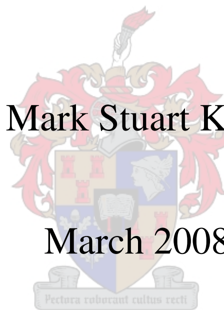
Towards the Identification and Isolation of VvPNP



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March 2008

Declaration

I hereby declare that " **Towards the Identification and Isolation of VvPNP** " is my own work, that has not been submitted for any degree or examination at any other university and that all the sources I have used or quoted have been identified and acknowledged by complete references

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.... Date: ....

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Abstract

We have identified a novel plant natriuretic peptide (PNP) like gene in *Vitis vinifera* cultivars of Chardonnay and Pinotage. The transcript of the gene was isolated from young leaves indicating that the peptide may function within these organs of the plant. The 15 kDa peptide, which we have putatively called *Vitis vinifera* plant natriuretic peptide (VvPNP), was expressed in a bacterial system as a recombinant fusion protein. This protein shares sequence similarity to other recognised natriuretic peptides, and was largely identified by using the primary sequence of the well characterised plant natriuretic peptide (AtPNP-A) from the model organism *Arabidopsis thaliana.* Previous studies have identified PNPs across a range of plant species such as *Dracena godseffiana, Hedera helix* and *Solanum tuberosum*, where the biological activity associated with water and solute homeostasis has been proven. Instrumental to the functionality of these peptides is the conservation of two cysteine residues which form a disulphide bridge, of no less than 23 amino acids apart, creating a secondary ring structure. Alignment of the VvPNP and AtPNP-A primary structures indicates that the two cysteine residues necessary for physiological function in AtPNP-A are in conserved positions within VvPNP. Also 14 identical amino acids and 7 conservative amino acids aligned within the active domain of the AtPNP-A molecule. The putative natriuretic peptide also displays two diagnostic amino acids motifs characteristic in PNPs and one other molecule CjBAp12, which is associated with citrus blight. On a nucleotide level the *VvPNP* contains a 100bp intron which is also found within the genomic sequence of *AtPNP-A*. Plant natriuretic peptides have sequence similarities to expansins, molecules which directly modify the mechanical properties of cell walls leading to turgor-driven cell extension. Although PNPs do not contain a wall-binding domain, evidence does suggest an evolutionary relationship between expansins and PNP molecules because of these similarities. The target site for PNPs being the cell membrane and not the cell wall, and it is for this reason that PNPs affect protoplasts. Although VvPNP shares some similarity with the domain organisation of expansins it lacks the tryptophan rich C-terminal domain. This domain also makes expansins larger ($\sim$25 kDa) than natriuretic peptides ($\sim$14 kDa). Attempts to show functionality of the recombinant fusion protein GST:VvPNP were unsuccessful thus far. Further inquiries into the role that VvPNP plays in the homeostasis of grapevine are needed to elucidate the potential enhancement for this important economic crop.

Opsomming

’n Nuwe plant natriuretiese peptied (PNP) is ontdek in die *Vitis vinifera* kultivars, Chardonnay en Pinotage. Die transkrip van die geen is van uit jong blare geisoleer wat aandui dat die peptied in hierdie organe van die plant uitgedruk word. Die 15 kDa proteien, wat *Vitis vinifera* plant natriuretiese peptied (VvPNP) in hierdie studie benoem is, is as ’n fusieproteien deur ’n bakteriele sisteem uitgedruk. Daar is bevind dat die aminosuurvolgorde van hierdie proteien heelwat ooreenstem met die van erkende natriuretiese peptiede. Hierdie proteien is oorspronklik gevind agv die ooreenkoms wat dit toon met die primere struktuur van ’n gekarakteriseerde *Arabidopsis thaliana* natriuretiese peptied (AtPNP-A). Instumenteel tot die funksionaliteit van hierdie peptiede is die konservering van sekere sisteien aminosure, wat nooit minder as 23 aminosure van mekaar af is nie, en wat ’n disulfiedbrug vorm om die proteien ’n sekondere struktuur ringkonformasie te gee. Vergelyking van die VvPNP en AtPNP-A volgordes toon dat sommige sisteien aminosuur residue met mekaar vergelyk. Daar is ook 14 aminosure wat identies is en 7 wat gekonserveer is in die vasgestelde biologiese aktiewe deel van die AtPNP-A molekule. Alle pogings om die funksionaliteit van die rekombinante fusieproteien GST:VvPNP aan te dui was tot dusver onsuksesvol. Dit was voorheen bewys dat PNP molekules plant protoplaste kan laat swel. Ongelukkig was dit nie in hierdie studie bevestig nie. PNP molekules is al vantevore gevind in ’n verskeidenheid van plantsoorte soos *Dracena godseffiana, Hedera helix* en *Solanum tuberosum* en dies meer, waar daar bevind is dat die biologiese aktiwiteit van die peptied met water en sout homeostase geassosieer is. Plant natriuretiese peptiede is na verwant aan ’expansins’, molekules wat die meganiese eienskappe van selwande direk verander en tot drukgedrewe selverlenging lei. Alhoewel PNPs nie in hierdie manier optree nie is daar bewyse van ’n evolusionere verwantskap tussen ’expansins’ en PNPs. Hierdie bewyse is versterk deurdat beide tipes molekules dieselfde teikenarea het en soortgelyke domein organisasies besit. VvPNP toon twee karakteriserende motiewe wat in beide PNPs en ’expansins’ voorkom, maar besit nie die triptofaanryke C-terminale streek van ’expansins’. Hierdie derde streek maak ’expansins’ ook groter ($\sim$25 kDa) as natriuretiese peptiedes ($\sim$14 kDa). Verdere studies oor die rol wat VvPNP in die homeostase van wingerd speel is nodig om die potensiele verbetering van hierdie ekonomiese gewas te bepaal.

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Abbreviations

A	: Adenine
aa	: Amino acid
Amp	: Ampicillin
ANF	: Atrial natriuretic factor
ANP	: Atrial natriuretic peptide
ATP	: Adenosine 5'-triphosphate
AtPNP-A	: *Arabidopsis thaliana* plant natriuretic peptide-A
BLAST	: Basic Logical Alignment Search Tool
β-ME	: 2-Mercaptoethanol
bp	: Base pair
BSA	: Bovine Serum Albumin
cDNA	: Complementary deoxyribonucleic acid
CI	: Chloroform:Isoamyl alcohol
C	: Cytosine
ddH_2O	: Double distilled water
DEPC	: Diethyl pyrocarbonate
DNA	: Deoxyribonucleic acid
dNTPs	: Deoxynucleoside triphosphate(s)
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetra-acetic acid
EST	: Expressed sequence tag
g	: Grams
GC	: Guanyly cyclase
GST	: Glutathione S-transferase

h : Hour(s)

IPTG : Isopropyl β-D thiogalactoside

irPNP : Immunoreactant plant natriuretic peptide

kDa : Kilodalton

KOAc : Potassium acetate

LB : Luria bertani

l : Litre

mA : Milliampere

MCS : Multiple cloning site

mM : Millimolar

min : Minute

ml : Millilitre

M : Molar

μg :Microgramme(s)

μl : Microlitre

μM : Micromolar

mRNA : Messenger RNA

NaOAc : Sodium acetate

ng : Nanogram(s)

NP : Natriuretic peptide(s)

$O.D._{600}$: Optical density (at 600nm)

ORF : Open reading frame

PAGE : Polyacrylamide gel electrophoresis

PBS : Phosphate buffered saline

PCI : Phenol:chloroform:isolamyl alcohol

PCR : Polymerase chain reaction

PEG : Polyethylene glycol

PIES : Protoplast Isolation Enzyme Solution

PIS : Protoplast Isolation Solution

PMSF : Phenylmethylsulphonyl fluoride

PNP : Plant natriuretic peptide

PVPP : Polyvinylpolypyrrolidone

RNA : Ribonucleic acid

rpm : Revolution(s) per minute

RT : Reverse transcription

RT-PCR : Reverse transcription polymerase chain reaction

SDS : Sodium dodecyl sulphate

SDS-PAGE : Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

STE : Sodium/Tris-HCl/EDTA

Sarkosyl : N-Lauryl sarcosine

SAP : Shrimp alkaline phosphatase

T_A : Primer annealing temperature

TAE : Tris-HCl/Acetic acid/EDTA

TE : Tris-HCl/EDTA

T_M : Primer melting temperature

Tris-HCl : Tris(hydroxymethyl)aminomethane

TEMED : N,N,N´N´- Tetramethylethylenediamine

U : Unit(s)

UV : Ultra violet

V : Volt

VvPNP : Putative *Vitis vinifera* plant natriuretic peptide

v/v : Volume per volume

w/v : Weight per volume

X-gal : 5-bromo-4-chloro-3-indocyl-β-D-galactoside

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Chapter 1

Literature Review

1.1 Introduction

One characteristic of all living organisms is the ability to maintain an internal equilibrium which is specialised, and in most cases relatively different from their external environment. This maintenance is accomplished by altering physiological processes and is referred to as homeostasis. Since plants are unable to escape their environment, when the abiotic conditions become challenging or they experience biotic stresses, they must have a well developed, robust and efficient homeostatic system. Such a system has been discovered in plants which is very distinct from the common pathways related to plant growth and development. Discoveries over the past decade indicated that plant cell signaling from stress stimuli may have close relation to the vertebrate hormonal system. And like the vertebrate system, peptide hormones and hormone specific receptors play a role in intracellular communications. With the completion of many whole genome sequencing initiatives numerous small peptide ligands and receptor-like kinase homologous encoding genes have been identified. In *Arabidopsis thaliana* alone 610 putative receptor-like kinase genes have been identified [The Arabidopsis Genome Initiative, 2000]. This discovery is an addition to the well known six non-peptide system of hormones which was thought to control growth and development of plants. These non-peptide hormones include the auxins, cytokinins, ethylene, gibberellin absisic acid and brassinolides [Matsubayashi, 2003]. Researchers have distinguished four peptide-ligand-receptor pathways in plants. Processes such as wound responses and cellular dedifferentiation have been attributed to these pathways [Matsubayashi, 2003]. It is clear that plants are challenged in different ways from vertebrates and as such there are few homologous peptide systems which occur across the kingdoms. However there are some similarities which have been discovered in the overall biochemical logic of peptide systems in plants and animals [Takayama and Sakagami, 2002]. Things like second messengers and phosphorylation/dephosphorylation events are similar yet, there are often very real differences to such an

extent that plant signaling systems often resemble bacterial signaling systems or some novel system altogether [Bowler and Chua, 1994]. Systemin was the first peptide hormone identified in plants [Pearce *et al.*, 1991] since then numerous small peptides have been associated with physiological responses ranging from root nodulation and cell division to antimicrobial peptides [Maryani *et al.*, 2003]. These finding are intriguing on their own however, for our research purposes the identification of proteins that have functions in modulating plant water and solute homeostasis are a lot more attractive [Gehring, 1999]. This novel class of plant peptide hormones are called plant natriuretic peptides and will be the focus of the following study.

1.1.1 Objectives

We undertook this study to identify and partially characterise a putative plant natriuretic peptide from *Vitis vinifera.* A previously discovered and well characterised peptide AtPNP-A, from the model organism *Arabidopsis thaliana* was used as a reference molecule during the study. Furthermore, the isolation of such a gene in grapevine along with its full length transcript would indicate not only the presence of a homologous gene in grapevine but a natriuretic peptide signaling system in the plant as well. The expression of the peptide within a bacterial system and isolation of the recombinant protein were also envisioned as stepping stones to the functional analysis of such a molecule.

1.2 Vertebrate Natriuretic Peptides

The study of natriuretic peptides (NP) started unwittingly in 1956 with the electron microscopical identification of secretory granules in the atrium of the Guinea pig [Kisch, 1956]. The first evidence of these peptides exhibiting actions related to natriuresis was reported in 1981 by de Bold *et al.* These findings were conducted using extracted protein from rat hearts and found to have a quick acting natriuretic effect when injected into myocardial extracts from rabbit. The primary structure of this protein, isolated from the atria of rats, was determined and published initially by Flynn *et al.* in 1983 and the biological activity shown by Misono [Misono *et al.*, 1984]. In the latter paper the isolated NP was shown to act on the kidneys by inhibition of sodium reabsorbtion as well as cause relaxation of smooth muscle contraction induced by noradrenalin. Other functions of NPs include the regulation of blood pressure, blood volume and solute transport [Takei, 2001]. The name atrial natriuretic factor was coined in a paper by de Bold which he published in 1985 [de Bold, 1985], a derivative no doubt of the location of isolation and its cardinal area of effect.

There are at least three predominant NPs found in vertebrates namely: atrial NP (ANP), brain NP (BNP) and C-type NP (CNP) natriuretic peptide with a possible fourth: ventricular NP

(VNP) in fish [Loretz and Pollina, 2000]. ANP and BNP are expressed almost exclusively in the heart whilst CNP is expressed in the brain and smooth muscle [Takei, 2006]. Each of these peptides are expressed independently by separate genes in the form of a prohormone, which is cleaved into various sized peptides, each with specific biological activity [Vesely, 2002].

The atrial natriuretic factor (ANF) gene organisation consists of three exons and two introns. The first exon encodes the 5' untranslated region, a signal peptide and 16 aa which forms part of the N-terminal domain of the long acting natriuretic peptide [Vesely, 2002]. Exon two translates ANF as a 126 aa precursor. Exon three encodes only a single amino acid in humans and three C-terminal amino acids in rodents [Pandey, 2005]. The preprohormone is cleaved enzymatically and circulates in the plasma as an N-terminus 98 aa (proANF 1-98) and the C-terminus 28 aa (proANF 99-126). The atrial natriuretic peptide (ANP) is derived from the carboxy terminus of the prohormone and is the 28 aa peptide [Inagami, 1989]. Both ANP and the N-terminus (proANF 1-98) are shown to have some actions on sodium excretion and vessel dilation [Vesely, 2002]. Production of ANF responds foremost to the distension of the atria [Edwards *et al.*, 1988].

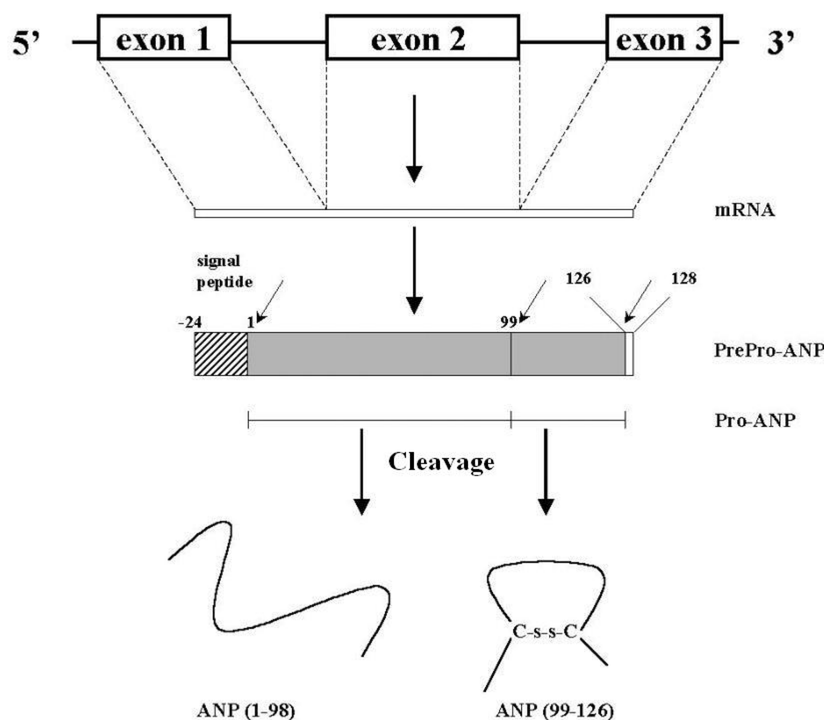

Figure 1.1: Representation of the gene structure and major processing steps of ANP. Small arrows mark the major cleavage sites. ANP (99-126) is the main and best established biologically active compound. The disulphide bridges between two cysteine residues of ANP (99-126) which are necessary for biological activity are shown. (from Gehring, 1999)

A ring structure formed by disulphide bridges between two cysteine residues is essential for activity of all NPs [Brenner *et al.*, 1990; Inagami, 1989; Misono *et al.*, 1984]. A carboxymethylated form of the peptide, when tested on an anesthetised rat, had no effect on natriuresis or smooth muscle relaxant activity as was shown by the purified peptide previously [Brenner *et al.*, 1990; Wang *et al.*, 2007]. The size of the ring structure may vary, however most are 17 aa in length with short carboxy- and amino-terminal tails. Once released, NPs act as ligands for three cell surface receptors called natriuretic peptide receptors A (NPR-A), B (NPR-B) and C (NPR-C), respectively. These receptors in turn are divided into two groups, those with guanylate cyclase activity R_1 (NPR-A, NPR-B) and without R_2 (NPR-C) [Anand-Srivastava and Trachte, 1993; Chinkers *et al.*, 1989]. Guanylate cyclase actively catalyses the conversion of guanosine tri-phosphate (GTP) producing, cyclic guanosine 3', 5' mono-phosphate (cGMP). Cyclic GMP acts as a second messenger by activating cGMP-dependent-protein kinases, cGMP-gated ion channels and cGMP-regulated cyclic nucleotide phosphodiesterases [Lincoln and Cornwell, 1993], which in turn produce cellular and physiological responses.

1.3 Natriuretic Peptides in Plants

It was reported by Vesely and Giordano (1991) that a system exists in plants, which is similar to the natriuretic peptide system in vertebrates. Immunological assays using antibodies directed towards the prohormone fragments proANF(1-98) and proANF(99-126) were recognised in *Dracena godseffiana.* Moreover, radioimmunoassays (RIA) based on I^{125}labeled rANP showed that the antibodies interacted with molecules in plant membranes [Gehring *et al.*, 1996]. Furthermore, studies have shown that alleged ANP and proANP from plants show affiliation to RIA generated standard curves of the human sequence [Vesely *et al.*, 1993]. In the same study it was shown that elution profiles and molecular weights from high-performance gel permeation chromatography (HPGPC) of plant-isolated truncated peptides covering the amino acids 1-30 and 31-67 and pure human sequences, of the same regions of the prohormone, were alike. [Vesely *et al.*, 1993]

Southern and Northern blot analysis of the roots stems and leaves of English ivy (*Hedera helix*) show the presence of an ANP-like gene and mRNA transcripts within plants [Vesely, 2001]. When DNA and RNA extracts from the roots stems and leaves were hybridised with full length rat ANP cDNA probe, results showed the presence of an ANP-like gene throughout the plant whilst the probe only detected transcripts of prohormone in the stems. The investigation led to conclusions that if a natriuretic peptide system existed in plants, and was as important as in vertebrates, it would be expected to find the product throughout the plant. The localisation of gene expression to the stems and leaves could be explained by

the argument that natriuretic peptides in the stems would help move water and solute to the leaves flowers and fruit [Vesely, 2001].

These arguments serve as evidence for the existence of a natriuretic peptide system in plants. The diverse functions of the system are considered in the following sections.

1.3.1 Effects of Vertebrate Natriuretic Peptides in Plants

Since the rate of transpiration in land plants is regulated by the pore size between stomatal guard cells; and a flux in turgor of the guard cells is associated with solute movements; changes in said pore size should indicate changes in transpiration. A question posed by Gehring *et al.* (1996) was, " does rat ANP induce stomatal aperture changes in a concentration dependent manner?" Using a microscope, the changes in stomatal aperture of *Tradescantia* spp. were measured over a 90 min interval when exposed to synthetic 28 aa rat ANP (rANP) in a concentration dependent manner. The results showed that rANP caused opening of the stomata at concentrations equal and greater than 10^{-6}M [Gehring *et al.*, 1996]. These results demonstrate that rANP can mitigate the opening and closing of guard cells through regulation of solute fluxes and therefore affect transpiration and homeostasis. The study raised another the question that since rANP regulated salt (NaCl) in animal cells what effect would be seen on stomatal aperture size of rANP treated guard cells in the presence of Na^+? Already known was that flux in sodium ions affect stomatal guard cell movements. Stomatal guard cells, treated with the auxin plant hormone indole acetic acid (IAA), swelled causing the aperture size of the pore to increase three fold from the control. The addition of either high or low NaCl concentrations accompanied with rANP or amiloride (Na^+/H^+inward channel inhibitor) affected the stomatal aperture in a precarious way. The treatment with IAA under low Na^+opened the aperture size as before, high salt concentrations caused stomatal closure, showing that high Na^+concentrations close the stomatal aperture and IAA has no effect in this situation. The amiloride treated leaf segments counteracted the high salt as would be expected since the Na^+ions were inhibited from entering the guard cells. Leaf segments treated with both IAA and rANP promoted stomatal opening even at high Na^+ concentrations [Gehring *et al.*, 1996]. Physiologically, influx of Na^+into cells goes hand in hand with water uptake which in turn is seen by stomatal swelling. Due to the high extracellular salt concentrations, water movement should have been out of the cell resulting in guard cell shrinkage and aperture closure to prevent further dessication through transpiration. However the fact that the guard cells swelled shows that the NP somehow interacted with receptors in the membranes effecting a similar response as amiloride. The observations of the study lead to investigation of the specific interactions between rANP and the plant membranes. The study involved the competitive binding of rANP antibodies to leaf membranes already bound specifically to I^{125}labeled antibodies [Gehring *et al.*, 1996]. The results suggested

that half the bound ligand was displaced once 0.1μM unlabeled antibody was added. This binding is higher than observed for vertebrates. These results indicate that leaf membranes contain binding sites albeit with low affinity for rANP.

1.3.2 NPs Enhance Solute Flow in Plants

It has been shown that the natriuretic peptides system plays a role in the regulation of solute flow in plants [Vesely *et al.*, 1993]. When the cut stems of White mums (*Chrysanthemum morifolium*) and Carnations (*Dianthus carophyllus*) were placed in coloured tap water containing 77ng of the respective NP the colour appeared in the flowers at a significantly increased rate compared to controls. Even once all the leaves of the stems had been removed, negating any water movement due to transpiration theory, the coloured water still reached flowers more rapidly when treated with the truncated peptide proANF (1-30).

The first plant natriuretic peptide (PNP) was isolated through immunoaffinity purification from the English ivy (*Hedera helix)* [Billington *et al.,* 1997]. Antisera directed against human ANP was shown to isolate peptides using a crude protein extraction protocol from the ivy. These protein molecules were deemed immunoreactant plant natriuretic peptides (irPNP). Furthermore, the isolated irPNPs were shown to induce stomatal guard cell swelling at a much lower concentration (2 x 10^{-8}M) than demonstrated previously using rANP (1 x 10^{-6}M) in a concentration dependent manner [Billington *et al.*, 1997]. Studies have shown that these novel irPNPs [Billington *et al.*, 1997] are located and effect radial water movements in the xylem of *Tradescantia multiflora* stems in a similar fashion as rANP and therefore play a role in plant homeostasis [Maryani et al., 2003; Suwastika and Gehring, 1998]. Complementing these findings it was seen that the addition of the cGMP analogue 8-Br-cGMP also increased radial solute movements out of xylem cells [Suwastika and Gehring, 1998]. Thus posing the question whether the mode of action of NP in plants is like vertebrates modulated by guanylate cyclase coupled receptors. Observations after the treatment with 6-anilinoquinoline-5,8-quinone (LY 83583), a guanylate cyclase inhibitor, and mercuric chloride ($HgCl_2$), a water channel inhibitor, showed decreased movement of water from the xylem cells in comparison to controls and previous addition of NPs [Suwastika and Gehring, 1998]. The concluding arguments were that several pathways could be interrelated to achieve NP-dependent solute movements including the regulation of signal transduction pathways through guanylate cyclases and modulation of ion channels directly by NP.

1.3.3 Plant Natriuretic Peptides Modulate cGMP Levels in *Zea mays* Root Stele

The similarity of the effects of rat ANP and plant NP led researchers to study the part natriuretic peptides played in plant homeostasis. The tangible evidence that natriuretic peptides bind to natriuretic peptide receptors and stimulate increases in cGMP levels through guanylate cyclase in vertebrates and the interesting observation that rANP has the same effect on plant tissues prompted investigations into plant natriuretic peptide effect on cGMP levels. Experiments conducted by Pharmawati *et al.* (1998b) documented the changes in cGMP in response to treatment of *Zea mays* root stele with PNP from ivy. Stele tissue with or without the pre-incubation in 3-isobutyl-1-methyl xanthine, a phosphodiesterase that inhibits cGMP catabolism to an extent was exposed to PNP and rANP for 10min. Cyclic GMP levels increased three fold in stele tissue which had been treated with irPNP within a ten minute period before returning to control levels in half an hour [Pharmawati *et al.*, 1998b]. The rANP treated tissues showed no changes in cGMP levels [Pharmawati *et al.*, 1998b]. The treatment of *Zea mays* root stele tissue with the LY 83583 in the presence or absence of 3-isobutyl-1-methyl xanthine showed similar results as above with irPNP increasing cGMP by three fold. This finding is an indication that a particulate guanylate cyclase may be present because LY 83583 do not inhibit this specific form of the enzyme in all instances. This is analogous to the receptors found in vertebrate species where the guanylate cyclase domain is intracellular [Pharmawati *et al.*, 1998b].

1.3.4 Natriuretic Peptide-Induced Stomatal Opening is Modulated by cGMP

It has been reported that an analogue of cGMP (8-Br-cGMP) induces stomatal opening in *Tradescantia albiflora* [Pharmawati *et al.*, 1998a]. In a study conducted by Pharmawati *et al.* (2001) it was shown that cGMP mediated stomatal opening of guard cell protoplasts is activated by compounds such as ANP and PNP. The first evidence for this statement is that stomatal aperture opening induced by NPs is prohibited by the addition of LY 83583 [Pharmawati *et al.*, 2001]. Not only does this inhibitor affect NP induced guanylate cyclase production of cGMP, it has been shown to reduce both kinetin and auxin induced stomatal opening [Pharmawati *et al.*, 1998a]. Moreover, guard cell protoplasts isolated from *Solanum tuberosum* show an increase in cGMP concentrations in response to treatment with either kinetin, ANP or irPNP. Interestingly the results obtained in the guard cell assay contrast those observed with *Zea mays* root stele [Pharmawati *et al.*, 1998b], where LY 83583 had little effect on cGMP concentrations. One possible explanation for this phenomenon is that root stele tissue is insensitive to LY 83583 whilst guard cell protoplast are not, suggesting the receptors may vary. There is overwhelming evidence that plant natriuretic peptide-induced

stomatal opening is triggered by a signal transduction cascade with cGMP as the second messenger. As reported by Pharmawati *et al.* (1998) this signaling cascade may affect K^+channels, which are responsible for the main cation movements regulating guard cells in connection with the hyperpolarising effects of the H^+ATPase [Kinoshita *et al.*, 1995; Pharmawati *et al.*, 2001; Suwastika and Gehring, 1999]. The question remains, which specific mechanisms effect the influx of solute and water causing guard cells to swell? When stomata were treated with ethylene glycol bis (β-aminoethyl ether)-N,N,N', N'-tetraacetic acid (a calcium chelator), ruthenium red (inhibits the release of intracellular Ca^{2+}) or procaine (acts on ryanodine receptor homologoues within plant membrane inhibiting Ca^{2+} release), it obstructed NP induced opening of the guard cells [Pharmawati *et al.*, 2001]. Furthermore, stomatal opening, stimulated by 8-Br-cGMP, was inhibited by these compounds but no effect was witnessed by the modulators individually [Pharmawati *et al.*, 2001]. Natriuretic peptide-induced stomatal opening is dependent on cGMP, which in turn requires Ca^{2+} [Pharmawati *et al.*, 2001]. However NP in turn stimulate H^+ATPase driven stomatal opening as well [Kinoshita *et al.*, 1995]. It has been shown previously that the cGMP analogue 8-Br-cGMP inhibits the activity of the H^+-ATPase cation pump [Pharmawati *et al.*, 1999; Suwastika and Gehring, 1998] and that irPNPs stimulate the influx of cations such as H^+, Na^+ and K^+ [Maryani *et al.*, 2000; Pharmawati *et al.*, 1999]. These processes contrast each other, leading to the explanation that PNPs affect stomatal opening through a cGMP dependent pathway that requires Ca^{2+} but not H^+-ATPase action.

1.3.5 irPNP Induces Osmoticum Dependent Volume Changes in Mesophyll Protoplasts

Mesophyll protoplasts were isolated from the leaves of *Solanum tuberosum* plants and incubated in solutions of varying sorbitol concentrations until the volume changes had stabilised. The addition of irPNP, which had been isolated from *Solanum tuberosum* leaves, showed swelling of the protoplasts in a concentration and time dependent manner [Maryani *et al.*, 2001]. The effect of the irPNP was fully evident after 4.5 minutes with volume changes increasing directly proportional to the amount of natriuretic peptide added [Maryani *et al.*, 2001]. The response dependence of the protoplasts to NP specificity was tested by pre-incubation with rabbit anti-human ANP (99-126). This treatment nullified any effect seen previously [Maryani *et al.*, 2001]. These results are consistent with natriuretic dependent effects on plant cell volume regulation and roles of homeostasis for peptide hormones. Interestingly, it was noted during the study that both the immunoaffinity purified bands from *Solanum tuberosum* shared significant sequence similarity to p12, a blight induced protein of unknown function in rough lemon (*Citrus jambhiri*) [Maryani *et al.*, 2001].

1.3.5.1 PNP Active Site Determination and Effects on cGMP and Cell Volume Regulation

Synthetic peptides of various lengths were prepared from the the *Arabidopsis thaliana* PNP-A (AtPNP-A) gene which is analogous to the human ANP primary structure [Wang *et al.*, 2007]. These ranged between amino acids 33 and 66 of the 126 amino acid primary structure. Peptide A (33-66), peptide B (33-44), peptide C (45-56) and peptide D (55-66) were added to *A. thaliana* mesophyll protoplasts at concentrations of one-hundred nanomolar. Peptide A was shown to be most effective and increased the volume of the protoplasts more than did a recombinant form of the whole AtPNP-A protein [Wang *et al.*, 2007]. The other three peptides had little effect, however peptides C and D were better than the control [Wang *et al.*, 2007]. Since peptide A contained two cysteine residues at positions seven and thirty it was linearised by a *S*-carboxymethylation reaction. The linearised peptide had no effect on protoplast volume [Wang *et al.*, 2007]. This confirms that the region required for activity lies within the homologous area to atrial natriuretic peptide [Morse *et al.*, 2004]. AtPNP-A induced protoplast volume increases modulated by cGMP. Inhibitors of animal soluble guanylate cyclase, LY 83583, ODQ and NS 2028 (ODQ and NS 2028 are analogues that are specific irreversible inhibitors of soluble guanylate cyclase *in vitro*) of were added to confirm that the volume changes were as a result of the increases in cGMP concentrations. Whilst ODQ caused a non-significant reduction in protoplast volume both LY 83583 and NS 2028 [Wang *et al.*, 2007] inhibited volume increases previously induced by AtPNP-A, confirming the hypothesis [Wang *et al.*, 2007].

1.3.5.2 A Protein with Guanylyl Cylase Activity in *Arabidopsis thaliana*

Until fairly recently no molecule with guanylyl cyclase (GC) activity has been identified within plants even though cGMP has been implicated in many responses from gene regulation to stress responses and the regulation of ion transport. One explanation given for this is that the high divergence of plant GCs has excluded them from detection by antibodies directed toward bacteria and animals; and even out of the scope of BLAST searches [Ludidi and Gehring, 2003]. In a paper by Ludidi and Gehring (2003) they report the identification, cloning and expression of the first functional guanylyl cyclase isolated from plants (*Arabidopsis thaliana*). The peptide called AtGC1 was shown to induce a greater than 2.5 fold increase in cGMP levels in transformed, induced bacteria compared to controls [Ludidi and Gehring, 2003]. Also noteworthy is that AtGC1 activity is not altered by the presence of nitric oxide, a known inducer of cGMP elevation in some stress responses.

1.3.6 Recombinant PNP Modulates Cation Flux in *Arabidopsis thaliana* Roots

The recombinant plant natriuretic peptide AtPNP-A from *Arabidopsis thaliana,* without the 26aa signaling molecule which would enable secretion in to the extracellular space, was expressed and purified for use in this study [Ludidi, 2004a]. The intention was to assess the influence of NPs on cations K^+, Na^+ and H^+ in root segments. Net ion fluxes from the elongation and mature zones of *Arabidopsis thaliana* roots, known to have distinctly different ion transport patterns, were measured in the presence or absence of AtPNP-A. The addition of 150ng/ml of AtPNP-A induced a rapid (< 5min) H^+ uptake in the elongation zone and an efflux in the mature zone [Ludidi *et al.*, 2004a]. These effects were coupled with an increase in cGMP (within 1min) concentrations; which occurred within 1min of exposure to AtPNP-A but were transient, returning to untreated levels within 60min. The elongation zone showed no flux changes in either Na^+or K^+in correlation to the large net influx of H^+ in this zone. Just the opposite was observed however for the mature zone with the net efflux of H^+ mimicked by efflux of both K^+ and Na^+ ions [Ludidi, 2004a]. Interpretation of these results deemed that the flux in H^+ was not significant enough to drive the changes seen in the other ion flux rates [Ludidi, 2004b]. A proposed explanation is that non-selective cation channels could be implicated due to the qualitatively similar character of the ions [Ludidi, 2004a]. One of the activation mechanisms associated with these channels are cyclic nucleotides. The indirect gating of these channels by activation of guanylyl cyclases would increase cGMP levels as shown before in *Zea mays* [Pharmawati *et al.*, 1998b] and *Solanum tuberosum* [Pharmawati *et al.*, 2001]. The regulation of salt and water fluxes indicates that AtPNP-A has a role in the homeostasis of the plant and in effect may respond to stresses placed on the plant by the environment such as drought or excessive solute content of soils i.e. osmotic stress [Ludidi, 2004a; Rafudeen *et al.*, 2003].

1.3.7 PNP expression responds to osmotic stress

The concentration of immunoaffinity purified AtPNP-A was increased by the application of 150mM NaCl and the osmo-equivalent, 300mM sorbitol, to the culture medium of a suspension of *Arabidopsis thaliana* cells. At these high concentrations of salt the growth of the cell suspension culture was arrested and the relative amounts of irPNP molecules were significantly increased [Rafudeen *et al.*, 2003]. Indications that irPNP respond to negate the effects of increasing osmotic stress rather than to modulate them is seen by this reaction of the cells. The control of threshold levels of cytoplasmic NaCl is essential to the plant as exceeding these concentrations is toxic [Blumwald *et al.*, 2000]. Known mechanisms used by plants during times of increased salinity stress are the compartmentalisation of excess sodium and active sodium extrusion. As the principal method for the generation of a potential difference

across the cell membrane is the H^+ATPase and it has been shown that PNP affect the rate of flux of ions [Ludidi *et al.*, 2004a; Pharmawati *et al.*, 1999] it is plausible that the increases in irPNP seen with the experiments go hand in hand with a homeostatic response mediated in part by these plant natriuretic peptides. This response also takes effect over a longer time period as is the case when peptides are synthesised. The response noticed by Rafudeen *et al.* (2003) was recorded 24h after the exposure to the increased solutes. Interestingly salt and osmotic stresses imposed on *Arabidopsis thaliana* seedlings cause rapid increases in cGMP levels [Donaldson *et al.*, 2004]. The evidence presented suggests that salt stress activates two cGMP dependent pathways, “an osmotic, calcium-independent pathway and an ionic, calcium dependent pathway” [Donaldson *et al.*, 2004]. Twenty four-day old transgenic seedlings of *Arabidopsis thaliana* expressing apoaequorin were treated with water, NaCl or sorbitol. The seedlings were snap frozen in liquid nitrogen and the cGMP content of each assessed [Donaldson *et al.*, 2004]. Results showed the salt and sorbitol (osmotic stress) treated plants had a rapid (< 5 seconds) increase in cGMP levels compared to the controls. This is an indication that salt stress did in fact have an effect on cGMP levels. These answers prompted researchers to ask what the interaction between cGMP and calcium would be during stress conditions? For this experiment the seedlings were reconstituted and placed into cuvettes with water for 30 minutes or pre-incubated either 10min or 1h prior to treatment in the guanylate cyclase inhibitor LY 83583 or neomycin, an IP_3-gated Ca^{2+} channel inhibitor, and then placed into the water. The seedlings in cuvettes were treated, while inside a digital luminometer, with either water, NaCl or sorbitol. Once baseline counter of luminescence had returned to normal the remaining aequorin was liberated using Ca_2Cl. The amount of Ca^{2+} was determined by the luminescence counts. The application of salt or osmoticum stress on the seedlings induced dose-dependent changes in Ca^{2+} concentrations. Addition of 50mM NaCl caused a large increase in cytosolic Ca^{2+} concentrations but, the equivalent of sorbitol did not [Donaldson *et al.*, 2004]. The seedlings which had been treated with LY 83583 showed a marked reduction of cGMP levels. The neomycin had massive effects by decreasing Ca^{2+} levels but little on the cGMP concentration. The results indicate that cGMP is not activated in the wake of an increase of Ca^{2+}but, the seedlings pre-treated with LY 83583 did show lower levels of Ca^{2+} [Donaldson *et al.*, 2004] indicating that the synthesis of cGMP is needed for the full Ca^{2+} flux [Donaldson *et al.*, 2004]. The conclusions drawn from these results are that cGMP rapidly downregulates Na^+ influx by acting on voltage independent channels due to its synthesis in response to salt [Donaldson *et al.*, 2004]. The observation that both Ca^{2+} and cGMP concentrations increased in the same time frame lends one to the idea of a connectivity. At high salt concentrations (150mM) increases in Ca^{2+} appear to be independent of cGMP but, it was seen that treatment with lower concentration of salt (50mM) corresponded to reduction of cGMP and the corollary amplitude of Ca^{2+} increase [Donaldson *et al.*, 2004]. The association between these signaling molecules could be cGMP-sensitive voltage independent channels [Donaldson *et al.*, 2004] strengthened by ob-

servations that the guanylate cyclase inhibitor caused a smaller Ca^{2+} concentration increase.

1.4 Expansins and AtPNP-A

Expansin is the name given to the protein molecules which promote cell wall loosening and extension. Isolated PNPs have shown similarity to CjBAp12, an undefined protein from citrus which is induced in response to blight infection [Ceccardi *et al.*, 1998]. This blight associated molecule shows significant similarity to expansins but lacks the characteristic cell wall loosening capabilities of expansins. Thus it is suggested that PNPs share some evolutionary relationship to expansins.

The cell wall is a network of polymers consisting of cellulose microfibrils (parallel linked β -1,4-glucans), embedded in a hydrophylic matrix of hemicellulose (branched glycans) and pectins (acidic polysaccharides). Growing cell walls possess impressive strength and pliancy, allowing for the large mechanical stresses imposed by turgor pressure, whilst permitting the distension associated with growth and enlargement of the protoplasts within. Cell wall expansion occurs by slippage of the polymers across each other rather than extension of the microfibrils as the cellulose fibrils have a tensile strength in excess of $10^{11}N.m^{-2}$ [Cosgrove, 2000a]. The process of cell wall extension was thought to be a result of hydrolysis of the polysaccharide matrix but the discovery of protein expansins that promote cell wall elongation and extensibility has altered this rationale. There are four lines of evidence implicating expansins with endogenous regulation of cell growth. Expansins induce elongation and stress relaxation of isolated cell walls in a pH dependent manner [McQueen-Mason and Cosgrove, 1995]. This phenomenon, known as acid growth shows that plant cells extend faster at lower pH values [Rayle and Cleland, 1992]. Secondly, when living cells are treated with expansins, the cells expand. Penultimately, expansin genes have been shown to be expressed at the right time and location for cell growth [Cho and Cosgrove, 2000] and finally, silencing of expansin expression inhibits growth [Cho and Cosgrove, 2000].

Presently two gene families the α- and β-expansins have been described [Cosgrove *et al.*, 1997]. In short, α- expansins are the largest subfamily with 26 genes being identified in *Arabidopsis thaliana* and 10 genes being identified for the β- expansin family. Both families of peptides function as cell wall loosening agents. What make expansins interesting is that without the hydrolytic activity they share structural motifs and alleged catalytic site similarities with endoglucanases of the family-45 glycosidases . However, the endoglucanases have no expansin activity which would suggest that these molecules posses an evolutionary relationship based on the site of action namely: the cell wall [Cosgrove, 1999; Cosgrove, 2000b; Ludidi, 2002].

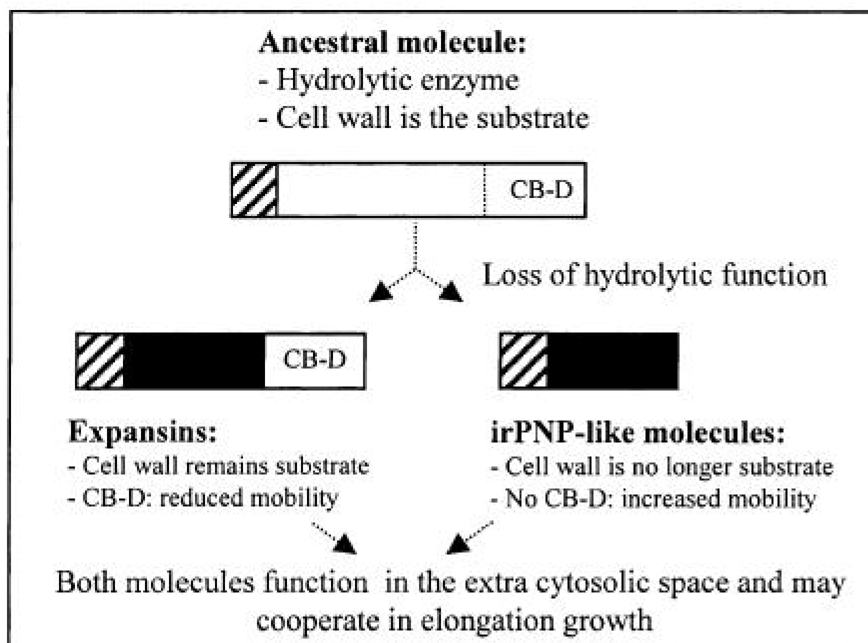

Figure 1.2: Model of molecular evolution of irPNP molecules (after Ludidi *et al.* (2002))

Analysis of expansins show that the genes encode three functional domains [Cosgrove, 1998]. The N-terminal domain has a signal peptide of 22 to 26 amino acids, much the same as natriuretic peptides. The central domain encodes a protein motif which is conserved across all families of expansins and has significant similarity to the catalytic site of the family-45 endoglucanases. This domain has a characteristic His-Phe-Asp (HFD) motif which is absent in all irPNP-like molecules [Ludidi, 2002]. While it is interesting to note the HFD motif, some expansins but not all have shown to posses minimal endoglucanase activity when tested. On the other hand the family-45 endoglucanases have a high enzyme activity but are proved not to posses the expansin's cell wall loosening capabilities [Cosgrove, 1999]. The C-terminal domain of expansins, which is lacking in all irPNP isolated molecules, is the cell binding domain (CB-D). This region of the peptide is characterised by several conserved tryptophan residues. These aromatic amino acids have been associated with protein-carbohydrate interactions and the conservation of their position as well as the phenylalanine at the centre of the catalytic site strengthen this view. Once again no plant natriuretic peptides have thus far been identified that have these motifs [Ludidi, 2002]. One such peptide possessing both sequence similarity and motif homology found in expansins and irPNP molecules has been identified. The protein, CjBAp12 was first isolated from *Citrus jambhiri* (rough lemon) trees infected with citrus blight [Ceccardi *et al.*, 1998]. Even though the ORF of p12 was shown to have a 49% similarity and 31% identity to expansins [Ceccardi *et al.*, 1998] they have no apparent expansin-like activity. Another similarity between PNPs and CjBAp12 is that the molecular mass of CjBAp12 is 12kDa which is much smaller than the ~25kDa classic expansin. This similarity between irPNP-like molecules

and this unknown citrus blight protein goes further than just the size. There are four cysteine residues in AtPNP-A and B which align perfectly to CjBAp12 [Ludidi, 2002]. The predicted secondary structure of both AtPNP-A and CjBAp12 show near conformity and both contain two distinct diagnostic motifs A and B. With A being K[VI]VD and B [LM]SxxAFxxI [Ludidi, 2002]. Interestingly, CjBAp12 is expressed in the roots and stems, as identified through Northern blot analysis, but has been isolated from roots stems and leaves [Ceccardi *et al.*, 1998]. This fact coupled with the lack of cell wall binding domain gives indications that these proteins are systemically mobile. Similarly AtPNP-A is by association potentially mobile in the plant, where it functions to regulate osmoticum dependent water uptake, opening of stomatal pores via guard cells and enhance solute flow in the xylem amongst other things all through a signal cascade mediated in part by cGMP. A proposition made by Ludidi *et al.* (2002) is that AtPNP-A and the expansin family of proteins may in fact exercise symbiotic physiological roles within the growing plant. It has been shown that natriuretic peptides cause the osmoticum-dependent swelling of protoplasts [Maryani *et al.*, 2001] coupled with evidence that stomatal guard cell opening is triggered by irPNP through cGMP/Ca^{2+}changes and the influx of solute and water [Pharmawati *et al.*, 2001], and presumably an increase in turgor pressure within the cell. The increased pressure within the cells would place stress on the cell wall and may signal loosening of the cell wall by expansins [Ludidi, 2002]. it is thus the suggestion that PNP induced swelling provides the force needed for cellular expansion. This hypothesis is an interesting prospect at the interplay between irPNP-like molecules and expansins and is being researched.

Chapter 2

Materials and Methods

2.1 Bioinformatic analysis

The AtPNP-A amino acid sequence (AAD08935) was used as a reference to perform a tBLASTn [Altschul *et al.*, 1997] against the grapevine database of the Computational Biology and Functional Genomics Laboratory (http://compbio.dfci.harvard/edu/tgi). Sequences (ESTs) with the highest scoring segment pairs were retrieved from GenBank using the associated accession numbers. These sequences were analysed with the open reading frame finder (ORF Finder) application (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to identify the largest continuous segments. The sequences were translated *in silico* and compared to the *Arabidopsis thaliana* primary structure using ClustalX [Thomson *et al.*, 1997] in conjunction with BioEdit (version 5.0.9)[Hall, 1999]. The sequence (VvPNP) was also BLAST [Altschul *et al.*, 1997] against the grape genomic database to identify possible genomic sequences.

2.2 Generation of cDNA fragments

2.2.1 Primer Design

Primers were designed based on the VvPNP sequence from the bioinformatic work conducted in section 2.1 and analysed using the program Oligo Analyzer version 1.0.2 (Copyright (C) 2000-2002 Teemu Kuulasmaa). Primers had to observe the following criteria: a GC content of 35-50% and melting temperature (T_M) between 45^oC and 60^oC. The melting temperatures were calculated based on the nearest neighbor parameters. Primers were synthesised by Inqaba Biotech (Pretoria, South Africa).

Table 2.1: Primers used to obtain genomic and transcript clones of the *VvPNP* gene

Primer Name	Sequence (5'-3')	$T_A (^oC)$
VvPNP-fwd	ATGAGGATTATTGAGACATTGC	52.0
VvPNP-rev	TCACACCTGTTGAAATTCAATG	52.0
VvPNP RE-fwd	CTCGAGGGATCCATGAGGATTATTGAGACATTGC	55.0
pGEX seq- 5'	GGGCTGGCAAGCCACGTTTGGTG	55.0
pGEX seq-3'	CCGGGAGCTGCATGTGTCAGAGG	55.0
T7	GTAATACGACTCACTATAG	55.0
Sp6	CATTTAGGTGCAACTATAG	55.0

2.2.2 Total RNA extraction

Total RNA from *Vitis vinifera* cv. Chardonnay and Pinotage was extracted using the perchlorate method with modifications [Davies and Robinson, 1996] Three grams of young leaf material, frozen in liquid N_2, were ground to a powder using a mortar and pestle. The frozen powder was added to 25ml of extraction buffer (3M sodium perchlorate, 0.2M Tris-HCl, pH 8.3, 5% [w/v] SDS, 8.5% [w/v] PVPP, 2% [w/v] PEG 6000, 1% [v/v] β-ME), and stirred at room temperature for 30 min. The mixture was then transferred to a centrifuge tube and spun at 14000rpm and 4^oC for half an hour. The intermediate phase was removed and added to 2.5 volumes 100% ethanol and precipitated overnight at -20^oC. Following sedimentation of the nucleic acids for 15 min at 8300rpm, the pellet was washed with 70% ethanol and resuspended in 2ml TE buffer containing β-ME (10mM Tris-HCl, pH 7.6, 0.1mM EDTA, 0.2% [v/v] β-ME). The suspension was extracted 4 times using an equal volume phenol:chloroform:isoamyl alcohol(PCI; 25:24:1 [v/v]) and once with chloroform:isoamyl alcohol (CI; 24:1 [v/v]). The nucleic acids were precipitated for 2 hours at -20^oC using 2.5 volumes ethanol and 0.1 volumes 3M sodium acetate, pelleted and rinsed as before. The pellet was dried before being resuspended in $100\mu l$ DEPC-treated water. Yield was checked by spectrophotometry using a Nanodrop® ND1000 spectrophotometer (NanoDrop Technologies, Inqaba Biotechnical Industries (Pty) Ltd, SA). RNA was differentially precipitated for 16 hours by adding 0.3 volumes 8M lithium chloride at 4^oC. An aliquot of the RNA was digested with RNase free DNase 1(Promega) according to the manufacturer's instructions. Ten micrograms of RNA were incubated (37^oC) with 10U DNase 1 and 1x DNase 1 reaction buffer for 30 min. The RNA was rinsed with PCI as above, and resuspended in $50\mu l$ DEPC-treated water and then stored at -80^oC until needed.

2.2.3 Reverse-transcription Polymerase Chain Reaction (RT-PCR)

2.2.3.1 Primer Annealing

The primer annealing cocktail (90-300ng RNA, 1.5μM reverse primer per 3.25μl reaction) was mixed in a 200μl centrifuge tube. The tube was incubated at 95^oC for 10min followed by 5min in an ice slurry.

2.2.3.2 cDNA Synthesis

The reverse transcription was performed with AMV reverse transcriptase (Promega) according to the manufacturer's instructions.

The RT-cocktail (1 x reaction buffer [50mM Tris-HCl, pH 8.3, 50mM KCl, 10mM $MgCl_2$, 0.5mM Spermidine, 10mM DTT], 4mM dNTPs, 10mM DTT, 1U RNase Out, 2.5U AMV reverse transcriptase per 9.75μl reaction) was added to the template and incubated at 42^oC for 2 hours. The resulting heteroduplex was stored at -80^oC until needed.

2.2.3.3 PCR Amplification

All PCR reactions were conducted in a Thermal cycler 2720 (Applied Biosystems).

PCR reactions were performed with Ex Taq^{TM}(Takara) DNA polymerase as directed by the manufacturer and used in a touchdown PCR protocol. This enzyme produces a 3' A overhang.

The reaction conditions for the PCR were: 2μl cDNA (approx. 20ng), 1 x Ex Taq Buffer, 20mM dNTPs, 0.5mM forward primer, 0.5mM reverse primer, 2.5U Ex Taq DNA polymerase per 50μl reaction.

The cycling conditioned for the touchdown PCR were: 94^oC for 5min followed by 5 cycles of (94^oC for 30", 65-1^oC for 30" and 72^oC for 45"), 4 cycles of (94^oC for 30", 60-2^oC for 30" and 72^oC for 45") 30 cycles of (94^oC for 30", 52^oC for 30" and 72^oC for 45") and final elongation of 72^oC for 5min.

All other PCR reactions were conducted by universal reaction conditions namely: $\pm$50ng template DNA, 1 x Bioline PCR NH_4 reaction buffer (16mM $(NH_4)_2SO_4$, 67mM Tris-HCl, pH 8.8, 0.01% [v/v] Tween-20), 2mM $MgCl_2$, 0.2mM dNTPs, 1μM of each primer, 1 x cresol/sucrose loading dye (1mM cresol red, 20% [w/v] sucrose) per 25μl reaction unless otherwise stated. Bioline Taq produces a 3' A overhang.

The universal PCR cycling conditions were: 94^oC for 5' then 25 cycles of (94^oC for 30", appropriate $T_A$for 30", 72^oC for 35") and a final elongation of 72^oC for 5min.

2.2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis was conducted according to instruction in Sambrook [Sambrook *et al.*, 1989].

VvPNP cDNA samples were resolved in 1-1.5% [w/v] agarose (D1 LE Hispanagar) TAE gels. Ethidium bromide (0.25μg/ml) was added to the gel for visualisation. A 50 x stock solution of TAE (2M Tris-HCl, 0.5M EDTA, pH 8.0, 5.71% [v/v] Glacial acetic acid) was used for the gels, and as running buffer at a 1 x dilution. Electrophoresis was carried out between 70 and 100 volt. The molecular markers HyperLadder I and O'Gene Ruler (both at 5μl/lane, BIOLINE) were used. Samples, unless already containing cresol/sucrose loading dye, were mixed in a 1:5 ratio with Loading dye (40% [w/v] sucrose, 0.25% [w/v] Bromophenol blue, 0.25% [w/v] Xylene cyanol)(Fermentas). The gels were viewed using a MULTI GENIUS^{TM} bio imaging system (SYNGENE).

2.2.5 Agarose Gel Purification of PCR Products and DNA Fragments

The use of the Wizard® SV Gel and PCR Clean-up System (Promega) or the Zymoclean Gel DNA Recovery Kit^{TM}(Zymo Research) were used to purify DNA fragments from agarose gels in accordance with the manufacturer's instructions.

The fragments were excised from the gel with a clean scalpel blade and placed into a microcentrifuge tube. The weight of the gel slice was determined.

Wizard protocol:

For every 10mg of gel slice, 10μl membrane binding solution was added to the centrifuge tube. The tube was placed into a 50-65°C water bath for 10min during which time it was vortexed frequently. Once the gel slice had completely dissolved the solution was transferred to a SV Minicloumn placed in a collection tube, incubated at room temperature for a minute and centrifuged at 13200rpm for a minute. After discarding the flow through, 700μl membrane wash solution was added and the assembly centrifuged (13200rpm, 30"). This step was repeated using 500μl membrane wash solution and spun for 5min, the flow through discarded and the assembly re-centrifuged (13200rpm, 1min). The SV minicolumn was transferred to a clean 1.5ml microcentrifuge tube, 50μl ddH_2O added, incubated at room temperature (1min) and centrifuged (13200rpm, 1min). The 1.5ml microcentrifuge tube containing the purified DNA was stored at $-20^{o}C$ until needed.

Zymo Protocol:

Three volumes of ADB buffer were added to every volume of gel excised. The mixture was vortexed and incubated (37°C) until the gel slice had completely melted. Following the transfer of the mixture to a Zymo-spin I^{TM}column in a collection tube, it was centrifuged

(13200rpm) for 30 seconds. The flow through was discarded, 200μl wash buffer added and the assembly centrifuged (13200rpm, 30"). Once the wash step had been repeated the column was centrifuged (13200rpm, 1min) again and 10μl ddH_2O added the column which was incubated at room temperature (1min). The Zymo-spin I^{TM} column was transferred to a clean 1.5ml microcentrifuge tube and centrifuged (13200rpm, 1min). The tube was then placed at -20^oC until needed.

Following purification samples were quantified spectrophotometrically using the NanoDrop® ND-1000 spectrophtometer.

2.3 Molecular Cloning

All fragments generated by PCR were cloned into the pDrive cloning vector (Qiagen) as directed by the manufacturer.

The T4 DNA ligase and 10 x Ligase buffer (Promega) were used as directed by the manufacturer.

2.3.1 Ligations

Ligation of PCR fragments into the pDrive cloning vector were in 10μl volume reaction creating the pDrive::VvPNP clone. An insert:vector ratio of 4:1 was used most often. The ligation reaction (1 x reaction buffer [30mM Tris-HCl, pH 7.8 at 25^oC, 10mM $MgCl_2$, 10mM DTT, 1mM ATP], 10% [v/v] PEG 4000, 100ng insert DNA, 25ng pDrive cloning vector, 4 Weiss units T4 DNA ligase) was incubated at either room temp (20^oC, 2h) or in a cooling water bath (16^oC, overnight).

2.3.2 *E. coli* Transformations and Screening of Recombinants by Colony PCR

2.3.2.1 *E. coli* Transformations

Transformations were performed according to directions in Sambrook *et al.* (1989). Competent *E. coli* DH5α cells were made according to the $CaCl_2$ method, and kept at -80^oC.

DH5αcells were plated on LB/Agar plates (without antibiotics) and incubated overnight (37^oC). A single colony was inoculated into 5ml LB medium and incubated (37^oC, 200rpm) overnight. The overnight culture was transferred to 500ml LB medium in a 2 litre flask and incubated (37^oC) with shaking until the OD_{600} of 0.6. The cells were pelleted (5000rpm, 10min, 4^oC) and resuspended in 100ml of 100mM $MgCl_2$through gently pipetting. After

a 20min incubation on ice, the cells were once again pelleted (4000rpm, 10min, 4^oC) and the supernatant discarded. The cells were carefully resuspended in 10ml ice cold 100mM $CaCl_2$with 15% [v/v] glycerol and aliquoted to pre-chilled 1.5ml microcentrifuge tubes in 100μl volumes. The cells were flash frozen with ethanol (-80^oC) and stored at -80^oC until needed.

The entire 10μl ligation reaction was added to 100μl *E. coli* competent cells, the tube gently flicked and incubated (20min) on ice. The mixture was heat shocked (42^oC) for 45 seconds and returned to the ice (2min). Luria-Bertani (LB) medium (900μl, MERCK) was added and the tube gently mixed. The cells were incubated (37^oC, 90min) and 100μl were plated onto LB/Agar (12g/*l,* agar bacteriological, MERCK) plates containing the appropriate antibiotics (100μg/ml Ampicillin), IPTG (0.2mM) and X-gal (40μg/ml). Remaining cells were pelleted, resuspended in 100μl LB medium and plated as above. Plates were incubated (37^oC) overnight and stored afterwards at 4^oC.

2.3.2.2 Screening of Recombinants by Colony PCR

Single white colonies were screened by colony PCR to confirm the presence of the insert and its orientation. A single blue colony was used as a negative control.

Fractions of the colonies were lifted from the LB/Agar plate, with a sterile toothpick, and the tip placed briefly into a pre-mixed 25μl PCR reaction cocktail. The cocktail was made as described by the universal reaction mixture in section 2.2.3.3. The final mixture was placed into the thermal cycler and subjected to the cycling conditions described in section 2.2.3.3. Results were authenticated by agarose gel electrophoresis. Positive clones were inoculated in 5ml LB medium with 100μg/ml Amp and incubated (37^oC) overnight.

2.3.2.3 Plasmid DNA Extractions

Both the Alkaline Lysis Minprep Method [Sambrook *et al.*, 1989] and the GeneJet^{TM}Plasmid Miniprep Kit (Fermentas) were used according to the manufacturer's instructions

Alkaline Lysis Miniprep Method [Sambrook *et al.*, 1989]:

One and a half millilitre of the overnight culture was pelleted (13200rpm, 1min), the supernatant discarded and the pellet resuspended in 100μl solution 1 (0.25M Tris-HCl, pH 8.0, 0.5M Glucose, 0.1M EDTA, pH 8.0) for 10min at room temperature. Following the addition of 200μl solution 2 (0.2M NaOH, 1% [w/v] SDS) and mixing, the tube was left on ice for 10min. One hundred and fifty microlitres chilled solution 3 (3M KOAc, 2M Acetic acid) were added, the tube inverted six times and incubated on ice for 10min. Once the tube had been centrifuged (13200rpm, 5min) the supernatant ($\pm$450μl) was carefully transferred to a clean 1.5ml microcentrifuge tube and 300μl isopropanol was added. The solution was

mixed gently and incubated (2min, room temp) before centrifugation (13200rpm, 10min). The supernatant was discarded and the pellet washed with 500μl of ethanol (70% [v/v]) and centrifuged (13200rpm, 5min). The pellet was air dried and finally resuspended in 50μl ddH_2O.

GeneJet^{TM}Plasmid minprep kit:

One and a half millilitre overnight culture was centrifuged (13200rpm, 1min) and the pellet resuspended by vortex in 250μl resuspension solution. Two hundred and fifty microlitre lysis solution was added and the tube inverted four to six times. Immediately after adding 350μl Neutralisation solution the tube was inverted and centrifuged (13200rpm, 5min). The supernatant was carefully transferred to a GeneJet spin column, centrifuged (13200rpm, 1min) and the supernatant discarded. To the spin column 500μl wash solution was added, the column centrifuged and the supernatant discarded. This wash step was repeated. The column was centrifuged (13200rpm, 1min) to remove any ethanol from the wash solution. The column was placed into a clean 1.5ml microcentrifuge tube, 50μl ddH_2O added, incubated on the bench for two minutes and centrifuged (13200rpm, 2min). Plasmid DNA was stored at -20^oC.

2.3.2.4 Freezer Cultures

Freezer cultures were made for all positive clones by adding 750μl 80% [v/v] glycerol to 750μl overnight culture in a 1.5ml cryo-tube. The tubes were stored at -80^oC.

2.4 Sequencing

2.4.1 Sequencing of Plasmid DNA Clones

Sequencing was undertaken using the Applied Biosystems ABI PRISM BigDye^{TM} Terminator v. 3.0 Ready Reaction Cycle Sequencing Kit according to the manufacturer's specifications for half reactions. The reaction conditions for the sequencing were: 2μl Terminator Ready Reaction Mix, 3.3pmol primer, 200-500ng DNA template for a final volume of 10μl.

The cycling conditions for the sequencing were: 94^oC for 5min followed by 25 cycles of (94^oC for 10", $T_A$of specific primer for 10", 60^oC for 4min) and a final stage of 4^oC. The primers used were combinations of those in Table 2.1.

Analysis was done at the Stellenbosch University DNA sequencing facility. Sequence alignment and editing were done using Chromas (version 2.13 Technelysium^C Pty Ltd) and BioEdit (version 5.0.9).[Hall, 1999]

2.4.2 Phylogenetic Analysis

Once sequencing of the clones had been completed, a phylogenetic tree was constructed based on similarity searches and BLAST [Altschul *et al.*, 1997] results against the EST database in GenBank. The data was processed and converted into fasta format whereby it was compiled into a nexus file. The computer programme PAUP was used to draw a distance based neighbour joining tree based on the nexus file with a bootstrap value of 1000. Finally the tree was analysed to establish relationships between the *Vitis inifera* sequences and those for *Arabidopsis thaliana* and other plant and animal species

2.5 Sub-cloning into pGEX 6p2 Expression Vector

2.5.1 Restriction Enzyme Digestion

The sequenced fragment was subcloned from the pDrive::VvPNP construct into the BamHI site of the glutathione S-transferase (GST) fusion expression vector pGEX6P-2 (Amersham Biosciences) to make the GST:VvPNP fusion expression construct pGEX::VvPNP.

The digestion reaction of pGEX6P-2 was performed in 10μl (1 x BamHI buffer [10mM Tris-HCl, pH 8.0 at 37^oC, 5mM $MgCl_2$, 100mM KCl, 0.02% [v/v] Triton X-100, 1mM β-mercaptoethanol, 0.1mg/ml BSA], ±300ng pGEX6P-2 and 5U BamHI) at 37^oC for 90min.

The digestion of pDrive::VvPNP was also performed in 10μl volumes as above. The digested fragments were separated on a 1% [w/v] Agarose TAE gel and the correctly sized fragment excised and purified (section 2.2.5).

2.5.2 SAP Treatment

The linearised pGEX6P-2 vector was dephosphorylated with Shrimp Alkaline Phosphatase (SAP; Promega) in a 10μl reaction, as directed by the manufacturer. The reaction (1 x SAP reaction buffer [0.05M Tris-HCl, pH 9.0, 10mM $MgCl_2$], 1U SAP [1U/μl]) was incubated (37^oC) for 15min and then heat inactivated (65^oC) for 15min. The linearised dephosphorylated vector was resolved in a 1% [w/v] Agarose TAE gel, the band excised and purified as before and stored at -20^oC until needed.

2.5.3 Ligation, Transformation and Screening of Recombinants

The ligation reactions were performed in 10μl with T4 DNA ligase (Promega) as in section 2.3.1. An insert:vector ratio of 5:1 was used for the sub-cloning. The ligation reaction was placed at 16^oC overnight.

Transformation of the construct into DH5α was performed (section 2.3.2.1) and since the pGEX6P-2 vector has no blue/white selection, no IPTG or X-gal were added to the LB/Agar plates. Colonies containing the pGEX::VvPNP construct were selected on Amp (100μg/ml) and the authenticity of the clones confirmed by colony PCR (section 2.3.2.2). The orientation of the insert was established by using a primer set which would only amplify if in the insert was correctly orientated.

Positive recombinants were inoculated into 5ml LB medium and incubated with shaking (160rpm, 37^oC) overnight. A plasmid extraction was performed using the kit described in section 2.3.2.3. Plasmids were also screened by restriction enzyme digestion with BamHI (Fermentas)(section 2.5.1).

The pGEX::VvPNP construct was sequenced (section 2.4) using the pGEX-5' primer and analysed to confirm correct codon frame position.

2.5.4 *E. coli* Rosetta Gami Transformation and Selection

Transformations were performed as directed in Sambrook et al., (1989). Two microlitres of the ligation (pGEX::VvPNP) was added to 100μl *E. coli* Rosetta-gami 2(DE3)pLysS competent cells (Novagen). The cells were incubated on ice (2min), heat shocked (42^oC, 45") and placed once more in an ice slurry (20min). Following the addition of 900μl LB medium, the cells were incubated (37^oC, 90min) before 100μl was streaked on LB/Agar plates with the appropriate antibiotics (Amp 100μg/ml). Plates were incubated (37^oC) overnight.

Single colonies were screened by colony PCR (section 2.3.2.2), inoculated in 5ml LB medium and incubated (37^oC) overnight. Freezer cultures were made as described in section 2.3.2.4.

2.6 Protein Expression and Purification

2.6.1 Protein Expression

Using a sterilised toothpick a small scraping of freezer culture was transferred to 10ml LB medium containing 100$\mu g/ml$ Amp. The culture was incubated (37^oC) overnight with shaking (200rpm). The saturated culture was diluted 1:100 in Terrific Broth (TB) (1.2% [w/v] Bacto Tryptone (Biolab, MERCK), 2.4% [w/v] Yeast Extract (Biolab; MERCK), 0.4% [v/v] Glycerol, 1% [w/v] D-glucose, 17mM KH_2PO_4, 73mM K_2HPO_4) with 100μg/ml Amp. The flask was incubated (37^oC) with shaking (100rpm) and the optical density (O.D.$_{600}$) measured once per hour until it reached O.D.$_{600}$= 0.4 and then every 15min until the O.D.$_{600}$= 0.6.

Once the O.D.$_{600}$= 0.6 was read, protein expression was induced by adding 0.1mM IPTG and the cultures were allowed to incubate for a further 3 hours. A sample of the culture was kept apart as an un-induced control. The culture was pelleted (4500rpm, 20min, 4°C), the supernatant discarded and the pellet washed in 1/10th the culture volume ice cold 1 x STE (0.01M Tris-HCl, pH 8.0, 0.001M EDTA, 0.15M NaCl). The cells were centrifuged (8000rpm, 5min, 4°C) and the pellet resuspended (1 x STE, pH 8.0, 100μg/ml PMSF, Complete Protease Inhibitor (Roche), 5mM DTT). N-Lauryl-Sarcosine (Sarkosyl, MERCK) was added to a final concentration of 0.3% [w/v]. The cells were snap frozen three times with either liquid N_2 or by submersion in cold ethanol ($-80^{o}C$) to lyse. The suspension was DNase treated (50μg/ml DNase1, 1h, 37°C) (Promega) and drawn through a needle (29G). Once the lysate had reached a consistency whereby it could be poured without any stringiness it was centrifuged (10000rpm, 10min, 4°C). The clarified supernatant was transferred to another tube and stored at 4°C. The insoluble pellet was resuspended in an equivalent volume STE.

Glutathione S-transferase was expressed in the same manner as a control for the purification step.

2.6.2 SDS-PAGE Analysis of Protein Samples

Samples were separated in a 12% [w/v] resolving gel with 4% [w/v] stacking gel. A 40% [w/v] acrylamide-bisacrylamide (38.9g acrylamide, 1.1g NN'-methylbisacrylamide per 100ml) concentrated stock solution was prepared and used at the appropriated dilutions.

Resolving gel: 2.5ml 4x resolving buffer (1.5M Tris-HCl, pH 6.8, 0.4% [w/v] SDS), 3ml 40% [w/v] acrylamide-bisacrylamide stock, 100μl 10% [w/v] ammonium persulphate, 10μl N,N,N´N´- Tetramethylethylenediamine(TEMED) per 10ml gel.

Stacking gel: 1.25ml 4x upper gel buffer (0.5M Tris-HCl, pH 8.3, 0.4% [w/v] SDS), 0.4ml 40% [w/v] acrylamide-bisacrylamide stock, 100μl 10% [w/v] ammonium persulphate, 10μl TEMED per 5ml gel.

Samples were prepared by heat denaturation in SDS-PAGE sample buffer (0.075M Tris-HCl, pH 6.8, 1% [w/v] SDS, 2.0% [v/v] β-mercaptoethanol, 15% [v/v] glycerol, 0.001% [w/v] bromophenol blue) at 95°C for 10min. The electrophoresis was conducted in a Hoefer MINIVE discontinuous buffer system (250V, 30mA). The use of either a low molecular weight ladder or the PageRuler^{TM}(5μl/lane, Fermentas) protein ladder were used as a molecular weight markers. The gels were stained with Coomassie Blue (0.25% [w/v] coomassie brilliant blue R-250, 45% [w/v] methanol, 10% acetic acid) for 45min and de-stained (30% [v/v] methanol, 10% [v/v] acetic acid) for 1 hour or until the protein bands were visualised.

2.6.3 Protein Purification

Proteins were purified by affinity chromatography on a non denaturing S-linked glutathione agarose (Sigma) column. The column was prepared according to the manufacturers instruction.

The lyophilised powder was left to swell overnight in water (200ml/g). The resin was washed with equilibration buffer (1x STE, $100\mu g/ml$ PMSF, 5mM DTT, Complete protease inhibitor) before loading the clarified supernatant. The sample was allowed to incubate (4^oC, 16h) with gentle shaking to facilitate binding. Once the lysate had been removed by gravity flow the column was washed with PBS (0.138M NaCl, 0.0027M KCl, pH 7.4). The GST bound VvPNP was eluted between one and four times by adding 1ml elution buffer (10mM reduced glutathione, 50mM Tris-HCl, pH 9.0) and shaking for 15min. Fractions were analysed by SDS-PAGE as in section 2.6.2. Samples were stored at at -20^oC with glycerol (20% [v/v]) added as cryo-preservative.

2.6.3.1 Protein Quantification

The protein from the isolated fractions was quantified by the method of Bradford [Bradford, 1976] using the Bio-Rad Protein Assay kit. Bovine Serum Albumin (BSA) was used as a standard ranging from 1μg/ml to 100μg/ml with blanks consisting of 50mM tris-HCl(pH 9.0). Ten microlitre BSA standard was added to $190\mu l$ Bradford reagent per well of a microtitre plate and left for at least 5 min for the colour to develop. Absorbance was recorded at 620nm on a Multiskan®BIOCHROMATIC v.1.8 (Labsystems) plate reader. All samples were in triplicate. A standard curve of absorbance against concentration was plotted.

2.6.3.2 Fusion Protein Cleavage

The GST was enzymatically cleaved from the fusion protein, GST:VvPNP, with PreScission Protease (Amersham Biosciences) as directed by the manufacturer. Fractions, eluted from the column showing protein presence, were dialysed against the PreScission cleavage buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 1mM DTT) to remove reduced glutathione. The samples were placed within dialysis tubing and incubated (4^oC) in the cleavage buffer for 2 hours and then with fresh cleavage buffer overnight. Five microlitres PreScission Protease (2U/μl) were added and the sample incubated (4^oC) with gentle shaking for 4 hours. The sample was placed onto the washed and equilibrated column and incubated (20^oC) for half an hour. The supernatant was eluted and analysed by SDS-PAGE (section 2.6.2).

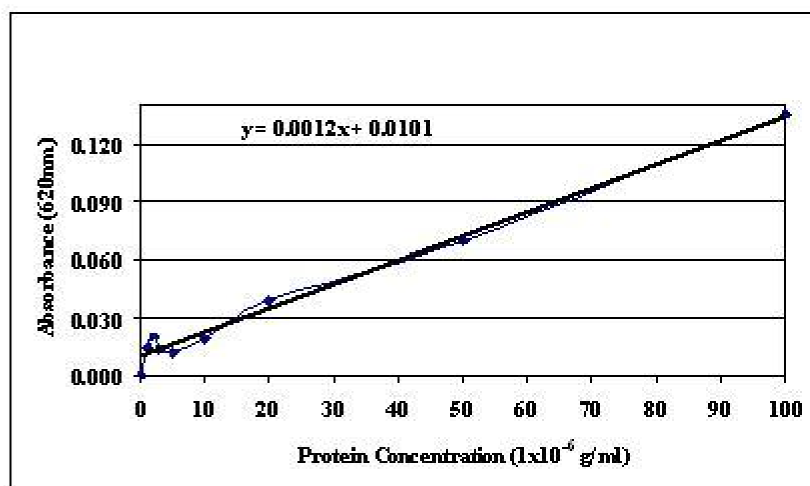

Figure 2.1: Bradford Assay Standard Curve

The standards used were BSA with concentrations of 1μg/ml, 2μg/ml, 3μg/ml, 4μg/ml, 5μg/ml, 10μg/ml, 15μg/ml, 20μg/ml, 40μg/ml, 50μg/ml, 80μg/ml and 100μg/ml respectively.

2.7 Biochemical Assays

2.7.1 Protoplast Isolation

Protoplasts were isolated from the leaves of broad bean (*Vicia faba)* seedlings using a combination of protocols [Babaoglu, 2000; Gross, 2001; Luan *et al.*, 1994; Spalding *et al.*, 1992]. Leaves were cut and placed into a container with tap water. The epidermis was peeled from the leaf with forceps and a scalpel. Both the peeled leaves and epidermal peels were placed peel side down into a beaker containing 5ml protoplast isolation enzyme solution (PIES; 0.6M mannitol, 1mM $CaCl_2$, 10mM KCl, 5mM MES, 2% [w/v] cellulase (Sigma), 0.5% [w/v] pectinase (Sigma), 0.1% [w/v] pectolyase (Sigma), 0.2% [w/v] BSA, pH 5.7) for 2 hours at 28^oC with gentle shaking (60rpm). Using a forceps, the bulk plant material was removed and the remaining material filtered through a 150μm sieve (Sigma) into a 15ml centrifuge tube. The filtrate was centrifuged (300x *g,* 20^oC, 5min) and the pellet resuspended in 2ml protoplast isolation solution (PIS; 0.6M mannitol, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM KCl, 5mM MES, pH 5.7). This was followed by filtering the solution through a 75μm sieve. The protoplasts were transferred to a 2ml microcentrifuge tube by using a cut 1ml pipette tip. The tube was centrifuged (300x *g,* 20^oC, 5min) and the protoplasts resuspended in 1ml PIS. The protoplasts were kept at 4^oC until used (max 8h).

2.7.2 Activity of GST:VvPNP

The Faba bean protoplasts were carefully pipetted onto a number of glass microscope slides and treated with either 150ng GST:VvPNP/ml, 150ng GST/ml, 50mM tris-HCl (pH 9.0) or left untreatd. The untreated, 150ng GST/ml and 50mM tris-HCl treated slides were used as controls. All slides were visualised after at least 5 minutes incubation ($20^{o}C$) by bright field microscopy at a magnification of 1000x and randomly selected protoplasts from all treatments were photographed under the same magnification using with a superimposed scale by the camera attached to the microscope. The diameter, or more correctly the volume, of 40 individual protoplasts were assessed to establish whether decisive any changes were caused due the addition of the protein. Results deemed to be statistically relevant were calculated by a paired Student's *t*-test. The Null hypothesis stated that no change in protoplast volume was observed and any change was due to the treatment with GST:VvPNP. The probability threshold for this test was one of 0.01.

Chapter 3

Results and Discussion

3.1 Bioinformatic Analysis

The detection of genes, with similar functions, from different organisms is facilitated by the fact that protein motifs are conserved. Therefore, for the identification of homologous genes between species with similar functions, the protein sequence can be used as reference. The AtPNP-A peptide, which was used as a model reference is 126 amino acids (approximately 14kDa) in length with the signal peptide of 26 aa still incorporated. Results from the tBLASTn [Altschul *et al.*, 1997] returned 11 candidate *Vitis vinifera* ESTs. The tBLASTn algorithm returns a nucleotide sequence for an amino acid input sequence [Altschul *et al.*, 1997]. These sequences were narrowed to 4 candidates (CB009659, CF609110, TC41780 (now has new accession EE108509), CB920937). Inclusion criteria of the candidate sequences were based on homology searches using BLAST [Altschul *et al.*, 1997]. Several of the returned sequences had significant homology to the family of expansins. It is suggested that PNP's may fall within a sub-group of this family and described by some as γ-expansins [Li *et al.*, 2003]. Plant natriuretic peptides are similar to the α and β-expansins but are truncated in regard to their C-terminal domain. This domain within expansins has been assigned as a cellulose binding region because of several tryptophans, in conserved positions on the outside of the folded protein, and as such a cell wall binding domain [Cosgrove, 1999]. No such wall binding domain has been identified within irPNP-like molecules and as such all sequences containing indications of this wall binding domain were ignored. The truncation also makes irPNP-like molecules considerably smaller ($\sim$12-16kDa) than expansins ($\sim$25kDa) [Ceccardi *et al.*, 1998]. Strikingly the majority of expansins contain two introns [Cosgrove, 1999], the second located close to the cellulose binding domain. IrPNP-like molecules without exception lack the second intron and a cellulose binding domain. It is for this reason that the proposition of evolutionary relations between these molecules was proposed [Ludidi *et al.*, 2002]. It is noteworthy to state that while expansins have very low

endo-glucanase activity they do posses a His-Phe-Asp (HFD) motif which is found in the catalytic site of family-45 endoglucanases [Cosgrove, 1999]. There is no HFD motif in any of the irPNP-like molecules identified thus far and may be a reason that PNP-like peptide do not exhibit the cell wall loosening properties of expansins. Query sequences were scanned for the highly conserved diagnostic motifs A and/or B (A: K[VI]VD, B: [LM]SxxAFxxI). These motifs have been identified within irPNP-like molecules and the blight associated protein CjBAp12 [Ceccardi *et al.*, 1998]. Motif B contains the phenylalanine and serine residues found across the families [Ludidi *et al.*, 2002].

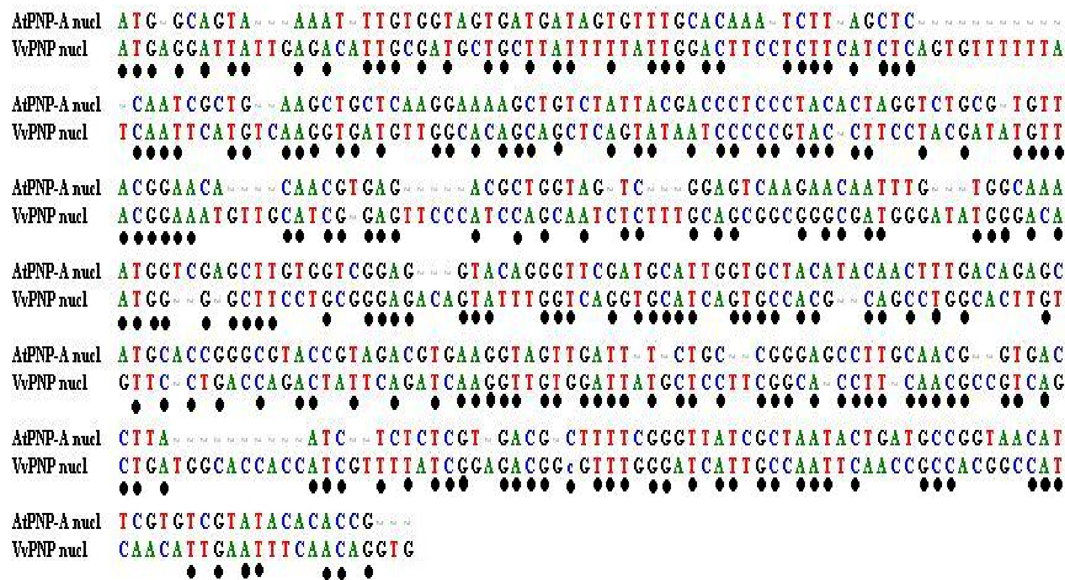

Figure 3.1: Transcript Nucleotide Alignment of AtPNP-A with VvPNP
Nucleotide alignment of AtPNP-A with VvPNP from Pinotage. The sequences have a similarity of 56% when aligned. Identical nucleotides are indicated by dots(•). Both sequences are in the frame +1.

Analysis of open reading frames of the four sequences showed that only the TC41780 EST contained significant length transcript in the +1 frame. Comparison of the nucleotide sequences revealed a 56% identity between the AtPNP-A and the putative VvPNP (Fig 3.1). Translation of the sequence *in silico* (Fig 3.2) produced a product comprising 144 amino acids, containing no HFD motif but including both a KVVD and a LSxxAFxxI motif [Ceccardi et al., 1998]. Several of the cysteine residues were also conserved, which according to Morse *et al.* (2004) delineate a domain critical for PNP-like molecule biological activity. Also noteworthy is that VvPNP and AtPNP-A share 14 identities and 7 conserved amino acids between the amino acids deemed to be critical for biological activity in *Arabidopsis thaliana*. The signal peptide which is required for release of this peptide into the intracellular

space.

```
Signal Peptide

AtPNP-A aa  -------MAVKFVVVMIVFAQILAPIAEAAQGKAVYYDPPYTRSACYG----TQRETLV
VvPNP aa    MRIIETLRCCLFLLDFLFISVFFINSCQGDVGTAAQYNPPYLPTICYGNVASEFPSSNLF

AtPNP-A aa  VGVKNNLWQNGRACGRRYRVRCIGATYNFDRACTGRTVDVKVVDFCR-EPCNGDLN----
VvPNP aa    AAAGDGIWDNGASCGRQYLVRCISAT-QPGTCVPDQTIQIKVVDYAPSAPSTPSADGTTI

AtPNP-A aa  -LSRDAFRVIANTDAGNIRVVYTP-
VvPNP aa    VLSETAFGIIANSTATAINIEFQQV
```

Figure 3.2: Nucleotide and amino acid comparison of AtPNP with VvPNP

(b) Amino acid alignment of AtPNP-A and the translated VvPNP sequence shows a 30% amino acid identity. Indicated in the figure is the 26aa signal peptide sequence for AtPNP-A. The brackets denote the domain within AtPNP-A responsible for water uptake in *A.thaliana.* The asterisks (*) designate identical amino acids, colons (:) are conservative amino acid substitutions and single dots (•) are semi-conservative amino acid substitutions. Squares beneath the alignment (■) indicate conserved cysteine residues. The KVVD and LSxxAFxxI motifs are underlined.

3.2 RNA Extraction and Amplification of the *VvPNP* Gene

Yields of approximately 4.5μg (90ng/μl) and 15.μg (300ng/μl) were obtained after RNA extraction from each of the Pinotage and Chardonnay *Vitis vinifera* cultivars. (Fig.3.2(a)). The figure also shows little smearing of the bands. An indication that is attributed to degradation of the RNA. The RT-PCR (Fig 3.2(b)) shows amplification of Pinotage fragments produced from various stages of extraction when using the primer set VvPNP-fwd and VvPNP-rev.. Lane 2 indicates amplification of the PCR product from RNA before differential precipitation with LiCl. The upper band at ±550 bp is possible amplification of DNA still present in the template. Once the precipitation had been completed the amount of DNA contamination had decreased significantly which is evident from lane 3, producing a fragment of ±450 bp. The final RNA product which had been treated with RNase free DNase 1 was devoid of contaminating DNA and a single band of ±450 bp was generated. The Chardonnay sample (Fig 3.2(c)) showed similar amplification to the Pinotage, however the

yield of nucleic acid was significantly lower and therefore no DNase 1 digestion was performed. Both the upper and lower bands were excised and cloned into pDrive from both varieties.

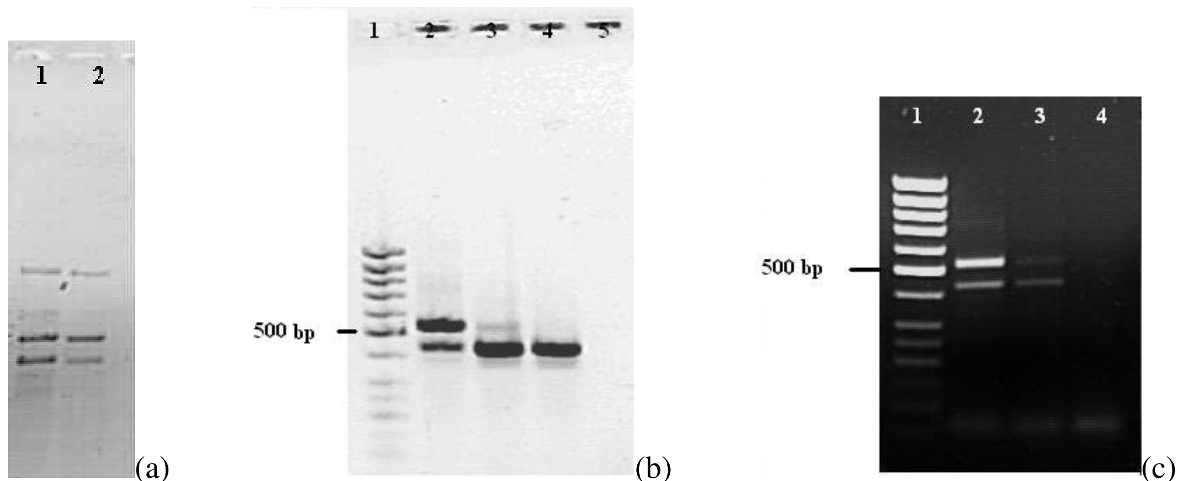

Figure 3.3: RNA Isolation and RT-PCR of the *VvPNP* gene from the *Vitis vinifera* cultivars of Chardonnay and Pinotage

(a) RNA extraction resolved on a 1.5% Agarose TAE gel. Lane 1: Chardonnay. Lane 2: Pinotage. (b) RT-PCR of Pinotage with RNA during various stages of extraction. Lane 1: 1 kb Ladder. Lane 2: PCR product before LiCl precipitation. Lane 3: PCR product after LiCl precipitation. Lane 4: PCR product once RNA had been precipitated and treated with DNase 1. Lane 5: Negative control. The two bands observed in lane 2 and 3 are the result of contaminating DNA within the RNA sample. This was removed completely with DNaseI and a clean transcript clone was generated. (c) RT-PCR amplification of Chardonnay sample. Lane 1: 1 kb Ladder. Lane 2: PCR product before LiCl Precipitation. Lane 3: PCR product after LiCl precipitation. Lane 4: Negative control.

The clones were maintained within the pDrive vector on LB Agar plates as described in section 2.3 and sequenced as described in section 2.4. The primers used for sequencing within the pDrive vector were either Sp6 and T7 or a combination of these with VvPNP-fwd or VvPNP-rev to establish orientation of the fragment. Analysis of the sequences revealed that the Pinotage transcript contained two nucleotide substitutions when compared to the Chardonnay and to the GenBank sequences. At position 149 an A to T and at position 361 a C to T were exchanged. The substitutions could be naturally occurring or have been caused by mismatching by the polymerase enzyme. However based on translational predictions the changes had no effect on the amino acid composition of the PNP molecule.

Analysis of the grapevine sequences revealed a 100bp intron in the genomic sequences when compared to the cDNA derived sequence. Figure 3.2 shows the organisation of the gene. It was seen that the gene contained two exons intersected by a single intron. Interestingly the individual exons would form incomplete peptides as they exist within the genomic sequence.

This is because there is a codon which is divided by the intron. This size of the intron corresponds with the intron size in the *AtPNP*-A gene. The length of the genomic putative *VvPNP* gene is 535bp, inclusive of the stop codon, compared to the 478 bp of the *AtPNP*-A gene. Once the intron is spliced a protein product of 144 amino acids in length would be made as predicted in Fig 3.2. The size of VvPNP is significantly larger than AtPNP-A and the predicted cysteine residues which would form the disulphide bridge are 27 amino acids apart as opposed to the 23 amino acid ring structure AtPNP-A forms.

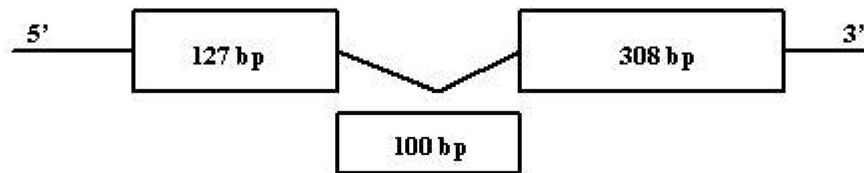

Figure 3.4: Intron/exon organisation of VvPNP
Intron and exon organisation of the VvPNP encoding gene from *Vitis vinifera.* (The 127bp and 308bp blocks represent the two exons identified within the *VvPNP* gene. The 100bp intron corresponds to the size of the intron in the *AtPNP*-A gene.)

As the GenBank sequence was derived from the Chardonnay variety and showed complete identity to the isolated gene it was not incorporated into Fig 3.5.

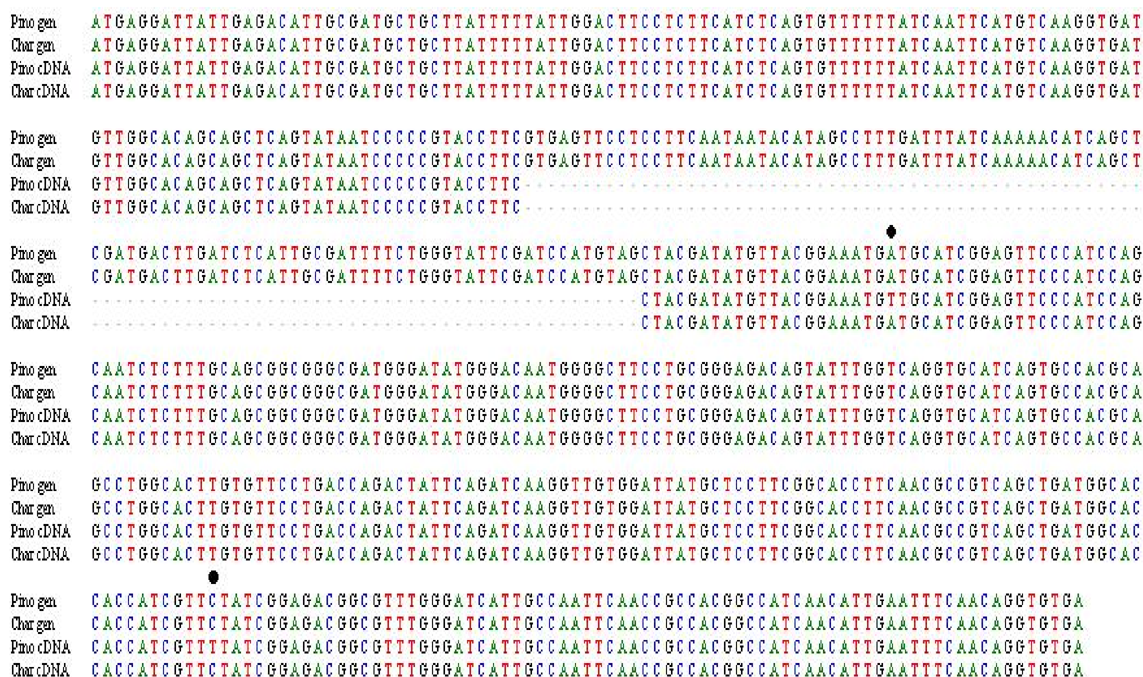

Figure 3.5: Sequence comparison of VvPNP genomic and transcript clones

Comparison of genomic and transcript clones from Pinotage and Chardonnay cultivars. The Chardonnay transcript has complete identity to the genomic sequence. The Pinotage transcript contains two nucleotide substitutions, shown by the dots (•) in the figure. Both genomic clones contain a 100bp intron.

A phylogenetic analysis of the putative *VvPNP* gene was conducted to establish the relationship of the putative VvPNP gene with other PNP-like molecules and expansins. The construction of a distance based neighbour joining tree exhibits the relationship of the putative *VvPNP* gene with other related and unrelated molecules which is shown in Figure 3.6

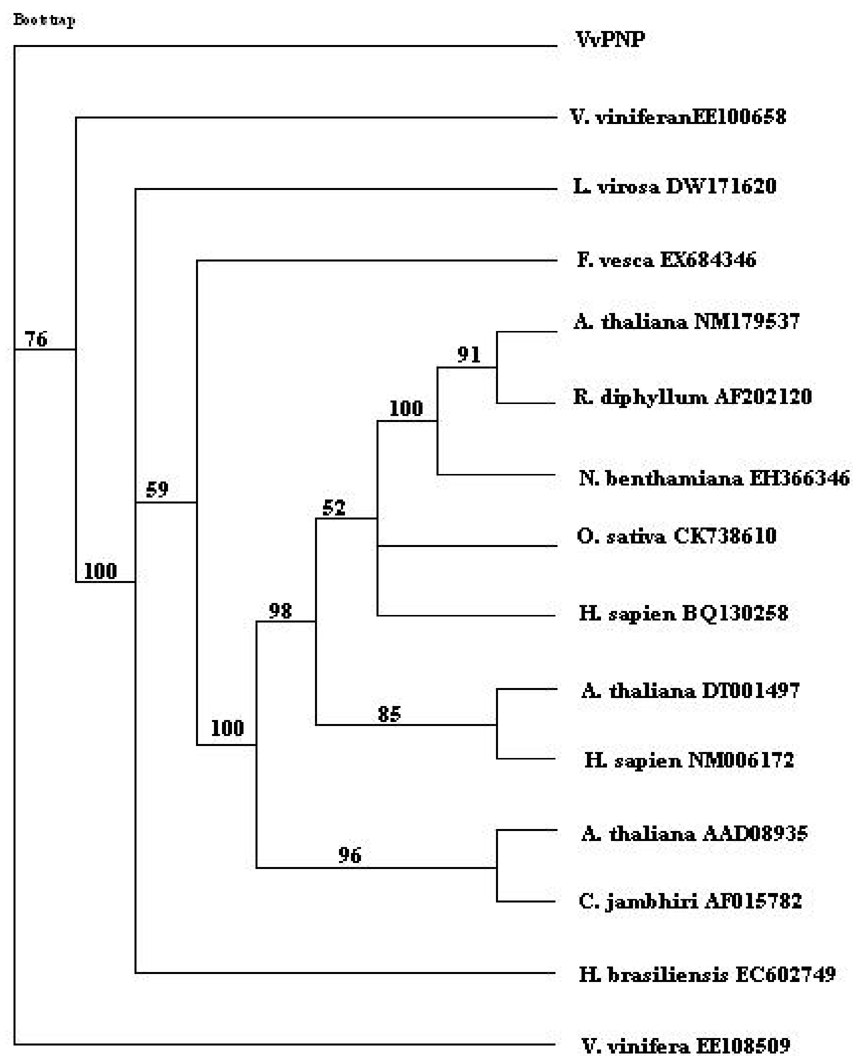

Figure 3.6: Phylogenetic tree representing PNP-like, CjBAp12-like, α-expansins and β-expansins
The numbers represent the distances between the represented nodes. The distance based neighbour joining tree has a bootstrap value of 1000. The sequences are derived from BLAST [Altschul *et al.*, 1997] results against ESTs in GenBank with the VvPNP nucleotide sequence as query and from homology searches identifying expansins. The accession numbers of the individual sequences are shown next to the name of the organism.

The VvPNP sequence identified numerous highly similar sequences from different organisms using the nucleotide BLAST algorithm [Altschul *et al.*, 1997]. The result identified a sequence which has a zero distance between the clades. This molecule's transcript has been identified within ripening berries from *Vitis vinifera.* The distance between VvPNP and AtPNP-A is 520 and incidentally this is the same for CjBAp12. It is seen that AtPNP-A and CjBAp12 exist from he same node, showing a high level of similarity. Interestingly the group containing the β-expansins (DT 001497, CK 738610) show a closer relation to VvPNP than that of the α-expansins (NM 179537, AF 202120, EH 366346).

3.3 Sub-cloning into pGEX 6P2

The sequenced clones were amplified using the primer pair VvPNP RE-fwd and VvPNP-rev in order to incorporate the restriction enzyme BamHI recognition site to the 5' terminal. The multiple cloning site (MCS) of pGEX6P2 (Fig 3.7) has a BamHI restriction site incorporated in frame with the GST gene. If VvPNP was incorporated into the pGEX6P2 vector in such a way that the start codon was in the frame with the amino acids shown in Fig 3.7 then the protein would be expressed as a fusion to GST.

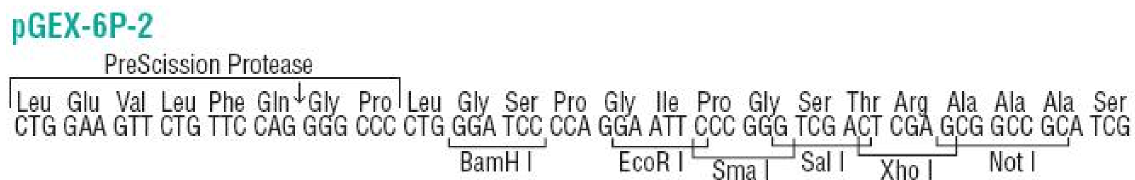

Figure 3.7: pGEX6P2 Bacterial Expression Vector Multiple Cloning Site
For proteins to be expressed the codon arrangement must coincide with those indicated. As the BamHI restriction endonuclease cuts between the A and T in the recognition site GGATCC the amino acid directly preceding the Methionine of the target will be Glycine.

The sub-cloning into pGEX6P2 was non-directional. Orientation of the fragment was confirmed by PCR of the construct using the primer set pGEX seq-5' and VvPNP rev. Sequencing of the construct, using the pGEX seq-5' primer (Fig 3.8 a) indicated positional reference with the start codon (ATG) of VvPNP directly following on the BamHI restriction site within the plasmid. The relevance of the positioning can be seen in Fig 3.7. Analysis of the data showed the VvPNP fragment was in frame with the *GST* gene. Digestion of pGEX:VvPNP with BamHI(Fig 3.8 b) produced a fragment size corresponding to the *VvPNP* gene (435bp). Once the the bacterial host Rosetta-gami 2(DE3)pLysS had been transformed, a colony PCR was performed as final reassurance (Fig3.8 c).

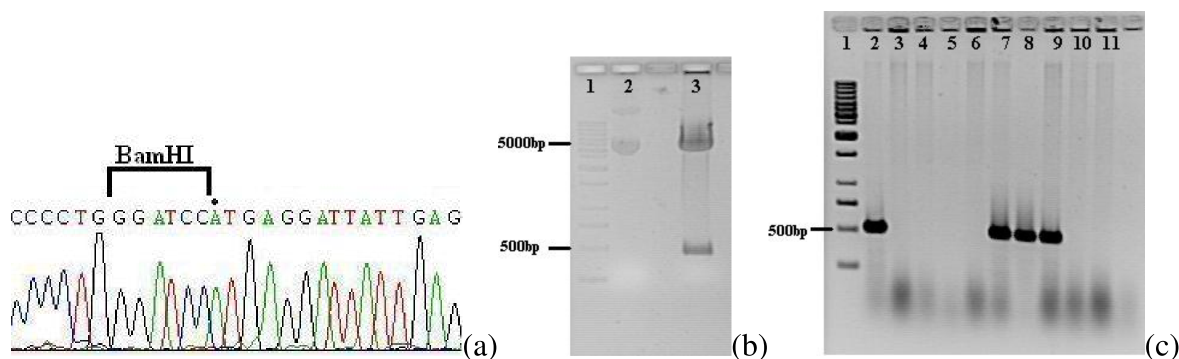

Figure 3.8: Sub-cloning VvPNP fragments

(a) In frame sequence confirmation of pGEX:VvPNP. Indicated also is the BamHI recognition site. The splice occurs between the A and T nucleotides. The start codon of VvPNP is designated by a dot(•). (b) BamHI digestion of pGEX:VvPNP confirms the presence of the fragment within the vector. (c) Colony PCR with primer pair pGEX seq-5' and VvPNP-rev to confirm incorporation of pGEX:VvPNP in the bacterial strain *E. coli* Rosetta-gami2(DE3)pLysS. Lane 1: 1kb DNA ladder, Lanes 2-10: Results of colony PCR. The presence of a band indicates a positive transformation. Lane 11: Negative control.

3.4 Protein Expression and Purification

3.4.1 Expression of GST:VvPNP

Given that both Chardonnay and Pinotage sequences translated to the same primary structure it was felt unnecessary to continue with the expression of both.

Initial expression was undertaken on a small scale (50ml) and later scaled up (1*l*). Fig 3.9 shows expression of the recombinant GST:VvPNP using the Rosetta gami2(DE3)pLysS cell line when induced with 0.1mM IPTG. Recombinant protein expression within the pGEX6P-2 vector is under control of the *tac* promoter. The plasmid contains the *lac*I^qgene whose protein product is a repressor which binds to the operator region of the *tac* gene and suppresses expression until the addition of the lactose analogue IPTG. Basal levels of expression (leakage) were further suppressed by the addition of D-glucose to the medium.

The fusion protein GST:VvPNP was successfully expressed (Fig 3.9) when induced with 0.1mM IPTG. When induced samples (lanes 2 and 4) were compared to the un-induced samples (lanes 3 and 5) the expression of a protein of approximately 41 kDa in size was observed in the induced samples. The molecular weight of GST is 26 kDa, it was therefore determined that the VvPNP moiety be approximately 15 kDa, which corresponds to the predicted size of VvPNP through bioinformatics.

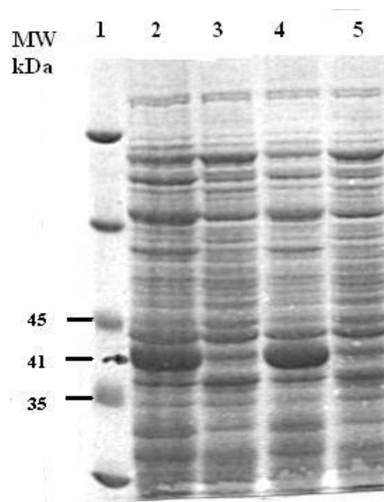

Figure 3.9: Protein expression profile of pGEX:VvPNP
Resolution of GST:VvPNP on a 12% polyacrylamide gel. The low weight molecular marker is represented in lane 1. Lane 2 and 4 indicates resolution of GST:VvPNP after induced expression in Rosetta gami2(DE3)pLysS. Lanes 3 and 5 represent the un-induced cultures of Rosetta gami2(DE3)pLysS.

3.4.2 Purification of GST:VvPNP

From the small scale induction studies it was seen that a significant portion of the expressed protein was present within inclusion bodies. However purification of a soluble fraction of the expressed protein was possible, albeit in very small quantities. The isolation of protein into inclusion bodies usually occurs when eukaryotic proteins expressed in *E. coli* are incorrectly folded. Another contributing factor may be that the cytoplasmic environment within the cells is reducing and thus the formation of the disulphide bonds is incorrect. These insoluble proteins are regarded by the bacterium as foreign and sequestered within inclusion bodies [Wingfield et al., 2000]. There are methods described in the literature and manuals which may improve the solubility of the protein. These include changing incubation temperatures, incorporation of molecular chaperons, such as the heat shock proteins, which help with folding and decreasing the concentration of IPTG during induction [Wingfield et al., 2000]. Multiple variations of these strategies were attempted with no distinctive change to the amount of soluble fraction (results not shown). It was decided to purify the protein under non-denaturing conditions as denaturing and refolding methods are time consuming and troublesome.

The soluble fusion protein was purified from the clarified supernatant on a S-linked glutathione agarose column under non-denaturing conditions (Fig 3.10 lanes 6-9). The extracted GST:VvPNP contained small amounts of contaminants which had been co-purified.

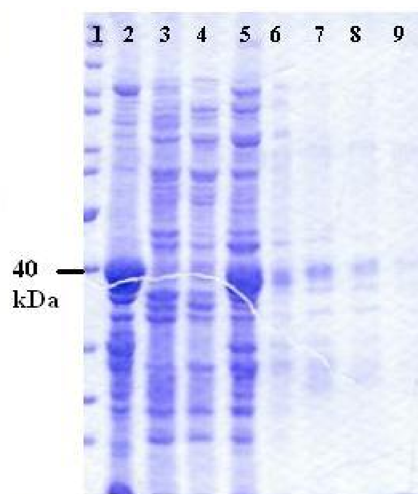

Figure 3.10: Purification of GST:VvPNP and GST by column chromatography

SDS-PAGE analysis of fractions obtained after affinity column chromatography. $5\mu l$ PageRuler^{TM} were loaded into lane 1. An aliquot of the induced sample before processing was loaded in lane 2. Lanes 3 and 4 contain $15\mu l$ of the washes with 1x PBS. Five microlitres of protein flow through were loaded into lane 5. Lanes 6-9 were loaded with $20\mu l$ of the eluted GST:VvPNP in 50mM tris-HCl (pH 9.0), 10mM reduced glutathione.

The contaminants were removed by re-chromatography of the eluant on the same column. Figure 3.11 denotes a 12% SDS-PAGE loaded with $40\mu l$ of the protein which had passed over the column for a second time. Unfortunately the recovery from the column was not 100% and some protein was lost during this process.

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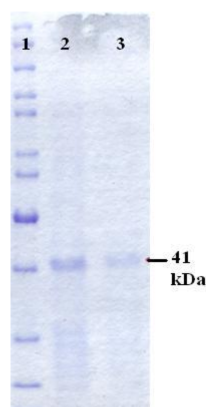

Figure 3.11: Second purification of GST:VvPNP

The amount of protein recovered was less than with the first stage, however the purity had increased significantly.

The column, with a bed volume of 2 ml, has a binding capacity of 10mg GST per millilitre of resin. Glutathione S-transferase was expressed by Rosetta gami2(DE3)pLysS cells

containing the pGEX6P-2 vector with no insert. The protein was used as a control for the column efficiency as well as further experiments (Fig 3.4.2). The 26 kDa protein appears as a soluble moiety when not bound as a fusion. This is established due to the observation that the clarified lysate did not need to be treated with denaturing substances. Also when there is a large amount of insoluble inclusion body debris present in a sample the pellet formed by the lysed cells created a black smear whereas a soluble lysate produced a white pellet. These black smears were cleaned and tested by washing them with Sodium deoxycholate and resolving the product on a 12% polyacrylamide gel. The size of the band witnessed in all instances was consistent with that of GST:VvPNP. These results are not represented here.

The cleavage of GST from VvPNP by PreScission protease was unsuccessful. Multiple digestions were performed using the prescribed buffer at pH 7.5, pH 8.5 and the column elution buffer for 4 hours or overnight at $4^{o}C$. The possibility that the enzyme had expired or that it was handled incorrectly previously was not discarded. A representative of the company selling the enzyme was consulted however the time taken to receive sufficient information was too long not to proceed with the uncleaved protein. Fortunately, it is suggested in the GST Fusion Handbook by Amersham biosciences the manufacturers of the system, that cleavage of GST from the fusion might not be essential for protein function.

It was seen that the expressed GST alone bound more efficiently than the fusion GST:VvPNP protein. This was an indication that the folding of the fusion was possibly incorrect and thus affecting binding to the column. GST was later used as a control for the biochemical assay.

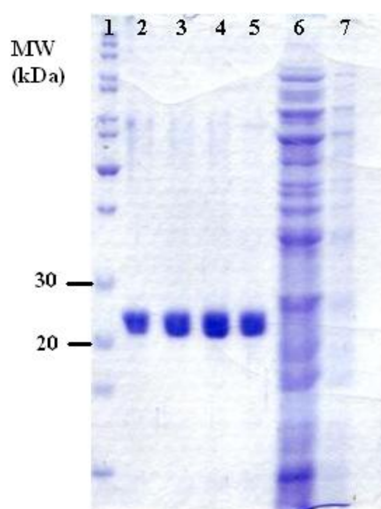

Figure 3.12: Purification of native GST by affinity column chromatography

A 12% SDS-PAGE of expressed GST. Lanes 2-5 were loaded with $20\mu l$ eluted GST. The protein flow through and wash are in lanes 6 and 7 respectively.

3.5 Biochemical Assay

The activity of GST:VvPNP was assessed by treating Faba bean protoplasts with the 150 ng/ml recombinant fusion protein and comparing the results to controls [Ludidi *et al.*, 2004]. A potentially prevalent idea would have been to use varying concentrations of VvPNP during the test. Although faba bean protoplasts have not been addressed in the preceding literature with regard to the ability to swell when treated with a PNP as have been *A.thaliana* [Ludidi *et al.*, 2004] and *S.tuberosum* [Maryani *et al.*, 2001], it was felt that the high level of conservation within the active domain of all PNPs identified thus far would negate this factor. Another reason for the use of faba bean plants is the speed and ease with which this plant grows and simplicity of the isolation protocol [Babaoglu, 2000; Gross, 2001; Luan *et al.*, 1994; Spalding *et al.*, 1992]. Due to time constraints at this point of the project, time saving applications were favoured.

Application of purified recombinant GST:VvPNP did not cause a statistically significant ($p < 0.01$) change in protoplast diameters when analysed using the paired Student's *t*-test (calculations not shown). The diameters of 40 randomly selected protoplasts were digitally analysed from repeated experiments . The impact of this result is that the null hypothesis stands true and no change in volume took place. These results were an indication that the recombinant fusion protein VvPNP produced in this study is probably functional under these circumstances. The 100x oil immersion objective was used to view the protoplasts. That combined with the 10x ocular magnification gave a final magnification of 1000x. Visual analysis of the photographs in figure 3.13 show that the protoplasts from the various experiments appear to have increased in size however, this apparent increase was not conclusive enough to provide a confirmed positive result. One may argue that the protoplasts were isolated from Faba bean leaves and that the putative natriuretic peptide from grapevine however, studies have shown that even natriuretic peptides from vertebrate origin have evoked swelling from protoplasts [Maryani *et al.*, 2001; Vesely and Giordano, 1991]. A very plausible explanation to the ineffectivity of GST:VvPNP may be attributed to the fusion protein obstructing the correct folding of VvPNP and not allowing the formation of necessary disulphide bridges to form. This possible inability to form the correct bonds can be explained by the reducing conditions within the bacterial host's cytoplasm where the protein is translated.

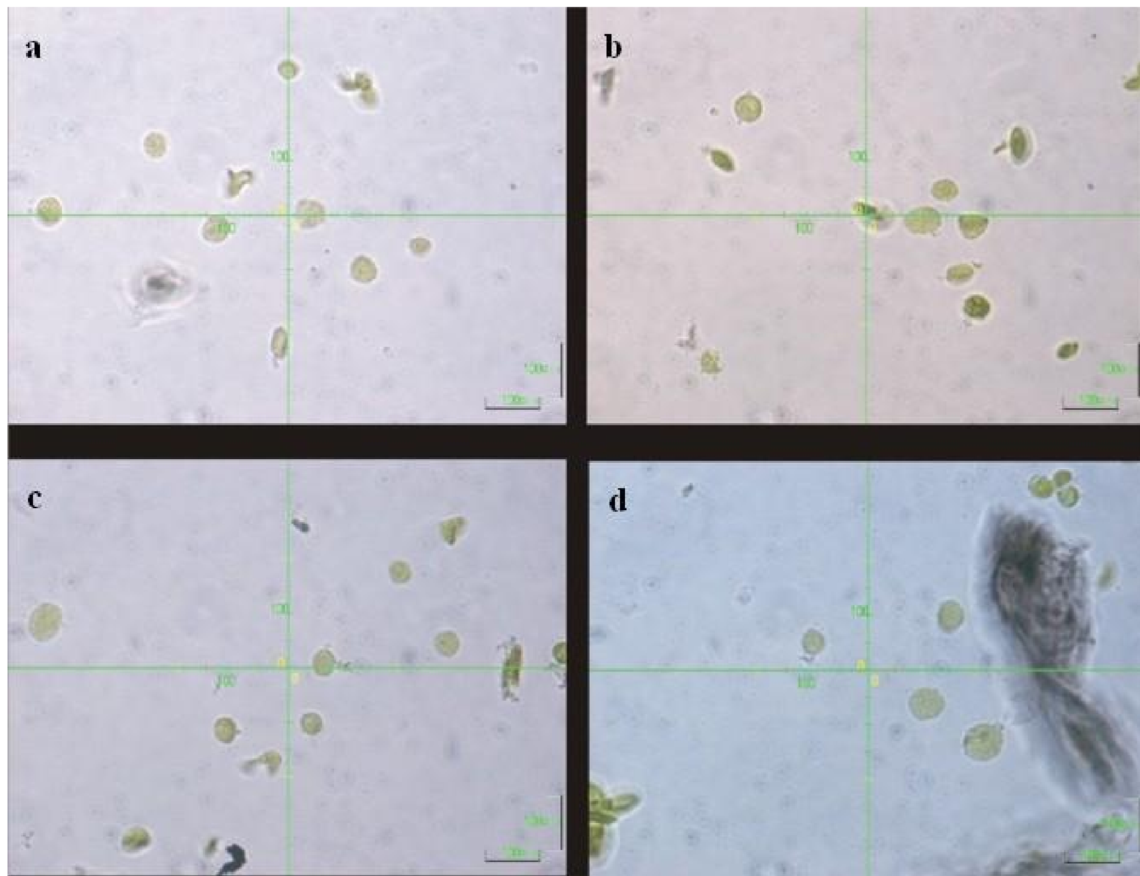

Figure 3.13: GST:VvPNP Activity Assay
The photographs shown are only scale representatives of larger experiments consisting of many more protoplasts. All the Faba bean protoplasts were initially suspended in 0.6M mannitol. Figure A: Untreated protoplast control. Figure B: Protoplasts treated with 50mM tris-HCl pH 9.0.Figure C: Protoplasts treated with 150ng GST/ml. Figure D: Protoplasts treated with 150ng GST:VvPNP/ml. All photographs were taken 5 minutes after the addition of any treatment but not exceeding 30min. The reference bar in the bottom right hand corner represents an arbitrary scale which was not needed because the diameters of the protoplasts at the same magnification (1000x) were compared to each other in scale, not physical units

The negative controls used provided only a basis from which to judge whether any changes had taken place. The use of a positive control such as a known functional AtPNP-A or any other functional PNP would have indicated the potential of not only the effect of a PNP on faba bean protoplasts but also shown significant swelling with which to compare the results.

Another factor which was unfortunately given little consideration was to measure the protoplasts at varying time stages of the experiments. It is possible that some changes may have been effected at a time frame which was not monitored. If the entire experiment was conducted in say intervals of 5min, a bracket may have developed in which changes with significance were observed.

The bioactivity of the protein may also have been assessed by means of a stomatal guard

cell assay [Gehring *et al*., 1996; Maryani *et al*., 2003; Pharmawati *et al*., 1998]. The use of this form of assay would have made it possible to utilise different species of plants and potentially even the *Vitis vinifera* cultivars from which the genes isolated to produce the peptide originated. It is extremely difficult to obtain viable grapevine protoplasts as the leaves are paper thin making it near impossible to remove the epidermis. They also contain high levels of polyphenolics. These mentioned stomatal guard cell assays are also easier to perform than the protoplasts assay.

Chapter 4

Conclusions and Prospectives

VvPNP is a small peptide molecule of 144 amino acids encoded by a gene consisting of a single intron flanked by two exons. The resulting 15 kDa peptide has been shown to have considerable homology to the molecule from *Arabidopsis thaliana*, AtPNP -A which was used as a template from the model organism. The gene encoding AtPNP-A has a 100bp intron, as does VvPNP, which has been conserved throughout natriuretic peptides. Isolation of the VvPNP transcript from the young leaves of grapevine show not only that the protein is likely to be found in the leaves but is synthesised there as well. Alignment of VvPNP with AtPNP-A shows that these two peptides share a 30% identity with the largest continuous stretch of identical amino acids in the regions of greatest importance. These being the designated biologically active domain [Wang *et al.*, 2007] and two diagnostic motifs [Ludidi et al., 2002] described to be found exclusively in irPNP molecules and one other peptide the , CjBAp12 in citrus. The is the first evidence of a natriuretic-like peptide gene found in *Vitis vinifera* exhibiting all major characteristics of previously described PNPs.

Confident that we have identified a natriuretic peptide in grapevine we turned to the task of acquiring the peptide for studies related to function of this molecule. The peptide was expressed in *E.coli* by an inducible system using the GST fusion protein as a coupling molecule to ease purification. Although the purification was successful, it is felt in lieu of the results that an alternate approach may have fared more to our advantage, as we encountered that the majority of the protein to be expressed as insoluble inclusion bodies. A denaturing purification using either guanidine-HCl or urea was an option however, with an approach such as this there are no guarantees of the functionality once the protein has been re-folded or the time frame necessary for optimization of the technique. This alternative method was seen as an option should the standard purification not succeed. Another very interesting procedure which could have been attempted was the expression of the protein within a eukaryotic system or even a cell free system which is a relatively new technology. Both these avenues offer an exciting opportunity for not only soluble but functional peptides as well. The ability for post translational processing is enhanced by the use of eukaryotic microorganisms which

contain the cellular machinery for such processes. Regardless of what could be done, the prescribed purification did work and subsequent functional test could be attempted albeit unsuccessful. The isolation of protoplasts was a novel experience. An interesting addition to the experiment would have been the addition of 8-Br-cGMP, the homologue of cGMP which was shown to induce protoplast swelling [Maryani et al., 2001; Wang et al., 2007]. Speculative reasons for the bioactivity assay not producing statistical significant results were that the GST may have obstructed the active region in VvPNP. Therefore a cleaved product should be tested during future research. Another is that the environment within a bacterial cell does not allow the formation of disulphide bridges and so would cause the protein to fold incorrectly. Finally the use of a different system of testing the functionality may well have provided alternative results.

The second messenger cGMP has been implicated in the signaling cascade with PNPs. With the completion of the grapevine genome sequencing initiative the search for a guanylate cyclase gene is not far behind. With the high level of divergence of these peptides, the identification will be a far more complex task than the *VvPNP* gene was. Until now we have little idea as to how critical a NP system is in plants. Future research using mutants or gene silencing studies would be a very exciting direction for exploration and could provide insight into biochemical pathways associated with natriuretic peptide signaling and the plant's dependency on such a system.

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