Physiological and Cellular characterization of a Plant Natriuretic Peptide

by

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Submitted in partial fulfilment of the requirement for the degree of Magister Scientiae (M.Sc.) in Biotechnology in the Department of Biotechnology, University of the Western Cape, South Africa.

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Declaration

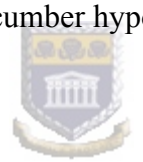
I, Monique Nonceba Maqungo declare that the thesis entitled 'Physiological and Cellular characterization of Plant Natriuretic Peptide' is my work and has not been submitted for any degree or examination at any other university and that all sources of my information have been quoted as indicated in the text and/or list of reference.

Name: Monique Nonceba Maqungo

Date: November 2005

TABLE OF CONTENTS

CHAPTER 2: MATERIALS AND METHODS



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ABBREVIATIONS

aa	Amino acids
AMP:	Ampicillin
ANP:	Atrial Natriuretic Peptide
APS:	Ammonium persulfate
ANOVA:	ANalysis Of VAriance
Bps	Base pairs
°C:	Celsius
$CaCl_2$:	Calcium chloride
CM:	Chloroamphenicol
8-Br-cGMP:	Cyclic guanosine monophosphate
EDTA	Ethylene Diamine Tetra-Acetic Acid Disodium Salt
g:	Gram
HCl:	Hydrochloric acid
IPTG:	Isopropyl-_-D-thiogalastopyranoside
irPNP:	immunoreactant plant natriuretic peptide

L: Liter

LB: Luria Broth

LY 83583: 6-anilino-5,8-quinolinequinone

kDa: kiloDaltons

μM: Micromolar

μL: Microliter

μg: Microgram

mg: Milligram

mM: Millimolar

mL: Milliliters

M: Molar

MES: 2–Morpholinoethanesulfonic acid

$MgCl_2$: Magnesium Chloride

MS: Murashige and Skoog Basal Salt Mixture

MSMO: Murashige and Skoog Basal Salt with Minimal Organics

nm:	Nanometers
TEMED:	N,N,N,N'- Tetra- methyl-ethylenediamine
NAA:	Naphthalene acetic acid
Ni-NTA:	Nickel-nitrilotriacetic acid
PAGE:	Polyacrylamide gel electrophoresis
K:	Potassium
KCl:	Potassium Chloride
PMSF:	Phenyl methyl sulfonyl fluoride
rpm:	Revolutions per minute
Na:	Sodium
NaCl:	Sodium Chloride
NaH_2PHO_4:	Sodium dihydrogen orthophosphate
SDS:	Sodium dodecyl sulphate
Tris-Cl:	Tris(hydroxymethyl)aminomethan
V:	Volts
YT:	Yeast

LIST OF FIGURES

Figure 3.2: Resolution of the AtPNP-A protein on an SDS-PAGE after its expression in pBL21DE3 star plysS cells.

Figure 3.3: SDS-PAGE analysis of fractions obtained during purification of AtPNPA using a Ni-NTA agarose under denaturing conditions.

Figure 3.4: *Arabidopsis thaliana* protoplasts suspended in 400 mM sorbitol and treated for 15 minutes with 150 ng/mL AtPNP-A.

Figure 3.4.1: Protoplasts were prepared from suspension cultures and subjected to different treatments, a representation of *Arabidopsis thaliana* protoplasts cells S.E.M.

Figure 3.5: A Dot plot representation of *A. thaliana* protoplasts resuspended in 400 mM sorbitol and then treated with 150 ng/mL AtPNP-A, a combination of 150 ng/mL AtPNP-A and 20 μM LY 83583, as well as 20 μM LY 83583 for 15 minutes.

Figure 3.6: *Arabidopsis thaliana* suspension culture cells were treated with 150 ng/mL AtPNP-A for 15 minutes and then viewed and photographed under a microscope.

Figure 3.6.1: A representation of *Arabidopsis thaliana* suspension cells S.E.M. after their diameters of randomly selected cells were measured.

Figure 3.7: *A. thaliana* protoplasts were treated with 150 ng/mL of expansin followed by measurement of cell volumes.

Figure 3.7.1: Extension of native and reconstituted cucumber hypocotyls walls.

Figure 3.8: Microarray expression profiling data on AtPNPA from the Genevestigator server.

Figure 3.8.1: Microarray expression profiling data on AtPNPA from the Genevestigator server.

ABSTRACT

Plants in the field are exposed to multiple stresses and their response to these various stresses determines their capacity to survive. Plants can use multiple signaling pathways and signals to mediate their response; for example, at least four different signal pathways have been identified for water-deficit stress (Shinozaki and Yamaguchi-Shinozaki, 1997; Xiong *et al*., 2002). Different forms of stress may activate or utilize the same components, including proteins and other signaling molecules. Signaling molecules such as jasmonic acid (JA) are involved in multiple stress response and development in plants (Creelman and Mullet, 1995, 1997; Turner *et al.,* 2002). However it is the specific combination of various components of the signaling network coupled with spatial and temporal factors that allows the plant to mount a directed response to any given stress factors. Systemic defense responses thus provide an attractive model for the study of cell-to-to cell signal transduction pathways that operates over long distances (Lucas and Lee, 2004).

Cellular and physiological evidence suggest the presence of a novel class of systemic mobile plant molecule that is recognized by antibodies against vertebrate atrial natriuretic peptides (ANPs). It has been demonstrated that a recombinant *Arabidopsis thaliana* natriuretic peptide analogue (AtPNP-A) molecule can induce osmoticum-dependent water uptake into protoplast at nanomolar concentrations thus affecting cell volume and hence plant growth. In this study we confirm that active recombinant protein causes swelling in Arabidopsis mesophyll cell protoplasts (MCPs); when

comparing with a Fluorescence-Activated Cell Sorter (FACS) technology based method the results obtained confirm previous findings. In particular, that swelling in response to AtPNP-A was observed in whole Arabidopsis suspension culture cells and that expansins do not cause swelling in protoplasts neither does AtPNP-A induce 'creep'.

LITERATURE REVIEW

CHAPTER ONE

1. Introduction

The plant and animal kingdoms are thought to have evolved independently from common ancestral eukaryotes, which appear to have existed around 1.6 billion years ago (Meyerowitz, 2002). In animal systems, peptide hormones and specific receptors plays a major role in cell-to-cell communication, as well as coordinating cell growth and differentiation in various organs. In contrast, most intracellular communications involved in plant growth and development have largely been explained on the basis of signaling by the six non-peptide plant hormones: auxin, cytokinin, ethylene, gibberellin, abscisic acid and brassinolides (Matsubayashi, 2003). There is no doubt about the significance of these hormones in plant homeostasis and growth, since discoveries over the past decade indicated that plant cell communication also made use of small peptide signals and specific receptors. Researchers to date have identified four peptide-ligand-receptor pairs in plants (Ryan *et al.,* 2002), which are involved in a number of processes, including wound responses, cellular differentiation and self incompatibility (Matsubayashi, 2003). Recent studies have further shown that plants also use complex signaling systems as major regulators affecting many aspects of their life cycles. Although most of the peptide signals in plants have no homologs in animals, and *vice versa* there are some similarities in the overall logic of peptide signaling systems in plants and animals (Takayama and Sakagami, 2002). However, plant genome sequencing has revealed many genes predicted to encode small peptide ligands and receptor-like kinases whose functions remain to be uncovered (The Arabidopsis Genome Initiative, 2000; Shiu and Bleecker, 2001). Here we are setting out to determine if plants, much like vertebrates, have a natriuretic peptides based signaling system and if so, to find out its structural basis and how it functions in plants.

1.1 The natriuretic peptide system in vertebrates

There are at least three different natriuretic peptides that have been characterized: ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide) and CNP (a C-type natriuretic peptide). ANP functions as a secreted hormone that is involved in regulating blood pressure and blood volume through direct effects on the kidney and systemic vasculature (Molkentin, 2003). ANP is a circulating hormone synthesized in cardiac atrial myocytes, and plays a role in the control of salt and water homeostasis and blood pressure (Hongkui, 1996). It has been argued that this hormone system consist of five peptide hormones derived from a 126 amino acids (aa) precursor or pro-hormone synthesized in the heart of vertebrates and invertebrates animals. These five units consist of amino acids 1 - 30, 31 - 67, 68 - 77, 78 - 98 and 99 – 126. The C-terminal 28-mer that forms a circular structure due to disulphide bonds formed between the cysteines in position 7 to 23 is believed to be the main biologically active ANP (Vesely, 1994 and 1998) (Figure1).

1.1.1 ProANP encoding gene in animals

The gene encoding the synthesis of atrial NP pro-hormone (proANP) consist of three exons (coding) sequences separated by two introns (intervening) sequences (Figure1). Exon 1 encodes the '5′ untranslated regions', the signal peptide and the first 16 aa of the ANP pro-hormone (Vesely, 2002). The signal peptide is cleaved from the pre-pro-hormone (151aa) in the endoplasmic reticulum. The resulting pro-hormone of the size 126 amino acids is the storage form for the four ANP hormones (Vesely, 1994 and 1998). Part of the intracellular mechanism of action(s) of the four peptide hormones encoded by the proANP gene is that after they bind to their specific receptors, may activate guanylate cyclases which is a part of the cytosolic portion of the receptors, and this then causes an increase in the intracellular messenger cyclic GMP (cGMP). Cyclic GMP then stimulates a cGMP-dependent protein kinases, which in turn phosphorylates protein(s) within the cell (Vesely, 2002).

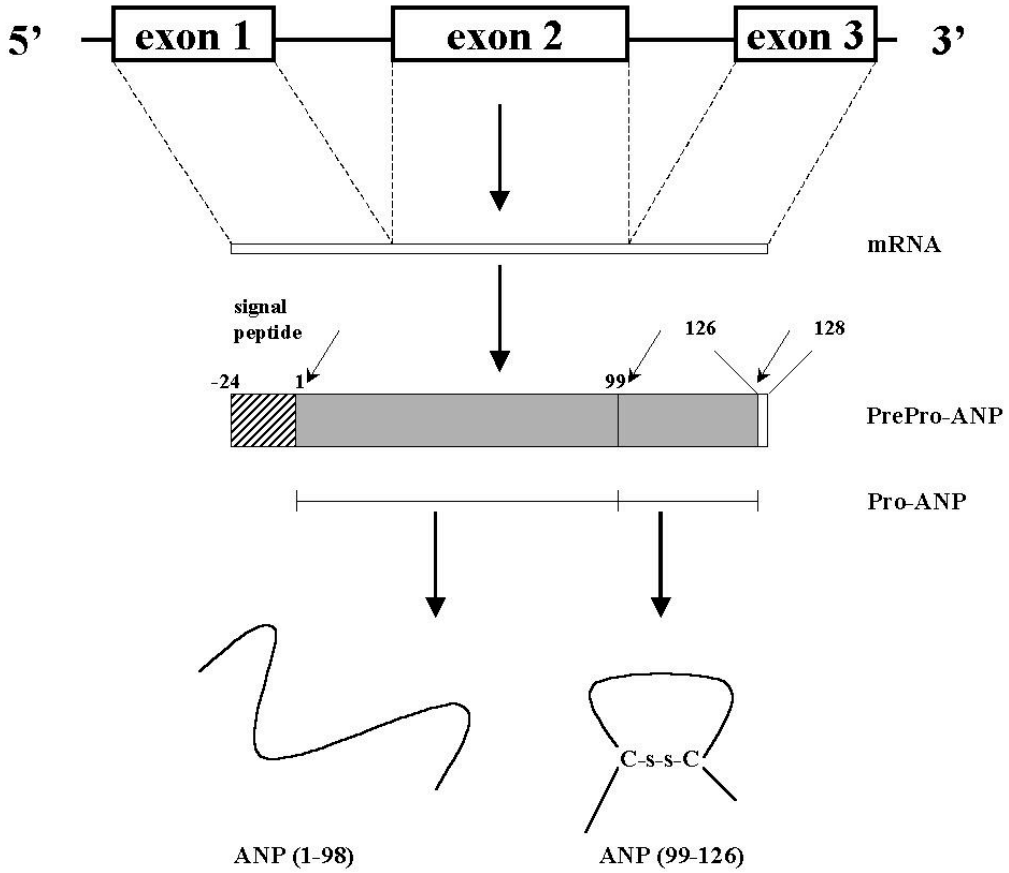

Figure 1.1: Representation of the gene structure and major processing steps of ANP. Small arrows mark the major cleavage sites. ANP (99-126) is the main and best established biologically active compound. ANP (1-98) has also been reported to be cleaved further into ANP (1-30), ANP (31-67), ANP (68-77) and ANP (78-98); the mode of action and function of these cleavage products is still debated (after Gehring 1999).

1.2 Evidence confirming the existence of natriuretic peptide-like molecules in plants

It was reported by Vesely *et al.*, (1993) that ANP – like peptides were present in the plant kingdom. His research group tested and confirmed that ANP peptides from the NH_2- terminus and/or COOH-terminus of the ANF pro-hormone 1 - 30, 31 - 67, 68 - 77, 78 - 98 and 99 - 126 increased solute flow upwards through the stem to the leaves and flowers. A further task was to determined if and how the discovered peptides

affected transpiration (i.e. water loss) by opening the stomatal pores in leaves and finally to study the effect of these peptides on solute absorption (Vesely, 1993; Gehring *et al*., 1996). Studies presented some evidence that NP-like transcripts existed in plants; these experiments used Southern and Northern blot hybridizations of English ivy (*Hedra helix*) DNA and RNA using the ANP gene sequence as a probe (Vesely, 2001). In addition, antibodies against the N - terminus (ANP 1-98), the mid-portion (ANP 31-67) and the C-terminus (ANP 99-126) recognized peptides in leaves and stems.

1.2.1 Ligand binding studies

It was also demonstrated that in an animal system, biological activity of ANP was lost after irreversible reducing the molecules and thus linearising ANP (99 - 126) (Misono *et al*., 1984). It was argued that ligand mediated receptor interaction depended on the circular configuration of the molecule. The ANP molecule contains six charged amino acids (five arginines and one aspartic acid) and forms a loop due to a disulphide bond between cyscteines in position 7 and 23 (Gehring, 1999). It was observed that the effect of rat ANP (rANP) in plants was not due to its charges since it required circular configuration. Reduced molecules irreversibly linearized by an S-carboxymethylation reaction (see Figure 1) were inactive. It had no biological activity in stomatal opening assays thereby excluding the possibility of a non-specific charge effect that reside in the primary structure. In plants, indirect evidence for specific receptor ligand interactions comes from binding experiments where isolated leaf membrane were exposed to rat (3-[^{125} I] iodotyrosyl ^{28} ANP. The specificity of binding was assessed by increasing the concentration of unlabelled competitor (rANP). The experiments suggested that 50 % of the labeled ligand could be displaced by 0.1μM rat ANP indicating that plant membranes contained a specific low affinity NP binding site for heterologous ANP. Since half maximal specific binding for peptide hormones in homologous vertebrate system is typical substantially lower (<0.1nM) and it will be instructive to learn what sort of binding constant will eventually be obtained with homologous plant NPs.

1.2.2 ANP induces stomatal opening in plants

It was also proven that synthetic rat ANP (rANP) could induce stomatal opening in *Tradescanta* sp. in a concentration dependent manner (of $\geq 10^{-6}$ M) (Gehring *et al*., 1996). The high concentration necessary to get a response to a peptide hormone could be due to large taxonomic divergence between hormone source and test organism. It was also noted that like in animal systems, rANP in plants also inhibited the amiloride sensitive Na^+/ H^+ channel. The mechanism by which rANP inhibits the Na^+/ H^+ channel in the plasmalemma is probably not as direct as the amiloride effect which is caused by a specific binding of the inhibitor to the channel (Canessa *et al*., 1993). This may imply that NPs in plants modulate K^+ transport, which is the major cation. In plants, stomatal movement is dependent on blue light, which activates a proton pump in the cell membrane which pumps out H^+. This creates a charge gradient and a pH gradient between the inside and the outside of the cell which then activate channels for K^+ ions to enter. Chloride (Cl^-) ions also counter the charge balance. The increase in solute concentration results in water uptake, swelling the guard cells and thus opening the stomata (for review see Assmann and Shimazaki, 1999).

1.2.3 ANP induces swelling in mesophyll cell protoplast

It was noted that a synthetic peptide identical to the C-terminus (amino acids 99- 126) of ANP modulates the osmotically induced swelling of potato (*Solanum tubersosum*) mesophyll cell protoplast (MCPs) in a concentration and time dependent manner (Maryani *et al*., 2001). Osmotically induced volume changes in MCP were also enhanced by plant extracts with immunoaffinity purified NP. In contrast, pretreatment of the plant extracts with rabbit anti-human ANP (99-126) antiserum suppressed enhanced osmoticum-induced swelling. These findings are consistent with a NP-dependent effect on the plant cell volume regulation and a role in homeostasis for peptides recognized by antibodies directed against the C-terminus of vertebrate ANPs.

1.2.4 Isolation of a biologically active NP immunoanalogues

An immunoaffinity purified plant NPs (irPNPs) from ivy (*Hedera helix*) was isolated by (Billington *et al*., 1997). The immunoaffinity column purified several different molecules indicating that plants contained more than one type of NP immunoanalogue and/or that different precursor molecules also contained the epitope(s) (Pharmawati *et al*., 1998b). Data from electrophoresis, electrospray mass spectroscopy and western analysis suggested the presence of immunoreactant molecules in the molecular weight range of the vertebrate proANP (approximately 10kDa) and C-terminus (approximately 3kDa). Immunoreactant plant NP (irPNP) like ANP can induce stomatal opening in a concentration dependent manner (Billington *et al*., 1997). The effect of the homologous peptide(s) is observed at concentration significantly smaller than those required to elicit a response with ANP (Billington *et al*., 1997). Plant hormones (growth regulator) isolated and characterized to date are small organic molecules many of which are derivates of amino acids (e.g. ethylene, indole acetic acids) or purines (e.g. kinetin). In order to elicit *in vivo* or *in vitro* responses to plant hormones such as auxin, abscisic acids (ABA) or kinetin concentrations $\geq 10^{-6}$ M are required (Gehring *et al*., 1990; Irving *et al*., 1992). Such concentrations are in fact several orders of magnitude higher than those required to trigger responses to peptide hormones in animal systems and therefore suggests different receptor and signaling systems. The discovery of a plant peptide hormone system that induces stomatal responses at concentrations $\leq 10^{-7}$ M does thus challenge current views on plant hormones and signaling.

1.2.5 Role of cyclic guanosine 3′, 5′- monophosphate (cGMP) in NP signaling

Since cGMP is a second messenger for ANP in vertebrate systems it was hypothesized that it may also played a role in ANP dependent signaling in stomata (Gehring *et al*., 1996). Pharmawati *et al.,* (1998) noted that ANP induced stomatal aperture increases are reversibly inhibited by guanylate cyclase inhibitors, methylene blue and LY 83583 (6-anilino-5, 8-quinolinequinone). The cell permeant cGMP

analogue 8-Br-cGMP (8 -bromo - guanosine 3´5´-cyclic monophosphate) had the effect to mimic ANP when applied alone. In addition, the measurements of dye movements and determinations of tissue water exchange ratios with ^{2}H NMR suggested that ANP significantly increased radial water movements out of the xylem of shoots of *Tradescantia multiflora* (Suwastika *et al.,* 2000). This enhancement was also observed in response to 8-Br-cGMP, a cell cGMP, while the aquaporin inhibitor, mercuric chloride and the guanylate cyclase inhibitor LY 83583 both significantly inhibited radial water movement. Thus, it is probable that NPs play a part in controlling radial water movement out of the xylem and that the effect may be mediated via regulation of guanylate cyclases, cGMP and water channels.

1.3 Role of cGMP as a second messenger

The second messenger cGMP is generated from an intracellular GTP either by soluble guanylyl cyclases (sGC) or particulate guanylyl cyclases (pGC). The sGCs are activated by nitric oxide (NO) or carbon monoxide, whereas the pGCs are receptors for a family of NPs consisting of atrial, brain and C-type natriuretic peptides (ANP, BNP and CNP, respectively) (Feil *et al*., 2003).

1.3.1 cGMP in vertebrate systems

All three peptides exert their main biological effects via binding to specific membrane-bound guanylyl cyclase (GC) receptors, GC-A (ANP and BNP) and GC-B (CNP), thereby increasing intracellular concentration of the second messenger cyclic GMP (cGMP) (Feil *et al*., 2003). GC-A and GC-B are also referred to as natriuretic peptide receptors type A and type B (NPR-A and NPR-B). In the bloodstream ANP activates GC-A receptors, which are expressed in a variety of tissues and thereby modulate blood pressure and blood volume, (Kuhn, 2004). Another target for cGMP includes the cyclic nucleotide-gated channels or CNG channels. CNG channels regulate the influx of Na^{+} and Ca^{2+} into cells and are opened by cGMP binding. CNG channel activation by cGMP is critical to regulate phototransduction and neurotransmission in the retina (Krumenacker *et al*., 2003).

1.3.2 NP and cGMP analogues modulate K^+, Na^+ and H^+ fluxes in plants

It was observed (Pharmawati *et al*., 1998) that within 30 seconds of adding PNP a rapid increase of cGMP levels occurred in *Zea mays* root stele. It was discovered that the transient after induction was similar to that of the ANP induced cGMP response in erythrocytes. Phamarwati *et al*., (1999) demonstrated in *Zea mays* roots that 8- Br-cGMP, a stable cell permeant cGMP analogue lead to a noticeable increase in K^+ uptake as well as shift towards net Na^+ influx. There was no evidence that 8-Br-cGMP regulated net H^+ flux from the stele tissue. It was concluded that cGMP can affects cation channels directly rather than driving e.g. K^+ uptake in response to an increased transmembrane H^+ gradient and it was suggested that some PNP effects on the channel might be mediated by cGMP. Maryani *et al*., (2000) also demonstrated that PNP consistently and significantly increased ATP hydrolysis *in vitro* in vesicles from *Solanum tuberosum L.* leaf tissue. When ATP-dependent H^+ gradient were monitored in the presence of low Cl^- concentrations in a medium where KCl has been replaced with KNO_3, H^+ gradients were not significantly affected by PNP. This observation suggested that PNP modulations of the transmembrane H^+ gradient were not due to a direct inhibitory effect of PNP on the ATPase but linked to PNP-modulated ion transport. Furthermore, it was observed that the ATPase was not inhibited by PNP but was directly or indirectly stimulated. It thus followed that PNP affected transmembrane H^+ gradient not by inhibiting ATPase activity but by modulating H^+ coupled co-transporters (see Figure 1.2).

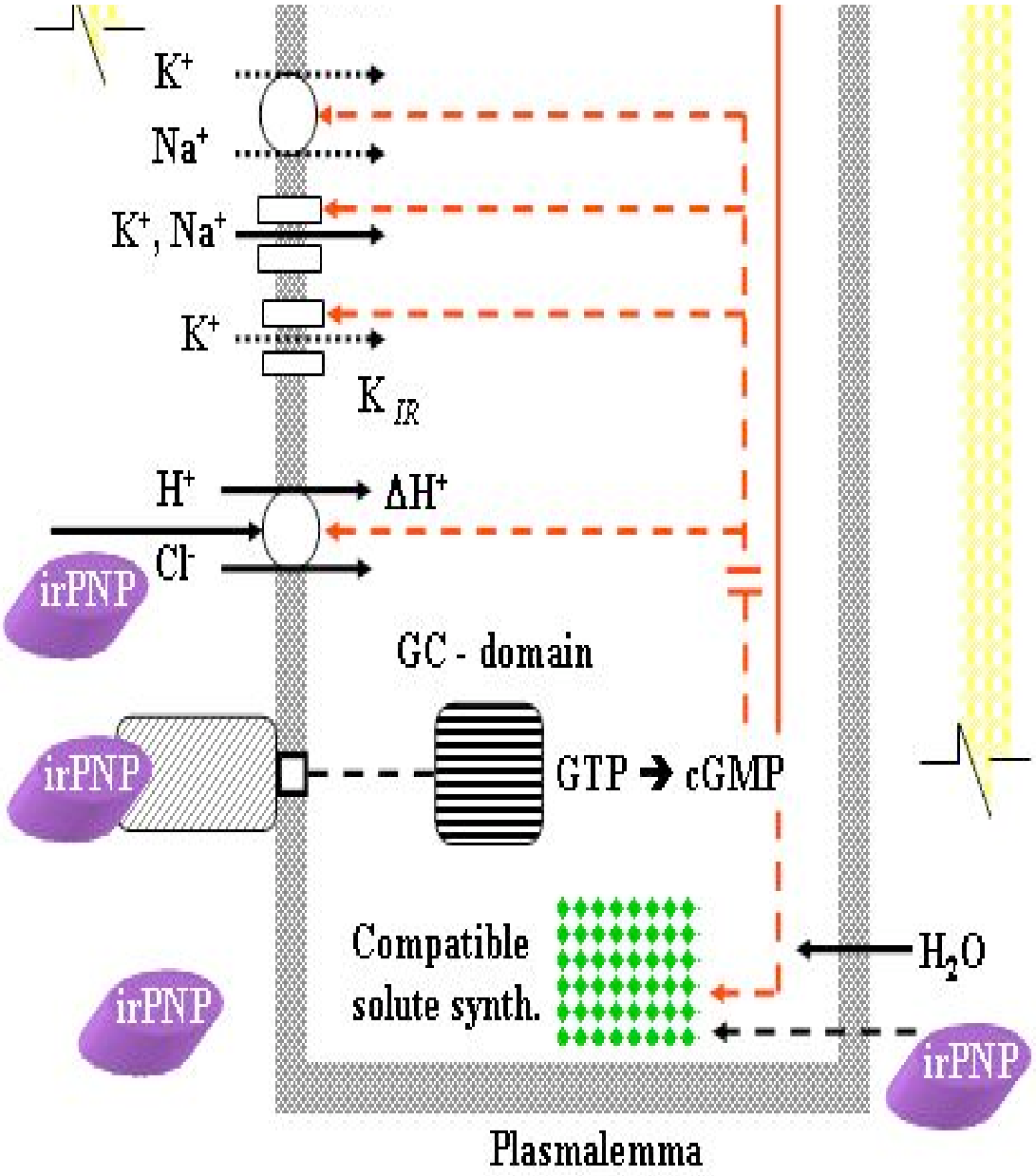

Figure 1.2: Model of interaction between irPNP-A, cGMP, H^+ - ATPase and ion channels in plant cells. Factors that stimulate activity are indicated by pointed arrows, those that inhibit by blunt arrows. Dashed lines signify currently hypothesized links (after Gehring and Irving, 2003).

1.4 What are expansins, and how do they relate to AtPNP-A?

Plant cells are contained by a rigid exoskeleton–like structure, the cell wall. The major components of the wall are cellulose microfibrils consisting of an unbranced β-1,4-glucan polymers, hemicelluose (branched glycans) that bind to cellulose to form a matrix and to acidic polysaccharides (pectins) that form ionic gels. Developmental processes (cell elongation) and any rapid physiological responses (stomatal movements) that require changes in cell shapes are necessitated by some temporary loosening of cell walls. Expansins are proteins that promote cell wall loosening and extension and recently two families of expansins, α-expansin and β-expansin, have been described (for review see Cosgrove 1999, 2000a and 2000b) (Figure 1.3).

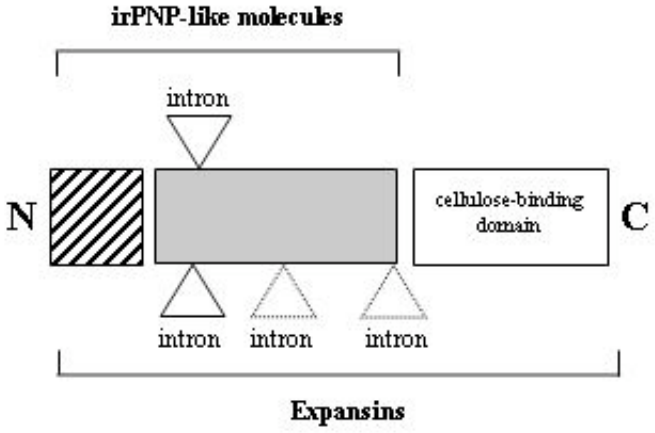

Figure 1.3: Comparisons of domain organization of irPNP-like molecules and expansin.

1.4.1 Structure and role of expansins and expansin-like molecules

A molecule, CjBAp12 (blight associated protein, p12), from citrus trees shares significant homology with expansins (Ceccardi *et al*., 1998). The molecule has a molecular mass of approximately 12 kDa and is thus considerably smaller than the classical expansins (molecular mass of ~ 25 kDa). The gene CjBAp12 is expressed in root and stem tissue in response to a challenge from citrus blight. The protein itself accumulates in root, stem and leaf tissues (Ceccardi *et al*., 1998) thus possibly suggesting systemic mobility. However, despite marked sequence similarity with

expansins, CjBAp12 has no apparent expansin-like activity (Ceccardi *et al*., 1998). An *Arabidopsis thaliana* transcript (AtPNP-A) encoding an immunoreactant plant natriuretic peptide (irPNP) analog was identified and isolated (Billington *et al*., 1997). The encoded protein showed similarity to CjBAp12 (Ludidi *et al.,* 2001). Recently Ludidi *et al*., (2001) have established the evolutionary and functional relationship of irPNP-like molecules within the superfamily of expansins, pollen allergens and distantly related molecules such as endoglucanases. They have shown that irPNP-like molecules are related to expansins and fall into two groups: one included CjBAp12 and the other AtPNP-A (Figure 1.4).

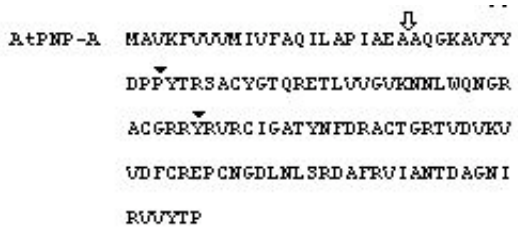

Figure 1.4: Sequence of AtPNP-A from *A. thaliana*. The cysteine conserved between AtPNP-A, AtPNP-B and CjBAp12. The symbol (X) delineates the sequence that is aligned with _- hANP. The symbol (⇓) marks the end of the signal sequence.

Members of both groups shared distinct sequence motifs (K[VI]VD and [LM]SxxAFxxI) but do not contain the tryptophan and tyrosine rich C-terminal putative polysaccharide-binding domain typical of expansins or bacterial cellulases and hemicellulases. They argued that both irPNP-like molecules and expansin had evolved from primitive/ancestral glucanases-like molecule that hydrolysed the cell wall. Importantly it was previously demonstrated that irPNP acted on protoplast (that is plant cells without cell walls) as well as microsomes, indicating that these novel proteins specifically interact with the plasma membrane. It therefore follows that the cell wall cannot be an obligatory substrate for irPNPs. Thus both irPNP function and domain structure may suggest that these molecules have a systemic role in water and solute homeostasis.

MATERIALS AND METHODS

CHAPTER TWO

2.1 The recombinant AtPNP-A construct

The pCRT7/NTAtPNP-A construct (stored at – 20 °C) used in the transformation for the over-expression of AtPNP-A was supplied by M. Morse (Department of Biotechnology, University of the Western Cape). It was maintained and propagated in TOP10'F cells. The transformation were prepared as described in the manual (pCR®T7 TOPO® TA Expression Kits, Invitrogen) (Figure 2.1(a) and Figure 2.1(b)), 2 µL of the TOPO® Cloning reaction was added to a vial of One shot® chemically competent *E.coli*, mixed gently and placed on ice for 5 minutes. The cells were then heat shocked for 30 seconds at 42 °C without shaking and immediately placed on ice. Two hundred and fifty micro liters of SOC (2 % Tryptone, 0.5 % Yeast extract, 0.05 % NaCl, 2.5 mM KCl, 10 mM $MgCl_2$) medium was added to the mix then placed at 37 °C shaking at 200 rpm for 30 minutes. The transformation mix was spread on selected plates (Luria Bertani media) containing 100 µg/mL ampicillin (AMP) and 34 µg/mL chloroamphenicol (CM) and incubated at 37 °C.

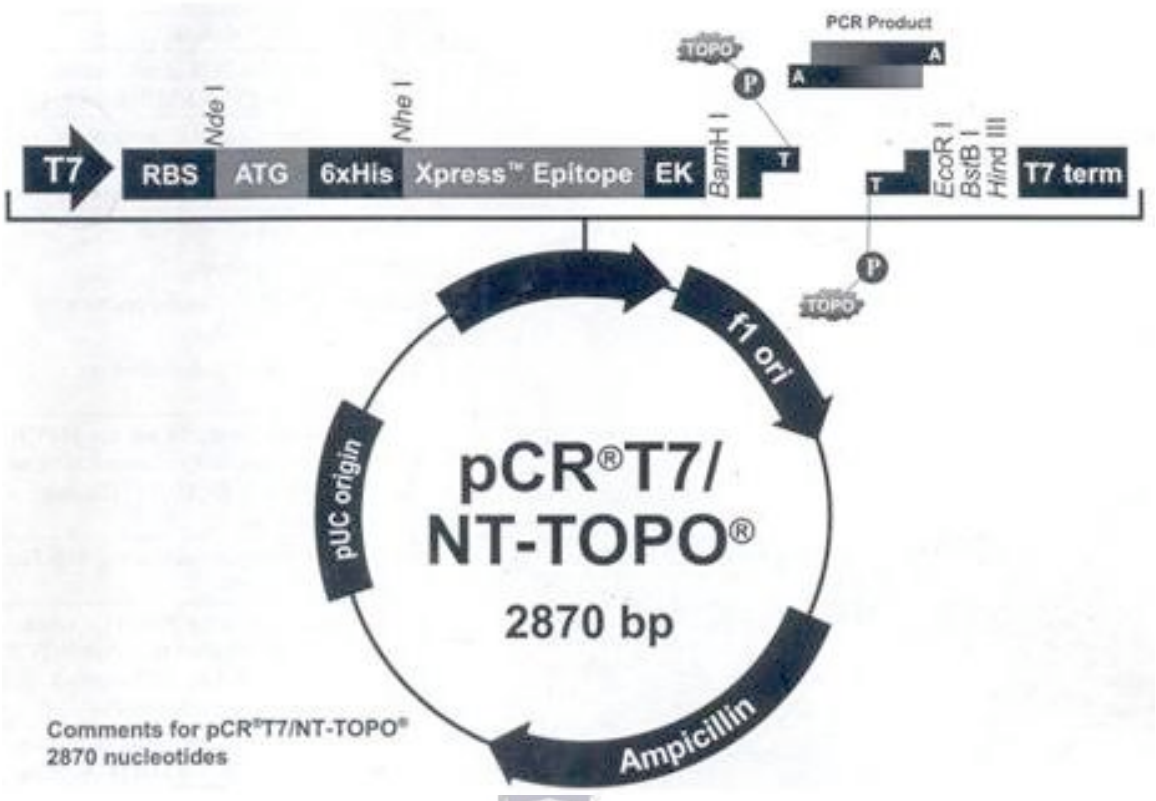

Figure 2.1(a): The map of pCR®T7 TOPO® TA vector showing the position of a T7 promoter, the various restriction sites, as well other features of the plasmid (AtPNP-A gene cloned into the multiple cloning site of this plasmid whereas double digestion of the plasmid (Studier *et al*., 1986).

Figure 2.1(b): The complete sequence map of pCR®T7 TOPO® TA vector showing the various features of the plasmid vector (Studier *et al*., 1986).

2.1.1 Isolation of plasmid DNA construct from bacterial culture

A single colony was picked from plates prepared in section (2.1) and used to inoculate 50 mL LB containing 100 μg/mL AMP and 34 μg/mL CM and placed in a 37 °C shaking incubator at 200 rpm until OD_{600} of the culture had reached 0.6. The culture was then spun at 10 000 rpm for 10 minutes to harvest the cells. Isolation of plasmid DNA was performed as described in the manual (Genopure Plasmid Midi Kit, Roche Applied Science). The pellet was resuspended in 4 mL of suspension buffer/RNase working solution and 4 mL of lysis buffer mixed well and incubated on ice for 3 minutes. Four mL of chilled neutralization buffer was added to the

suspension, inverted 8 times and incubated on ice for 5 minutes. The lysate was centrifuged at 10 000 rpm for 15 minutes and the supernatant was passed through a funnel containing folded filter (added a few drops of equilibration buffer) inserted into a 50 mL polypropylene tube. The flow through was discarded and the column was washed twice with 5 mL of washed buffer. The column was re-inserted into a fresh 50 mL polypropylene tube and 3.6 mL of elution buffer was added. The eluted plasmid was precipitated with 3 mL of isopropanol and spun at 10 000 rpm for 30 minutes. The plasmid was then washed with chilled 70 % ethanol and spun at 10 000 rpm for 15 minutes. The pellet was dissolved in 100 µL of sterile distilled water. Quantification and purity of the isolated DNA was determined spectrophotometrically.

2.1.2 Restriction enzyme digestion

One µg/µL plasmid DNA (pCRT7/NT AtPNP-A) was cut with 10U BamHI and EcoRI (Fermentas), 10 X buffer R (Fermentas) in a double digest at 37 ºC for 2 hours and analyzed on a 2 % agarose gel.

2.1.3 Transformation of pCRT7/NT AtPNP-A for over-expression

For over-expression of AtPNP-A, the pCRT7/NT AtPNP-A construct was transformed into chemically competent BL21 (DE3) Star pLysS *E. coli* cells (Invitrogen). As described in the manual (pCR®T7 TOPO® TA Expression Kits, Invitrogen), one shot vial of BL21 (DE3) Star pLysS (Invitrogen) cells per transformation was thawed on ice and 2 µL of plasmid DNA (pCRT7/NT AtPNP-A at 5 ng/µL) was added and gently mixed by stirring with a pipette tip. The vial was incubated on ice for 30 minutes and a heat-shock treatment was applied for 30 seconds at 42 °C without shaking. The vial was immediately placed on ice and 250 µL of room temperature SOC medium was added. The tube was tightly capped, placed by its side for better aeration and incubated for an hour at 37 °C while shaking at 200 rpm. The entire transformation reaction was added to 10 mL of YT (8 g Tryptone, 5 g Yeast extract, 2.5 g NaCL, 4 g glucose; pH 7.0 made up in 1 L)

medium containing 100 μg/mL (AMP) and 34 μg/mL (CM). Some of the transformation mix was plated on YT plates with antibiotics and the remainder was left to grow overnight at 37 °C while shaking at 200 rpm.

2.1.4 Small scale – expression of recombinant protein

Ten milliliters YT containing 100 μg/mL AMP and 34 μg/mL CM was inoculated with 500 μL of overnight culture (from transformation mix). The culture was incubated at 37 °C shaking for two hours until the OD_{600} was 0.6. 500 μL of the culture was taken and the cells harvested by centrifugation at maximum speed (13 000 rpm) for 30 seconds. The supernatant was discarded and the pellet stored at -20 °C. This was time 0 point. The culture was then split into two with one culture being induced by the addition of IPTG (Isopropyl-_-D-thiogalastopyranoside, Fermentas) to a final concentration of 0.5 mM. The remaining culture (uninduced sample) was left incubating under the same conditions and at every hour for 3 hours a 500 μL sample was taken from both induced and uninduced cultures. Cells were harvested by centrifugation and the pellets stored at - 20 °C before analysis by SDS PAGE. Glycerol stocks were prepared using the overnight culture and stored at -70 °C till needed.

2.1.5 Large scale expression of recombinant protein

In a 250 mL Erlenmeyer flask, 50 mL of YT containing 100 μg/mL AMP with 34 μg/mL CM was inoculated with a glycerol stock of an overnight culture of BL21DE3 Star pLysS transformed with pCRT7/NT AtPNP-A and incubated at 37 °C shaking at 200 rpm and left to grow overnight. In a 3 L Erlenmeyer flask, 1 L YT containing 100 μg/mL AMP with 34 μg/mL CM was inoculated with 20 mL overnight culture (pAtPNP-A+ BL21DE3 Star pLysS) and incubated at 37 °C shaking at 200 rpm. The culture was left growing until the OD_{600} reached 0.6 and induced with a final concentration of 0.5 mM IPTG and left to grow for 3 hours. A 1 mL sample was taken, spun at 13 000 rpm for 1 minute, the supernatant discarded and the pellet were

stored at -20 °C. The remainder of the culture was spun down at 10 000 rpm (Beckmann centrifuge) for 10 minutes. The supernatant discarded and the pellet stored at -20 °C for further analysis.

2.2 SDS-PAGE analysis of protein samples

Protein samples were analysed in a discontinuous buffer system according to the method of Laemmli (1970) on a 15 % resolving and 4 % stacking gel. The composition of resolving and stacking gels are described below:

Resolving gel: 5.0 mL 30 % Acrylamide (Sigma), 1.25 mL 0.8 % SDS (Sodium Dodecyl Sulfate) 1.25 mL 3 M Tris-HCl buffer (pH 8.8), 2.43 mL sterile distilled water, 50 µL 10 % APS (Ammonium persulfate) (Sigma) and 20µL Temed (N,N,N´N´- Tetramethylethylenediamine).

Stacking gel: 0.8 mL 30 % acrylamide (Sigma), 0.625 mL 0.8 % SDS, 0.625 mL 1 M Tris-HCl buffer (pH 6.8), 2.905 mL sterile distilled water, 25 µL 10 % APS (Sigma) and 20 µL Temed (Sigma).

2.2.1 Sample preparation for SDS-PAGE

Cell pellets were thawed on ice and re-suspended in 200 µL lysis buffer (50 mM potassium phosphate; pH 7.8, 40 mM NaCl, 100 mM KCl, 10 % glycerol, 0.5 % Triton-X, 10 mM imidazole). The suspension was snap frozen using liquid nitrogen and then thawed at 42 °C, this freeze/thaw cycle was repeated at least 3 times. Samples were spun at 13 000 rpm in a microcentrifuge for 1 minute and the supernatant was transferred into a sterile 1.5 mL Eppendorf tube and stored on ice. Four µL of 5 x sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 0.5 % (w/v) bromophenol blue, deionized water) was added to 40 µL of the supernatant and mixed. The samples were then placed in boiling water for 5 minutes after which 20µL of each sample was loaded on the gel and run at 200 Volts for 45 minutes. The gels were stained with Coomassie blue (0.1 % Coomassie-R250, 10 % acetic acid, 40 % ethanol) for 30 minutes and destained with destaining solution (10 % methanol, 10 % ethanol, 10 % acetic acid) for 30 minutes or until protein bands were visualized.

2.3 Purification of expressed protein under denaturing conditions

Protein purification was performed under denaturation conditions since it was determined that the recombinant protein was expressed largely as insoluble inclusion bodies. The pellet (obtained as described in section 2.1.5) was weighed and thawed on ice and then re-suspended with buffer B (100 mM NaH_2PHO_4, 10 mM Tris-Cl, 8 M urea; pH 8.0) at 5 mL per gram wet weight. The cells were stirred at room temperature for an hour and then spun at 10 000 rpm for 20 minutes to pellet cell debris. Hundred μL of the cleared lysate was kept at -20 °C for analyses on SDS-gel electrophoresis while the remaining supernatant (cleared lysate) was used in the purification. Two mL of 50 % Ni-NTA slurry (Qiagen) was added to 8 mL of the cleared lysate and gently mixed on a rotary shaker for an hour. The lysate-slurry mix was transferred to a column (12mm X 135mm) and the flow through collected and stored. The column was then washed twice with 4 mL of buffer C (100 mM NaH_2PHO4, 10 mM Tris-Cl, 8 M urea; pH 7.0). Eight M urea (500 mM NaCl, 20 % glycerol, 20 mM Tris-Cl; pH 7.4) was also added and 2 times 4 mL were collected and placed on ice. Six M urea (500 mM NaCl, 20 % glycerol, 20 mM Tris-Cl; pH 7.0) was then added and 4 mL was collected and about 2 mL were left above the resin. The protein was refolding on an agarose matrix using a linear gradient (6 M to 1 M urea; 500 mM NaCl, 20 % glycerol, 20 mM Tris-Cl; pH 7.0) over a period of 1.5 hours. Five milliliters of eluted protein was eluted with 1 M urea; 500 mM NaCl, 20 % glycerol, 20 mM Tris-Cl; pH 7.0 containing 250 mM imidazole and 0.5 mM PMSF, (Phenylmethanesulfonyl, Sigma).

2.3.1 Concentration and desalting of protein

The eluent obtained in section (2.3) was concentrated by pouring it into a Centriplus Filter device (YM-3) (Millipore) with a molecular weight cut off of 3000 as described in the instruction manual (Centriplus YM-3, Millipore). The column was spun at 4 400 rpm for 2 hours (using a swing-bucket rotor, Eppendorf) and washed with sterile

distilled water for 2 hours. The protein was further spun at 3 600 rpm for 4 minutes and the collected protein quantified before freeze-drying and storage at -80 °C.

2.3.2 Protein determination: Bradford assay

Protein concentration of samples was determined according to the Bradford method (Bradford, 1976). BSA (Bovine Serum Albumin) was used as a standard to construct a standard curve ranging from 0.01 mg/mL to 0.6 mg/mL of BSA. Twenty micro liter of BSA standard was added to 200 µL of Bradford reagent per well in a microtitre plate. Absorbance was recorded at 620 nm on a Multiskan® BIOCROMATIC (Labsystems) plate reader and a standard curve obtained where samples were being assayed for protein concentration. Blanks consisted of sterile water and all assays were performed in duplicates. The remainder of the sample was placed on a freeze-drier overnight and the resultant powder was re-suspended in 500 µL of sterile distilled water.

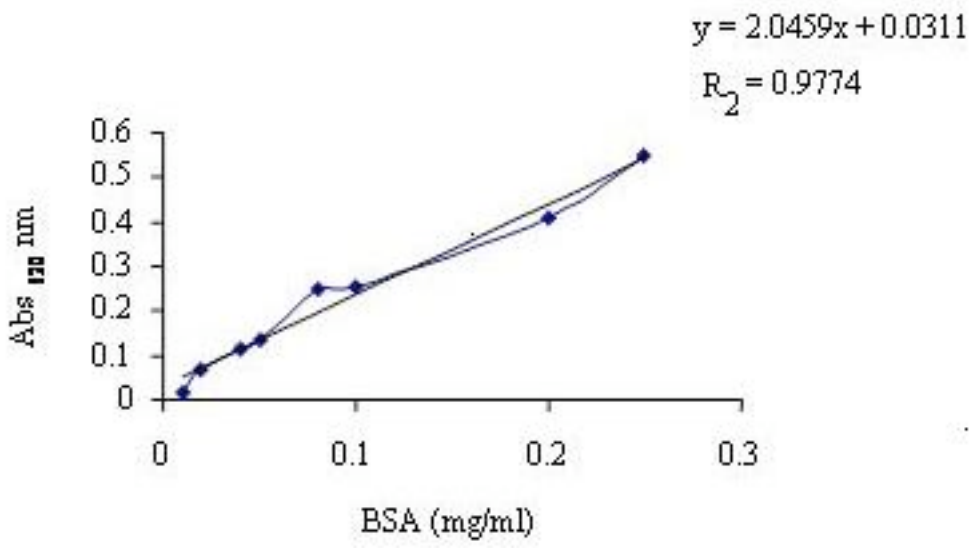

Figure 2.2: A standard curve using Bovine Serum Albumin (0.01 mg/mL-0.6 mg/mL) as standard.

2.4 Tissue culture

2.4.1 Germination of Arabidopsis seeds

Fifty *A. thaliana* seeds were transferred into a 1.5 mL Eppendorf tube submerged in 1 mL of 95 % ethanol and shaken for 60 seconds. Seeds were left to settle and the ethanol was removed and seeds were then repeatedly washed (4 times) with sterile distilled water. The seeds were then submerged into a sterilization solution (10 % bleach, a drop of Tween-20 and 17 mL of sterile water) was mixed with the seeds for 10 minutes by shaking followed by 10 minutes without shaking. The sterilizing solution was removed and the seeds were again washed (4 times). On an open Petri dish covered with sterile filter paper in the laminar flow, the 1.5 mL Eppendorf tube was inverted and seeds were left to dry out for an hour. The seeds were placed onto _ MS10 germination medium (2.2 g Murashige and Skoog Basal Salt Mixture (MS) media; Sigma, 10 g sucrose, 8 g agar, 0.5 g MES (2–Morpholinoethanesulfonic acid, Sigma), in 1 L, pH 5.7 with 2 M KOH/HCl) plate. The plates were sealed with parafilm covered in foil and placed at 4 °C for 3 days. After 3 days they were then placed under light cycle of 16 hours light/ 8 hours dark at 25°C. The seeds were then left to germinate. In a jar, MS 30 medium (4.4 g MS, 30 g sucrose, 8 g agar, 0.5 g MES in 1 L, pH 5.8 with 2 M KOH/HCl) was poured and allowed to set. The 4 weeks old seedlings were then transferred using a sterile forceps into the jar. A minimum of two seedlings per jar was transferred to allow the seedlings to develop into a full plant.

2.4.2 Preparation of callus from A. thaliana

The plants were removed from the jar onto a sterile Petri dish and the leaves were detached. The leaves were then finely chopped and the clumps of chopped leaves were transferred onto solid callus inducing medium (30 g sucrose, 4.43 g Murashige and Skoog with Minimal Organics (MSMO), 1 mg/mL Naphthalene acetic acid (NAA), 1 mg/mL kinetin, 0.8 % agar in 1 L, pH 5.8 with 1 M KOH/HCl). The plates

were incubated at light cycle of 16 hours light/8 hours dark. The resulting calli was also sub-cultured every 4 weeks and the old callus discarded.

2.4.3 Preparation of A. thaliana cell suspension

Callus from the callus induction plates was aseptically transferred to a sterile Petri dish. The callus was then divided into small pieces and transferred to suspension culture medium (30 g sucrose, 4.43 g MSMO, 1 mg/mL kinetin, 1 mg/mL NAA, in a 1 L, pH 5.7 with 1M KOH/HCL). The suspension was incubated at 23 °C while shaking at 118 rpm. After 7 days, the suspension culture (10 mL) was sub-cultured into fresh suspension media (90 mL) under sterile conditions and incubated under the same conditions as described above.

2.4.4 Protoplast preparation

The suspension culture was transferred into 50 mL polypropylene tube spun at 4 400 rpm at 23 °C for 10 minutes. The supernatant was discarded and an enzymatic solution (0.8 % w/v cellulase (Fluka), 0.2 % w/v pectinase (Worthington), 0.08 % w/v pectolyase (Sigma) made up in 400 mM sorbitol; pH 4.8) was added to the pellet. The tube was covered in foil and placed in a 37 °C shaking incubator at 135 rpm, for 3 hours. A further 1 mL of the same enzymatic solution was added to the cells and the suspension left incubating under the same conditions for a further 2 hours. The digested cell walls were filtered off through gauze and the filtrate spun down at 3500 rpm room temperature for 15 minutes. The supernatant was discarded and the pellet (protoplasts) were re-suspended in 2 mL of 400 mM sorbitol, pH 4.8 and placed on ice until used.

2.4.5 Stomatal guard cell assay

A. thaliana leaves were rinsed and submerged in stomatal assay solution (50 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 6.5) at 25 °C under an incandescent light (430 nm at 35 W m^{-2}) in a microtiter plate and treated with AtPNP-A at a final concentration of 150 ng/mL for an hour. As a control, an equivalent amount of sterile

water to protein was mixed with the assay buffer (90 μL) to cover the leaves under the above-mentioned condition. Stomatal guard cell pore widths were viewed using a microscope fitted with a calibrated ocular micrometer (data not shown).

2.5 A. thaliana protoplasts volume measurements

2.5.1 Microscope

Protoplasts used were prepared from 3 day old *A. thaliana* cell suspension cultures as described in section 2.4.4 and treated with either 150 ng/mL AtPNP-A or 20 μM LY 83583 (6-anilino-5,8-quinolinequinone, CalbioChem) or 10 μM 8-Br-cGMP and incubated at 23 °C for 15 minutes. The protoplasts were visualized under a microscope with a calibrated ocular micrometer and pictures were taken. The volume of > 50 randomly selected controls and treatments were calculated and the results analyzed by one-way analysis of variance (ANOVA) or paired Student's *t*-test.

2.5.2 FACScan system

Protoplasts used were prepared from 3 day old *A. thaliana* cell suspension cultures as described in section 2.4.4 and treated with either 150 ng/mL AtPNP-A, a combination of 150 ng/mL AtPNP-A and 20 μM LY 83583, 10 μM 8-Br-cGMP (8 -bromo - guanosine 3´5´-cyclic monophosphate, Sigma) and 20 μM LY 83583 and incubated at 23 °C for 15 minutes. Two hundred μL of protoplast suspension from each treatment was analyzed by flow cytometry at 488 nm wavelength using a FACS SCAN (Becton Dickinson, California, USA) and FACS Calibur™ soft ware (Vermeulen *et al*., 2002; Herzig *et al.*, 2003; Nile, 2003).

Flow cytometry is used to detect size, granularity or fluorescence whereas size is the easiest of all to be detected. Inside a flow cytometer, the laser beam is projected at a specific point in the system. Particles (cells) pass through this beam and some of the incident light will be scattered, and the amount as well as the intensity of this scattered light will be proportional to the characteristic that is being measured. Most light will scatter to the forward direction (FSC), which is dependant of the size

particle. The amount of light scattered at right angles to the incident light beam is dependent on the internal complexity of the particle, and this is known as side scatter (SSC). The FSC as a measurement of size was set at a threshold. A fluidic system is used to carry the particle (cells) one by one towards the sensing region. The optical system generates and collects the light pulses according to size, granularity and fluorescence's. The fluidic elements in a flow cytometer are also used to position the sample particles in the center of the laser beams and this is known as hydrodynamic focusing. The flow cell chamber is where the cell is introduced and below it is a sample uptake needle that goes into the sample. Firstly, a continuous flow sheath of fluid is created inside the cell chamber and when pressure is applied to the sample tube, the cells in suspension are gradually forced into the center thereby creating a co-actual. The sample stream remains separate within the sheath fluid stream and the laser can interrogate a particle at a time (for details see FACScan manual, Becton Dickinson).

2.6 A. thaliana cell volume measurements

2.6.1 Microscope

Five milliliters of 3-day-old suspension cells were transferred into a 50 mL polypropylene tube and spun at 4 400 rpm at room temperature for 15 minutes. The supernatant was discarded and 400 mM sorbitol; pH 4.8 was added to the cell pellet until further treatments. The cells were treated with either 150 ng/mL AtPNP-A or 10 μM 8-Br-cGMP and incubated at 23 °C for 15 minutes. The cells were visualized under a microscope with a calibrated ocular micrometer and pictures were taken. The volume of > 50 randomly selected controls and treatments were calculated and the results analyzed by one-way analysis of variance (ANOVA) or a paired Student's *t*-test.

2.7 ***Effect of expansins***

2.7.1 Expansin measurements on A. thaliana protoplasts

Protoplasts were isolated using the method described by Pandey *et al*., (2002). *A. thaliana* leaves major veins were cut using a razor blade and the leaves blended for 1 minute and repeated for 30 seconds (depending on the number and toughness of the leaves) in 100 mL of cold tap water using a commercial Waring blender that was connected to a Variac set to 85 V. The blender mixture was poured through a 200 µm mesh to remove broken mesophyll and epidermal cells. The peels which are pale green were transferred into a 50 mL Erlenmeyer flask containing 10 mL of enzyme 1 solution (0.7 % (w/v) cellulysin cellulose from *Trichoderma viride,* (Calbiochem, La Jolla, CA, USA), 0.1 % (w/v) polyvinyl pyrrolidone 40 000 (PVP- 40), 0.2 % (w/v) bovine serum albumin (BSA Fraction V, Sigma) and 0.5 mM L-ascorbic acid dissolved in 55 % (v/v) basic solution (5 mM 2 –[N-morpholino] ethane sulfonic acid -Tris (Mes-Tris), pH 5.5, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 µm KH_2PO_4, 0.5 mM ascorbic acid (Sigma, St Louis, MO, USA) and 0.55 M sorbitiol) and 45 % (v/v) distilled water. The flask was placed in a shaking water bath at 27 °C for 30 minutes with a shaking speed set at 140 excursions per minute. Digestion was performed in darkness. After 30 minutes, 30 mL of basic solution was added and the mixture was shaken for an additional 5 minutes under the same conditions. Digested peels were collected on a 200 µm nylon mesh and rinsed gently with basic solution and placed in 10 mL of enzyme 2 solution (1.3 % (w/v) Onozuka RS cellulose (Yakult Honsha, Tokyo, Japan), 0.0075 % (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), 25 % (w/v) BSA and 0.5 mM L-ascorbic acid in basic solution) in a clean 50 mL Erlenmeyer flask. The flask was shaken at 60 excursions per minute in the dark at 20 °C for 30 minutes. The shaking speed was further reduced to 40 excursions per min and digestion was stopped at 45 minutes. At the end of the digestion period the flask was swirled gently by hand for a few seconds. The mixture was filtered through 20 µm nylon mesh and the peels remaining on the mesh were washed with 100 mL of basic solution to release GCP (guard cell protoplast) adhering to them. The filtrate

was collected in a four 50 mL tubes and the protoplast were subsequently spun in a bench top centrifuge for 5 minutes at 1 100 rpm. All but 2 mL of supernatant were carefully removed from each loose pellet and the (pellet) re-suspended in 25 mL basic solution with a second identical centrifugation is performed. After removal of supernatant, each GCP pellet was gently re-suspended yielding a volume of 3 mL. The protoplasts were then treated with 150 ng/mL expansin for 20 and 40 minutes. The protoplasts were visualized under a microscope with a calibrated ocular micrometer and pictures were taken. Volume of > 50 randomly selected controls and treatments (depending on the quality of cells) were calculated and the results analyzed by computer program ImageQuant 5.0 Windows NT.

2.7.2 Extension measurements of cucumber hypocotyls

Cucumber hypocotyls were prepared as described in McQueen-Mason *et al*., (1992). The hypocotyls were frozen and thawed and abraded with carborundum to disrupt the cuticle. The hypocotyls were then boiled in water for 15 seconds and secured between two clamps with about 5 mm between the two clamps under a constant tension of 20 g. The walls were then filled with bathing solution (50 mM sodium acetate, pH 4.5) for 20 minutes. Movements of the lower clamps were detected with an electronic position transducer and recorder on a microcomputer. Extensions of the cucumber hypocotyl walls were measured in the same fashion, except that the apical 1 cm region of the hypocotyl was fixed to the clamps. After 20 minutes, the bathing solution was changed and the same buffer was added and subsequently treated with 150 ng/mL of AtPNP-A and expansin, and 'native' hypocotyls. The walls are referred to as 'native' because they have been frozen, thawed and abraded.

RESULTS

CHAPTER THREE

3.1 Conformation of correct insert

Figure 2.1(a) and (b) shows the pCRT7/NT AtPNP-A construct obtained from M. Morse (UWC etc). AtPNP-A gene (without its signal peptide) was cloned into the linearised pCR®T7 TOPO® TA vector (Figure 2.1(a) and Figure 2.1 (b)).

In order to confirm the presence of the gene, a double digestion of the construct with BamHI and EcoRI was carried out. This digestion was aimed at liberating the AtPNP-A gene fragment. The digested material was resolved on an agarose gel (Figure 3.1) and the results confirmed the presence of the AtPNP-A gene (bp) on the pCR®T7 TOPO® TA vector.

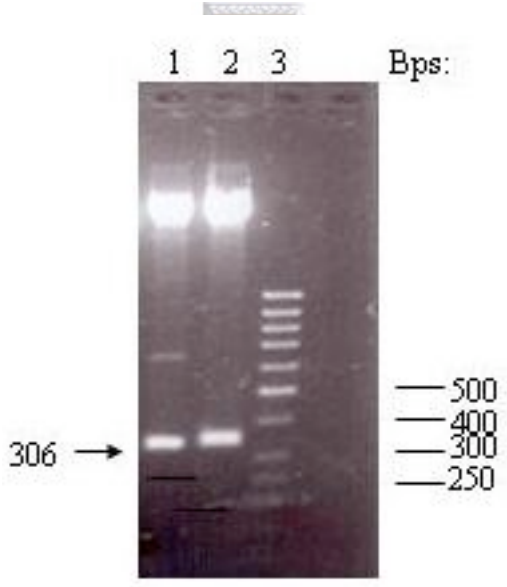

Figure 3.1: Double restriction enzyme (RE) digestion of pCRT7/NT AtPNP-A using BamHI and EcoRI. The size fractionation was resolved on a 2 % agarose gel stained with 0.5μg/mL ethidium bromide. Lane 1 represent RE digestion of BamHI and EcoRI releasing an insert size of 306 base pairs. Lane 2 represents RE digestion of BamHI and Hind III and lane 3, a 50 base pair DNA ladder.

3.1.2 Verification of expression of gene

Figure 3.2 shows the expression of recombinant AtPNP-A using the BL21 (DE3) Star pLysS cell line. The expression of AtPNP-A was induced by the addition of IPTG. Expression of any gene cloned into pCR®T7 TOPO® TA vector is controlled by a very strong T7 phage promoter present on this plasmid (Figure 2.1(a) and Figure 2.1(b)). This promoter is specifically recognized by a T7 polymerase for control of expression. It is therefore always necessary to deliver a T7 RNA polymerase to those cells that are transformed with this plasmid through either induction of expression on the polymerase gene found on the plasmid or by infecting the transformed cells with a phage that has the ability to express the polymerase (Invitrogen, pCR®T7 TOPO® TA Expression Kit). In this study, the former approach was employed and AtPNPA was successfully expressed as shown in Figure 3.2. When comparing the induced with the uninduced culture, the protein of expected molecular mass (approx. 18.7 kDa) is seen in induced culture and is absent from the uninduced culture.

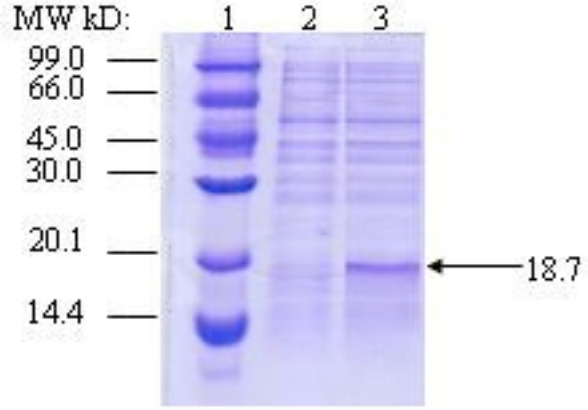

Figure 3.2: Resolution of the AtPNP-A protein on an SDS-PAGE after its expression in pBL21DE3 star plysS cells. Lane 1 represents the low molecular weight marker, lane 2 represents the uninduced culture of BL21DE3 star pLysS cells and lane 3 represents the induced BL21DE3 Star pLysS cells.

3.2 Discarding impurities and restoring folding of protein using Ni-NTA matrix

Purification of AtPNP-A was performed under denaturing conditions (Figure 3.3) since the recombinant was found to be present as insoluble inclusion bodies. The sequestering of proteins into inclusion bodies normally occurs when eukaryotic proteins expressed in *E.coli* are incorrectly folded. The improper formation of disulphide bonds in the reducing environment of the *E.coli* cytoplasm may also contribute to incorrect folding and formation of inclusion bodies (Wingfield and Palmer, 1995). After denaturation and refolding of the protein on the Ni-NTA matrix, the recombinant was successfully eluted using 250 mM imidazole. A total of 0.2 mg/mL perhaps we should give a total value recombinant was obtained from 1L of bacterial culture.

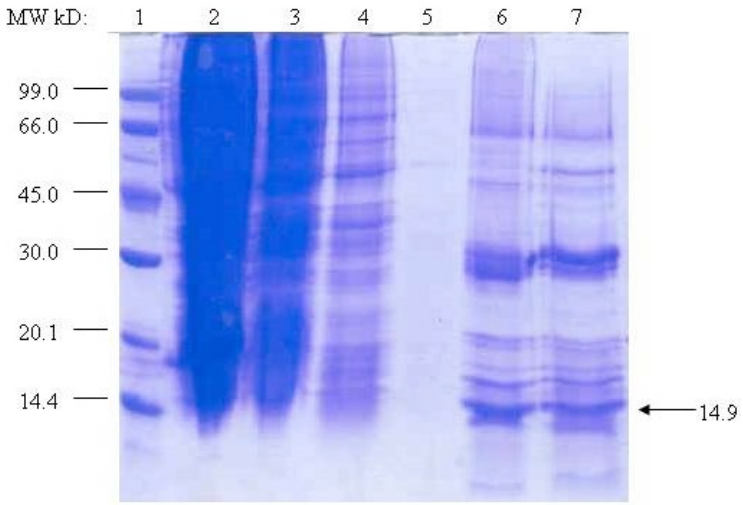

Figure 3.3: SDS-PAGE analysis of fractions obtained during purification of AtPNPA using a Ni-NTA agarose under denaturing conditions. 5 μL of the protein marker were loaded in lane 1. In lane 2, 20 μL of the cleared lysate of cell culture after denaturation in buffer B were loaded whereas the same volume of the flow-through was loaded in lane 3. Lanes 4, 5 and 6, each contains 20 μL of the different washes of the bound resin with buffer B and buffer C respectively. Lanes 6 and 7 was loaded with 20 μL of the eluted AtPNPA protein with 250 mM imidazole in 1 M urea, pH 7. Several bands were resolved in lane 7 indicating the capabilities of the AtPNPA protein (14.96 kDa) and its C-terminus fragment (3.74 kDa) to form different interactions with each other.

3.3 Confirmation on the activity of the protein

The activity of the purified recombinant protein was assessed by a stomatal guard cell assay and by measuring protoplast volume changes. Purified recombinant was shown to increase the stomatal pore widths of *A. thaliana* leaves treated with 150 ng/mL of the protein (data not shown) and this was taken as an indication that the recombinant protein was active.

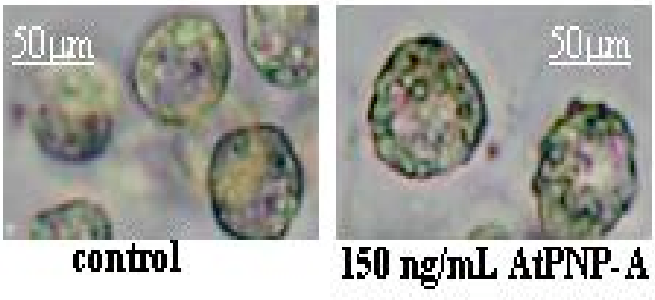

Figure 3.4: *A. thaliana* protoplasts suspended in 400 mM sorbitol. The protoplasts were treated with 150 ng/mL AtPNP-A for 15 minutes and photographed under a microscope. Diameters of randomly selected cells were then measured and their volumes calculated assuming spherical shapes of protoplasts.

When examining the effect of the recombinant AtPNP-A on *A. thaliana* protoplasts prepared from suspension culture cells, the treatment of protoplasts with AtPNP-A resulted in an increase in volume (Figure 3.4), while the treatment of protoplasts with a combination of 150 ng/mL AtPNP-A and 20 µM LY 83583 had no effect on protoplast volume (Figure 3.4.1). Treatment of protoplasts with 10 µM 8-Br-cGMP, however, resulted in a slight increase in volume (Figure 3.4.1).

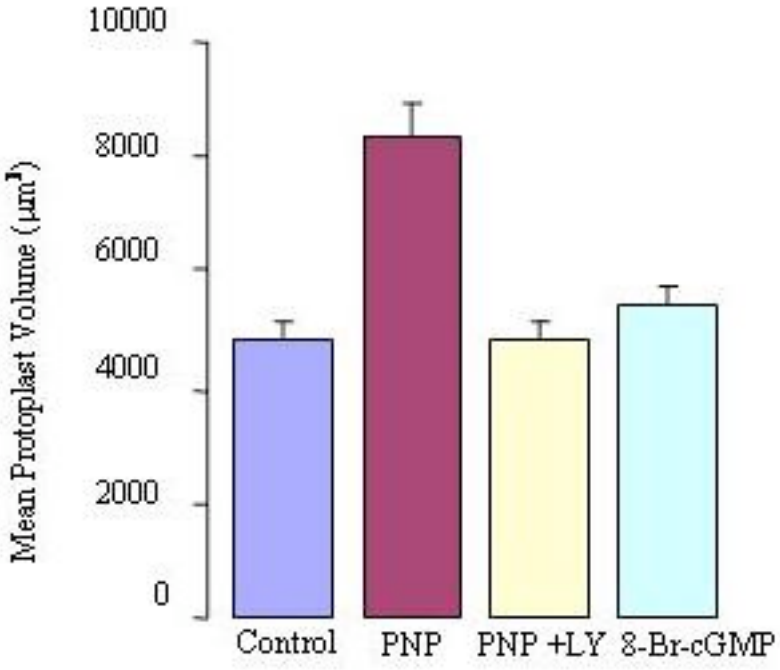

Figure 3.4.1: A representation of *A. thaliana protoplasts* S.E.M. Protoplasts were prepared from suspension cultures and subjected to different treatments. After treatment, cells were photographed under a microscope and diameters of randomly selected protoplasts were measured. Volume of protoplasts was calculated assuming spherical shape of protoplasts.

3.4 Comparing results between microscope and FACScan system

Flow cytometry analysis of protoplasts treated with either 150 ng/mL AtPNP-A, a combination of 150 ng/mL AtPNP-A and 20 μM LY 83583, or 10 μM 8-Br-cGMP confirmed the results obtained by visual analysis of protoplasts. In the FACScan system, a shift in volume between Region 1 and Region 2 signifies either an increase or decrease in protoplasts volume, depending on the direction of the shift. The data represented in the dot plot (Figure 3.5) clearly shows a shift in protoplasts suspended in 100 mM sorbitol, indicating an increase in volume. This increase in volume is expected since the suspension media was changed from 400 mM to 100 mM, a change in osmolarity that results in net water uptake. This shift in volume is also observed when protoplasts are treated with the recombinant AtPNP-A, confirming an increase in volume in response to the peptide. The dot plot presentation does not indicate an increase in shift between R1 and R2 regions for 20 μM LY 83583, the

combination of 20 μM LY 83583 and 150 ng/mL AtPNP-A or 10 μM 8-Br-cGMP treatments, suggesting no change in volume under these conditions.

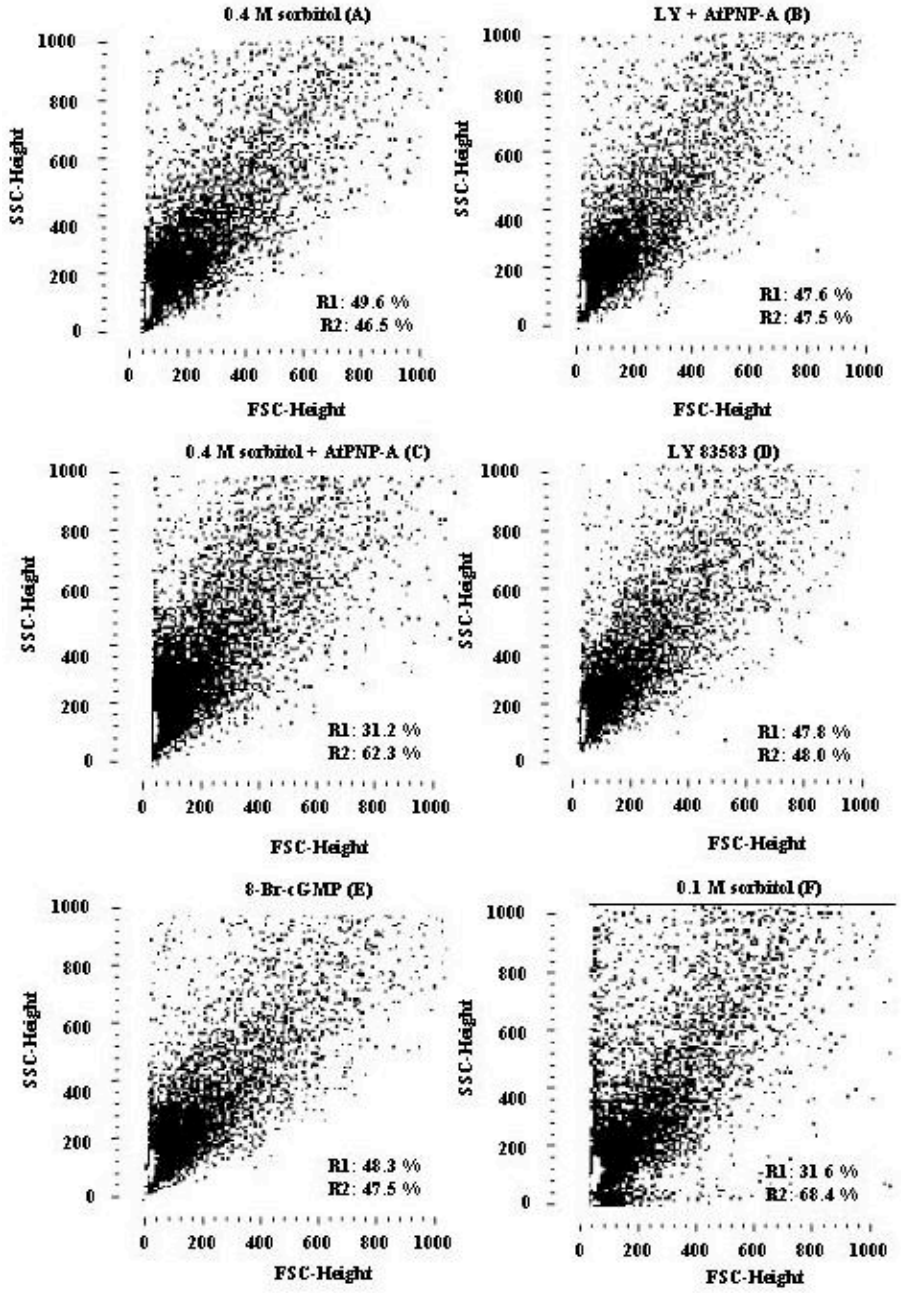

Figure 3.5: A Dot plot representation of *A. thaliana* protoplasts resuspended in 400 mM sorbitol (A), treated with 150 ng/mL AtPNP-A (C), a combination of 150 ng/mL AtPNP-A and 20 μM LY 83583 (B), as well as 20 μM LY 83583 (D) and 10 μM 8-Br-cGMP (E) for 15 minutes. The plot also shows the effects of dilution on sorbitol concentration from 0.4 M to 0.1 M (F) before a flowcytometric analysis**.**

3.5 Determining the effects of AtPNP-A on whole cells

When examining the effect of the recombinant AtPNP-A on *A. thaliana* suspension culture cells, treatment of cells with recombinant protein resulted in an increase in volume (Figure 3.6) while treatment of cells with AtPNP-A and 8-Br-cGMP had an increase in volume respectively (Figure 3.6.1). These results could not be confirmed by flow cytometry analysis on suspension culture cells since suspension cells are clumped which would make it difficult to accurately measure the volume changes of single cells.

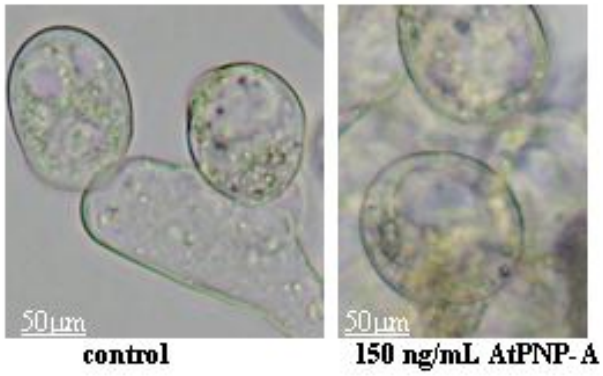

Figure 3.6: *A. thaliana* suspension culture cells were treated with 150 ng/mL AtPNP-A for 15 minutes. The cells were then viewed and photographed under a microscope.

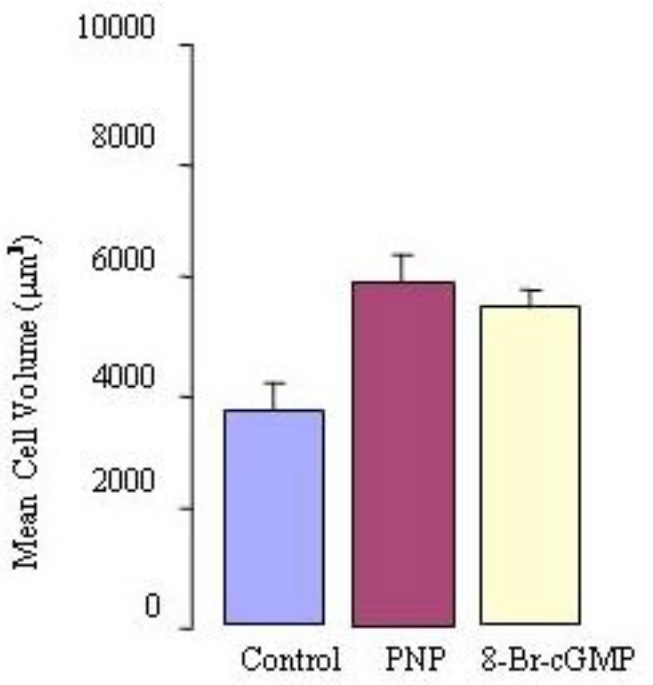

Figure 3.6.1: A representation of *A. thaliana* suspension culture cells S.E.M. After treated cells were photographed under a microscope and diameters of randomly selected cells were measured. Volume of cells was calculated assuming spherical shape of cells.

3.6 Determining if recombinant AtPNP-A has expansin activities

When examining the effect of expansins on cell volume, no changes in cell volume are observed when protoplasts are treated with 150 ng/mL expansin (Figure 3.7). The effect of AtPNP-A on cucumber hypocotyls was analyzed using an extensometer and showed that recombinant AtPNP-A did not produce a 'creeping' response (Figure 3.7.1) when compared with expansin controls.

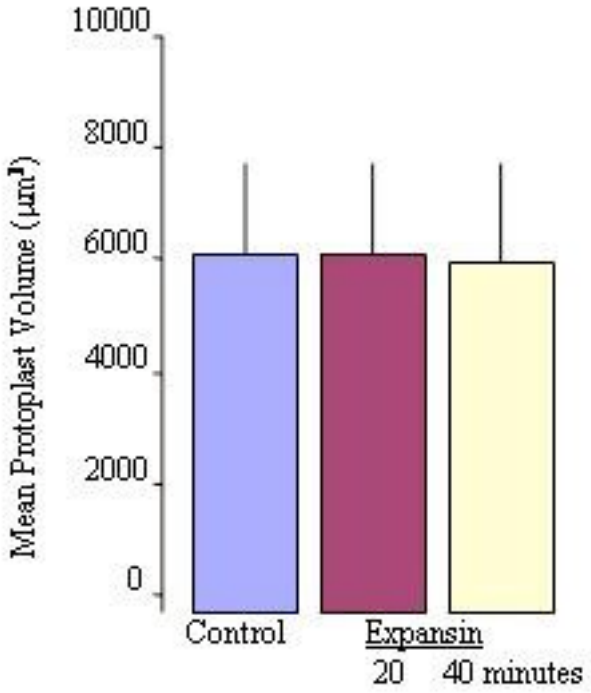

Figure 3.7: *A. thaliana* protoplasts were treated with 150 ng/mL expansin followed by measurement of cell volumes. Protoplasts were untreated for 0 min (control) or treated with expansin for either 20 min or 40 min before volume determination. In all cases, diameters of randomly selected cells were measured and their volumes calculated assuming spherical shapes of cells.

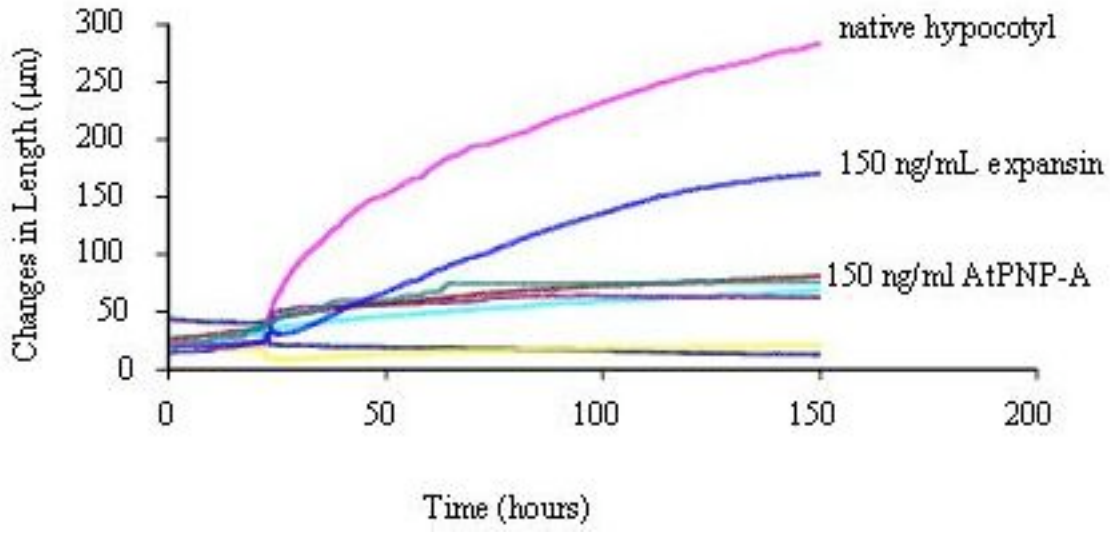

Figure 3.7.1: Extension of native and reconstituted cucumber hypocotyls walls. Apical 1-cm tissues were frozen, thawed, abraded and then pressed prior to suspension in the extensometer. Samples were clamped under applied force of 20 g and extension was recorded using a linear voltage displacement transducer (Cosgrove, 1998). The specimen length between the clamps was 5mm.

3.7 Expression profile of AtPNP-A

Microarray data found on genevestigator server (www.genevestigator.ethz.ch) indicates the expression profile of AtPNP-A (Figure 3.8(a) and 3.8(b)). The data shows that AtPNP-A is strongly up-regulated in response to K+ and Zn+ treatment. Experimental evidence has suggested that irPNP-A and AtPNP-A could indeed promote H_2O net up-take even under conditions of low K^+ availability (Maryani *et al*., 2001; Morse *et al*., 2004). If PNPs do a play a role in the compensatory mechanism, one would expect the encoding gene(s) to be up-regulated under conditions osmotically unfavourable for H_2O up-take. Zinc also provides a structural role in many transcription factors and is a cofactor of RNA polymerase, making it vital to cells. Zn^+ is involved in the homeostasis of Zn^+ ions in the plant cell by affecting ion transporters in the cell membrane.

Factor	# of Chips	Mean	Std-Dev	Std-Err	Ratio	Control samples
Biotic: A. tumefaciens (+)	2	963	345	345	4.01	Biotic: A. tumefaciens (-)
Biotic: A. tumefaciens (-)	2	240	19	19		
Biotic: E. cichoracearum (+)	4	3272	469	271	10.18	Biotic: E. cichoracearum (-)
Biotic: E. cichoracearum (-)	4	321	68	39		
Biotic: M. persicae (+)	3	1123	1072	758	2.05	Biotic: M. persicae (-)
Biotic: M. persicae (-)	3	549	320	226		
Biotic: mycorrhiza (+)	1	79	0	0	0.5	Biotic: mycorrhiza (-)
Biotic: mycorrhiza (-)	1	157	0	0		
Biotic: nematode (+)	1	466	0	0	1.27	Biotic: nematode (-)
Biotic: nematode (-)	1	366	0	0		
Biotic: P. infestans (+)	6	744	268	120	4.34	Biotic: P. infestans (-)
Biotic: P. infestans (-)	6	171	117	52		
Biotic: P. syringae (+)	12	1020	1286	388	0.88	Biotic: P. syringae (-)
Biotic: P. syringae (-)	12	1158	1430	431		
CO2: control	2	2460	805	805		
CO2: high	2	2111	374	374	0.86	CO2: control
Hormone: ABA (+)	4	677	428	247	1.16	Hormone: ABA (-)
Hormone: ABA (-)	4	584	506	292		
Hormone: ACC (+)	4	735	404	233	1.26	Hormone: ACC (-)
Hormone: ACC (-)	4	584	506	292		
Hormone: BL (+)	4	1729	2124	1226	2.96	Hormone: BL (-)
Hormone: BL (-)	4	584	506	292		
Hormone: GA3 (+)	4	910	665	384	1.56	Hormone: GA3 (-)
Hormone: GA3 (-)	4	584	506	292		
Hormone: IAA (+)	4	807	689	398	1.38	Hormone: IAA (-)
Hormone: IAA (-)	4	584	506	292		
Hormone: MJ (+)	4	757	781	451	1.3	Hormone: MJ (-)
Hormone: MJ (-)	4	584	506	292		
Hormone: zeatin (+)	4	559	411	237	0.96	Hormone: zeatin (-)
Hormone: zeatin (-)	4	584	506	292		

Figure 3.8(a): Microarray expression profiling data of AtPNPA from the Genevestigator server.

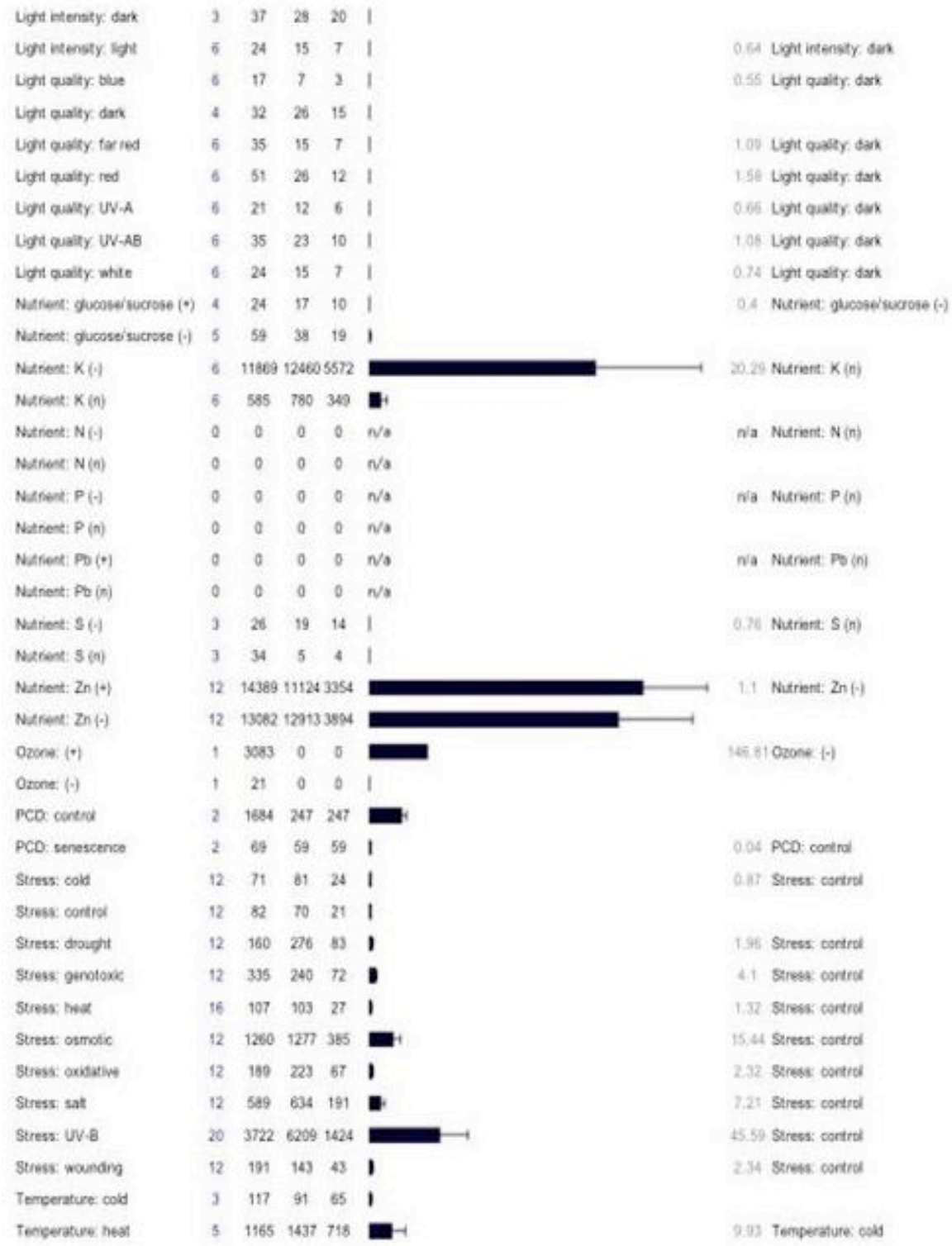

Figure 3.8(b): Microarray expression profiling data on AtPNPA from the Genevestigator server.

DISCUSSION

CHAPTER FOUR

4.1 Relating recombinant AtPNP-A in this study

Natruiretic peptides are a family of peptide hormones that occur in the brain and periphery of vertebrates and are strongly implicated in the regulation of salt and water balance (Rosenzweig and Seidman, 1991). Atrial natriuretic peptides (ANPs) also referred to as atrial natriuretic factors or atriopeptins were first discovered in extracts of rat atria (deBold *et al*., 1981). Biological responses in plants to vertebrate ANP have been reported and led to the hypothesis that plants may also contain ANP-like molecules that play a role in homeostasis (Vesely and Giordano, 1991; Gehring *et al*., 1996, 1999). Maryani *et al*., (2000) reported the immunaffinity purification of novel biologically active proteins from ivy (*Hedera helix*) and potato (*Solanum tubersum*) with antibodies directed against vertebrate ANP. These were termed immunoreactant plant natriuretic peptides. Immunoaffinity purified PNP from *Solanum tubersum* (StPNP) was partially sequenced and two closely related molecules identified in *A. thaliana* (AtPNP-A and AtPNP-B) (Ludidi *et al*., 2002).

AtPNP-A is a small protein of 126 aa in length (MW: 14016kDa; pI: 9.22) that is encoded by a gene with a single intron of 100 bp (Ludidi *et al*., 2002). AtPNP-A, its related sequence AtPNP-B and orthologues in other higher plant species, shares a domain with the cell wall loosening expansin. RT-PCR has revealed AtPNP-A transcripts in leaf tissue from unstressed *Arabidopsis* plants (Ludidi *et al*., 2002), indicating that the protein is not only found, but also synthesized, in leaves. An alignment of AtPNP-A sequence with vertebrate ANPs shows that the common epitope is not in the signal peptide, but the N-terminus of the cleaved peptide/protein (Ludidi *et al*., 2002). Synthesis and processing pathways of vertebrate NPs is complex and NPs are synthesized from precursors; vertebrates ANPs are highly conserved molecules and consist of 28 amino acids that are the C-termini cleaved

from 126 aa precursor molecules with a molecular mass ≥10 kDa. The cleaved C-terminus ANP (99-126) seems to be present only at exceedingly low concentrations and the presence of two bands in the molecular weight range of the vertebrate precursors might suggests that in plants also there is more than one molecular species that contains the epitope of the C-terminus (Pharmawati *et al*., 1998b). The term irPNP is used to define all plant molecules that are specifically recognized by the antiserum raised against the ANP C-terminus and thus share an epitope with ANP. The C-teminus (99-126) of the molecule is associated with some biological activities and hence it was chosen in this study.

4.2 Isolated and purified recombinant protein

In this study AtPNP-A was expressed in an IPTG- inducible bacterial expression system. The pCRT7 expression vector was chosen to produce an N-terminal His-tag fusion recombinant protein and thus facilitate easy purification using a Ni-NTA matrix. The recombinant protein was expressed in the BL21 DE3 STAR pLysS cell line and upon analysis, the protein of molecular mass (14.9 kDa) was found to be insoluble. This is not uncommon as eukaryotic proteins expressed intracellularly in *E.coli* are frequently sequestered into insoluble inclusion bodies. The intermolecular association of hydrophobic domains during folding is believed to play a role in the formation of inclusion bodies. For proteins with cysteine residues, improper formation of disulphide bonds in the reducing environment of the *E.coli* cytoplasm may also contribute to incorrect folding and formation of inclusion bodies (Wingfield and Palmer, 1995). Since biologically active recombinant AtPNP-A was required, it was decided to follow the protein refolding recommendation as described in literature. As such, the recombinant protein was purified under denaturating conditions on the Ni-NTA matrix (Wahle *et al*., 1999), with a yield of successfully refolded protein at a concentration of 0.2 mg/mL protein per litre of bacterial culture.

Rafudeen *et al*., (2003) had successfully expressed (AtPNP-A) in a yeast expression system and established the recombinant as a true plant immunoanalogue of ANPs. The recombinant was also shown to be biologically active and lead to a concentration-dependent increase in protoplast volume (Rafudeen *et al*., 2003).

4.3 Activity of purified protein is established

Stomatal aperture changes play a role in plant homeostasis and is/are caused by guard cell volume changes whereby volume increases lead to the opening of the pore and volume reduction leads to closure. This process is turgor-based and modulated by environmental conditions, plant hormones, ion channels and second messengers (Pharmawati *et al*., 2001; Blatt, 2000). Stomatal opening has previously been reported in response to both rat ANP and immunoaffinity-purified PNPs and these findings have suggested that an endogenous natriuretic peptide-like system does have a role in plant homeostasis (Gehring *et al*., 1996; Billington *et al*., 1997). In this study the biological activity of the recombinant protein was tested in a stomatal guard cell assay (Gehring *et al*., 1996; Billington *et al*., 1997). When *A. thaliana* leaves were treated with the purified recombinant an opening of the stomatal pores was observed when leaves were viewed under a microscope, confirming that the protein was active (data not shown).

When treating *A. thaliana* protoplasts with the recombinant protein at a concentration of 150 ng/mL, an increase in protoplasts volume was observed (Figure 3.4). Previous studies had shown that protoplasts treated with irPNP, ANP and recombinant PNP induced an increase in protoplast volume (Billington *et al*., 1997, Gehring *et al*., 1996, Morse *et al*., 2004).

4.4 Confirm results between different experimental systems

In addition to the microscopic analysis of protoplast volume changes in response to the recombinant protein, another method was employed to determine volume changes in protoplasts. One of the major parameters (measurement of light scatter signal) of the FACscan is to determine cell size/volume as described previously in section (3.5). FACScan analysis indicated an increase in volume (or swelling) of protoplasts in response to 150 ng/mL of the recombinant protein, confirming results obtained by microscopic analysis of protoplasts.

Several PNP-dependent processes have been observed in experimental systems that did not contain cell walls such as protoplasts or microsomal and plasma membrane vesicles. The processes include *in vitro* PNP binding to isolated *Tradescantia multiflora* leaf microsomes, PNP-dependent modulation of plasma membrane H^+ gradients in potato leaf tissue vesicles, increases of cGMP levels in response to PNP in potato guard cell protoplasts and PNP-dependent volume changes in protoplasts (Suwastika *et al*., 2000; Maryani *et al*., 2000; Pharmawati *et al*., 2001; Maryani *et al*., 2001).

PNPs have also been shown to promote stomatal opening to rapidly and specifically induce transient elevation of the second messenger cGMP and to modulate cation fluxes in maize (*Zea mays*) root stele tissue (Billington *et al*., 1997; Pharmawati *et al*., 1998 Pharmawati *et al*., 1999).

4.5 Relationship between AtPNP-A and cGMP

The guanylate cyclase inhibitor LY 83583 has extensively been used as a tool in NP research in animals even though there is considerable contradiction about its precise mode of action. Some reports suggest that LY 83583 antagonises the soluble guanylate cyclase whereas others claim that LY 83583 is specific for the particulate form of the enzyme (von Ruecker *et al*., 1989; Malta *et al*., 1988). The discussion is further complicated by different responses in different species and tissues; a situation

which could be similar in plants. LY 83583 inhibits different cGMP dependent processes such as GA dependent gene induction in barley aleurone layers and stomatal openings in *Tradescantia sp.* (Penson *et al*., 1996; Pharmawati *et al*., 1998). The treatment of protoplasts with the GC inhibitor LY 83583 resulted in a decrease in protoplast volume (as determined microscopically). These results are consistent with previous reports where Maryani *et al*., (2001) showed that LY 83583 as an inhibitor of soluble rather than particulate guanulate cyclase on intact MCP are NP-dependent activation of an LY 83583-resistant membrane bound enzyme.

While it is not known how NPs function in osmoticum-dependent volume regulation in plant cells and what the role of cGMP is in the process (Maryani *et al*., 2000) have put forward the possibility of two direct modes of action leading to enhanced osmoticum-dependent volume increases (Figure 1.2). The first is an effect on aquaporins while the second is an aquaporin-independent effect on plasma-membrane water permeability. The model also accounts for the cGMP-dependent reduction of osmoticum-dependent volume increases proposing that cGMP might exert this effect via down-regulating aquaporin activity and it includes NP-effects on chloride channels transport (Maryani *et al*., 2000). A conserved family of chloride channels has been implicated in cell volume regulation in plants and chloride channels like CiC-2 have been shown to be activated by cell swelling (Hechenberger *et al*., 1996; Lurin *et al*., 1996). It is noteworthy that irPNP has earlier been shown to induce net Cl^- uptake into plasma-membrane vesicles and it remains to be seen if the response was induced by irPNP acting directly on chloride channels or occurred as a secondary effect of irPNP-induced swelling (Maryani *et al*., 2000).

4.6 Conclusive evidence that AtPNP-A are not expansins

The most closely related molecules to irPNPs are expansins. Expansins have extended C-termini when compared to irPNP-s. Expansins are in turn related to glucanases and cellulases and in the case of glucanses and cellulose, the C-termini have been shown to be cell wall binding (Linder and Teeri 1997; Linder *et al*., 1998)

and the same function has been suggested for the expansin C-terminus (Cosgrove, 2000). Since expansin, the closest relatives of irPNPs, and the more distantly related glucanases and cellulose contain the C-terminus, it was reasonable to argue that irPNP-like molecules had in fact lost this domain. Such a domain loss could lead to increased mobility of the molecule, an argument supported by the fact that another related molecule CjBAp12 appeared to be systemically mobile protein present but not synthesized in leaves (Ceccardi *et al*., 1998). IrPNP molecules also appeared to be functionally further diverged from the ancestral endoglucanases since the HFD (His-Phe-Asp) motif shared by expansins and the endoglucanases was absent from the irPNP-like protein (Ludidi *et al*., 2001). Based on the sequence comparisons it was proposed that ancestral glucanases had given rise to the expansins and also had been recruited to serve in capacities entirely different from cell wall loosening. These recruited molecules may play a part in stress responses and possibly function as extracellular signaling molecules that directly or indirectly affect water and ion transport. Such conclusions were not supported by the domain structure but by the fact that PNPs were initially isolated using an antibody directed against ANP, a vertebrate signal peptide with a role in water and solute homeostasis (Billington *et al*., 1997; Gehring *et al*., 1999).

GENERAL CONCLUSION

Maintenance of water and solute homeostasis is a key requirement for living systems and in vertebrate homeostasis is in part achieved by natriuretic peptides (NPs), a family of peptide hormones (Anand-Srivastavaand and Trachte, 1993; Zeidel, 1993).

A recombinant plant natriuretic peptide from *A*. *thaliana*, AtPNP-A, which has a role in homeostasis, has been partially characterized in this study. While AtPNP-A induces net water uptake in protoplast it also induces net water uptake in cells and with intact protoplast. This finding is consistent with evidence that both atrial natriuretic peptides have been shown to affect water net uptake into potato mesophyll protoplasts (Maryani *et al*., 2001) and thus provide more details evidence for the role of plant natriuretic peptides in plant growth and homeostasis while AtPNP-A induces net water uptake in protoplasts.

As a second messenger to NP pathway (i.e. AtPNP-A), guanylate cyclase has shown to contribute to plant homeostasis. Expansin are involved in loosing cell wall when turgor pressure is applied within the cell, AtPNP-A, on the other hand increases cell volume. The results obtained here shows that AtPNP-A, on its own does not induce cell wall extension 'creeping'. A finding that supports the 'idea' that AtPNP-A and expansin work together. Interestingly, AtPNP-A induces net water uptake in cells with intact cell walls while exerting no effect on hypocotyls where native expansin have been inactivated. Future research will involve the elucidation of the functional relationship between expansin and PNPs making use of both expansin and AtPNP-A mutants.

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