous resurrection plants are *C. wilmsii* and *M. flabellifolia* (Sherwin and Farrant, 1996). Poikilochlorophyllous plants lose their chlorophyll upon drying and rebuild their photosynthetic machinery upon rehydration (Sherwin and Farrant, 1998), e.g. *Xerophyta viscosa* (Baker) (Figure 1.2). Anthocyanins and carotenoids are accumulated in *X. viscosa* when its chlorophyll is degraded, which is thought to be linked to protection of the plants against UV-light and from damage as a result of reactive oxygen species generation during desiccation (Sherwin and Farrant, 1998).

Figure 1.2 *Xerophyta viscosa* (Baker) flowering under greenhouse conditions.

1.4.1 *Xerophyta viscosa*

Mundree and Farrant (2000) have elucidated *X. viscosa*'s response to some of the key stresses during desiccation of vegetative tissues, including:

- mechanical stress associated with volume reduction
- oxidative stress as a consequence of unregulated metabolism, especially those of photosynthesis and respiration
- disruption of membrane and macromolecular integrity due to the loss of structure-associated water.

In the plant cell the plasmalemma is attached to a very rigid cell wall via plasmodesmata, and upon severe water loss the plasmalemma would normally tear, releasing hydrolytic enzymes into the cytoplasm, resulting in the collapse of the cell wall. This is an example of mechanical stress as a consequence of a reduction of cell volume upon desiccation. It was observed by Mundree and Farrant (2000) that *X. viscosa* subdivides its large central vacuole (Figure 1.3A) into smaller vacuoles (Figure 1.3B), which could act as a preventative mechanism for mechanical stress sustained during dehydration. It was proposed that the smaller vacuoles become filled with non-aqueous material as water exits them. The contents of these vacuoles are unlikely to be water, because vacuole structures are present in leaves dried to 2% RWC. Instead, the vacuoles could be filled with sucrose, sorbitol, anthocyanins and other polyphenolics, unknown compatible solutes and even proteins.

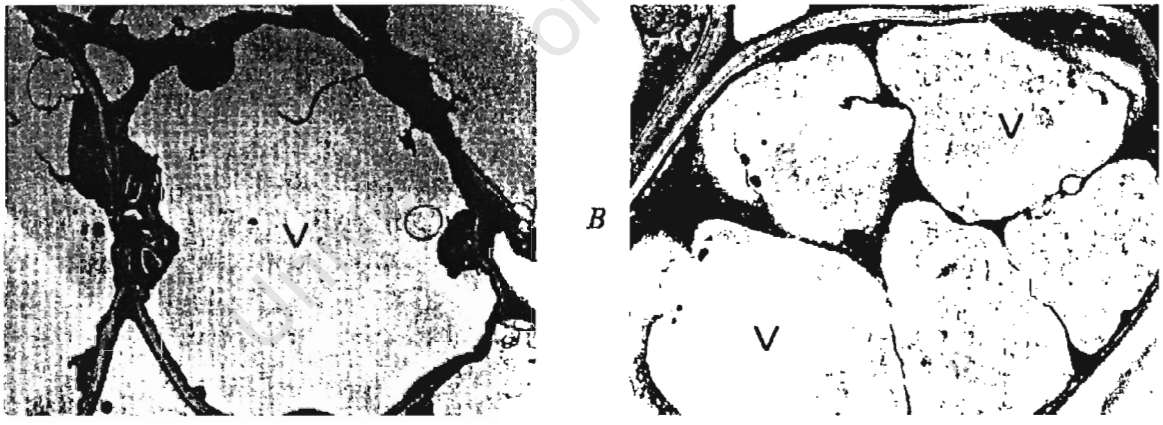

Figure 1.3 **(A)** Electron micrographs of hydrated (100% RWC) mesophyll cell from *X. viscosa*. Note the large central vacuole (V). **(B)** Ultrastructural detail of a cell from a dehydrated (5% RWC) leaf of *X. viscosa*. Note that the central vacuole has divided into smaller vacuoles (V) which have electron dense content. Magnification X8000. Mundree and Farrant (2000).

Vander Willigen *et al.* (2001) showed an accumulation of mostly proline in the vacuoles of desiccation tolerant vegetative tissue of the resurrection grass *Eragrostis nindensis.*

Oxygen free radicals cause subcellular damage and loss of viability in angiosperms that are desiccation sensitive. Desiccation sensitive plants accumulate oxygen free radicals when water deficit disrupts their photosynthetic and respiration machinery. *X. viscosa* has the mechanisms in place to prevent free radical formation and to alleviate their activity. The presence of chlorophyll during desiccation leads to an excess of excitation energy that is not quenched by photosynthesis, and hence oxygen free radicals are generated. By degrading its chlorophyll and dismantling the thylakoid membranes into smaller vesicles, *X. viscosa* prevents light-chlorophyll interactions, minimising the formation of free radicals while the plant is in its dried state. Evidence has shown that photosynthesis ceases at roughly 55% RWC in *X. viscosa* (Sherwin and Farrant, 1998; Mundree and Farrant, 2000). This is proposed to minimise free radical production in the chloroplasts during drying.

The existence of a reactive oxygen quencher has been displayed by work conducted on a 1-Cys peroxiredoxin, XvPer1, isolated from *X. viscosa*. *XvPer1* had been identified as a thiol-specific antioxidant enzyme, that unlike traditional 1-Cys peroxiredoxins which are seed specific, shows activity in the vegetative tissue of *X. viscosa*. XvPer1 displayed an increase in transcript when *X. viscosa* was subjected to dehydration stress, heat stress at 42°C, high light intensity stress at 1 500 μmol photons $m^{-2}s^{-1}$, when treated with abscisic

acid (100 μM ABA) and subjected to salinity stress of 100 mM NaCl (Mowla *et al.*, 2002)

Additional enzymes associated with antioxidant metabolism are active during dehydration of *X. viscosa*. Ascorbate peroxidase, glutathione reductase and cytoplasmic superoxide dismutase show increased activity as vegetative tissue dehydrates. On the other hand, it was found that chloroplastic superoxide dismutase activity decreases during dehydration. Hence it was suggested that these enzymes allow antioxidant protection against oxygen free radicals produced in the mitochondria, given that respiration is ongoing in the presence of low water content (Sherwin and Farrant, 1998).

1.5 The plant vacuole

When it is part of the apical meristem tissue, the plant cell has hundreds of small provacuoles that originate from the budding off of the *trans* Golgi network. As the plant cell grows in size, the provacuoles fuse, resulting in a large central vacuole. Vacuolar sap contains the cellular complement of K^+, Ca^{2+}, sugar, organic acids, and other solutes; many of these requiring active transport against the electrochemical gradients (Figure 1.4). A large amount of energy is therefore required to maintain the solute concentration inside the vacuole. The enzymes that are directly involved in supplying tonoplastic energy are the vacuolar H^+ ATPase (V-ATPase) and the vacuolar H^+-pyrophosphatase (V-PPase; Taiz, 1992).

In mature non-vascular tissue, the vacuole occupies 90% of the cell volume, playing a role in maintaining cell size and structure, which increases the cell's surface area exposure to sunlight (in the case of leaves), and facilitating photosynthesis. It is energetically cheaper to increase surface area through water uptake than to synthesise protein. Vacuoles are capable of storing sugars, polysaccharides, organic acids, proteins, flavour compounds, and colour compounds.

Since plants are immobilised and do not possess an excretory system, the vacuole acts as a micro-kidney by filtering and sequestering potentially toxic ions from the cytosol. V-ATPases and V-PPases help maintain a homeostatic balance in the plant cell by inducing or facilitating the transport of ions into the vacuole. These enzymes primarily translocate protons out of the cytosol into the lumen of the vacuole. The pH of the vacuole in higher plants is generally around 5.0-5.5, but it can decrease as in the case of the lemon fruit vacuole to 2.5. The most acidic vacuole recorded is that of a brown alga *Desmerestia sp.* with a lumenal pH of 0.6 (Taiz, 1992).

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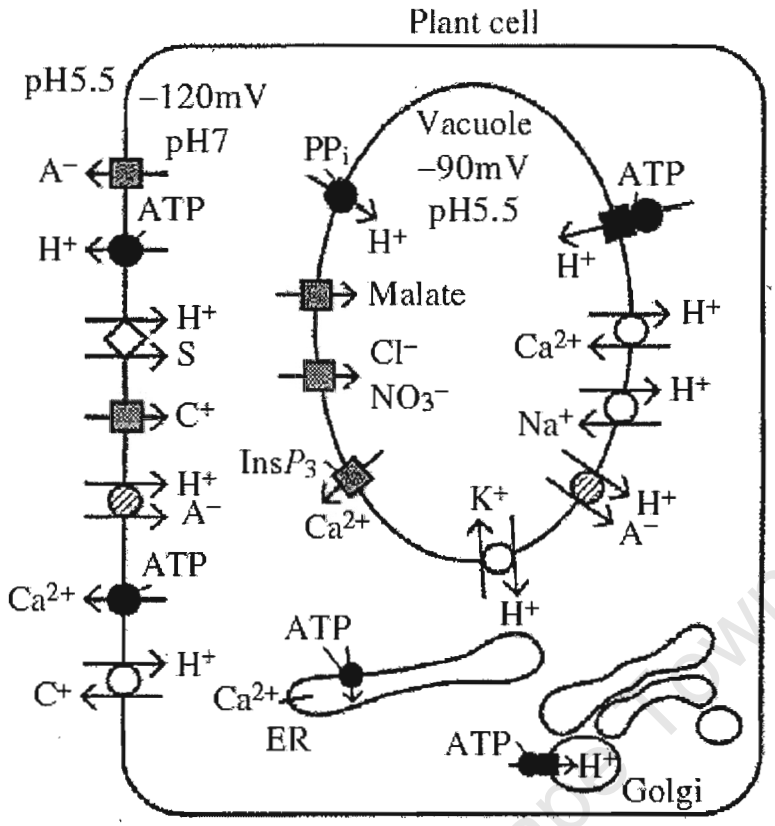

Figure 1.4 A model of primary H^+ pumps, H^+-coupled transporters and channels in a simplified plant cell. A plasma membrane ATPase (P-type) pumps H+ out of the cell, generating a proton electrochemical gradient (inside 2120 mV relative to the outside). An electrogenic V-ATPase and an H+-PPase (pyrophosphatase) acidify the vacuole. The proton-motive force provides energy for uptake and release of solutes across the tonoplast through antiporters (open circles), symporters (hatched circles) and channels (squares). Primary ion pumps are shown as filled circles. C+, A2 and S refer to cations, anions and organic solutes, respectively. The V-ATPase also acidifies endomembrane compartments, such as the Golgi body and coated vesicles. InsP3, inositol triphosphate; ER, endoplasmic reticulum. (Sze *et al.*, 1992).

Vacuolar acidification is vital for a variety of cellular processes which include ligand-receptor dissociation and receptor recycling that follows receptor-mediated endocytosis, intracellular targeting of newly synthesised lysosomal enzymes, protein processing and degradation and coupled transport of small molecules (Nishi *et al.*, 2002, and Forgac, 1999).

1.6 V-ATPases - an insight into their structure, subunit composition and function

Vacuolar proton adenosine triphosphatases (V-ATPases) (Figure 1.5) are a family of highly conserved ATP-dependant proton pumps responsible for the acidification of intracellular compartments in all eukaryotic cells (Kawasaki-Nishi *et al.*, 2003). They are present in all eukaryotic endomembrane systems which include vacuoles, lysosomes, endoplasmic reticulum and the Golgi bodies. All endomembrane systems need to be energized through the activity of electrogenic pumps such as V-ATPases (Finbow and Harrison, 1997). The physiological roles of V-ATPases such as control of cytosolic pH homeostasis, driving secondary transport of nutrients and ions, and regulating protein sorting are only beginning to be appreciated.

1.6.1 *Saccharomyces cerevisiae* V-ATPase subunit composition and structure and its comparison to F-ATPases

The *S. cerevisiae* V-ATPase has served as a model in trying to understand the function, subunit composition, and macro- and micro-structure of the macromolecule for all biologists. *S. cerevisiae* cultures are easy to cultivate, have a relatively fast reproduction cycle, are easy to isolate proteins from and *S. cerevisiae* was one of the first eukaryotes whose genome was completely sequenced.

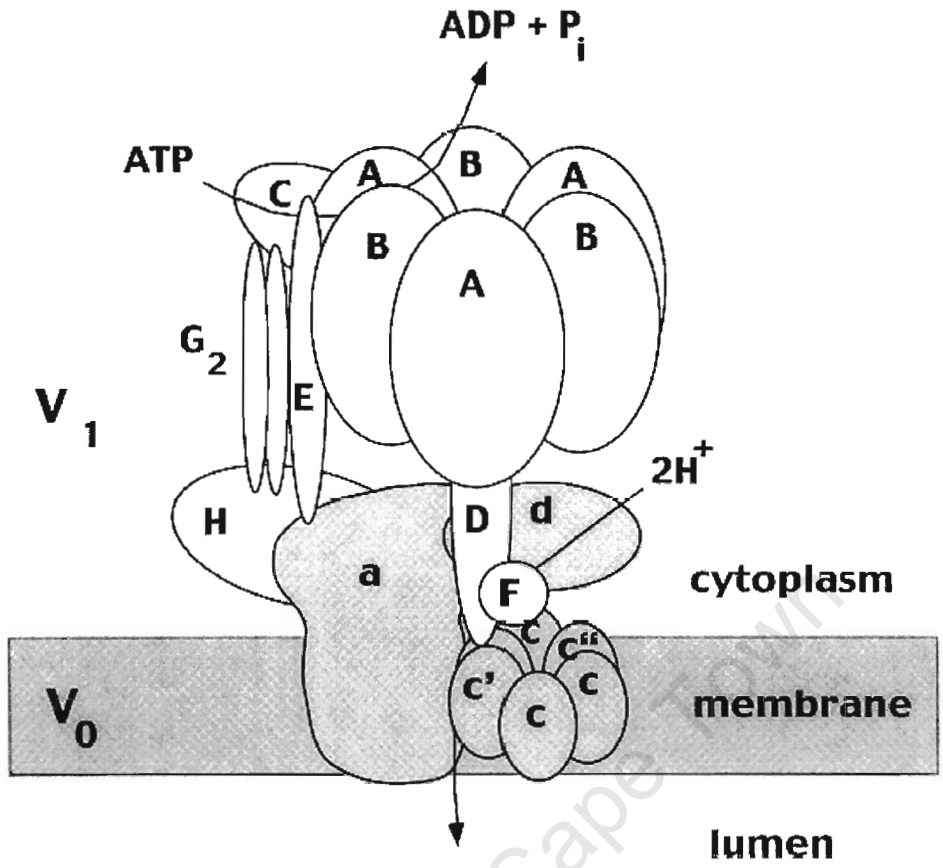

Figure 1.5 Structural model of the V-ATPase complex from *S. cerevisiae*. The V1 domain (shown in white) is responsible for ATP hydrolysis whereas the V0 domain (shaded) is responsible for proton translocation. ATP hydrolysis at the catalytic nucleotide-binding sites (located on the A subunits) is proposed to drive rotation of a central stalk (composed of the D and F subunits), which in turn drives rotation of the ring of proteolipid subunits (c, c`, c``) relative to subunit a. Subunit a is held fixed relative to the A3B3 head by a peripheral stalk composed of subunits C, E, G, and H and the soluble domain of subunit a. Movement of the ring of proteolipid subunits past subunit a is thought to drive unidirectional proton transport across the membrane. (Arata *et al.*, 2002b).

Additionally, deletion studies are readily conducted on viable mutants, allowing the phenotypic characterisation of the knock-out mutants and contributing to the functional understanding of each subunit in a multimeric complex such as the V-ATPase.

V-ATPases are composed of two domains. V1 is the peripheral domain (also referred to as the head) and is typically responsible for ATP hydrolysis. It is a 640-kDa multimeric complex with eight different subunits (A-H) that typically have an $A_3B_3CDEFG_2H_{1-2}$ stoichiometry. The integral domain V0 (also referred to as the membrane anchor) is a 260-kDa multimeric complex composed of five subunits (a, b, c, c`, c``) with a stoichiometry typically following ad $c_4c`c``$.

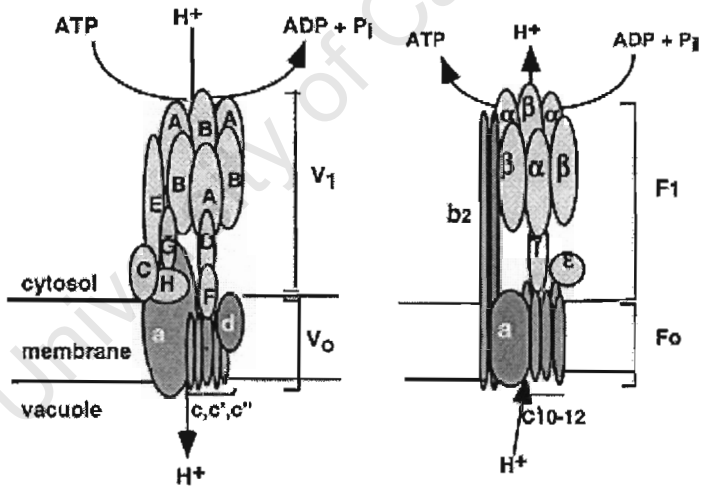

Figure 1.6 A comparison of structure and subunit composition between *S. cerevisiae* V-ATPase (left) and the *Escherichia coli* F-ATPase (right). V1 and F1 domains are shown in light grey, V0 and F0 domains are shown in dark grey. (Modified from Kane and Smardon 2003)

V-ATPases are structurally similar to F-ATPases (Figure 1.6) but they differ in function. F-ATPases are involved in ATP synthesis in mitochondria, chloroplast and bacteria. In both the case of V-ATPases and F-ATPases the peripheral and integral domains are attached by central and peripheral stalks.

Table 1.1 displays the subunit composition of the *S. cerevisiae* V-ATPase tabulating the domain localisation, subunit common name, the corresponding *S. cerevisiae* name and that subunit's function and/or location.

Table 1. 1 Subunit composition of the *S. cerevisiae* V-ATPase. (?) depicts unknown.

Domain	Subunit	Molecular weight (kDa)	Apparent stoichiometry	Yeast gene	F-ATPase homologue	Subunit function/location
V1	A	70	3	*VMA1*	β	Catalytic site, regulation
	B	60	3	*VMA2*	α	Non-catalytic site, targeting (?)
	C	40	1	*VMA5*	?	Peripheral stalk
	D	34	1	*VMA8*	γ	Central stalk
	E	33	2	*VMA4*	?	Peripheral stalk
	F	14	1	*VMA7*	ε	Central stalk
	G	13	2	*VMA10*	b	Peripheral stalk
	H	50	1	*VMA13*	?	Peripheral stalk
V0	a	100	1	*VPH1/ STV1*	a	H^+ transport, targeting
	d	38	1	*VMA6*	?	Cytoplasmic side of V0
	c	17	4	*VMA3*	c	H^+ transport, DCCD binding
	c`	17	1	*VMA11*	c	H^+ transport
	c``	21	1	*VMA16*	c	H^+ transport

1.6.2 The subunit c proteolipids

The V0 domain contains three different proteolipid subunits; subunit c (the product of the *VMA3* gene), subunit c` (the product of the *VMA11*gene) and subunit c`` (the product of the *VMA16* gene). Both subunit c and c` are approximately 17 kDa while the c`` proteolipid is 23 kDa (Mandel *et al*., 1988; Umemoto *et al*., 1990; Hirata *et al*., 1997). All of the proteolipid subunits are largely hydrophobic, are similar in sequence to one another and subunit c of the F-ATPase, and each contain four transmembrane domains. Subunit c`` was previously thought to have five transmembrane domains, but recent results that included the use of mutagenised cysteine residues and labelling by membrane permeant- and impermeant- maleimides, confirmed the presence of four transmembrane domains, with both the amino- and carboxy-termini on the cytosolic side of the membrane (Nishi *et al*., 2003). The F-ATPase subunit c is only 8 kDa in mass with two transmembrane helices arranged in a helical pin structure. This formed the basis of the theory that the V-ATPase subunit c evolved from the F-ATPase subunit c through gene duplication and fusion of the F-ATPase subunit c gene.

The subunit c proteolipids in the V0 domain arrange themselves in a proton translocating pore. The arrangement pattern has recently been elucidated via co-immunoprecipitation of epitope-tagged proteins, which demonstrated that c` and c`` both exist as single copies in V0, whereas subunit c is present in two or more copies (Powell *et al*., 2000). Recent data suggests c`` exists in two or more copies per V0 complex, but this could have been due to the over-expression of the vma16p.

Each of the subunit c proteolipids contains a vital proton-binding and translocating glutamate residue situated in a hydrophobic transmembrane domain. In subunits c and c` the glutamate is in the fourth transmembrane domain, whereas the c`` subunit has its glutamic acid residue in the second transmembrane domain. Mutagenesis of any of the glutamate residues completely inhibits proton translocation, and the glutamate residues of subunit c (and possibly subunit c`) are the site of covalent modification by the inhibitor dicyclohexylcarbodiimide (DCCD), where only one DCCD complex interacts with a subunit c residue (Arai *et al.*, 1987). Cross linking studies and labelling by a membrane-permeant carboxyl reagent have helped understand the arrangements of the subunit c and their orientation. It was determined that the fourth transmembrane domain containing the glutamate residue faces the hydrophobic phase of the membrane bi-layer, while the first transmembrane domain lines the centre of the proteolipid doughnut-like core. Subunit c has also been elucidated as the binding site contributor for the V-ATPase inhibitors bafilomycin and concanamycin, since mutations mapped to the subunit c gene conferred resistance to bafilomycin and concanamycin (Bowman and Bowman, 2002; Huss *et al.*, 2002).

1.6.3 Proposed V-ATPase rotary mechanism

The rotary mechanism of operation used by V-ATPases is thought to be similar to that of the F-ATPases since there is a great deal of structural similarity between the two enzymes. The proposed mechanism (Figure 1.7) follows the following sequence of events. ATP hydrolysis in V1 is proposed to drive the rotation of the central D and F subunits. Subunit a is held fixed relative to the A_3B_3 hexamer by a peripheral stalk

composed of C, E, G, H and the domain of a located in the cytoplasm. The peripheral stalk's complexity has hinted that it may act as the stator in the rotary catalysis, but may also control reversible dissociation of the complex during regulation of the V-ATPase activity. Subunit a residues Glu789 and His743 are projected to line the access channels leading to the c subunit carboxyl groups, whereas the Arg735 is proposed to function in releasing the proton into the luminal access channel. The V-ATPase inhibitor bafilomycin may prevent rotation of the proteolipid ring by disrupting the binding interface between the a and c subunits. Even though this hypothesis may hold ground since it is based on the known mechanism of F-ATPases, it still needs to be proved by direct experimental demonstration (Xu *et al.*, 1999; Arata *et al.*, 2002a, b).

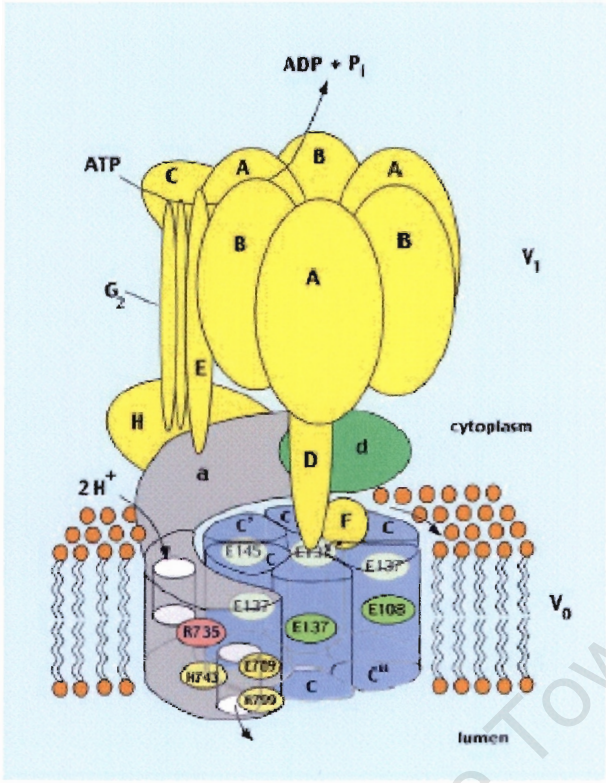

Figure 1.7 Proposed rotary mechanism of ATP-driven proton translocation by the V-ATPases. ATP hydrolysis in the V1 domain is proposed to drive rotation of the central stalk (composed of the D and F subunits), which in turn drives rotation of the ring of the proteolipid subunits (c, c`, c``) relative to subunit a in V0. Subunit a is held fixed relative to the A_3B_3 head of V1 by the peripheral stalk (or stator) composed of subunits C, E, G and H. Subunit a is shown forming two hemi-channels in communication with either the cytoplasmic or luminal side of the membrane. As each of the protonated carboxyl groups on the proteolipid subunits encounters the luminal channel, it is induced to lose a proton through interaction with Arg735 of subunit a. By contrast, these acidic residues must pick up a proton from the cytoplasmic channel before re-entering the hydrophobic phase of the bilayer. This model is based upon that originally proposed for F-ATPases. (Kawasaki-Nishi *et al.*, 2003).

1.6.4 Plant V-ATPases - similarities and differences to the *S. cerevisiae* V-ATPase

How similar would the structure of a plant V-ATPase complex be to that of *S. cerevisiae?* Figures 1.8 and 1.9 summarize the efforts by Domgall *et al.*, (2002) to demonstrate the detailed structure of the V-ATPase from the plant *Kalanchoë daigremontiana* (common name is Mother-of-Thousands) at a resolution of 2.2 nm. Not only did the authors manage to collect information about the three-dimensional structure of the V-ATPase, but they also noted the change in structure in the presence and absence of a non-hydrolyzable ATP analogue, AMP-PNP. AMP-PNP mimicked nucleotide conditions comparable to cellular conditions under normal growth and proved useful in ensuring a stable, active conformation of the V-ATPase from a structural point of view. By removing AMP-PNP, conditions of completely deprived nucleotide conditions were copied.

In the presence of the AMP-PNP analogue the details of the stalk region between V0 and V1 were revealed for the first time. The central stalk was surrounded by three peripheral stalks of different sizes and shapes. Interestingly, the three peripheral stalks were each found to have individual sizes and shapes and were located in different positions with respect to the AB-subunits. In the absence of AMP-PNP, the tilt of V0 changed relative to V1 and the stalk region was less clearly defined. This could have arisen due to an increased flexibility and partial detachment of some of the peripheral stalks. These structural changes corresponded to decreased stability of the complex and might be the initial step in a controlled disassembly.

Domgall *et al.*, (2002) concluded that based on their results, plant V-ATPases employ a rotary mechanism of functioning similar to that of F-ATPases based on the structural

similarity of the subunits. One has to make a distinction however between the differences presented between the V-ATPase from *K. daigremontiana* and *S. cerevisiae* such as the presence of three peripheral stalk structures in the former and one peripheral stalk in the latter. Differences of this nature highlight possible regulatory distinctions that very similar structures from diverse organisms may have.

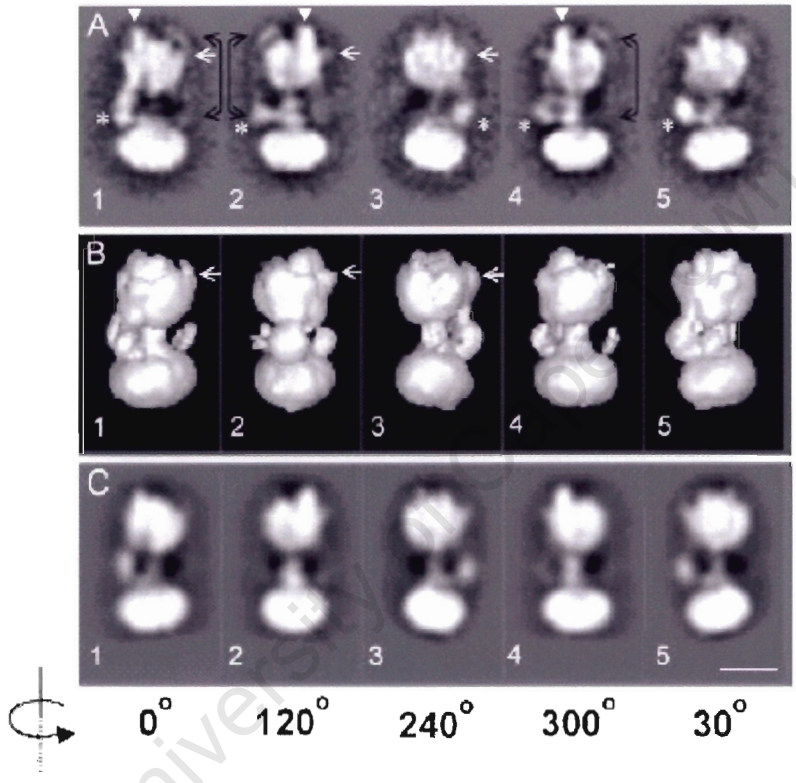

Figure 1.8 Two-dimensional projection maps and different representations of the three-dimensional map of the V-ATPase from *K. daigremontiana* with added AMP-PNP. (A) Selected class averages. (B) Surface representation of the final three-dimensional map viewed in the same directions as calculated for the projection directions of the class averages. (C) Projections of the three-dimensional map into the directions of the class averages shown in (A). The respective projection angles are given in the bottom panel. Labels: White arrowheads point toward the "spike", white arrows point toward the

"knobs"; asterisks depict the prominent peripheral stalk density, black arrows point toward a faint connection. Bar = 10 nm. (Adapted from Domgall *et al.*, (2002).

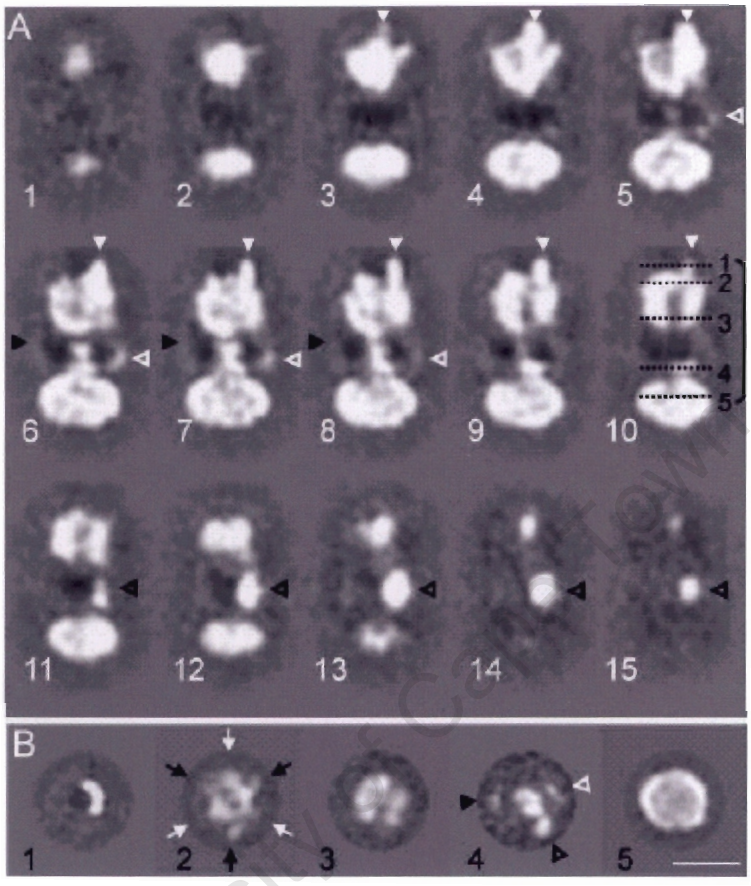

Figure 1.9 Slices through the three-dimensional map of the V-ATPase from *K. daigremontiana* with added AMP-PNP. (A) Slices of V-ATPase (0.8 nm thick) perpendicular to the supposed plane of the membrane. (B) Selected slices (0.4 nm thick) parallel to the supposed plane of the membrane at the positions indicated in (A) slice 10. Labels: Black arrows are A-subunits, white arrows are B subunits, white arrow heads point toward the "spike", black arrowheads point towards the faint peripheral stalks, open black arrowheads point toward the prominent stalk, open white arrowhead points toward the intermediate stalk. Bar = 10 nm. Adapted from Domgall *et al.*, (2002).

1.6.5 C- source dependent regulation of V-ATPases

The components of V-ATPase show a strong functional interdependence with one another. Without V1, V0 cannot translocate protons, and similarly without the V0 component, V1 cannot catalyse the hydrolysis of ATP. Disassembly of the components from one another is an efficient means of regulating V-ATPase activity without sacrificing ATP or compromising the PMF. It was observed that if *S. cerevisiae* shifts its primary carbon source preference from glucose to a less preferred sugar such as raffinose or galactose, V1 would rapidly dissociate from V0. Then as the glucose availability is restored, the complexes reassemble without requiring *de novo* synthesis of subunits (Finbow & Harrison, 1997).

The dissociation process is therefore fully reversible. Also reported is the observation that higher plants respond to salt stress by increasing their expression of V-ATPase subunits, and in particular the highest expression was of subunit c (the 16-kDa proteolipid). The promoter region of the gene encoding the catalytic subunit from carrot has been found to contain an ABA response element (Taiz, 1992). In addition, it has been shown that ABA increases V-ATPase gene expression in tobacco cells.

1.6.6 **PMF, V-ATPase and water deficit stress tolerance**

Previously, the proposed stoichiometry for proton translocation per ATP hydrolysis was 2:1 as determined by kinetic methods. The problem with this method is that it does not take into account unquantified proton recirculation and uncoupling of ATP hydrolysis and it is commonly restricted to a single pH at which scalar production or consumption of protons is minimised (Davies *et al.,* 1994). A patch-clamp configuration applied by Davies *et al.* (1994) however overcomes these limitations. Using the red beet V-ATPase as a model, it was determined thermodynamically that the ratio of protons transported per every ATP hydrolyzed ranges from 1.75 to 3.28 and was strictly dependent on the relative pH between the cytoplasm and vacuolar lumen.

The PMF generated by V-ATPases is essentially used as a reservoir of potential energy to drive the secondary transport of solutes into the vacuole lumen. The PMF generated by V-ATPases consist of a Δ pH of approximately 1.5-2.0 and a $\Delta\psi$ of +30mV relative to the cytosol. Antiporters (Figure 1.10) in particular have been known to use the pH gradient to drive the uptake of Ca^{2+}, Na^{+} and sugars in exchange for protons. The Na^{+}/H^{+} antiporter is present in high copy numbers in halophytic, salt-tolerant plants and glycophytes, such as beets, barley and corn. Mechanisms used to tolerate high salt stress in these plants include the accumulation of sodium in the vacuolar lumen and the pumping of salt out of the cell via secondary active transport by means of a Na^{+}/H^{+} antiporter on the plasma membrane.

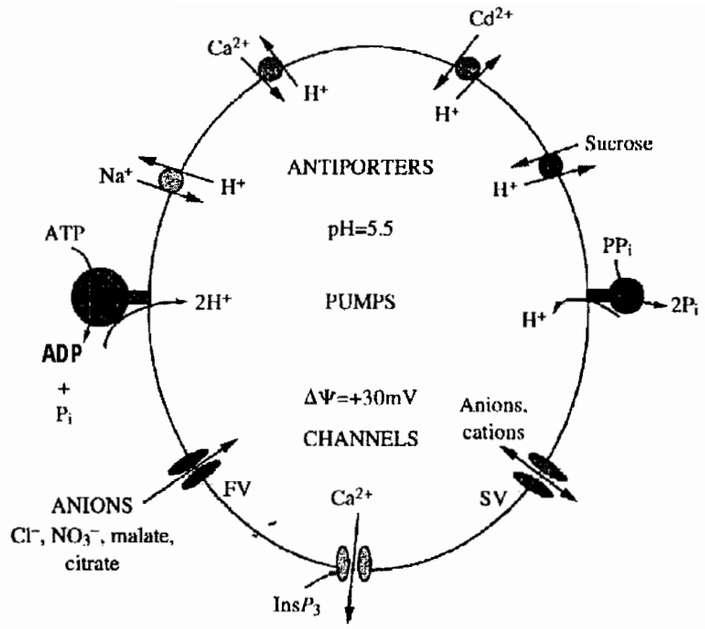

Figure 1.10 Transport systems on the tonoplast of plant cells. Two electrogenic pumps driven by ATP hydrolysis, V-ATPase and the pyrophosphatases (PPi) generate ΔpH and ΔΨ for secondary transport of other solutes into the vacuole. SV, slow vacuolar channel; FV, fast vacuolar channel; $InsP_3$, inositol triphosphate; Pi, inorganic phosphate. (Taiz, 1992).

Cellular accumulation of sugars in plant cells is accomplished by a combined use of a plasma membrane H^+/hexose symporter and tonoplast sucrose or hexose/H^+ antiporter. A tonoplast sucrose/H^+ antiporter may be the primary driving force for sugar accumulation in sugar beet sugar-storing cells, since uptake across the membrane seems to be largely passive.

When water deficit is imposed upon plants, water is passively extracted from the plant cell, concentrating the solutes within the cell, which could lead to hyperosmotic tension across the tonoplast. By increasing the expression of the subunits and/or stimulating the assembly of V-ATPase, the vacuolar lumen can be acidified which will increases the PMF across the tonoplast. Automatically, secondary transport will make use of the energised membrane to transport solutes into the vacuolar lumen until the elevated PMF is consumed and osmotic equilibrium is restored across the tonoplast.

1.7 Aims of this dissertation

This dissertation forms part of a larger research programme directed at understanding the mechanisms used by resurrection plants, and in particular *Xerophyta viscosa*, to tolerate abiotic stress.

Mundree and Farrant (2000) described the strategies of complementation by functional sufficiency and differential screening used to isolate genes that are differentially expressed in *X. viscosa* during dehydration. The strategy of complementation by functional sufficiency was adapted from Mundree (1996). Messenger RNA was extracted from dehydrated leaf tissue from *X. viscosa* ranging from 85 to 5% RWC. A cDNA library was created from the expressed mRNA and the resulting cDNAs were cloned into a Lambda ZAPII vector. Lambda ZAPII allowed for the directional cloning of cDNAs and the efficient rescue of phagemids from this vector using a helper phage-mediated *in vivo* excision.

The cDNA clones were inserted into pBluescript phagemid vectors. These were used to transform *Escherichia coli* (*srl*::Tn10) cells, and the transformed cells were grown on minimal media agar supplemented with varying concentrations of sorbitol. Nine cDNA clones were isolated from *X. viscosa* and each allowed *E. coli* (*srl*::Tn10) to grow in the presence of 1.25 M sorbitol (Mundree and Farrant, 2000).

One such cDNA clone, *XV5,* was 779 bp, with an open reading frame (ORF) of 753 bp and a predicted coding sequence of 251 amino acids. These findings led to a calculated *M*r of 25.3 kDa. A database search for the nucleotide and protein identities using the BLAST network service resulted in the identification of XV5 as a homologue of the 16-kDa proteolipid subunit of V-ATPase. At the time, XV5 showed a 50% identity to PPA1 (VMA16), a hydrophobic 23-kDa protein in *S. cerevisiae* that is highly homologous to the proteolipid subunit of yeast ATPase. The second highest homology was 45% identity at the amino acid level with HATPL, a hydrophobic ATPase-like protein from *Homo sapiens* (Mundree and Farrant, 2000). *XV5* will now be referred to as *XvVHA-c``1* to comply with international nomenclature standards for genes encoding subunits of the V-ATPase (Sze *et al.*, 2002)

The DNA sequence of *XvVHA-c``1* was subjected to a hydropathy plot using an 11 amino acid window, which demonstrated the presence of four transmembrane domains. Additionally, the four domains were highly conserved, and one of them contained a glutamate residue. Southern hybridisation analysis of the *X. viscosa* genome using *XvVHA-c``1* as a probe revealed three bands, suggesting the possible presence of a small

gene family. Northern hybridisation analysis was also conducted on hydrated and dehydrated tissue, showing that the *XvVHA-c``1* mRNA is present in both tissue types, but an increase in expression had occurred in dehydrated tissue (Mundree and Farrant, 2000).

The aim of this work was the further molecular characterisation of *XvVHA-c``1* in trying to elucidate its potential role in the *X. viscosa* stress response. This was achieved by completing the bioinformatics analyses on the *XvVHA-c``1* DNA sequence, repeating the Southern hybridisation analysis to ascertain the correct copy number of *XvVHA-c``1* in the *X. viscosa* genome, note the *XvVHA-c``1* transcript level response in *X. viscosa* when the plant is subjected to various abiotic stresses and complementation studies in *S. cerevisiae* to conclude XvVHA-c``1's functional role.

It is hoped that this work will ultimately in some way contribute to the development of a genetically enhanced crop variety that will be able to tolerate periods of drought and/ or improve the yield of that crop.

Chapter Two

Sequence and Southern hybridisation analyses of *XvVHA-c``1*

2.1 Summary

*XvVHA-c``1* full length cDNA was sequenced and results showed that the ORF consisted of 534 bp. The predicted protein contained 177 amino acids and its size was 19 580 Da. A BLAST search revealed that XvVHA-c``1 showed significantly high homology to the proteolipid c`` subunit of the V-ATPase complex. A hydrophobicity plot was conducted, revealing four potential transmembrane domains within the amino acid sequence. To further characterise XvVHA-c``1 analysis was performed using SOSUI software from the *Mitaku's Lab* web page. The results confirmed putative hydrophilic and hydrophobic domains within the XvVHA-c``1 sequence, providing insight into its secondary structure. Additionally, putative functional domains were identified which included potential phosphorylation sites and a conserved glutamic acid residue. It was therefore concluded that XvVHA-c``1 is a novel orthologue of a proteolipid subunit of the V-ATPases. Furthermore, Southern hybridization analysis was conducted on genomic DNA isolated form the *X. viscosa* genome. Using *Bam*H1, *Eco*R1, *Eco*RV, *Hind*III and *Xho*1 which do

not cut within the *XvVHA-c``1* sequence, it was hypothesised that *XvVHA-c``1* exists in the *X. viscosa* genome at a single locus.

2.2 Introduction

Mundree and Farrant (2000) sequenced the *XvVHA-c``1* cDNA and showed it had significant similarity to a proteolipid subunit of the vacuolar ATPase (V-ATPase) complex. The V-ATPase proteolipid subunit is indispensable to the functioning and assembly of the V-ATPase complex.

Proteolipid subunits exhibit common characteristics that include the presence of four hydrophobic transmembrane domains in the secondary protein structure and a highly conserved glutamic acid residue which is typically in the fourth transmembrane domain for subunits c and c`, but is present in the second transmembrane domain in subunit c`` proteolipids according to the *S. cerevisiae* model (Hirata *et al.*, 1997). The conserved glutamic acid is thought to bind the inhibitor DCCD and aids proton translocation (Finbow *et al.*, 1997).

During the *XvVHA-c``1* cloning procedure the possibility of contamination cannot be ignored. By confirming the presence of *XvVHA-c``1* within the *X. viscosa* genome this could be ruled out. It was also of interest to establish the gene copy number of *XvVHA-c``1*.

In this chapter, analyses are described that were used to identify the signature characteristics of a proteolipid subunit of the V-ATPase complex in the *XvVHA-c``1* sequence and Southern hybridisation results are described.

2.3 Materials and methods

Plant material

X. viscosa plants were collected from their natural habitat on Cathedral Peak in the Drakensberg mountain range (Kwazulu-Natal Province, South Africa). All plants were maintained in and grown under glass house conditions which include regular watering and fertilization, as described by Sherwin and Farrant (1996).

Sequence Analyses

All sequencing was conducted on a MegaBASE 500 (Molecular Dynamics, Amersham Pharmacia Biotech, UK), which uses an automated capillary DNA sequencing system. The sequencing kit used in the MegaBASE was the DYEnamic ET Dye terminator Cycle sequencing Kit for the MegaBASE and the sequencing reaction is based on the traditional dideoxynucleotide chain termination chemistry (Sanger *et al.*, 1977). All sequencing reactions were performed according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems, UK). The primers used to amplify *XvVHA-c``1* in the PCR reaction were the standard pBluescript SK reverse and forward primers, T3 and T7 respectively. The gel matrix used

was the LPA long-read. All resulting sequences were analysed using the MegaBASE 500 Sequence Analyser v2.0 software.

The amino acid sequences of related proteins were retrieved from the Internet after conducting a BLAST search of *XvVHA-c``1* cDNA against other genes within the NCBI database (http://www.ncbi.nih.nlm.gov).

The forward sequence of *XvVHA-c``1* was subjected to translation in three possible reading frames using DNAMAN software (v 4.13 Lynnon Biosoft © 1994- 1999). The multiple sequence alignment was conducted using DNAMAN software. In the multiple sequence alignment, homology was drawn between XvVHA-c``1 and similar sequences retrieved from the BLAST search.

The XvVHA-c``1 sequence was subjected to a hydrophobicity plot forecast using DNAMAN, which predicts possible hydrophobic regions within the amino acid sequence. The pI and total protein charge were also predicted from the amino acid sequence using DNAMAN software.

On-line software was used to predict the localisation of hydrophobic and hydrophilic domains of XvVHA-c``1 using *Mitaku's Lab* (http://sosui.proteome.bio.tuat.ac.jp/welcomeE.html). From this page, the SOSUI program (ver. 1.0 / 10, Mar., 1996) was elected. NetPhos software was used to predict potential phosphorylation sites in XvVHA-c``1 (Blom *et al.*, 1999).

Southern hybridisation

The protocol used to extract genomic DNA from *X. viscosa* was as described by Dellaporta *et al.*, (1983). Five to six leaves cut from a fully hydrated *X. viscosa* plant had all the woody tips removed and were washed briefly in sterile distilled water. The leaves were further cut into smaller pieces to facilitate grinding and these were then flash-frozen in liquid nitrogen. Quantitation of the genomic DNA was conducted spectrophotometrically using the relationship A_{260}=1 for 50 µg/ml of double stranded DNA. Restriction enzyme digestion was conducted on the extracted genomic DNA with the appropriate buffers and incubated at 37°C overnight. Digested genomic DNA was electrophoresed at 15V overnight on a 0.8% TBE gel and transferred its contents, according to the protocol (Coyne *et al.,* 2001), on to a nylon membrane (Hybond XL, Amersham Pharmacia Biotech). Five microlitres of [α-^{32}P] dCTP, which displayed a specific activity of approximately 3000 Ci/mmol, was used to PCR radiolabel *XvVHA-c``1*. The cycling conditions used were 95°C for 3 mins, denaturation at 94^{0}C for 1 min, annealing at 56°C for 1 min, elongation at 72°C for 10 mins and a final elongation at 72°C for 10 mins. The PCR cycle was repeated 15 times. The primers used in the amplification were T7 and T3. Radioactive hybridisation was followed according to (Coyne *et al.*, 2001) and the stringency conditions applied in the membrane washing steps were 10 min wash with wash buffer A (2xSSC, 0.5% SDS) at 65°C with shaking. The radiolabel-bound nylon membrane was exposed to X-ray film (Hyperfilm^{TM}, Amersham Pharmacia Biotech) at -70^{0}C for 5-10 days and thereafter developed manually.

2.4 Results

ORF and translation of *XvVHA-c``1*

Once the gene sequence of *XvVHA-c``1* (AY462241) had been translated, putative start and stop codons were identified within each of the three translated reading frames, and the resulting approximate protein size was determined by multiplying the number of amino acids by the average molecular weight of an amino acid (110 Da). Together with this information, the alignment analysis between each reading frame and a consensus of proposed homologues retrieved from the NCBI website; the most likely ORF was determined.

The sequence information from both forward and reverse sequencing events was used to rectify any potentially ambiguous bases that could have resulted from anomalous sequencing data. Sequence analysis and comparison with the result from the BLAST search revealed that the original cDNA contained a putative un-translated upstream region. As a result, the original cDNA was 779 bp in length, with an ORF of 753 bp. After the original cDNA was edited, the nucleotide sequence of *XvVHA-c``1* was 534 bp in length (Figure 2.1). When translated, XvVHA-c``1 encoded 177 amino acids, with a predicted size of 19 580 Da.

```
1       ATGATGGCTGATTCGAGCTCATGGGGACGAGCGCTTGTTCAGATCTCGCCGTACACTTTC
1        M  M  A  D  S  S  S  W  G  R  A  L  V  Q  I  S  P  Y  T  F

61      GCCGCCATCGGCATCGCAATCTCCATCGGCGTCTCTGTCCTCGGCGCCGCCTGGGGGATC
21       A  A  I  G  I  A  I  S  I  G  V  S  V  L  G  A  A  W  G  I

121     TTCATAACGGGTAGCAGTTTGATCGGTGCGGCGATCAAAGCGCCGAGAATCACTTCTAAG
41       F  I  T  G  S  S  L  I  G  A  A  I  K  A  P  R  I  T  S  K

181     AACCTCATCAGCGTCATCTTCTGTGAGGCAGTTGCTATATATGGAGTCATAGTTGCAATT
61       N  L  I  S  V  I  F  C  E  A  V  A  I  Y  G  V  I  V  A  I

241     ATCTTGCAAACGAAGTTAGAAAGTGTTCCAGCGGCACAGATTTACACCGCAGAGTCACTT
81       I  L  Q  T  K  L  E  S  V  P  A  A  Q  I  Y  T  A  E  S  L

301     AGAGCTGGTTATGCAATCTTTGCTTCTGGGATTATTGTGGGTTTTGCAAATCTTGTATGT
101      R  A  G  Y  A  I  F  A  S  G  I  I  V  G  F  A  N  L  V  C

361     GGGCTTTGTGTCGGAATAATCGGAAGCAGTTGCGCACTGTCAGATGCTCAAAATTCCTCC
121      G  L  C  V  G  I  I  G  S  S  C  A  L  S  D  A  Q  N  S  S

421     CTCTTTGTGAAGATTTTGGTAATTGAAATCTTTGGCAGCGCACTCGGTTTGTTTGGAGTT
141      L  F  V  K  I  L  V  I  E  I  F  G  S  A  L  G  L  F  G  V

481     ATCGTAGGAATCATTATGTCATCTCAAGCTACTTGGCCGGCAAGGGGAGCGTGA
161      I  V  G  I  I  M  S  S  Q  A  T  W  P  A  R  G  A  *
```

Figure 2.1 The DNA and corresponding amino acid sequence of *XvVHA-c``1*. The amino acid sequence consisted of 177 amino acids and the predicted protein has a molecular weight of 19 580 Da. The * represents a stop codon.

Multiple sequence alignment

The multiple sequence alignment revealed that the *XvVHA-c``1* amino acid sequence displayed high identity to other proteolipid subunit c`` proteins which included a putative H^+-transporting ATPase from *Oryza sativa* NM_192733.1 (90.96%), the V-ATPase subunit c`` proteolipid subunit from *Citrus limon* AY 226999.1 (87.91%), a putative vacuolar ATP synthase proteolipid subunit c`` from *Arabidopsis thaliana* At2g25610 (86.67%) and a subunit c`` H^+-transporting ATPase-like protein from *A. thaliana* At4g32530 (85.71%) (Figure 2.2)

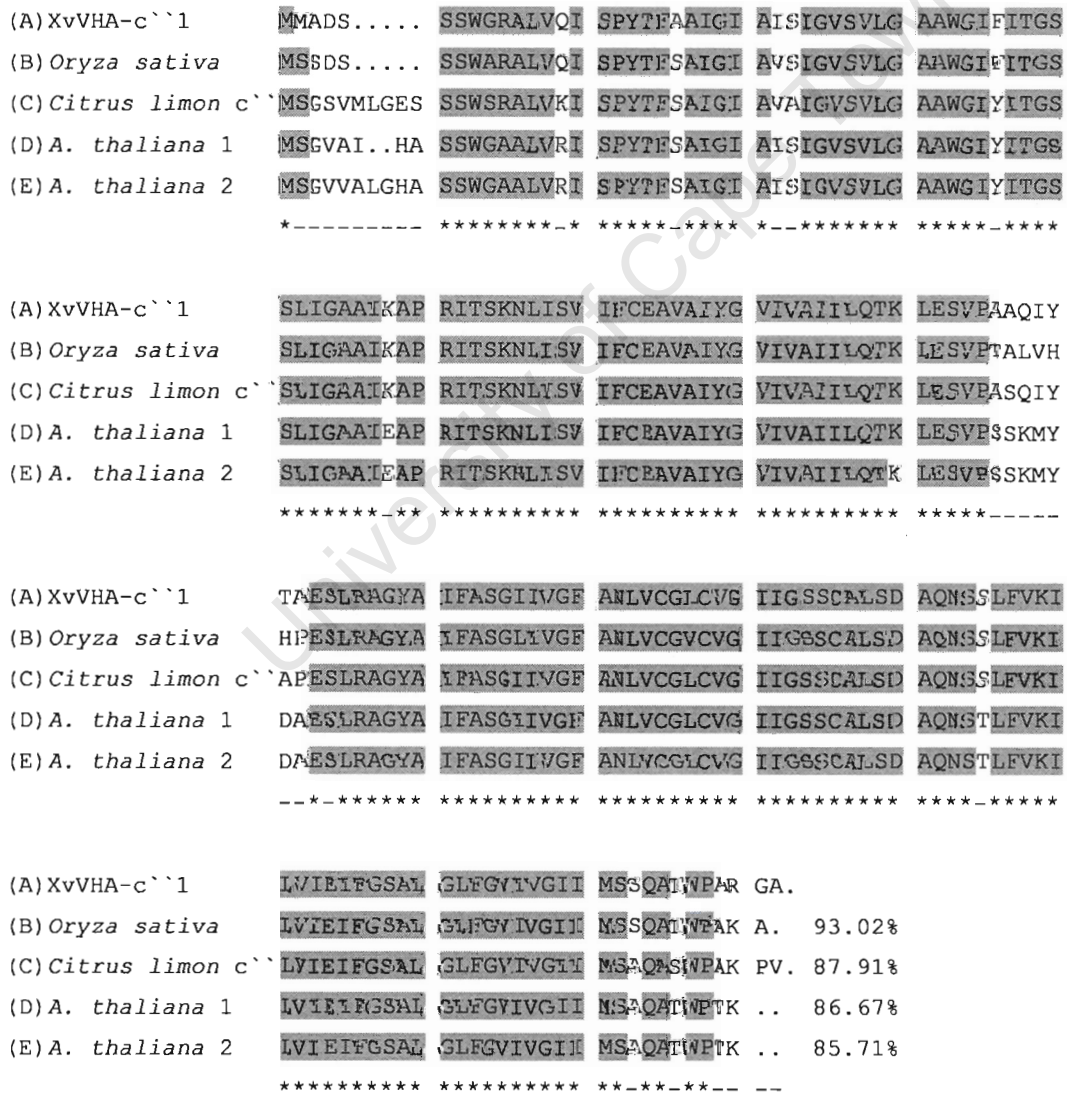

Figure 2.2. Multiple amino acid sequence alignment of XvVHA-c``1 with other subunit c`` proteolipids (A) XvVHA-c``1 from *X. viscosa* (AY462241) (B) *Oryza sativa* (NM_192733.1), (C) *Citrus limon* (AY 226999.1), (D) *A. thaliana* (At2g25610) and (E) *A. thaliana* (At4g32530). Identity is depicted with (*) and similarity is depicted with (-).

Hydrophobicity plot, pI and total protein charge prediction

Hydrophobicity analysis of XvVHA-c``1 showed that it has four putative hydrophobic domains interspersed with short hydrophilic domains (Figure 2.3). An eleven amino acid window was selected on DNAMAN, which informs the software to calculate the hydrophobicity profile of eleven amino acids at a time and then plot these values graphically.

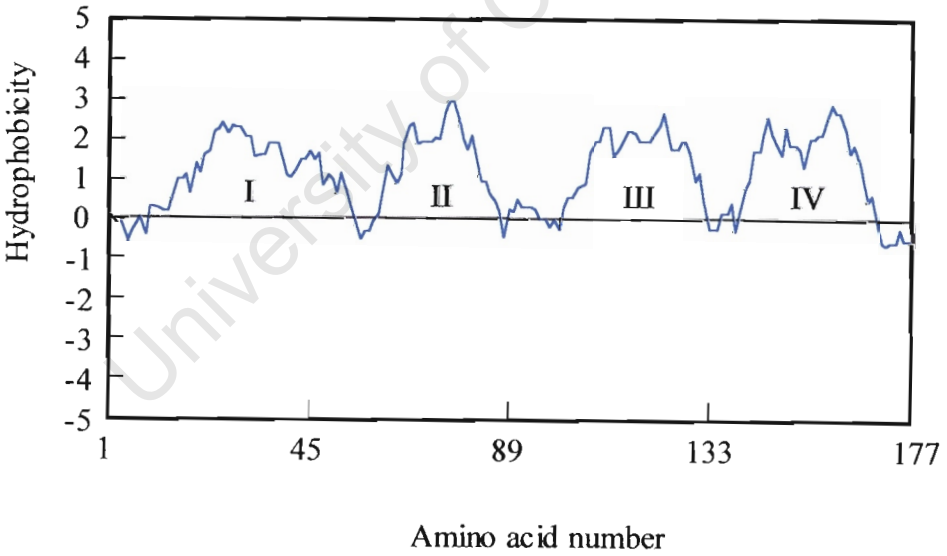

Figure 2.3 A hydrophobicity profile of XvVHA-c``1. Peaks above the meridian line display amino acid sequences that are potentially hydrophobic and peaks below the meridian line are amino acids that are potentially hydrophilic.

The predicted pI of XvVHA-c``1 is 8.23 in a buffer of pH 7. The total protein charge was predicted to be 1.82 and no disulfide bonds were predicted.

Protein domain localisation and putative functional domain identification

The SOSUI on-line programme is a tool for predicting the secondary structure of membrane proteins from an amino acid sequence. Prediction is based on the physiochemical properties of amino acid sequences such as hydrophobicity and charges. The system deals with three types of prediction: discrimination between soluble and membrane proteins, calculation of transmembrane helices and determination of transmembrane helical regions. The information from the *Mitaku Lab* website was compiled and displayed in Figure 2.4.

The accuracy of this programme for the discrimination of membrane proteins, determining the existence of transmembrane helices and transmembrane helical regions, were approximately 99%, 96% and 85%, respectively (Hirokawa *et al.*, 1998; Mitaku *et al.*, 1999; Mitaku *et al.*, 1999).

```
                                   •
(A)XvVHA-c``1        .....MMADS SSWGRALVQI SPYTFAAIGI AISIGVSVLG AAWGIFITGS
(B)Oryza sativa      .....MSSDS SSWARALVQI SPYTFSAIGI AVSIGVSVLG AAWGIFITGS
(C)Citrus limon c``MSGSVMLGES SSWSRALVKI SPYTFSAIGI AVAIGVSVLG AAWGIYITGS
(D)A. thaliana 1     MSGVAI..HA SSWGAALVRI SPYTFSAIGI AISIGVSVLG AAWGIYITGS
(E)A. thaliana 2     MSGVVALGHA SSWGAALVRI SPYTFSAIGI AISIGVSVLG AAWGIYITGS

                                   ★          ▼
(A)XvVHA-c``1        SLIGAAIKAP RITSKNLISV IFCEAVAIYG VIVAIILQTK LESVPAAQIY
(B)Oryza sativa      SLIGAAIKAP RITSKNLISV IFCEAVAIYG VIVAIILQTK LESVPTALVH
(C)Citrus limon c``SLIGAAIKAP RITSKNLISV IFCEAVAIYG VIVAIILQTK LESVPASQIY
(D)A. thaliana 1     SLIGAAIEAP RITSKNLISV IFCEAVAIYG VIVAIILQTK LESVPSSKMY
(E)A. thaliana 2     SLIGAAIEAP RITSKNLISV IFCEAVAIYG VIVAIILQTK LESVPSSKMY
```

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                    •
(A)XvVHA-c``1       TAESLRAGYA IFASGIIVGF ANLVCGLCVG IIGSSCALSD AQNSSLFVKI
(B)Oryza sativa     HPESLRAGYA IFASGLIVGF ANLVCGVCVG IIGSSCALSD AQNSSLFVKI
(C)Citrus limon c``APESLRAGYA IFASGIIVGF ANLVCGLCVG IIGSSCALSD AQNSSLFVKI
(D)A. thaliana 1    DAESLRAGYA IFASGIIVGF ANLVCGLCVG IIGSSCALSD AQNSTLFVKI
(E)A. thaliana 2    DAESLRAGYA IFASGIIVGF ANLVCGLCVG IIGSSCALSD AQNSTLFVKI

                    ▼
(A)XvVHA-c``1       LVIEIFGSAL GLFGVIVGII MSSQATWPAR GA
(B)Oryza sativa     LVIEIFGSAL GLFGVIVGII MSSQATWPAK A.   93.02%
(C)Citrus limon c``LVIEIFGSAL GLFGVIVGII MSAQASWPAK PV   87.91%
(D)A. thaliana 1    LVIEIFGSAL GLFGVIVGII MSAQATWPTK ..   86.67%
(E)A. thaliana 2    LVIEIFGSAL GLFGVIVGII MSAQATWPTK ..   85.71%
```

Figure 2.4 Multiple amino acid sequence alignment of XvVHA-c``1 with other subunit c`` proteolipid; with emphasis on novel domains in XvVHA-c``1. (A) XvVHA-c``1 from *X. viscosa* (AY462241) (B) *Oryza sativa* (NM_192733.1), (C) *Citrus limon* (AY 226999.1), (D) *A. thaliana* (At2g25610) and (E) *A. thaliana* (At4g32530). Bioinformatics software was used to identify hydrophobic regions in the XvVHA-c``1 sequence (shaded sequences) as well as the presence of the glutamate in the fourth and second hydrophobic region (▼). NetPhos software predicted potential serine (•) and threonine (★) phosphorylation sites.

NetPhos online software was used to predict which amino acids are most likely to be phophorylated. Three potential phosphorylation sites were identified. Serine (7), threonine (58) and serine (99) displayed potential phosphorylation scores of 0.982, 0.953 and 0.972, respectively (Figure 2.4).

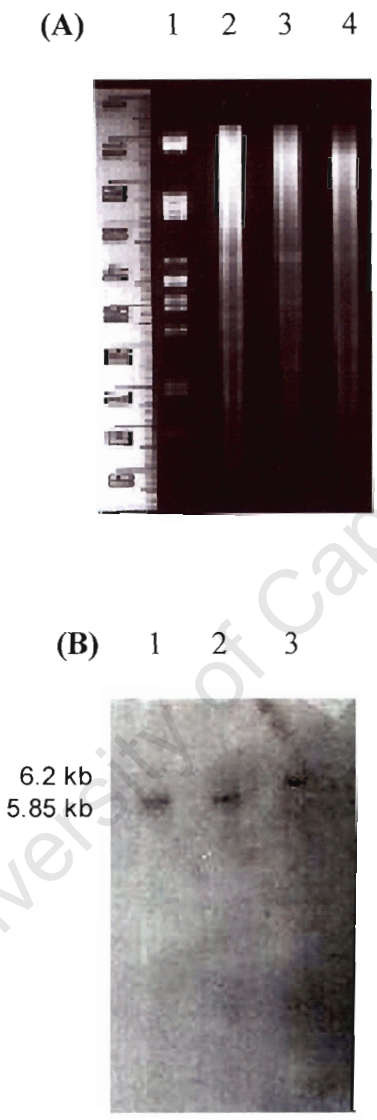


Figure 2.5 **(A)** Restriction digest of *X. viscosa* genomic DNA electrophoresed on a 0.8% Agarose gel. Its contents were transferred to a nylon membrane as part of a Southern hybridisation assay. In lane 1 Lambda *Pst*1 molecular weight markers were loaded. The next three lanes show restriction digested *X. viscosa* genomic DNA with *Eco*R1 (lane 2);

*Eco*RV (lane 3) and *Bam*H1 (lane 4). **(B)** Southern hybridisation analysis of restriction digested *X. viscosa* genomic DNA, probed with α^{32}P dCTP radioactively labelled *XvVHA-c``1*. Lane 1, *X. viscosa* genomic DNA digested with *EcoR*1; lane 2, *EcoR*V and lane 3, *Hind*III. Predicted sizes of DNA marker ladder are displayed on the left-hand side in kilo base pairs (kbp).

Southern hybridisation results

The Southern hybridisation result demonstrates that *XvVHA-c``1* is present on the *X. viscosa* genome and that it exists at a single locus (Figure 2.5 **(B)**). The high stringency implemented while washing off the excess transcript when the nylon membrane was probed, assisted in establishing a more exact result. The presence of single bands in each lane in Figure 2.5 proves that the single locus result was reproducible.

2.5 Discussion

The resultant information represented here, which include the identity of XvVHA-c``1 in relation to similar proteins, the localisation of the four transmembrane domains in the XvVHA-c``1 sequence, identification of potential glutamate residues that help bind protons for their translocation and the introduction of putative phosphorylation sites, is proof of the novelty of XvVHA-c``1. No other publication has highlighted or identified potential phosphorylation domains or residues on any other subunit c and c`` proteolipid. We can therefore only hypothesise their function in the regulation of XvVHA-c``1 by using our current understanding of these putative domains.

The first functional domain identified from the N-terminus, and later repeated toward the C-terminus, were putative serine phosphorylation residues, which were suggested to be phosphorylated by protein kinase C (PKC) (Figure 2.4 •). PKC's are found in animal cells and are involved in intracellular signalling, transducing the cellular signals that promote lipid hydrolysis. This 80-kDa enzyme is targeted to the plasma membrane by diacylglycerol and in many cases, by calcium which is bound by a Ca^{2+}-binding C2 domain rather than the EF-hand motif common among plant Ca^{2+}-binding proteins such as Calcium-dependent protein kinases (CDPK) and SNK-1 related kinases (SnRK) (Harmon, 2003). PKC phosphorylates a variety of target proteins which control growth and cellular differentiation (http://cti.itc.virginia.edu/~cmg/Demo/pdb/pkc/pkc.html). Plant PKC-type proteins, or derivatives that recognise serine phosphorylation sites, could potentially phosphorylate XvVHA-c``1 to regulate the V-ATPase activity and its function in cell growth and differentiation. Hypothesised consequences of this potential phosphorylation include increasing the association rate of the multimeric proton translocating proteins, which will in return promote the association of the *X. viscosa* V-ATPase holoenzyme. Phosphorylation may induce the rate of proton translocation into the vacuolar lumen. The significance of the first putative PKC phosphorylation site being located on the hydrophilic amino acid region is that it is localised within the vacuolar lumen. It suggests that if phosphorylated and therefore activated, then the source of regulation of XvVHA-c``1 activity originates from the vacuolar lumen. The second possible PKC phosphorylation site was predicted to be localised on the second cytoplasmic localised hydrophilic domain (SOSUI prediction). This leads to the

assumption that, as opposed to the first PKC phosphorylation site, the second is phosphorylated by PKC from the cytoplasm.

A probable threonine phosphorylation site was localised to the first hydrophilic region exposed to the cytoplasm (Figure 2.4 ★). The phosphorylation of this potential site may add to the regulation of XvVHA-c``1 activity in conjunction with or as the sole regulatory element of the proteolipid subunit.

Additional functional studies, such as generating deletions within these sites, have to be conducted to test which PKC phosphorylation site or threonine is active. This will negate or confirm the hypothesis that XvVHA-c``1 may be phosphorylated and that the energised protein could change the efficiency of V-ATPase activity.

The first glutamic acid residue which is implied to be integral to proton translocation, was identified in the second hydrophobic transmembrane domain and the second glutamic acid residue thought to have had significance in proton binding and translocation was identified in the fourth transmembrane domain (both depicted in Figure 2.4 ▼). The presence of this residue confirms the identity of XvVHA-c``1 as a V-ATPase proteolipid subunit according to the findings and characterisation in *S. cerevisiae* of the Vma16p protein. To date, no other information has been published concerning the

similar sequences presented in Figure 2.4, and hence the importance or significance of either the second or fourth glutamic acid residue has not been established.

Besides the four transmembrane domains which are common to all subunit c, c` and c`` proteolipids, an additional domain was uncovered in the *S. cerevisiae* subunit c`` proteolipid (vma16p) on its amino terminus (Nishi *et al.*, 2003). It is thought that the new domain is localised in the cytoplasm. The presence of this additional domain has no role in the assembly and functioning of the V-ATPase complex, as proven by deletion studies (Nishi *et al.*, 2003).

This Southern blot analysis confirms the presence of *XvVHA-c``1* on the *X. viscosa* genome at a single locus as a result of applying higher stringency washes compared to those used by Mundree and Farrant (2000). Mundree and Farrant (2000) showed that *XvVHA-c``1* (referred by them as XV5) exists as a small gene family on the *X. viscosa* genome. What they most likely had shown was either the presence of multiple copies of *XvVHA-c``1* isogenes, the presence of all the proteolipid subunit homologues on the genome or both.

Evidence of the existence of multigene families of the subunit c proteolipid is common among a few species of plants analysed (Sze *et al.*, 1992, Averzier-Hagai *et al.*, 2000). Four subunit c proteolipid isogenes have been analysed on the oat genome, where the isogenes have demonstrated 97-99% identity at the amino acid level. Southern

hybridisation of this genome has identified as many as seven genes that could comprise this multi-gene family (Lai *et al.*, 1991). Similarly, a subunit c proteolipid has been identified in *A. thaliana*, by initially using the oat gene cDNA as a probe where two *A. thaliana* cDNA were isolated, AVA-P1 and AVA-P2 (Lai *et al.*, 1991). Later when AVA-P1 was used as a probe three to four fragments hybridised, identifying another small gene family in *A. thaliana*.

A small gene family of four subunit c isoforms exist in the *Avena sativa* (common oat) genome. Two-gene families have been localised in the *Gossypium hirsutum*, *M. crystallinum*, *Zea mays* and *Citrus limon* genomes (Averzier-Hagai *et al.*, 2000)

In order to verify the exact copy number of *XvVHA-c``1* in the *X. viscosa* genome, a genomic reconstruction would have to be done that includes sequencing. Also, deletion studies have to be conducted on the XvVHA-c``1 sequences, to verify the role of the proposed domains identified.

Chapter Three

Northern hybridisation analysis

3.1 Summary

Northern hybridisation analysis was conducted on total RNA isolated from *X. viscosa* plants subjected to stress treatments that included a dehydration stress, salinity stress and a -20^{0}C shock. Each RNA- bound membrane was probed with radiolabelled *XvVHA-c``1*. An increase in *XvVHA-c``1* RNA transcript was seen in response to salinity, dehydration and severe cold shock stresses at sampling times specific to each of the stresses. The results were conclusive of the stress responsive expression of *XvVHA-c``1* and hints at a potential role for this gene in *X. viscosa*'s response to the stresses.

3.2 Introduction

In order to determine the role of a gene and its protein within a cell, and possibly tissues, expression analysis of these important molecular messages is necessary. To molecular biologists there are at least two levels at which one can quantitate the expression profile, the messenger RNA and protein. The cell is capable of using finer control mechanisms in the regulation of these molecules *viz.* post-transcriptional modification (e.g. RNA interference and hairpin-loop structure on the mRNA strand), post-translational

modification of the primary protein structure and sequestration of the protein product, to name a few. Nevertheless, the presence of a transcript, or protein for that matter, may hint at some understanding of the potential stimuli that affect a specific gene's expression.

A more accurate means of determining the expression of genes in response to stress is by means of extracting the polysomal mRNA, as this class of RNA is destined for translation. Nevertheless, the detection of mRNA transcript in a total RNA extraction is significant, especially when observing an increase in transcript where there was none before. The northern hybridisation analysis data should be followed by a western blot analysis to understand the full nature of the expression response of a gene.

Drought, salinity and extreme temperatures are environmental features that adversely affect plant growth and crop productivity. They also have a common effect by imposing a water deficit stress in plants. Drought and salinity stress both inflict hyperosmotic stress which can decrease the chemical activity of water and cause loss of cell turgor. The effects of salinity stress in particular could result in the accumulation of cytotoxic levels of Na^+ and Cl^- and ultimately ion disequilibrium (Jones and Pollard 1983, Serrano 1996, Serrano *et al.,* 1999). In the case of low temperature stress below 0^0C, cellular water changes conformation into a glass-like solid state which may disrupt internal-membranes. This poses a great threat to the cell, as the glass-like state of water could rupture membranes and lead to the disintegration of the cell.

This chapter describes the expression profile of the subunit c`` proteolipid homologue, *XvVHA-c``1*, in *X. viscosa* with an intention to understand its role within the cell. Because it is thought that *XvVHA-c``1* is an integral V0 domain subunit, it was intended to assess its expression in response to stresses that could affect the osmotic potential across membranes within the cell.

3.3 Materials and methods

Plant material

X. viscosa plants were collected from their natural habitat on Cathedral Peak in the Drakensberg mountain range (Kwazulu-Natal Province, South Africa). All plants were maintained in and grown under glass house conditions as described by Sherwin and Farrant (1996).

Plant stress treatments

All stress treatments were conducted in a phytotron at a constant temperature of 25°C, 50% relative humidity, a photon flux density (PFD) of 300 µmol $m^{-2}s^{-1}$ and a day/night cycle 16/8h, unless otherwise stated. Plants were moved from glasshouse conditions to the phytotron, where they were equilibrated for a week. Whole plants were subjected to dehydration stress, 150 mM NaCl solution application and low temperature shock stress. At regular intervals after initiation of the stress, harvested plant tissue was placed into foil pockets, flash frozen and stored at -70°C until RNA extraction. Unless otherwise indicated, leaves were randomly sampled. The dehydration stress entailed withholding

water from a whole plant for over a 30d period. Leaves were sampled at various RWC during drying, the control sample being hydrated leaves before the dehydration stress commenced. Salinity stress was imposed by watering the soil with 150 mM NaCl solution at the beginning of the stress. The *X. viscosa* plants were potted in a similar volume of soil, whose composition had a sandy soil:vermiculite:loam soil ratio of 1:1:1, respectively. Samples were collected every 24 h for 4 days. The extreme temperature shock was conducted by moving a *X. viscosa* plant from ambient temperature to -20°C, and collecting leaf material at half-hourly intervals for 2 h. The leaves of the -20°C stress were frozen after the first 60 mins.

RNA extraction and northern hybridisation

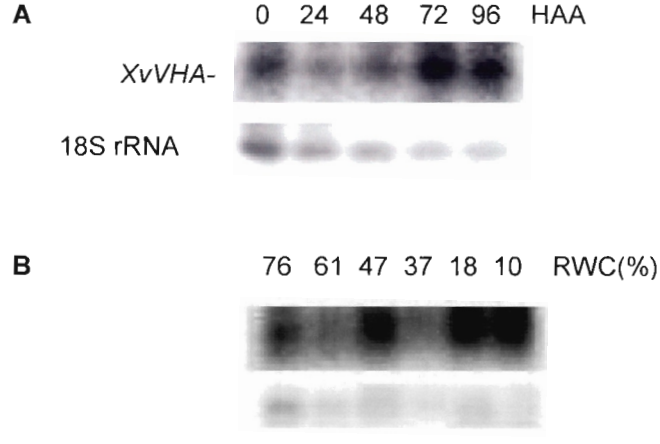
Total RNA was extracted from *X. viscosa* tissue using TRIzol LS Reagent (Life Technologies, GibcoBRL) according to the manufacturer's instructions. Plant tissue was flash frozen using liquid N_2 and ground in a sterile mortar and pestle. Extracted RNA was quantified spectrophotometrically using the relationship $A_{260} = 1$ for 40 µg/ml of single stranded RNA. Approximately 10 µg of RNA was loaded equally in 1.2% agarose gels. RNA sample loading buffer (10xMOPS, 3.1 µl formaldehyde, 10 µl formamide and 2 µl sample buffer) was added to each RNA sample then the mixture was boiled for 5 min. All reagents were prepared using RNAse-sterile techniques, which included the use of 0.01% diethyl pyrocarbonate (DEPC) and ethanol rinses of equipment where possible. Electrophoresed RNA was transferred onto nylon membranes (Hybond XL, Amersham Pharmacia Biotech) by capillary transfer. The RNA-bound membranes were probed with [α-^{32}P]dCTP labelled *XvVHA-c``1* using Megaprime DNA Labelling System (Amersham

Biosciences) to the manufacturer's specifications, except that 100 ng of probe was labelled in the initial step. Thereafter, membranes were re-probed with the *X. viscosa* 18S gene that was radiolabelled using the Megaprime DNA Labelling System (Amersham Biosciences) to the manufacturer's instructions. In both cases, hybridisation was conducted at 65^0C in hybridisation buffer. Excess un-bound probe was washed off using wash buffer A (2 x SSC, 0.5% SDS) and wash buffer B (0.5 x SSC, 0.1% SDS). Membranes were exposed to storage phosphor screen (Kodak) and scanned using the BioRad Personal Molecular Imager Fx.

3.4 Results

XvVHA-c``1 expression profile

XvVHA-c``1's expression in *X. viscosa* in response to dehydration, 150 mM NaCl application and -20^0C treatments was ascertained by northern analysis (Figure 3.1). An increase in the transcript was evident 72 and 96 hours after application (HAA) of 150 mM NaCl stress (Figure 3.1 A). When the dehydration stress was implemented on *X. viscosa* an increase in the transcript was also noted at 18% and 10% RWC, with a transient increase in transcript at 47% RWC (Figure 3.1 B). Similarly, mRNA levels had increased significantly after 60 min exposure to -20^0C (Figure 3.1 C). The loading control used for all treatments was the native *X. viscosa* 18S rRNA gene.



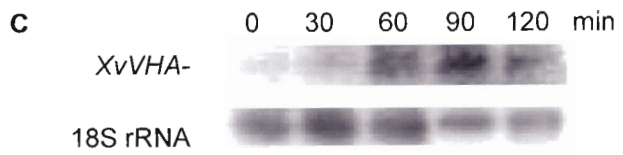


Figure 3.1 Northern hybridization analysis of *XvVHA-c``1* expression by *X. viscosa* in response to stress. **A** 150 mM NaCl; **B** dehydration and **C** low temperature shock (-20$^0$C). *XvVHA-c``1* indicates that radiolabelled *XvVHA-c``1* cDNA was used to probe membrane. 18S indicates where radiolabelled *X. viscosa* 18S rRNA was used as a constitutively expressed loading control. These results have shown to be reproducible.

3.5 Discussion

To date information on the expression patterns of plant subunit c`` homologues in response to stress has not been published. Most of the published work deals with the expression of subunit c proteolipids, and their relevance in the V-ATPase stress response. This is understandable since the role and copy number of the c`` subunit in V-ATPase has only recently been established. It is only therefore possible to relate the gene regulation response of the c`` to the c proteolipid subunit, and assume that their gene expression to the stresses conducted is comparable.

It was evident from the results that basal amounts of mRNA transcript were present at time 0 in the case of the salinity and extreme temperature shock treatments, or hydrated tissue in the case of the dehydration treatment. The significance of this observation highlights XvVHA-c``1's potential role as a house-keeping protein in the functioning of

X. viscosa's V-ATPase. This observation also substantiates the fact that subunit c`` proteolipids are known to exist as part of the V0 domain, meaning that *X. viscosa* is no exception to the rule.

It is a common response in plants to increase steady state transcript levels of the subunit c proteolipid subunit in response to salinity stress and this is true too in *X. viscosa* (Figure 3.1 A). In the halophyte *Mesembryanthemum crystallinum* (common ice plant) it was shown that the activity of V-ATPase increases when the plant was treated with NaCl (Ratajczak *et al.*, 1994; Tsiantis *et al.*, 1996). Subunit c transcripts were found to be upregulated in leaves and roots of 6-week-old *M. crystallinum* treated with 350 mM NaCl (*Tsiantis et al.*, 1996). In a whole plant and in a cell suspension culture of *Beta vulgaris*, subunit c transcripts were upregulated in response to NaCl stress (Kirsch *et al.*, 1996; Lehr *et al.*, 1999). Subunit c homologue Vac1 transcripts in the moss *Tortula ruralis* increased in the polysomal RNA fraction, but the VAC1 protein levels remained steady (Chen *et al.*, 2002).

Not only would salinity stress have an impact on the osmotic potential across internal membranes, but it may also have an effect on the vacuolar size. The vacuolar volume has been recorded to increase drastically in the mangrove plant *Brugruiera sexangula* and in *Hordeum vulgare* root meristematic cells under salt stress. In both cases an increase in V-ATPase activity was also measured. *Pisum sativum* however, did not display an increase in vacuolar size in response to salinity stress (Mimura *et al.*, 2003).

Little is known about proteolipid subunit c homologue expression in relation to water deficit stress. In all of the cases presented above, subunit c proteolipid's role in salinity tolerance was established as a key contributor in the regulation of the secondary transport system. Here we show increased *XvVHA-c``1* mRNA levels (Figure 3.1B) when *X. viscosa* is subjected to dehydration stress. Water deficit and salinity stresses have common effects, *viz.* the decrease in cell turgor. It is therefore not surprising that *XvVHA-c``1* steady state mRNA levels increase in *X. viscosa* in response to dehydration. Subunit c homologues' expression, and V-ATPase activity in response to dehydration in other plant forms have not been characterized as well as it has been in response to salinity stress. Because *XvVHA-c``1* cDNA was isolated from dehydrated *X. viscosa* and an increase in transcript levels was seen in the plant at low RWC (Figure 3.1 B), we hypothesize that the *X. viscosa* V-ATPase plays a role in its stress tolerance given the functional importance subunit c proteolipids have for V-ATPases.

Yoshida *et al.* (1999) showed that there are three factors that affect V-ATPase activity during low temperature stress: (1) chilling injury appears to inhibit V-ATPase activity; (2) as a result the formation of pH gradients is inhibited and probably the compartmentation of solutes is disrupted and (3) the fluidity of membranes has to be adjusted by an increase of unsaturated fatty acids in the membranes. These effects are probably manifested in *X. viscosa* in response to low temperature stress, given its natural environment where extremes in temperature are very common in winter. When a low temperature shock was conducted on *X. viscosa*, it was intended to quantitate the amount of *XvVHA-c``1* steady-state mRNA when the plant's membrane stability is compromised.

The results presented here suggest that increasing *XvVHA-c``1* levels could be a critical mechanism to counteract the effects of low temperature stress by increasing internal membrane stability upon the expression of its protein. However, further experiments need to be conducted to ascertain the degree of membrane damage incurred upon extreme temperature stress at -20^{0}C by measuring leakage and possibly visualising tissue cross sections under an electron microscope. Together with this, cellular localisation studies need to be conducted to show the predicted accumulation of XvVHA-c``1 within internal membrane systems.

Judging by these results, *XvVHA-c``1*'s expression is responsive to osmotic-related stresses. In order to ascertain its expression response more accurately, northern hybridisation analysis could be conducted on the polysomal rRNA on *X. viscosa* leaf material subjected to the same stresses. In addition to this, western hybridisation needs to be conducted on proteins isolated from *X. viscosa* that was subjected to stresses.

Chapter Four

Yeast complementation

4.1 Summary

A *Saccharomyces cerevisiae* strain was received with a knockout mutation in the *VMA3* gene. This strain was transformed via electroporation with the pYES2 shuttle expression vector into which *XvVHA-c``1* had been cloned. The *VMA3* knockout mutant displays a phenotype sensitive to 100 mM $CaCl_2$. The transformed strains were subsequently subjected to 100 mM $CaCl_2$ stress in order to demonstrate functional complementation. The *XvVHA-c``1* transformed strain was capable of growing on the 100 mM $CaCl_2$ stress media, unlike the pYES2 transformed and untransformed mutants. The results were reproducible and demonstrated that *XvVHA-c``1* could complement *VMA3*'s role in the V-ATPase.

4.2 Introduction

S. cerevisiae is an excellent model organism for molecular studies. It is easy to culture, its genome has been sequenced and readily available on internet sites and it possesses a V-ATPase whose subunits' structures, roles and inter-subunit interactions have been well characterised (Nishi and Forgac, 2002). Access to the genomic *S. cerevisiae* genomic sequence has allowed scientists to map the position of the genes whose proteins make up

the subunits of the V-ATPase. Knockout mutants of *S. cerevisiae* are also readily available on request and easily generated by those who know how. It was therefore on these grounds that a complementation study using a *S. cerevisiae VMA3* knockout mutant, which is viable and readily manipulated, was a feasible assay.

As mentioned in the general introduction, *VMA3* encodes the subunit c proteolipid subunit, which is integral to the functioning and assembly of the entire V-ATPase complex (Nuomi *et al.*, 1991). It contributes four subunits to the membrane anchored V0 domain, forming the proton translocating pore together with one copy each of subunits c` (*VMA11*) and c`` (*VMA16*) proteolipids (Arata *et al.*, 2002b). The reason for electing subunit c and not c`` for the functional complementation, is because it has been the subject of scientist investigation into the role and structure in the V-ATPase complex. Additionally, reports have been noted on the increase of subunit c homologue transcript levels in different plant systems in response to stress (Ratajczak *et al.*, 1994; Tsiantis *et al.*, 1996; Kirsch *et al.*, 1996; Lehr *et al.*, 1999; Chen *et al.*, 2002). Recent publications have highlighted subunit c` and c`` roles in the V0 domain, which were previously thought of as secondary proteolipids to the subunit c proteolipid.

The aim of this chapter is to present the results obtained when *XvVHA1* was transformed on a shuttle vector into S. *cerevisiae VMA3* knockout and complementation studies were conducted on the transformants.

4.3 Materials and methods

S. cerevisiae strains, vectors and media

All strains and vectors were received as a gift from Nathan Nelson (Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University). The *S. cerevisiae* strains received were wild-type W303 (MATaα *trp1 ade2 leu2 his3 ura3*) and Δ*VMA3* (*MATaα trp1 ade2 his3 ura3 VMA3::LEU2*). Strains were cultured in YPD medium (1% yeast extract (Difco), 2% Bactopeptone (Difco), 2% dextrose and, where applicable, 2% Bactoagar (Difco) for solid media). The stress media used post-transformation in the complementation assay was YPD containing 100 mM $CaCl_2$ (Ohya *et al.*, 1991).

Cloning *XvVHA-c``1*

*XvVHA-c``1* was directionally cloned into pYES2 (Invitrogen, Figure 4.1) plasmid from XvVHA-c``1::pGEM®-T Easy (Promega) using the *EcoR*1 and *Xho*1 restriction sites. The selection of successfully subcloned plasmids was verified via sequencing (by using the T7 primer) and restriction enzyme digestion using *Eco*R1 and *Xho*1 to release the insert and visualising the result on a 1% agarose gel.

S. cerevisiae transformation and selection

The *S. cerevisiae* strains were transformed using a modified electroporation protocol from Ausubel *et al.* (1995) where the only difference was substituting DTT with twice the volume of 2-mercaptoethanol. Successfully transformed cells were selected on URA$^-$

minimal media (0.67% yeast nitrogen base, 2% dextrose, 2% agar and 0.76% CSM-URA (Bio 101 Inc.)).

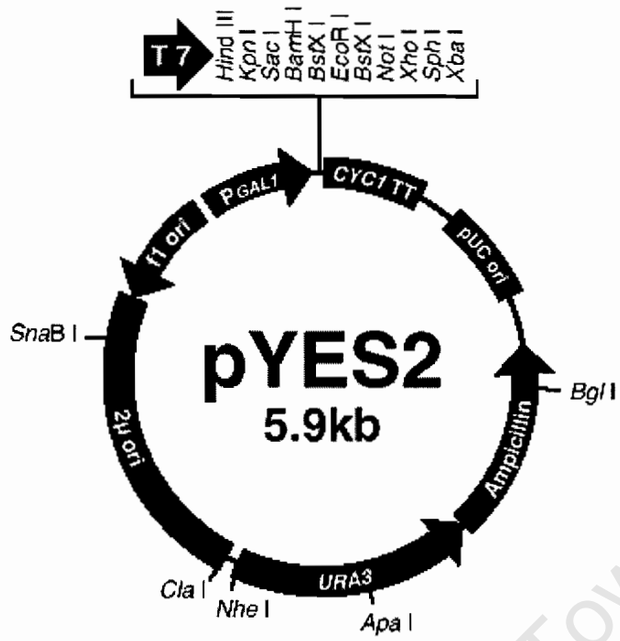


Figure 4.1 Vector map of pYES2. *XvVHA-c``1* was cloned between the *Eco*R1 and *Xho*1 restriction sites and the T7 primer binding site was used to sequence potential cloned vectors.

4.4 Results

The viability of the relative strains used in the complementation assay was assessed by growth on YPD media (Figure 4.2 A). It was evident by the amount of growth seen that both of the transformed strains as well as the untransformed control were viable. Their stress sensitivity was tested by exposing them to 100 mM $CaCl_2$ (Figure 4.2 B).

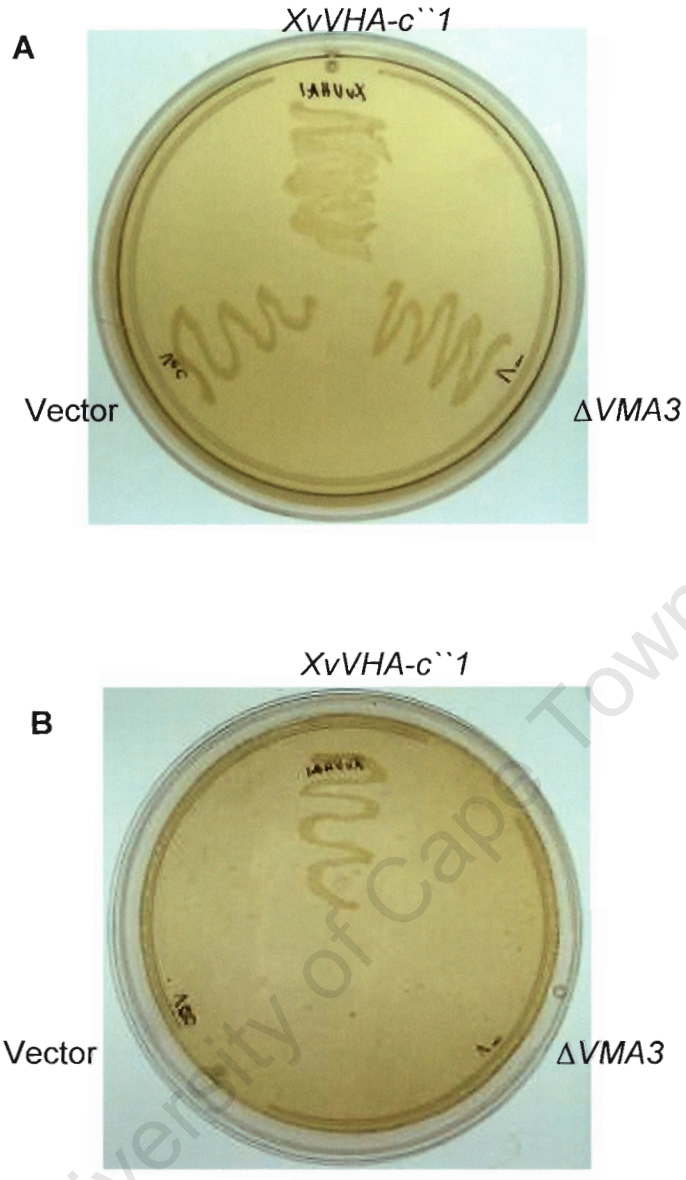


Figure 4.2 Photographs capturing the complementation studies involving *S. cerevisiae VMA3* knockouts. Strains were cultured on **A** YPD media and **B** YPD supplemented with 100 mM $CaCl_2$. *XvVHA-c``1* represents the *S. cerevisiae VMA3* knockout transformed with pYES2::*XvVHA-c``1*, vector refers to *S. cerevisiae VMA3* knockout transformed with pYES2 vector only, and Δ*VMA3* refers to untransformed *S. cerevisiae VMA3* knockout.

The result was that only the *S. cerevisiae VMA3* knockout strain transformed with pYES2::*XvVHA-c``1* could tolerate the excess $CaCl_2$, whereas the untransformed *S. cerevisiae VMA3* knockout and pYES2 transformed control could not. It is therefore conclusive that the pYES2 vector does not afford the *S. cerevisiae VMA3* knockout any protection or degree of tolerance to the stress; neither did the untransformed *S. cerevisiae VMA3* knockout strain.

4.5 Discussion

XvVHA-c``1 was capable of rescuing the *VMA3* mutant *S. cerevisiae* strain from 100 mM $CaCl_2$ stress, which is proof of its ability to associate with *S. cerevisiae* V-ATPases and assist the restoration of its function in acidifying the vacuole (Figure 4.2).

Vma3 mutants are defective in efficient assembly of the proton translocating pore that is integral to V-ATPase functioning. In its absence, it is hypothesised, that native subunits c` and c`` may contribute in some manner to pore formation and correct assembly of the V-ATPase. This explains why *S. cerevisiae VMA3* knockouts are viable when grown on full strength YPD media.

Ohya *et al.* (1991) have shown that although *VMA3* mutant strains are viable, they are nevertheless deficient in Ca^{2+} homeostasis and as a result their vacuoles contain considerably less Ca^{2+} than WT strains which is an indication of their inability to acidify the vacuolar lumen. The presence of trace amounts of Ca^{2+} in *VMA3* mutant vacuoles could also prove that c` and c`` provide sufficient functionality to the V-ATPase in order

to acidify the vacuolar lumen and therefore establish some sort of proton motive force for crucial secondary transport. Secondary transport systems in *S. cerevisiae* play an important role in Ca^{2+} homeostasis, in particular the vacuolar Ca^{2+}/H^{+} antiport proteins which are driven by proton motive force generated by V-ATPases.

Hence, the stress sensitivity response of *S. cerevisiae VMA3* knockouts to 100 mM $CaCl_2$ can be explained as follows. In presence of 100 mM $CaCl_2$ presented by the media, the VMA3 deficient V-ATPase cannot cope with the excess Ca^{2+}, even though subunits c` and c`` are present. The lack of viability under these conditions proves that the role subunit c` and c`` can complete under ideal growing conditions, becomes inefficient in the presence of excess Ca^{2+}. For *XvVHA-c``1* to restore the *S. cerevisiae VMA3* mutant's V-ATPase activity under conditions of excess $Ca^{2+}$, suggests that *XvVHA-c``1*'s role as an integral component of the V0 domain is correct and it contributes to the functioning of the V-ATPase.

Work done by Aviezer-Hagai *et al.* (2000) describes complementation studies that entailed cloning subunit c proteolipids from the lemon fruit and *A. thaliana* cDNA libraries. Their work was aimed at determining the differences in V-ATPase, and which subunits were vital in maintaining such different trans-tonoplast pHs. They reported the cloning of two subunit c proteolipid isoforms from *Citrus limon* fruit, LPL-I and LPL-II, which differed only by an additional amino acid in the predicted LPL-II protein sequence. The lemon cDNA clones were expressed in *S. cerevisiae VMA3* knockouts, and later *VMA11* (subunit c` proteolipid) knockouts since their identity was most similar to that of

VMA11. The results showed that only LPL-II could partially rescue *VMA3* knockouts but did not show any acidification of the vacuole under the fluorescent assay, and neither clone could complement *VMA11* knockouts in response to the stress conditions. It was assumed that if the proteolipid isoforms cause the hyper-acidification of lemon fruit vacuoles, then expressing it in *S. cerevisiae* may slow down the cell growth rate and the vacuole acidification could not be detected. Further work was necessary to prove their hypothesis.

*XvVHA-c``1* encodes a subunit c`` proteolipid homologue which is capable of complementing *S. cerevisiae VMA3* knockout mutants when subjected to a differential stress condition 100 mM $CaCl_2$. Experiments should be conducted to quantify the accumulation of Ca^{2+} in the vacuole of *VMA3* knockout strains transformed with X*vVHA1*, relative to the untransformed knockout and wild type *S. cerevisiae*. This could be achieved by staining live *S. cerevisiae* cultures with commercially available Ca^{2+}-specific stains and visualising them with fluorescent microscopes. In addition the change in luminal acidification could be visualised in *VMA3* knockout *S. cerevisiae* mutants transformed with *XvVHA-c``1*.

Chapter Five

General discussion

The resurrection plant *X. viscosa* serves as a good model system to discover novel genes that could be used for the genetic enhancement of crop varieties. This is based on the following observations: because it has the unique ability to resurrect after desiccation it must have a gene pool containing many genes that might play a vital role in tolerating desiccation and resurrecting; it is monocotyledonous which is a characteristic shared with many crops; it is easily subjected to stress treatments, access is relatively easy in South Africa and a *X. viscosa* tissue culture system has been developed to increase the ease of laboratory manipulation (unpublished).

Here we present the novel *XvVHA-c``1*, a gene encoding for a subunit c`` proteolipid which plays a vital role in the assembly and functioning of the vacuolar-type ATPase (V-ATPase). The V-ATPase has been localised to many internal membrane systems that include the vacuolar tonoplast, Golgi body, endoplasmic reticulum and vesicles, and its primary function is to acidify the internal compartments. It achieves this by pumping protons across the endo-membrane in which it is anchored, at the expense of hydrolysing ATP. The uses of an acidic internal compartment are varied and may include protein regulation, secondary transport across the membrane by an increase in proton motive

force and the sequestration and storage of hazardous and beneficial compounds, to name a few.

Subunit c`` shares it function with two other proteolipids, subunit c` and c. Subunit c has been the focus of expression studies in plant systems, because it is often shown as integral to the functioning, assembly and regulation of the V-ATPase holoenzyme. The novelty of this study was the isolation of a subunit c`` proteolipid homologue when *X. viscosa* underwent a dehydration stress, and the identification of its gene having a stress responsive expression pattern.

Sequence analysis of *XvVHA-c``1* revealed signature characteristics common among all subunit c homologues, and a few novel protein domains. The identification of four transmembrane domains (Figure 2.3) and conserved glutamate residues (Figure 2.4) are among the classic signature characteristics of subunit c, c` and c`` homologues. Novel characteristics included the localisation of three potential phosphorylation target sites (Figure 2.4) that may play a role in *XvVHA-c``1* regulation. The implication of being able to regulate the function of a membrane-bound proteolipid through phosphorylation is novel and implies a potential regulatory point for V-ATPase function in *X. viscosa*.

XvVHA-c``1* has been localised to a single genomic locus after a Southern hybridisation analysis was conducted on *X. viscosa* genomic DNA (Figure 2.5). The presence of *XvVHA-c``1 in the *X. viscosa* genomic DNA confirms that there was no contamination during its cloning process. To determine the exact copy number of the gene, a complete

genomic re-construction would have to be performed which includes sequencing and bioinformatics analysis. This exercise will prove valuable in determining the presence of subunit c and c` proteolipid homologues in order to draw some sort of relationship between the V-ATPase from *X. viscosa* and other higher plants- such as *A. thaliana* and *M. crystallinum*- in which orthologue and isoform copy numbers have already been determined. *X. viscosa* genomic analysis will also allow the dissection of promoter regions for *XvVHA-c``1* and its homologues, in order that the regulatory elements which control its expression can be identified.

XvVHA-c``1's expression in *X. viscosa* in response to abiotic stress was ascertained by northern analysis (Figure 3.1). The stresses were designed to elicit an osmotic stress at the cellular level. Whole plants were subjected to dehydration stress, application of 150 mM NaCl and a freezing shock stress at -20^{0}C. An increase in the transcript was evident 72 and 96 hours after application (HAA) of 150 mM NaCl stress (Figure 3.1 A), at 18% and 10% RWC when the plant was subjected to dehydration stress (Figure 3.1 B) and after 60 min exposure to -20^{0}C (Figure 3.1 C). A transient increase in transcript was noticed at 47% RWC during the dehydration stress. It was concluded that *XvVHA-c``1* had demonstrated stress responsive gene expression in *X. viscosa* and that western hybridisation analysis needed to be conducted to fully understand the protein expression response within the cell. Subunit c`` proteolipids have not been reported to display similar expression levels as presented here. It is possible for *XvVHA-c``1* to act as a important proteolipid that allows the cell to implement finer control of endo-membrane compartment acidification.

A functional complementation study was conducted where *XvVHA-c``1* was cloned into a shuttle expression vector and transformed into *S. cerevisiae VMA3* mutant. The result clearly demonstrated that *XvVHA-c``1* was able to restore the *S. cerevisiae* V-ATPase function in the cell. This finding conclusively identifies *XvVHA-c``1* as a V0 associated subunit, and suggests that the *X. viscosa* subunit c`` homologue can successfully substitute for the *S. cerevisiae* subunit c proteolipid in function, by restoring Ca^{2+} homeostasis in the assay.

The following model represents a hypothesis of how *XvVHA-c``1* could assist *X. viscosa* to tolerate osmotic stress based on the fact that vacuole structures are present in the resurrection plant cell at 5% RWC (Figure 1.3), that *XvVHA-c``1*is a subunit c`` homologue and its gene expression and assumed protein availability increases in response to osmotic stress.

As free water leaves the cytoplasm of dehydrating cells in *X. viscosa* tissue, solutes within the cytoplasm become concentrated. The osmotic pressure across the tonoplast membrane therefore increases and the plant has to employ some kind of cellular response to counter the increase in osmotic pressure, which probably amounts to water moving out of the vacuole into the cytoplasm. If the plant is unable to respond, the vacuole could collapse, releasing an array of cytotoxic enzymes.

XvVHA-c``1 transcripts are upregulated in response to the water deficit stress and its protein associates itself with the V0 domain of the *X. viscosa* V-ATPases. An increase in the number of proton-translocating structures in the tonoplast leads to an increase in the number of assembled of V0 components. This cascade, in turn, leads to an increased rate of V1 component assembly, with these attaching to the V0 components. The fully assembled V-ATPase will begin to hydrolyse ATP, using the energy of removing the inorganic phosphate group to translocate two protons into the vacuolar lumen. An accumulation of protons in the vacuolar lumen acidifies it and results in the rise in proton motive force (PMF). An increased in PMF could facilitate secondary transport of cations, sugars, anions, amino acids and small proteins across the tonoplast into the vacuolar lumen. Here an increase in solute concentration will allow the establishment of sustainable osmotic pressure equilibrium across the tonoplast. Also, an increase of V0 domains in the tonoplast would assist in the stabilisation of the membrane when it cannot associate with water molecules because of their absence. It is thought that compartmentalising the tonoplast under such high hydrostatic pressure is an additional stabilisation mechanism used by the cell to prevent damage. Vander Willigen *et al.* (2001) demonstrated that proline accumulated in the vacuoles of the resurrection plant *Eragrostis nindensis* during drying. It is possible that the same occurs in *X. viscosa.*

XvVHA-c``1 is as an excellent candidate for the genetic enhancement of crop varieties. It could assist the native V-ATPase in regulating its function in response to osmotic stresses. If in the instance that it does not fulfil this role, then it could always integrate

into the target organism's endo-membranes to assist in their stabilisation and recovery from any osmotic stress to which it is subjected.

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