CHARACTERIZATION OF TWO *Arabidopsis thaliana* GENES WITH ROLES IN PLANT HOMEOSTASIS

Ndomelele Ndiko Ludidi

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Supervisor: Prof. C. A. Gehring

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DECLARATION

I declare that "**CHARACTERIZATION OF TWO *Arabidopsis thaliana* GENES WITH ROLES IN PLANT HOMEOSTASIS**" is my own work that has not been submitted for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Ndomelele Ndiko Ludidi January 2004

Signed.....

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Dedicated to my mother, my daughter and my late father,

With love!

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ABBREVIATIONS

2,4-D: 2,4-Dichlorophenoxyacetic acid

3-D: 3-dimensional

8-Br-cGMP: 8-bromoguanosine 3',5'-cyclic monophosphate

ABA: Abscisic acid

ANP: Atrial natriuretic peptide

ATP: Adenosine 5'-triphosphate

BAPH: Blight-associated protein homologue

BAPTA: 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

BNP: Brain natriuretic peptide

cADPR: Cyclic adenosine diphosphate ribose

cAMP: Adenosine 3',5'-cyclic monophosphate

CAMV: Cauliflower mosaic virus

cGMP: Guanosine 3',5'-cyclic monophosphate

CNG: Cyclic nucleotide-gated

CPTIO: Carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide

Da: Dalton

DAF: 4,5-diamino-fluorescein

EGTA: Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

fmol: femtomole

GA: Gibberellic acid

GDP: Guanosine 5'-diphosphate

GFP: Green fluorescent protein

GMP: Guanosine 5'-monophosphate

GST: Glutathione S-transferase

GTP: Guanosine 5'-triphosphate

GUS: β-glucuronidase

IAA: Indole-3-acetic acid

IBMX: 3-isobutyl-1-methyl xanthine

IPTG: Isopropyl-β-D-thiogalactopyranoside

irPNP: Immunoreactant plant natriuretic peptide

kDa: Kilodalton

L-NAME: N_{ω}-nitro-L-arginine methyl ester

LY83583: 6-(phenylamino)-5,8-quinolinedione

MIFE: Microelectrode ion flux estimation

Min: min

NAA: Naphthaleneacetic acid

NOS: Nitric oxide synthase

NP: Natriuretic peptide

NPR: Natriuretic peptide receptor

ODQ: 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one

PCR: Polymerase chain reaction

Pk G: Protein kinase G

PNP: Plant natriuretic peptide

PPi: Inorganic pyrophosphate

PR-1: Pathogenesis-related-1

rGC: Receptor/particulate guanylyl cyclase

RT-PCR: Reverse transcription-polymerase chain reaction

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

sGC: Soluble guanylyl cyclase

SNAP: S-nitroso-N-acetyl penicillamine

SNOG: S-nitroso-L-glutathione

TMV: Tobacco mosaic virus

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SUMMARY

A novel *Arabidopsis thaliana* gene (*AtPNP-A*) and its product (the protein we refer to as AtPNP-A), which directly or indirectly up-regulates guanylyl cyclase activity and thus leads to cGMP elevation, has been identified and partially characterized in this study. AtPNP-A is closely and evolutionarily related to the cell wall-loosening expansins that are responsible for cell expansion but has not been shown to have expansin activity. Furthermore, AtPNP-A induces elevation of cGMP levels in plants, modulates ion fluxes across roots, regulates stomatal aperture by affecting guard cell movements and regulates cell volume by promoting water uptake into plant cells. These functional features of AtPNP-A are thus similar to the effects elicited by atrial natriuretic peptides. It is interesting to note that a plant pathogen (*Xanthomonas axonopodis*) has been discovered to have a gene encoding a protein (Xac 2654) with significant similarity to AtPNP-A and no homologue in other bacteria. This finding has implications for plant-pathogen interactions, as putative horizontal gene transfer may be useful for a pathogen's success in colonizing its host.

The second messenger, cGMP, is known to have a role in salinity and osmotic stress. The fact that AtPNP-A regulates ion fluxes and induces elevation of cGMP levels in plants, coupled with the recent finding that expression of this protein is up-regulated in response to salinity and osmotic stress, indicates that AtPNP-A may play a role in responses to salinity and drought (osmotic stress). Identification of AtPNP-A and establishment of its role in inducing an increase in cGMP levels implies that it activates guanylyl cyclase(s) in plants, since it is the activity of these enzymes that is responsible for cGMP

biosynthesis. Several stimuli affect processes that are vital for plant survival and some of these processes are mediated by cGMP. These processes include: gene expression, responses to and establishment of resistance to diseases, normal responses to light in order to facilitate proper plant development and nutrition, responses to and establishment of tolerance to salinity and drought and responses to other environmental conditions. It is thus important to investigate the effect of such stimuli on the expression of genes whose products stimulate/up-regulate the activity of guanylyl cyclases (GCs). Furthermore, it is important to identify and fully characterize GCs in plants if the role of cGMP in plants is to be well understood. However, identification of a plant guanylyl cyclase has previously not been achieved despite the well-established roles of cGMP in plants. This study identifies a plant protein, named AtGC1, which has guanylyl cyclase activity. AtGC1 contains a domain similar to parts of the catalytic domain of known guanylyl cyclases and its activity is regulated by Mg^{2+} in a manner similar to that of known guanylyl cyclases, although AtGC1 activity does not appear to be regulated by Mn^{2+} whereas other guanylyl cyclases are. AtGC1 is, however, significantly different in amino acid sequence and domain organization from the known guanylyl cyclases. The identification of both a guanylyl cyclase-stimulating protein (AtPNP-A) and a guanylyl cylase (AtGC1) opens another important avenue for the study of cGMP-mediated signaling processes and is a step towards understanding plant responses to biotic and abiotic stimuli that are mediated by cGMP. Such an understanding has potential for use in establishment of plants with enhanced tolerance to biotic and abiotic stress. Current research is aimed at studying the spatial and temporal expression pattern of both AtPNP-A and AtGC1, studying the

cellular localization of these proteins and performing full characterization of *in planta* biological functions of these proteins.

GENERAL ABSTRACT

Plants are continuously exposed to varying conditions in their environment, to which they have to adapt by manipulating various cellular processes. Environmental (abiotic) and pathogen (biotic) stress are challenges against which plants have to defend themselves. Many plant responses to stress stimuli are a result of cellular processes that can be divided into three sequential steps; namely signal perception, signal transduction and execution of a response. Stress signal perception is, in most of these cases, facilitated by cell surface or intracellular receptors that act to recognize molecules presented to the cell. In several cases, hormones are synthesized in response to stress signals and in turn these hormones are perceived by cellular receptors that trigger signal transduction cascades. Propagation of signal transduction cascades is a complex process that results from activation of various signaling molecules within the cell. Second messengers like calcium (Ca^{2+}) and guanosine 3', 5'-cyclic monophosphate (cGMP) play a vital role in mediating many signal trasduction processes. The result of these signal transduction cascades is, in most instances, expression of genes that contribute to the plant's ability to cope with the challenges presented to it. Plant natriuretic peptides (PNPs) are novel plant hormones that regulate water and salt homeostasis via cGMP-dependent signaling pathways that involve deployment of Ca^{2+}. The aim of this study is to partially characterize a PNP and a guanylyl cyclase, both from *Arabidopsis thaliana*. Guanylyl cyclases synthesize cGMP from the hydrolysis of guanosine 5'-triphosphate (GTP) in the cell. The study also aims

to investigate the effect of drought and salinity on cGMP levels in plants, using sorbitol to mimic the osmolarity/dehydration effect of drought and NaCl as a source of salinity stress and thus link NaCl and sorbitol responses to both AtPNP-A and cGMP up-regulation.

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Natriuretic peptides (NPs) are polypeptide hormones that function to regulate blood pressure, blood volume and salt transport (Beltowski and Wójcicka, 2002; Takei, 2001). Natriuretic peptides such as atrial natriuretic factor (ANF, ANP_{99-126}), long-acting natriuretic peptide (ANP_{1-30}), vessel dilator (ANP_{31-67}) and kaliuretic peptide (ANP_{79-98}), as well as brain or B-type natriuretic peptide (BNP) act on target cells through A-type and B-type atrial natriuretic peptide receptors and stimulate cGMP biosynthesis by up-regulating the activity of guanylyl cyclases (Beltowski and Wójcicka, 2002). The role of NPs in modulation of cell growth, cell proliferation and cardiomyocyte hypertrophy has been demonstrated (Oliver *et al*, 1997). Binding of NPs to specific cell-surface receptors results in a signaling cascade that leads to the physiological responses affecting blood volume and cardiomyocyte growth (Oliver *et al*, 1997). Two types of NP receptors are known: R1 receptors that contain a guanylyl cyclase catalytic domain at the C-terminus (NPR-A, NPR-B) and R2 receptors that have no guanylyl cyclase activity (NPR-C). One

of the major biochemical effects of NP signaling is the generation of cGMP (Takei, 2001). However, the NPR-C clearance receptor, lacking in a guanylyl cyclase domain, is proposed to perform biological functions that are cGMP-independent (Anand-Srivastava, 1997).

1.2. Vertebrate natriuretic peptide immunoanalogues exist in plants

Radioimmunoassays, based on I^{125} labeling, have been used to demonstrate that antibodies against atrial natriuretic peptides interact with molecules in plant cells (Gehring *et al*, 1996). This observation has lead to the proposal that plants possess a system that is analogous to the natriuretic peptide system that occurs in vertebrates. It has been shown that this natriuretic peptide system plays a role in the regulation of solute flow in plant cells (Vesely *et al*, 1993). Treatment of plant leaf segments with rat atrial natriuretic peptide leads to opening of stomata in a manner dependent on *Rattus norvegicus* atrial natriuretic peptide concentration, resulting from swelling of guard cell pairs (Gehring *et al*, 1996). This demonstrates that rat atrial natriuretic peptide can modulate guard cell movements via regulation of water and solute fluxes across guard cells. The observation that vertebrate natriuretic peptides regulate salt (Na^+) fluxes in animal cells (DeBold, 1985) prompted an investigation into the effect of rat atrial natriuretic peptide on stomatal aperture size in the presence of salt (NaCl), with the view that if rat atrial natriuretic peptide has an analogous function in plant cells then rat atrial natriuretic peptide would be expected to influence stomatal opening in response to Na^+ since Na^+ ions affect guard cell movements. This question was addressed by pre-incubating leaf segments in indole-3-acetic acid, a hormone that induces stomatal

opening, in the presence of low or high NaCl concentration combined with addition of rat atrial natriuretic peptide and/or the Na^+/H^+ inward channel inhibitor amiloride (Gehring *et al*, 1996). Results from this study showed that application of indole-3-acetic acid results in maximal opening of stomata under low Na^+ concentrations (Gehring *et al*, 1996). However, high Na^+ levels inhibited indole-3-acetic acid-induced stomatal opening (Gehring *et al*, 1996). This indicates that high salt levels lead to stomatal closure and that such closure is not reversed by indole-3-acetic acid. Amiloride reversed this NaCl-induced stomatal closure (Gehring *et al*, 1996). However, *Rattus norvegicus* atrial natriuretic peptide promotes indole-3-acetic acid-induced stomatal opening even in a high salt environment (Gehring *et al*, 1996). Taken together, these observations indicate that stomatal opening is induced by indole-3-acetic acid but high NaCl levels prevent this effect. Furthermore, the observations also demonstrate that rat atrial natriuretic peptide serves to promote stomatal opening even in high salt. The biophysical response to solute influx into plant cells is water uptake and this water uptake is responsible for guard cell swelling and the resulting opening of stomata. However, the presence of high extracellular salt leads to water loss from the cells and results in guard cell shrinkage and stomatal closure. This stomatal closure is intended to prevent further water loss from the cells. The observation that rat atrial natriuretic peptide exerts its effects in animals by binding to membrane-spanning receptors, namely natriuretic peptide receptors (Anand-Srivastava and Trachte, 1993), prompted an investigation of the interaction between rat atrial natriuretic peptide and plant cells. Results from such a study, based on competition between I^{125}-labeled rat atrial natriuretic peptide and unlabeled rat atrial natriuretic peptide for specific binding to isolated cell membranes from leaves, showed that I^{125}-

labeled rat atrial natriuretic peptide binds specifically to the cell membranes and its binding can be displaced by increasing amounts of unlabeled rat atrial natriuretic peptide (Gehring *et al*, 1996). These observations serve as an indication of the presence of plant cell membrane receptors with specific affinity, albeit low, for rat atrial natriuretic peptide and further suggest that plants themselves have peptides immunologically, if not even functionally, similar to rat atrial natriuretic peptides. This thought necessitated attempts to isolate natriuretic peptide analogues in plants. Crude protein extracts from *Hedera helix* were subjected to immunoaffinity purification using rabbit anti-alpha atrial natriuretic peptide antibodies (Billington *et al*, 1997). These purified plant natriuretic peptides have a similar effect on guard cells as rat atrial natriuretic peptide does, namely induction of stomatal opening in a natriuretic peptide concentration-dependent manner (Billington *et al*, 1997). This serves as evidence for the existence of plant peptides capable of mimicking the effect of atrial natriuretic peptide in plants and strengthens the idea that plants express endogenous plant peptides with natriuretic function.

1.3. Plant natriuretic peptides modulate cGMP levels in *Zea mays* root stele

The common effects of rat atrial natriuretic peptide and plant natriuretic peptide described above prompted further study into the role of plant natriuretic peptides in plants. Knowing that atrial natriuretic peptides bind to natriuretic peptide receptors to stimulate cGMP biosynthesis by the guanylyl cyclase domain of the natriuretic peptide receptors, combined with the fact that atrial natriuretic peptides appear to exert the same cGMP-elevating effect on plants, it was necessary to investigate the effect of plant natriuretic peptides on cGMP levels in plant cells. To this end, plant natriuretic peptide

from *Hedera helix* was used to treat root tissue from *Zea mays* in the presence or absence of 3-isobutyl-1-methylxanthine, a phosphodiestrease (an enzyme that breaks down cGMP) inhibitor. Treatment of *Zea mays* root stele with *Hedera helix* plant natriuretic peptide resulted in elevation of cGMP levels by three times (Pharmawati *et al*, 1998b). This increase in cGMP levels was transient and returned to the same levels as untreated *Zea mays* root tissue after approximately 3 hours (Pharmawati *et al*, 1998b). This transient cGMP increase was reversed back to a level similar to that of untreated root tissue when the tissue was treated with phosphodiesterase in the presence of *Hedera helix* plant natriuretic peptide (Pharmawati *et al* 1998b). Treatment of *Zea mays* root tissue pre-incubated in 6-(phenylamino)-5,8-quinolinedione, a guanylyl cyclase inhibitor that blocks cGMP-mediated intracellular Ca^{2+} release, with *Hedera helix* plant natriuretic peptide also resulted in elevation of cGMP level (Pharmawati *et al* 1998b). This indicates that *Zea mays* roots may contain guanylyl cyclases that are insensitive to 6-(phenylamino)-5,8-quinolinedione. Taken together with the expectation that plant natriuretic peptides most likely bind to receptors on the cell membrane in the same manner as atrial natriuretic peptides bind to natriuretic peptide receptors, it is reasonable to expect plant natriuretic peptides to interact with membrane-bound receptors that mediate cGMP synthesis via activation of guanylyl cyclase activity.

1.4. Natriuretic peptide-induced stomatal opening is modulated by cGMP

Establishment of the natriuretic role of plant natriuretic peptides opens a new field of focus in hormone-mediated signal transduction. The observations, described above, that plant natriuretic peptides induce elevation of cGMP levels in *Zea mays* root stele

prompted an investigation into the effect of these hormones on guard cells. Treatment with plant natriuretic peptides resulted in promotion of stomatal opening (Pharmawati *et al*, 2001) and it appeared that the plant from which the plant natriuretic peptide is isolated does not affect the activity of the peptide, that is, plant natriuretic peptides are not species specific in their action because a plant natriuretic peptide from one species had the same effect on different plant species (Pharmawati *et al*, 2001). The stomatal opening induced by plant natriuretic peptides in guard cells was inhibited by 6-(phenylamino)-5,8-quinolinedione (Pharmawati *et al*, 2001). This stomatal opening effect of plant natriuretic peptide is possibly mediated by cGMP because the level of cGMP was elevated in guard cells treated with plant natriuretic peptide (Pharmawati *et al*, 2001). The inhibition of the observed plant natriuretic peptide-induced cGMP elevation by 6-(phenylamino)-5,8-quinolinedione in guard cells contrasts with the observation in *Zea mays* root stele where the plant natriuretic peptide-induced cGMP elevation is not affected by 6-(phenylamino)-5,8-quinolinedione. This may suggest that the guanylyl cyclase linked receptors for plant natriuretic peptides in guard cells or leaves are sensitive to 6-(phenylamino)-5,8-quinolinedione while those in roots are insensitive to 6-(phenylamino)-5,8-quinolinedione. The fact that both the stomatal opening and the cGMP elevation induced by plant natriuretic peptides are inhibited by 6-(phenylamino)-5,8-quinolinedione serves as an indication of the dependence of plant natriuretic peptide-induced stomatal opening on activation of guanylyl cylclase-catalyzed synthesis of cGMP by plant natriuretic peptides. Plant natriuretic peptides therefore most likely stimulate guanylyl cyclase activity, which elevates cGMP levels, triggering a signal transduction pathway that leads to stomatal opening. This can occur via promotion of K^+ influx followed by water uptake

into guard cells, probably by direct or Ca^{2+}-mediated inward gating of K^+ channels, resulting in stomatal opening. The opening of stomata, induced by plant natriuretic peptides, appears to require the presence of intracellular cytosolic free Ca^{2+} because ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (a calcium chelator) and ruthenium red (an inhibitor of intracellular Ca^{2+} release) inhibited stomatal opening by plant natriuretic peptides (Pharmawati *et al*, 2001). These Ca^{2+}-specific inhibitors also inhibited stomatal opening induced by application of 8-bromoguanosine 3',5'-cyclic monophosphate, a cell-permeable analog of cGMP (Pharmawati *et al*, 2001). It has been shown that the enzyme H^+-ATPase plays a role in promoting stomatal opening via adenosine 5'-triphosphate hydrolysis (Kinoshita *et al*, 1995) and consequent promotion of H^+ efflux out of guard cells (Pharmawati *et al*, 2001; Suwastika and Gehring, 1999). The resulting cellular hyperpolarization lead to K^+ influx into guard cells, followed by water uptake that lead to stomatal opening (Briskin and Gawienowski, 1996; Kinoshita *et al*, 1995; Pharmawati *et al*, 2001; Suwastika and Gehring, 1999). Elevation of intracellular Ca^{2+} levels inhibited this H^+-ATPase effect (Kinoshita *et al*, 1995; Shimazaki *et al*, 1999) and this observation is supported by the observed closure of stomata upon exposure of guard cells to high Ca^{2+} concentrations (Shimazaki *et al*, 1999) but is in contrast with Ca^{2+}-dependent stomatal opening induced by plant natriuretic peptides (Pharmawati *et al*, 2001; Suwastika and Gehring, 1999). However, plant natriuretic peptides appeared to stimulate both the H^+-ATPase and Ca^{2+}-dependent cGMP-mediated stomatal opening (Pharmawati *et al*, 2001; Suwastika and Gehring, 1999). It thus appears that plant natriuretic peptides affect stomatal opening via a cGMP-dependent signaling pathway that requires Ca^{2+} but is independent of the stimulation of

the H^+-ATPase. The independence of plant natriuretic peptide-induced stomatal opening from the H^+-ATPase-induced stomatal opening is supported by two observations. First, cGMP inhibited the H^+-ATPase that leads to stomatal opening (Pharmawati *et al*, 2001; Suwastika and Gehring, 1999). Therefore, the cGMP-mediated stomatal opening has to be independent of the H^+-ATPase-mediated stomatal opening. Second, Ca^{2+} inhibited the H^+-ATPase but has been shown to be required for the cGMP-mediated stomatal opening induced by plant natriuretic peptides (Pharmawati *et al*, 2001; Suwastika and Gehring, 1999).

1.5. The cell membrane is the target for natriuretic peptides

I^{125}-radiolabeled rat atrial natriuretic peptide (ANP) has been shown to bind to plasma membranes from leaf and stem tissue of *Tradescantia multiflor*a and this binding could be competitively displaced by unlabeled rat ANP and plant natriuretic peptide (Suwastika *et al*, 2000). Furthermore, *in situ* autoradiography of leaf and stem tissue demonstrated that I^{125}-labeled rat atrial natriuretic peptide binds specifically to these tissues (Suwastika *et al*, 2000). Binding of *Rattus norvegicus* atrial natriuretic peptide and plant natriuretic peptides to membranes isolated from plant tissues indicates the presence of plasma membrane-localized receptors for these natriuteric peptides and further strengthens the concept of a natriuretic peptide hormone and receptor system in plants.

1.6. Natriuretic peptides and cGMP modulate ion fluxes in roots

The discovery of a natriuretic peptide hormone system in plants promted an investigation of the role of plant natriuretic peptides in ion fluxes in plants, since the natriuretic peptide

system in animals is known to mediate ion fluxes (Beltowski and Wójcicka, 2002). Plant natriuretic peptides were found to promote rapid H^+ influx into root stele, as measured using non-invasive ion-selective vibrating microelectrodes (Pharmawati *et al*, 1999). This H^+ influx has been linked to the activation of an H^+/Cl^- symporter system and thought to be independent of H^+-ATPase activity. Changes in H^+-ATPase activity *in vitro* have been shown to be inhibited by cGMP (Pharmawati *et al*, 1999; Suwastika and Gehring, 1999). Interestingly, cGMP on its own appeared not to induce H^+ influx into roots (Pharmawati *et al*, 1999). This implies that the pathway through which plant natriuretic peptides induce H^+ influx, probably via an H^+/Cl^- symporter system, is independent of cGMP. Furthermore, both plant natriuretic peptides and cGMP induced K^+ and Na^+ influx into roots and this influx was delayed as it occured only after approximately 30 min after treatment with plant natriuretic peptides (Pharmawati *et al*, 1999). The fact that the K^+ and Na^+ influxes are induced by both the plant natriuretic peptides and cGMP after a time delay, while the H^+ influx is a rapid response induced only by plant natriuretic peptides and not cGMP, does support the idea of two independent plant natriuretic peptide pathways for modulation of K^+/Na^+ and H^+ fluxes. The observed K^+/Na^+ influx cannot be due to a cGMP/plant natriuretic peptide-mediated activity of the H^+-ATPase since cGMP has been shown to inhibit the H^+-ATPase and plant natriuretic peptides appeared not to affect H^+ pumping by the H^+-ATPase, even though plant natriuretic peptides promoted adenosine-5'-triphosphate hydrolysis by the H^+-ATPase (Pharmawati *et al*, 1999; Suwastika and Gehring, 1999). It is therefore likely that cGMP acts directly on inward rectifying non-selective K^+/Na^+ channels to promote K^+/Na^+ influx. This being the case, it is sensible to speculate that plant natriuretic peptide binding to its membrane receptor

leads to activation of guanylyl cyclases to elevate cGMP levels, which would subsequently act on the K^+/Na^+ channels to promote influx of these cations. The idea that plant natriuretic peptides affect K^+/Na^+ fluxes in root cells via a cGMP-mediated pathway is supported by the observation that both the plant natriuretic peptides and cGMP appeared to have similar K^+/Na^+ flux responses in roots (Pharmawati *et al*, 1999) and the reports that showed that plant natriuretic peptides elevate cGMP levels in roots (Pharmawati *et al*, 1998a). Further evidence pointing to the independence of H^+-ATPase stimulation and H^+ fluxes mediated by plant natriuretic peptides is that plant natriuretic peptides stimulated net Cl^- uptake by root cells (Maryani *et al*, 2000). This observation supports the concept that plant natriuretic peptides promote H^+ influx by stimulating an H^+/Cl^- symporter instead of directly acting on the H^+-ATPase for mediation of H^+ influx.

1.7. Plant natriuretic peptides are located in conductive tissue and stomata

The probable role of a protein can at least in part be deduced from its localization in specific tissues or cell types. To examine tissue localization of plant natriuretic peptides (PNPs) in plants, *Solanum tuberosum* and *Hedera helix* natriuretic peptides were isolated and purified using immunoaffinity columns prepared with rabbit anti-human ANP and anti-potato PNP antibodies (Maryani *et al*, 2003). Moreover, recombinant *Arabidopsis thaliana* PNP (AtPNP-A) expressed in *Saccharomyces cerevisiae* strain Y294 using the Yep352 expression vector was immunoaffinity purified on an anti-potato natriuretic peptide antibody column (Maryani *et al*, 2003). These purified recombinant PNPs promoted stomatal guard cell opening in a concentration-dependent manner (Maryani *et al*, 2003) and this effect was abolished if the PNPs were incubated with anti-human ANP

antibodies prior to treatment of the guard cells with the PNPs (Maryani *et al*, 2003). This is not just proof of the biological activity of the recombinant but also, at least indirect proof of the fact that PNPs are *bona fide* immunoreactants of ANP. Immunoreactivity with anti-human ANP antibodies occured in conductive tissue of *Solanum tuberosum* (Maryani *et al*, 2003), suggesting localization of PNPs in the phloem. Tissue prints of *Hedera helix* and *Solanum tuberosum* probed with anti-human atrial and anti-potato natriuretic peptide antibodies generated strong immunoreaction signals in vascular tissue, with the strongest signal in the phloem (Maryani *et al*, 2003). Immunoreactivity also occurred in the parenchyma of the leaf midrib, spongy parenchyma and leaf epidermis (Maryani *et al*, 2003). Immunolabeling signal strength found in the phloem was the strongest with anti-potato PNP antibodies but did not occur in the xylem (Maryani *et al*, 2003). Such observations confirm that PNPs are localized in conductive tissue and are concentrated in the phloem. Immunofluorescence in embedded *Hedera helix* tissue sections labeled with anti-potato plant natriuretic peptide antibodies indicated strong fluorescence in the stomatal ledge wall, with distinct fluorescence in guard cells restricted to the vacuolar membrane and absent in the cytoplasm and cell wall (Maryani *et al*, 2003). This supports the hypothesis that PNPs may be ligands acting specifically on the cell membrane. Fluorescence was evident in bundle sheath cells from leaf vacuolar bundles and in phloem and xylem of stem vacuolar bundles (Maryani *et al*, 2003). Exudate obtained from the xylem sap of *Pectranthus ciliatus* was subjected to anti-human artial natriuretic peptide antibody immunoaffinity purification and tested for natriuretic peptide activity on *Arabidopsis thaliana* stomatal guard cells (Maryani *et al*, 2003). The

purified exudate promoted stomatal opening, supporting the idea that this peptide is systemically mobile (Maryani *et al*, 2003).

1.8. Plant natriuretic peptides regulate water flow

AtPNP-A was found to induce stomatal opening in *Arabidopsis thaliana* leaves at nanomolar concentrations (Morse *et al*, 2004). Stomatal opening is a result of net water uptake by guard cells that results from solute influx into or compatible solute biosynthesis in the cell. Furthermore, *Arabidopsis thaliana* protoplasts treated with nanomolar concentrations of AtPNP-A were found to undergo significant swelling (Morse *et al*, 2004) which results from water uptake by the protoplasts. AtPNP-A induced water uptake in the cell wall-free protoplasts, further confirming that AtPNP-A is interacting with the cell membrane (Morse *et al*, 2004). AtPNP-A was also found to prevent a decrease in cell volume caused by high sorbitol concentration (Morse *et al*, 2004) that would otherwise induce water loss and result in cell shrinkage. AtPNP-A prevented sorbitol-induced water loss, illustrating that this molecule may play a significant role in drought responses. AtPNP-A-induced swelling was inhibited in AtPNP-A-treated protoplasts that had been pre-incubated in cyclohexamide, an inhibitor of protein synthesis (Morse *et al*, 2004). This indicates that active protein synthesis is required for AtPNP-A-induced water uptake and further suggests that AtPNP-A likely binds to a receptor that triggers a signaling pathway resulting in active synthesis of new proteins. The mechanism of action of AtPNP-A still needs further elucidation, but it is clear that AtPNP-A is an important component in maintenance of water homeostasis. Salinity stress induced with NaCl has been demonstrated to up-regulate the expression of

plant natriuretic peptides and an even more pronounced up-regulation of the expression of plant natriuretic peptides has been observed in response to a sorbitol concentration of equivalent osmolarity (Rafudeen *et al*, 2003). This serves as further indication that plant natriuretic peptides have an important role in salinity and osmolarity/drought stress tolerance in plants. In addition, the work done on plant natriuretic peptides indicates that there is a role of guanylyl cyclases in plant natriuretic peptide-mediated signaling, since some of the responses resulting from plant natriuretic peptide activity lead to elevation of cGMP.

IN SEARCH OF A ROLE FOR cGMP IN PLANT RESPONSES

1.9. Phototransduction in plants is mediated by cGMP

A number of phototransduction pathways, regulated by light receptors known as phytochromes, are mediated by cGMP (Bowler and Chua, 1994; Bowler *et al*, 1994a, Bowler *et al*, 1994b). Amongst these processes are the regulation of the expression of the chalcone synthase gene involved in anthocyanin biosynthesis and the regulation of the expression of genes encoding components of photosystem I and cytochrome b 6-f complexes (Bowler and Chua, 1994; Bowler *et al*, 1994a, Bowler *et al*, 1994b). Phototransduction studies showed that anthocyanin production is regulated by cGMP and reciprocally controlled by Ca^{2+} since a high cytosolic free Ca^{2+} level resulted in repression of anthocyanin accumulation (Wu *et al*, 1996). This suggests that cGMP stimulates release of Ca^{2+} from intracellular stores (probably via stimulation of cyclic adenosine diphosphate ribose biosynthesis), which then functions in the regulation of cellular processes including gene expression. It would be expected that once the Ca^{2+}

levels excede a threshold level in the cell, the high Ca^{2+} levels trigger repression of expression of anthocyanins. Ca^{2+} is thought to regulate the expression of the ribulose-1,5-bisphosphate carboxylase gene that is involved in chloroplast development, a gene that is reciprocally controlled by cGMP since high cGMP levels repress chloroplast development (Wu *et al*, 1996). This demonstrates signal interaction between cGMP and Ca^{2+} in the regulation of genes involved in chloroplast development. Phytochrome A is known to regulate expression of the asparagine synthase gene, a gene expressed in the dark and repressed in the light (Tsai and Coruzzi, 1990; Neuhaus *et al*, 1994). Phytochrome PfrA down-regulated the expression of the asparagine synthase gene (Neuhaus *et al*, 1997). This down-regulation was achieved via a signal transduction pathway that requires G proteins, Ca^{2+} and low concentrations of cGMP (Neuhaus *et al*, 1997). Interestingly, a high concentration of cGMP inhibited the down-regulation of asparagine synthase gene expression by PfrA (Neuhaus *et al*, 1997). A 17 base pair *cis*-acting element, known as RE3 in the asparagine synthase gene promoter has been identified as a binding site for a repressor whose binding results in down-regulation of the asparagine synthase gene (Neuhaus *et al*, 1997). The RE3 element contains a motif with the sequence TGGG, found in promoters of all light-down-regulated genes characterized so far (Neuhaus *et al*, 1997). It is likely that light triggers cGMP synthesis by activating guanylyl cyclases, which in turn possibly promotes elevation of cytosolic free Ca^{2+}, thus stimulating PfrA to activate a repressor that binds to RE3 in the asparagine synthase promoter. Binding of the repressor to RE3 would result in the down-regulation of asparagine synthase gene expression in the light (Neuhaus *et al*, 1997).

1.10. Cytosolic free Ca^{2+} release is mediated by cGMP

The role of cGMP in triggering increases in cytosolic free Ca^{2+} release in plants has been demonstrated using protoplasts from *Nicotiana tabacum* plants expressing the Ca^{2+}-activated chemilumescent protein, aequorin (Volotovski *et al*, 1998). Addition of membrane-permeable analogues of cGMP to the protoplasts resulted in large transient elevation of cytosolic free Ca^{2+} (Volotovski *et al*, 1998). This increase in cytosolic free Ca^{2+} occurred in the absence of external Ca^{2+} in the medium used to maintain the protoplasts (Volotovski *et al*, 1998). This suggests that cGMP can invoke intracellular release of Ca^{2+} from intracellular Ca^{2+} stores into the cytosol. Additionally, the transient cytosolic free Ca^{2+} elevation seen in cGMP-treated protoplasts in the presence of external Ca^{2+} were higher than those observed in the absence of external Ca^{2+} (Volotovski *et al*, 1998). This indicates that cGMP also signals promotion of Ca^{2+} influx, possibly by promoting opening of Ca^{2+} inward channels, leading to Ca^{2+} uptake. Further evidence of the role of cGMP in trigerring and promoting transient elevation of cytosolic free Ca^{2+} is provided by the finding that the cyclic nucleotide monophosphate diesterase inhibitor 3-isobutyl-1-methyl-2,6-(1H,3H)-purinedione, which inhibits conversion of cGMP to GMP, also lead to large transient increases in cytosolic free Ca^{2+} (Volotovski *et al*, 1998). The Ca^{2+} channel inhibitor verapamil blocked the induction of an increase in cytosolic free Ca^{2+} by cGMP (Volotovski *et al*, 1998), confirming that cGMP, in addition to promoting Ca^{2+} release from intracellular stores, also activates Ca^{2+} channels to promote Ca^{2+} influx into the cell. The physiological role of this Ca^{2+} elevation in plants seems to include the promotion of water uptake by the cell since cGMP induced elevation of cytosolic free Ca^{2+} levels were followed by an increase in cell volume evidenced by

protoplast swelling (Volotovski *et al*, 1998). An increase in cell volume due to water uptake appears to occur via a Ca^{2+}-regualted cGMP-dependent signaling pathway in which cGMP is the trigger for cytosolic free Ca^{2+} release.

1.11. Na^+ uptake is regulated by cGMP in roots

K^+ and Na^+ are the major ions that can cause salinity stress and they also affect water flow across plant cells (Flowers and Yeo, 1995). Ion channels spanning the plant cell membrane regulate ion flow across the cells (Amtmann and Sanders, 1999; Demidchik *et al*, 2002; Essah *et al*, 2003; Tyerman and Skerrett, 1999). Plant inward- and outward-rectifying voltage dependent channels are specific in their ion transport in the sense that they are highly selective for K^+ and have very limited permeability to Na^+ (Amtmann and Sanders, 1999; Tyerman and Skerrett, 1999). Voltage independent cation channels are thus believed to be the major transport system for Na^+ transport across plant cells (Amtmann and Sanders, 1999; Tyerman and Skerrett, 1999). Comparison of K^+:Na^+ current ratios in root protoplasts maintained in media containing either K^+ or Na^+ have been used to confirm that the major pathway for Na^+ uptake by roots is most likely not via voltage-dependent channels (Maathuis and Sanders, 2001). Non-selective monovalent cation channels have been found to have very little or no dependence on voltage across the cell membrane and it has been shown that they are the major transport channels for Na^+ (Maathuis and Sanders, 2001). Addition of micromolar concentrations of cGMP to the cytosol lead to low activity of voltage-independent channels (Maathuis and Sanders, 2001). This inactivation of voltage-independent channels occurred in an instant immediately after application of cGMP (Maathuis and Sanders, 2001), indicating that

cGMP possibly binds directly to the channels. However, it appeared that only a subset of the cells was sensitive to cGMP (Maathuis and Sanders, 2001). *Arabidopsis thaliana* plants grown in the presence of 100 mM NaCl normally die within 7 days but this effect could be prevented application of micromolar concentrations of cGMP (Maathuis and Sanders, 2001). This suggested that cGMP plays a role in enhancing plant tolerance to NaCl toxicity by inhibiting voltage-independent cation channels, resulting in the inhibition of Na^+ uptake by roots. Plant growth or survival upon exposure to an equimolar (200 mM) concentration of sorbitol was not improved by cGMP (Maathuis and Sanders, 2001). Thus suggesting that the effects of NaCl on the plants are most likely due to salinity-induced toxicity rather than an osmolarity-induced toxicity and that, at low concentrations, cGMP functions to enhance salinity tolerance but not osmotic stress/drought tolerance. Reduction in Na^+ uptake, achieved through inactivation of voltage-independent cation channels by cGMP, and/or enhanced Na^+ efflux could be responsible for the prevention of intracellular Na^+ accumulation in plants. Measurements of unidirectional Na^+ uptake by plant cells have been performed using the $^{22}Na^+$ radioisotope and it was demonstrated that micromolar concentrations of cGMP inhibit Na^+ uptake by roots (Maathuis and Sanders, 2001), further confirming that cGMP enhances salinity tolerance by preventing Na^+ uptake in roots.

1.12. Regulation of gene expression by gibberellic acid is mediated by cGMP

Gibberellic acid is a plant hormone that acts on receptors at the plasma membrane, altering the levels of various signaling molecules (Zentella *et al*, 2002; Hoffmann-Benning and Kende, 1992; Kende and Zeevaart, 1997). Many of the processes affected

by gibberellic acid are antagonistically regulated by abscisic acid (Gómez-Cadenas *et al*, 2001; Zentella *et al*, 2002; Hoffmann-Benning and Kende, 1992; Kende and Zeevaart, 1997). One of the prominent events occurring as a result of gibberellic acid action is a change in the level of expression of a number of genes (Fridborg *et al*, 2001; Hoffmann-Benning and Kende, 1992; Kende and Zeevaart, 1997; Zentella *et al*, 2002). Aleurone layers from barley have been shown to have low levels of cGMP, ranging between 0.065 fmol/mg (0.038 nM) and 0.08 fmol/mg (0.04 nM) fresh weight (Penson *et al*, 1996). Treatment of aleurone layers with gibberellic acid in the presence of Ca^{2+}, supplied as $CaCl_2$ in the culture medium, resulted in rapid and transient increases in the levels of cGMP in the aleurone layers (Penson *et al*, 1996). The gibberellic acid-induced cGMP elevation peaked to almost three times more compared to untreated tissue over a period of approximately 2 hours, after which the cGMP levels returned to the same levels as the untreated aleurone layers within 4 hours (Penson *et al*, 1996). Abscisic acid did not induce a change in the levels of cGMP in aleurone layers (Penson *et al*, 1996). Pre-incubation of aleurone layers in an inhibitor of guanylyl cyclases, 6-(phenylamino)-5,8-quinolinedione, lead to a reduction of cGMP levels by approximately 60 % in the aleurone layers upon treatment with gibberellic acid (Penson *et al*, 1996). Expression of the α-amylase gene is regulated by gibberellic acid (Zentella *et al*, 2002). Pretreatment of aleurone layers with 6-(phenylamino)-5,8-quinolinedione had a dose-dependent inhibitory effect on α-amylase activity in gibberellic acid-treated aleurone cells (Penson *et al*, 1996). The notion that guanylyl cyclase-dependent production of cGMP is responsible for gibberellic acid-regulated expression of the α-amylase gene was tested by measuring α-amylase mRNA levels upon pretreatment of aleurone layers with 6-

(phenylamino)-5,8-quinolinedione, followed by treatment with either gibberellic acid or abscisic acid (Penson *et al*, 1996). Northern blots showed that gibberellic acid increases α-amylase transcript levels (Penson *et al*, 1996). 6-(phenylamino)-5,8-quinolinedione resulted in a strong dose-dependent decrease in α-amylase mRNA levels despite the presence of gibberellic acid (Penson *et al*, 1996). Abscisic acid-induced gene expression was not significantly affected by 6-(phenylamino)-5,8-quinolinedione (Penson *et al*, 1996); suggesting that abscisic acid-mediated signaling in aleurone layers is not regulated by guanylyl cyclases.

1.13. Regulation of protein kinase activity by cGMP

The activity of a soluble protein kinase purified from *Pharbitis nil* seedlings was shown to increase two-fold in the presence of cGMP, with *Pharbitis nil* histones used as substrates for phosphorylation (Szmidt-Jaworska *et al*, 2003). The *Pharbitis nil* protein kinase cross-reacted with antibodies raised against the animal protein kinase G (Szmidt-Jaworska *et al*, 2003), suggesting a structural similarity between the *Pharbitis nil* protein kinase and the animal Pk-G. Incubation of *Pharbitis nil* protein kinase without any substrate but in the presence or absence of cGMP showed that the protein kinase does not undergo significant autophosphorylation in the absence of cGMP but high levels of autophosphorylation of the protein kinase were observed in samples containing cGMP (Szmidt-Jaworska *et al*, 2003). Furthermore, the level of autophosphorylation of *Pharbitis nil* protein kinase increased in a cGMP concentration-dependent manner (Szmidt-Jaworska *et al*, 2003). Maximal autophosphorylation of the *Pharbitis nil* protein kinase was reached at a cGMP concentration of 1 μM (Szmidt-Jaworska *et al*, 2003). The

fact that the activity of protein kinases in plants appears to be regulated by cGMP further demonstrates the importance of cGMP in plant signal transduction.

1.14. Regulation of cation channel activity in plants is mediated by cGMP

α-Subunits of voltage-gated K^+ channels like the *Arabidopsis thaliana* KAT1 channel (Anderson *et al*, 1992) have a structural fold that results in the formation of a pore-like structure. This pore is selective for K^+ and it was demonstrated that K^+ flow through these channels is triggered and driven by changes in voltage across the cell membrane in a process modulated by direct binding of cGMP or adenosine 3',5'-cyclic monophosphate (cAMP) to the channels (Hoshi, 1995). Cyclic nucleotide gated channels regulate cation flow across the cell membrane in a cation non-selective amnner with an activity triggered and regulated by cyclic nucleotides like cGMP and adenosine 3',5'-cyclic monophosphate (Zagotta and Siegelbaum, 1996). Cyclic nucleotide gated ion channels also form a structural fold that allows for K^+, Ca^{2+} and Na^+ transport in a non-selective manner (Zagotta and Siegelbaum, 1996). AtCNG2 is an *Arabidopsis thaliana* cyclic nucleotide gated channel with a putative hydrophobic pore-forming region, a putative cyclic nucleotide binding motif and a putative calmodulin-binding domain (Leng *et al*, 1999). The function of AtCNG2 as a cyclic nucleotide gated ion channel was studied using expression of AtCNG2 in *Saccharomyces cerevisiae* CY162, a yeast mutant that is deficient in K^+ uptake and grows poorly or does not grow at low external K^+ concentrations (Ko and Gaber, 1991). In media containing low external K^+ concentrations, *Saccharomyces cerevisiae* CY162 expressing AtCNG2 showed increased growth in the presence of cGMP or cAMP compared to *Saccharomyces cerevisiae*

CY162 expressing AtCNG2 in the absence of cGMP or cAMP (Leng *et al*, 1999). This serves as an indication that cGMP and cAMP trigger K^+ uptake by the yeast mutant through stimulation and regulation of AtCNG2 activity. *Xenopus laevis* oocytes transfected to express AtCNG2 showed cGMP- and cAMP-dependent K^+ currents in voltage clamp studies (Leng *et al*, 1999). This is additional evidence showing that cGMP and cAMP modulate the activity of AtCNG2. However, depolarizing voltage had no effect on the current in the *Xenopus laevis* oocytes (Leng *et al*, 1999) which suggests that AtCNG2 activity is not driven by voltage. AtCNG2 also promoted Ca^{2+} influx into the cell in a cGMP- and cAMP-triggered and dependent manner (Leng *et al*, 1999). However, AtCNG2 excluded Na^+ even in the presence of cGMP or cAMP (Leng *et al*, 1999). It follows that AtCNG2 is not directly involved in the regulation of Na^+ fluxes in or out of the cell.

1.15. Auxin-induced root formation is mediated by cGMP

Auxin is required for root formation and the ratio of auxin to cytokinin is an important determining factor for proper regulation of root formation (Reinhardt *et al*, 2000). It has been reported that cGMP plays a role in auxin-induced root formation in *Commelina communis* calli (Cousson, 2003). A combination of 2,4-dichlorophenoxyacetic acid, a cell impermeant auxin, and the cell membrane permeable cGMP analogue 8-bromoguanosine 3',5'-cyclic monophosphate increased the number of roots formed from *Commelina communis* calli in the absence of the active auxin 1-naphthaleneacetic acid (Cousson, 2003). This implies that 1-naphthaleneacetic acid can be replaced with cGMP in combination with 2,4-dichlorophenoxyacetic acid to achieve the same root-generating

effect seen when both auxins are used and serves to indicate that interaction between cGMP and auxin are required to promote root formation. However, 8-bromoguanosine 3',5'-cyclic monophosphate failed to induce root formation in the absence of 2,4-dichlorophenoxyacetic acid (Cousson, 2003). This means that cGMP alone is not sufficient to trigger root formation without auxin. The guanylyl cyclase inhibitor 6-(phenylamino)-5,8-quinolinedione inhibited the 2,4-dichlorophenoxyacetic acid-induced root formation but this inhibition was reversed by addition of 8-bromoguanosine 3',5'-cyclic monophosphate, although fewer roots were formed compared to those formed from calli treated with a combination of 2,4-dichlorophenoxyacetic acid and 8-bromoguanosine 3',5'-cyclic monophosphate (Cousson, 2003). This shows that the inhibition of root formation by 6-(phenylamino)-5,8-quinolinedione is a result of inhibition of guanylyly cyclase activity by 6-(phenylamino)-5,8-quinolinedione and not a result of 6-(phenylamino)-5,8-quinolinedione toxicity on the calli, as callus growth was unaffected by 6-(phenylamino)-5,8-quinolinedione (Cousson, 2003). The highly selective Ca^{2+} chelator [1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid], the inhibitor of intracellular Ca^{2+} release (ruthenium red) and the antagonist of the synthesis of the cytosolic free Ca^{2+} releasing cyclic adenosine diphosphate ribose (nicotinamide) all completely inhibited root formation (Cousson, 2003). Addition of 8-bromoguanosine 3',5'-cyclic monophosphate to calli treated with these Ca^{2+} inhibitors resulted in restoration of normal root formation (Cousson, 2003). The finding indicates that cGMP acts via stimulation of cyclic adenosine diphosphate ribose (cADPR) biosynthesis to promote release of intracellular Ca^{2+} and perhaps promotion of Ca^{2+} influx into the cell,

resulting in elevation of cytosolic free Ca^{2+} that consequently acts as a signal for auxin-induced root formation.

1.16. Nitric oxide stimulates cGMP biosynthesis in plants

Two general classes of guanylyl cyclases are known in animals, namely soluble guanylyl cyclases and particulate guanylyl cyclases (Lucas *et al*, 2000). Particulate guanylyl cyclases are transmembrane proteins with an extracellular domain of approximately 450 amino acids that binds ligands such as natriuretic peptides, a hydrophobic transmembrane domain of approximately 25 amino acid residues, a kinase homology domain of approximately 250 amino acids with amino acid sequence similarity to the active domain of protein kinases, an amphipathic coiled coil domain of approximately 41 amino acids known as the hinge region, and lastly an intracellular domain of approximately 250 amino acids at the C-terminus of the protein on the cytoplasmic side of the cell in which the guanylyl cyclase catalytic domain resides (Potter and Hunter, 2001; Lucas *et al*, 2000). Soluble guanylyl cyclases are cytoplasmic proteins that form heterodimers made up of two sub-units, namely the α- and β-subunits (Lucas *et al*, 2000). Generally, both subunits are required for catalytic activity of soluble guanylyl cyclases and each of the subunits has a regulatory domain at the N-terminus and a catalytic domain at the C-terminus (Lucas *et al*, 2000). The β-subunit of soluble guanylyl cyclases also contains a heme domain that acts as a binding site for nitric oxide (Namiki *et al*, 2001). The activity of soluble guanylyl cyclases increases by at least 100 times upon binding of nitric oxide to the heme domain (Stone and Marletta, 1994). It appears that plants express nitric oxide responsive guanylyl cyclase given that external application of nitric oxide to *Picea abies*

lead to a rapid increase of cGMP levels in this plant (Pfeiffer *et al*, 1994). This nitric oxide effect on *Picea abies* affected only cGMP levels and not cAMP levels (Pfeiffer *et al*, 1994). A number of processes in plants are known to be mediated by nitric oxide (see below) and it is perhaps not surprising that the stimulation of nitric oxide biosynthesis in plants should lead to cGMP elevation, which would then act as a second messenger that mediates signaling pathways triggered in response to nitric oxide.

The nitric oxide donor sodium nitroprusside has been shown to induce root organogenesis in *Cucumis sativa* root explants in the same manner as indole-3-acetic acid does (Pagnussat *et al*, 2003). Nitric oxide may thus play a role in mediating a pathway that regulates root development. The length and number of roots generated by treatment with sodium nitroprusside was similar to that generated by treatment with indole-3-acetic acid, but auxin-depleted (i.e. in which auxin was removed) explants developed 3 times less adventitious roots than auxin-non-depleted explants (Pagnussat *et al*, 2003). Treatment of auxin-depleted explants with either sodium nitroprusside or indole-3-acetic acid reversed the arrest of root development in auxin-depleted root explants (Pagnussat *et al*, 2003). These results show that indole-3-acetic acid and nitric oxide, a stimulant of soluble guanylyl cyclase-catalyzed cGMP biosynthesis, both regulate root development. Pretreatment of auxin-depleted explants with carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide, which is a nitric oxide scavenger, resulted in arrest of adventitious root development even in the presence of sodium nitroprusside and externally applied indole-3-acetic acid (Pagnussat *et al*, 2003). The arrest of adventitious root organogenesis was also observed in auxin-non-depleted root explants pretreated with

carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (Pagnussat *et al*, 2003). Indole-3-acetic acid thus functions as a trigger for root organogenesis via a pathway that requires the induction of nitric oxide. Using 4,5-diamino-fluorescein diacetate, the generation of nitric oxide in hypocotyls of *Cucumis sativa* explants was monitored to examine the role of indole-3-acetic acid in stimulation of nitric oxide biosynthesis and it was demonstrated that indole-3-acetic acid-non-depleted explants accumulate nitric oxide in the basal region of the hypocotyls (Pagnussat *et al*, 2003). Addition of the nitric oxide scavenger carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide to the indole-3-acetic acid-non-depleted explants resulted in reduced nitric oxide accumulation (Pagnussat *et al*, 2003), indicating that carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide is indeed an effective agent for removal of nitric oxide. Depletion of indole-3-acetic acid in hypocotyls of the *Cucumis sativa* explants severely reduced the levels of nitric oxide compared to nitric oxide levels in indole-3-acetic acid-non-depleted explants (Pagnussat *et al*, 2003). This shows that indole-3-acetic acid triggers production of nitric oxide in *Cucumis sativa* roots, probably by stimulating nitric oxide synthase activity. Addition of exogenous indole-3-acetic acid to the auxin-depleted explants reversed the inhibition of nitric oxide production that is otherwise seen in the auxin-depleted explants (Pagnussat *et al*, 2003), confirming that indole-3-acetic acid is the agent responsible for the stimulation of nitric oxide biosynthesis in the roots explants. A delay in the emergence of roots in auxin-non-depleted explants was observed when the explants were treated with the nitric oxide donor sodium nitroprusside after preteatment with the guanylyl cyclase inhibitor 6-(phenylamino)-5,8-quinolinedione (Pagnussat *et al*, 2003). This combined sodium

nitroprusside treatment and 6-(phenylamino)-5,8-quinolinedione pretreatment also reduced root length and root number (Pagnussat *et al*, 2003). Such effects on root development upon these treatments show that cGMP, synthesized by guanylyl cyclases, is required for root organogenesis and that the guanylyl cyclases involved in this process are nitric oxide-responsive. Addition of the cell-permeable cGMP analogue 8-bromoguanosine 3',5'-cyclic monophosphate to the auxin-non-depleted 6-(phenylamino)-5,8-quinolinedione pretreated and sodium nitroprusside treated root explants reversed the inhibition of root development caused by 6-(phenylamino)-5,8-quinolinedione (Pagnussat *et al*, 2003) confirming that cGMP is required for adventitious root development.

1.17. cGMP and nitric oxide in processes regulated by light

Light-sensitive *Lactuca sativa* seeds do not germinate in the dark at temperatures above 25°C but will germinate at such temperatures if they are exposed to light (Bewley and Black, 1982). *Lactuca sativa* seeds treated with 100 μM of the nitric oxide donor, sodium nitroprusside, were able to germinate at 26°C in the dark (Beligni and Lamattina, 2000). This finding implicates nitric oxide in the induction of seed germination. It could be argued that the nitric oxide effect on seed germination is due to the decomposition products of nitric oxide, namely NO_3^- or NO_2^-, but this was shown not to be the case because neither H_2O^-- nor NO_3^--, nor NO_2-treated seeds germinate in the dark (Beligni and Lamattina, 2000). Further proof that nitric oxide is responsible for induction of seed germination in sodium nitroprusside-treated dark-grown seeds came from the observation that these seeds failed to germinate when they were pretreated with the nitric oxide scavenger carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (Beligni and

Lamattina, 2000). Treatment of 26°C-dark-grown seeds with gibberellic acid was found to induce seed germination (Beligni and Lamattina, 2000) an observation that may suggest that gibberellic acid induces seed germination via a nitric oxide-mediated pathway. One might interpret the fact that 26°C-dark-grown seeds fail to germinate without exogenous application of gibberellic acid (Beligni and Lamattina, 2000) as an indication that light plays a role in stimulating gibberellic acid biosynthesis, which in turn would stimulate nitric oxide biosynthesis and consequent nitric oxide-induced seed germination. Sodium nitroprusside was also found to prevent etiolation of seedlings in the dark (Beligni and Lamattina, 2000), suggesting that nitric oxide stimulates chlorophyll biosynthesis. The fact that etiolation occurs in the dark and is prevented by nitric oxide suggests that light stimulates chlorophyll biosynthesis via a nitric oxide-dependent pathway. Furthermore, treatment of dark-grown plants with sodium nitroprusside resulted in reduction of hypocotyl and internode lengths (Beligni and Lamattina, 2000), indicating that nitric oxide has a role in promoting the reduction of hypocotyl and internode lengths. Reduction of hypocotyl and internode length are processes stimulated by light (Bertram and Lercari, 1997). In addition, there is a link between cGMP and nitric oxide. It would be expected that cGMP is likely to be the second messenger mediating these nitric oxide-induced responses of plants to light (Trejo *et al*, 1995) since it is known that nitric oxide stimulates cGMP production in plants (Pfeiffer *et al*, 1994) and several cGMP production-dependent pathways in plants have been linked to nitric oxide production (Beligni and Lamattina, 2001; Wendehenne *et al*, 2001).

1.18. Nitric oxide and cGMP in guard cell movements

The hormone abscisic acid induces closure of stomata (Trejo *et al*, 1995) and this closure is mediated by signaling pathways that are either dependent or independent of Ca^{2+} (Assmann and Shimazaki, 1999; Webb *et al*, 2001). Treatment of *Pisum sativa* leaf epidermal cells with abscisic acid in the presence of the nitric oxide scavenger carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide inhibited abscisic acid-induced stomatal closure (Neill *et al*, 2002). The inhibitory effect of carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide on stomatal closure suggests that nitric oxide is required for abscisic acid-induced stomatal closure. The requirement for nitric oxide in abscisic acid-mediated signaling for stomatal closure is further confirmed by the observation that the nitric oxide synthase inhibitor N_{ω}-nitro-L-arginine methyl ester inhibited abscisic acid-induced stomatal closure in *Pisum sativum* epidermal cells (Neill *et al*, 2002). Furthermore, exogenous application of nitric oxide to *Pisum sativum* leaf epidermal strips also induced stomatal closure (Neill *et al*, 2002), indicating that nitric oxide has a role in inducing stomatal closure. *Pisum sativum* leaf epidermal cells treated with nitric oxide donors, namely sodium nitroprusside and S-nitrosoglutathione, also induced stomatal closure but this induction was prevented if the cells were pre-incubated in carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (Neill *et al*, 2002). These results serve as further indication that nitric oxide is required in signaling mediated by abscisic acid in induction of stomatal closure via regulation of guard cell movements. Significant increases in nitric oxide synthesis were observed using the cell-permeable fluorescent nitric oxide probe diaminofluorescein diacetate upon treatment of the *Pisum sativum* epidermal cells with absciscic acid and this abscisic acid-induced nitric oxide

accumulation was inhibited in cells pretreated with carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide or N_{ω}-nitro-L-arginine methyl ester (Neill *et al*, 2002). This further strengthens the concept that abscisic acid-induced stomatal closure is mediated by nitric oxide. Treatment of *Pisum sativum* leaf epidermal cells with a cell permeable analogue of the Ca^{2+} chelator, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid did not affect abscisic acid-induced nitric oxide generation (Neill *et al*, 2002). This suggests that uptake of extracellular Ca^{2+} by the cell is not required for abscisic acid-induced nitric oxide biosynthesis and it is thus not required for abscisic acid-induced stomatal closure. Pretreatment of the epidermal peels with the nitric oxide-responsive soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one alone did not affect stomatal closure (Neill *et al*, 2002). However, pretreatment of the epidermal peels with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one followed by treatment with abscisic acid and sodium nitroprusside inhibited stomatal closure (Neill *et al*, 2002). These findings support the concept that abscisic acid is a signal for induction of nitric oxide biosynthesis and that this nitric oxide biosynthesis is the trigger for stimulation of soluble guanylyl cyclase activity to produce cGMP, which in turn induces stomatal closure. Further evidence to confirm that abscisic acid-induced stomatal closure mediated by nitric oxide requires cGMP is provided by the fact that *Pisum sativum* leaf epidermal peels treated with the cell permeable cGMP analogue 8-bromoguanosine 3',5'-cyclic monophosphate exhibited stomatal closure even in the presence of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one in abscisic acid- and nitric oxide-pretreated epidermal peels (Neill *et al*, 2002). However, 8-bromoguanosine 3',5'-cyclic monophosphate alone did not affect stomatal closure in epidermal peels that were

pretreated with either abscisic acid or sodium nitroprusside (Neill *et al*, 2002). These further observations suggest that cGMP is required but not sufficient for abscisic acid-induced stomatal closure. Nicotinamide, an antagonist of the Ca^{2+}-mobilizing cyclic adenosine diphosphate ribose, inhibited the stomatal closure normally seen in abscisic acid- or sodium nitroprusside-treated guard cells (Neill *et al*, 2002). This inhibitory effect on Ca^{2+} indicates that intracellular release of Ca^{2+} is required for abscisic acid- and nitric oxide-induced stomatal closure. It thus appears that abscisic acid activates nitric oxide synthase to synthesize nitric oxide, which then stimulates soluble guanylyl cyclase to synthesize cGMP, consequently followed by cGMP-stimulated biosynthesis of cyclic adenosine diphosphate ribose that stimulates release of Ca^{2+} from intracellular stores. This would lead to elevation of cytosolic free Ca^{2+}, resulting in stomatal closure. These explanations are however contradictory to the observations on the effect of plant natriuretic peptides, which also appear to lead to elevation of cGMP levels in *Zea mays* roots (Pharmawati *et al*, 1998b) and guard cell protoplasts (Pharmawati *et al*, 2001), because these peptides lead to stomatal opening (Pharmawati *et al*, 2001; Morse *et al*, 2004). Perhaps the contrasts seen in these cases are due to tissue specificity or even level of induction of cGMP elevation because there seem to be cases where a low level of cGMP elevation results in an effect opposite to the effect of a high level of cGMP elevation, *e.g.* the regulation of the asparagine synthase gene by cGMP levels (Neuhaus *et al*, 1997). Studying the PNP-dependent cGMP elevations in leaves and even in whole seedlings may help to clarify the role of this important second messenger.

1.19. Nitric oxide and cGMP in iron homeostasis

Cellular levels of iron (required for many cellular functions but toxic at levels higher than required) are regulated by ferritin, a protein that binds iron, the accumulation of which is induced by high levels of cellular iron (Lobreaux *et al*, 1993; Prouhdon *et al*, 1996). Infiltration of *Arabidopsis thaliana* leaves with the nitric oxide donor, sodium nitroprusside induced accumulation of ferritin mRNA 1 hour after infiltration followed by a decrease in ferritin mRNA levels only after 24 hours (Murgia *et al*, 2002). This proves that nitric oxide induces ferritin gene expression. Sodium nitroprusside-infiltrated *Arabidopsis thaliana* leaves were shown, using Western blot analysis with anti-*Pisum sativum* ferritin polyclonal antibodies, to accumulate high levels of ferritin protein within 18 to 24 hours after sodium nitroprusside infiltration (Murgia *et al*, 2002). Ferritin accumulation upon infiltration with sodium nitroprusside shows that nitric oxide induces biosynthesis of ferritin protein. This induction of ferritin biosynthesis by nitric oxide was inhibited by treatment of *Arabidopsis thaliana* cell suspensions with the nitric oxide scavenger carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (Murgia *et al*, 2002), confirming that nitric oxide is responsible for the induction of ferritin accumulation. Iron, supplied as iron citrate, induced ferritin accumulation and this iron-induced ferritin accumulation was inhibited by carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (Murgia *et al*, 2002), suggesting that nitric oxide acts downstream of iron in the induction of ferritin accumulation. Iron depletion of *Arabidopsis thaliana* suspension cultures with the iron chelator deferoxamine mesylate resulted in accumulation of ferritin upon treatment of the cells with sodium nitroprusside (Murgia *et al*, 2002), indicating that nitric oxide can induce ferritin accumulation even in

the absence of available iron in the cells confirming that nitric oxide acts downstream of iron in the signaling pathway leading to ferritin accumulation. A *cis*-acting element of the *Arabidopsis thaliana Atfer1* promoter (the promoter of the ferritin gene), named Iron Dependent Regulatory Sequence, was shown to be the target for nitric oxide-mediated ferritin mRNA accumulation (Murgia *et al*, 2002). Iron thus triggers nitric oxide biosynthesis, which then signals to activate ferritin gene expression by modulating the activity of the ferritin gene promoter through the Iron Dependent Regulatory Sequence. Furthermore, the protein synthesis inhibitor cyclohexamide inhibited both iron citrate- and sodium nitroprusside-induced ferritin accumulation (Murgia *et al*, 2002), pointing to a *de novo* protein synthesis requirement for for iron- or nitric oxide-induced ferritin accumulation. Given the established role of nitric oxide in stimulating cGMP biosynthesis by soluble guanylyl cyclase in plants (Pfeiffer *et al*, 1994; Beligni and Lamattina, 2001; Wendehenne *et al*, 2001), it would be expected of a cGMP-regulated pathway to mediate nitric oxide-induced ferritin accumulation in response to high levels of iron. A possible sequence of signaling is that high levels of iron stimulate nitric oxide biosynthesis, leading to elevation of cGMP levels as a result of stimulation of a soluble guanylyl cyclase by nitric oxide. cGMP would then regulate ferritin supply in order to maintain iron homeostasis.

1.20. Plant disease resistance is mediated by cGMP

Infection of Tobacco Mosaic Virus resistant *Nicotiana tabacum* with the Tobacco Mosaic Virus has been shown to increase nitric oxide synthase activity by at least 5 times compared to uninfected *Nicotiana tabucum* plants or Tobacco Mosaic Virus susceptible

plants (Durner *et al*, 1998; Klessig *et al*, 2000). This demonstrates that recognition of a pathogen by plants can lead to elevation of nitric oxide levels and hence implies that nitric oxide is involved in virus response. Artificially induced nitric oxide elevation caused accumulation of pathogenesis-related protein 1 (PR-1), a marker for disease resistance in plants, whereas treatment of infected *Nicotiana tabacum* with both the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine monoacetate and the nitric oxide scavenger carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide inhibited PR-1 expression (Durner *et al*, 1998). This supports the idea that nitric oxide stimulates the expression of PR-1 and implies that nitric oxide is one of the signals mediating disease resistance in plants. Further evidence for the induction of PR-1 gene expression by nitric oxide was obtained from experiments that showed that the nitric oxide donors S-nitroso-L-glutathione and S-nitroso-N-acetylpenicillamine induce PR-1 accumulation (Durner *et al*, 1998). Salicylic acid is known to induce PR-1 gene expression (Ryals *et al*, 1996; Durner *et al*, 1998). Total salicylic acid levels increased upon treatment of *Nicotiana tabacum* with S-nitroso-L-glutathione and S-nitroso-N-acetylpenicillamine (Durner *et al*, 1998; Klessig *et al*, 2000). The expression of *PAL*, a gene expressed early in response to a number of stress stimuli, was increased independently of salicylic acid accumulation in response to infiltration with nitric oxide synthase (Durner *et al*, 1998; Klessig *et al*, 2000). These observations confirm that nitric oxide plays a role in establishing of disease resistance. *PAL* gene expression and activity was increased more than 8 times upon treatment of *Nicotiana tabacum* with S-nitroso-L-glutathione and this increase was mimicked by the cell-permeable cGMP analogue 8-bromoguanosine 3',5'-cyclic monophosphate (Durner *et al*, 1998; Klessig *et al*, 2000) implying that cGMP acts

downstream of nitric oxide. The guanylyl cyclase inhibitors 6-(phenylamino)-5,8-quinolinedione and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one inhibited induction of *PAL* gene expression by S-nitroso-L-glutathione (Durner *et al*, 1998; Klessig *et al*, 2000) further strengthening the idea that nitric oxide stimulates guanylyl cyclase activity in response to pathogen attack resulting in elevation of cGMP levels and consequent activation of defense gene expression by cGMP. This is consistent with the observation that induction of nitric oxide synthesis resulted in more than 10 times elevation of cGMP levels in *Nicotiana tabacum* and a concomitant rapid transient increase in *PAL* gene induction (Durner *et al*, 1998). Treatment of *Nicotiana tabacum* with cyclic adenosine diphosphate ribose (cADPR) induced the expression of *PAL* and *PR-1* and this cADPR effect was inhibited by ruthenium red, an inhibitor of Ca^{2+} release from intracellular stores (Durner *et al*, 1998). Interestingly, the cyclic adenosine diphosphate ribose effect on *PAL* gene expression was not observed in *NahG* mutant plants, which are insensitive to salicylic acid (Durner *et al*, 1998). It can thus be concluded that cADPR activates *PAL* and *PR-1* expression by stimulating Ca^{2+} release from intracellular stores leading to elevation of cytosolic free Ca^{2+} in a pathway that is dependent on salicylic acid for *PR-1* gene expression and salicylic acid-independent for *PAL* gene expression. Simultaneous treatment of *Nicotiana tabacum* cells with both 8-bromoguanosine 3',5'-cyclic monophosphate and cADPR resulted in enhanced induction of both *PAL* and *PR-1* gene expression and this effect was suppressed by ruthenium red (Durner *et al*, 1998). A cADPR antagonist suppressed nitric oxide-mediated *PR-1* gene expression (Klessig *et al*, 2000), suggesting that cADPR mediates nitric oxide-induced expression of the *PR-1*gene. These observations, taken together, imply that recognition of a pathogen by a plant

triggers activation of nitric oxide synthase to synthesize nitric oxide, which in turn stimulates cGMP biosynthesis by soluble guanylyl cyclase. The cGMP would then act as a signal for cADPR biosynthesis, resulting in elevation of cytosolic free Ca^{2+} that in turn induces defence gene expression and consequent disease resistance.

1.21. Drought tolerance may be mediated by cGMP

The relative water content of detached leaves subjected to drought through dehydration for 2 hours on water-absorbent paper was found to decrease from 53 % to 42 %. Similarly treated detached leaves had also been induced to produce nitric oxide by treatment with sodium nitroprusside (Mata and Lamattina, 2001). This points to a role of nitric oxide in enhancing drought tolerance. Seedlings subjected to drought conditions in the presence of sodium nitroprusside-generated nitric oxide also showed enhanced drought resistance compared to seedlings subjected to the same conditions without sodium nitroprusside treatment (Mata and Lamattina, 2001). This shows that nitric oxide can confer drought tolerance in plants. Nitric oxide was also found to decrease the transpiration rate in leaves and this effect was inhibited by the nitric oxide scavenger carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (Mata and Lamattina, 2001). Water loss in plants occurs mostly as a result of transpiration and it thus makes sense for plants to limit transpiration rate in drought conditions in order to facilitate water retention. Opening and closing of stomata in guard cells regulates transpiration, where water influx into the guard cells leads to opening of stomata whereas water efflux out of the guard cells leads to closing of stomata. It is thus useful to investigate the effect of nitric oxide on guard cell movements. Nitric oxide was found to induce stomatal closure

and this nitric oxide-induced stomatal closure was inhibited by carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (Mata and Lamattina, 2001), indicating that the observed stomatal closure is due to the effect of nitric oxide on guard cells. Chelation of intracellular Ca^{2+} by treatment with a membrane-permeable form of the Ca^{2+} chelator ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid inhibited nitric oxide-induced stomatal closure (Mata and Lamattina, 2001) pointing to a downstream Ca^{2+} requirement. Stomatal closure induced by osmotic stress as a result of treatment of cells with 20 % (w/v) polyethylene glycol was more pronounced in cells treated with sodium nitroprusside than in untreated cells but was similar in sodium nitroprusside-treated and untreated cells that were pre-incubated with the Ca^{2+} chelator ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (Mata and Lamattina, 2001). It is clear that nitric oxide enhances water retention in response to drought and hyperosmotic stress by inducing and amplifying the degree of stomatal closure, thus limiting transpiration via a signaling pathway that is mediated by Ca^{2+}. It has already been described here (previous sections) that the generation of nitric oxide in plant cells stimulates cGMP biosynthesis by guanylyl cyclases and that cGMP stimulates cyclic adenosine diphosphate ribose biosynthesis, leading to increased cytosolic free Ca^{2+}. The fact that drought and osmotic stress tolerance is mediated by nitric oxide through a Ca^{2+}-dependent pathway indicates a strong possibility that nitric oxide enhances drought and osmotic stress tolerance via stimulation of guanylyl cyclase activity to synthesize cGMP, which in turn causes Ca^{2+} elevation via stimulation of cADPR biosynthesis.

The effect of plant natriuretic peptides on cGMP levels, coupled with the role of plant natriuretic peptides in water/ion homeostasis in plants and the diverse yet important processes mediated by cGMP in plants necessitate full characterization of plant natriuretic peptides and identification and characterization of guanylyl cyclases in plants. This thesis describes a study in which sequence analyses in *Arabidopsis thaliana* have been used to identify and partially characterize both a plant natriuretic peptide and a guanylyl cyclase from this plant.

1.22. AIMS

The aim of this study is to partially characterize a plant natriuretic peptide and a plant guanylyl cyclase, both from *Arabidopsis thaliana*. The study aims to establish the evolutionary and functional relationships of irPNP-like molecules within the superfamily of expansins, pollen allergens, and distantly related molecules such as endoglucanases. It also aims to investigate the effect of drought and salinity on cGMP levels in plants, using sorbitol to mimic the osmolarity/dehydration effect of drought and NaCl as a source of salinity stress and thus link NaCl and sorbitol responses to both AtPNP-A and cGMP up-regulation.

CHAPTER 2

MOLECULAR EVOLUTION OF PLANT NATRIURETIC PEPTIDES

Abstract

An *Arabidopsis thaliana* transcript (*AtPNP-A*) encoding an immunoreactant plant natriuretic peptide (irPNP) was identified and isolated. The encoded protein shows similarity to CjBAp12, a functionally undefined protein from citrus that is induced in response to blight infection. CjBAp12 shows significant sequence identity to domains found in the cell wall loosening expansins but has tested negative for cell wall loosening activity. It has thus been undertaken to establish the evolutionary and functional relationships of irPNP-like molecules within the superfamily of expansins, pollen allergens, and distantly related molecules such as endoglucanases. It is shown that irPNP-like molecules are related to expansins and fall in two groups; one includes CjBAp12 and the other AtPNP-A. Members of both groups share distinct sequence motifs (K[VI]VD and [LM]SxxAFxxI) but do not contain the tryptophan and tyrosine rich C-terminal putative polysaccharide-binding domain typical of expansins or bacterial cellulases and hemicellulases. It is argued that both irPNP-like molecules and expansins have evolved from ancestral glucanase-like molecules that hydrolyzed the cell wall. Importantly, we have previously demonstrated that irPNPs act on protoplasts, i.e. plant cells without cell walls as well as microsomes, indicating that these novel proteins specifically interact with the plasma membrane. It follows that the cell wall cannot be an obligatory substrate for irPNPs. Thus, both irPNP function and domain structure point to these molecules having a systemic role in H_2O and solute homeostasis.

2.1. Introduction

Plant cells are contained by a rigid, exoskeleton-like structure, the cell wall. The major components of the wall are cellulose microfibrils consisting of an unbranched β-1,4-glucan polymer, hemicelluloses (branched glycans) that bind to cellulose to form a matrix, and acidic polysaccharides (pectins) that form ionic gels. Developmental processes (e.g. cell elongation) and any rapid physiological responses (e.g. stomatal movement) that require changes in cell shape thus necessitate temporary loosening of the cell walls. The expansins are proteins that promote cell wall loosening and extension and recently two families of expansins, α-expansins and β-expansins, have been recognized (Cosgrove, 1999; Cosgrove, 2000a; Cosgrove, 2000b). Expansins are functionally defined to induce long-term pH dependent extension and enhance stress relaxation of plant cell walls. What makes expansin activity particularly remarkable is that the enzymes do not seem to have any apparent hydrolytic activity against the major cell wall constituents (McQueen-Mason *et al*, 1992; McQueen-Mason and Cosgrove, 1995). However, expansins share structural motifs with the putative catalytic sites of endoglucanases of the family-45 glycosidases, which in turn do not have expansin activity (Cosgrove, 1999; Cosgrove, 2000b). It would thus appear that the structural similarity between glucanases and expansins reflects an evolutionary relationship between two classes of enzymes that operate differently on a common substrate, the cell wall. There has long been an interest in allergenic components from pollen and grass pollen, in particular, that induce hay fever and allergic asthma. A group of proteins termed the group-1 allergens were identified as the main causative agents of hay fever

and allergic asthma elicited by grass pollen (Knox and Sophioglu, 1996). The group-1 allergens are glycoproteins abundantly expressed on the surface of pollen grains and released upon hydration (Knox and Sophioglu, 1996). The discovery that expansins bore some sequence similarity with group-1 pollen allergens hinted at a biological role for these allergenic proteins (Shcherban *et al*, 1995). Cell wall loosening may indeed be an advantageous strategy for the pollen tube growing along the stigma and through the style as a prelude to fertilization. Such a role might also explain the ubiquity and abundance of the group-I pollen allergens. Subsequent experiments have demonstrated that maize pollen group-I allergens have expansin activity and that this activity is essentially limited to cell walls from grasses (Cosgrove *et al*, 1997). This type of expansin is termed β-expansin and its preferred substrate is the monocotyledons, and specifically the grass cell wall. β-Expansins occur in monocotyledons and dicotyledons but seem more efficient on grass cell walls (Cosgrove, 2000a). However, an intriguing question remains. If β-expansin catalytic activity is limited to monocot cell walls, why would they occur in dicots? Different substrate specificities and biological roles for structurally related molecules from two classes (monocotyledons and dicotyledons) provide an opportunity to study the evolution of this family of molecules. Furthermore, a molecule, CjBAp12 (previously referred to as a blight-associated protein, p12), from *Citrus jambhiri* shares significant homology with expansins (Ceccardi *et al*, 1998). The molecule has a molecular mass of approximately 12 kDa and is thus considerably smaller than the classic expansins (molecular mass of ~25 kDa). The gene *CjBAp12* is expressed in root and stem tissue in response to a challenge from citrus blight. The protein itself accumulates in root, stem, and leaf tissues (Ceccardi *et al*, 1998), suggesting systemic mobility. However,

despite marked sequence similarity with expansins, CjBAp12 has no apparent expansin-like activity (Ceccardi *et al*, 1998). We have previously reported the immunoaffinity purification of novel, biologically active proteins from *Hedera helix* and *Solanum tuberosum* with antibodies directed against vertebrate atrial natriuretic peptide (ANP) (Billington *et al*, 1997; Maryani *et al*, 2001) and termed them immunoreactant plant natriuretic peptides (irPNPs). Here we identify an *Arabidopsis thaliana* gene and transcript that encodes a plant natriuretic peptide. We investigated the evolutionary relationship between this protein and expansin-like molecules. Finally, these findings are discussed in the light of the increasing body of functional data available on irPNPs.

2.2. Materials and Methods

Total RNA from *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg erecta was extracted from tissue ground in liquid N_2 using the hot phenol extraction procedure (Verwoerd *et al*, 1989). Ground tissue was placed into microfuge tubes containing 0.5 ml NETS buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris pH 7.4, 0.1 % SDS) and 0.5 ml H_2O-saturated phenol and incubated at 80°C for 3–5 min. Samples were spun at 12 000*g* for 10 min and supernatant transferred to tubes containing 0.5 ml NaCl saturated phenol. Tubes were vortexed, spun at 12 000*g* for 10 min, and the supernatant collected. RNA was phenol extracted with salted phenol followed by a salted phenol/chloroform mix and lastly, chloroform. The RNA was ethanol precipitated and resuspended in 50–100 µl TE buffer. RT-PCR was performed using Superscript II (Gibco BRL) according to the manufacturer's instructions. The specific primers were: 5'-AAG AAA ATG ATA AAA ATG GCA G-3' (forward), 5'-AAG AAT GAA ACT TAC GGT GTG T-3' (reverse). All

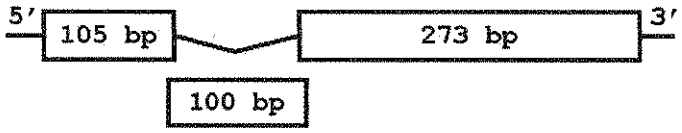
reactions were denatured at 94°C for one min and then 35 cycles of amplification were performed (30 s denaturation at 94°C, 30 s annealing at 52°C, and 60 s extension at 72°C), with a final extension at 72°C for 10 min. The PCR-isolated gene and RT-PCR isolated cDNA fragments were bluntended using T4 DNA Polymerase (Gibco BRL) and cloned into the *Sma* I restriction site of the plasmid vector pBluescript SK (+/-) (Stratagene). Sequencing was undertaken using the ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and analyzed using the facilities at the Australian Genome Research Facility (AGRF). Sequence alignments and phylogenetic trees were constructed using ClustalX (Thompson *et al*, 1997) and motif searches were performed against the Prints and Prosite databases (Attwood *et al*, 1994; Hofman *et al*, 1999). Protein secondary structures were predicted using position-scoring matrices (Jones, 1999). MODELLER (Sali and Blundell, 1993) and MOLSCRIPT (Kraulis, 1991) were used to generate a 3-D structure model of AtPNP-A, based on the crystal structure of the N-terminal domain of Phl P 1 Timothy Grass Pollen Allergen (Accession no. P43213) determined to 2.9Å (pdb code = 1n10).

2.3. Results

We have previously reported the isolation of irPNPs with antibodies directed against the C-terminal of atrial natriuretic peptides (α-hANP) (Billington *et al*, 1997; Gehring, 1999). We have since partially sequenced C- and N-termini of several of these immunoaffinity purified plant natriuretic peptide immunoanalogues (irPNPs) from *Solanum tuberosum* (Maryani *et al*, 2001), and identified a homologous sequence in *Arabidopsis thaliana* (accession no. AAD08935) that we term AtPNP-A. Figure 2.1a

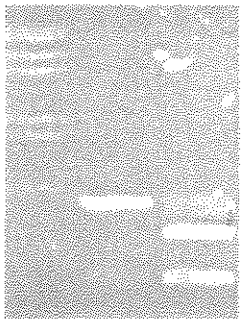
shows the genomic organization of AtPNP-A as predicted from the genomic sequence data. The genomic sequence is 478 bp long and contains one predicted 100 bp long intron. The predicted protein is thus expected to consist of 126 amino acids (approximately 14 kDa). Figure 2.1b confirms the length of the genomic PCR product and importantly shows a RT-PCR product of the predicted length (upper band). The sequence is not annotated in the database and the presence of a RT-PCR product implies that the gene is transcribed in unstressed leaves. Figure 2.1c shows a second sequence from *Arabidopsis* thaliana that shows similarity (37 % identity) with AtPNP-A. Hence, we refer to this sequence as AtPNP-B. This sequence is annotated as a blight associated protein homologue (BAPH, accession no. CAB79756) since it shows a high degree (54 % identity) of sequence similarity with CjBAp12 (accession no. AAD03398). CjBAp12 is a blight-induced protein of a yet unknown function, isolated from *Citrus jambhiri* (Ceccardi *et al*, 1998). The cysteine residues in both AtPNP-A and AtPNP-B (Fig. 2.1 c) are aligned which might suggest a conserved fold in these proteins. While CjBAp12 contains an additional cysteine in the putative signal peptide, all other cysteine residues align with the AtPNP-A and AtPNP-B (Fig. 2.1 c and Fig. 2.2). IrPNPs were first isolated through immunoaffinity purification with an antibody directed against the biologically active C-terminus of human α-ANP (α-hANP). The alignment of AtPNP-A and *Homo sapines* ANP (α-hANP, the antigen of the antibody used to isolate the *Solanum tuberosum* homologue) (Fig. 2.1 d) shows considerable similarity between the two molecules, with nine of the 28 amino acids of ANP being conserved and four conservative replacements.

(a)



(b)

M PCR RT-PCR



(c)

```
                                                                  30
AtPNP-A  --MAVKFVVVMIVFAQILAPIAEAAQGKAV
AtPNP-B  ---MSKSIVFFSTVLVFLFSFSYATPGIAT

AtPNP-A  YYDPPYTRSACYG-TQRETLVVGVKNNLWQ
AtPNP-B  FYTS-YTP--CYRGTQEGVMIAAASDTLWD

AtPNP-A  NGRACGRRYRVRCIGATYNFDRACTGRTVD
AtPNP-B  NGRVCGKMFTVKCSGPRNAVPHPCTGKSVK

AtPNP-A  VKVVDFCREPCNGDLNLSRDAFRVIANTDA
AtPNP-B  VKIVDHCPSGCASTLDLSREAFAQIANPVA

AtPNP-A  GNIRVVYTP
AtPNP-B  GIINIDYFP
```

(d)

```
AtPNP-A  PYTRSACYGTQRETLVVGVKNNLWQNGRACGRRY
α-hANP   SLRRSSCFGGR--MDRIGAQSGLGCN----SFRY
            **:*:* :      :*.:..*  *     **
```


Fig. 2.1. (**a**) Intron and exon organization of the *AtPNP-A* encoding gene from *Arabidopsis thaliana*. (**b**) PCR and RT-PCR products from *Arabidopsis thaliana* DNA and RNA templates. M denotes molecular weight marker, in base pairs, made from λ DNA digested with HindIII. (**c**) Sequence alignment of AtPNP-A and AtPNP-B from *Arabidopsis thaliana*. The conserved cysteines are in bold (**C**), the solid triangles (▼) delineate the sequence that is aligned with α-hANP in (**d**). The sequence between the open arrows (⇩) has been used to construct the dendrogram in Fig. 2.3. (**d**) Alignment of AtPNP-A and α-hANP; asterisks (*) identify identical amino acids, colons (:) are conservative amino acid substitutions, and dots (.) are semi-conservative amino acid substitutions.

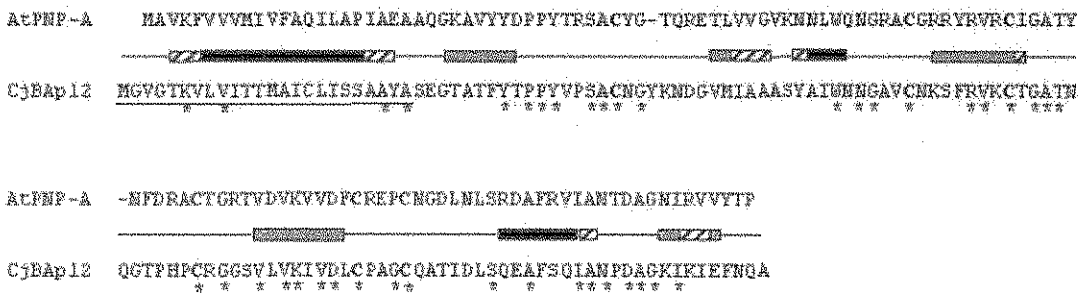


Fig. 2.2. Sequence comparison and predicted secondary structures of AtPNP-A from *Arabidopsis thaliana* and CjBAp12 from *Citrus jambhiri*; asterisks (*) indicate conserved amino acid residues. Black boxes indicate α-helices (■), gray boxes are strands (β-sheets) (■), and lines (——) represent random coils. The boxes are hashed when the predictions for the two molecules, AtPNP-A and CjBAp12, differ.

A secondary structure prediction program (Jones, 1999) was applied to test for similarities in coil, β-sheet and helix domains (Fig. 2.2). The comparison between AtPNP-A and CjBAp12 shows near congruence in predicted secondary structure and might imply common, albeit unknown, functionality. In order to obtain further information on the extent of this family of small proteins we queried the TIGR Gene Indices (http://www.tigr.org/tdb/tgi.shtml). The result of the search revealed a number of indices from different plant species notably *Oryza sativa* and *Glycine max*. An index is defined as a number of (partially overlapping) transcripts that represent either a single gene or possibly two or more genes with identical coding subregions. It is noteworthy that no related transcripts were found in either *Saccharomyces cerevisiae* or *Dictyostelium discoideum*. How then are these molecules, the irPNP-like proteins and CjBAp12, interrelated and related to other proteins, and can similarities with proteins of known function provide a key to understanding their hitherto unknown function?

Homology searches using BLAST (Altshul *et al*, 1997) identified the expansin family as the only proteins with significant sequence similarity to irPNP-like molecules. The phylogenetic tree in Fig. 2.3, constructed with subsequences (see Fig. 2.1 c) representing a best guess at the region common to both irPNP-like molecules and expansins, suggests firstly that irPNP-like proteins and CjBAp12 form a distinct group of proteins to the α-expansins and β-expansins. Secondly, AtPNP-B clusters with CjBAp12 in a group that contains both members of the mono- and dicotyledons. AtPNP-A is part of a different subgroup. IrPNP-like and CjBAp12-like sequences are considerably shorter than the expansin sequences: ~12–14 kDa rather than ~25 kDa (Fig. 2.4 a). Importantly, the sequence alignments reveal that the C-terminus of the expansins is consistently absent in irPNP-like molecules. The C-terminus of the expansins contains several tryptophans (four residues in ~70), which are also in conserved positions and exposed on the surface of the folded protein. This domain was assigned as a putative cellulose binding, and thus cell wall binding region of expansins (Cosgrove, 1999). The latter is inferred by similarity to cellulose-binding domains of many microbial cellulases and hemicellulases; these domains have a role in enzyme immobilisation on insoluble carbohydrate substrates, and can confer carbohydrate-binding specificity but are not necessary for catalysis (Linder and Teeri, 1997; Linder *et al*, 1998). Incidentally, fungal family-45 glycosidases (e.g. the β-1,4-endoglucanse from *Trichoderma reesei*) also contain a conserved C-terminal cellulose-binding domain (Saloheimo *et al*, 1994). The cellulose-binding domain from the fungus shows 38 % sequence identity with α-expansins. More importantly the cysteine residues are aligned and two of the tryptophan residues align with tyrosine, another aromatic amino acid. This C-terminal domain of the ancestral

family-45 glycosidases (glucanases) and the expansin family is absent in all irPNP-like molecules identified so far. It is noteworthy that expansins have two introns (Cosgrove, 1999), the second close to the border of the two functional domains (endoglucanase-like and cellulose/polysaccharide-binding) (Fig. 2.4 a). The second intron and the C-terminal cellulose-binding domain are both absent in the irPNP-like molecules. The sequence alignment (Fig. 2.4 b) reveals a number of interesting features. First, the conservation of the cysteines is remarkable. Of the six cysteines in the irPNP-like molecules, the first four (solid arrows) are conserved in the representatives of all sequences. In addition, two glycine residues (solid squares) are highly conserved across all molecules and a third is conserved in most irPNP-like molecules (open square in square brackets). Furthermore, two amino acids, tryptophan and valine (open squares) are present in all irPNP-like molecules. Two highly conserved diagnostic motifs: A and B (A: K[VI]VD and B: [LM]SxxAFxxI), have been identified in irPNP-like molecules. The second motif (B) includes the serine and phenylalanine that are conserved across the families.

While expansins have only barely detectable (endo-) glucanase activity (Cosgrove, 1999), most nevertheless contain a His-Phe-Asp (HFD) motif that is part of the catalytic site of the family-45 endoglucanases. This HFD motif is absent in irPNP-like molecules pointing to a further functional divergence of the group. IrPNP-like molecules also share similarities with barley wound-induced (Barwin) proteins and it is remarkable that the region of closest homology between the irPNP-like molecules and Barwin proteins comprises both modified versions of the motifs A and B. In Barwin proteins, the K in motif A is replaced by an R (A: K/R[VI]VD) and motif B is modified to LxxxxFxxI. In

addition, the glycine (open square) and the cysteine closest to motif A (open arrow) are also conserved between the Barwin proteins and irPNP-like molecules.

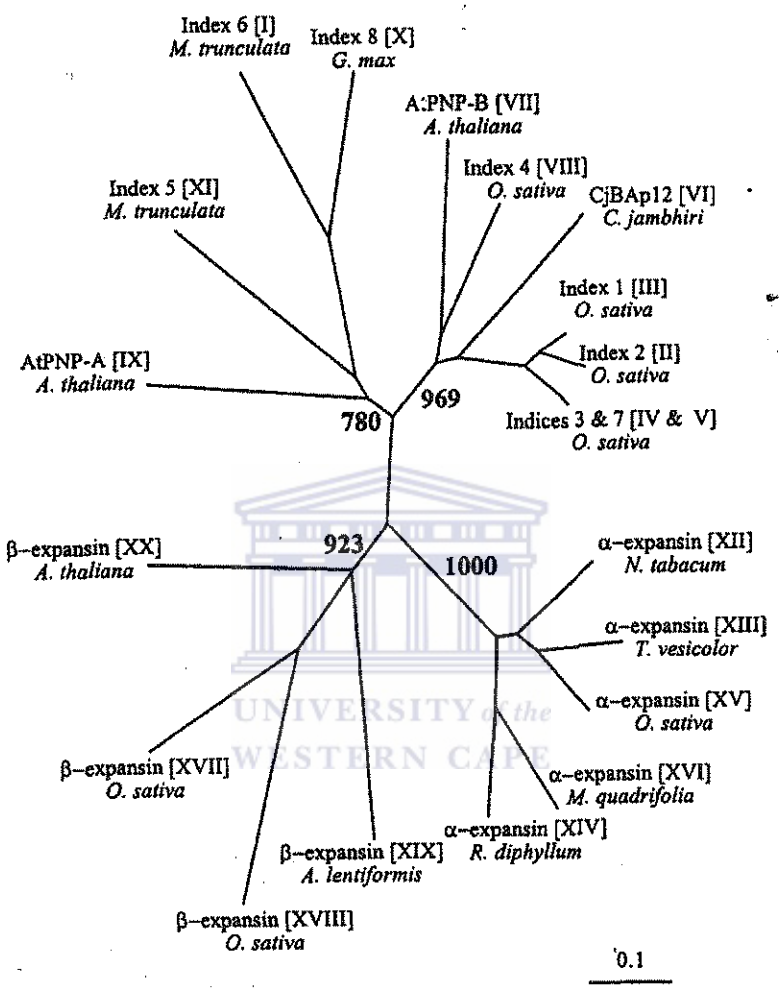


Fig. 2.3. Phylogenetic tree representing irPNP-like and CjBAp12-like molecules, α-expansins and β-expansins. The numbers represent bootstrap values from 1000 replicates. Indices are clustered EST sequences representing transcripts from one gene (or identical copies of one gene). Querying the TIGR database (http://www.tigr.org/tdb/tgi.shtml) with the complete AtPNP-A sequence (AAD08935) identified the indices. Sequence identification: I, TC29780; II, OSM110636; III, OSM11811; IV, OSM11298; V, AU101292; VI, AAD03398; VII, CAB79756; VIII, OSM110634; IX, AAD08935; X, TC64839; XI, BE943215; XII, AAC96081; XIII, T50660; XIV, AAF17571; XV, AAF62180; XVI, AAF17570; XVII, AAF72983; XVIII, AB61710; XIX, BAB20817.

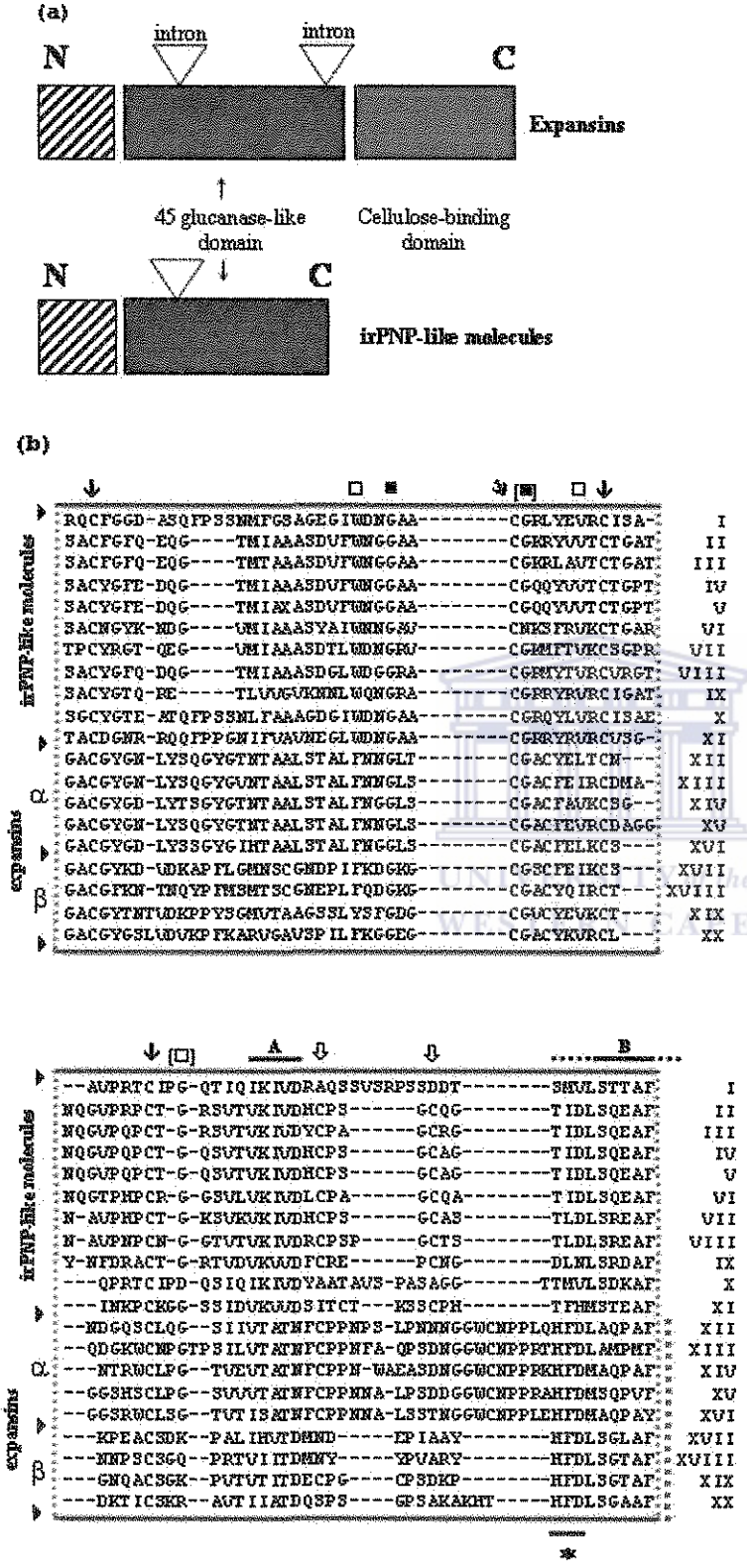


Fig. 2.4. (a) Domain comparisons of expansins and irPNP-like molecules. The open triangles (▽) signify introns. (b) Alignment of sequences represented in the dendrogram (Fig. 2.3). Solid arrows (⇓) are cysteines conserved in all molecules but the out-group. The open arrow (⇓) indicates cysteines present in most irPNP-like molecules, but not expansins. Solid squares (■) indicate amino acids other than cysteines conserved across all molecules, solid squares in square brackets ([■]) indicate a high degree of conservation across all molecules. Open squares (□) are conserved amino acids other than cysteines in irPNP-like molecules, open squares in square brackets ([□]) indicate a high degree of conservation within the irPNP-like molecules and α-expansins. The asterisk (*) shows the HFD motif conserved between many expansins and the endoglucanases of the family-45 glycosidases and (A) and (B) delineate two conserved motifs in irPNP-like molecules.

Structural modeling (Fig. 2.5) reveals that AtPNP-A has the same fold as the N-terminal domain of a Phl P 1.

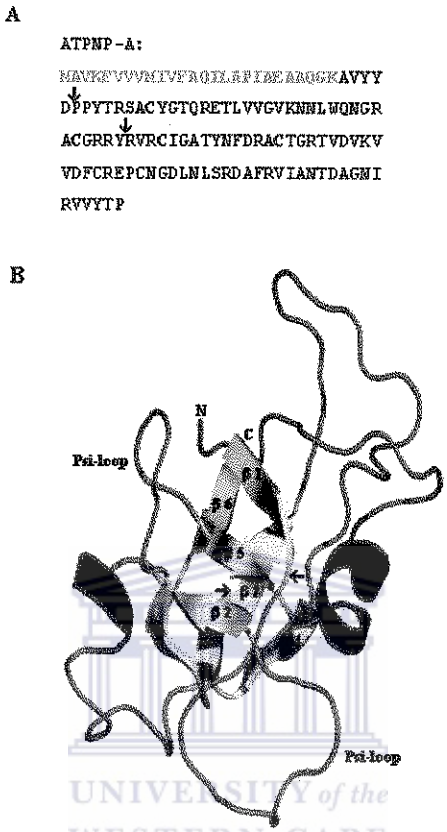


Fig. 2.5. Structural model of AtPNP-A. **A**, Amino acid sequence of AtPNP-A. The signal peptide (amino acid 1 – 26) that is not required for biological function outside the cell (Morse *et al*, 2004) is shown in gray, the cysteines (C) that are highly conserved in PNP-like molecules and expansins are in red and the two arrows delineate the domain critical for natriuretic peptide-like biological activity of the molecule (Morse *et al*, 2004). **B**, Structural model of AtPNP-A (amino acids 27 to 126). The model shows the six stranded double-psi β barrel structure that assumes a pseudo-twofold axes with parallel strands forming two psi structures. The α-helices are in red, the 6 β-strands are in yellow. The signal peptide was not included in the model and the domain conferring activity is between arrows.

The basic common fold for Phl P 1 and AtPNP-A is a double-psi β barrel structure (Fig. 2.5 B) where a six-stranded β barrel assumes a pseudo-two-fold axes in which the parallel strands form two psi structures (Fedorov *et al*, 1997). Such a psi structure comprises a loop and a strand that resemble the Greek letter psi (ψ). The first psi loop connects strands β1 and β2, whereas the second psi loop connects strands β4 and β5 (Fig. 2.5 B). In AtPNP-A the first psi loop connects strands β1 and β2, whereas the second psi loop connects strands β4 and β5 (Fig. 2.5 B). Interestingly, the domain spanning the second and third β strand flanking the α helix (amino acid 33 to 66) has been demonstrated in AtPNP-A to be both critical and sufficient for conferring homeostatic activity (Morse *et al*, 2004).

2.4. Discussion

What are natriuretic peptides and how do irPNPs and in particular AtPNP-A relate to them? Maintenance of water and solute homeostasis is a key requirement for all living systems. In vertebrates water and solute homeostasis is, in part, achieved by natriuretic peptides (NPs), a family of well-characterized peptide hormones (Thibault *et al*, 1999). Several lines of evidence suggest the presence of a structurally related and biologically active natriuretic peptide hormone-like system in plants (Gehring, 1999). Most importantly, a number of biologically active proteins were isolated and purified by immunoaffinity chromatography from species including *Hedera helix* (Billington *et al*, 1997) and *Solanum tuberosum* (Maryani *et al*, 2001). IrPNPs have been shown to promote stomatal opening (Billington *et al*, 1997), to rapidly and specifically induce

transient elevation of the second messenger cGMP (Pharmawati *et al*, 1998b) in *Zea mays* root stele tissue and to modulate cation fluxes (Pharmawati *et al*, 1999). Several irPNP-dependent processes have been observed in experimental systems that do not contain cell walls, such as protoplasts or microsomal and plasma membrane vesicles. The processes include *in vitro* irPNP binding to isolated *Tradescantia multiflora* leaf microsomes (Suwastika *et al*, 2000), irPNP-dependent modulation of plasma-membrane H^+ gradients in potato leaf tissue vesicles (Maryani *et al*, 2000), increases of cGMP levels in response to irPNP in *Solanum tuberosum* guard cell protoplasts (Pharmawati *et al*, 2001) and irPNP-dependent volume changes in protoplasts (Maryani *et al*, 2001). It thus follows that irPNPs can act directly on the plasma membrane. IrPNP-like molecules from potato have been isolated and partially sequenced irPNPs (Maryani *et al*, 2001) in order to study their molecular nature. An *Arabidopsis thaliana* transcript (*AtPNP-A*) encoding an irPNP was identified and isolated (Fig. 2.1). As expected, a section of a sequence with homology to vertebrate ANP was found within AtPNP-A (Fig. 2.1 d), thus linking the protein to the antibody and epitope used for its initial isolation. Sequence similarity of AtPNP-A was also observed to a functionally uncharacterized protein from *Citrus jambhiri*, CjBAp12. Since CjBAp12 in turn shows significant homology with the expansins, it was decided to undertake a detailed survey of irPNP-like sequences deposited in databases and thus establish the phylogenetic position and domain organization of a potentially novel family of proteins that contain at least one member (AtPNP-A) that shares an epitope with ANP. Such an analysis may help to explain the absence of expansin activity in CjBAp12 and further interpret our results from the functional assays with ANP and irPNPs in cell wall-free systems (Suwastika *et al*, 2000;

Maryani *et al*, 2000; Pharmawati *et al*, 2001; Maryani *et al*, 2001). The most closely related molecules to irPNPs are expansins. Expansins have extended C-termini when compared to the irPNPs. Expansins are in turn related to glucanases and cellulases and in the case of glucanases and cellulases, the C-termini have been proven to be cell wall binding (Linder and Teeri, 1997; Linder *et al*, 1998) and the same function has been suggested for the expansin C-terminus (Cosgrove, 2000). Recently, phylogenetic analysis of expansins has placed AtPNP-A, a plant natriuretic peptide from *Arabidopsis thaliana*, in a new class of expansis-related molecules named γ-expansins, in which AtPNP-A is referred to as Ath-Expγ-1.2 (Li *et al*, 2002). Since expansins, the closest relatives of irPNPs, and the more distantly related glucanases and cellulases contain the C-terminus, it is reasonable to argue that irPNP-like molecules have in fact lost this domain. This conclusion is also in keeping with the fact that the second intron and third exon are absent in irPNP-like molecules. Such a domain loss, we speculate, would result in absence of wall binding and thus increased mobility of the molecule. The concept of increased mobility is supported by the fact that CjBAp12 appears to be a systemically mobile protein (Ceccardi *et al*, 1998), present, but not synthesized, in leaves. In addition, *in situ* localization data in *Solanum tuberosum* also identifies irPNP in conductive tissue (Maryani *et al*, 2003). The conductive tissue is a highly unlikely place for irPNP synthesis but not for its transport. The above observations and conclusions in themselves do not explain the absence of expansin activity of CjBAp12 since the catalytic activity of expansin resides in the N-terminus. However, irPNP-like molecules also appear to be functionally further diverged from the ancestral endoglucanases since the HFD motif shared by the expansins and the endoglucanases is absent from the irPNP-like proteins

(see Figure 2.4 b). Based on the sequence comparisons, it is proposed that ancestral glucanases have given rise to the expansins and irPNP-like molecules and the latter have been recruited to serve in capacities entirely different from cell wall loosening. These recruited molecules may be part of stress responses and possibly function as extracellular signaling molecules that directly or indirectly affect water and ion transport. Such a conclusion is not only supported by the domain organization but also by the fact that irPNPs were initially isolated through the use of immunoaffinity to an antibody directed against atrial natriuretic peptide, a vertebrate signaling peptide with a role in water and solute homeostasis (Billington *et al*, 1997; Gehring, 1999). Natriuretic peptides, ANPs, and irPNPs have been shown to affect ion transport (Pharmawati *et al*, 1999; Maryani *et al*, 2000; Pharmawati *et al*, 2001) across plant cell membranes. Furthermore, water transport has been shown to be affected by ANP in vertebrate systems (Wolfensberger *et al*, 1994; Han *et al*, 1998) and both ANP and irPNP modulate osmoticum dependent water transport (Maryani *et al*, 2001) in protoplasts, an experimental system without cell walls. In summary, all evidence points towards a unique set of physiological functions for irPNP-like molecules that is entirely different from cell wall loosening (Fig. 2.6).

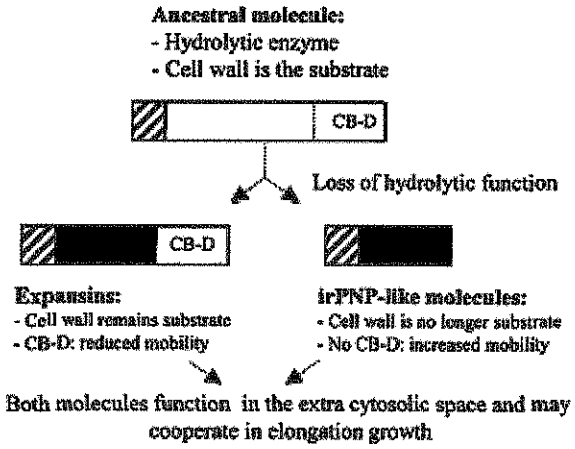


Fig. 2.6. Model of molecular evolution of irPNP-like molecules.

The data and hypothesis presented here further strengthen the case for the presence of a functional peptide based extracellular signaling system. This system may be derived from ancestral molecules that also gave rise to expansins. Furthermore, irPNP-like molecules from plants have been identified and isolated due to an epitope shared between the signaling vertebrate ANP and irPNPs. Moreover, ANP and irPNP induce a remarkable number of common physiological responses in plants. An interesting feature is that the irPNP-like molecules can cause enhanced osmoticum-dependent water uptake (Maryani *et al*, 2001; Pharmawati *et al*, 2001) and hence increases in cell turgor. Increasing cell turgor will pose stress on cell walls and may in turn signal the need for expansin-dependent wall loosening. It is conceivable, that irPNP-dependent swelling exerts the force required for cellular expansion. This observation provides fuel for the provocative suggestion that despite the divergent functions of expansins and irPNP-like molecules, their action is in fact cooperative.

An adjunct to this characterization of the molecular evolution of irPNP-like molecules is the recent finding that AtPNP-A shares significant sequence similarity with a protein from the *Xanthomonas axonopodis* pv. citri str. 306 (Fig. 2.7) named Xac2654 (da Silva *et al*, 2002) (GenBank accession no. AAM37501; E-value = $8.9e^{-10}$ from BLAST against the *Xanthomonas axonopodis pv. citri 306* peptide database). The polypeptide domain shared by AtPNP-A and Xac2654 has previously been shown to be sufficient to cause H_2O uptake into protoplasts (Maryani *et al*, 2001). While the origin of the bacterial gene is difficult to ascertain since it could have been either transferred horizontally from plants or it could be the product of convergent evolution, clues can be obtained to its role by

examining the biology of *Xanthomonas axonopodis*. *Xanthomonas axonopodis* is a plant pathogen and the causal agent of citrus canker. The symptoms include corky lesions surrounded by watersoaked margins (Civerolo, 1984). These watery margins must be a consequence of enhanced cellular H_2O uptake and this is precisely one of the effects that have been observed in response to both ANP and PNPs. It is thus very possible that the pathogen uses its PNP-like molecule to induce plant tissue hyper-hydration ensuring its own supply while disturbing the homeostasis of its host.

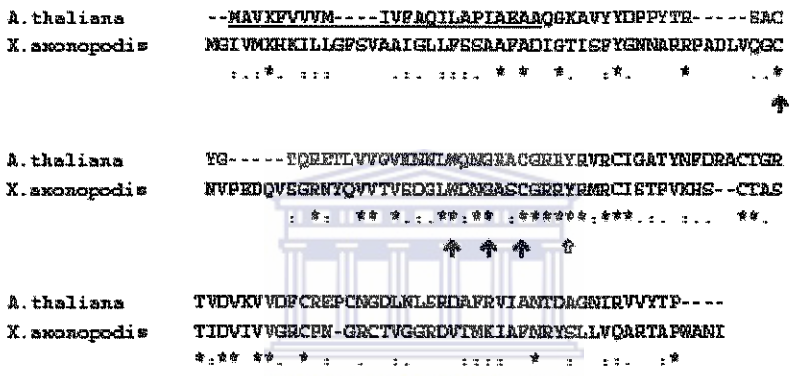


Fig. 2.7 Alignment of a plant natriuretic peptide from *Arabidopsis thaliana* (AtPNP-A; Accession No. AAD08935) and Xac2654 from *Xanthomonas axonopodis* (Accession no. AAM37501). The red letters delineate the sequence that shows similarity to vertebrate atrial natriuretic peptides and the underlined sequence represents the signal peptide. Asterisks (*) identify identical amino acids, colons (:) are conservative amino acid substitutions and dots (.) are semi-conservative amino acid substitutions. Solid arrows (↟) are conserved amino acids within the ANP-like domain that are also conserved in PNP-like molecules from other plant species while the open arrow (⇧) marks a position where other PNP-like molecules have a tyrosine or a phenylalanine.

Part of the work described in the above chapter has been published as cited below:

Ludidi N.N., Heazlewood J.L., Seoighe C., Irving H.R. & Gehring C.A. (2002) Expansin-like molecules: Novel functions derived from common domains. *Journal of Molecular Evolution* **54**, 587-594.

CHAPTER 3

IDENTIFICATION OF A PLANT GUANYLYL CYCLASE IN *Arabidopsis thaliana*

Abstract

Guanylyl cyclases catalyze the formation of the second messenger guanosine 3',5'-cyclic monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). While many cGMP mediated processes in plants have been reported, no plant molecule with guanylyl cyclase activity has been identified. When the *Arabidopsis thaliana* genome is queried with guanylyl cyclase sequences from cyanobacteria, lower and higher eukaryotes no unassigned proteins with significant similarity are found. However, a motif search of the *Arabidopsis thaliana* genome based on conserved and functionally assigned amino acids in the catalytic center of annotated guanylyl cyclases returns one candidate that also contains the adjacent glycine-rich domain typical for guanylyl cyclases. In this molecule, termed AtGC1, the catalytic domain is in the N-terminal part. AtGC1 contains the arginine or lysine that participates in hydrogen bonding with guanine and the cysteine that confers substrate specificity for GTP. When AtGC1 is expressed in *Escherichia coli*, cell extracts yield >2.5 times more cGMP than control extracts and this increase is not

nitric oxide dependent. Furthermore, purified recombinant AtGC1 has Mg^{2+}-dependent guanylyl cyclase activity *in vitro* and >3 times less adenylyl cyclase activity when assayed with adenosine 5'-triphosphate (ATP) as substrate in the absence of GTP. Catalytic activity *in vitro* proves that AtGC1 can function either as a monomer or homo-oligomer. AtGC1 is thus not only the first functional plant guanylyl cyclase but also, due to its unusual domain organization, a member of a new class of guanylyl cyclases.

3.1. Introduction

Guanylyl cyclases (EC 4.6.1.2) catalyze the formation of guanosine 3',5'-cyclic monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). The cyclic nucleotide, cGMP, acts as second messenger in many prokaryotes and all eukaryotes including plants (Lucas *et al*, 2000). In higher plants cGMP-mediated processes control phytochrome-dependent gene expression required for chloroplast development and anthocyanin biosynthesis (Bowler *et al*, 1994; Bowler *et al*, 1997; Chamovitz and Deng, 1996). The light-downregulated gene, asparagine synthetase, has been shown to be controlled by a Ca^{2+}/cGMP-dependent pathway that activates other light responses and complementary loss- and gain-of-function experiments have identified a 17 base pair *cis*-element within the asparagine synthetase promoter that is both necessary and sufficient for this regulation (Neuhaus *et al*, 1997). This *cis*-element may well be the target for a conserved phytochrome-generated repressor whose activity is regulated by calcium and cGMP (Neuhaus *et al*, 1997). In plants as well as in animals, nitric oxide is operating as a redox-active signaling molecule, and nitric oxide donors have been shown to induce expression of some defense-related genes, and tobacco mosaic virus-dependent increases of nitric

oxide synthase activity occur in resistant plants only (Durner *et al*, 1998). Nitric oxide does elevate cGMP levels in plants, and nitric oxide-induced expression of some defense related genes was found to be mediated by the second messengers cGMP and cyclic adenosine diphosphate ribose, both of which also operate in animal responses to nitric oxide (Durner *et al*, 1998). Cellular cGMP levels are also increased transiently after application of the plant hormone gibberellic acid (GA) in barley aleurone layers and guanylyl cyclase inhibition prevents the GA-induced increase in cGMP and inhibits GA-induced α-amylase synthesis and secretion (Penson *et al*, 1996). Both processes can be restored by exogenous application of membrane-permeable analogues of cGMP, thus establishing cGMP as a second messenger critical for α-amylase synthesis and/or secretion. The regulation of ion transport is also, in part, dependent on cyclic nucleotides (Hoshi, 1995; Maathuis and Sanders, 2001). Such regulation can occur in plant voltage-gated K^+ channels where binding of cGMP modulates the voltage/current relationship (Maathuis and Sanders, 2001). Plants also contain cyclic nucleotide-gated low affinity cation channels where binding of cAMP and cGMP to the intracellular portion leads to direct gating (Leng *et al*, 1999). Recently, voltage-independent channels without selectivity for particular monovalent cations have been characterized in *Arabidopsis thaliana*. Voltage-independent channels showed no selectivity among monovalent cations, and their gating was found to be voltage-independent, while micromolar concentrations of cAMP or cGMP at the cytoplasmic side of the plasma membrane caused rapid decreases in channel open probability (Maathuis and Sanders, 2001). It was shown that short-term unidirectional Na^+ influx is reduced in the presence of cyclic nucleotides and that membrane-permeable cyclic nucleotide can improve salinity

tolerance presumably by reducing net Na^+ uptake (Maathuis and Sanders, 2001). Cell-permeable cGMP and cAMP analogs elicit elevation of cytosolic Ca^{2+} in tobacco protoplasts and cause a physiological swelling response in plant protoplasts (Volotovski *et al*, 1998). Opening of the stomatal pore, which results from a swelling of the two neighboring guard cells, has been observed in response to cell-permeable cGMP analogs and is suppressed by guanylyl cyclase inhibitors (Pharmawati *et al*, 1998a). Like protoplast swelling, stomatal aperture regulation is likely to be tuned by Ca^{2+} and cGMP cross-talk (Pharmawati *et al*, 2001). Since significant and transient increases in intracellular cGMP levels, *e.g.* in response to the plant hormones gibberellic acid (Penson *et al*, 1996) and cytokinins (Pharmawati *et al*, 2001) as well as vertebrate atrial natriuretic peptides and immunoreactant plant natriuretic peptides (Pharmawati *et al*, 1998a; Pharmawati *et al*, 1998b; Pharmawati *et al*, 2001; Ludidi *et al*, 2002), have been reported, it is reasonable to presuppose guanylyl cyclse activity in plants. However, no plant molecule with guanylyl cyclase activity has been identified to date. An unusually high level of divergence in plant guanylyl cyclases could explain this failure. Such a high divergence would put guanylyl cyclases outside the detection limit of "Blast" searches or biochemical tools such as specific antibodies against guanylyl cyclases from e.g. bacteria or animals. Alternatively, plant guanylyl cyclasess may not be homologous to currently annotated guanylyl cyclases and thus not easily identified. In both cases, however, it was hypothesized that plant guanylyl cyclases may contain a significant degree of similarity to the catalytic center from previously identified nucleotide cyclases and guanylyl cyclases in particular. Consequently, designated catalytic domains (Wedel and Gabers, 1997; Thompson and Gabers, 1995; Liu *et al*, 1997; McCue *et al*, 2000) from vertebrates,

lower eukaryotes and prokaryotes were aligned with a view to deduce a guanylyl cyclase catalytic domain search motif. Such a motif would then be used to do pattern searches of the complete *Arabidopsis thaliana* genomic sequence to identify candidate proteins for functional testing.

3.2. Materials and methds

Guanylyl cyclases were retrieved from NCBI, and their catalytic domains were used for Blast (Altschul *et al*, 1997) queries of "The *Arabidopsis* Information Resource" database (www.arabidopsis.org) and GenBank. The catalytic domains were aligned using Clustal X (Thompson *et al*, 1997), and the alignment at the catalytic center of the catalytic domain was used to derive the search motif. The derived search motif was tested for accurate and specific detection of nucleotide cyclases by querying the Protein Information Resource (www-nbrf.georgetown.edu) using the Pattern Match option on the PIR-NREF link. The search motif was used to query The *Arabidopsis* Information Resource database using the Patmatch link in The *Arabidopsis* Information Resource. Total RNA was isolated from 3-week-old seedlings using the RNeasy plant mini kit (Qiagen) in combination with DNase treatment using RNase free DNase Set (Qiagen) according to the manufacturer's instructions. First strand AtGC1 cDNA was synthesized from total RNA with 1 µM Primer GC1fwd (5'-CAC TGT GGA TCC ATG TGG CCT CTT TGT TTT CTG-3') incorporating a 5' *Bam*HI restriction site (underlined), 1 µM Primer GC1rev (5'-CTG ACT CTC GAG CTA ATA TCC GTT CTG GTT CC-3') incorporating a 5' *Xho*I restriction site (underlined) using reverse transcriptase (Promega). Double-stranded AtGC1 cDNA synthesis was done by PCR on the first strand

cDNA from above, with 0.4 μM Primer GC1fwd and 0.4 μM Primer GC1rev, using the expand high fidelity PCR system kit (Roche Diagnostics) as instructed by the manufacturer, except that the deoxynucleotide triphosphate concentration was changed to 100 μM for each deoxynucleotide triphosphate. The *AtGC1* cDNA was cloned as a *Bam*HI/*Xho*I fragment into pBluescript SK(+/-) (Stratagene) to construct pBS:AtGC1 and sequenced. The *AtGC1* cDNA was subcloned from the pBS:AtGC1 construct into the *Bam*HI/*Xho*I sites of the glutathione *S*-transferase (GST) fusion expression vector pGEX-6P-2 (Amersham Biosciences) to make the GST:AtGC1 fusion expression construct pGEX:AtGC1. *Escherichia coli* BL21 (DE3) pLysS cells (Invitrogen) were transformed with pGEX:AtGC1 to express of the recombinant protein and with pGEX-6P-2 (Amersham Biosciences) as a positive control experiment. *Escherichia coli* BL21 (DE3) pLysS cells were grown at 30 °C to an OD_{600} of 0.8. The cells were then induced for expression of GST:AtGC1 or GST by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega) to a final concentration of 0.6 mM and growth at 30 °C for 60 min with 3-isobutyl-1-methylxanthine (Sigma-Aldrich) added to a final concentration of 1 mM to inhibit resident bacterial phosphodiesterases. To determine nitric oxide dependence of AtGC1, the nitric oxide donor sodium nitroprusside (Sigma-Aldrich) was added to a final concentration of 1 mM to both uninduced and induced cells 10 min before harvesting of the cells. Cells were harvested by centrifugation at 4 °C at 12 000*g* for 10 min, then resuspended in ice-cold phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3). Total *Escherichia coli* proteins were extracted by three freeze-thaw cycles in phosphate-buffered saline with freezing done in liquid nitrogen and thawing done at 42 °C. Purification of both recombinant

GST:AtGC1 and GST was performed on a glutathione-sepharose 4B affinity column (Amersham Biosciences) and eluted in glutathione elution buffer made up of 10 mM reduced glutathione (Sigma-Aldrich) in 50 mM Tris-HCl at pH 8.0 according to the protocol supplied by the manufacturer. The eluted proteins were desalted and concentrated using Amicon® Ultra 10 000 Da molecular weight cut-off 15 ml centrifugal filter devices (Millipore). The levels of cGMP were measured by radioimmunoassay using the cGMP (^{125}I) assay system kit (Amersham Biosciences) as described in the supplier's manual for the acetylation protocol. Purified GST:AtGC1 recombinant fusion protein (140 µg) was used to determine AtGC1 guanylyl cyclase activity *in vitro* (Thorpe and Morkin, 1990). Adenylyl cyclase activity of purified GST:AtGC1 was determined using the same method (Thorpe and Morkin, 1990) except that the substrate used was 1 mM ATP instead of 1 mM GTP. Production of cGMP by GST:AtGC1 was assayed using the cGMP (^{125}I) assay system kit (Amersham Biosciences). Generation of cAMP by GST:AtGC1 from the adenylyl cylase reaction was determined using the cAMP Biotrak enzyme immunoassay (Amersham Biosciences).

3.3. Results

Sequence alignment of representatives of annotated catalytic domains of guanylyl cyclases from prokaryotes and eukaryotes are shown in Fig. 3.1. The residues implicated in catalysis (Liu *et al*, 1997; McCue *et al*, 2000) are indicated in red, and a 14-amino acid-long search motif spanning the catalytic center was deduced (Fig. 3.1). An isoleucine (Ile) and leucine (Leu) was added to positions 4 and 9, respectively, to include all three aliphatic amino acids with non-polar side chains; this is in keeping with

requirements of these positions, since they are part of the hydrophobic pocket where the purine moiety binds (Liu *et al*, 1997). The glutamic acid (Glu) implied in Mg^{2+} binding (Liu *et al*, 1997) that is conserved in the present alignment (Fig. 3.1) was not included in the search motif, since it is not conserved in all guanylyl cyclases (McCue *et al*, 2000). The guanylyl cyclase catalytic domain alignments also revealed that guanylyl cyclases contain a glycine-rich domain N-terminal of the catalytic center (Fig. 3.1), and this is the case in all currently annotated guanylyl cyclases (not shown). This glycine-rich domain can be expressed as (G-{X}3,4-G-X{2,3}-G) and will subsequently be referred to as glycine-rich motif and used as secondary search parameter. A pattern search of PIR with the catalytic center motif identifies 263 protein sequences of which 208 are annotated guanylyl cyclases and the rest are either hypothetical proteins or annotated proteins with different functions. A pattern search of the *Arabidopsis thaliana* genome database with no substitution allowed returns no candidates. If a replacement with any amino acid was permitted at the functionally unassigned, but conserved, glycine (Gly) residue in the catalytic center, seven candidates molecules are returned. Of the seven *Arabidopsis thaliana* proteins returned only one also contains the N-terminal glycine-rich motif. This *Arabidopsis thaliana* molecule of unassigned function was termed AtGC1. AtGC1 is 274 amino acids in length and contains the search motif in its N-terminal region between residues 32 and 46 (Fig. 3.2). The functionally unassigned but conserved glycine (Gly) in the catalytic center is replaced by an aspartic acid (Asp) (Fig. 3.1). Homology searches with AtGC1 return guanylyl cyclases, albeit with *e*-values ≥0.19, as well as more closely related ($<e^{-20}$) *Homo sapiens*, *Mus musculus*, *Anopheles gambiae* and *Drosophila melanogaster* proteins without commonly known guanylyl cyclase domains and of

currently unknown function. AtGC1 contains the conserved arginine (Arg) or lysine (Lys) (Fig. 3.1) that participates in hydrogen bonding with guanine (Liu *et al*, 1997; McCue *et al*, 2000) and the cysteine (Cys) that confers substrate specificity for GTP (Liu *et al*, 1997; McCue *et al*, 2000; Tucker *et al*, 1998). Finally, one of the amino acids that have been reported to stabilize the transition state in the conversion of GTP to cGMP, namely arginine (Arg), is conservatively replaced by lysine (Lys) (Liu *et al*, 1997; McCue *et al*, 2000). Such a replacement is also seen in the *Takifugu rubripes* guanylyl cyclase (Fig. 3.1). AtGC1 contains a glycine-rich domain N-terminal of the catalytic center (Fig. 3.1) and 17 amino acids N-terminal of the motif an arginine (Arg) (Fig. 3.2 *A*) that is implicated in inorganic pyrophosphate binding (Liu *et al*, 1997). This arginine is flanked by hydrophobic amino acids, and this together with the distance to the catalytic center is common in annotated class III nucleotide cyclases (Liu *et al*, 1997; McCue *et al*, 2000). The modeled secondary structure (Jones, 1999) (Fig. 3.2 *A*) predicts a β-sheet spanning the purine binding hydrophobic pocket, a feature that has been reported previously (Liu *et al*, 1997). Three-dimensional structure prediction (Kelley *et al*, 2000) (not shown) indicates 18 % identity of AtGC1 with the P-loop in nucleotide triphosphate hydrolases. A domain comparison (Fig. 3.2 *B*) between classical guanylyl cyclases and AtGC1 reveals the unusual N-terminal location of the catalytic motif as well as the reduced size of the domain. Pairwise BLAST sequence comparison of AtGC1 against sequences from the dimerization domain, kinase-homology domain, transmembrane domain, ligand-binding domain, and heme-binding domain that interacts with nitric oxide indicated that AtGC1 does not have significant sequence similarity with any of these domains.

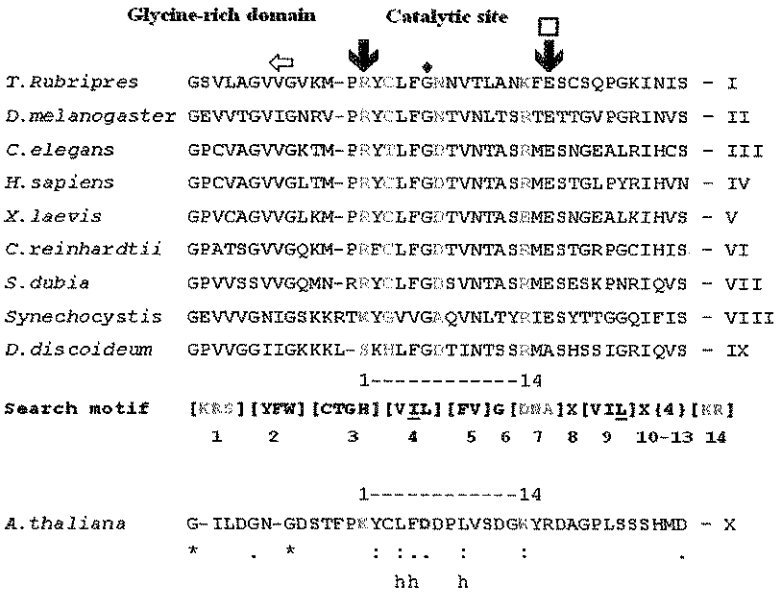


Fig. 3.1. Alignment of guanylyl cyclase catalytic domains. Edited Clustal X alignment of catalytic domains of guanylyl cyclases is shown. The catalytic center is delineated by two solid arrows (⬇), and the glycine-rich motif is delineated by an open arrow (⇦). The deduced 14-amino acid-long search motif is in bold, and substitutions are in square brackets([]); X represents any amino acid, and curly brackets ({}) define the number of amino acids. Red amino acids are functionally assigned residues of the catalytic center, the blue amino acid is the replacement of the conserved glycine (♦), and the underlined amino acids in positions 4 and 9 are the third branched aliphatic amino acid not appearing in the alignment. The glutamic acid (E) implied in Mg^{2+} and Mn^{2+} binding (□) is not included in the search motif. Asterisks mark conserved residues, colons are conservative replacements, and periods are semiconservative substitutions, and the letter h stands for hydrophobic residues forming the hydrophobic pocket. Accession numbers of aligned sequences are as follows:

I, BAB60905; II, NP_524603; III, NP_494995; IV, NP_000171; V, BAA83786; VI (expressed sequence tag), BI717053; VII (expressed sequence tag), AL132834; VIII, NP_440289; IX, CAB42641; X, AY118140.

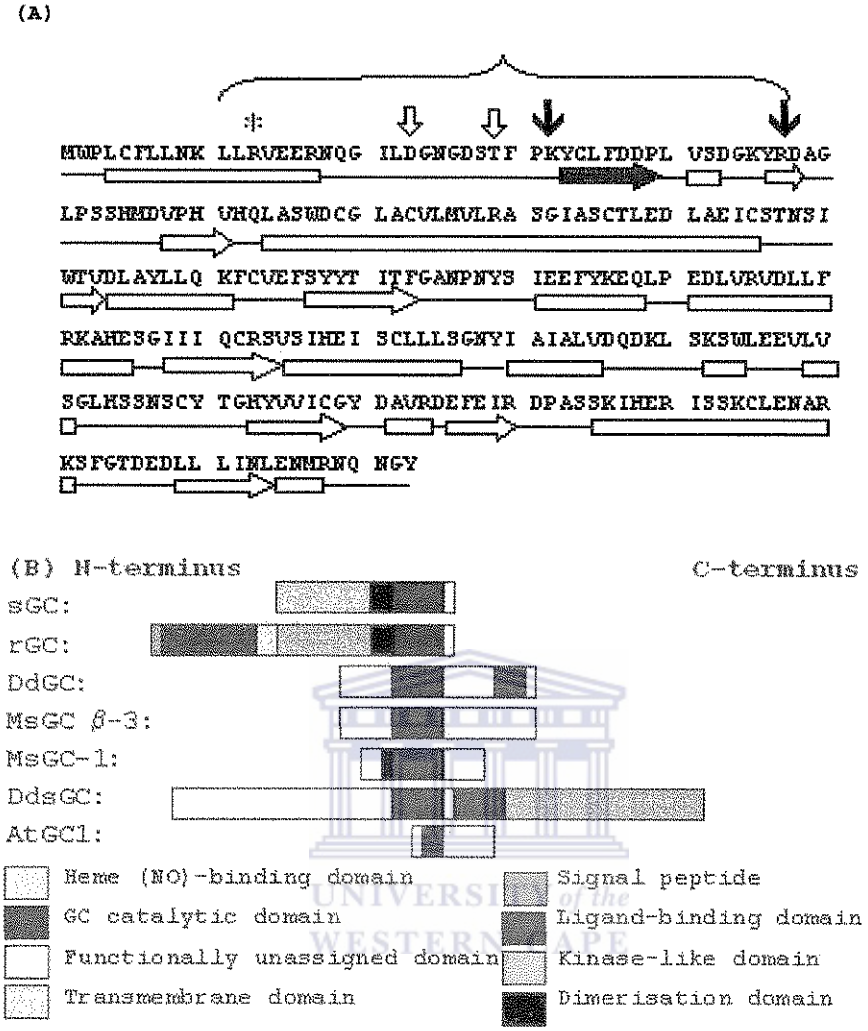


Fig. 3.2. Secondary structure and domain organization of AtGC1. **A**, predicted secondary structure of AtGC1 with the designated catalytic domain under the bracket, the catalytic center between solid arrows, the glycine-rich motif delineated by open arrows, and the putative inorganic pyrophosphate (PPi)-binding arginine (R) residue marked by an asterisk. A line under the amino acid symbols signifies a predicted coil, open boxes are α-helices, and open arrows are β-sheets. The red arrow marks the designated purine-binding hydrophobic pocket (Liu *et al*, 1997; McCue *et al*, 2000). **B**, comparison of domain arrangements between different types of guanylyl cyclases.

To test biological activity of the candidate molecule, we have obtained the cDNA by reverse transcriptase-PCR and expressed it in a prokaryotic system (Fig. 3.3 A). Only in protein extracts from transformed induced bacteria do we observe a fusion protein (GST:AtGC1) of the predicted molecular mass of 57 kDa. When equal amounts of cells were extracted and assayed for cGMP with a radioimmunoassay, it was observed that the cGMP levels were more than 2.5 fold elevated in extracts from transformed induced bacteria as compared with both extracts from non-induced cells and the control (Fig. 3.3 B). The findings would suggest that the *Arabidopsis thaliana* protein is either a *bona fide* guanylyl cyclase or stimulates resident *Escherichia coli* guanylyl cyclases. Fig. 3.4 shows the purified recombinant AtGC1 and results from *in vitro* testing of the recombinant molecule. The guanylyl cyclase activity of total extracted protein from untransformed, but IPTG-induced, *Escherichia coli* is less than 10 fmol/μg of protein and higher in the presence of Mn^{2+} than Mg^{2+}. The purified fusion protein (Fig. 3.4 A) has an activity of >20 fmol/μg in the presence of Mg^{2+} and a drastically reduced activity (>50×) in the presence of Mn^{2+} (Fig. 3.4 *B*). Both control extract from untransformed induced *E. coli* obtained after glutathione-Sepharose 4B column purification and GST on its own show no activity, thus indicating that the cGMP measured in the recombinant protein preparation is solely due to the catalytic function of AtGC1. A time course of GST:AtGC1-dependant cGMP formation shows that > 80 % of the product was generated in less than 10 min and that no significant amount of cGMP was produced from heat-inactivated recombinant protein under the same experimental conditions (Fig. 3.4 *C*).

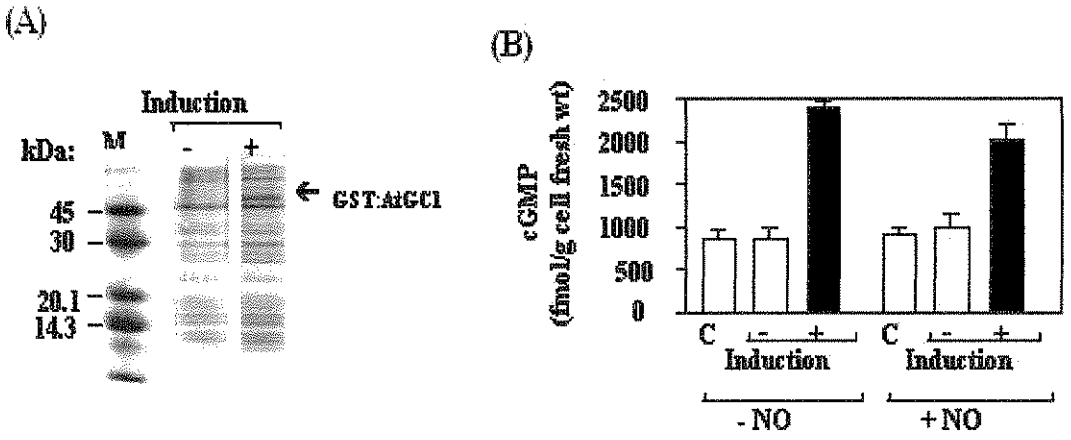


Fig. 3.3. Expression of AtGC1 as a GST recombinant fusion protein in *Escherichia coli*. **A**, SDS-PAGE gel of uninduced (-) and induced (+) proteins from *Escherichia coli* BL-21 (DE3) pLysS containing the expression construct (pGEX:AtGC1). The solid arrow marks the expressed recombinant protein (GST:AtGC1) after 60 min induction with 0.6 mM IPTG at 30 °C. **B**, cGMP levels in extracts from *Escherichia coli* (0.1 g) determined with a cGMP (^{125}I) radioimmunoassay system (see "Experimental Procedures"). The control (C) is the cGMP value from *Escherichia coli* extracts containing vector (pGEX-6P-2) only, induced with 0.6 mM IPTG for 60 min; - is the value of the uninduced and + is the value of the induced cells. Nitric oxide induction was stimulated with 1 mM nitroprusside (see "Experimental Procedures"). cGMP levels of the control (C) and the non-induced (-) are not different, and both are significantly different from the induced (+) ($p < 0.01$). The bars (•) represent a mean of quadruplicate data points, with S.D. indicated by bars (•). The data are representative of three independent experiments.

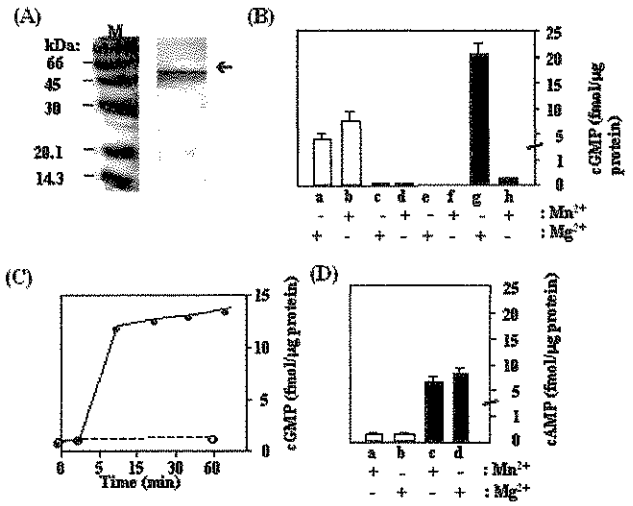


Fig. 3.4. Expression of AtGC1 as a GST recombinant fusion protein and *in vitro* testing. **A**, SDS-PAGE gel of recombinant GST:AtGC1. **B**, cGMP levels in fmol per µg of protein in the presence of 5 mM Mg^{2+} or Mn^{2+} and 1 mM IBMX. Open bars (lanes a and b) represent cGMP values from untransformed, IPTG-induced *Escherichia coli* protein extracts (see "Experimental Procedures"). Solid bars are cGMP values from GST column-purified extracts of non-transformed, but induced, *Escherichia coli* (lanes c and d), a GST only control (lanes e and f), and GST:AtGC1 recombinant protein (lanes g and h). The bars represent a mean of quadruplicate data points, with S.D. indicated by bars (•). The data are representative of three independent experiments. **C**, time course of GST:AtGC1 guanylyl cyclase activity performed *in vitro.* Control reactions were performed in the same way except that GST:AtGC1 was heat-inactivated. Data points are means of duplicate measurements. **D**, cAMP levels in fmol per µg of recombinant GST:AtGC1 in the presence of 5 mM Mg^{2+} or Mn^{2+}. Open bars (lanes a and b) represent cAMP values from purified GST. Solid bars are cAMP values from GST:AtGC1 recombinant protein (lanes c and d). The bars (•) represent a mean of triplicate data points with S.D. indicated by bars (•).

The results obtained from *in vitro* testing are thus consistent with monomeric or homo-oligomeric catalytic function that is independent of adenosine 5'-triphosphate (ATP), activating proteins or co-factors other than Mg^{2+}. Finally, GST:AtGC1 was also tested for adenylyl cyclase activity *in vitro* (Fig. 4.4 *D*). The result indicates that with ATP as substrate and in the absence of GTP, AtGC1 has adenylyl cyclase activity that is ≥ 3 times lower than the guanylyl cyclase activity and not significantly different in the presence of either Mg^{2+} or Mn^{2+}.

3.5. Discussion

Since there is compelling evidence that plants operate cGMP-dependent signaling pathways, it was decided to use the resource of the complete *Arabidopsis thaliana* genome sequence to search for guanylyl cyclases. The approach was based on the observation that known guanylyl cyclase catalytic domains appear highly conserved between highly diverged organisms. It was reasoned that plant guanylyl cyclases might contain at least some of the amino acid residues that are conserved in the catalytic center of known guanylyl cyclases, specifically the residues that are directly implicated in catalytic activity. Furthermore, the obligatory presence of the glycine-rich motif found adjacent to the N-terminus of the catalytic center of all known guanylyl cyclases suggested inclusion of this feature as additional search criterion. In guanylyl cyclases the glycine-rich motif may play a similar role as in the P-loop of GTP-binding proteins where it gives flexiblity to the part of protein that interacts with the phosphate group in GTP, thus facilitating substrate binding (Saraste *et al*, 1990). It was anticipated that plant guanylyl cyclases would be significantly evolutionarily distinct and different from known

guanylyl cyclases, since homology searches have failed to identify functional molecules in plants. Consequently, a search motif was constructed to allow conservative and semiconservative replacements of amino acid residues in the catalytic center. The glutamic acid (Glu) implied in Mg^{2+} binding (Fig. 3.1) (Liu *et al*, 1997) was not included in the search motif, since it is substituted by a wide range of different amino acids (Asp, Ala, Gln, Cys, Thr, Arg, and Tyr) in prokaryotic guanylyl cyclases (McCue *et al*, 2000). In addition, the glutamic acid (Glu) is also replaced by aspartic acid (Asp) in some type I and type II adenylyl cyclases (Liu *et al*, 1997). This substitution pattern would thus suggest that polarity and charge of the amino acid side chain in this position is not restrictive for catalysis. The fact that no *Arabidopsis thaliana* protein contains the unsubstituted search motif prompted a decrease of the stringency of the motif allowing for a replacement of the functionally unassigned but highly conserved glycine in the catalytic center (Fig. 3.1). The modified search parameters yield seven *Arabidopsis thaliana* proteins, and only one, AtGC1, fulfils the secondary criteria of the presence of the adjacent glycine-rich motif. It is concluded that the motif search with one substitution in the catalytic center is not selective enough for guanylyl cyclases, since 20 proteins out of 260 proteins identified in PIR that contain the motif are implicated in functions not related to known guanylyl cyclase activity. However, the combination of the two search parameters, the catalytic center search motif and the glycine-rich motif, identify annotated guanylyl cyclases only. This would suggest that the use of both parameters combined is a reliable tool for the identification of candidates. AtGC1 contains the conserved residues that participate in hydrogen bonding with guanine (Liu *et al*, 1997; McCue *et al*, 2000) and the cysteine (Cys) that confers substrate specificity for GTP

rather than adenosine 5'-triphosphate (ATP) (Liu *et al*, 1997; McCue *et al*, 2000). This structural feature is reflected in the *in vitro* activity of AtGC1 that shows significantly more guanylyl than adenylyl cyclase activity. The arginine (Arg) that stabilizes the transition state in the conversion of GTP to cGMP is conservatively replaced by lysine (Lys) (Liu *et al*, 1997; McCue *et al*, 2000) and such a substitution is also observed in *Takifugu rubripes* (Fig. 3.1). AtGC1 also contains the hydrophobic pocket to which nucleotide triphosphate purine moieties can bind (Liu *et al*, 1997) and the secondary structure modeling predicts that this pocket is part of a β-sheet (Fig. 3.2 *A*). A further N-terminal feature of guanylyl cyclases is the presence of a pyrophosphate (PPi) binding motif that consists of an arginine (Arg) flanked by aliphatic amino acids (Liu *et al*, 1997). AtGC1 contains such a motif 22 amino acids from the catalytic center and hence in a position that is conserved in PPi binding sites of known guanylyl cyclases (Fig. 3.2 *A*). The similarity between the predicted three-dimensional structure of AtGC1 and that of the P-loop structure contained in nucleotide triphosphate hydrolases (Saraste *et al*, 1990; Prakash *et al*, 2000) is noteworthy, since nucleotide triphosphate hydrolases can convert GTP to both guanosine 5'-diphosphate (GDP) and guanosine 5'-monophosphate (GMP) (Schwemmle and Staeheli, 1994; Neun *et al*, 1996). In triphosphate hydrolases, the P-loop interacts with Mg^{2+} for GTP binding and hydrolysis (Schwemmle and Staeheli, 1994; Neun *et al*, 1996; Saraste *et al*, 1990; Prakash *et al*, 2000), and it may be argued that the P-loop-like structure in AtGC1 has an analogous function, and this is supported by the observed dependence of the catalytic activity of AtGC1 on Mg^{2+} (Fig. 3.4 B). AtGC1 contains a number of features that are unusual in currently annotated guanylyl cyclases. First, the catalytic center motif is found close to the N-terminal rather than the

C-terminal or central part of the molecule where it is located in both particulate and soluble guanylyl cyclases (Wedel *et al*, 1995; Koesling *et al*, 1991; Garbers and Lowe, 1994). C-terminal location is the norm in particulate guanylyl cyclases, where the N-terminus functions as receptor and the C-terminal guanylyl cyclase domain generates the cytosolic signal (Lucas *et al*, 2000). In addition, there is no evidence of a signal sequence in AtGC1 for direction to the membrane and the designated PPi-binding arginine (Arg) is only 12 amino acids removed from the N-terminal end of the molecule. Other features typical of particulate guanylyl cyclases, the ligand-binding domain, transmembrane domain, kinase homology domain, and dimerization domain are also absent. Contrary to the homodimeric particulate guanylyl cyclases, the soluble guanylyl cyclases form α/β heterodimers (Koesling *et al*, 1991; Zhao *et al*, 1999). Soluble guanylyl cyclases are the principal effector of the gaseous messenger nitric oxide. Nitric oxide dependence of soluble guanylyl cyclases in turn requires a heme-binding region, and this region is N-terminal of the catalytic domain (Koesling *et al*, 1991; Zhao *et al*, 1999). Nitric oxide binds covalently via two cystine-thioether bonds that are provided by a conserved C-*X*-*X*-C-H motif in soluble guanylyl cyclases (Lawson *et al*, 2000). In AtGC1 there is no evidence of a heme-binding motif that is required for nitric oxide binding. The absence of such a heme-binding motif is thus consistent with the lack of nitric oxide inducibility observed in AtGC1. From the sequence analysis, one would thus predict that AtGC1 is not a membrane protein, not similar in domain structure to known receptor or soluble guanylyl cyclases, and its lack of a heme-binding group implies activity that is insensitive to nitric oxide, while catalytic activity *in vitro* implies that it is active either as a monomer or homooligomer. The assay result obtained from transformed induced

Escherichia coli demonstrates a significant increase in cGMP (Fig. 3.3). Since in this assay cGMP was generated in intact bacteria and in the absence of added substrate, it can be concluded that at least the N-terminus of the recombinant was present in the cytosol and thus supports the predictions based on structural features. Since AtGC1 is likely to be located in the cytosol, it was tested whether the activity *in vivo* in *Escherichia coli* cells was affected by the nitric oxide donor sodium nitroprusside, and the observed nitric oxide insensitivity is consistent with the structural features of AtGC1. Nitric oxide independence in soluble guanylyl cyclases does not appear to be the norm; however, an unusual soluble guanylyl cyclase from *Manduca sexta* (MsGC-β3) that does not require dimerization has been reported (Nighorn *et al*, 1999). In this molecule amino acids thought to participate in heme binding are substituted with non-similar amino acids (Nighorn *et al*, 1999). AtGC1 and MsGC-β3 are thus both soluble guanylyl cyclases that do not depend on heterodimer formation for activity. Taken together, our results imply that AtGC1 is active as a monomer, homodimer, or homo-oligomer, with no obligatory dependence on membrane associations, activation by ligands, ATP, or other co-factors with the exception of Mg^{2+}. While homodimeric and oligomeric activity is common in particulate guanylyl cyclases (Lucas *et al*, 2000; Thompson and Garbers, 1995; Garbers and Lowe, 1994), monomeric activity in a soluble guanylyl cyclase has only recently been reported (Koglin *et al*, 2001). Monomeric guanylyl cyclase activity has also been demonstrated in a soluble guanylyl cyclase (DdsGC) from *Dictyostelium discoideum* (Roelofs *et al*, 2001) that has in fact been identified as a homologue of an adenylyl cyclase. It is in keeping with this ancestry that DdsGC has two catalytic domains within a single molecule. The discovery of another guanylyl cyclase from *Manduca sexta* (MsGC-

I) has further complicated attempts to categorize guanylyl cyclases (Simpson *et al*, 1999; Nighorn *et al*, 2001). MsGC-I shows highest sequence identity with receptor guanylyl cyclases throughout its catalytic and dimerization domains but does not contain the ligand-binding, transmembrane, or kinase-like domains typical receptor guanylyl cyclases (Simpson *et al*, 1999). Both AtGC1 and MsGC-I contain C-terminal extensions that are not present in other known guanylyl cyclases. MsGC-I shows no similarity to domains typical of soluble guanylyl cyclases but appears to exist as a soluble homodimer insensitive to nitric oxide stimulation (Simpson *et al*, 1999); however, despite the absence of a transmembrane domain, an as yet undefined membrane association *in vivo* has been suggested (Simpson *et al*, 1999). AtGC1 is adding to the steadily growing number of structurally diverse molecules with guanylyl cyclase activity and it appears that many more and diverse guanylyl cyclase-dependent physiological responses await discovery.

The work described in this chapter has been published as cited below:

Ludidi N.N. & Gehring C. (2003) Identification of a novel protein with guanylyl cyclase activity in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **278**, 6490-6494.

CHAPTER 4

THE ROLE OF AtPNP-A IN CATION HOMEOSTASIS AND MODULATION OF cGMP LEVELS

Abstract

Plant natriuretic peptides (PNPs) belong to a novel class of systemically mobile molecules that are structurally similar to the N-terminal domain of expansins and affect physiological processes such as stomatal movement and cell volume regulation at nanomolar concentrations. Here we demonstrate that AtPNP-A, a recombinant *Arabidopsis thaliana* natriuretic peptide, causes rapid H^+ influx in the elongation zone of *Arabidopsis thaliana* roots while H^+ efflux is observed in the mature zone of the root. AtPNP-A also induces significant K^+ and Na^+ efflux and this effect is seen in the mature root zone only. Furthermore, AtPNP-A induces elevation of cGMP levels in *Arabidopsis thaliana* seedlings by up to 3 times compared to seedlings that are not treated with AtPNP-A. This AtPNP-A-induced cGMP elevation is partially reduced by the guanylyl cyclase inhibitor 6-(phenylamino)-5,8-quinolinedione. Elevation of cGMP levels induced by AtPNP-A is rapid and transient since it occurs within 1 min and lasts for at least 10 min but reverts to levels of the untreated seedlings within 60 min. These observations suggest that AtPNP-A has a complex signaling role in plant homeostasis.

4.1. Introduction

A PNP from the brassicaceus weed *Erucastrum strigosum* has been isolated and its relative amounts, expressed as a percentage of extracted total proteins, are increased when plants are exposed to 300 mM NaCl (Rafudeen *et al*, 2003). AtPNP-A was also significantly up-regulated in *Arabidopsis thaliana* suspension culture cells in response to 150 mM NaCl and even more so in response to iso-osmolar amounts of sorbitol (Rafudeen *et al*, 2003). Plants respond to stress conditions by maintaining homeostasis, detoxifiying harmful elements and recovering growth. Salt stress triggers responses that are due not only to impaired ion homeostasis but also due to disturbance in cellular osmotic balance, both of which lead to other forms of stress conditions, e.g. oxidative stress (Xiong *et al*, 2002; Zhu, 2003). Ion-specific signals in salt stress are presumed to be more important than hyperosmolarity in the regulation of Na^+ transport, but osmotic stress is also believed to play a role in this regulation (Zhu, 2003). It has also been shown that high Na^+ levels disturb K^+ uptake by roots (Hasegawa *et al*, 2000). Changes in AtPNP-A accumulation as a result of exposure of plants to NaCl (Rafudeen *et al*, 2003) suggest that this protein may affect Na^+ and K^+ fluxes in roots. Collectively, these findings thus point to a role for PNP-like molecules in salinity and drought responses. Plant natriuretic peptides have also been shown to induce elevation of cGMP levels in *Zea mays* roots (Pharmawati *el at*, 1998b). The regulation of ion transport is partially dependent on cyclic nucleotides (Hoshi, 1995; Maathuis and Sanders, 2001). Such regulation can occur in plant voltage-gated K^+ channels where binding of cGMP modulates the voltage/current relationship (Maathuis and Sanders, 2001). Plants also contain cyclic nucleotide-gated low affinity cation channels where binding of cAMP and

cGMP to the intracellular portion leads to direct gating (Leng *et al*, 1999). Recently, voltage-independent channels without selectivity for particular monovalent cations have been characterized in *Arabidopsis thaliana* (Maathuis and Sanders, 2001). Voltage-independent channels showed no selectivity among monovalent cations, and their gating was found to be voltage-independent, while micromolar concentrations of cAMP or cGMP at the cytoplasmic side of the plasma membrane caused rapid decreases in channel open probability (Maathuis and Sanders, 2001). It was shown that short-term unidirectional Na^+ influx is reduced in the presence of cyclic nucleotides and that membrane-permeable cyclic nucleotides can improve salinity tolerance presumably by reducing net Na^+ uptake (Maathuis and Sanders, 2001). Cell-permeable cGMP and cAMP analogs have been shown to elicit cADPR-dependent elevation of cytosolic free Ca^{2+} in *Nicotiana tabacum* protoplasts and cause a physiological swelling response in plant protoplasts as a result of enhanced water uptake (Volotovski *et al*, 1998). One would speculate that this response is likely to be a consequence of Ca^{2+}-mediated signaling for promotion of water uptake by the cells. A guanylyl cyclase has recently been identified in *Arabidopsis thaliana* (Ludidi and Gehring, 2003). This study was aimed at characterizing the effects of AtPNP-A on ion transport and cGMP levels in *Arabidopsis thaliana*, with the objective to elucidate the mechanisms underlying the homeostatic role of PNP-like molecules.

4.2. Materials and Methods

Recombinant AtPNP-A without the signal peptide required for secretion into the extracellular space was obtained from M. Morse (Department of Biotechnology,

University of the Western Cape). For ion flux measurements *Arabidopsis thaliana* Columbia (Col-0) seeds were placed, approximately 3 cm from the end of the filter paper, between two layers of wetted filter paper that were cut so that they are approximately 7 cm high when rolled. The filter paper layers were then rolled so that they fit into a 50 ml beaker containing 20 ml tap water. The seeds were germinated at room temperature under a 16 hours/8 hours light/dark cycle and seedlings were allowed to grow until use when they were 10 days old, with the water level in the beaker maintained at approximately 20 ml. Measurement and quantification of net H^+, K^+ and Na^+ ion fluxes in roots of 10 days old *Arabidopsis thaliana* seedlings were measured non-invasively using the microelectrode ion flux estimation (MIFE) technique (Shabala *et al*, 1997; Shabala and Newman, 1999). The microelectrodes were back filled with solutions (0.15 mM NaCl + 0.4 mM KH_2PO_4 adjusted to pH 6.0 using NaOH for proton electrodes; 0.5 M KCl for potassium electrodes; 0.5 M NaCl for sodium electrodes), tips filled with hydrogen 95297, potassium 60031 or sodium 7117 (Fluka Chemical, Buchs, Switzerland) and calibrated in sets of standards before and after use. An *Arabidopsis thaliana* seedling was secured on a small platform by covering only the stem of the seedling with cooled (± 28°C) molten 0.8 % agar and placed on a measuring chamber containing 1 ml of bath solution composed of 0.1 mM KCl, 0.1 mM CaCl, 0.2 mM NaCl, 1 mM 2-[N-morpholino]ethanesulfonic acid (Sigma-Aldrich) at pH 5.5 and left to equilibrate at room temperature for 20 min. The seedling in the measuring chamber was transferred onto a microscope stage and the ion selective electrodes were mounted on a manipulator, above the microscope stage, providing 3-dimensional positioning 50 μm above the surface and parallel to the root axis and moved between 50 to 90 μm at a frequency of 0.1 Hz with a

3-way hydraulic micromanipulator (Narashige WR-88). The seedling was left to equilibrate in the measuring environment for 30-40 min after which 150 ng AtPNP-A were added, with mixing achieved by careful pipetting in the bath solution. A control was set up in the same manner for flux measurement with 150 ng AtPNP-A in the absence of seedlings in the measuring chamber to determine if the protein on its own would significantly alter the electrophysical properties of the bath solution. The recorded potential differences were converted into electrochemical potential differences using the calibrated Nernst slope of the electrodes. For cGMP assays, *Arabidopsis thaliana* Columbia (Col-0) seeds were surface-sterilized with 10 % commercial bleach (v/v) followed by 5 rinses with sterile distilled H_2O and germinated at 23 °C under 16/8 hours light/dark cycle in solid 0.5×MS10 made up of 2.2 g Murashige and Skoog basal medium (Sigma-Aldrich)/l, 10 g sucrose/l, 7 g agar/l, 0.5 g 2-[N-morpholino]ethanesulfonic acid/l, pH 5.5. Seedlings were transferred to transparent 15 ml polypropylene tubes (2 seedlings per tube) containing 5 ml of liquid MS30 (4.4 g Murashige and Skoog basal medium/l, 30 g sucrose/l, 0.5 g 2-[N-morpholino]ethanesulfonic acid/l, pH 5.5) when they were 1 week old and allowed to grow at 23 °C under 16/8 hours light/dark cycle until use when they were 2 weeks old. For treatment with 6-(phenylamino)-5,8-quinolinedione, seedlings were pre-incubated in this guanylyl cyclase inhibitor for 30 min before addition of NaCl or sorbitol. Seedlings were treated with 150 ng of AtPNP-A or a combination of 150 ng of AtPNP-A and 20 μM of the guanylyl cyclase inhibitor 6-(phenylamino)-5,8-quinolinedione (Sigma-Aldrich) or 6-(phenylamino)-5,8-quinolinedione alone over different lenghths of time. Seedlings were snap-frozen in liquid nitrogen immediately after each treatment and

homogenized in ice-cold 6 % trichloroacetic acid (Merck) to make a homogenate of 10 % w/v. The homogenate was centrifuged at 4000 rpm for 5 min at room temperature and the aqueous extract (supernatant) was then recovered and washed 4 times in 5 volumes of diethyl ether (Merck). The diethyl ether layer was discarded after every wash and 500 µl of the final aqueous extract was dried on the SpeedVac (Savant) under vaccum at 25 °C overnight. The dried extract was used to determine cGMP content in the *Arabidopsis thaliana* seedlings using radioimmunoassay according to the manufacture's instructions for the cGMP (^{125}I) Assay System (Amersham Biosciences).

4.3. Results

Addition of 150 ng of AtPNP-A/ml of bath solution to *Arabidopsis thaliana* roots leads to rapid (less than 5 min) and sustained changes in net H^+ fluxes (Fig. 4.1). The direction of the induced H^+ fluxes are however different in different zones of the root. In the elongation zone AtPNP-A causes net H^+ up-take (Fig. 4.1 A) whereas in the mature zone the recombinant triggers increased H^+ efflux (Fig. 4.1 B). No significant K^+ and Na^+ flux changes are observed in the elongation zone (Fig. 4.2 A and Fig. 4.2 B). AtPNP-A alone, without the seedlings, did not result in any oscillation signal in MIFE measurements. AtPNP-A induces rapid (less than 5 min) net K^+ and Na^+ efflux in the mature zone (Fig. 4.2 C and Fig, 4.2 D). A rapid (within 1 min) transient increase in cGMP levels occurs upon treatment of *Arabidopsis thaliana* seedlings with AtPNP-A, which starts to decrease within 1 hour (Fig. 4.3). The cGMP elevation induced by AtPNP-A is inhibited by 6-(phenylamino)-5,8-quinolinedione, indicating that the guanyly cyclase(s) activated by AtPNP-A is sensitive to 6-(phenylamino)-5,8-quinolinedione.

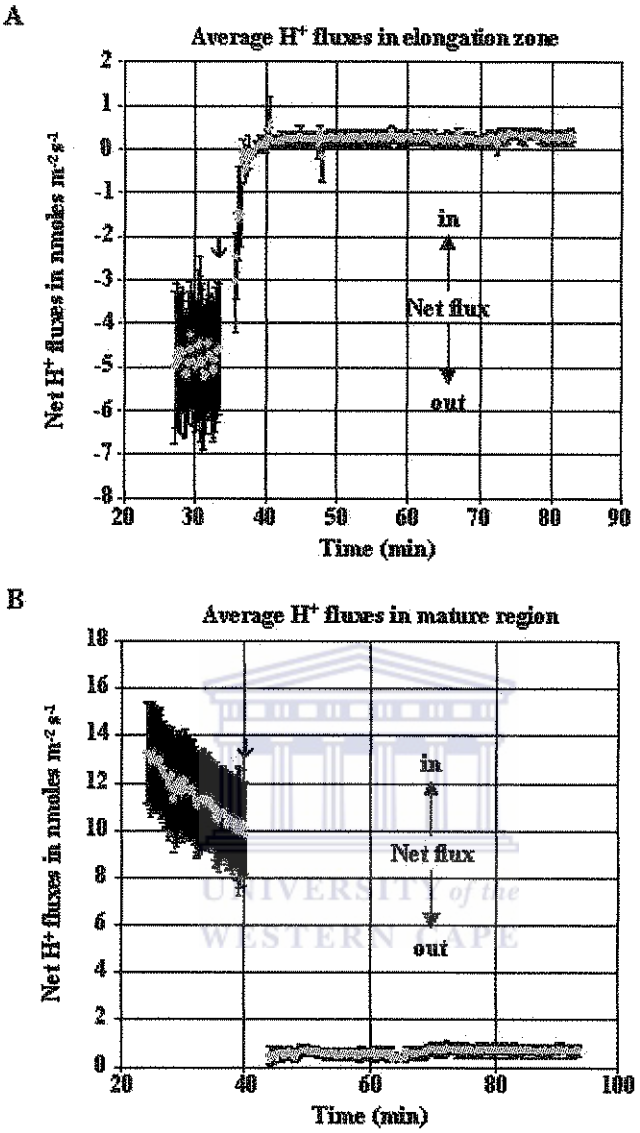


Fig. 4.1. Average net H^+ fluxes induced by AtPNP-A in *Arabidopsis thaliana* roots. Net H^+ flux measurements in *Arabidopsis thaliana* root elongation (**A**) and mature (**B**) zone with 1 ml of bath solution (see Methods and Materials) used as the medium for ion flux measurements. Transient changes in net H^+ flux (nmoles.m^{-2}.s^{-1}) near the tissue before and after the addition (⬇) of AtPNP-A (final concentration of AtPNP-A = 150 ng/ml) were measured at 5 second intervals and error bars are SEs of measurements on roots from 6 seedlings.

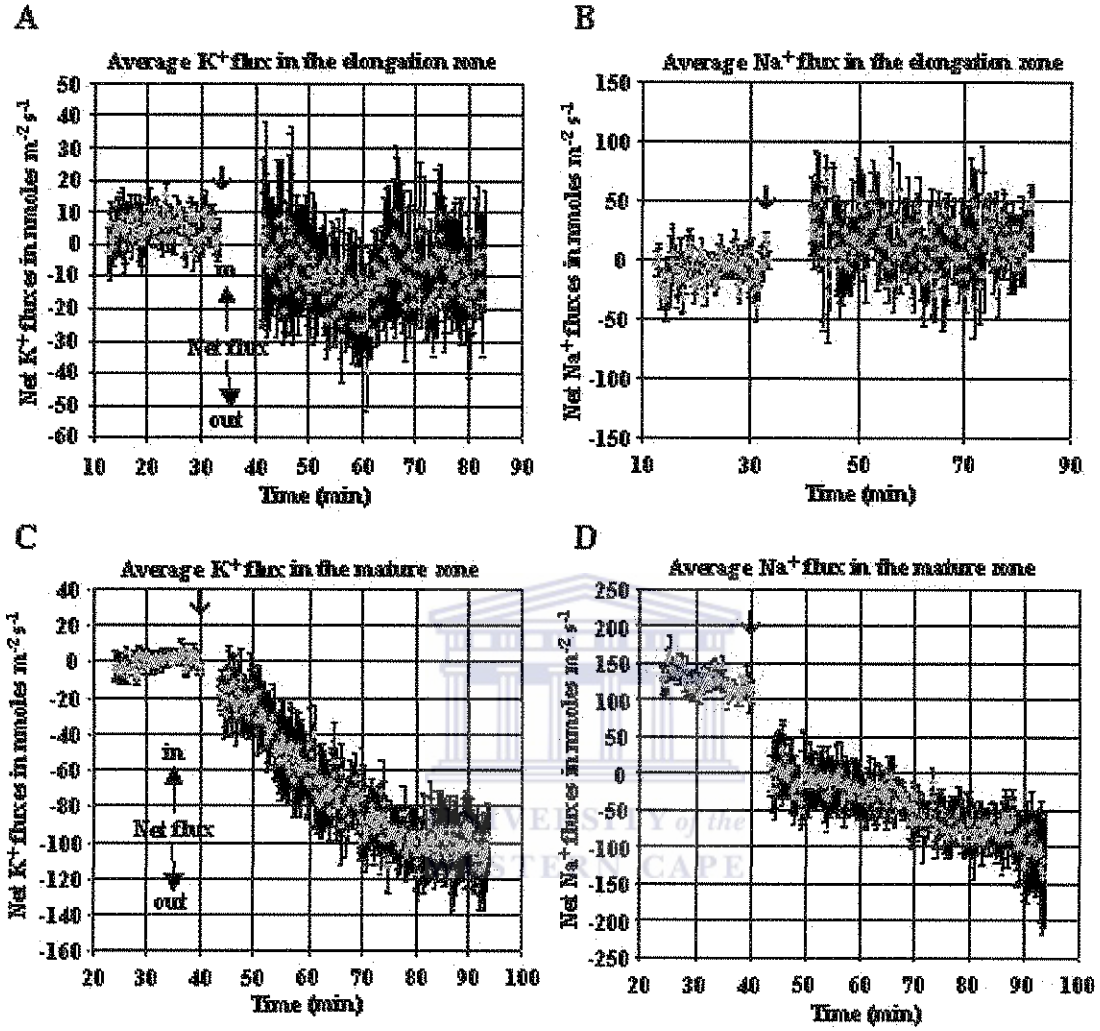


Fig. 4.2. Average net cation fluxes induced by AtPNP-A. Effect of AtPNP-A on K^+ (**A** and **C**) and Na^+ (**B** and **D**) net fluxes in *Arabidopsis thaliana* root elongation (**A** and **B**) and mature (**C** and **D**) zone. Transient changes in net cation flux (nmoles.m^{-2}.s^{-1}) near the tissue before and after the addition (⬇) of AtPNP-A (final concentration = 150 ng/ml) were measured at 5 second intervals and error bars are SEs of measurements on roots from 6 seedlings.

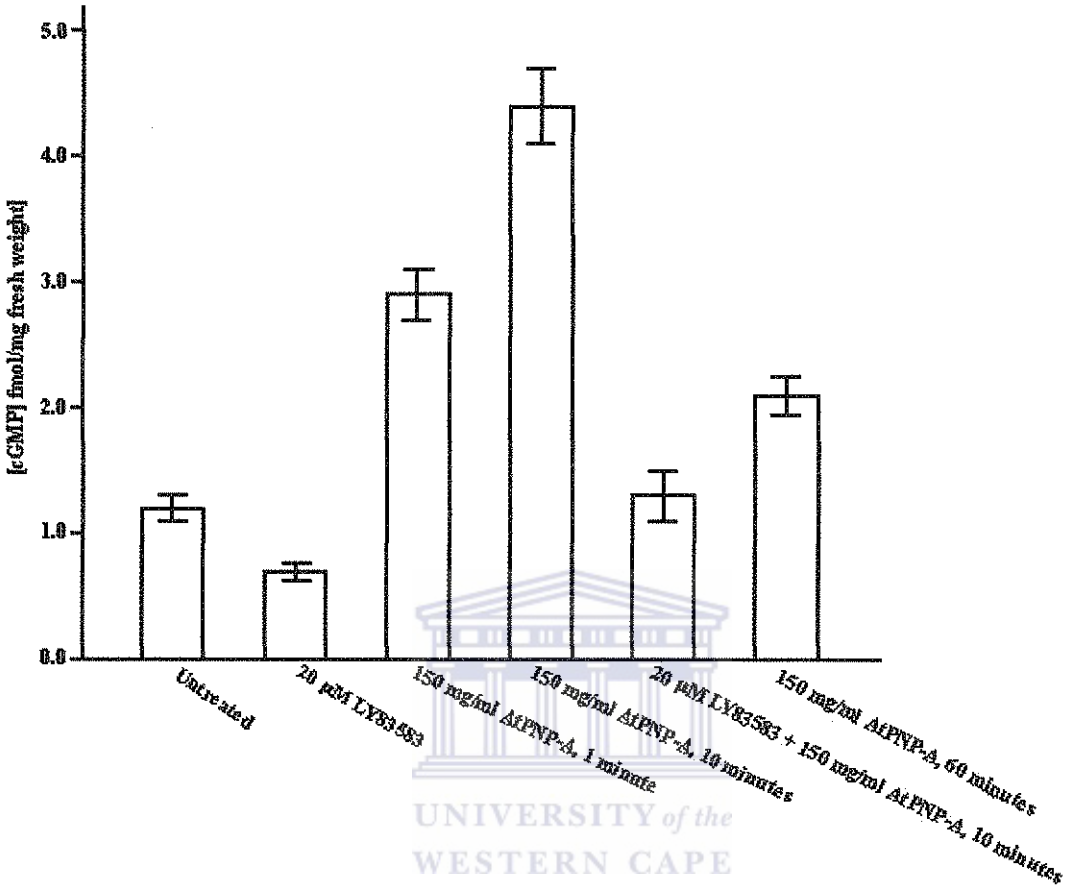


Fig. 4.3. Modulation of cGMP levels by AtPNP-A. Seedlings in 5 ml of liquid MS30 (see Materials and Methods) were treated with either 6-(phenylamino)-5,8-quinolinedione (LY83585) at a final concentration of 20 μM for 10 min or AtPNP-A at a final concentration of 150 ng/ml of MS30 for 1, 10 and 60 min or a combination of 6-(phenylamino)-5,8-quinolinedione and AtPNP-A at a final concentration of 20 μM and 150 ng/ml of MS30 respectively for 10 min. The levels of cGMP (fmol/mg fresh weight) shown are means of duplicate measurements from 3 independent treatments and the bars represent the standard deviation (SD).

4.4. Discussion

Since AtPNP-A-dependant changes in ion fluxes are spatially distinct in different root zones, it is conceivable that this molecule plays a role in plant growth and other processes. Plant natriuretic peptides appear to induce transient elevation of cGMP levels in roots (Pharmawati *et al*, 1998b) and the role of cGMP in regulating ion channel activity has been established (Leng *et al*, 1999; Maathuis and Sanders, 2001). The fact that AtPNP-A induces changes in net H^+, K^+ and Na^+ fluxes, taken together with the observation that it induces elevation of cGMP levels in seedlings, suggests that AtPNP-A may directly or indirectly induce changes in net cation fluxes by triggering elevation of cellular cGMP levels, which would alter the activity of cGMP-gated ion channels and also induce elevation of cytosolic free Ca^{2+}, all of which are factors known to lead to changes in net ion fluxes. Involvement of AtPNP-A in the regulation of water and salt homeostasis implicates AtPNP-A as having a role in plant responses to salinity and drought stress and it has been demonstrated that the expression of AtPNP-A changes in response to high salt and under osmotic stress (Rafudeen *et al*, 2003).

CHAPTER 5

EFFECT OF NaCl AND OSMOTIC STRESS ON cGMP LEVELS IN *Arabidopsis thaliana*

Abstract

The addition of cGMP to the cytoplasm of plant cells has been shown to reduce Na^+ uptake by roots and this process appeares to be mediated by Ca^{2+} (Maathuis and Sanders, 2001). Furthermore, this inhibition of Na^+ uptake results in enhanced survival of cGMP-treated plants under conditions of high Na^+. Hyperosmotic stress also induces nitric oxide biosynthesis, followed by elevation of Ca^{2+} levels in plant and nitric oxide is known to induce cGMP elevation in plants. These observations imply that cGMP affects Na^+ uptake but do not show directly what the effects of Na^+-generated salinity and osmotic stress are on cGMP levels in plants. Here we demonstrate that both NaCl and sorbitol induce elevation of cGMP levels in a concentration- and time-dependent manner in *Arabidopsis thaliana* seedlings and that Ca^{2+} is not required for Na^+/hyperosmolarity-induced cGMP accumulation.

5.1. Introduction

Co-ordinated regulation of intracellular ion concentrations is essential for living cells. Regulation of ion flow is necessary for cells to keep the concentrations of toxic ions low and to accumulate essential ions. A high concentration of K^+ and a low concentration on Na^+ are maintained by plant cells in conditions of salt stress (Hasegawa *et al*, 2000).

Plants use two mechanisms to maintain high K^+ concentrations and low Na^+ concentrations in the cytosol. These mechanisms are primary active transport driven by H^+-ATPases, and secondary transport mediated by ion channels and ion co-transporters (Hasegawa *et al*, 2000). Maintainance of membrane potential, an appropriate osmoticum for cell volume regulation and the activity of many enzymes are affected by intracellular K^+ and Na^+ homeostasis in the cytosol (Hasegawa *et al*, 2000). Under salt stress, the maintenance of K^+ and Na^+ homeostasis becomes even more crucial because Na^+ stress prevents K^+ uptake by roots, disrupting ion homeostasis and affecting enzyme funtion (Hasegawa *et al*, 2000). Plant cells have to maintain low cytosolic Na^+ concentrations in high salt environments to avoid Na^+ toxicity effects. Na^+/H^+ antiporters play a role in regulating intracellular Na^+ levels (Nass *et al*, 1997; Apse *et al*, 1999; Shi *et al*, 2000; Blumwald, 2000, Apse *et al*, 2003). Plant H^+-ATPases generate H^+ electrochemical gradients that drive the transport of ions and nutrients across the cell membrane (Barkla *et al*, 1995; Blumwald, 2000) and ion ratios in plants are altered by the influx of Na^+ through K^+ pathways (Xiong and Zhu, 2002; Blumwald, 2000; Blumwald *et al*, 2000). Na^+ and K^+ have similar hydrated ionic radii, which could account for the limited ability of plant cells to discriminate between these two cations and this is thus a cause for Na^+ toxicity in plants. Protein synthesis requires physiological K^+ concentrations in the range of 100–150 mM (Wyn-Jones and Pollard, 1983). Plants use low- and high-affinity transporters to take up K^+ from the extracellular medium (Sentenac *et al*, 1992; Spalding *et al*, 1999; Wegner and Raschke, 1994). However, protein synthesis is inhibited if Na^+ concentrations exceed 100 mM which leads to competition between Na^+ and K^+ for K^+-binding sites in cation channels (Wyn-Jones and Pollard, 1983). Plants can however

respond to high Na^+ levels by reducing cytosolic Na^+ accummulation and increasing cytosolic K^+/Na^+ ratio (Blumwald, 2000; Xiong *et al*, 2002; Zhu, 2001; Zhu, 2003). Plants maintain a high K^+/Na^+ ratio in the cytosol by increasing efflux of Na^+ from the cell and/or by intracellular compartmentalization of Na^+, mainly in the vacuole (Blumwald, 2000; Zhu, 2003). These two processes are required for cytosolic Na^+ detoxification and cellular osmotic adjustment needed to tolerate salt stress. In theory, Na^+ can be sensed either before or after entering the cell, or both (Blumwald, 2000; Zhu, 2003). Extracellular Na^+ may be sensed by a membrane receptor, whereas intracellular Na^+ may be sensed either by membrane proteins or by Na^+-sensitive enzymes in the cytoplasm (Blumwald, 2000; Zhu, 2003). A cell membrane Na^+/H^+ antiporter with a possible role in Na^+ sensing has been identified (Shi *et al*, 2000). This Na^+/H^+, named SOS1 (Salt Overly Sensitive1), has 10–12 transmembrane domains and a cytoplasmic domain of approximately 700 amino acids (Shi *et al*, 2000). SOS1 Na^+/H^+ antiporter activity is essential for Na^+ efflux out of *Arabidopsis thaliana* cells (Qiu *et al*, 2002; Quintero *et al*, 2002). Plants respond to salt in several ways, regulated both at a genetic/molecular level and physiological level. Some responses involve changes in ion movement both into the cell and across intracellular membranes to re-establish ion and osmotic homeostasis (Hasegawa *et al*, 2000; Zhu, 2000; Zhu, 2003). Furthermore, changes in gene expression and metabolism can be triggered to limit or repair damage to cells (Hasegawa *et al*, 2000; Zhu, 2003). Plants may also utilize changes in the rate of cell division to maintain a growth rate suitable for the environmental conditions (Koiwa *et al*, 2003). The reduction in growth rate in response to salt and osmotic stress is often utilised as a measure of tolerance and ultimately, is the essential parameter in agriculture.

One established salt stress signalling pathway is the SOS pathway (Zhu, 2000; Zhu, 2003). A calcium binding protein, SOS3, is thought to interact with and activate a serine/threonine protein kinase, SOS2, in response to salt stress (Quintero *et al*, 2002; Zhu, 2000; Zhu, 2003). Together SOS3 and SOS2 activate the ion transport activity and expression of SOS1, a plasma membrane Na^+/H^+ antiporter (Quintero *et al*, 2002; Zhu, 2000; Zhu, 2003). This pathway is mediated by the binding of calcium to SOS3 and consistent with cytosolic free Ca^{2+} concentration increases after exposure to salt (Knight *et al*, 1997; Quintero *et al*, 2002; Zhu, 2003). The increase in cytosolic free Ca^{2+} in response to salt involves both influx of calcium across the plasma membrane and release of calcium from the vacuole (Knight *et al*, 1997). The use of inhibitors suggests the latter is via inositol 1,4,5-trisphosphate -gated channels and inositol 1,4,5-trisphosphate levels increase rapidly after salt stress (DeWald *et al*, 2001). However, a similar calcium increase occurs in response to osmotic stress but the SOS pathway is not activated (Scrase-Field and Knight, 2003). In this SOS-independent pathway, it is presumed that calcium is acting as a switch to prime the SOS pathway but is not responsible for its specific activation in response to salt stress (Scrase-Field and Knight, 2003). Water is required for biochemical activities by all known life forms. For plant cells, water also functions to generate the turgor pressure that is a driving force of cell expansion (Bray, 1997). Plant growth can only occur at a certain range of water status, which can be measured by the free energy state of water molecules, that is the water potential (ψ_w). In a given cell, ψ_w mainly consists of pressure and osmotic potential. While maintaining a positive turgor pressure, in most cases plant cells adjust their osmotic potential to meet the requirement of the whole plant in balancing its water distribution (Bray, 1997; Xiong

and Zhu, 2002). Significant changes in water potential in the environment can lead to osmotic stress in plants, which disrupts normal cellular function, or even causes plant death (Bray, 1997). High salt concentration in the soil and water deficits caused by drought conditions are the major causes of osmotic stress in plants (Bray, 1997; Xiong *et al*, 2002; Xiong and Zhu, 2002).

There is evidence that cGMP plays a role in salt stress through affecting response mechanisms. The regulation of ion transport is partially dependent on cyclic nucleotides (Hoshi, 1995; Maathuis and Sanders, 2001). Such regulation can occur in plant voltage-gated K^+ channels where binding of cGMP modulates the voltage/current relationship (Maathuis and Sanders, 2001). Plants also contain cyclic nucleotide-gated low affinity cation channels where binding of cAMP and cGMP to the intracellular portion leads to direct gating (Leng *et al*, 1999). Recently, voltage-independent channels without selectivity for particular monovalent cations have been characterized in *Arabidopsis thaliana*. Voltage-independent channels showed no selectivity among monovalent cations, and their gating was found to be voltage-independent, while micromolar concentrations of cAMP or cGMP at the cytoplasmic side of the plasma membrane caused a rapid decrease in channel open probability (Maathuis and Sanders, 2001). It was shown that short-term unidirectional Na^+ influx is reduced in the presence of cyclic nucleotides and that membrane-permeable cyclic nucleotide can improve salinity tolerance presumably by reducing net Na^+ uptake (Maathuis and Sanders, 2001). Cell-permeable cGMP and cAMP analogs were shown to elicit elevation of cytosolic Ca^{2+} in *Nicotiana tabacum* protoplasts and cause a physiological swelling response in plant

protoplasts (Volotovski *et al*, 1998). It has been shown that nitric oxide enhances water retention in response to drought and hyperosmotic stress by inducing and amplifying the degree of stomatal closure, thus limiting transpiration via a signaling pathway that is mediated by Ca^{2+} (Mata and Lamattina, 2001). It has also been shown that nitric oxide stimulates cGMP biosynthesis by guanylyl cyclases in plant cells (Pfeiffer *et al*, 1994) and that cGMP stimulates cyclic adenosine diphosphate ribose biosynthesis, leading to increased cytosolic free Ca^{2+} levels (Volotovski *et al*, 1998). The fact that the drought and osmotic stress tolerance seen in plants, achieved through restriction of stomatal closure, is mediated by nitric oxide through a Ca^{2+}-dependent pathway, indicates a strong and almost certain possibility that nitric oxide enhances drought and osmotic stress tolerance via stimulation of guanylyl cyclase activity to synthesize cGMP. This chapter investigates the involvement of cGMP in salt and osmotic stress responses in plants and whether there is crosstalk between cGMP and calcium signalling pathways.

5.2. Materials and Methods

Arabidopsis thaliana Columbia (Col-0) seeds were surface-sterilized with 10 % commercial bleach (v/v) followed by 5 rinses with sterile distilled H_2O and germinated at 23°C under an 16/8 hours light/dark cycle in 12-well Costor® cell culture clusters (Corning Incoporated) (4 seedlings per well) containing liquid MS30 [4.4 g Murashige and Skoog basal medium (Sigma-Aldrich)/l, 30 g sucrose/l, 0.5 g 2-[N-morpholino]ethanesulfonic acid/l, pH 5.5] and allowed to grow until use when they were 2 weeks old. Seedlings were treated for 15 min with either 50 mM NaCl, 150 mM NaCl, 100 mM sorbitol or 300 mM sorbitol. For the treatments with 6-(phenylamino)-5,8-

quinolinedione and neomycin, seedlings were pre-incubated in these inhibitors for 30 min before addition of NaCl or sorbitol. Seedlings were also treated with a combination of either 50 mM NaCl, 150 mM NaCl, 100 mM sorbitol or 300 mM sorbitol and 20 μM of the guanylyl cyclase inhibitor 6-(phenylamino)-5,8-quinolinedione (Sigma-Aldrich) or 150 μM neomycin (an inhibitor of polyphosphoinositide-specific phospholipase C, the enzyme required for inositol 1,4,5-trisphosphate biosynthesis that triggers cytosolic free Ca^{2+} release) for 15 min. Seedlings were snap-frozen in liquid nitrogen immediately after each treatment. Frozen tissue was ground to a fine powder with a pre-chilled motar and homogenized in ice-cold 6% trichloroacetic acid (Merck) to make a homogenate of 10 % w/v. The homogenate was centrifuged at 3000*g* for 5 min at room temperature and the aqueous extract (supernatant) was recovered and washed 4 times in 5 volumes of diethyl ether (Merck). The top (diethyl ether) phase was discarded after every wash and 500 μl of the final aqueous extract were dried on the SpeedVac (Savant) under vaccum at 25°C overnight. The extract was used to determine cGMP content according to the manufacture's instructions for the cGMP (^{125}I) Assay System (Amersham Biosciences).

5.3. Results

NaCl at two different concentrations leads to elevation of the level of cGMP in *Arabidopsis thaliana* seedlings (Fig. 5.1). Low salt (50 mM NaCl) induces a rapid (within 7 seconds) increase in cGMP level by 1.7 times compared to the levels of cGMP in control (H_2O-treated) seedlings over the same period (Fig. 5.1). The level of cGMP in seedlings treated with 50 mM NaCl for 15 min increases by more than 3 times compared to seedlings treated with H_2O for 15 min. A three-fold increase in salt level (150 mM

NaCl) induces an even higher increase in cGMP level in the seedlings (Fig. 5.1). Treatment of the seedlings with 150 mM NaCl for 7 seconds increases cGMP level by more than 2 times compared to H_2O treatment (Fig. 5.1). This rapid 150 mM NaCl-induced increase in cGMP level is almost 1.5 times more than the increase induced by 50 mM NaCl (Fig. 5.1). The pattern of cGMP elevation induced by both low (50 mM NaCl) and high (150 mM NaCl) salinity is similar over a period of 15 min, although high salinity induces a higher increase in cGMP level than low salinity. Sorbitol was also found to lead to an increase in cGMP levels in *Arabidopsis thaliana* seedlings (Fig. 5.1). Osmotic stress (100 mM sorbitol) induces a rapid (within 7 seconds) increase in cGMP level 3 times more than the levels of cGMP in control (H_2O-treated) seedlings over the same period (Fig. 5.1). This sorbitol-induced increase is almost twice the increase induced by an equiosmolar level of NaCl (100 mM sorbitol versus 50 mM NaCl). The level of cGMP in seedlings treated with 100 mM sorbitol for 15 min increases 4 times compared to level of cGMP in seedlings treated with H_2O for 15 min. A three-fold increase in sorbitol concentration (300 mM sorbitol) induces an even higher increase in cGMP level in the seedlings (Fig. 5.1). Treatment of the seedlings with 300 mM sorbitol for 7 seconds increases cGMP level approximately 3.5 times compared to H_2O mock treatment (Fig. 5.1) and 300 mM sorbitol induces an increase in cGMP level approximately 1.2 times more than the increase induced by 100 mM sorbitol (Fig. 5.1). The pattern of cGMP elevation induced by both low (100 mM sorbitol) and high (300 mM sorbitol) osmotic stress is similar over a period of 15 min, although high osmotic stress induces a higher increase in cGMP level than low osmotic stress.

The effect of the guanylyl cyclase inhibitor 6-(phenylamino)-5,8-quinolinedione (LY83583) and the inhibitor of cytosolic free Ca^{2+} release neomycin on salinity- and osmotic stress-induced elevation of cGMP levels in *Arabidopsis thaliana* seedlings were also invesigated. Three times higher cGMP levels are induced by treating seedlings with 50 mM NaCl than treating the seedlings with H_2O (Fig. 5.2). This NaCl-induced elevation of cGMP is partially inhibited by 6-(phenylamino)-5,8-quinolinedione because the guanylyl cyclase inhibitor results in a significant decrease in cGMP elevation in 50 mM NaCl-treated seedlings that were pre-incubated with 6-(phenylamino)-5,8-quinolinedione (Fig. 5.2).

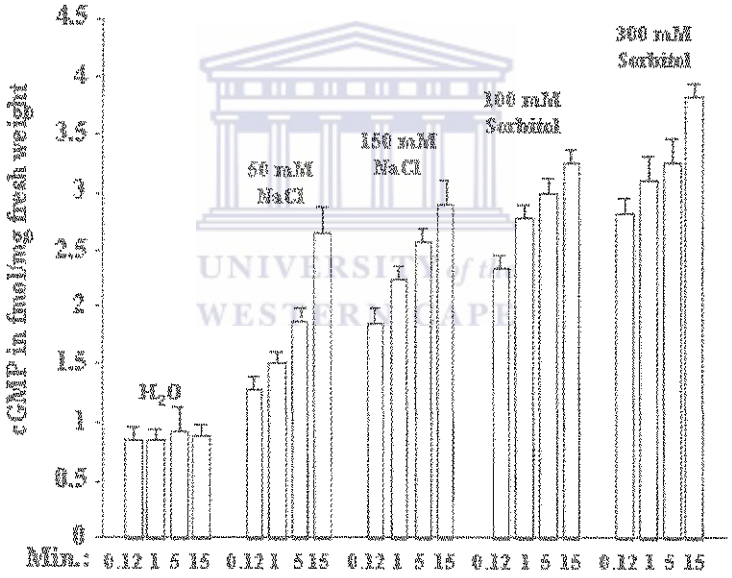


Fig. 5.1. Effect of salinity and osmotic stress on cGMP levels in *Arabidopsis thaliana*. Seedlings were treated with either water (H_2O), 50 mM NaCl, 150 mM NaCl, 100 mM sorbitol or 300 mM sorbitol for a period of 0.12 min (7 seconds), 1 min, 5 min, and 15 min in all treatments, then snap-frozen in liquid nitrogen before extraction of cGMP. Data represent mean of duplicate measurements from 2 independent experiments for each treatment. Bars signify standard errors.

The induction of elevation of cGMP by 50 mM NaCl is not affected by neomycin (Fig. 5.2). Treatment of seedlings with 150 mM NaCl results in more than 3.6 times higher cGMP levels than treating the seedlings with H_2O and this increase is 1.3 times higher than the increase induced by 50 mM NaCl (Fig. 5.2). This NaCl-induced elevation of cGMP is partially inhibited by 6-(phenylamino)-5,8-quinolinedione (Fig. 5.2). The induction of elevation of cGMP by 150 mM NaCl is not significantly affected by neomycin since the levels of cGMP in the treatment with or without neomycin are similar (Fig. 5.2). Levels of cGMP 4 times higher than control levels are induced by treating seedlings with 100 mM sorbitol than treating the seedlings with H_2O (Fig. 5.2). Sorbitol is thus more potent in inducing cGMP elevation than NaCl at equi-osmolar concentrations. Partial inhibition of the sorbitol-induced elevation of cGMP by 6-(phenylamino)-5,8-quinolinedione also accurs because treatment with the guanylyl cyclase inhibitor results in a significant decrease in cGMP elevation in 100 mM sorbitol-treated seedlings in the presence of 6-(phenylamino)-5,8-quinolinedione (Fig. 5.2). The induction of elevation in cGMP level by 100 mM NaCl is not inhibited by neomycin (Fig. 5.2). A higher concentration of sorbitol (300 mM) results in 5 times more cGMP levels than that seen in H_2O-treated seedlings, an effect even more pronounced than that of 100 mM sorbitol (Fig. 5.2). 6-(phenylamino)-5,8-quinolinedione partially inhibits induction of cGMP elevation by 300 mM sorbitol in a manner similar to that seen for 100 mM sorbitol (Fig. 5.2). The effect of 300 mM sorbitol on elevation of cGMP is not significantly affected by neomycin (Fig. 5.2).

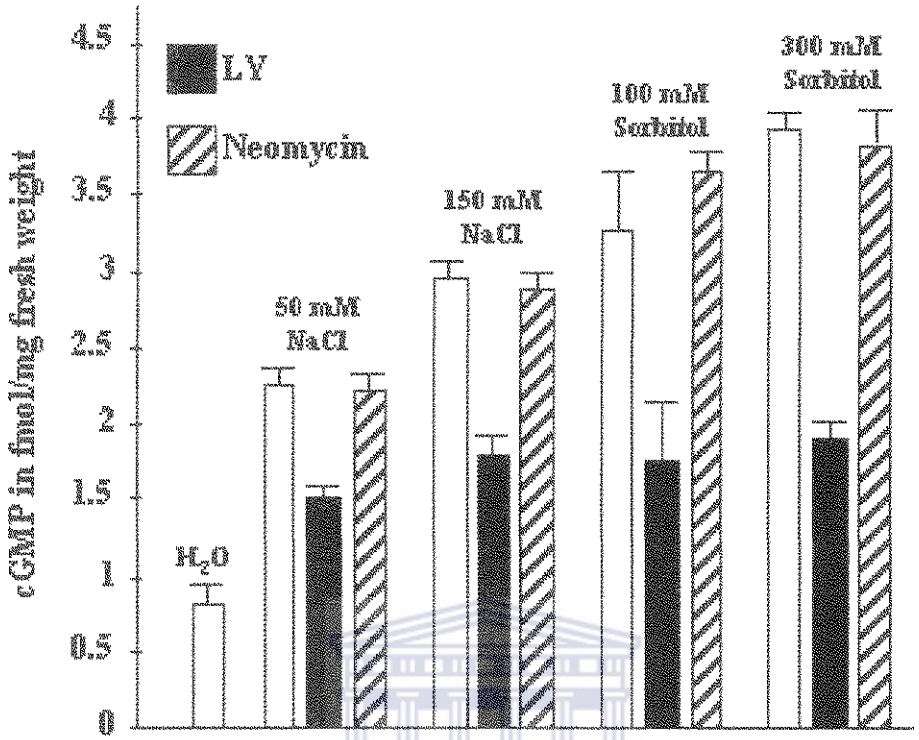


Fig. 5.2. Effect of inhibition of cytosolic free Ca^{2+} release and inhibition of guanylyl cylase activity on salinity- and osmotic stress-induced cGMP elevation in *Arabidopsis thaliana* seedlings. Treatment of seedlings with NaCl or sorbitol were carried out for 15 min, followed by immediate freezing of the seedlings in liquid nitrogen before cGMP extraction. LY denotes pretreament with 6-(phenylamino)-5,8-quinolinedione for 30 min before addition of NaCl or sorbitol. Data represent mean of duplicate measurements from 2 independent experiments for each treatment. Bars signify standard errors.

5.4. Discussion

Plant cells have to maintain low cytosolic sodium concentrations in order to grow in high salt concentrations because of the toxicity of high cytosolic Na^+ to plant cells, caused by the osmotic and ionic effect of salinity. The results reported here demonstrate that NaCl

induces rapid elevation of cGMP levels in plants in a manner directly propotional to NaCl concentration. The finding that salt-induced cGMP accumulation is inhibited by a guanylyl cyclase inhibitor indicates that NaCl induces cGMP biosynthesis by triggering a signaling pathway that leads to activation of guanylyl cyclases. It has previously been shown that cGMP functions to limit NaCl uptake by roots (Maathuis and Sanders, 2001) and the findings on the effect of NaCl on cGMP levels demonstrate the importance of cGMP, and thus of guanylyl cyclases, in salinity tolerance. The finding that osmotic stress caused by treatment of seedlings with sorbitol also leads to elevation of cGMP level in the seedlings and that 6-(phenylamino)-5,8-quinolinedione inhibits this cGMP elevation shows that osmotic stress also triggers cGMP biosynthesis via activation of guanylyl cyclases. It has previously been demonstrated that drought mimicking conditions that lead to osmotic stress trigger nitric oxide biosynthesis (Mata and Lamattina, 2001). It has been shown that nitric oxide stimulates cGMP biosynthesis by guanylyl cyclases in plant cells (Pfeiffer *et al*, 1994). Furthermore, cGMP stimulates cyclic adenosine diphosphate ribose biosynthesis, leading to an increase in cytosolic free Ca^{2+} levels (Volotovski *et al*, 1998). The results reported here further demonstrate that both the salt- and hyperosmolarity-induced cGMP elevation do not require increases in Ca^{2+} since an inhibitor of cytosolic free Ca^{2+} release does not affect this cGMP elevation. Hyperosmotic stress appears to be a stronger trigger for cGMP biosynthesis than salinity stress since sorbitol leads to higher cGMP levels than NaCl. This may be puzzling since NaCl induces both ionic stress effects and hyperosmotic effects. It will thus be interesting to study these two components of abiotic stress further in relation to cGMP-mediated signaling in plants. Recent findings demonstrate that both NaCl and sorbitol at the

concentrations used in this study induce Ca^{2+} elevation and that this Ca^{2+} increase requires cGMP since it is inhibited by a guanylyly cyclase and the 6-(phenylamino)-5,8-quinolinedione effect is reversed by a cell-permeable analog of cGMP (L. Donaldson and K. Deny, Department of Molecular and Cell Biology, University of Cape Town, personal communication). The fact that salt- and hyperosmolarity-induced cGMP elevation does not require Ca^{2+} implies that Ca^{2+} elevation as a result of salt and osmotic stress is downstream of cGMP synthesis.

GENERAL CONCLUSION AND OUTLOOK

It is evident that many cell signaling processes vital for plant survival under various conditions are mediated by cGMP, including gene expression, responses to and establishment of resistance to diseases, normal responses to light in order to facilitate proper plant development and nutrition, responses to and establishment of tolerance to salinity and drought and responses to other environmental conditions. All of these factors have considerable implications for agriculture with respect to plants and plant-based products. It is thus important to investigate the effect of stress stimuli on the expression of genes whose products regulate the activity of guanylyl cyclases, which are the enzymes catalyzing cGMP biosynthesis. Of equal importance is the study of these guanylyl cyclase-activating molecules. Furthermore, it is important to identify and fully characterize guanylyl cyclases in plants if the role of cGMP in plant cell signaling and function is to be well understood. A novel *Arabidopsis thaliana* gene (*AtPNP-A*) and its product (the protein we refer to as AtPNP-A), which up-regulates guanylyl cyclase activity and thus leads to cGMP elevation, has been identified and partially characterized

in this study. AtPNP-A is closely and evolutionarily related to the cell wall-loosening expansins that are responsible for cell expansion in elongation growth (Chapter 2 of this thesis) but has not been shown to have expansin activity. Furthermore, AtPNP-A induces elevation of cGMP levels in plants (Chapter 4 of this thesis), modulates ion fluxes across roots (Chapter 4 of this thesis), regulates stomatal aperture by affecting guard cell movements and regulates cell volume by promoting water uptake into plant cells (Morse *et al*, 2004). These functional features of recombinant AtPNP-A are similar to the features of immunoaffinity purified plant natriuretic peptides (Gehring and Irving, 2003). It is interesting to note that a plant pathogen (*Xanthomonas axonopodis*) has also been found to have a gene encoding a protein (Xac 2654) with significant similarity to AtPNP-A (Chapter 2 of this thesis). This finding has implications for plant-pathogen interactions, as such gene transfer may be useful to the pathogen's success in colonizing its host. The role of cGMP in salinity and osmotic stress has been described in this thesis (Chapter 5). The fact that AtPNP-A regulates ion fluxes and induces elevation of cGMP levels in plants, coupled with the finding that expression of this protein is up-regulated in response to salinity and osmotic stress (Rafudeen *et al*, 2003), indicates that AtPNP-A plays a role in responses to salinity and drought (osmotic stress). Identification of AtPNP-A and establishment of its role in inducing an increase in cGMP levels implies that it activates guanylyl cyclase(s) in plants. However, identification of a plant guanylyl cyclase has previously not been achieved despite the well-established diverse roles of cGMP in plants. This study identifies a plant protein, named AtGC1, which has guanylyl cyclase activity (Chapter 3 of this thesis). AtGC1 contains a domain similar to parts of the catalytic domain of known guanylyl cyclases and its activity is regulated by Mg^{2+} in a

manner similar to that of known guanylyl cyclases (Chapter 3 of this thesis), although AtGC1 activity does not appear to be regulated by Mn^{2+} (Chapter 3 of this thesis) whereas other guanylyl cyclases are (Lucas *et al*, 2000). AtGC1 is, however, significantly different in amino acid sequence and domain organization from the known guanylyl cyclases (Chapter 3 of this thesis). The identification of both a guanylyl cyclase-stimulating protein (AtPNP-A) and a guanylyl cylase (AtGC1) opens another important avenue for the study of cGMP-mediated signaling processes and is a step towards understanding plant responses to biotic and abiotic stimuli that are mediated by cGMP levels in plants. Such understanding has potential for use in establishment of plants with enhanced tolerance to biotic and abiotic stress. Our current research is aimed at studying the spatial and temporal expression pattern of both AtPNP-A and AtGC1 using the β-glucoronidase reporter gene technology, studying the cellular localization of these proteins using Green Fluorescence Protein (GFP) and performing full characterization of the *in planta* biological functions of these proteins by using CaMV 35S promoter overexpression and RNAi gene silencing technologies.

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