Isolation and identification of PNP-A homologues from *Xerophyta viscosa* Baker

by

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PREFACE

"I declare that, ISOLATION AND CHARACTERAZATION OF PNP-A in *Xerophyta viscosa* BAKER, is my own work and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references."

Sithembiso Mabuto May 2004

Signed.....

percentage similarity margins of 96 %. The phylogenetic tree proposes that this group of orthologs is closely related to p12 (classified as PNP-B) molecules, β-expansins and α-expansins.

XvPNP-A and *AtPNP-A* active domain sequences comparisons show homology of 99.05%, with a single nucleotide difference on the third base of the coding sequence and 99.05% similar at the amino acid level. The close homology between *XvPNP-A* and *AtPNP-A* may suggest that they share the same functional attributes. Thus, the key question is weather AtPNP-A can open stomata in both *X. viscosa* and in *A. thaliana.* AtPNP-A was cloned and expressed in *E. coli* BL21 DE3* pLys (transformed with pCRAtPNP-A). Here we report that AtPNP-A which is a very close homolog of XvPNP-A induces significant stomatal opening in fully hydrated *X. viscosa* and this is taken as (albeit indirect) evidence for a function of PNP-like molecules in the regulation of homeostasis in *X. viscosa.*

LIST OF FIGURES

ABBREVIATIONS

Amp	Ampicillin
APS	Ammonium Persulfate
bp	Base pair
X-Gal	5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
^{o}C	Celsius
CTAB	Cetyltrimethyl ammonium bromide
CM	Chloramphenicol
EDTA	Ethylene Diamine Tetra-Acetic Acid Disodium Salt
g	Gram
IPTG	Isopropylthio-ß-D-galactoside
K	Potassium
KCl	Potassium Chloride
L	Liter
LB	Luria broth
µM	Micromolar
µL	Micro liter
mA	Milliamps
mg	Milligrams
mM	Millimolar
ml	Milliliter
M	Molar
nm	Nanometers

1. INTRODUCTION

Maintenance of growth, development and normal physiological processes remains crucial for the survival of plants. Plant hormones are described as integrators of external signal and internal developmental process in plants. They also attribute as mediators of physiological and developmental adaptation throughout the plant life cycle (Wilkinson *et al.*, 1997).

It has been well over 50 years since Went and Thimann (1937) published their classical book *Phytohormones*. At that time, the term phytohormone was synonymous to auxin, although compounds like cell division factors were anticipated to be phytohormones based on physiological experiments. Since then a number of plant hormones were discovered and a series of studies concluded that plant hormones are at least partly responsible for plant growth, development, cell elongation, cell division, differentiation and stomatal movement in response to environmental stimuli. Plant hormones are categorized into three groups, known as *classical*, *non-classical* and *peptide* hormones. Here, Plant Natriuretic Peptide (PNP) will be reviewed as a proposed additional peptide hormone.

1.1 Classification of plant hormones

1.1.1 Classical Hormones

Since 1937, cytokinin, gibberellin (GA), ethylene and abscisic acid (ABA) together with auxin are regarded as classical hormones. In the 1950s cytokinin was classified as plant hormone, due to its ability to induce plant cell division (Miller *et al.*, 1955). Shortly after

are encoding for these enzymes have been transformed with *A. tumefaciens* to alter the level of IAA in transgenic plants. (Klee and Romano, 1994). IAA was reported to conjugate to amino acids, peptides or carbohydrates to form biological inactive IAA, which functions as IAA storage in seeds and hormonal homeostasis (Szerszen *et al.*, 1994)

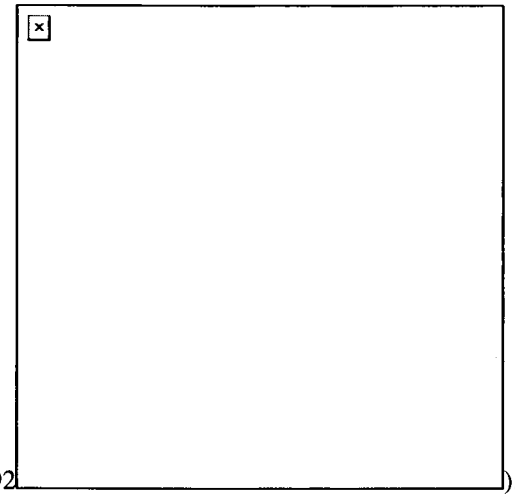
Gibberellin (GA) is now considered as one of the important classical plant hormone involved in the regulation of processes from seed germination through to the development and reproduction of plants (Hooley, 1994). Since the first GA from higher plants was identified 46 years ago, more than 112 GAs have been identified (Hisamastu *et al.*, 1998). However of those, only a few are bioactive whereas others are precursors or deactivated GAs (Ingram *et al.*, 1984). Studies have shown that the GA biosynthetic pathway can be divided into three stages. The first stage is considered to be the cyclization of geranylgeranyl diphosphate to *ent*-copalyl diphosphate, which in turn is converted to *ent*-kaurene (MacMillan, 1997). The second stage of the pathway involves sequential oxidation of C19 of *ent*-kaurene via *ent*-kaurenol and *ent*-kaurenal to form *ent*-kaurenoic acid to give out GA_{12}-aldehyde (Kende and Zeevaart, 1997). The last stage involves oxidation of GA_{12}-aldehyde to GA_{12}, and GA $_{12}$ is oxidized further to G_{20} and then hydrolase converts GA_{20} to the bioactive GA_1 (Xu *et al.*, 1995; Wu *et al.*, 1996). Three classes of enzymes catalyze GA biosynthesis: terpene cyclases catalyze the synthesis of *ent*-kaurene, cytochrome P450 monooxygenases catalyze the second step of the pathway and soluble dioxygenases catalyze the final steps of the pathway. Genes encoding enzymes for the two-terpene cyclase-catalyzed steps, copalyl diphosphate

epoxidation of zeaxanthin to antheraxanthin and violaxanthin, indicating that the epoxycaretoids volaxanthin and neoxanthin are essential for ABA production (Zeevaart *et al.,* 1991). Moreover, ABA biosynthesis increases when plant cells lose turgor (Zeevaart and Creelman, 1988), raising the question of which step in the pathway is activated by water stress. Epoxycaretenoid to xanthoxin reaction is the most likely regulatory step in the pathway since epoxycaretenoid precursor is always in large excess relative to ABA (Norman *et al.,* 1990). Recently, a viviparous mutant of maize, vp14, has been isolated and gene cloned. Moreover, a VP14 fusion protein catalyses the cleavage of 9-*cis*-epoxycaretenoids to form xanthoxin (Schwartz *et al.,* 1997b), which in turn is converted to the active isomer of ABA.

Ethylene biosynthesis pathway remained a mystery until 1979 when 1-aminocyclopropane-1-carboxylic acid (ACC) was discovered to be an immediate precursor of ethylene (Yang and Hoofman, 1984). Ethylene biosynthesis starts with the conversion of S-adenosyl-L-methoinine to 5'-methylthioadenosine and then ACC. ACC synthase is the enzyme that catalyses this reaction and it is encoded by a multigene family (Kende, 1993). The final step in ethylene biosynthesis is the conversion of ACC to ethylene, catalysed by ACC oxidase. Ethylene can be metabolised by plant tissues to ethylene oxide and ethylene glycol (Sanders *et al.,* 1989), but the physiological significance of this metabolism remains to be established (Kende and Zeevaart, 1997).

main groups of oligosaccharins that modulate plant growth and development. Both are generated from the cell walls or cell wall linked compounds (Creelman and Mullet, 1997). Biologically active pectic oligosaccharins are generated by chemical or by enzymatic hydrolysis of cell walls. Exogenous pectic oligosaccharins antagonize auxin action when excised tissues or tobacco TCL explants are utilized. However, not much is currently known about the role of pectic oligosaccharins in whole plant growth and development.

1.1.3 Peptide Hormones

Peptide hormones are well established in animal systems but have only recently been discovered in plants (Ryan *et al.*, 2002). In tomato leaves, an 18-amino acid polypeptide called systemin (SYS), that signals the systemic synthesis of several wound-responsive proteins that seem to participate in plant defense and wound healing was discovered in 1991 (McGurl *et al.*, 1992 ). Several other plant polypeptide hormones like ENOD40, rapid alkalinization factor (RALF),

hormones (like natriuretic peptides) and factors, in that it is processed from a larger precursor by proteolytic cleavage (McGurl *et al.,* 1992). In plants, prosystemin (PS) has been shown to be associated with the vascular tissue, placing the synthesis of the SYS precursor in the tissue necessary for systemic signaling (Jacinto *et al.,* 1997).

RALF was identified and isolated from tobacco leaves as a 49 amino acid polypeptide and shown to be active in alkalinization assays (Pearce *et al.,* 2001a; Pearce *et al.,* 2001b). RALF induces MAP kinase activity in cultured cells and causes alkalinization in tobacco leaves (Pearce *et al.,* 2001b).

Two small polypeptide were reported to regulate plants cell proliferation in dispersed asparagus suspension cultured cells by Matsubayashi and Sakagami (1996). Later structural analyses studies revealed that these small peptides were modified with sulfate groups (Matsubayashi and Sakagami, 1996) and they were called phytosulfikines (Hanai *et al.,* 2000b).

CLAVAT3 (CLV3) is an extracellular signaling polypeptide of 79 amino acid in length, involved in the determination of cell fate in the shoot apical meristem (SAM) of *A thaliana* (Clark *et al.,* 1993; Nakajima and Benfey, 2002).

Although only a small number of plant polypeptide signals and their receptors have been identified, major research efforts in this area are necessary. Botanists and plant physiologists are faced with the task of elucidating plant hormone biosynthesis pathways and its evolutionary links, if any, with animal polypeptide hormone signaling systems.

effect of natriuretic peptide via the generation of cGMP. Clearance receptors play an

1.2.2 The Function of Natriuretic Peptides

Three different natriuretic peptides have been characterized as ANP, BNP and C-type natriuretic peptide, all of which function as secreted hormones involved in regulating blood pressure and blood volume through direct effects on the kidney and systemic vasculature (Silberbach and Roberts, 2001; Misono, 2002; Hamet *et al.*, 2002). ANP and BNP are both produced and released from the heart in response to atrial stretching, and has immediate effects on increasing electrolyte and water excretion in the kidney. They also regulate and modulate cellular growth, cellular proliferation and most recently hypertrophy (Silberbach and Roberts, 2001; Misono, 2002; Hamet *et al.*, 2002).

These natriuretic actions of ANP are explained by its combination with ANP receptors on kidney cells that convert GTP into cGMP by activating particulate guanylate cyclases (GC). This form of GC is part of a cell surface receptor for ANP (Ruskoaho, 1992; Hamet *et al.*, 2002). A cGMP cascade activation by natriuretic peptides such as atrial natriuretic peptide (ANP) has been shown to stimulate natriuresis and diuresis in the kidney by increasing glomerular filtration rate, inhibiting renin release in juxtaglomerulosa cells, and inhibiting Na^+ and water re-absorption in the collecting duct (Ballermann *et al.*, 1992; Gambaryan *et al.*, 1996). In mammalian kidney, active Ca^{2+} reabsorption takes place primarily in the distal part of the nephron and involves the coordinated processes of passive Ca^{2+} influx across the luminal membrane by an as yet unidentified transporter. This process is followed by diffusion through the cytosol of Ca^{2+} bound to calcium-binding proteins, and active extrusion across the opposing basolateral membrane (Bindels *et al.*, 1991; Friedman and Gesek, 1995; Friedman *et al.*, 1996). It is well established that this transcellular Ca^{2+} transport is under the control of hormones, but

1.3.1 Structural evidence of plant natriuretic peptide

Radioimmunoassay (RIA) with antibodies to the 126-amino acid prohormone of atrial natriuretic peptide (proANP) and ANP recognize similar molecules in plants from different divisions (Vesely and Giordano, 1991). Furthermore, a study based on RIA standard curves to the human sequence suggested that putative proANP and ANP isolated from plants show high degree of similarity with the vertebrate analogues. Data from high performance gel permeation chromatography (HPGPC) indicates that proANP and ANP elution profiles and molecular weights are nearly identical between plant immunoreactants and the human peptides (Vesely *et al.,* 1993). Isolation and immunoaffinity purification of active plant natriuretic peptide using rabbit anti-ANP antibodies on isolated plant membranes has been reported (Billington *et al.,* 1997). This immunological evidence suggests the presence of natriuretic peptides in plants. Immunoreactants Plant Natriuretic Peptide (irPNP) has been partially sequenced on their C-terminal and N-terminal (Maryani *et al.,* 2001) and since then a homologous sequence from *A. thaliana* has been identified. This *Arabidopsis* sequence is termed *Arabidopsis thaliana* Plant Natriuretic Peptide (AtPNP-A), it is 478 nucleotides long and contains one intron of 100 bp and the predicted protein contains 126 amino acids and has molecular mass of approximately 14 kD (Ludidi *et al.,* 2002). *AtPNP-A* encoded protein displays similarities to AtPNP-B, expansins, pollen allergens and distant relation with endoglucanases (Ludidi *et al.,* 2002). AtPNP-B displays domain similarities with cell wall loosening expansins, but lack cell wall loosening activity of expansins (Ludidi *et al.,* 2002). Ludidi *et al* (2002) further reports that expansins share domain similarities with

1996). Later irPNP was isolated and immunoaffinity purified from *Hedera helix* leaf extracts with rabbit anti-α-ANP(1-28) antiserum (Billington *et al.,* 1997). Pharmawati *et al* (1998a) further reports that irPNP induced stomatal aperture is inhibited by LY 83583, a guanylate cyclase inhibitor. Pharmawati *et al.,* 1998 further reported that a rapid and specific transient elevations cGMP levels was observed in response to irPNP in maize (*Zea mays* L.) root stele tissue. The irPNP fraction showed a three-fold increase in cGMP levels whereas 1μM ANP failed to increase cGMP levels, even when the concentration was raised to 10μM (Pharmawati *et al.,* 1998). IrPNP, rANP and cGMP also proved to promote lateral water movement out of xylem of *Tradescantia* shoots (Suwastika and Gehring, 1998). These findings suggest the presence of NP receptors in plant tissues and the presence of guanylate cyclases possibly similar to the domains that have been identified in the receptors NPR-A and NPR-B in vertebrates (Chinkers *et al.,* 1989). Figure 1.3 shows a proposed model of the interaction between irPNP, cGMP, H^+-ATPase and ion channels in plant cells.

Therefore if plant immunoaffinity purified natriuretic peptides can modulate osmotically induced swelling of mesophyll cell protoplasts (MCPs) that can result in water and salt homeostasis, then there is a possibility that a natriuretic peptide hormone similar to vertebrates may exist in plants. IrPNP would also require receptors as a binding site prior to guanylate cyclase production.

1.4 Drought Tolerance in plants

Drought stress remains a major impeding-factor for plant growth and development. Synthesis and accumulation of osmoprotectants, up-regulation of gene expression implicated in repair of desiccation injury and late-embryogenesis-abundant (LEA) protein may contribute to an increase in the drought tolerance of plant (Song and Wang, 2002). Earlier it was noted that auxins, GAs, and ABA are also involved in regulating grain development (Kende and Zeevaart 1997) and ABA is necessary for seed maturation and dormancy and adaptation to a variety of environmental stresses. The role of ABA in these processes is mediated by changes in gene expression and stomatal closure (Zeevaart, 1999). It has been shown in bean (*Phaseolus vulgaris*) that the gene encoding the cleavage enzyme (*PvNCED1*) is up-regulated by water stress, preceding accumulation of ABA. These plants showed a marked increase in their tolerance to drought stress (Qin and Zeevaart, 2002). Transgenic wild tobacco (*Nicotiana plumbaginifolia* Viv.) plants were produced that overexpresses the *PvNCED1* gene either constitutively or in an inducible manner. *PvNCED1* gene from bean was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and introduced into wild tobacco. The transgenic plants had a low stomatal conductance and presumably as a result they remained much longer turgid than wild-type plants when deprived of water (Qin and Zeevaart, 2002). These findings suggest that water use efficiency in plants can be improved by genetic manipulation of ABA levels.

PNP effects such as increased cell volume, increase in water movement in xylem and promotion of stomatal opening in responses to plant NP's (Gehring and Irving, 2003)

(Radin, 1983). Due to the reported involvement of irPNP in plant homeostasis and abiotic stress response, it is further hypothesized that the presence and regulation of irPNP in *X. viscosa* may be assisting in the drought resistance or tolerance of the *X. viscosa* plant.

A further investigation that encompasses a search for the transcriptional pattern and gene regulation during dehydration of *X. viscosa* is required. A set of experiment such as western blots, quantitative RT-PCR or northern blots would help to elucidate the role of PNP-like molecules in resurrection plants.

supernatant was removed and the genomic DNA pellet was washed with 1 ml 75%(v/v) ethanol. Lastly the DNA pellet was centrifuged at 7500g for 5 minutes at 4°C, supernatant removed and the pellet was air dried for 5-10 minutes. The genomic DNA pellet was re-suspended by pipeting in 0.3ml sterile distilled water. Genomic DNA was stored at -20°C and before use, thawed on ice.

2.2 RNase treatment of *Xerophyta viscosa* genomic DNA

Isolated genomic DNA was further purified using an RNase treatment procedure. This was performed in as described in the Promega, Wizard® SV genomic DNA purification system. The DNA pellet washed with 0.3ml cold 70% ethanol and centrifuged again at 13,000g for 5 minutes at 4°C. The DNA pellet was dried and dissolved in 50µl sterile distilled water and then analyzed after agarose gel electrophoresis. Spectrophotometric readings were taken at OD_{260} and OD_{280} respectively to determine the DNA concentration and protein contamination.

2.3. Primer selection

All oligonucleotides primers were purchased from the UCT DNA synthesis laboratory (UCT, Cape Town). The oligonucleotides were synthesized on a Oligo 1000M DNA Synthesizer (Beckman Instruments Inc.). The primers were designed flanking the AtPNP-A main domain excluding the signal peptide (Figure 2.1).

containing 2µl of genomic DNA (0.4µg/µl) and 5µl of the forward and the reverse primers (0.5µM each) aafter mixing by pipeting, 30µl of mineral oil was added into each tube to overlay the sample and the tubes were placed in a pre-heated Eppendorf Master thermal-cycler. The negative control reaction contained all the PCR reaction components except the genomic DNA.

The PCR cycle was as follows: 1st stage- denaturation step, 1 cycle at 94°C for 2 minutes. 2nd stage, 30 cycles at 94°C for 1 minute (denaturation), 55°C for 1 minute (annealing), 72°C for 1 minute (extension), 3rd stage- 72°C for 10 minutes. The samples were stored at 4°C after the PCR reaction was completed.

2.5 PCR DNA band purification

The PCR product obtained from the enhanced PCR reaction was run on an agarose gel and a band of ±315bp was excised from the gel and purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences GFX™ PCR DNA and Gel Band Purification Handbook) according to the manufacturers' instructions.

2.6 Ligation using pGEM®-T Easy Vector

The ligation reaction of pGEM-T Easy vector and the PCR product contained:
1µl of T4 Ligase 10X Buffer, 1µl of PGEM®-T Easy Vector (50ng), 2µl of PCR product, 1µl of T4 Ligase (3Weiss units/µl) and 5µl of distilled H_2O to make up a 10µl reaction.

2.8 Isolation of a XvPNP plasmid clone

A single bacterial colony was transferred from a selective plate of transformed bacteria and inoculated into a starter culture in 5ml LB medium containing 100ug/ml Amp in a 50ml flask. The culture was incubated at 37°C for 12 hours with vigorous shaking at 200rpm. 1ml of the starter culture was inoculated into a 50ml LB (0.5g Yeast extract, 1g Tryptone, 1g NaCl, 100ml dH2O for 100ml) medium containing Amp (100ug/ml) and incubated at 37°C overnight with vigorous shaking at 200rpm. 30ml of the overnight *E.coli* XL1-blue culture was centrifuged at 5000g for 10 minutes at 4°C and the supernatant discarded. The pellet was carefully resuspended in 4ml Suspension buffer as described in Roche, Geno Pure Plasmid Midi Kit with RNase (10mg/ml). Suspension buffer was mixed well and 4ml of Lysis Buffer as described in Roche, Geno Pure Plasmid Midi Kit was added and mixed gently by inverting the tube. It was allowed to stand at room temperature for 3 minutes after which, 4ml of chilled Neutralization Buffer as described Roche, Geno Pure Plasmid Midi Kit) was added to the suspension and mixed gently. The suspension was incubated on ice for 5 minutes. The suspension was centrifuged at 12000g for 30 minutes at 4°C to clear the lysate. Immediately after centrifugation the supernatant was carefully removed from the tube and stored on ice in a sterile tube.

The binding column as described in Roche, Geno Pure Plasmid Midi Kit was fixed and equilibrated with Equilibration Buffer as described in Roche, Geno Pure Plasmid Midi Kit. The supernatant was loaded into the equilibrated column and the column was

3. EVOLUTIONARY ANALYSIS OF THE PNP MOLECULE

3.1 Homology searches

The National center for Biotechnology Information (NCBI) nucleotide-to-nucleotide blast search engine was used to search for homologous sequence in the nucleotide database (Madden *et al.,* 1996).

XvPNP gene was used as a query to retrieve and identify plant homologues. Homology searches of the PNP-A amino acid against the NCBI nucleotide database were performed using nucleotide-to-nucleotide BLAST searches. A set of nucleotide sequences with significant sequence similarities to the submitted queries formed the basis for the selection of homologous sequences to be included in the alignment and subsequently coding sequences were retrieved. Entries shorter than 50% or longer than 300% of the length of the query sequence were removed from the final set of homologues.

3.2 Phylogenetic analysis

All potential homologues were automatically retrieved, edited and loaded into Bioedit (Hall, 1999). The coding sequences were toggle (reserve to nucleotide option) translated into protein sequences. Full inframe multiple alignments of the coding sequences under bootstrap value of a 1000 was done with ClustalW (Higgins *et al.*, 1996). The inframe alignment was saved as a FASTA (*.fas,*.fst,*.fsa) format document to construct

heat shocked at 42°C for 2 minutes. Cell aliquots of 200µl were prepared in 1.5ml sterile tubes, frozen in liquid nitrogen and stored at –80°C.

4.3 Growth and induction of *E.coli* BL21 DE3* pLys Expression cultures

E .coli BL21 DE#*pLyS transformed with PCRAtPNP-A plasmid was inoculated into 50ml of 0.4% glucose YT broth with 35µg/ml CM and 200µg/ml Amp and incubated overnight at 37°C under vigorous 220rpm shaking. 4ml of the overnight culture was subcultured into 200ml of 0.4% glucose YT broth with 200µg/ml Amp. The subculture was incubated for 2-3 hours until the OD_{600} reading was 0.5-0.7 and 1ml of culture was taken, centrifuged at 10000g for 30 seconds and supernatant was discarded carefully. The cell pellet was redissolved in 50µl 5x SDS-PAGE sample buffer and 300µl (0.3mM) IPTG was added into the expression culture and incubated for 3 hours at 37°C under vigorous shaking of 220rpm. Every 1 hour, 1ml aliquot was taken for OD_{600} reading and 1ml for SDS-PAGE analysis. Expression culture was transferred to 50ml centrifuge tubes and centrifuged at 4000g for 10 minutes at 4°C. Supernatant was poured off carefully and the pellets were stored at –20°C.

4.4 Preparation of cleared *E. coli* lysates and active AtPNP-A purification.

The cell pellet was thawed for 15 minutes on ice and resuspended in buffer B (100mM NaH_2PO_4, 10mM Tris-Cl and 8M Urea at pH 8) at 5ml of buffer B per g-wet weight. Resuspended cells were stirred carefully for 60 minutes at room temperature and the cell

Running Gel

Stock	15% Acrylamide gel
30% Acrylamide	5.0ml
0.8% SDS	1.25ml
3M Tris buffer, pH8.8	1.25ml
Sterile distilled water	2.43ml
10% Ammonium persulphate	50µl
TEMED	20µl

Stacking Gel

Stock	5% Acrylamide gel
30% Acrylamide	0.8ml
0.8% SDS	0.625ml
1M Tris buffer, pH6.8	0.625ml
Sterile distilled water	2.905ml
10% Ammonium persulphate	25µl
TEMED	20µl

An aliquot of 25µl elution sample was loaded in SDS-PAGE electrophoresis gel and the samples were ran at 50V for 20 minutes and then 200V for 45 minutes. SDS-PAGE gel was soaked in coomassie staining buffer (45% methanol, 10% acetic acid, 1% brilliant blue R-250 in distilled water) for 30 minutes. The staining buffer was poured off and

RESULTS AND DISCUSSION

5. Isolation and characterization of PNP from *Xerophyta viscosa* Baker

5.1 Isolation of PNP-A homologue

Since drought stress remains a major prohibitive factor for plant growth and development the synthesis and accumulation of osmoprotectants, up-regulation of gene expression implicated in repair of desiccation injury and late-embryogenesis-abundant (LEA) protein could increase the drought tolerance of plants (Song and Wang, 2002). The second messenger cGMP is reported to modulate proton pumping in the plasma membrane (PM) vesicles from *Tradescantia multiflora* stem and leaf cells (Suwastika and Gehring, 1999). Cation uptake, driven by plasma membrane H^+-ATPase activity is utilized by plants as one way to regulate cell volume or water uptake (Suwastika and Gehring, 1999). Recently, it has been reported that irPNP modulates the plasma membrane H^+ gradient in *Solanum tuberosum* tissue vesicles (Maryani *et al.*, 2000). This functional evidence suggests that in plants, as in vertebrates, NPs can influence water and solute homeostasis possibly via modulating ATPase-dependant ion transport. Rafudeen *et al.* (2003) reports that NaCl and osmotic stress induce irPNP increase in *A. thaliana* and NaCl causes an irPNP increase in *E. strigosum* at 300mM NaCl, thus linking irPNP increases to changes in ionic (and homeostatic) conditions.

nucleotide sequence (Accession no: AY142603). The AtPNP gene is 492bp long including a single 100bp intron. The AtPNP gene is subdivided into two parts, the signal peptide and main domain (family-45 endoglucanase-like domain) respectively. The primer set design omitted the signal peptide using only the flanking regions of the main domain to design the primers. A band of ± 392bp including the intron in the main domain was expected upon PCR amplification. The nucleotide and primer sequences are shown in figure 3.2.

(A)

ATGATAAAAATGGCAGTAAAATTTGTGGTAGTGATGATAGTGTTTGCACAAATCTTAGCTCCA
ATCGCTGAAGCT**GCTCAAGGAAAAGCTGTCTATTACG**ACCCTCCCTACACTAGGTCTGCGTG
TTACGGAACACAACGTGAGACGCTGGTAGTCGGAGTCAAGAACAATTTGTGGCAAAATGGTC
GAGCTTGTGGTCGGAGGTACAGGGTTCGATGCATTGGTGCTACATACAACTTTGACAGAGCAT
GCACCGGGCGTACCGTAGACGTGAAGGTAGTTGATTTCTGCCGGGAGCCTTGCAACGGTGACC
TTAATCTCTCTCGTGACGCTTTTCGGGTTATCGCTAATACTGATGCCGGTAA**CATTCGTGTCG**
TATACACACCGATATGA

(B)

Forward primer: 5' **GAT[3] ATC G[7]GA TCC** GCT CAA GGA AAA GCT GTC TAT TAC G 3'

3 EcoRV and 7 BamHI

Reverse primer: 5'**ACT AAG AAG[2]** CTT TTA CGG TGT GTA TAC GAC ACG AAT G 3'

2 Hind III

Figure 3.2: The primer set was designed from the main domain of the known full length PNP sequences isolated from *A. thaliana* (Accession no: AY142603). (A) The full-length *A. thaliana:AtPNP-A* gene sequence. The primer set is indicated in bold. (B) Nucleotide sequences of the forward and reverse primer set. Both primers were designed to have restriction site to facilitate ligations into plasmid vectors.

The band that was amplified using PCR generated a band less than the predicted size. The amplified DNA band was ± 100bp less that expected size, suggesting that the amplified

plasmid (pGEMXvPNPA) was also performed using the T7 and SP6 primers. The resultant nucleotide sequence result was sent to the NCBI and TIGR database to search for similarity and alignment to known irPNP gene sequences.

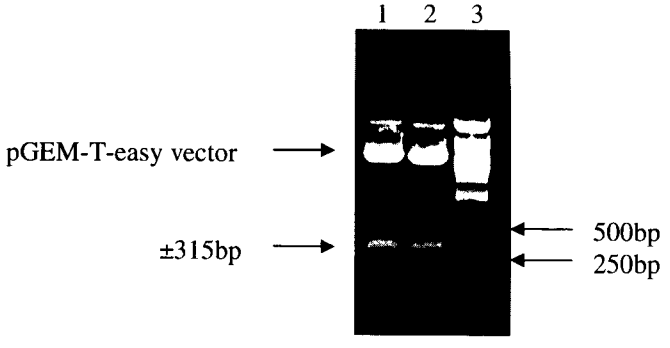


Figure 3.4: XvPNP plasmid enzyme digest separated on 1% agarose electrophoresis gel. Lane 1:10µl of digested XvPNP plasmid (0.38µg/µl) with EcoR I; Lane 2: 10µl of EcoR I digested XvPNP plasmid (0.38 µg/µl) ; Lane 3: 10µl of Pst I DNA marker (0.1µg/µl).

A nucleotide sequence of 315bp was elucidated from the sequencing results (Figure 3.5). The sequence was termed *Xerophyta viscosa* Plant Natriuretic Peptide (XvPNP), since it was a homologous/orthologous sequence to the AtPNP-A nucleotide sequence. The nucleotide sequence had a 96.0 % homology with AtPNP, EsPNP and HhPNP genes.

We further report that *XvPNP-A* contains no intron as compared to *AtPNP-A* (Ludidi *et al.*, 2002). This finding can be interpreted in the light of recent scientific findings and arguments that propose that introns arose as "selfish" elements that play no constructive role in evolution and thus gene function (Fedorov *et al.*, 2002). The "introns-late hypothesis" postulates that, introns appear relatively late in the evolution of eukaryotes

Logsdon *et al* (2002) reports that if the "introns-early theory" was true, then the common animal-plant intron positions should be also enriched in fungal genes, because the evolutionary separation of fungi and animals occurred after the separation of animals and plants. The result presented here - *XvPNP-A* lacking an intron compared to the *AtPNP-A* – would be consistent with the "intron-late hypothesis".

5.2 Phylogenetic analysis of XvPNP-A

XvPNP sequence was used as a query sequence to the NCBI database. In all retrieved sequences 15 coding domain sequence (CDS) were identified as homologues (determined by BLAST E-value) to *XvPNP* as described on the material and methods. All retrieved nucleotide sequences were translated to amino acid sequence and a multiple sequence alignment was done using ClastalW (Higgins *et al.*, 1996). The sequence alignment was performed to determine the sequence similarity or homologous relations between the sequences. Similarity in this case is defined as an observable quantity that is expressed as percentage or other suitable measures such as amino acid of the same group (Baxevanis and Ouellette, 2001). The multiple alignment displayed domain conservation between PNP-A, p12 (classified as PNP-B) and expansin molecule (See figure 3.6).

Conserved positions or regions are often of functional importance, since it is assumed or presumed that homologous sequences have diverged from the common ancestral sequence (Baxevanis and Ouellette, 2001). An alignment of well known trypsin protein of mouse (SWISS-PROT P07146) and crayfish (SWISS-PROT P00765) displays a sequence identity of 41%. They have conserved regions, which are important for disulfide bonds formation (Baxevanis and Ouellette, 2001). Disulfide bonds are imperative for proper folding of these proteins and substitution of cysteine in the conserved positions will result in protein misfolding. Conservative substitution is possible in some areas of the gene, due to the fact that it allows easy substitution of amino acid that are similar in physiochemical properties. Examples of conservative substitutions include isoleucine for valine (both are small and hydrophobic) and serine for threonine, both polar (Baxevanis and Ouellette, 2001).

Ludidi *et al.* (2002) report that the most closely related molecules to irPNPs are expansins. Expansins have extended C-termini when compared to the irPNPs. Ludidi *et al* (2002) further argue that irPNPs have in fact lost the C-termini and thus gained increased mobility. Expansins have been implicated in the cell wall loosening activity. IrPNP dependent processes have been observed in experimental systems that did not contain cell walls. The processes include irPNP-dependent modulation of plasma-membrane H^+ gradients in potato leaf tissue vesicles (Maryani *et al.,* 2000), elevation of cGMP levels in response to irPNP in potato guard cell protoplasts (Pharmawati *et al.,* 2001). This functional evidence thus follow that irPNP can act directly with the plasma membrane.

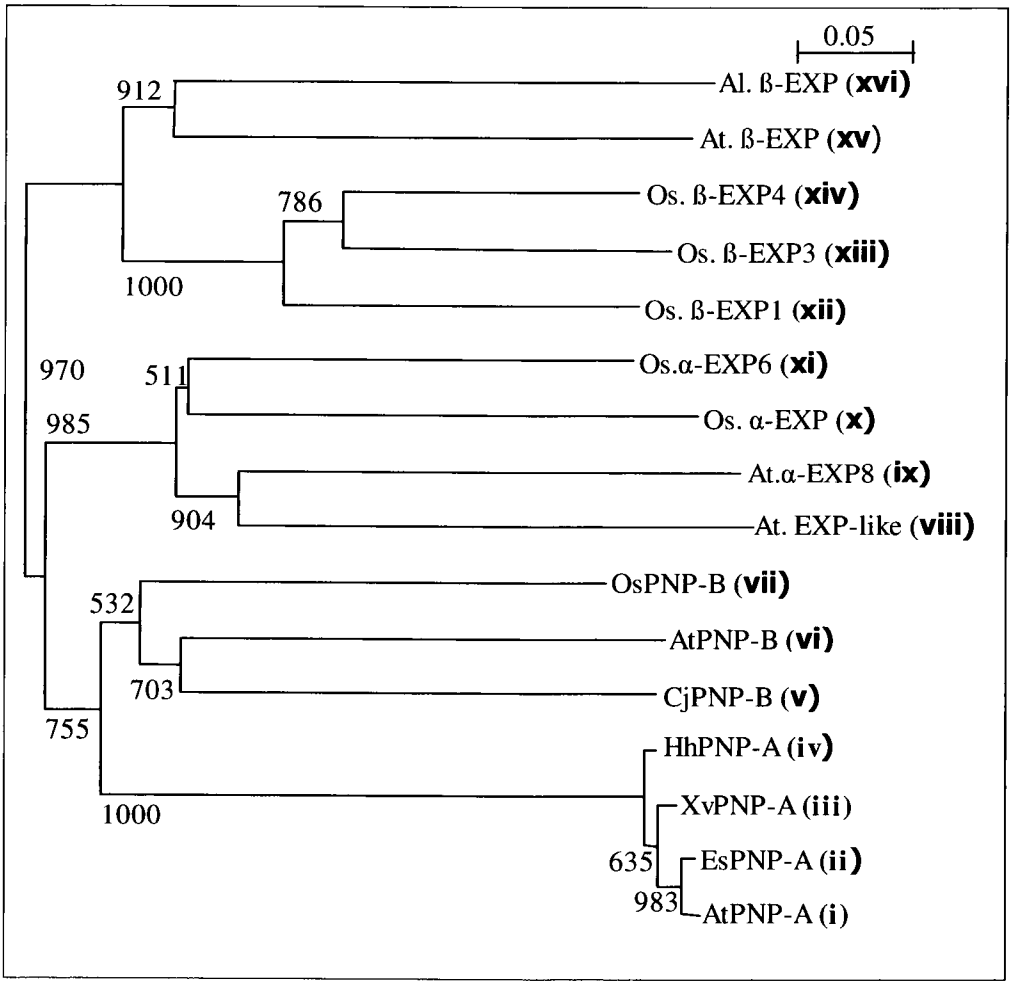


Figure 3.7: The phylogenetic tree representing relationship between PNP-A, PNP-B molecules, α–expansins and β–expansins (scale of 0.05). Branch lengths are approximately proportional to the amount of sequence difference. All PNP molecules display a close evolutionary relationship and are closely related to the blight response protein p12α–expansins and β-expansins are both distant related to PNP molecules. Sequence identification: **(i)** NM179648 **(ii)** AY093582 **(iii)** isolated **(iv)** AY243474 **(v)** AF015782 **(vi)** NM_119184 **(vii)** AK063132 **(viii)** TC190653 **(ix)** NM_129623 **(x)** NM_197539 **(xi)** TC140249 **(xii)** AF261273 **(xiii)** AF1011313 **(xiv)** AF391109 **(xv)** NM_105241 **(xvi)** AB051899

Figure 3.8: Phylogenetic relationships among PNP-A, PNP-B and expansins. Rooted Neighbor-jointing (NJ) trees are shown. Number of branches indicates percent support to the bootstrap analyses and the branch length are proportional to the distance between sequences. **(A)** Displays similarity among the PNP-A molecules and their bootstrap score. **(B)** Evolutionary relationship of PNP-A and PNP-B molecules display similarity, but distant evolutionary relations. The PNP-A molecules cluster together with high bootstrap values, indicating orthology relationships. **(C)** Evolutionary relationship of β expansins display paralogy relationships. The rice β expansins cluster together with high bootstrap value than other expansins, indicating paralogy relationship. **(D)** Evolutionary relationship among the α expansins also displays paralogy relationships in both rice and *arabidopsis* sequences.

5.4 Expression of recombinant AtPNP-A protein in *E. coli* BL21 DE3* pLys.

Recombinant AtPNP-A was expressed to be utilized in guard cell assays experiment on *X. viscosa* leaves to ascertain the activity of PNP-A in *X. viscosa*. AtPNP-A and XvPNP-A are almost identical, both on a nucleotide and amino acid level. XvPNP and AtPNP-A main domain sequences displays close homology of 99.05%, with a single nucleotide difference on the third base of the coding sequence and 99.98% similarity on the amino acid level. PCRAtPNP-A plasmid clone was utilized for AtPNP-A recombinant protein expression. AtPNP-A was expressed in *E. coli* BL21 DE3*, *E. coli* BL21 DE3* pLys and *E. coli* BL21 DE3* pLysE respectively after transformation with pCRAtPNP-A plasmid.

The expressed AtPNP-A protein lacked the signal peptide sequence. Cells of transformed *E. coli* BL21 De3 were grown at 37°C and the expression of the AtPNP-A protein was

A protein was produced as inclusion bodies in the *E. coli.* The formation of inclusion bodies is a frequently consequence of high-level protein production in the cytoplasm (Tsumoto, 2003).

Refolding of proteins is affected by several factors, including solubilisation of proteins by denaturants. 8M Urea was preferred as an efficient solubilisation and cell lysis reagent. Urea and other detergents are regarded as important since they play an important role in solubilisation efficiency, in the structure of the protein during denaturing state (Tsumoto, 2003). Urea shows concentration dependent binding to the proteins and in most cases, 6-8 M urea is required to achieve extensive binding efficiency that is sufficient to unfold and solubilise the proteins. It is reported that even at higher concentrations intra and inter-molecular interactions can occur (Tsumoto, 2003), often resulting in non-native structure. The proteins were subjected to both a stepwise dialysis and affinity column chromatography to renature the proteins. During affinity column chromatography 8M urea was administered and the concentration was decrease using a gradient as the solubilised proteins were applied into the affinity column to avoid any misfolding. The solubilised protein was purified in a single chromatography step on Ni-NTA agarose. AtPNP-A protein bound to the affinity matrix was eluted by adding imidazole in the final elution buffer. Homodimers that formed during renaturation would not be retained on the NTA–agarose resin because of the lack of a His-tag. In both the stepwise dialysis and affinity column chromatography procedures some protein was lost. Analyses of the purified fractions by SDS-PAGE gel identified only minor contaminations were in the

(Billington *et al.*, 1998 *b*) that stomatal cells open when exposed to AtPNP-A protein whereas the control the stomatal cells remain closed. *X. viscosa* stomatal guard cells were opened in response to AtPNP-A (0.2mg/ml), which indicated that the recombinant protein was active. The findings also suggest that a PNP-A derived system exist in *X. viscosa* plants and that this system may affect homeostasis in Xerophytes. Figure 3.11 displays an increase of *X. viscosa* stomatal aperture in response to AtPNP-A.

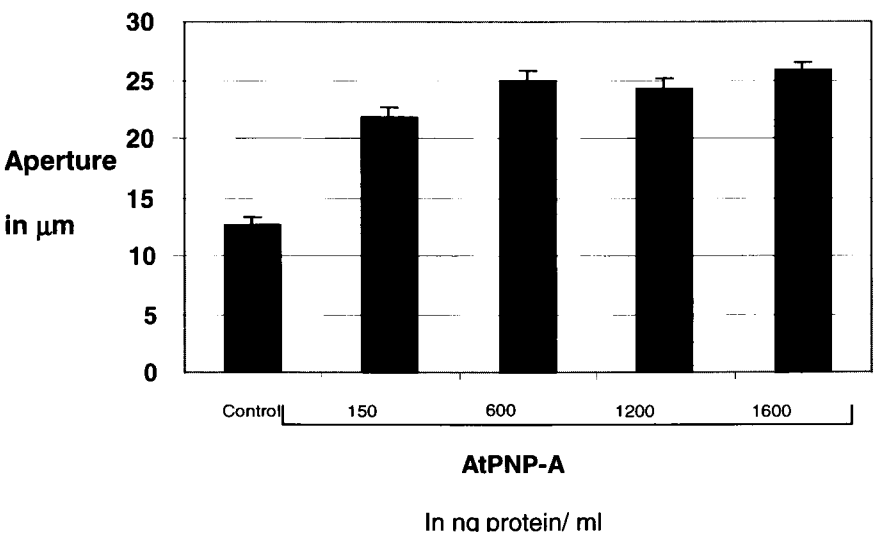


Figure 3.11: Graph of stomatal aperture measured on fully hydrated leaves of *X. viscosa* plants during exposure to different ATPNP-A concentration of 150ng/ml, 600ng/ml, 1200ng/ml and 1600ng/ml respectively. The plant leaves were incubated in microtitre plate and exposed to incandescent light (430nm at 35W m^{-2}) for 1 hour. The Y-axis represents stomatal aperture in µm, the X-axis is the control and AtPNP-A concentration in ng/ml. Bars are the mean ±SE of aperture of >60 stomata.

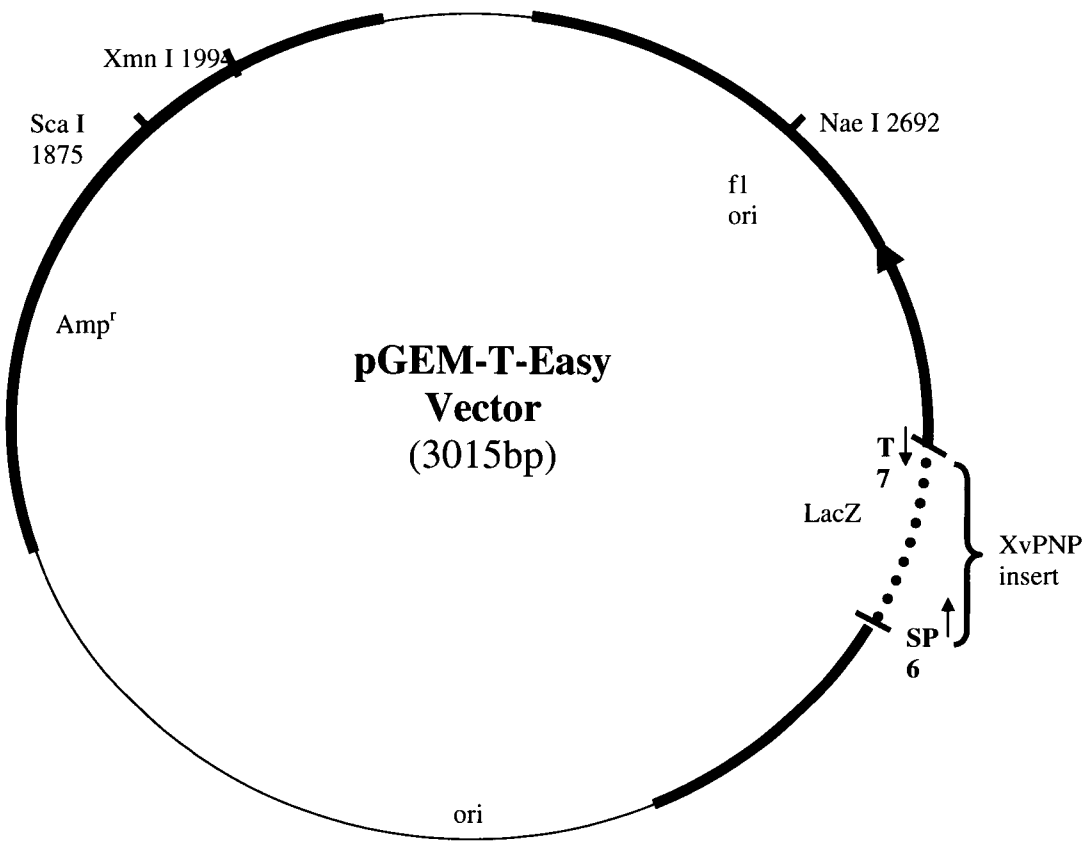


Figure 3.12: The recombinant representation of pGEM-T-Easy vector circle map and sequence reference points with the XvPNP insert. pGEM-T-Easy is contains T7 and S6 promoter, T7 and S6 RNA Polymerase transcription initiation site and multiple cloning sequence. Lac Z gene and β lactamase coding region are also contain to promote direct identification of plates by color screening.

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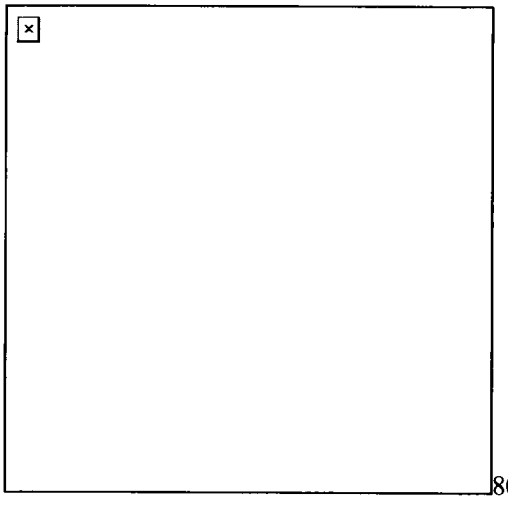

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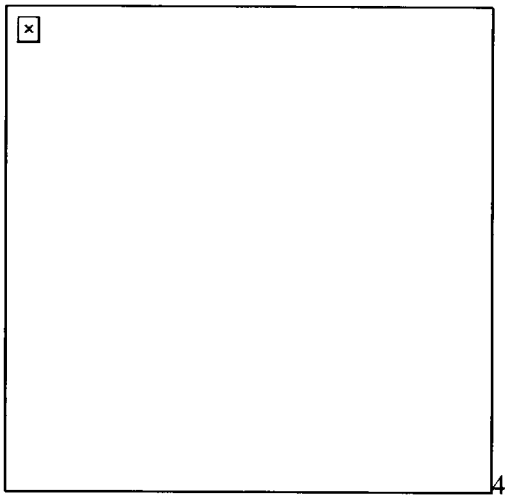

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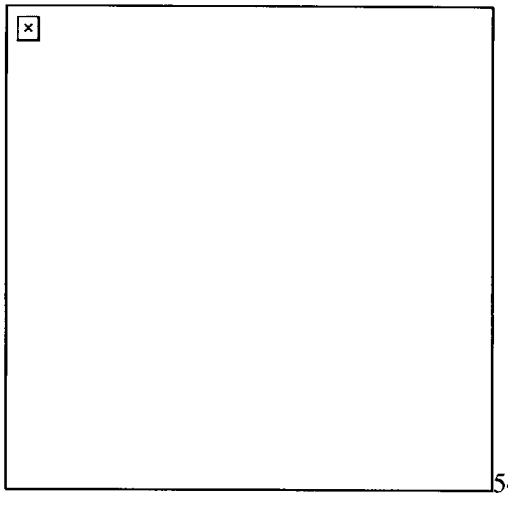

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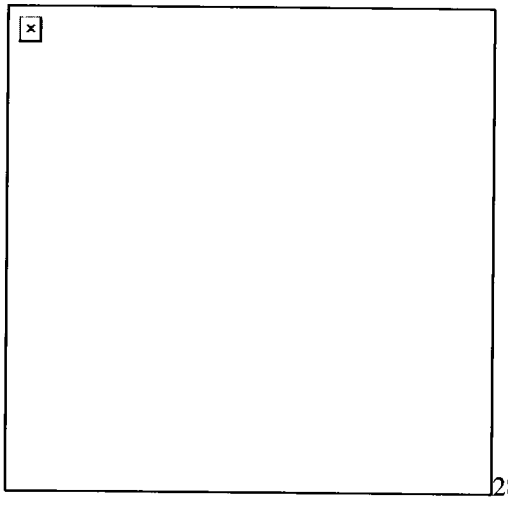

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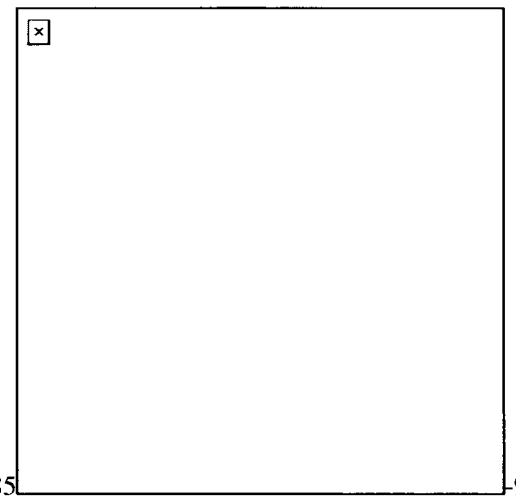

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