

**Molecular Characterisation of the Brassinosteroid,
Phytosulfokine and cGMP-dependent Responses in
Arabidopsis thaliana.**

by

Lusisizwe Kwezi



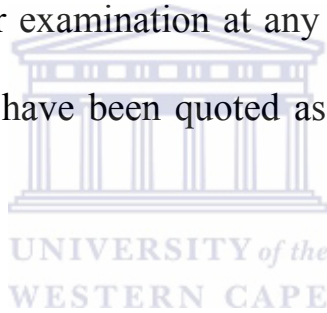
**Submitted in fulfilment of the requirements for the degree of Doctor of
Philosophy (PhD) in Biotechnology in the Department of Biotechnology,
University of the Western Cape, South Africa**

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November 2010

Declaration

I, Lulisizwe Kwezi, declare that the thesis entitled "Molecular characterization of the Brassinosteroid, Phytosulfokine receptors and cGMP-dependent Responses in *Arabidopsis thaliana*" is my work and has not been submitted for any degree or examination at any other university and that all sources of my information have been quoted as indicated in the text and/or list of references.



Name: Lulisizwe Kwezi

Date: November 2010

Signature:

Date:

Dedicated to Amampandla, ooMbona, ooKhipha, ooTshayingwe.

Ndiyabulela !!



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WESTERN CAPE

Table of Contents.

Title.....	page
Acknowledgements.....	1
Abbreviations.....	2
Lists of Figures and Tables.....	6
Summary	9
Chapter 1: Literature review.	11
1.1 Introduction.	11
1.2 Signalling in plants.	14
1.2.1 Classical hormones.	14
1.2.2 The non-classical hormones.....	15
1.3 Peptide hormones signalling.....	17
1.4 Perception of hormones signals in plants.	19
1.5 The family of guanylyl cyclases.	25
Chapter 2: The <i>Arabidopsis thaliana</i> Brassinosteroid Receptor (AtBRI1) contains a Domain that functions as a Guanylyl Cyclase <i>in vitro</i>.	27



Abstract.....	27
2.1 Introduction	28
2.2 Materials and Methods.	29
2.2.1 Identification of the GC catalytic domain.....	29
2.2.2 Extension of the GC search motif to identify AtBRI1.....	29
2.2.3 Preparation of <i>Arabidopsis thaliana</i> plant material.	30
2.2.4 Genomic DNA extraction.....	30
2.2.5 Isolation of the AtBRI1 GC catalytic center.....	32
2.2.5.1 Primer design.	32
2.2.5.2 Polymerase Chain Reaction (PCR) amplification of AtBRI1 from genomic DNA.	32
2.2.6 Preparation of pCR-T7/NT-TOPO-AtBRI1 construct.....	33
2.2.6.1 Restriction digest of AtBRI1.	33
2.2.6.2 Restriction digest of pCR [®] T7/NT-TOPO [®] vector.....	34
2.2.7 Construction of a pCRT7/NT-TOPO-AtBRI1 expression vector.....	34
2.2.7.1 Ligation of AtBRI1 into pCR [®] T7/NT-TOPO [®] vector.....	34
2.2.8 Preparation of an expression host.	35

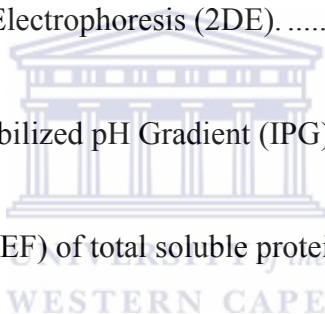


2.2.8.1 Transforming pCRT7/NT-TOPO-AtBRI1 construct into <i>E.coli</i> BL21(DE3)pLysS.	35
2.2.8.2 Colony PCR.	35
2.2.9 Heterologous expression of the AtBRI1 recombinant protein.	36
2.2.9.1 Culture enrichment and induction.	36
2.2.10 Purification of recombinant pCR T7/NT-TOPO-AtBRI1.	36
2.2.11 Cyclic nucleotide activity assays on the recombinant AtBRI1 catalytic.	37
2.2.11.1 Enzyme immunoassay.	37
2.2.11.2 Mass spectrometry.	38
2.3 Results.	39
2.4 Discussion.	47
Chapter 3: The <i>Arabidopsis thaliana</i> Phytosulfokine (AtPSKR1) Receptor has dual Enzymatic function <i>in vitro</i>.	50
Abstact.	50
3.1 Introduction.	51
3.2 Methods and Materials.	55
3.2.1 Preparation of the expression host.	55



3.2.2 Synthesis of the recombinant AtPSKR1-KD2 and cytoplasmic kinase domains...	55
3.2.2.1 Expression of kinase domains under native condition.....	55
3.2.2.2 Protein extraction and preparation of lysate.	56
3.2.2.3 Batch purification of Kinase domain under native conditions.....	57
3.2.2.4 Cyclic nucleotide assays.	58
3.2.2.5 Determination of the kinase activity of AtPSKR1-KD2 and the effects of cGMP on Substrate directed phosphorylation.....	58
3.2.2.6 Determination of the effects of cyclic nucleotides on the autophosphorylation state.	59
3.2.2.7 Western Blot analysis	60
3.3 Results.	62
3.4 Discussion.....	69
Chapter 4: The <i>Arabidopsis thaliana</i> Brassinosteroid and Phytosulfokine Receptors have Guanylyl Cyclase activity <i>in vivo</i>.	74
Abstract.....	74
4.1 Introduction.	75
4.2 Methods and Materials.	77
4.2.1 Preparation of plant material for protoplast isolation.	77

4.2.2 Preparation of plant material for phosphoproteomics.....	77
4.2.3 Preparation and isolation of mesophyll protoplasts from <i>Arabidopsis thaliana</i> leaves.....	78
4.2.3.1 Treatment of protoplast and measurement of intracellular cGMP.....	78
4.2.4 Protein sample preparation for phosphoproteome analysis.....	80
4.2.4.1 Protein extraction.....	80
4.2.4.2 Sample Preparation for Two-Dimensional Gel Electrophoresis.....	81
4.2.5 Two Dimensional Gel Electrophoresis (2DE).....	81
4.2.5.1 Rehydration of Immobilized pH Gradient (IPG) strips.....	81
4.2.5.1 Isoelectric focusing (IEF) of total soluble proteins.....	82
4.2.6 Phosphoproteome Analysis with Pro-Q Diamond stain.....	83
4.2.7 Mass spectrometric analysis.....	83
4.2.7.1 In-gel tryptic digestion.....	83
4.2.7.2 Protein identification by MALDI-TOF mass spectrometry.....	84
4.2.7.3 Confirmation of the expression of AtBIKI in Arabidopsis leaves with RT-PCR.....	85
4.3 Results.....	86
4.4 Discussion.....	92



General conclusion and outlook..... 99

References..... 103



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Abbreviations.

ABA: Abscisic acid

ABP1: Auxin binding protein 1

ANP: Atrial natriuretic peptide

APS: Ammonium persulfate

ARF: Auxin response factor

AtBRI1: *Arabidopsis thaliana* Brassinosteroid receptor 1

AtCNG2: *Arabidopsis thaliana* cyclic nucleotide-gated 2

AtGC1: *Arabidopsis thaliana* guanylate cyclase 1

ATP: Adenosine 5'-triphosphate

AtPSK1: *Arabidopsis thaliana* Phytosulfokine

AtWAKL10: *Arabidopsis thaliana* wall associated kinase-like 10

Aux: Auxin

BPB: Bromophenol blue

bp: base pair

cADPR: Cyclic adenosine diphosphate ribose

cAMP: Cyclic 3',5'-adenosine monophosphate

cGMP: Cyclic 3',5'-guanosine monophosphate

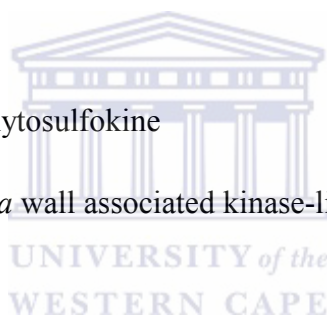
CHCA: α -cyna-hydroxy-cinnamic

CKI1: Cytokinin independent-1

CLV: Clavata

CNG: Cyclic nucleotide-gated

CNGC: Cyclic nucleotide-gated channel



COI1: Coronatine Insensitive1

DTT: Dithiothreitol

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

EIA: Enzyme immunoassay

ET: Ethylene

ETR1: Ethylene response 1

FPLC: Fast protein liquid chromatography

GA: Gibberellic acid

GC: Guanylate cyclase

GTP: Guanosine 5'-triphosphate

IAA: Indole-3-acetic acid

IBMX: 3-isobutyl-1-methyl xanthine

IEF: Isoelectric focusing

IPG: Immobilised pH gradient

IPTG: isopropyl- β -D-thiogalactopyranoside

JA: Jasmonic acid

kDa: Kilodalton

LB: Luria broth

LRR: Leucine-rich repeat

MALDI-TOF: Matrix-assisted laser desorption/ionization-time of flight

MES: 4-Morpholineethanesulfonic acid

MSMO: Murashige and Skoog basal salt with minimum organics

Ni-NTA: Nickel-nitrilotriacetic acid



NP: Natriuretic peptide

NPR: Natriuretic peptide receptor

NMR: Nuclear magnetic resonance

PCR: Polymerase chain reaction

PMSF: Phenylmethanesulfonylfluoride

PNP: Plant natriuretic peptide

PSK: Phytosulfokine

PSB: Phosphate buffered saline

PSBT: Phosphate buffered saline-Tween[®] 20

RALF: Rapid alkalisation factor

RLK: Receptor-like kinase

RFU: Relative fluorescent unit

RT-PCR: Reverse transcriptase polymerase chain reaction

Rubisco: Ribulose-1,5-biphosphate carboxylase

SA: Salicylic acid

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SE: Standard error

SP: Signal peptide

SCR: Secreted Cys-rich proteins

TEMED: 1,2-Bis(dimethylamino)ethane

TIR1: Transport Inhibitor Response1

TMB: 3,3',5,5'-Tetramethylbenzidine TMV: Tobacco mosaic virus



UV: Ultraviolet

WAK: Wall-associated kinase

WAKL: Wall associated kinase-like

WAKL10: Wall associated kinase-like 10



Lists of Figure and Tables.

Table 1.3.1: Summary of plant peptide molecules present in <i>A. thaliana</i> their receptors and function.....	17
Figure 1.4.1: Structural topology of receptor-like kinases (RLKs).....	23
Figure 2.3.1: Alignment of <i>Arabidopsis</i> GC catalytic domains with the original search motif.....	38
Figure 2.3.2: Structural features of the GC catalytic domain and the <i>Arabidopsis thaliana</i> Brassinosteroid receptor AtBRI1.....	39
Figure 2.3.3: Isolation of AtBRI1-GC and preparation of expression vector.....	40
Figure 2.3.4: Expression and purification of recombinant AtBRI-GC.....	41
Figure 2.3.5: Preparation of recombinant AtBRI1-GC and functional testing <i>in vitro</i>	43
Figure 3.2.1: Structural features of the Gateway [®] pDEST [™] 17 expression vector.....	53
Figure 3.3.1: Structural features of the guanylate cyclase catalytic domain of AtPSKR1.....	60

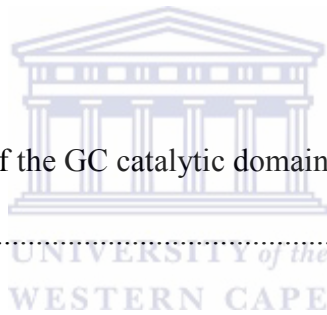


Figure 3.3.2: Expression and purification of the complete cytoplasmic domain of AtPSKR1-KD.....	61
Figure 3.3.3: Demonstration of GC activity of the complete cytoplasmic domain of PSKR1-KD2.....	62
Figure 3.3.4: Demonstration of the recombinant AtPSKR1-D2 kinase activity with Ser/Thr Peptide 1.....	63
Figure 3.3.5: Demonstration of the effect of cGMP on the kinase activity of AtPSKR1.....	64
Figure 3.3.6: Demonstration of the effects of cyclic nucleotides on the autophosphorylation state of AtPSKR1.....	65
Figure 4.3.1: Demonstration of the <i>in vivo</i> GC activity of AtPSKR1 and AtBRI1.....	83
Figure 4.3.2: Demonstration of cGMP mediated changes in the phosphoproteome of <i>A. thaliana</i>	85
Figure 4.3.3: Two dimensional protein analysis of cGMP induced phosphophorylation and MALDI-TOF mass spectrometry identification in <i>A. thaliana</i>	86

Figure 4.3.4: Determination of the expression of AtBIKI in *A. thaliana* leaves using RT-PCR.....87

Figure 4.4.1: Clustal W alignment of AtBIKI with AtBRI1 and AtPSKR1.....92

Figure 4.4.2: A speculative model of the activation of AtPSKR1 by α -PSK and how this may relate to kinase and GC activity.....94



Summary.

Guanylyl cyclases (GCs) catalyze the formation of the second messenger guanosine 3',5'-cyclic monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). Cyclic GMP has been implicated in an increasing number of plant processes, including responses to abiotic stresses such as dehydration and salt, as well as hormones. However, the identification of cGMP generating molecules in higher plants has been elusive. To identify such molecules, we propose a rational search strategy based on conserved and functionally assigned residues in the catalytic centre of annotated GCs in animal species and use the resulting search terms to query the *Arabidopsis thaliana* proteome. A number of the identified candidate molecules belong to the family of leucine rich repeat receptor like kinases and include the Brassinosteroid (AtBR1) and the Phytosulfokine receptors (AtPSKR1). The typical architecture of these receptors is such that they have an extracellular domain with which to perceive the signal, a single transmembrane domain and an intracellular kinase domain within which the GC domain is found. Brassinosteroids are polyhydroxylated plant steroid hormones with an essential role in co-regulating many processes including embryogenesis and cell elongation. Brassinosteroids have potential use in industry due to their characteristics as plant growth promoters for the increase of crop yields and increases in resistance to biotic and abiotic stresses. In turn, Phytosulfokines (PSK) are peptidic plant growth factors that are perceived by the Phytosulfokine receptor (AtPSKR1). PSK has been shown to stimulate development of sugar beet (*Beta vulgaris* L.) mesophyll protoplasts therefore suggesting applications in agriculture.

In this thesis, we have firstly cloned and expressed the domains that harbours the putative catalytic GC domain in these receptor molecules and demonstrate that these molecules can convert GTP to cGMP *in vitro*.

Secondly, we show that exogenous application of both Phytosulfokine and Brassinosteroid increase changes of intracellular cGMP levels in Arabidopsis mesophyll protoplast demonstrating that these molecules have GC activity *in vivo* and therefore provide a link as second messenger between the hormones and down-stream responses.

In order to elucidate a relationship between the kinase and GC domains of the PSK receptor, we have used the AtPSKR1 receptor as a model and show that it has Serine/Threonine kinase activity using the Ser/Thr peptide 1 as a substrate. In addition, we show that the receptor's ability to phosphorylate a substrate is affected by the product (cGMP) of its co-domain (GC) and that the receptor autophosphorylates on serine residues and this step was also observed to be affected by cGMP. When Arabidopsis plants are treated with a cell permeable analogue of cGMP, we note that this can affect changes in the phosphoproteome in Arabidopsis and conclude therefore that the cGMP plays a role in kinase-dependent downstream signalling.

The obtained results suggest that the receptor molecules investigated here belong to a novel class of GCs that contains both a cytosolic kinase and GC domains, and thus have a domain organisation that is not dissimilar to that of atrial natriuretic peptide receptors NPR1 and NPR2. The findings also strongly suggest that cGMP has a role as a second messenger in both Brassinosteroid and Phytosulfokine signalling. We speculate that other proteins with similar domain organisations may also have dual catalytic activities and that a significant number of GCs, both in plants and animals, remain to be discovered and characterised.

Chapter 1: Literature review.

1.1 Introduction.

Life depends on the maintenance of physiological processes like growth and development in single cellular and multicellular organisms, plants and animals. The life-supporting state of homeostasis is often disturbed by abiotic and biotic stresses. Enzymes and hormones produced by an organism play crucial roles in stress responses, their regulation and transduction of these signals at the intracellular and intercellular level. Signal transduction of environmental or developmental signals commonly activate a signalling cascade that involves downstream processes, which in turn carry out the required response (Vogler and Kuhlemeier, 2003). Hormones play a role in the regulation of a number of cellular processes ranging from cell division, cell elongation to cell differentiation (Johri and Mitra, 2001; Kende and Zeevaart, 1997). Auxin was the first hormone discovered and subsequently other hormones were identified and these include Gibberellins, Cytokinins, Abscisic acid and Ethylene. These five hormones are commonly known as the “classical” five and are non-protein molecules that have specific effects on a variety of developmental and physiological processes (Johri and Mitra, 2001). In plants there are other compounds known as the “non-classical” hormones and they have been shown to affect plant growth and development and include Oligosaccharides, Jasmonates, Brassinosteroids, Salicylic acid and Polyamines (Creelman and Mullet, 1997; Gaspar *et al.*, 1996). In addition, another group of signalling molecules has been shown to be present in plants and these are peptide hormones. These peptidic signalling compounds include Systemin (Pearce *et al.*, 1991), Phytosulfokines (PSK) (Matsubayashi and Sakagami 1996), Rapid Alkalinization Factor (RALF) (Pearce *et al.*,

2001), CLAVATA 3 (Trotochaud *et al.*, 1999), Secreted Cys-rich proteins (SCR) (Schopfer *et al.*, 1999) and plant natriuretic peptides (PNP) (Gehring, 1999).

In plants, the action of a hormone involves its perception to initiate a specific response pathway that may be independent of transcriptional or translational control (Johri and Mitra, 2001; Vogler and Kuhlemeier, 2003). One of the mechanisms through which cells communicate in multicellular organisms is through the secretion of ligands, examples of which are mentioned above. These may then bind to cell surface receptors amongst some of which contain and signal through protein kinase catalytic activities (Dievart and Clark, 2004). The family of enzymes known as the Receptor Like Kinases (RLK's) are widely distributed in the plant kingdom and are known to be involved in the perception and modulation of a number of cellular processes including responses to environmental and development signals such as light (Deeken and Kaldenhoff, 1997), hormones (Li and Chory, 1997), expression of defence related genes in tobacco (Durner *et al.*, 1998). The major subgroup of RLKs is the leucine-rich repeat receptor like kinases (LRR-RLKs). The early responses would typically involve changes at the level of ion fluxes and generation of second messengers such as Ca^{2+} , cyclic adenosine diphosphate ribose (cADPR) or cyclic guanosine 3',5'-monophosphate (cGMP) (Johri and Mitra, 2001; Vogler and Kuhlemeier, 2003).

In this thesis will be examining the downstream signalling of hormone signalling by Brassinosteroids and Phytosulfokines. We will begin by providing some brief descriptions of the functions of the five classical plant hormones and plant peptide signalling molecules. We shall consider how these molecules are perceived and the types of receptors that are involved their signalling. We will then briefly describe Guanylyl cyclases which have been implicated in downstream hormone signalling and signal transduction and finally outline the roles of the second messenger guanosine 3', 5'-monophosphate in plants.

1.2 Signalling in plants.

1.2.1 Classical hormones.

The classical plant hormones are amino acid derivatives that have specific effects on a large number of developmental and physiological processes (Vogler and Kuhlemeier, 2003). Plant hormones play pivotal roles in plant growth, development, and response to biotic and abiotic cues (McCourt, 2001). Plant hormones are structurally diverse compounds that are grouped into the following major classes: Auxins, Cytokinins, Abscisic acid, Gibberellins and Ethylene. Though each class of these hormones elicits characteristic biological effects, multiple plant hormones often mediate development and stress responses through synergistic and/or antagonistic actions (Wolters and Jurgens 2009).

Auxin, whose primary form is indole-3-acetic acid (IAA), was the first plant hormone identified and is synthesized primarily in the shoot tips and growing tissues (Bartel, 1997; Palme and Nagy, 2008). Auxins promote plant growth by stimulating formation of lateral root primordia and through the promotion of cell elongation (Kende and Zeevaart, 1997). The hormone has also been shown to act synergistically with Cytokinins in the regulation of cell division (John *et al.*, 1993).

Cytokinins on the other hand are also found in sites of active cell growth in plants where they play a role in cell division and are important in cell differentiation as well as in organogenesis e.g. in plant tissue cultures (John *et al.*, 1993; Pernisova *et al.*, 2009).

Gibberellic acids are a large family of tetracyclic compounds widespread throughout the plant kingdom with over 112 members identified (Hisamatsu *et al.*, 1997). Among other responses, Gibberellins regulate the mobilization of soluble sugars from the starch in cereal grains and co-regulate germination and growth (Kende and Zeevaart, 1997).

Abscisic acids are synthesized from carotenoids and are involved in the regulation of stomatal closure, adaptation to various stresses, induction of dormancy and seed formation (Davies, 2010; Mundy and Chua, 1988; Walton, 1980). Abscisic acids also controls seed maturation and synthesis of storage proteins (Xu and Bewley, 1995).

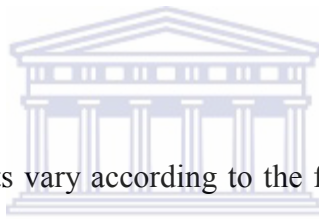
Ethylene is a simple gaseous hydrocarbon produced from an amino acid and appears in most plant tissues in large amounts when they are stressed. The hormone Ethylene also has effects on plant growth and development (Chang and Shockey, 1999), and is involved in promotion or inhibition of flowering which evokes the classical triple response in *Arabidopsis thaliana* seedlings grown in the dark. These responses are characterized by an exaggerated curvature of the apical hook, a radial swelling of the hypocotyl and an inhibition of hypocotyl and root elongation which is critically associated with ripening of fruits (Johri and Mitra, 2001). Environmental stresses such as wounding, pathogen attack, and flooding can induce Ethylene production and this in turn can lead to defence responses such as accelerated senescence, apoptosis and abscission of infected organs as well as the induction of specific defence proteins (Chang and Shockey, 1999).

1.2.2 The non-classical hormones.

Brassinosteroids, Jasmonate and Salicylic acid, are now also being viewed as phytohormones (Boller, 2005) and are classified as the “non-classical” hormones. In addition to the classical hormones, which also included Polyamines and Oligosaccharins (Gross and Parthier, 1994). Polyamines are involved in a wide range of growth and developmental processes and plant tissues that are deficient in Polyamines exhibit abnormal growth. At a physiological pH, Polyamines act as polycations and complexing agents that bind strongly to phospholipid groups and to other anionic sites on membranes thus affecting membrane fluidity (Schuber *et*

al., 1983). It has been reported that Polyamines can also compensate for ionic deficiencies or the damaging effects of some stresses on membranes (Gaspar *et al.*, 1996).

Oligosaccharins as the name implies are complex carbohydrates (short chains of sugar residues connected by glycosidic linkages) that are capable of modulating plant growth and development (Creelman and Mullet, 1997). These type of hormone at low concentration affect biological processes in plant tissues other than the break-down of the carbon to generate ATP (Gaspar *et al.*, 1996). Some Oligosaccharins, such as Oligogalacturonids have been reported to act as elicitors and raise the pathogen defence responses which results in the accumulation of proteinase inhibitors and peroxidases amongst others components (Darvill *et al.*, 1992; Eder *et al.*, 1994).



Responses to Jasmonates in plants vary according to the function of the tissue and cell type and in response to several different environmental stimuli (Creelman and Mullet, 1997). Jasmonates have been reported to be involved in the cellular transduction processes between external stress and macromolecular components involved in the stress responses that in turn involve the expression of defence genes and production of Jasmonate induced proteins (Reinbothe *et al.*, 1994).

To-date many different steroids have been identified, but only Brassinosteroids (BR`s) are widely distributed throughout the plant kingdom and are known to effect plant growth (Li and Chory, 1997). Brassinosteroids are involved in various growth processes such as stem elongation, leaf development and pollen tube growth (Li and Chory, 1997; Li and Chory, 1999). Brassinosteroids are naturally occurring polyhydroxy steroids which were originally isolated from *Brassica napus* L (Grove *et al.*, 1979). In addition to their growth-promoting

activities, these hormones have been reported to inhibit root growth, enhance gravitropism (Kim *et al.*, 2000), retard leaf abscission (Lawlor 2004), enhance resistance to stress (Nakashita *et al.*, 2003), and promote xylem differentiation (Sakurai and Fujioka 1993).

1.3 Peptide hormones signalling.

Defensive and development cues in plants can be activated and mediated by several different types of signalling molecules and we have briefly reviewed some of these above. Plants have also evolved a number of mechanisms to respond rapidly to environmental changes to continue normal growth and development. In addition to the classical and non-classic hormones, they also have signalling processes mediated through networks of regulatory peptidic hormones and these signalling molecules (ranging in size from ~60 to 180 amino acids) usually containing an N-terminal secretory signal sequence (Denecke *et al.*, 1990). The first peptide signalling molecule identified was Systemin, which was isolated from wounded tomato leaves (*Solanum lycopersicum* L.) where it induces the synthesis of proteinase inhibitors (Pearce *et al.*, 1991).

Systemin is a proteolytically processed form of a 200 amino acid residue precursor called pro-Systemin (Pearce *et al.*, 1991). In addition to Systemin, two more Prosystemin isoforms produced by an alternative splicing mechanism were also shown to be active as signals in the wound response (McGurl and Ryan, 1992). Several other non-classic hormones identified recently include S-locus cysteine rich (SCR) proteins that acts in self-incompatibility (Schopfer *et al.*, 1999), the rapid alkalization factor (RALF) which arrests root growth and development (Pearce *et al.*, 2001), and other molecules that are involved in antimicrobial activities (Garcia-Olmedo *et al.*, 2001). Further examples of signalling peptides with their site of action and function are listed in Table 1.3.1. One group of these and of particular

interest here are the Phytosulfokines (PSK). Phytosulfokines are signalling peptides that have been purified from dispersed asparagus mesophyll suspension cell culture medium (Fang and Hirsch, 1998) and to-date, are the only group of peptides reported to be produced from post-translational sulfation of tyrosine residues, sulfated in the active pentapeptide region in plants (Hanai *et al.*, 2000; Takayama and Sakagami, 2002). PSK is widely distributed in higher plants and is present in both monocotyledon and dicotyledon cell lines (Fang and Hirsch 1998). The peptide can induce dedifferentiation and callus growth at very low concentrations ($\geq 10^{-8}$ M) and at low cell density (~ 300 cells/ml) (Matsubayashi and Sakagami 1996). However it has been suggested that at these concentration levels PSK alone does not induce cellular dedifferentiation and re-differentiation and may do so in conjunction with Auxins and Cytokinins (Matsubayashi *et al.*, 1999).

Propeptide ^A	Gene family	Propeptide size	Processed peptide, size	Function	Site of action	Receptor
CEP	5	8.5–11.5 kDa	CEP1, 14 aa	Inhibits root growth	Lateral root primordia	Unknown
CLE (and CLV3)	31	7.8–14.5 kDa	CLE or mCLV3, 12–14 aa	Stimulates organogenesis and inhibits meristematic growth; can stimulate vascular development	Floral, shoot and root meristems; vascular	CLV1, BAM, CLV2,
EPF	7	11.5–14.3 kDa	Unknown	Promotes epidermal cell division leading to guard cell (stomatal) formation	Epidermis and meristemoid mother cells	TMM, ER, ERL1, ERL2
IDA and IDL	6	8.4–13 kDa	EPIP	Inhibits floral abscission	Abscission zone	HAS, HSL
PROPEP	7	9.3–12.3 kDa	Pep1, 23 aa	Promotes innate immune responses (a danger signal)	Widespread, leaves	Pep1R
PNP	2	13–14 kDa	Unknown	Extracellular, Cell expansion, water or ion movement, stomatal opening, inhibits ABA induced stomatal closure	Mesophyll protoplasts, guard cells, root stele, stem	Unknown
PSK	6	8.7–9.7 kDa	PSK- α , 5 aa	Promotes cell proliferation and longevity, root elongation	Mesophyll cells, roots	PSKR1
PSY	1	7.9 kDa	PSY1, 18 aa	Promotes cellular expansion and proliferation, upregulated by wounding	Mesophyll cells, roots	PSYR1
RALF and RALFL	34	7–14 kDa	RALF, 25–30 aa	Associated with danger signals, affects growth – inhibits root growth	Widespread in plants	Unknown
SCRL	27	9.2–11.5 kDa	Not processed	Prevents self-fertilisation (but not in <i>A. thaliana</i>)	Pollen	SRK
TPD	1	19.5 kDa	TPD1	Anther development promoting tapetum formation	Anthers	EMS1

Table 1.3.1 Summary of plant peptide molecules present in *A. thaliana* their receptors and function. The table represents the receptors and as well as the biological activities into which these different signal peptides are involved. [Table adapted from (Wheeler and Irving, 2010)].

1.4 Perception of hormones signals in plants.

Integration of the message carried by the hormone into a physiological response requires both perception and relay. Plants perceive and respond to a variety of environmental and endogenous signals, some of which have been described in Sections 1.2 and 1.3 and these modulate their growth and development (Hooley, 1998). Receptors for both the non-peptidic phytohormones and the peptidic signalling molecules have been proposed and identified.

Ethylene has been shown to be perceived by a family of high-affinity receptors, the Ethylene response 1 (ETR1) protein in *Arabidopsis* (Chang *et al.*, 1993; Hua *et al.*, 1998).

The ETR1 gene encodes a protein which contains a hydrophobic N-terminal domain that binds Ethylene and a C-terminal domain that is related in sequence to the histidine kinase response regulator two-component signal transducers of environmental stimuli in a variety of adaptive responses in bacteria (Chang *et al.*, 1993; Parkinson and Kofoed 1992). The ETR1 amino-terminal harbours a sensor domain which consists of three hydrophobic sub-domains that are membrane-associated and has been shown to contain elements necessary and sufficient high affinity for binding Ethylene (Schaller and Bleecker, 1995). The ETR1 has been demonstrated to be an Ethylene receptor based on the observations that an *etr1* mutant is dominant and insensitive to Ethylene in all the Ethylene responses (Bleecker *et al.*, 1988), and the ETR1 gene acts upstream of the rest of the Ethylene signal transduction pathway (Kieber *et al.*, 1993).

The receptor Cytokinin independent-1 (CKI1), a histidine kinase homolog has been suggested to perceive Cytokinins (Kakimoto 1996). Overexpression of CKI1 in *Arabidopsis* calli derived from hypocotyl results in the characteristic effects of Cytokinin action,

suggesting that the product acts in regulation of Cytokinin levels, in Cytokinin recognition or at an early stage of signal transduction (Kakimoto 1996).

In many other instances where receptors have been identified, they have the typical architecture of receptor-like kinases (RLKs) or at least seem to signal through a kinase mediated process. The exceptions are Auxin and Jasmonate with the F-box protein Transport Inhibitor Response1 (TIR1) for the former (Dharmasiri *et al.*, 2005; Gray *et al.*, 2001; Kepinski and Leyser, 2005) and the F-box protein Coronatine Insensitive1 (COI1) (Yan *et al.*, 2009) for the latter. Previous studies had implicated the Auxin binding protein 1 (ABP1) as the most likely candidate receptor for Auxin due to the fact that it could bind Auxin (Napier *et al.*, 2002).

Although ABP1 shows characteristic receptor function, its role in Auxin signalling is not entirely clear (Dharmasiri *et al.*, 2005). In addition, two families of transcription factors, the Auxin response factor (ARF) and Aux/IAA proteins had also been implicated in Auxin signalling. The ARF proteins bind DNA directly to either activate or repress transcription whilst Aux/IAA proteins exert their effects by binding to the ARF proteins through a conserved dimerization domain (Liscum and Reed 2002; Reed 2001). Auxin regulates transcription by stimulating the degradation of the Aux/IAA proteins (Gray *et al.*, 2001; Tian *et al.*, 2003; Tiwari *et al.*, 2001; Zenser *et al.*, 2001). Some studies have indicated that Auxin acts by promoting an interaction between the Aux/IAA proteins and the ubiquitin protein ligase SCFTIR1 (Gray *et al.*, 2001) and substrate recognition requires an Auxin-dependent modification of TIR1 or an associated protein, rather than the substrate (Kepinski and Leyser, 2004). When an *in vitro* pull-down assay of total protein was performed on proteins extracted from Arabidopsis seedlings, the interaction between the transport inhibitor response 1 (TIR1) and Aux/IAA proteins was shown not to require stable modification of either protein. Instead Auxin promotes the Aux/IAA-SCF (TIR1) interaction by binding directly to

SCF (TIR1). Furthermore it was shown that the loss of TIR1 and three related F-box proteins eliminates Auxin binding in plant extracts thereby confirming that TIR1 is the Auxin receptor that mediates Aux/IAA degradation and Auxin-regulated transcription (Gray *et al.*, 2001).

The Jasmonate receptor was identified by first constructing a high-quality structural model of COI1 followed by a molecular modelling of COI1–Jasmonate interactions. The obtained results implied that COI1 has the structural traits necessary for binding Jasmonate. The direct binding of these molecules with COI1 was further examined using a combination of molecular and biochemical approaches. Secondly, immobilized Jasmonate was shown to be able to bind the COI1 protein from crude leaf extracts. Together, these results with additional confirmatory assays demonstrated that COI1 directly binds to Jasmonate (Yan *et al.*, 2009).

Receptors for some of the known signalling peptides are listed in Table 1.3.1. Perhaps the best of characterised receptor super-family in plants is the Receptor-like kinase family. It has been shown to have roles in a variety of biological processes including growth, development, hormone perception, and plant-microbe interactions (Afzal *et al.*, 2008; He *et al.*, 2000; Li and Chory, 1997; Matsubayashi *et al.*, 2002). Receptor-like kinases belong to a large gene family with at least 610 members that represent nearly 2.5 % of Arabidopsis protein coding genes (Shiu and Bleeker, 2001b). The major subgroup of RLK is the leucine-rich repeat receptor like kinases (LRR) RLK's and makes up of at least 120 genes in Arabidopsis (He *et al.*, 2000). This family of receptors has a characteristic imperfect repeat of 24-amino acid leucine-rich motif in the extracellular domain, a single trans-membrane domain and an intracellular kinase domain (Braun and Walker, 1996; Zhang, 1998). A diagrammatic representation of the architecture of the family of receptors is presented in Figure 1.2.1.

Though the most common length of an LRR is 24 residues, repeats containing any number between 20 and 29 residues are also found (Kobe and Deisenhofer 1994). The leucine-rich repeats form amphipathic α helices that are believed to mediate protein–protein interactions

including the binding of protein ligands with their receptors with each repeat forming a loop which is partially embedded in an exposed β -sheet (Kobe and Deisenhofer 1994).

The LRRs whose ligands have been identified in plants are the Arabidopsis Brassinosteroid insensitive receptor1 (AtBRI1, At4g39400) (He *et al.*, 2000; Li and Chory 1997) and the *Arabidopsis thaliana* Phytosulfokine receptor 1 (AtPSKR1, AT2G02220) (Matsubayashi *et al.*, 2002). In living organisms, many different steroids have been identified, but only Brassinosteroids (BR`s) are widely distributed throughout the plant kingdom and are known to affect plant growth (Li and Chory 1997). The AtBRI1 receptor is ubiquitously expressed and plasma membrane-localized in all tissues in plants (Friedrichsen *et al.*, 2000).

The organization of the AtBRI1 receptor is such that it has an extracellular domain containing 21 tandem amino-terminal LRRs, a 70-amino acid island domain and four additional LRRs preceding the transmembrane domain (Friedrichsen *et al.*, 2000). It was reported that (Li and Chory, 1997) loss of function mutations in both the extracellular and the intracellular kinase domains of AtBRI1 lead to BR-insensitive dwarf phenotypes identical to those of BR-deficient mutants, providing evidence that AtBRI1 is a BR receptor. More recently, when the extracellular leucine-rich repeat (LRR) and transmembrane domains of the Arabidopsis Brassinosteroid receptor were fused to the Serine/Threonine kinase domain of XA21, a rice disease resistance receptor (Song *et al.*, 1995) it was shown that the chimeric receptor was able to initiate plant defence responses in rice cells upon treatment with Brassinosteroids indicating that the extracellular domain of AtBRI1 perceives Brassinosteroids (He *et al.*, 2000).

Using ligand based affinity chromatography, with PSK as the ligand, a 120-kilodalton membrane protein, specifically interacting with the immobilised PSK, from carrot microsomal fractions was purified (Matsubayashi *et al.*, 2002). This provided evidence for

the existence of high-affinity binding sites for PSK that was subsequently identified as an LRR. The Phytosulfokine receptor has an extracellular LRR domain that contains 21 tandem copies of a 24 amino acid LRR and in addition also exhibits an island domain of 36 amino acids at LRR 18 (Matsubayashi *et al.*, 2002).

In RLK-LRR mediated hormonal signalling ligand binding induces dimerization of the intracellular kinase domains into proximity and allows them to transphosphorylate and activate one another (Becraft, 2002). However there are exceptions to this general model as several documented plant RLKs are multimers in their inactive form (Giranton *et al.*, 2000; Trotochaud *et al.*, 1999).

Another common manner through which these molecules function is through an intramolecular kinase activity phosphorylation signalling cascade (Schulze-Muth *et al.*, 1996).

We have recently reported that the intracellular kinase domains of the Brassinosteroid and Pytosulfokine and another RLK-LRR, AtWAKL10, receptors harbour guanylyl cyclase (GC) domains that are active in *in vitro* (Kwezi *et al.*, in revision) (Kwezi *et al.*, 2007; Meier *et al.*, 2010). Although signal transduction through the intracellular kinase domain has been well documented, the role of the GC domains in the RLK-LRR kinase transduction pathways remains to be defined.

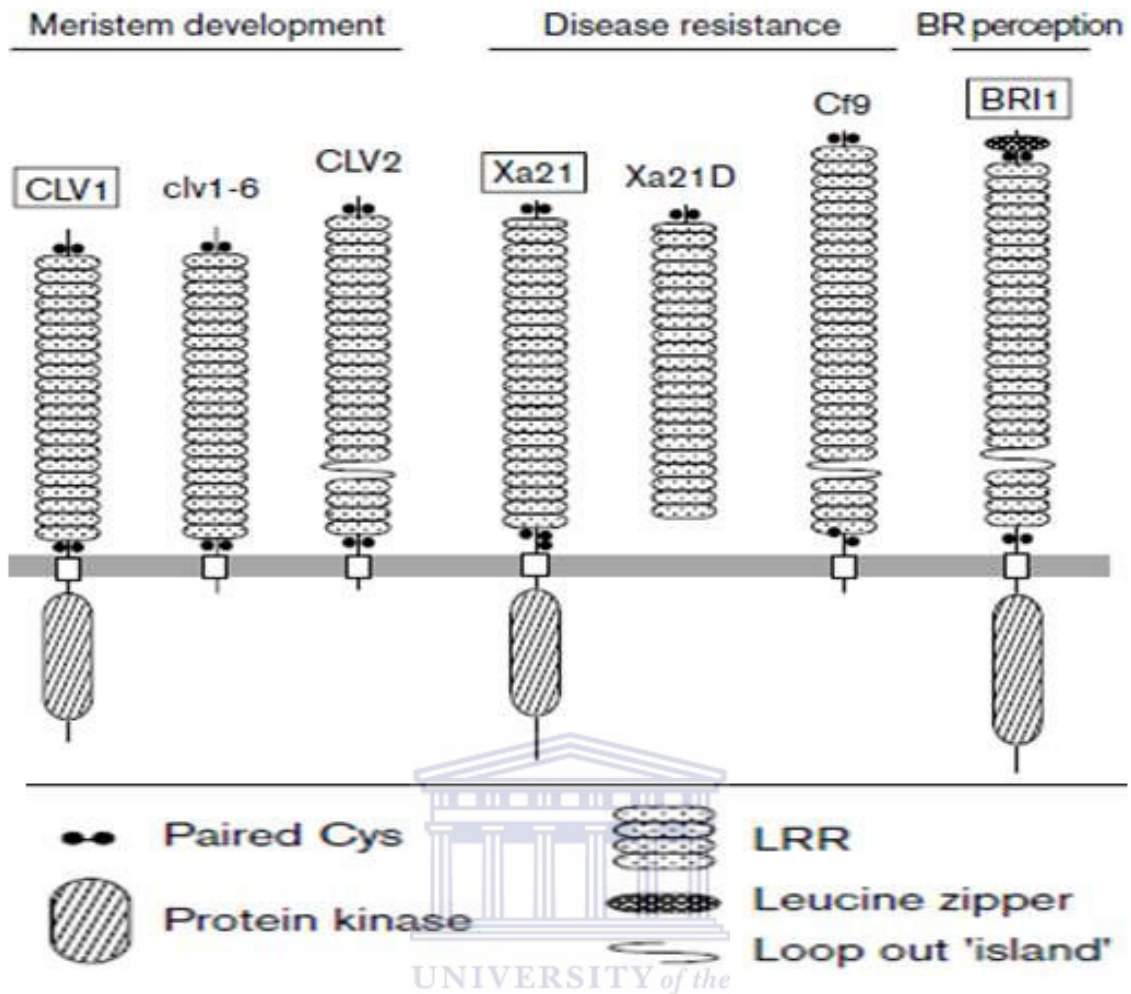


Figure 1.4.1 Structural topology of receptor-like kinases (RLKs). The figure represents the architecture of a RLK, with the extracellular, transmembrane and intracellular domains represented. The names of examples of receptors and their biological functions are shown at the top of the figure. The extracytoplasmic LRR domain of CLV2, Cf9, and BRI1 are intercepted at an identical position, between the fourth and fifth LRRs from the transmembrane domain. In the LRR-RLKs a pair of cysteines exists at the beginning and the end of the LRR domain and the paired cysteines are presumed to act as a receptor dimerization motif. Figure adapted from Torii (2000).

1.5 The family of guanylyl cyclases.

Guanylyl cyclases (GCs) are enzymes that catalyze the formation of the second messenger guanosine 3',5'-cyclic monophosphate (cGMP) from guanosine 5'-triphosphate (GTP) and the first GC in higher plants has been identified recently (Ludidi and Gehring, 2003a). Guanylate Cyclase (GC) activity is found in both the soluble and particulate fractions of most prokaryotes and all eukaryotes including plants (Lucas *et al.*, 2000; Ludidi and Gehring, 2003a).

The particulate GCs have been reported to be receptors for natriuretic peptides (NP) and exhibit highly conserved domain structures which include an N-terminal extracellular binding domain, a hydrophobic transmembrane domain, a regulatory domain that shows homology to protein kinases, a hinge region and an intracellular domain at the C-terminal of the protein on the cytoplasmic side of the cell (in which the GC catalytic domain resides) (Lucas *et al.*, 2000). Soluble GCs are heterodimeric proteins consisting α and β subunit of ~70kDa and 73-82 kDa respectively (Gerzer *et al.*, 1981; Lucas *et al.*, 2000) where generally both subunits are required for the catalytic activity of the molecule (Lucas *et al.*, 2000). Each of the subunits has a regulatory domain at the N-terminus and a catalytic domain at the C-terminus (Lucas *et al.*, 2000) while the β -subunit also contains a heme-domain that acts as a binding site for nitric oxide (Gerzer *et al.*, 1981; Namiki *et al.*, 2001).

Cyclic GMP is increasingly being reported to be involved in a large number of plant processes including responses to abiotic stresses such as dehydration and salt (Donaldson *et al.*, 2004), hormone dependent signalling (Penson *et al.*, 1996) and changes of the transcriptome of *A. thaliana* (Maathuis 2006). GCs synthesize cGMP in response to diverse signals such as nitric oxide (NO), peptide ligands and fluxes in intracellular Ca^{2+} (Lucas *et al.*, 2000; Cann, 2003; Lucas *et al.*, 2000).

Recently (Maathuis 2006) characterized a voltage-independent channel in *Arabidopsis* roots, the opening of which decreases with the presence of micromolar concentrations of cytoplasmic cGMP. Cyclic GMP has also been shown to be involved in plant defences since it has been shown that NO in tobacco suspension cells triggers the expression of defence related genes which are also induced by cGMP (Durner *et al.*, 1998).

The role of cyclic nucleotides as second messengers in animals has been well-established where cAMP and cGMP have, as main targets, specific kinases whose activity is modulated by binding of ligands to particular regulatory subunits (Newton and Smith 2004). Though there have been reports of cGMP-dependent kinase signalling in plants this mechanism is not as well characterised as in animals.



Chapter 2: The *Arabidopsis thaliana* Brassinosteroid Receptor (AtBRI1) contains a Domain that functions as a Guanylyl cyclase *in vitro*.

Abstract.

Guanylyl cyclases (GCs) catalyze the formation of the second messenger guanosine 3',5'-cyclic monophosphate (cGMP) from guanosine 5'-triphosphate (GTP). Cyclic GMP has been implicated in an increasing number of plant processes, including responses to abiotic stresses such as dehydration and salt, as well as hormones. Here we have used a rational search strategy based on conserved and functionally assigned residues in the catalytic centre of annotated GCs to identify candidate GCs in *Arabidopsis thaliana* and show that one of the candidates is the Brassinosteroid receptor AtBRI1, a leucine rich repeat receptor like kinase. We have cloned and expressed a 114 amino acid recombinant protein (AtBRI1-GC) that harbours the putative catalytic domain, and demonstrate that this molecule can convert GTP to cGMP *in vitro*. The obtained results suggest that AtBRI1 may belong to a novel class of GCs that contain both a cytosolic kinase and GC domain, and thus have a domain organisation that is not dissimilar to that of atrial natriuretic peptide receptors, NPR1 and NPR2. The findings also suggest that cGMP may have a role as a second messenger in Brassinosteroid signalling. In addition, it is conceivable that other proteins containing the extended GC search motif may also have catalytic activity, thus implying that a significant number of GCs, both in plants and animals, remain to be discovered, and this is in keeping with the fact that the single cellular green alga *Chlamydomonas reinhardtii* contains over 90 annotated putative CGs.

2.1 Introduction

The family of proteins, Guanylyl cyclases (GCs), find a wide and diverse distribution amongst organisms from prokaryotes to eukaryotes, where they catalyse the synthesis of the second messenger guanosine 3',5'-cyclic monophosphate (cGMP) from guanosine 5'-triphosphate (GTP) (Schaap, 2005). In higher plants cGMP has been shown to act as a second messenger in a large number of physiological responses (Newton and Smith, 2004) that include cGMP-mediated changes of the transcriptome (Maathuis 2006), NO-dependent signalling (Prado *et al.*, 2004) as well as gravitropic responses (Hu *et al.*, 2005) and plant hormone-dependent responses (Pagnussat *et al.*, 2004; Penson *et al.*, 1996; Pharmawati *et al.*, 2001). Furthermore, significant and transient increases in intracellular cGMP levels have also been reported in response to plant natriuretic peptides (PNPs) (Pharmawati *et al.*, 1998; Pharmawati *et al.*, 2001) as well as NaCl and drought stress (Donaldson *et al.*, 2004). The first functional GC in higher plants was identified with a search motif based on several functionally assigned amino acids in the catalytic domain of known GCs from lower eukaryotes and animals (Ludidi and Gehring, 2003a). Here we show that a rationally designed search motif of the catalytic domain identifies several members of the family of Leucine Rich Repeat Receptor-Like Kinases (LRR RLKs) including an *Arabidopsis thaliana* Brassinosteroid receptor (AtBRI1). A recombinant domain protein was made which tested positive for GC activity *in vitro*. The implications of this finding for both the projected number of different classes of GCs and the role of cGMP in Brassinosteroid signalling are discussed.

2.2 Materials and Methods.

2.2.1 Identification of the GC catalytic domain.

Catalytic domains (McCue *et al.*, 2000) of protein sequences annotated as GCs in the “National Center for Biotechnology Information” (NCBI) were retrieved and used for Blast searches of “The Arabidopsis Information Resource” (TAIR) database and GenBank. Multiple alignments of the retrieved catalytic domains were done using Clustal X, with alignments at the catalytic centre of these domains used to deduce the original search motif (Ludidi and Gehring, 2003a). Since an ideal search motif will have to have stringent detection parameters, the derived search motifs were tested for accuracy and specificity in detecting nucleotide cyclases. This was done through querying both the Protein Information Resource (www-nbrf.georgetown.edu) using the Pattern Match option on the PIR-NREF link and the Arabidopsis genome using the “Patmatch” option on the Arabidopsis server (www.arabidopsis.org) (Ludidi and Gehring, 2003a).

2.2.2 Extension of the GC search motif to identify AtBRI1.

When the Arabidopsis genome was queried with the original search motif it returned seven candidate proteins including AtGC1 that has been demonstrated to have GC activity *in vitro* (Ludidi and Gehring, 2003a). Amongst the seven, two were annotated kinases, with one of these: (At1g79680) belonging to the group of Wall Associated Kinase-Like proteins (WAKLs).

To further identify other candidate GCs in plants, position seven in the original search motif was mutated from an Aspartic acid [D] to a Leucine [L] and we subsequently added [L] at

position seven to give [DNAL]. This extended motif retrieves 123 *Arabidopsis thaliana* proteins including the Brassinosteroid receptor AtBRI1.

2.2.3 Preparation of *Arabidopsis thaliana* plant material.

Arabidopsis seeds were obtained from Dr Shane Murray from the Centre for Proteomic and Genomic Research (CPGR), Cape Town, South Africa. Plants were germinated in seedling germination soil from the Environmental Education Resource Unit (EERU - University of the Western Cape, Cape Town, South Africa) under measured light intensity and humidity at 23 °C. A light and dark regime of 16 hours light and 8 hours dark photoperiods were applied.

2.2.4 Genomic DNA extraction.

Plants were grown to three weeks and 0.10g of leaf tissue was harvested and homogenised with pestle and sand in 500 µL buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5 % SDS) in an eppendorf tube. The homogenate was incubated at 60 °C for 10 minutes and genomic DNA was extracted with 500 µL chloroform: isoamyl alcohol (24:1) by briefly vortexing followed by centrifuging at 12 x 4 g for 10 minutes at room temperature on a bench top centrifuge (Eppendorf, Hamburg, Germany). The supernatant was collected into a clean eppendorf tube and DNA was precipitated with 2x volume ice cold 70 % Ethanol and a tenth the volume with 3 M Sodium Acetate; pH 5.2 for one hour at -20 °C. Precipitated DNA was collected by centrifuging at 12 x 4 g for 10 minutes, supernatant was discarded as waste, and the pellet was washed with 500µL of 70 % Ethanol and air dried at room temperature for 10 minutes. Genomic DNA was resuspended in 50 µL of sdH₂O and treated with 10 mg/mL RNase (Fermentas International Inc., Burlington, Canada) and this was incubated for one hour at 37 °C. Genomic DNA was the purified using the GFX DNA

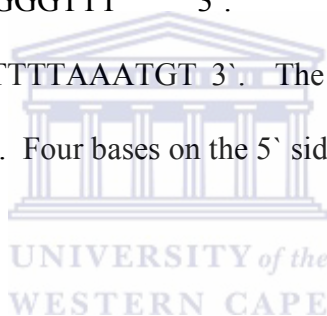
purification kit (GE Healthcare, Wisconsin) as per manufacturer`s instructions and stored at 4 °C short term and -20 °C long term.



2.2.5 Isolation of the AtBRI1 GC catalytic center.

2.2.5.1 Primer design.

The genomic sequence of AtBRI1 gene as retrieved from The Arabidopsis Information Resource (TAIR) revealed that the gene has no introns. Primers were designed to amplify, directly from genomic DNA, the catalytic center that would subsequently give a gene product of 50 amino acids on either side (N and C terminus). Based on the sequence flanking position 3132 and 3638 of the Brassinosteroid gene, the following forward and reverse primers could be designed to specifically prime and amplify this region: Forward primer: 5' GCT**AGGATCCT**TGGAAGCTCGGGTTT 3'. Reverse primer: 5' TCC**AGAATTCT**CAAGCAACTTTTAAATGT 3'. The bold and underlined regions are BamH1 (Fwd.) and EcoR1 (Rev.). Four bases on the 5' side were added to provide a scaffold for restriction enzymes.



2.2.5.2 Polymerase Chain Reaction (PCR) amplification of AtBRI1 from genomic DNA.

Genomic DNA from Arabidopsis was used as the DNA template in the PCR reactions. Amplifications were performed on a Mastercycler[®] personal (Eppendorf, Hamburg, Germany) in a 50 μ L reaction. Each reaction contained 1.5 mM MgCl₂, 200 μ M dNTPs, 0.5 μ M reverse and forward primer, 7.4 ng of genomic DNA, and 2.5 units Econo *Taq* polymerase (Fermentas International Inc., Burlington, Canada). The thermal cycling parameters were: initial denaturation at 96 °C for 3 minutes, followed by 30 seconds at 96 °C, 50 °C for 45 seconds and 72 °C for 1 minute for 32 cycles, followed by a final extension at 72 °C for 10 minutes. PCR products were resolved on a 0.8 % agarose at 7V/cm for an hour.

Visualisation was done under short wavelength UV illumination using the Alphaimager™ Gel Imaging System (Alpha Innotech Corp., California). DNA fragments corresponding to 342 base pairs (bp) in size were excised from the gel and purified using the GFX purification kit as per manufacturer's instruction (GE Healthcare, Wisconsin). Concentrations of the AtBRI1 PCR product were quantified using a Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts).

2.2.6 Preparation of pCR-T7/NT-TOPO-AtBRI1 construct.

2.2.6.1 Restriction digest of AtBRI1.

To prepare the AtBRI1 insert for ligation into pCR[®]T7/NT-TOPO[®] (Invitrogen Corp., California) the insert was digested in two steps. The insert was first digested in a 50 µL reaction containing, 32 ng of insert, 1 X Tango Buffer and 3 units of BamHI (Fermentas International Inc., Burlington, Canada). The reaction was incubated at 37 °C for 3 hours. After the 3 hour incubation the Tango Buffer concentration was increased to two times (2x) in the same reaction tube after which 3 units of EcoRI (Fermentas International Inc., Burlington, Canada) were added and the reaction volume was increased to 60 µL with sdH₂O and incubated at 37 °C for 3 hours. The digest was resolved on a 0.8 % agarose gel at 7V/cm an hour and a band corresponding to 342 bp was excised out of the gel and purified using the GFX purification kit (GE Healthcare, Wisconsin) as per manufacturer's specifications.

2.2.6.2 Restriction digest of pCR[®]T7/NT-TOPO[®] vector.

The same two step procedure as described in section 2.2.6.1 was performed using the BamHI and EcoRI enzymes to digest 60 ng of the pCR[®]T7/NT-TOPO[®] vector (Invitrogen Corp., California). The digest was then resolved on a 0.8 % agarose at 7V/cm 100 V for 45 min. Digested vector was excised and purified with the GFX purification kit (GE Healthcare, Wisconsin).

2.2.7 Construction of a pCRT7/NT-TOPO-AtBRI1 expression vector.

2.2.7.1 Ligation of AtBRI1 into pCR[®]T7/NT-TOPO[®] vector.

The ligation of AtBRI1 into pCR[®]T7 TOPO[®] vector was performed in a final reaction volume of 50 μ L in an eppendorf tube, with reaction mixture containing: 2 units of T₄ DNA ligase (Fermentas International Inc., Burlington, Canada), 1X Ligase Buffer, 6 ng of EcoRI/BamHI (Fermentas International Inc., Burlington, Canada) digested vector and 42 ng of EcoRI/BamHI digested AtBRI1 PCR product. The reaction mixture was incubated at 4 °C over night. Aliquots were resolved on a 0.8 % agarose at 7V/cm for an hour and the construct excised and purified using the GFX purification Kit (GE Healthcare, Wisconsin). Plasmid DNA was eluted in sterile dH₂O and store at 4 °C short term, -20 °C long term. Recombinant vectors were confirmed by sequencing and PCR with gene specific primers as described in section 2.2.5.1.

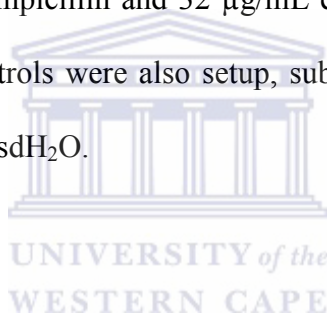
2.2.8 Preparation of an expression host.

2.2.8.1 Transforming pCRT7/NT-TOPO-AtBRI1 construct into *E.coli*

BL21(DE3)pLysS.

Expression host of choice, *E.coli* BL2 (DE3) pLysS (Invitrogen Corp., California) competent cells were transformed with 10ng of pCRT7/NT-TOPO-AtBRI1 in a 100 μ L reaction.

This was heat shocked at 37 $^{\circ}$ C for 5 minutes and was supplemented with 900 μ L of Luria Broth (LB) (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl and 0.4 % glucose) and cells grown at 37 $^{\circ}$ C, with shaking for 1 hour. Aliquots of 100 μ L were spread plated on LB agar supplemented with 100 μ g/mL ampicillin and 32 μ g/mL chloramphenicol, and incubated at 37 $^{\circ}$ C over night. Negative controls were also setup, substituting recombinant vector with either non-recombinant vector or sdH₂O.



2.2.8.2 Colony PCR.

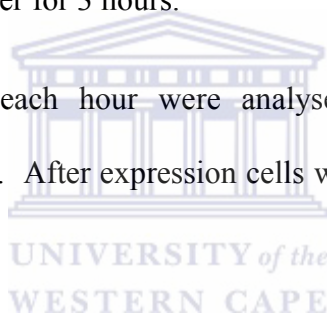
Selected colonies were resuspended in 10 μ L of sdH₂O, boiled at 94 $^{\circ}$ C for 2 minutes and 1 μ L of the lysate was used as a template in gene specific primer verification PCR. Cycling conditions were as described in section 2.2.5.2. Positive colonies were subsequently inoculated in ten 10 mL of LB, supplemented with 100 μ g/mL ampicillin and 32 μ g/mL of chloramphenicol and these were grown over night at 37 $^{\circ}$ C and glycerol stocks were prepared for long term storage at -80 $^{\circ}$ C.

2.2.9 Heterologous expression of the AtBRI1 recombinant protein.

2.2.9.1 Culture enrichment and induction.

Ten mL of LB containing 100 µg/mL of ampicillin and 32 µg/mL chloramphenicol were inoculated with 200 µL of cells carrying the pCR T7/NT-TOPO-AtBRI1 construct and grown at 37 °C over night with shaking at 200 rpm. Following over night incubation, enriched culture was used to inoculate 2 ml into 500 mL of fresh LB supplemented with 100 µg/mL ampicillin and 32 µg/mL chloramphenicol. Inoculated culture was allowed to grow to OD₆₀₀ of 0.5 at which expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and allowed to grow further for 3 hours.

Aliquots from time zero and each hour were analysed on sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). After expression cells were harvested by centrifugation at 12 x 4 g for 30 minutes at 5 °C.



2.2.10 Purification of recombinant pCR T7/NT-TOPO-AtBRI1.

Recombinant pCR T7/NT-TOPO-AtBRI1 was purified under denaturing conditions as described in (Kwezi *et al.*, 2007). Cells were lysed in buffer (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-Cl; pH 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol, and 15 % (v/v) glycerol, 45 mM Imidazole), cellular debris were then separated through centrifugation and the lysate kept for downstream purification. Cleared lysate was purified with 50 % NI-NTA slurry according to the QIAexpressionist denaturing protocol (Qiagen, Venlo, Netherlands). In a 50 mL polypropylene tube 5 mL of NI-NTA was mixed with 5 column volumes (25 mL) of cleared lysate and this was mixed at room temperature for 1 hour.

Unbound protein was washed off with a buffer containing 8 M urea, 100 mM NaH₂PO₄, 10 mM Tris-Cl; pH 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol and 15 % (v/v) glycerol, 45 mM Imidazole, pH 7.0. Bound protein was refolded through a controlled gradient system, using Fast Protein Liquid Chromatography (FPLC) (GE Healthcare, Wisconsin). The gradient gradually reduces the urea concentration and replaces it with refolding buffer [200 mM NaCl, 50 mM Tris-Cl; pH 8.0, 500 mM glucose, 0.05 % (w/v) poly-ethyl glycol (PEG), 4 mM reduced glutathione, 0.04 mM oxidized glutathione, 100 mM non-detergent sulfobateine, and 0.5 mM phenylmethanesulfonylfluoride (PMSF)]. Recombinant protein was eluted and subsequently de-salted and concentrated using Centriplus filtration devices (Millipore Corp., Massachusetts).

2.2.11 Cyclic nucleotide activity assays on the recombinant AtBRI1 catalytic.

2.2.11.1 Enzyme immunoassay.

Enzymatic activity of the recombinant AtBRI1 catalytic domain to convert GTP to cGMP was assessed with two methods, a cGMP enzyme immunoassay Biotrak (EIA) system (GE Healthcare, Wisconsin) and Mass Spectrometry (Kwezi *et al.*, 2007). To determine activity 10 µg of purified recombinant AtBRI1 were assessed in a 50 µL a reaction system containing 50 mM Tris-HCl; pH 8.0, 2 mM IBMX, 5 mM Mg²⁺ and/or 5 mM Mn²⁺ and 1 mM GTP. Reactions were incubated at room temperature for 5 to 20 minutes, followed by termination of the reaction by adding 4 mM EDTA and rapid chilling at 4 °C for 10 minutes. Cyclic nucleotide levels were assessed using the cGMP enzyme immunoassay Biotrak (EIA) system based on the acetylation protocol as is described in the manufacturer's manual (GE Healthcare, Wisconsin).

2.2.11.2 Mass spectrometry.

Mass spectroscopic determinations of cGMP were done with a Waters API Q-TOF Ultima in the W-mode. The samples were introduced with a Waters Acquity UPLC (Waters Microsep, Johannesburg, South Africa) at a flow rate of 180 mL/min and separation was achieved by a Phenomenex Synergi (Torrance, California) 4 mm Fusion -RP (250x62.0 mm) column. A gradient of solvent “A” (0.1 % formic acid) and solvent “B” (100 % Acetonitrile) over 18 minutes was applied. During the first 7 minutes the solvent composition was kept at 100 % “A” followed by a linear gradient of 3 minutes to 80 % “B” and re-equilibration to the initial conditions. Electrospray ionisation in the negative mode was used at a cone voltage of 35 V. The running parameters were optimised for sensitivity and specificity.



2.3 Results.

The design of a search motif that can specifically identify GC's was based on the assumption that the catalytic centre of known GCs should at least in part be conserved across kingdoms. Consequently a search motif (Figure 2.3.1 A) based on several functionally assigned amino acids in the catalytic domain of known GCs from lower eukaryotes and animals was designed (Ludidi and Gehring, 2003a). Two of the seven candidate proteins are annotated kinase (Figure 2.3.1 B) and one of the two (At1g79680) belongs to the group of wall associated kinase-like proteins (WAKLs) that are closely related to the wall-associated kinases (WAKs) (Verica and He, 2002). A BLAST search against the NCBI database with the catalytic centre containing the immediate fourteen N-terminal and thirteen C-terminal amino acids of both candidate kinase-GC identified over 50 plant kinases with e-values < 0.001. The alignment (Figure 2.3.1 B) suggests firstly a high degree of conservation at the catalytic centre and secondly, that none of these kinase molecules contains an asparagine [D], aspartic acid [N] or alanine [A] in the seventh position of the GC motif which is proposed to be responsible for the stabilisation of the dimer interphase. Instead, these molecules contain a [L] and when [D] in position 7 is substituted by [L] in a 100 amino acid recombinant AtGC1 (1–100), no significant loss in catalytic GC activity was observed (Kwezi *et al.*, 2007).

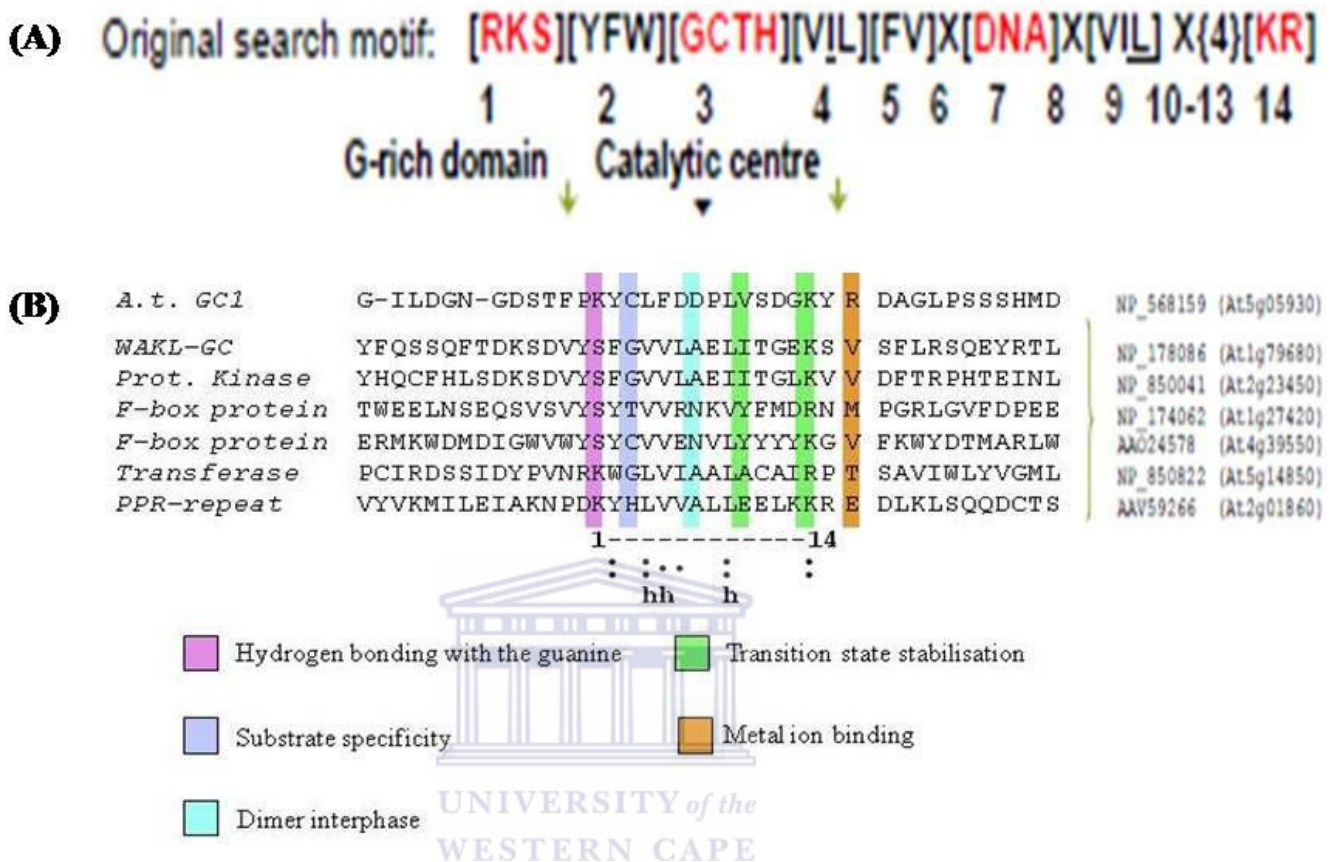


Figure 2.3.1 Alignment of *Arabidopsis* GC catalytic domains with the original search motif. (A) The 14 amino acid long original search motif. Red amino acids are functionally assigned residues of the catalytic centre. The residue in position 1 does the hydrogen bonding with the guanine, the amino acid in position 3 confers substrate specificity and the residues in positions 10 and 14 stabilise the transition (GTP/cGMP). Amino acid substitutions are represented by [], X= any amino acid and {n} is number of amino acids. (B) Alignment of seven catalytic domains that were identified with the original search motif.

A query of the *Arabidopsis* genome with the extended motif (Figure 2.3.2 A) identifies 123 *Arabidopsis* protein including the Brassinosteroid receptor AtBRI1 (*Arabidopsis thaliana* Brassinosteroid insensitive 1 (At4g39400.1)). The Brassinosteroid receptor is a members of the family of leucine rich repeat receptor-like kinases (LRR RLKs) (Li and Chory, 1997) and has an architecture as depicted in Figure 2.3.2 B. Closer inspection of the receptor reveals

that the GC catalytic center is embedded within the cytosolic kinase domain, a characteristic that is also observed in AtWAKL10 (Meier *et al.*, 2010).

Brassinosteroids, are physiologically well characterised growth regulators and their receptors have been identified in several other species and these also contain the conserved GC motif (Figure 2.3.2 D). Considering that the derived search motif is based on functionally assigned amino acids in the catalytic center of several GC's, AtBRI1 was chosen as a good model to investigate whether the extended GC motif can identify an active GC.

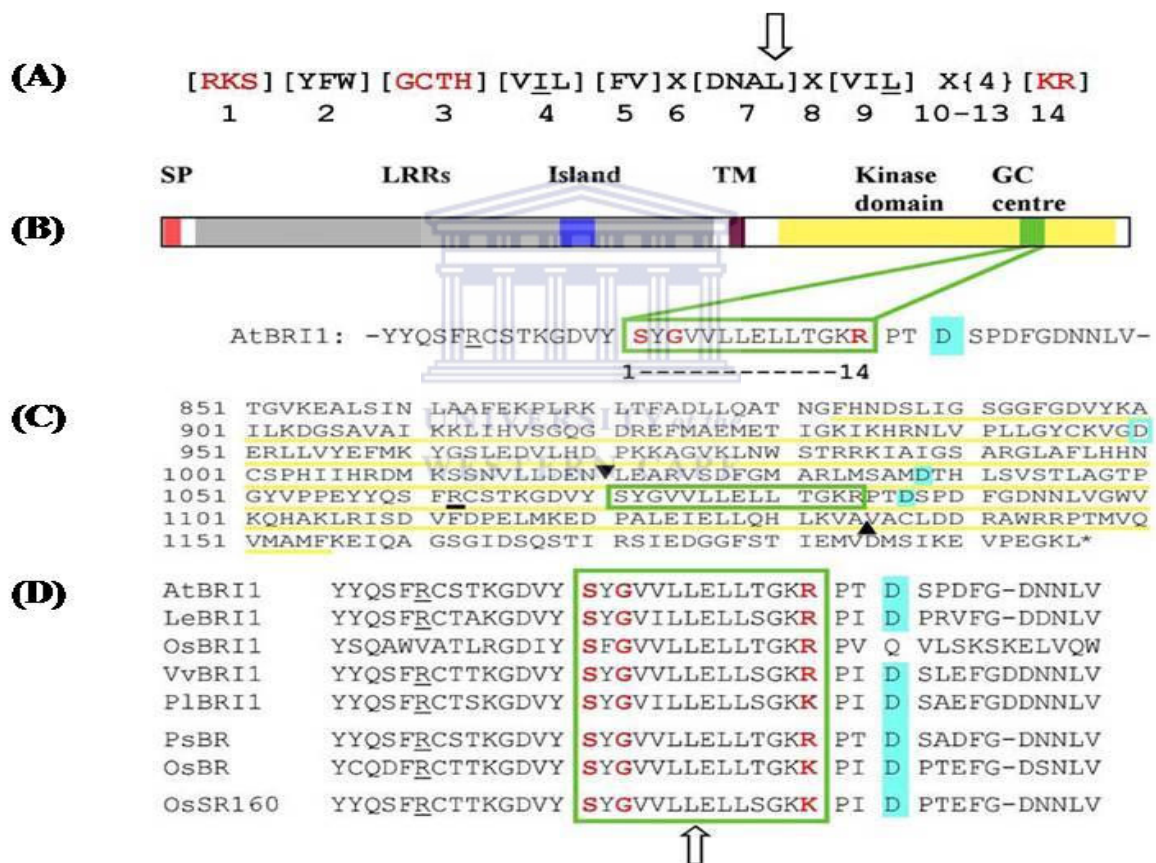


Figure 2.3.2 Structural features of the GC catalytic domain and the *Arabidopsis thaliana* Brassinosteroid receptor (AtBRI1). (A) The 14 amino acid long original search motif (modified after (Ludidi and Gehring, 2003b) with an inclusion of [L] in position 7). Red amino acids are functionally assigned residues of the catalytic centre. The residue in position 1 does the hydrogen bonding with the guanine, the amino acid in position 3 confers substrate specificity and the residues in positions 10 and 14 stabilise the transition (GTP/cGMP). (B) Representation of the domain organisation of AtBRI1 containing a signal peptide (SP), leucine rich repeats (LLRs) including an island, a transmembrane domain (TM), a GC centre embedded in the kinase domain. The position (16 or 17) outside the catalytic centre is implicated in Mg^{2+}/Mn^{2+} -binding (aquamarine). (C) Amino acid sequence of the intracellular C-terminal region of AtBRI1. The kinase domain is underlined (yellow),

the GC domain is boxed in green, putative Mg²⁺/Mn²⁺-binding sites are highlighted (aquamarine), the proposed PPi binding is underlined in black, and the recombinant protein (AtBRI1-GC) tested for GC activity *in vitro* is delineated by solid triangles (*s*). **(D)** Alignment of AtBRI1-like sequences. AtBRI1 (At4g39400), LeBRI1 (tomato|TC185049, Q9LJF3), OsBRI1 (Os06g0691800), VvBRI1 (grape |TC70352, Q9ZWC8), PIBRI1 (poplar|TC57820, Q9ZWC8), PsBR (BAC99050), OsBR (Os08g25380), OsSR160 (BAD34326.1, AP006156.2). doi:10.1371/journal.pone.0000449.g002.

To test whether a candidate protein that has been identified and conforms to the criteria as specified by the extended search motif (Figure 2.3.2.A), the catalytic domain fragment of the AtBRI1 gene was isolated from genomic DNA (Figure 2.3 A) and cloned into pCR T7/NT-TOPO. The recombinant vector was transformed into BL21 (plysS) (*DE3*) and positive transformants were confirmed with gene specific PCR (Figure 2.3 B) and sequencing.

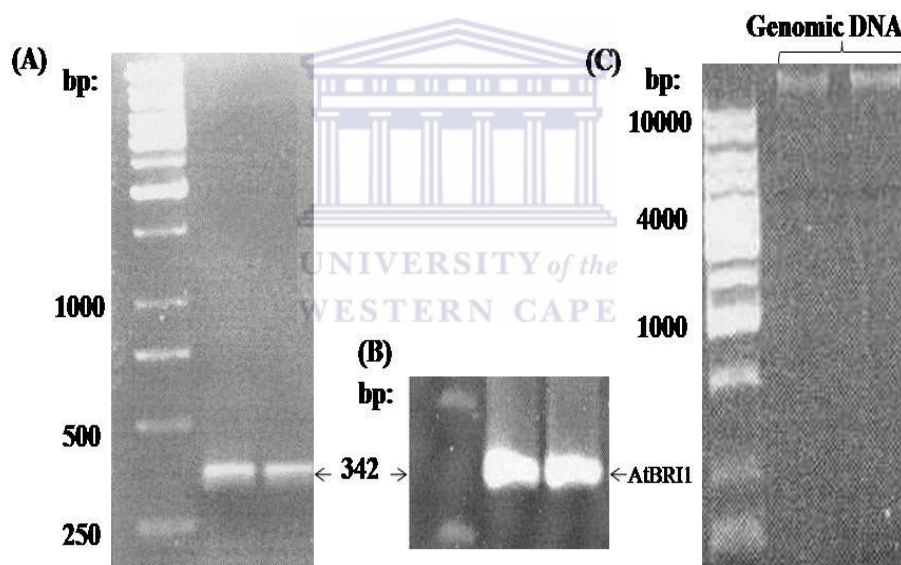


Figure 2.3.3 Isolation of AtBRI1-GC and preparation of expression vector. Genomic DNA (C) was extracted from Arabidopsis and used as a template to specifically amplify the 342 base pair long gene fragment (A). The gene was cloned into of pCR-T7/NT-TOPO (Invitrogen). The construct was used to transform E. coli BL21 (pLysS) (*DE3*) (Invitrogen). **(B)** Verification of recombinant vector using gene specific primer was subsequently used to identify cell that were successfully transformed with the recombinant pCR T7/NT-TOPO-AtBRI1.

The recombinant protein that was synthesised and tested for *in vitro* activity contains the GC catalytic centre as predicted for AtBRI1 (At4g39400) and 50 additional amino acids on both the N-terminus and the C-terminus (Fig. 2.2 C). The 114 amino acid long peptide (AtBRI1-

GC) is part of the cytoplasmic kinase domain containing the N-terminal part aspartic acid ([D] at 233 from the catalytic centre) implicated in metal binding (Tang and Hurley, 1998) as well as a metal binding [D] in position 17 relative to the C-terminus of the motif.



The expression vector that was used allows expression of a His-tagged recombinant protein. The protein was over-expressed and purified from inclusion bodies (Lilie *et al.*, 1998; Suttnar *et al.*, 1994) under denaturing conditions and purified on an NI-NTA affinity column.

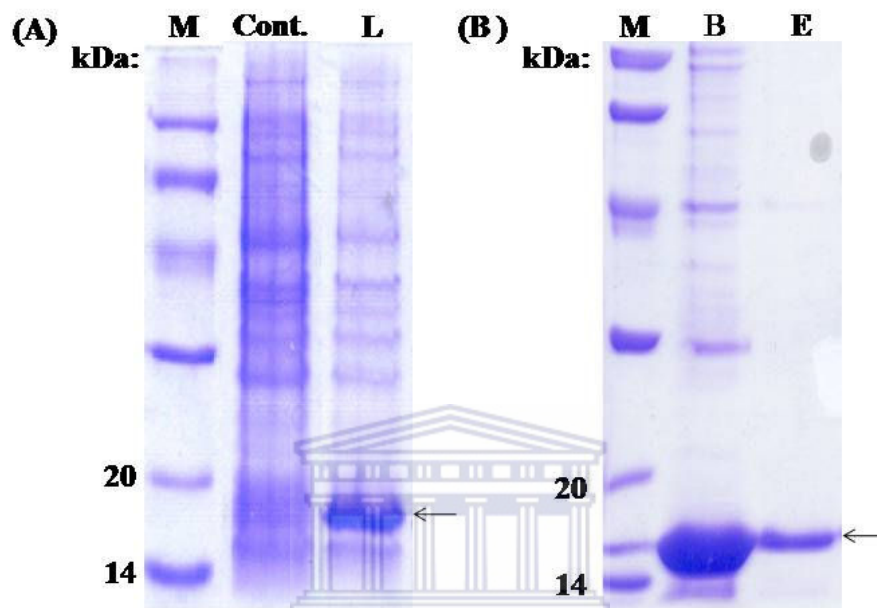


Figure 2.3.4 Expression and purification of recombinant AtBRI1-GC. (A) SDS PAGE analysis of uninduced (Cont) and induced (L) proteins from *E. coli* BL21 (pLysS) (*DE3*) (Invitrogen) harbouring the pCRT7/NT-TOPO-AtBRI1 expression construct. (B) Purification of the recombinant AtBRI1 at different steps (B) is protein bound to Ni-NTA agarose matrix, (E) is the eluted protein after it had been refolded. The arrows mark the recombinant AtBRI1 bands while (M) represents the low molecular weight markers.

The results indicate that the recombinant protein can cyclase GTP and does so preferably in the presence of Mg^{2+} (Fig. 2.3.5 A). In order to verify the result obtained with this anti-body based detection method we also used mass spectrometry. Firstly, we established that the Q-TOF mass chromatogram could detect cGMP at fmol concentrations (Fig. 2.3.5 D, right inset) much like the enzyme immunoassay. We detected neither cGMP in the solution containing the recombinant protein only (Fig. 2.3.5 B) nor in the reaction mix in the absence of the protein (Fig. 2.3.5 C).

The recombinant generates cGMP in a time dependent way (Fig. 2.3.5 D), within just after 5 minutes of incubation in the presence of 1 mM GTP, 100 fmoles cGMP/mg and increased amounts after 20 minutes (3 pmoles cGMP/mg protein (Fig. 2.3.5 D).

Notably, the values of the amount of cGMP generated, as obtained with the mass spectroscopy read higher than those obtained with the enzymatic assay and this observation has been made consistently in independent *in vitro* experiments with recombinant proteins (Kwezi *et al.*, 2007). In addition, it has been noted that plant GC activities are reportedly low and not at the levels observed for some soluble animal GCs (Newton *et al.*, 1999). The proposed reason for this is that higher activities may require co-factors (e.g. Ca^{2+} , chaperones or co-proteins) or post-translational modifications that do not occur in the recombinant tested *in vitro*. Mass spectrometric analysis was also used to assess the capacity of AtBRI1 recombinant to act as an adenylyl cyclase in the presence of 1 mM ATP as the substrate and no significant amounts of cAMP were generated after 20 minutes (Kwezi *et al.*, 2007). These observations indicate that, at least *in vitro*, the recombinant protein has the predicted substrate preference for GTP rather than ATP.

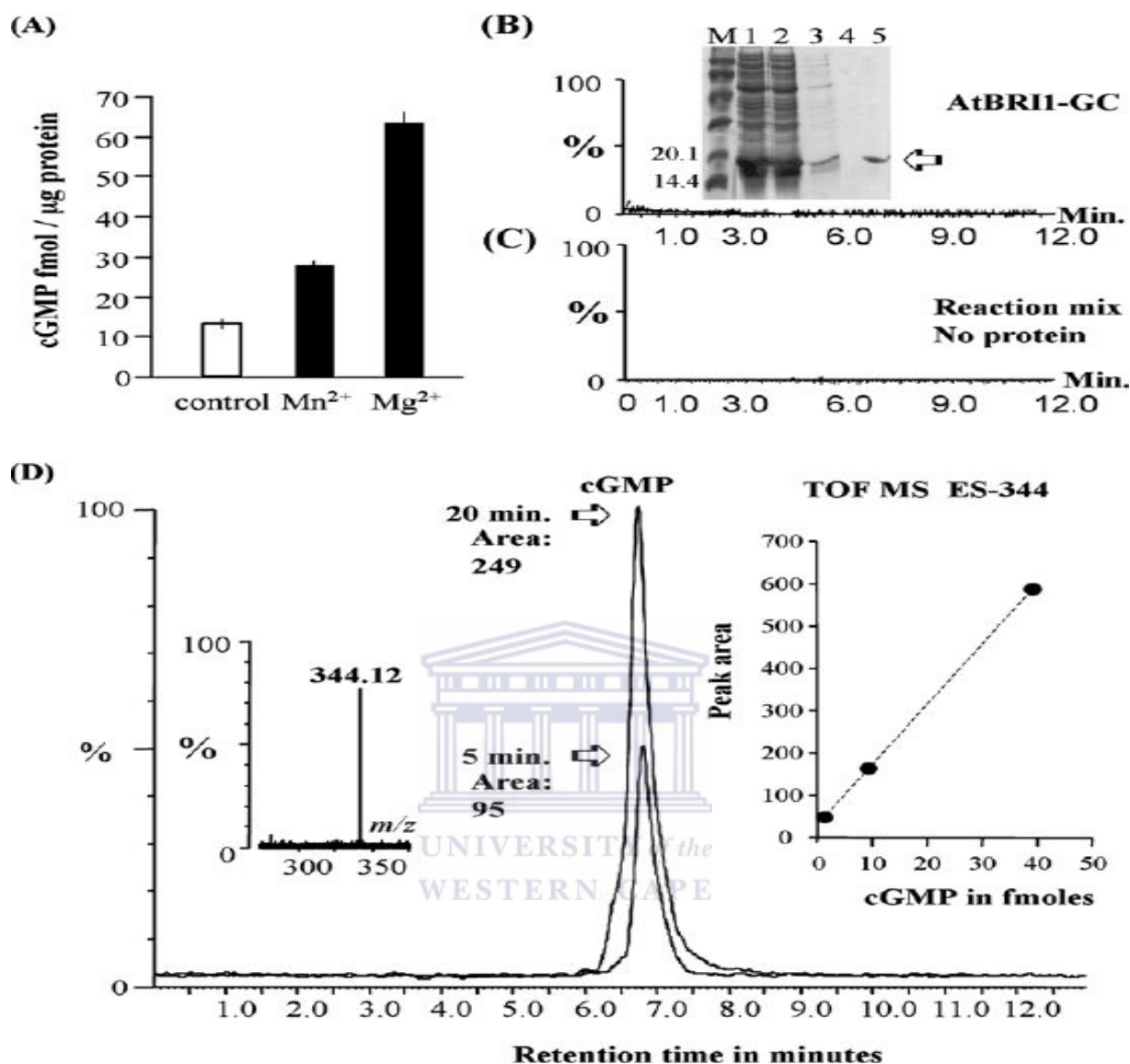


Figure 2.3.5 Preparation of recombinant AtBRI1-GC and functional testing *in vitro*. (A) Testing of GC activity with an enzyme immunoassay. The control contains the reaction mixture without the substrate (10 mg recombinant protein in 50 mM Tris-HCl (pH 7.5), 2 mM isobutyl methylxanthine (IBMX), 5 mM Mg²⁺ and 5 mM Mn²⁺), the other columns represent cGMP generated in the presence of 1 mM GTP and either Mn²⁺ or Mg²⁺ after 20 min. The bar values represent the mean (+/2SEM). (B) Extracted mass chromatogram of *m/z* 344 [M-1]21 ion of cGMP generated by 10 mg recombinant protein. The inset shows an SDS-PAGE of AtBRI1-GC expressed in *E. coli* BL21 (pLysS) (*DE3*) and purified with Ni-NTA agarose under denaturing conditions. Cleared lysate (lane 1), flow through (lane 2), first wash (lane 3), second wash (lane 4) and eluted recombinant protein (lane 5). ‘M’ is the molecular weight marker. (C) Extracted mass chromatogram of *m/z* 344 [M-1]21 ion of the reaction mix without AtBRI1-GC. (D) Two superimposed extracted mass chromatogram of *m/z* 344 [M-1]21 ion of cGMP generated by 10 mg recombinant protein after 5 and 20 min. respectively in the presence of 5 mM Mg²⁺. (Note that the sample was diluted 200 times as compared to the experiment presented in Fig. 2A). The left inset represents the mass of the peak in the chromatogram, the right inset is the calibration curve with 1.25, 10 and 50 fmoles on the column. doi:10.1371/journal.pone.0000449.g003 Brassinosteroid Receptor PLoS ONE.

2.4 Discussion

Brassinosteroids (BRs) are polyhydroxylated plant steroid hormones and have been shown to induce diverse cellular responses such as stem elongation, pollen tube growth, leaf bending (Clouse *et al.*, 1996; Mandava 1988). Brassinosteroid Insensitive-1 (BRI1) was first identified from mutant analysis and then cloned and found to be a leucine rich repeat receptor like kinase (Li and Chory, 1997) located in the plasma membrane (Friedrichsen *et al.*, 2000). Based on the binding of the ligand BR to the leucine rich repeat extracellular domain, BRI1 has been proposed as a BR receptor in Arabidopsis (Kinoshita *et al.*, 2005; Wang *et al.*, 2001) and therefore is potentially a critical signal component. AtBRI1 is ubiquitously expressed in Arabidopsis and potential AtBRI1 kinase substrates have been identified such as transthyretin-like protein which is phosphorylated *in vitro* by the kinase domain of AtBRI1 (Nam and Li 2004). Several models have been developed to describe the signalling events following perception of BR by AtBRI1 (Goda *et al.*, 2002) involving other membrane associated proteins and activation of transcription factors (Wang *et al.*, 2006). The observation that AtBRI1 harbours a functional GC domain within the cytosolic part of the molecule suggests that cGMP is a second messenger in some BR dependent processes. However, this hypothesis remains to be tested. Several genes that regulate physiological functions are stimulated by BR and these may be dependent on the generation of cGMP. An example for this dual dependence is plant cell elongation (Haubrick and Assmann, 2006). Microarray studies reveal that genes involved in cell wall expansion such as Expansins and Pectinesterases are up-regulated by both BR (Goda *et al.*, 2002) and membrane permeable cGMP treatments (Maathuis 2006). Both BR and Gibberellin interact to regulate plant growth and it is conceivable that both hormones signal via cGMP. Some of these interactions are antagonistic but in other cases, BR can potentiate Gibberellin activity (Bouquin *et al.*, 2001).

Gibberellin itself causes increases in cGMP (Penson *et al.*, 1996). It is possible that in some instances the GC domain of AtBRI1 could stimulate cGMP production and so potentiate gibberellin activity. On a speculative note, there may be key molecules within specific cells that specify decreased cytoplasmic kinase activity and enhance the GC activity of the AtBRI1 receptor. There are several recessive alleles of AtBRI1 with mutations in the cytoplasmic kinase domain. One of these mutants, *bri-101* is the only mutant in the GC catalytic region (Glutamic acid [E] at position 1078 to a Leucine [L]) and it is insensitive to BR and also has reduced kinase activity when tested in a heterologous system (Friedrichsen *et al.*, 2000; Li and Chory, 1997). This mutation should not affect the GC activity as it occurs at position 8 which can be any amino acid. Three other mutants have been found in the region that is shown to confer GC activity *in vitro* and they are : *bri-103,104* from an Alanine [A] at position 1031 to a Threonine, *bri-105-107* has a Glutamine [Q] at position 1059 which introduces a stop codon (which would exclude the GC catalytic domain from the truncated protein) and *bri-115* mutates from a Glycine [G] at position 1048 to an Aspartic acid [N] (Friedrichsen *et al.*, 2000). The GC domain, in AtBRI1 that has been identified occurs within the kinase domain (Friedrichsen *et al.*, 2000). We demonstrate that the isolated 114 amino acid recombinant peptide (AtBRI1-GC) has GC activity *in vitro* (Fig. 2.3.5 A). The relative importance of the two functions in the action of the receptor remains to be demonstrated bearing in mind that previously work has focused on the kinase domain as the GC domain had not been identified. Interestingly, a number of enzymes have recently been identified as “moonlighting” proteins with dual functions (Jeffery, 2003); the kinase and GC activity of AtBRI1 could be yet another example. On a more general level, the finding implies that functional GC domains may be part of a large variety of different multifunctional signalling molecules and receptors in particular.

It is noteworthy that the atrial natriuretic peptide receptors NPR1 and NPR2 both signal through cGMP and have an AtBRI1-like domain organisation with an extracellular ligand-binding domain, a transmembrane domain and an intracellular kinase and GC domain (Chinkers *et al.*, 1989; Garbers and Lowe, 1994). Finally, the fact that two recombinant proteins AtGC1(1–100) and AtBRI1 - GC of less than 120 amino acids have GC activity *in vitro* begs a re-examination of the minimal catalytic requirement for GCs and may suggest that the number of different potentially functional GC domains is significantly higher than currently assumed. This is in keeping with the fact that the single cellular green alga *Chlamydomonas reinhardtii* contains a surprisingly large number (up to 90) annotated putative GCs (Schaap, 2005). It is possible that an increasing number of biological processes will be discovered that are modulated by the second messenger cGMP (Meier *et al.*, 2007).



Chapter 3: The *Arabidopsis thaliana* Phytosulfokine (AtPSKR1) Receptor has dual Enzymatic function *in vitro*.

Abstract.

Cell proliferation is important for the growth and development of plants and this process is mediated by a number of factors. One such factor is the five amino acid peptide Phytosulfokine (PSK). Phytosulfokines (PSKs) are sulphated pentapeptides that stimulate plant growth and differentiation mediated by the PSK receptor (AtPSKR1) which is a leucine rich repeat receptor like kinase. We identified a putative guanylate cyclase (GC) catalytic centre in PSKR1 that is embedded within the kinase domain and hypothesised that the GC works in conjunction with the kinase in downstream PSK signalling. We expressed the complete cytoplasmic kinase domain of AtPSKR1 as a recombinant protein and showed that it has Serine/Threonine kinase activity using the Ser/Thr peptide 1 as a substrate with an approximate K_m of 7.5 μM and V_{max} of 1800 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein. This same recombinant protein also has GC activity *in vitro* that is dependent on the presence of either Mg^{2+} or Mn^{2+} . In addition, we show that the receptor's ability to phosphorylate a substrate is affected by the product (cGMP) of its co-domain (GC) and that the receptor autophosphorylates Serine residues. We have also observed that autophosphorylation is affected by cGMP. Together these results indicate that the AtPSKR1 receptor contains dual GC and kinase catalytic activities that operate *in vitro* and that this receptor constitutes a novel class of enzymes with overlapping catalytic domains that could be co-regulating each other.

3.1 Introduction.

In order for plants to maximize access to essential resources, responses to environmental factors and regulation of physiological processes (e.g. development and growth) and facilitation of cell to cell communication have evolved and they are capable of detecting the direction of incoming physical, chemical, and light signals (Braun and Walker, 1996; Deeken and Kaldenhoff, 1997). Many of these signals are initially perceived by transmembrane receptors, a large number of which function by activation of an intrinsic protein kinase domain activity (Braun and Walker, 1996). A number of these well characterized receptors are kinases (Trewavas and Malho, 1997). Receptor kinases are known to be present and to play important roles in animal cellular signalling processes (Zhang, 1998). In plants, a number of membrane localized receptor proteins with architectures that are characteristic of receptor kinases have been identified (Yin *et al.*, 2002). These are the Receptor-Like Kinases (RLKs) whose sequence homology and structural similarity with that of animal receptor kinases suggests that they have a similar biological function and use similar mechanisms (Zhang, 1998). Receptor Like-Kinases mediate signal transduction of a variety of cellular processes, amongst these are responses to environmental signals such as light (Deeken and Kaldenhoff, 1997) and hormones (Li and Chory, 1997). However even though these receptors share structural similarities current insight indicates that plant RLKs which generally show Serine/Threonine kinase activity have evolved independently of animal Receptor Tyrosine Kinases (RTKs) and Receptor Serine/Threonine Kinases (RSKs) (Johnson and Ingram, 2005). The major sub-group of RLKs are the leucine-rich repeat (LRR) RLKs, which contain imperfect repeats of a 24-amino acid leucine-rich motif in the extracellular domain (Braun and Walker, 1996; Zhang, 1998). The classical model that typically describes kinase activation and subsequent signal transduction as reported by (Tichtinsky *et al.*, 2003; Torii, 2000) includes ligand binding to the extracellular domain which causes the receptor to

dimerise. This in turn triggers the subsequent activation of the intracellular kinase domain. The activated kinases then phosphorylate, by transfer of a phosphate group from a donor, typically a tri-phosphate e.g. Adenosine 5'-triphosphate (ATP) to substrate proteins within the cell. This action may result in transduction of a signal that forms part of the signal transduction cascade. Downstream responses include further activation of other proteins, synthesis and transduction of signals by second messengers which in turn may lead to changes in the patterns and levels of gene expression (Maathuis, 2006). Receptor protein kinases (RPKs) are known to activate a number of intracellular signalling pathways in response to the extracellular environment (van der Geer *et al.*, 1994). Receptor Protein Kinases (RPKs) are single-pass transmembrane proteins that contain an amino-terminal signal sequence, extracellular domains unique for each receptor and a cytoplasmic kinase domain (Shiu and Bleecker, 2001a). Generally in this type of receptor, ligand binding will induce homo or heterodimerization of RPKs, and the resultant close proximity of the cytoplasmic domains results in kinase activation by autophosphorylation and/or transphosphorylation (Friedrichsen *et al.*, 2000) and/or the synthesis of signal transduction intermediates.

In eukaryotes some of the key signalling intermediates are cyclic nucleotides amongst these are cGMP and cAMP. Particularly in higher plants cGMP has been identified as an important molecule involved in regulating a wide variety of physiological effects ranging from chloroplast development, plant hormone dependent responses to the induction of plant defence responses (Kaplan *et al.*, 2007; Newton *et al.*, 1999; Newton and Smith, 2004). Nitric oxide (NO) signalling is thought to stimulate cGMP production in defence and stress responses but how it generates cGMP is still uncertain (Leitner *et al.*, 2009; Wilson *et al.*, 2008). Cyclic GMP can act on cyclic nucleotide gated ion channels (Leng *et al.*, 1999),

various other downstream molecules and the transcriptome (Kaplan *et al.*, 2007; Maathuis, 2006) to modulate plant responses.

In a previous study we hypothesised that several different functional GCs exist in higher plants and embarked on a quest to search for them. In summary, our strategy was based on the assumption that the catalytic centre of known GCs is at least in part conserved across the different kingdoms. Consequently, we designed and tested a search motif based on several functionally assigned amino acids in the catalytic domain of known GCs from lower eukaryotes and animals (Figure 3.3.1 A) and identified and experimentally confirmed the first three molecules with GC activity in higher plants (Ludidi and Gehring, 2003a; Kwezi *et al.*, 2007; Meier *et al.*, 2010). The first GC to be identified in higher plants, AtGC1 is a soluble protein with the GC domain towards the N terminus but does not contain a haem binding motif essential for nitric oxide binding (Ludidi and Gehring, 2003a). Since NO had been shown to stimulate synthesis of cGMP we concluded that other GCs are likely to be present. Recently, a GC1 homologue has been characterised in *Pharbitis nil* (Morning Glory) and the expression of this molecule is regulated by light (Szmidi-Jaworska *et al.*, 2009). The second functional GC was identified by relaxing the initial search motif and, surprisingly, is the Brassinosteroid receptor AtBRI1. The GC domain is found within the intracellular kinase domain and it is this region that has been shown to have activity *in vitro* (Kwezi *et al.*, 2007). AtBRI1 is a leucine rich repeat receptor like kinase (Oh *et al.*, 2009) and was one of several LRR RLKs identified in the screen which all shared a similar kinase-GC domain structure (Kwezi *et al.*, 2007).

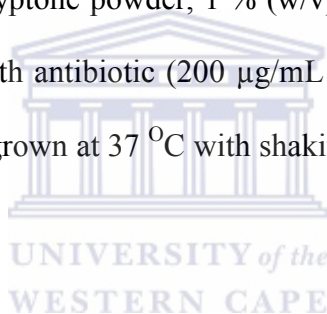
In addition, we have recently reported another membrane associated kinase molecule, the wall associated kinase-like 10 (WAKL10) and shown that it has GC activity *in vitro* as well as being transcriptionally up-regulated in response to biotic stress (Meier *et al.*, 2010). Amongst the other LRR RLKs detected in this screen for novel GCs we identified the

Phytosulfokine (PSK) receptor. AtPSKR1 and its domain organization are shown in (Figure 3.3.1 B). AtPSKR1 is a typical LRR-RLK with an island that binds PSK in its extracellular leucine rich repeat domain, a single transmembrane spanning domain and an intracellular kinase domain (Matsubayashi *et al.*, 2006a; Matsubayashi *et al.*, 2002). AtPSKR1 has been identified in several other species where the GC domain is also present (Figure 3.3.1 C) indicating that this domain organisation is widespread. Mutant studies indicate that AtPSKR1 is involved in regulating root elongation. Here we show that the kinase domain of AtPSKR1 has both kinase and GC activity and that its kinase activity is affected by cGMP *in vitro*. It is a well know characteristic of kinases that they do not only phosphorylate one or more intracellular target proteins (heterophosphorylation) but can phosphorylate themselves, a process termed autophosphorylation (Smith *et al.*, 1993). Protein kinase autophosphorylation is functionally important, since it can also play a major role in the protein kinase function. Therefore, we also tested a possible modulation of the kinase by the cyclic nucleotide. Together the results presented here indicate that AtPSKR1 contains dual functioning GC and kinase catalytic activity and that this type of activity represents a novel class of kinases with overlapping catalytic domains that may co-regulate each other.

3.2 Methods and Materials.

3.2.1 Preparation of the expression host.

The recombinant Gateway[®] vector pDEST-PSKR1-KD2 harbouring the full cytoplasmic kinase domains of AtPSKR1 insert was provided by Dr. Sylvana Iacuone (Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Australia) and processed further as detailed elsewhere (Kwezi *et al*, under revision). The construct was transformed into *E. coli* BL21-AI[™] One Shot[®] Chemically Competent cells (Invitrogen Corp., California) and selected colonies were used for protein expression. Recombinant cells were enriched in 10 mL of double strength yeast-tryptone medium (1.6 % (w/v) tryptone powder, 1 % (w/v) yeast extract, 0.5 % (w/v) NaCl) supplemented with double strength antibiotic (200 µg/mL ampicillin) for stringent selection and propagation. Cultures were grown at 37 °C with shaking on an orbital shaker at 200 rpm over night.



3.2.2 Synthesis of the recombinant AtPSKR1-KD2 and cytoplasmic kinase domains.

3.2.2.1 Expression of kinase domains under native condition.

Following overnight enrichment, 100 mL of double strength yeast-tryptone (YT) medium were inoculated with 2 mL of the overnight culture under “relaxed” antibiotic selection conditions (100 mg/mL of ampicillin). This was grown further at 37 °C with shaking at 200 rpm until an OD₆₀₀ of 0.4 to 0.5 was reached. Cultures were cooled down on ice for 5 minutes and protein expression was induced with 0.2 % L-Arabinose (Sigma-Aldrich Corp., Missouri) and 100 µM isopropyl-β-D-thiogalactopyranoside [(IPTG), (Sigma-Aldrich Corp., Missouri)] and expressed at 30 °C for 8 hours. Aliquots for each hour were analysed on

SDS-PAGE and the rest of culture was harvested by centrifugation at 12 x 4 g for 30 minutes at 5 °C.

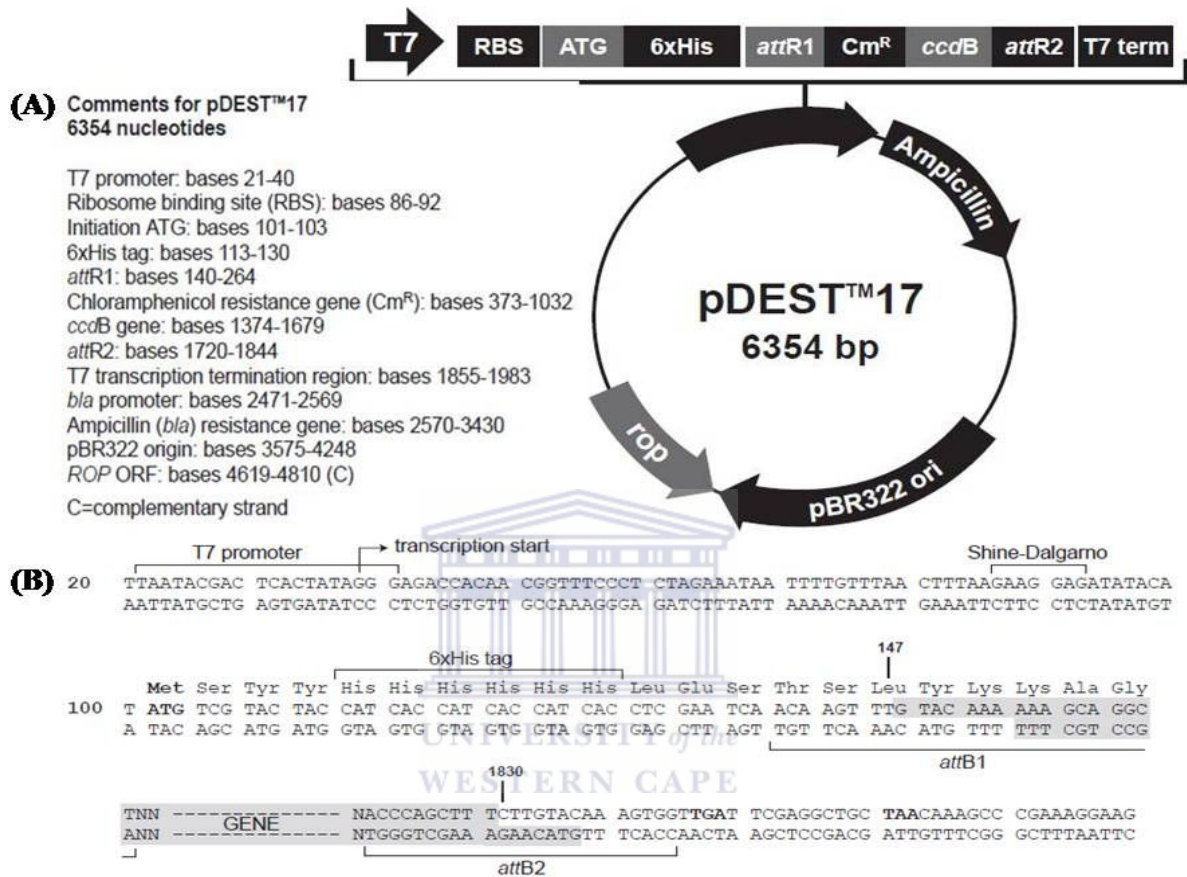


Figure 3.2.1 Structural features of the Gateway® pDEST™17 expression vector. (A) Physical map depicting the elements of the Gateway® pDEST™17 expression vector (Invitrogen Corp., California), which allows expression of a His-tagged fusion protein. (B) The sequence map of the vector shows the cloning site into which the insert was recombined in between the *attB1* and *attB2* and other *cis*-elements. (Figure from Invitrogen Gateway® manual)

3.2.2.2 Protein extraction and preparation of lysate.

Soluble proteins were purified under native conditions on Ni-NTA beads (Qiagen, Venlo, Netherlands) following a modified protocol 12 in the QIAexpressionist manual (Qiagen, Venlo, Netherlands). Harvested cells were resuspended in ice cold Lysis buffer [50 mM NaH₂PO₄:H₂O, 300 mM, NaCl pH 8.0, 45 mM imidazole supplemented with 1 mM PMSF

(phenylmethylsulphonyl fluoride)] and incubated on ice for 30 minutes with 50 µg/mL Lysozyme (Sigma-Aldrich Corp., Missouri). The crude lysate was further homogenised through sonication (5 second pulses for 2 minutes at 50% power on an Ultrasonic homogenizer (Cole-Palmer, Illinois). The lysate was clarified by centrifuging at 12 x 4 g for 10 minutes on a bench top centrifuge at 5 °C. The supernatant was decanted and kept as clarified lysate from which aliquots were analysed on SDS-PAGE.

3.2.2.3 Batch purification of Kinase domain under native conditions.

All steps in the purification procedure were either carried out in a 5 °C cold room or on ice. The recombinant kinase domain was purified under native conditions on Ni-NTA beads (Qiagen, Venlo, Netherlands). A 4 mL volume of 50 % Ni-NTA bead slurry was first equilibrated with 5 X column volumes (20 mL) ice cold Lysis buffer and mixed on a rotary mixer for 30 minutes, beads were collected by centrifugation at 12 x 4 g for 1 minute and the supernatant was discarded as waste, equilibration was repeated three times before the beads were used for purification. In a 50 mL tube, 4 mL of beads were mixed with 5 X column volumes (20 mL) of lysate for 1 hour and beads were collected by centrifugation at 12 x 4 g for 1 minute. The supernatant was decanted and kept as Flow through (FL). The beads were transferred to a Glass Econo-Column (Bio-Rad Laboratories, Inc., California) and unbound protein was washed off three times with 5 X column volumes wash buffer (50 mM NaH₂PO₄:H₂O, 300 mM, NaCl pH 7.0, 45 mM imidazole and 1 mM PMSF). Bound recombinant kinase was then eluted in (50 mM NaH₂PO₄:H₂O, 300 mM, NaCl pH 8.0, 300 mM imidazole and 1 mM PMSF). The eluted protein was concentrated using Centriplus[®] filtration columns (3 000 MWCO PES, Millipore Corp., Massachusetts) by centrifugation (2 hours at 4300×g at 5 °C) and de-salted with washing buffer (20 mM Tris, pH 8.0, 1 mM

PMSF) at $43\,300 \times g$ for 60 minutes at $5\text{ }^{\circ}\text{C}$. The protein concentration was determined using a Quant-iT[™] protein assay kit in a Qubit[®] fluorometer (Invitrogen Corp., California).

3.2.2.4 Cyclic nucleotide assays.

The GC activity of AtPSKR1-KD2 was measured *in vitro* by incubating 10 μg of protein in 50 mM Tris-HCl (pH 8.0), 5 mM MgCl_2 or 5 mM MnCl_2 and 1 mM GTP in a final volume 100 μL and 2 mM isobutyl methylxanthine (IBMX). Incubations were performed for 5, 10, 15 and 20 minutes at room temperature ($\sim 25\text{ }^{\circ}\text{C}$) and terminated by the addition of 10 mM EDTA. Tubes were then boiled for 3 minutes, cooled on ice for 2 minutes and centrifuged at $2300 \times g$ for 3 minutes and the supernatant was kept to assess cGMP content. The cGMP content produced by the recombinant protein was analysed using the Amersham cGMP enzyme immunoassay (EIA) Biotrak[™] System following the acetylation (protocol 4) as described in the supplier's manual (GE Healthcare, Wisconsin). The optical density was read at 450 nm using an Envision 2101 plate reader (Perkin Elmer Inc., Massachusetts). Cyclic GMP levels were then calculated as fmol / μg protein. All assays in the described experiments were carried out in triplicate.

3.2.2.5 Determination of the kinase activity of AtPSKR1-KD2 and the effects of cGMP on Substrate directed phosphorylation.

The kinase activity of the recombinant AtPSKR1-KD2 was assessed *in vitro* by measuring its capacity to phosphorylate a substrate peptide, a fluorophore namely SOX (Ser/Thr peptide 1) (Shults and Imperiali, 2003) as described in the Omnia[™] Ser/Thr-Recombinant Kit1 (BioSource, Nivelles, Belgium; code KNZ2011). Reactions were set up in white FluoroNunc[™] Maxisorp[™] 96 well plates (Thermo Fisher Scientific, Massachusetts).

In a 50 μ L reaction, 1 μ g of purified recombinant AtPSKR1-KD2 was incubated in 1 X reaction buffer (20 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$) with 1 mM ATP, 0.2 mM DTT, with 0 to 50 μ M of Ser/Thr substrate peptide1 to prepare the standard curve. Under the same experimental conditions but in a different reaction system recombinant AtPSKR1-KD2 was incubated with either 0.1 or 1 μ M cGMP followed by measuring the levels of phosphorylation on an Envision 2101 plate reader (Perkin Elmer Inc., Massachusetts) at an excitation of 405 nm (λ_{ex} 405) and emission of 492 nm (λ_{em} 492). Readings were recorded in relative fluorescence units (RFUs) every 10 seconds for 30 minutes at room temperature (~ 25 $^{\circ}C$).

3.2.2.6 Determination of the effects of cyclic nucleotides on the autophosphorylation state.

To determine the effects of cyclic nucleotides on the autophosphorylation state of the kinases and its specificity, cGMP, cAMP and 8-bromo-cGMP (Sigma-Aldrich Corp., Missouri) were used. Experiments were setup in 50 μ L reaction containing 1 X buffer (20 mM Tris-HCl, pH 7.5 and 15 mM $MgCl_2$) and with either 0.05 μ M, 0.1 μ M or 1 μ M of cyclic nucleotide. The reaction mix was incubated at 30 $^{\circ}C$ for 5 minutes and 10 μ g of kinase were added and incubated further for 10 minutes at room temperature (~ 25 $^{\circ}C$). The reaction was initiated by adding 1mM ATP and incubated at room temperature for 30 minutes. Reaction were halted by adding SDS sample buffer [125 mM Tris-HCl pH 6.8, 20 % (v/v) glycerol, 0.01 % (w/v) bromophenol blue, 4 % (w/v) SDS and 200 mM DTT] and inactivated for 5 minutes at 95 $^{\circ}C$. The samples were then cooled down on the bench and then centrifuged at 12 x 4 g for 1 minute. Samples were loaded on a 12 % SDS polyacrylamide gel composed of a 5 % stacking gel [0.5 M Tris-HCl, pH 6.8, 40 % Acrylamide/Bis solution (37.5:1) (Sigma-Aldrich Corp., Missouri) , 10 % (v/v) SDS, 10 % (v/v) APS (Ammonium persulfate) and TEMED

[1,2-Bis(dimethylamino)ethane] (Sigma-Aldrich Corp., Missouri) and 12 % Resolving gel composed of 1.5 M Tris-HCl pH 8.8, 40 % Acrylamide/Bis solution (37.5:1) (Sigma-Aldrich Corp., Missouri) , 10 % (v/v) SDS, 10 % (v/v) APS and TEMED (Sigma-Aldrich Corp., Missouri).

Electrophoresis was carried out in 1 X SDS PAGE running buffer (SDS, Tris, Glycin and H₂O), using a Bio-Rad Mini-Protean 3 electrophoresis module assembly system and a Bio-Rad PowerPac™ Basic power supply. The gels were first run at 100 V for the samples to reach the resolving gel then at 120 V for +/- 45 minutes.

3.2.2.7 Western Blot analysis

The Western blotting experiments were performed to detect possible cyclic nucleotide mediated changes in the autophosphorylation state of the cytoplasmic kinase domain. Proteins were transferred using Protean II™ system (Bio-Rad Laboratories Inc., California) at 100V and 350 amps for 1hour onto PVDF-P nitrocellulose membrane (GE Healthcare, Wisconsin). The membrane was transferred to a clean container and left to dry over night to fix the protein on the membrane. The membranes were briefly rehydrated with 1 X Phosphate Buffered Saline (PBS) (150 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ 1.4 mM KH₂HPO₄, pH 7.3) and placed in blocking solution [1 % (w/v) Bovine serum albumin (BSA), 0.1 % Tween 20 in 1x PBS] for 1 hour. The blocking solution was decanted and the membranes were probed with the primary (1°) antibody solution [1/50000 dilutions of 0.1 µg/mL Anti-Phosphothreonine Mouse mAB and PhosphoDetect™ Anti-Phosphoserine Mouse mAb (Merck®, Darmstadt, Germany) in blocking solution] on a shaker for 2 hours at room temperature (~25 °C). The membranes were then washed in 1x PBS containing 0.1 % (v/v) Tween 20 (PBST) solution for 3 x 5 minutes at room temperature followed by treatment of the membranes with secondary (2°) antibody [1/ 3000 dilutions of 1 µg/µl Anti-Mouse IgG

(H+L), HRP conjugate (Biomol international, U.S.A.) in blocking solution] and incubated on a shaker for 1 hour on a shaking platform at room temperature ($\sim 25^{\circ}\text{C}$) in the dark. The membranes were then washed with PBST for 3 x 5 minutes followed with 1 x 5 minutes with PBS. Detection was done by pouring 5 mL of 3,3',5,5'-Tetramethylbenzidine (TMB)(Millipore Corp., Massachusetts) and the signal was developed for 5 minutes by monitoring visually until signal strength was sufficient for protein bands to be detected.



3.3 Results.

The domain organization of both AtPSKR1-KD2 is a typical of LRR RLKs with a highly conserved leucine rich repeat domain in the extracellular space with a single transmembrane domain and a carboxyl-terminal intracellular kinase (Shiu and Bleecker, 2001a; Torii, 2004) which forms the majority of the intracellular protein (Shiu and Bleecker, 2001a). The protein has similar domain organisation as reported for AtBRI1 (Kwezi *et al.*, 2007) in that a putative GC catalytic centre is also found in the latter part of the kinase domain just downstream of the key kinase catalytic residues and the predicted activation loop (Figure 3.3.1 B)



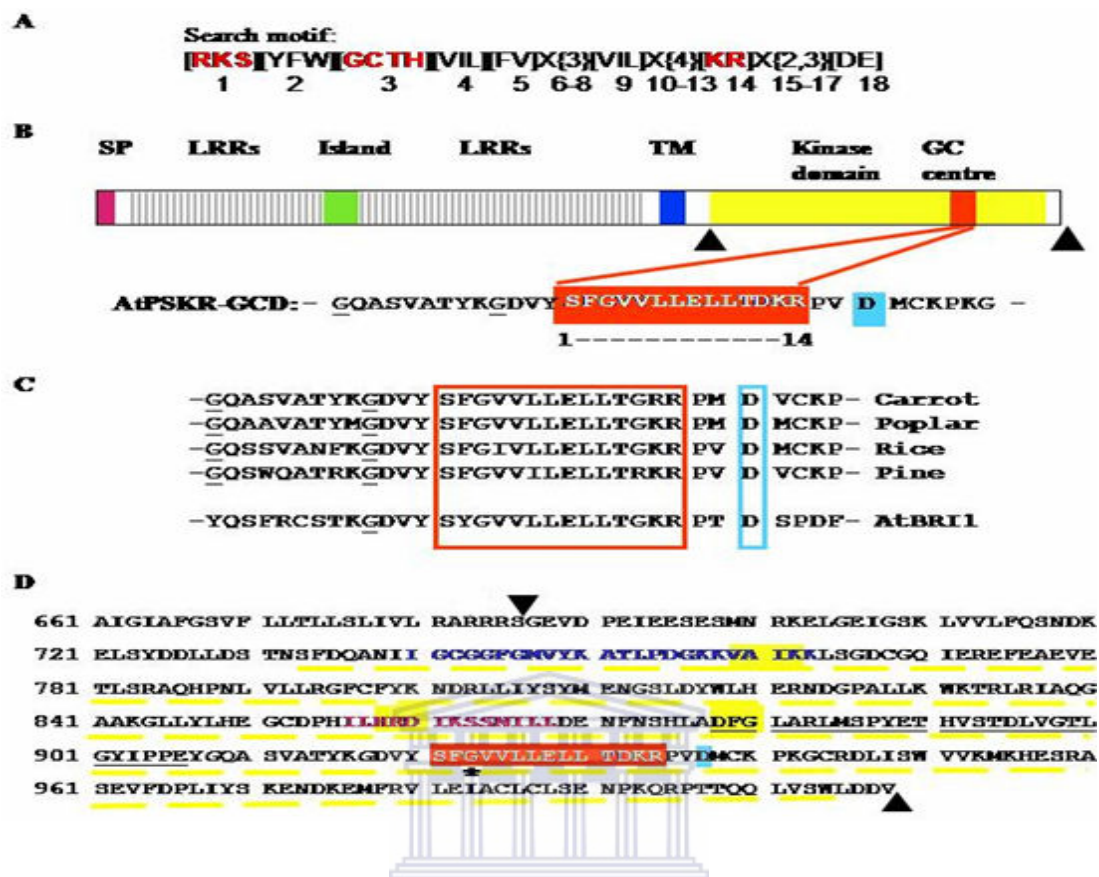


Figure 3.3.1 Structural features of the guanylate cyclase catalytic domain of AtPSKR1. (A) The 14 amino acid core catalytic centre motif generated diagnostic for guanylate cyclases (GC) is found in the cytoplasmic carboxyl terminus of AtPSKR1 starting at amino acid number 920. Amino acids in red are functionally assigned residues in the catalytic centre. Square brackets ([]) contain different possible amino acids in this position, “X” represents any amino acid and the number in the curly brackets ({}) indicates the number of residues that could be any amino acid. The search motif has been extended at the C terminal to include at position 17/18 [D] or [E] proposed to be involved in metal (Mg^{2+} / Mn^{2+}) binding. (B) Representation of domain organisation of AtPSKR1 containing a signal peptide (SP, magenta), an extracellular domain (grey) leucine rich repeats (LRR) region inclusive of an island (green) region, a transmembrane domain (TM, blue), and a GC catalytic centre (red) embedded in the kinase domain (yellow). The glutamic acid at position 17 is implicated in Mg^{2+} / Mn^{2+} binding (aquamarine). The glycine residues (underlined) N-terminal to the catalytic centre may have a role in stabilizing the centre. The two black triangles indicate the kinase domain that was expressed as a recombinant protein. (C) Alignment of the GC catalytic centres of PSKR1 (AT2G02220) orthologues present in genomes of other plant species: *Daucus carota*, BAC00995.1; *Populus trichocarpa*, XP_002312507.1; *Oryza sativa*, OS02G0629400; *Sorghum bicolor*; XP_002454207.1. Also aligned is the functionally confirmed GC catalytic centre of AtBRI1 (AT4G39400). The putative GC catalytic domain is highlighted in the red box and the metal binding site in the blue box and the N terminal glycine residues are underlined as they can a characteristic feature of GCs. (D) The amino acid sequence of the intracellular C terminal region of PSKR1. The two black triangles demarcate the kinase domain that was cloned into pDEST17 for expression of the recombinant AtPSKR1 kinase domain (PSKR1-KD2) His-tag protein. The sequence highlighted in red represents the GC catalytic centre with the aquamarine being the metal binding residue. The sequences highlighted in yellow indicate amino acids that form essential components of the kinase catalytic domain and the black underlined sequence indicates the activation loop. The asterisk points to the position that was mutated from glycine (G) to a lysine (K). The sequences in blue indicate the protein kinase ATP-binding region signature and those in pink the Serine / Threonine protein kinases

active-site signature (prosite scan (November 1994) and the yellow underlined sequence corresponds to the complete kinase domain.

To test if the putative GC domain in AtPSKR1-KD1 was functional a pDEST17 construct that contains the complete intracellular domain of AtPSKR1 (AtPSKR1-KD2) which constitutes the kinase domain was used to express the recombinant protein in BL21-AI cells as an N terminal His-tag protein with a predicted molecular weight of 38.9 kDa (Figure 3.3.2). The recombinant protein was expressed in BL21-AI cells as an N terminal His-tag protein and purified under native conditions.

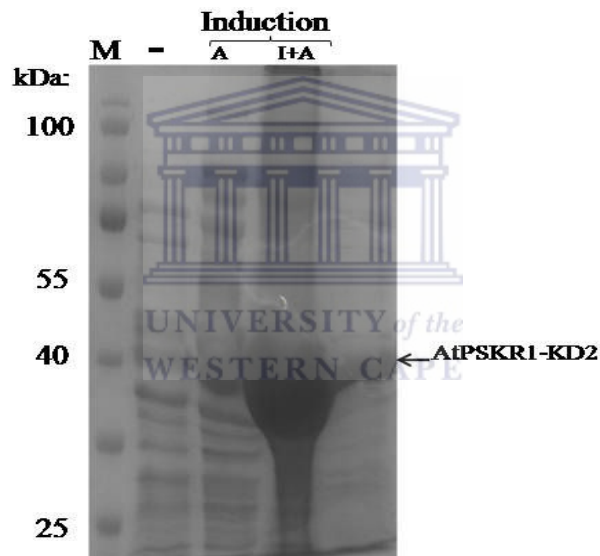


Figure 3.3.2 Expression and purification of the complete cytoplasmic domain of AtPSKR1-KD. AtPSKR1-KD2 was cloned into pDEST17 (Invitrogen Corp., California) to express it as a N-terminal His-tagged protein. The vector contains an IPTG inducible promoter, the expression hosts that was used BL21-AI (Invitrogen Corp., California) in addition has a genomic insertion of an Arabinose inducible promoter. The expression of the fusion protein was done under two condition i.e induced with 0.2% L-Arabinose (**I**) only at 30⁰C shaking 200 rpm. Expression was also done under the combination of 100µM IPTG and 0.2 % L-Arabinose (**AI**) at 30⁰C, shaking at 250 rpm. The SDS PAGE shows the uninduced (-) and both expression conditions. Purification was under native conditions on a Ni-NTA matrix (Qiagen, Venlo, Netherlands), the arrows marks the purified recombinant AtPSKR1-KD2 bands with (M) representing the low molecular weight marker (Fermentas International Inc., Burlington, Canada).

To test if the kinase and GC domain were both functional, the constructs were also used for *in vitro* GC and phosphorylation studies. First the GC activity was assessed and the obtained

results demonstrate that AtPSKR1-KD2 also exhibited GC activity as shown for AtBRI1 (Kwezi *et al.*, 2007) (Figure 3.3.3).

Unlike the AtBRI1 GC domain, the AtPSKR1 GC catalytic center shows no preference in metal ion selectivity between Mn^{2+} or Mg^{2+} ions however the presence of one or the other metal ions was essential to observe activity (Figure 3.3.3).

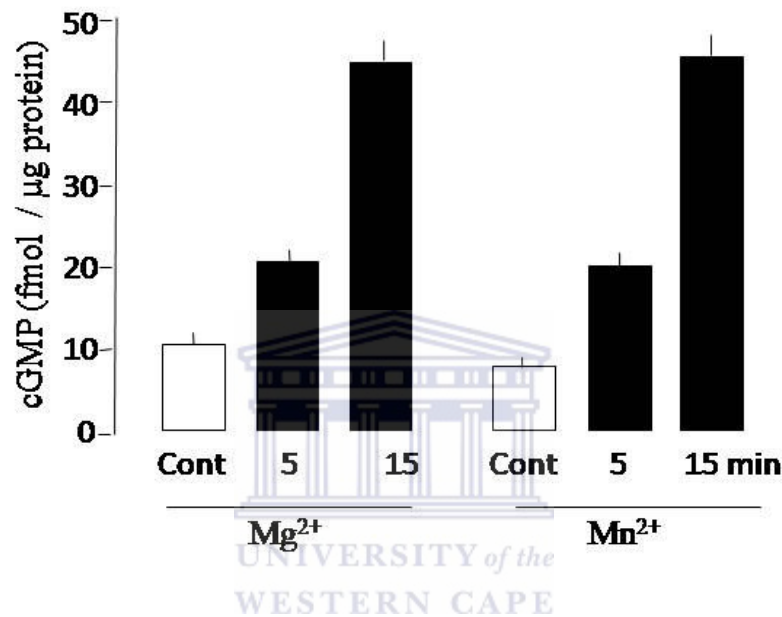


Figure 3.3.3 Demonstration of GC activity of the complete cytoplasmic domain of PSKR1-KD2. GC activity of PSKR1-KD2 (residues 686 to 1008). The control contained all reaction components without the protein and reactions contained 10 μ g protein with 1 mM GTP in the presence of either 5 mM Mn^{2+} or Mg^{2+} . The cGMP levels were measured after two time intervals using the enzyme immunoassay and all experiments were done in triplicate and error bars represent the error.

Secondly, using the OmniaTM kinase assay, we also show that AtPSKR1-KD2 has serine/threonine kinase activity through its capacity to phosphorylate the Ser/Thr peptide 1. Under these conditions, AtPSKR1-KD2 has a K_m of $\sim 7.5 \mu M$ and a V_{max} of $\sim 1800 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein (Figure 3.3.4 B). To our knowledge, this is the first reported kinetic value for AtPSKR1 kinase activity and it is similar to the K_m values reported for WAKL10-KD using the same substrate (Meier *et al.*, 2010).

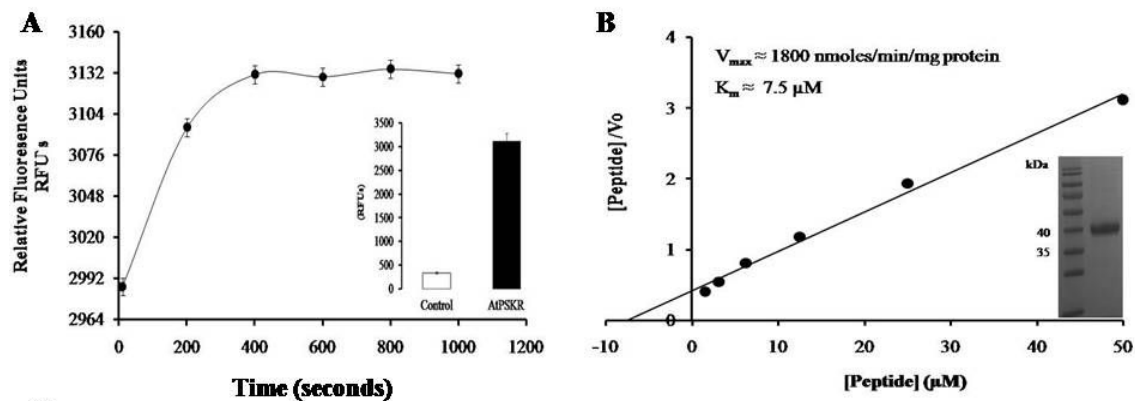


Figure 3.3.4 Demonstration of the recombinant AtPSKR1-D2 kinase activity with Ser/Thr Peptide 1. (A) Calibration curve produced with 1 μg of recombinant AtPSKR1-KD2 in a reaction mixture containing 1x reaction buffer, 1 mM ATP, 0.2 mM DTT, and 12.5 μM Ser/Thr-peptide 1, followed by measurement of the peptide phosphorylation levels with an OmniaTM Ser/Thr-Recombinant system (BioSource, Nivelles, Belgium). Peptide phosphorylation levels generated with no protein (control) and with 1 μg recombinant AtPSKR1-KD2 as was determined by an OmniaTM Ser/Thr-Recombinant system. (B) Hanes-Woolf plot of the kinetic determinants of serine-threonine kinase activity of the recombinant AtPSKR1-KD2 (shown) using the Ser/Thr peptide 1 as a substrate and measuring activity with the OmniaTM kinase assay (BioSource, Nivelles, Belgium). Peptide phosphorylation levels were measured on an Envision 2101 Multilabel plate reader (Perkin Elmer Inc., Massachusetts) with excitation at 405 nm (λ_{ex} 405) and emission at 492 nm (λ_{em} 492). Readings taken every 10 seconds for 15 minutes and the values represent the means of three experiments with standard error bars.

The catalytic end-product of the GC domain, cGMP was tested to determine if it had any effects on the enzymatic activity of the kinase domain as is shown in (Figure 3.3.5 B). The presence of 0.1 and 1.0 μM cGMP in a 30 minute kinase reaction resulted in a decrease of AtPSKR1 kinase activity. The obtained results indicate that the effect of cGMP on the kinase activity of the recombinant AtPSKR1 are not only concentration dependent but are also time-dependent (between 0.1 to 1.0 μM cGMP) (Figure 3.3.5 A).

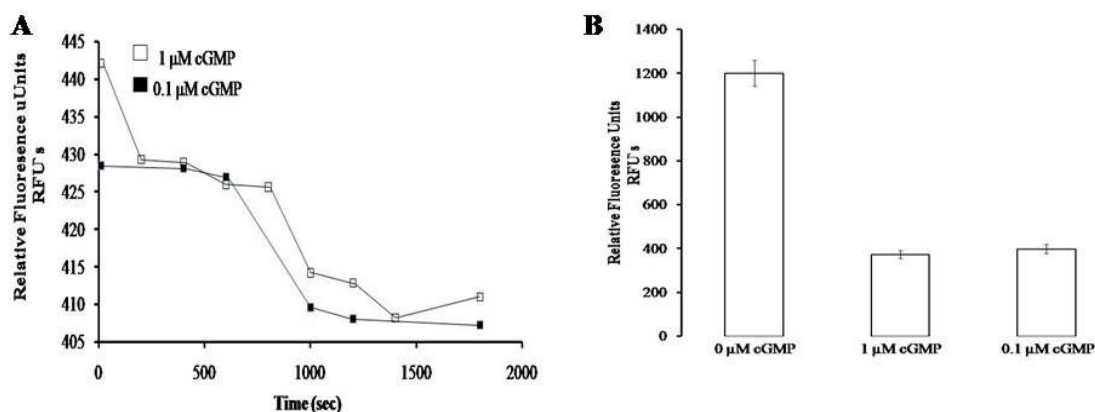


Figure 3.3.5 Demonstration of the effect of cGMP on the kinase activity of AtPSKR1. (A) A time dependent curve of the effect of cGMP on AtPSKR1 kinase activity. In a reaction system, 1 μg of recombinant AtPSKR1-KD2 was incubated with either 0.1 or 1 μM cGMP for 10 minutes in 1 X reaction buffer with Ser/Thr peptide 1(BioSource, Nivelles, Belgium). The reaction was initiated by adding 1 mM ATP and readings were monitored over time. (B) End point determination of the effect of cGMP on AtPSKR1. Peptide phosphorylation levels were measured on an Envision 2101 Multilabel plate reader (Perkin Elmer Inc., Massachusetts) with excitation at 405 nm (λ_{ex} 405) and emission at 492 nm (λ_{em} 492). Readings taken every 10 seconds for 15 minutes and the values represent the mean of three experiments with standard error bars.

Protein kinases play a critical role as regulators for most cellular processes and autophosphorylation is a characteristic regulatory mechanism in these phosphorylation-dependent signalling cascades (Pike *et al.*, 2008). This reversible phosphorylation can influence its activity by inducing a conformational change that in turn could expose and therefore activate or hide, thereby deactivating binding sites for interaction partners (Hutti *et al.*, 2004). When AtPSKR1 was incubated with cGMP, cAMP and 8-bromo-cGMP in a kinase autophosphorylation reaction we observed a capacity to autophosphorylate *in vitro* that was inhibited by cGMP but not cAMP indicating that this reaction is cGMP specific (Figure 3.3.6).

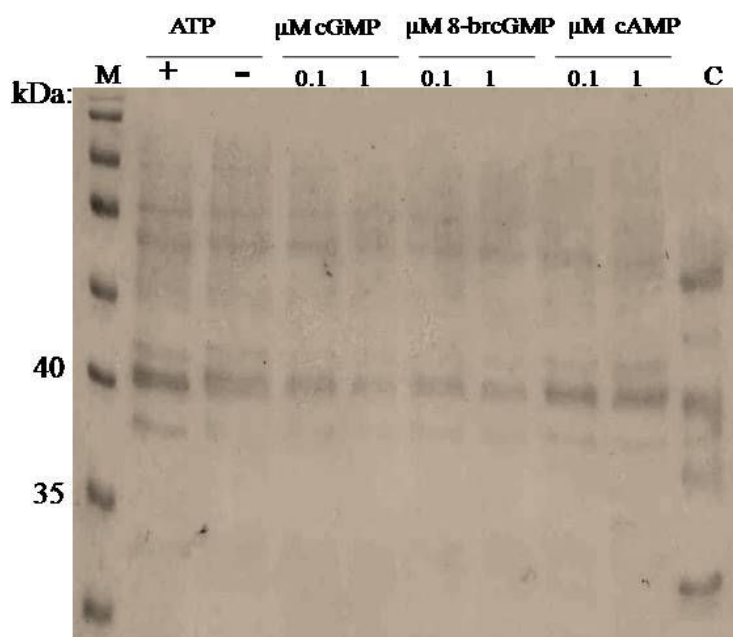


Figure 3.3.6 Demonstration of the effects of cyclic nucleotides on the autophosphorylation state of AtPSKR1. Recombinant AtPSKR1-KD protein was expressed and purified under native conditions and the effects of cyclic nucleotides were assessed. In a reaction system containing 1 X reaction buffer (20 mM Tris pH 7.5 15 mM MgCl₂, 0.2 mM DTT) recombinant protein was pre-incubated with 01 or 1 μM of either cGMP, cAMP or 8-bromo-cGMP (Sigma-Aldrich Corp., Missouri) for 10 minutes. Kinase reaction was initiated by addition of 1 mM ATP followed by a 20 minute incubation at room temperature. AtPSKR1 autophosphorylation controls contained either protein only with no cyclic nucleotide or ATP (-) or protein with ATP and no cyclic nucleotide (+) and positive control contained a phosphopeptide mixture (C)(Merck®, Darmstadt, Germany) Reaction were halted by adding SDS loading buffer and resolved on 12 % SDS-PAGE. Gels were blotted on to PVDF-P nitrocellulose membrane (GE Healthcare, Wisconsin) and probed with Anti-Phosphoserine Mouse mAb (Merck®, Darmstadt, Germany) and band were visualised with 3,3',5,5'-Tetramethylbenzidine (TMB)(Millipore Corp., Massachusetts).

3.4 Discussion

In a previous study we demonstrated that the AtBRI1 receptor has a functional GC domain within its kinase domain (Kwezi *et al.*, 2007), these findings prompted the suggestion that the GC or at least the catalytic product of this domain may also be involved or contribute to downstream signalling cascade of the receptor. We had also identified a number of other candidate GC e.g. WAKL10 (Wall Associated Kinase-like 10) a member of the family of LRR-RLKs, that contain a functional GCs embedded within kinase domains (Meier *et al.*, 2010).

This could be an indication that we are looking at a widespread and novel molecular architecture that may play a complex role in second messenger signalling. In order to further investigate this possibility we undertook to investigate both the GC and kinase activity of another of these molecules, namely AtPSKR1. We demonstrated that the kinase domain of the PSKR1 has both functional kinase and GC activity *in vitro* and that mutating a key residue in the GC catalytic centre removes GC activity (Kwezi *et al.*,; under revision). In animals all characterised GCs contain sequence similarity to adenylyl cyclase class III cyclases and are predicted to function as dimers (Schaap, 2005). Changes in two residues (Asp and Lys to Glu and Cys) can convert a mammalian membrane bound GC, GUCY2D (human retGC-1) to an adenylate cyclase (Tucker *et al.*, 1998). The mammalian receptor GCs are well characterised and include receptors for natriuretic peptides and the guanylin peptide family as well as GUCY2D (retinal specific membrane Guanylate cyclase 2D) (Aparicio and Applebury 1996; Chinkers *et al.*, 1989). In a recent study (Biswas *et al.*, 2009) analysed the relationship between the receptor GC catalytic domains and kinase homology domains and suggested that these domains co-evolved. The kinase domain is separated from the GC domain by a linker domain whose length appears to have been evolutionary conserved (Biswas *et al.*, 2009).

Animal receptor GC domain topology is thus distinctly different from that observed in the plant receptor GCs where the kinase domain encapsulates the GC catalytic centre (Figure 3.3.1 B). Interestingly, GUCY2D appears to be the only receptor GC to contain an active kinase domain that autophosphorylates (Aparicio and Applebury, 1996) as the kinase homology domains in other receptor GCs are predicted to be inactive (Biswas *et al.*, 2009). Membrane bound GCs also occur in lower eukaryotes and have a topology more similar to mammalian adenylyate cyclases with two cassettes of six transmembrane spans that fold together to form the functional GC (Linder and Schultz 2002). Unicellular GCs generally do not contain kinase homology domains (Biswas *et al.*, 2009). Hence, the plant receptor GCs AtPSKR1, AtBRI1 (Kwezi *et al.*, 2007) and AtWAKL10 (Meier *et al.*, 2010) appear to be unique molecules as they contain overlapping functional kinase and GC catalytic domains.

To the best of our knowledge, this is the first report of kinase activity established for the PSKR1 receptor. The recombinant kinase domain has a K_m value of $\sim 7.5 \mu\text{M}$ obtained using a standard commercial substrate developed for serine / threonine kinases (Figure 3.3.4 B). This value is comparable to the values obtained for WAKL10-KD of $2.7 \mu\text{M}$ (Meier *et al.*, 2010) and for mouse cAMP-dependent kinase (PKA) catalytic subunit of $1.8 \mu\text{M}$ (Shults and Imperiali, 2003) using the same substrate. However, it is lower than the K_m values of 71 or $82 \mu\text{M}$ reported for the related LRR RLK kinase domain BRI1-KD using the BRI12 peptide substrate (Oh *et al.*, 2000; Wang *et al.*, 2005). Hence the kinase domain has a typical functioning catalytic ability at least *in vitro*. To date there are no reports on the natural downstream substrates of PSKR1 kinase activity. One possibility might be the BRI1-associated kinase 1 (BAK1) which partners other LRR RLKs such as BRI1 and FLAGELLIN SENSING 2 (FLS2) (Chinchilla *et al.*, 2009; Chinchilla *et al.*, 2007; Li *et al.*, 2002; Nam and Li, 2002).

BAK1 is a promiscuous LRR RLK that interacts with AtBRI1 or FLS2 and other receptors to form heterodimers that initiate trans- or cross-phosphorylation of the intracellular domains of the receptor and this in turn generates a downstream signalling cascade (Chinchilla *et al.*, 2009).

The same recombinant protein preparations of AtPSKR1-KD were used to demonstrate both kinase and GC activity. Initial experiments revealed a relatively low GC activity *in vitro* with no preference for either Mn^{2+} or Mg^{2+} although their presence was critical for activity (Figure 3.4). It is noteworthy that we have noticed before that the low GC activity we observe in these *in vitro* experiments are possibly due to the lack of other essential co-factors in the *in vitro* assay buffer (Kwezi *et al.*, 2007) and we have subsequently showed that inclusion of Ca^{2+} does enhance GC activity *in vitro* (Kwezi *et al.*, under revision). Residues at position 3 in the catalytic centre of the GC that are predicted to enable specificity for GTP in the active site include Gly, Cys, Thr, His and Ser (Biswas *et al.*, 2009; Linder and Schultz, 2002; Liu *et al.*, 1997; McCue *et al.*, 2000; Schaap, 2005; Sunahara *et al.*, 1998; Tucker *et al.*, 1998). AtPSKR1 contains a Gly at this position in the GC catalytic centre and when it was mutated to a Lys, GC activity was removed (Kwezi under revision). This is a significant finding as it shows that GC activity is dependent upon the predicted catalytic centre and the presence of specific amino acids predicted to directly interact with the guanine moiety. Since both these domain show activity *in vitro* we investigated whether these domains can co-regulate each other. Low concentrations (0.1 and 1.0 μM) of cGMP (a product of the GC domain) were added to a kinase reaction with ATP as the substrate for the kinase activity. The observations were such that the ability of the kinase to phosphorylate its substrate was significantly reduced (Figure 3.3.5 A and B).

Cyclic guanosine 3',5'-monophosphate has been shown to promote phosphorylation in a soluble protein kinase that was purified from *Pharbitis nil* seedlings where such a promotion was also concentration-dependent with a maximal activity level reached at 1.0 μ M cGMP concentration (Szmidt-Jaworska *et al.*, 2003). Protein kinases have diverse roles in a physiological system and phosphorylation of a target protein usually results in a functional change of that target protein. Although this inhibitory effect of cGMP on the AtPSKR1 kinase activity is not a common trait of most characterized cGMP dependent/regulate kinases, here we suggest a possibly unconventional manner in which cGMP can affect a protein kinase. The mechanism of action of cGMP in the AtPSKR1 signalling pathway may involve a negative feedback loop to down-regulate transphosphorylation and possibly autophosphorylation to either inhibit kinase activity or the GC domain. If this turns out to be the case for these receptors with this novel architecture it may mean that they regulate directed phosphorylation more specifically. We also investigated the effects of cGMP on autophosphorylation. The protein family of RLKs are known to phosphorylate either Serine or Threonine residue and recently for AtBRI1 kinase it has been shown to autophosphorylate Tyrosine residues (Oh *et al.*, 2009). To test this we chose an antibody based strategy to investigate the effects of cGMP on the autophosphorylation state of the receptor in order to gain insights as to whether cGMP can mediate autophosphorylation in a residue specific manner. When Anti-Phosphothreonine Mouse mAB and PhosphoDetect™ Anti-Phosphoserine Mouse mAb (Merck®, Darmstadt, Germany) were used to probe AtPSKR1 after it had been incubated with cGMP during kinase autophosphorylation activity assessment, only a signal for the PhosphoDetect™ Anti-Phosphoserine could be detected and none for the Anti-phosphothreonine could be detected (Figure 3.3.6).

This could indicate that AtPSKR1 autophosphorylates on Serine rather than Threonine residues and this is affected by cGMP. It has been shown previously that a cAMP-dependent kinase can be altered to bind cGMP by mutating some key residues (Shabb *et al.*, 1991).

In order to determine which cyclic nucleotide is selectively bound by the AtPSKR1 kinase domain, cAMP, cGMP and 8-bromo-cGMP, an analogue of cGMP were used (Figure 3.3.6). The obtained results indicate that cyclic nucleotide inhibition of AtPSKR1 autophosphorylation is specific for cGMP. Compared to the strength that is obtained when cGMP and its analogue are incubated with AtPSKR1 when incubated with cAMP and to that of the control, the signal is lower. Therefore cAMP seems to have no effect on the kinase or at least the autophosphorylation of the kinase domain (Figure 3.3.6). These results implicate cGMP in the kinase signalling cascade of AtPSKR1 and so raise the possibility that cGMP dependent proteins kinases as well as their substrates form part of the signal relay network. This in turn highlights the role of cGMP in PSK signalling and is in keeping with the fact that it has been reported that cGMP modulates various intracellular processes i.e. cyclic nucleotide gated ion channel gating (Kaupp and Seifert, 2002), the transcriptome (Maathuis, 2006) and chloroplast development (Bowler *et al.*, 1994). In addition, cGMP has been implicated as a signalling molecule in cell expansion (Volotovski *et al.*, 1998; Wang *et al.*, 2007b) which is one of the described functional responses to α -PSK (Kutschmar *et al.*, 2009; Matsubayashi *et al.*, 2006b). It is also conceivable that cGMP has a role in regulating the kinase activity of AtPSKR1 itself possibly by allosterically modulating the receptor.

Chapter 4: The *Arabidopsis thaliana* Brassinosteroid and Phytosulfokine Receptors have Guanylyl Cyclase activity *in vivo*.

Abstract.

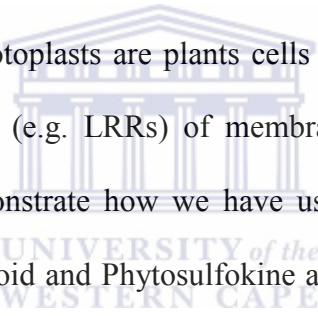
Intercellular communication is a crucial trait in multicellular organisms as it facilitates not only growth and development but also localised and systemic responses to environmental stimuli. In plants this communication is at least in parts modulated by peptide hormone signalling. Amongst some of the well characterised signalling molecules in plants are Brassinosteroids and Phytosulfokines which are involved in development and cell proliferation respectively. The receptors for both these signalling peptides have been identified in plants as the AtBRI1 for Brassinosteroids and AtPSKR1 for Phytosulfokines. To signal from the point of stimulus perception to organelles and molecules within the cell where the appropriate response can occur, plants use receptors and one of the most abundant receptor families are the Leucine Rich Repeat Receptor-like kinases (LRR-RLK) and they include AtBRI1 and AtPSKR1. Here we demonstrate how exogenous application of both Phytosulfokine and Brassinosteroid increases changes of intracellular cGMP levels in *Arabidopsis* mesophyll protoplasts demonstrating that these molecules have GC activity *in vivo* and therefore provide a link as second messenger between the hormones and downstream responses. In addition, *Arabidopsis* plants were treated with a cell permeable analogue of cGMP and it was noted that this can affect changes in the phosphoproteome in *Arabidopsis*. We therefore conclude that cGMP plays a role in kinase-dependent downstream signalling possibly from cGMP generated as a result of ligand binding.

4.1 Introduction.

For sessile multicellular organism like plants, it is important for their cells to be able to communicate, to coordinate and systematically respond to stimuli. In plants some of the ways through which this intercellular communication is possible is via peptide hormone and phytohormones signalling. The first identified plant signalling peptide in plants was the tomato Systemin, a peptide hormone that is involved in the modulation of the wound response (Pearce *et al.*, 1991). Since then a number of other signalling molecules have been identified, amongst them are the phytohormone Brassinosteroids (Boller, 2005) and the pentapeptides Phytosulfokines. Brassinosteroids are growth promoting steroids in plants with Brassinolide being the most bioactive form (Bishop and Koncz, 2002). The latter is the most widely distributed in the plant kingdom and perceived by the Brassinosteroid receptor (AtBRI1) (Li and Chory, 1997). Phytosulfokine was first discovered as a cell proliferation agent essential for low density cell cultures and several precursor proteins for PSK (proPSK) have been identified in different species that contain an N terminal secretory signal sequence and a PSK sequence near the C terminus (Lorbiecke and Sauter, 2002; Matsubayashi and Sakagami, 1996; Yang *et al.*, 2001).

The action of a hormone involves perception and Receptor-Like Kinases (RLKs) that have been implicated in peptide signalling and of particular interest are the RLKs with an extracellular leucine rich repeat (LRR) domain implicated in protein-protein or protein-peptide interactions (He *et al.*, 2000; Kinoshita *et al.*, 2005; Kobe and Deisenhofer, 1994). These receptors typically have an island ligand binding domain embedded within the leucine repeats and it is thought that the non-globular shape of these domains are such that they expose sufficient surface area for optimum interaction with smaller globular proteins or other ligands, making them optimal for protein binding by facilitating more interactions, and as a result increased affinity for the ligand (Kobe and Deisenhofer, 1994).

The Brassinosteroid and Phytosulfokine receptors (AtBRI1 and AtPSKR1 respectively) are amongst the LRRs whose natural ligands have been characterized (Li and Chory, 1997; Matsubayashi *et al.*, 2002). α -PSK is the natural ligand for AtPSKR1 and it is a pentapeptide that is sulphated on its two tyrosine residues (Y(SO₃H)IY(SO₃H)TQ) with cellular activity dependent on sulphation of the tyrosine residues (Matsubayashi and Sakagami 1996). It is likely that pro-PSK is sulphated by tyrosyl protein sulphotransferase as the protein is processed through the Golgi network before secretion (Hanai *et al.*, 2000).

Arabidopsis mesophyll protoplasts have been used and have provided a reliable tool for conducting cell-based experiments using molecular elicitors followed by biochemical analysis to characterise the functions and molecular components within diverse signalling pathways (Yoo *et al.*, 2007). Protoplasts are plants cells with cell walls removed and thus having the extracellular domains (e.g. LRRs) of membrane bound receptors (e.g. RLKs) exposed directly. Here we demonstrate how we have used protoplast as an experimental system to administer Brassinosteroid and Phytosulfokine and report for the first time *in vivo* GC activity for both the Brassinosteroid and Phytosulfokine receptors. Responses to stimuli in organisms include signalling networks that often rely on post-translational modifications and amongst these is protein phosphorylation. In Chapter 3, (Figure 3.3.6), we showed how the ability of AtPSKR1 to phosphorylate a substrate is affected by cGMP *in vitro*. In order to gain further understanding as to what are the effects of cGMP on the global phosphorylation within a cell we have taken a phosphoproteomics approach. Phosphoproteomics coupled with mass spectrometry is versatile technique for large scale characterization of complex proteins samples (de la Fuente van Bentem and Hirt, 2007). Using this approach we demonstrate the effects of cGMP on the *Arabidopsis thaliana* phosphoproteome.

4.2 Methods and Materials.

4.2.1 Preparation of plant material for protoplast isolation.

Arabidopsis thaliana Columbia (Col-0) seeds were surface-sterilized by washing in 70 % Ethanol through a brief vortexing cycle, seeds were allowed to settle at the bottom and the supernatant was discarded as waste. This was followed by washing with a 10 % solution of commercial bleach (Sodium hypochlorite) (v/v) and subsequently rinsed 5 times with sterile distilled H₂O. Seeds were transferred onto MS-agar plates [0.44 % Murashige and Skoog basal medium (w/v), 3 % Sucrose (w/v) and 0.4 % Agar (w/v), pH 5.7 (all reagents were from Sigma-Aldrich Corp., Missouri)] and vernalised at 4 °C for three days. The seeds were germinated at 23 °C with a light and dark regime of 16/8 hours light/dark photoperiod cycle for two weeks. Seeds were transferred to seed trays (15 cm x 20 cm) filled with $\frac{2}{3}$ commercial pot mix at the bottom filled with seedling germination soil and further grown under the same light and temperature conditions until they were 4 weeks old.

4.2.2 Preparation of plant material for phosphoproteomics.

Arabidopsis thaliana Columbia (Col-0) seeds were surfaced sterilized as described in (Section 4.2.1) and germinated in MS media [0.44 % Murashige and Skoog basal medium (w/v), 3 % Sucrose (w/v) and, pH 5.7 (all reagents were from Sigma-Aldrich Corp., Missouri)]. The seeds were germinated at room temperature in sterile 250 mL conical flasks capped with 4 layers of foil with shaking on an orbital shaker shaking at 130 rpm under long day conditions (16 hours light and 8 hours dark) until use when they were 4 weeks old. At this stage plants were treated with cell permeant 8-bromo-cGMP and allowed to grow further for 3 hours after which growth was halted by flash freezing the plants in liquid Nitrogen.

4.2.3 Preparation and isolation of mesophyll protoplasts from *Arabidopsis thaliana*

leaves.

Protoplasts were prepared based on a protocol by (Yoo *et al.*, 2007). Leaves were sliced in a Petri dish with a sterile surgical blade into thin strips in W5 buffer [0.4 M Mannitol, 7 mM CaCl₂ and 3 mM MES (4-Morpholineethanesulfonic acid), pH 5.7]. Buffer was discarded as waste and leaf strips were incubated in enzyme solution [1.5 % (w/v) Cellulase R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.4 % (w/v) Macerozyme R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.4 mM D-Mannitol, 20 mM KCl, 20 mM MES, 10 mM CaCl₂ and 0.1 % (w/v) Bovine Serum Albumin (BSA), pH 5.7 (all reagents excluding the enzyme were from Sigma-Aldrich Corp., Missouri)]. The Petri dishes were covered with foil and digestion was carried in the dark at room temperature with slow shaking at ~40 rpm on an orbital shaker for 2 hours. The solution was filtered through a nylon mesh, pore size 60 µm (Millipore Corp., Massachusetts), the filtrate was centrifuged at 100g for 3 minutes to pellet the protoplast. Protoplast pellets were resuspended in W1 buffer [0.4 M D-Mannitol, 4 mM MES, 20 mM KCl, pH 5.7 (all reagents were from Sigma-Aldrich Corp., Missouri)] and cell density was counted using a Hemocytometer.

4.2.3.1 Treatment of protoplast and measurement of intracellular cGMP.

The prepared protoplasts were subjected to two treatments. Firstly with the active sulphated form of Phytosulfokine- α (A-PSK) and the inactive non-sulphated form (n-PSK) as the control. The Phytosulfokine peptides were obtained from Professor Helen Irving (Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash University, Australia) and were synthesised as detailed elsewhere (Kwezi *et al.*, under revision).



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The protoplasts (2.9×10^5 / treatment) were treated with $0.1 \mu\text{M}$ of either A-PSK or n-PSK for intervals of 2, 5 and 15 minutes. Secondly protoplasts (2.5×10^5 /treatment) were treated with $10 \mu\text{M}$ Epibrassinolide for the same time intervals. Protoplasts were then collected by briefly spinning at $100 g$ and flash frozen in liquid nitrogen. The cGMP content from protoplast assays was analysed using the Amersham cGMP enzyme immunoassay (EIA) Biotrak System following protocol “4”, an acetylation protocol which allows for measurement of intracellular cGMP, as described in the supplier’s manual (GE Healthcare, Wisconsin). The optical density was read at 450 nm using an Envision 2101 plate reader (Perkin Elmer Inc., Massachusetts). Cyclic GMP levels were calculated and expressed in $\text{fmol} / \mu\text{g}$ protein or $\text{fmol} / \text{well}$ (treatment).



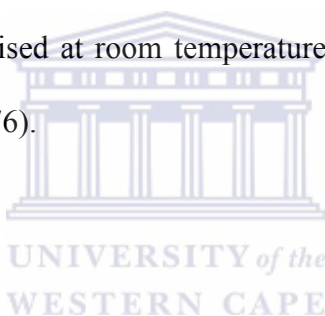
4.2.4 Protein sample preparation for phosphoproteome analysis.

4.2.4.1 Protein extraction.

Proteins were extracted based on a method for preparation of microsomes by (Komatsu and Hirano, 1993) in which protein fractionation and enrichment can be achieved by centrifuging in sucrose and in the presence of detergents and buffering salts. Plant tissues were ground in liquid N_2 and homogenized at 4°C with a homogenization buffer composed of [20 mM Tris-HCl pH 8.8, 0.25 M Sucrose, 10 mM EGTA, 1 mM DTT, Phosphatase Inhibitor Cocktail Set II (Merck , Darmstadt, Germany)] and Protease inhibitors (Merck , Darmstadt, Germany). all reagent except those specified were from Sigma-Aldrich Corp. Missouri]. The homogenates were then centrifuged at $3000 g$ for 15 minutes. The supernatant was decanted and kept as total soluble protein (TSP) and aliquots from these were subsequently analysed on SDS-PGE.

4.2.4.2 Sample Preparation for Two-Dimensional Gel Electrophoresis.

Total soluble protein were precipitated from extraction buffer using 4 X volume 80 % ice cold Acetone and a tenth of the final volume 1 mM Tris-HCl, pH 8.8 and by incubating at -20 °C over night. Precipitated proteins were collected through centrifugation at 3000 g at room temperature for 15 minutes. The pellets were washed three times with in ice cold 80 % (v/v) acetone equivalent to the amount of acetone precipitated with by vortexing for 5 x 3 minutes and then centrifuged at max speed using a bench centrifuge at 4 °C for 15 minutes. The resulting pellets were air dried on to remove excess Acetone. All pellets were solubilized in urea lysis buffer suitable for 2-DE analysis composed of [9 M Urea, 2 M Thiourea and 4 % (w/v) CHAPS, all reagents for the urea lysis buffer were from Sigma-Aldrich Corp., Missouri]. Proteins were solubilised at room temperature for an hour and quantified using the Bradford assay (Bradford, 1976).



4.2.5 Two Dimensional Gel Electrophoresis (2DE).

4.2.5.1 Rehydration of Immobilized pH Gradient (IPG) strips.

Protein samples were prepared by mixing the protein samples with 0.8 % (v/v) DTT, 0.2 % (v/v) pH 3-10 Ampholytes (Bio-Rad Laboratories Inc., California), 5 µl 0.1 % (w/v) Bromophenol Blue (BPB) and made up to a final volume of 125 µl with urea lysis buffer [9 M Urea, 2 M Thiourea and 4 % (w/v) CHAPS, all reagents for the urea lysis buffer were from Sigma-Aldrich Corp., Missouri]. This mixture was vortexed for 10 seconds and centrifuged for 1 minute at 12.4 x g to pellet the insoluble material. Protein samples (200 µg) were rehydrated passively into 7 cm IPG (Immobilized pH gradient)(Gorg *et al.*, 2004) strips of a pH gradient of pH 4 - 7 (Bio-Rad Laboratories Inc., California) on a re-swelling tray (GE

Healthcare, Wisconsin) and overlaid with mineral oil (GE Healthcare, Wisconsin) overnight at room temperature.

4.2.5.1 Isoelectric focusing (IEF) of total soluble proteins.

Isoelectric focusing is the first dimension of the two dimensional electrophoresis. Rehydrated strips were removed from the re-swelling tray and rinsed with a stream of de-ionized water to remove crystallized urea and unabsorbed sample. The strips were gently blotted on filter paper. Isoelectric focusing was done on an Ettan™ IPGphor II™ IEF machine (GE Healthcare, Wisconsin).

The ends of the gel strips covered with 0.5 cm damp IEF (Isoelectric focusing) electrode strips (GE Healthcare, Wisconsin) to ensure conductivity and collection of salts and other ionic contaminants in the sample. Isoelectric focusing was carried out at 20 °C in three steps - Step 1: 0 – 250 V for 10 minutes, step 2: 250 V – 4000 V for 1 hour and step 3: 4000 V – 4000 V for 12000 Vhrs (Volt hours). Strips were then removed and rinsed with distilled H₂O, followed with an equilibration step to bring the protein to their reduced state by incubating in equilibration buffer 1 [6 M Urea, 0.375 M Tris-HCl pH 8.8, 2 % SDS, 20 % Glycerol, 2 % (w/v) Dithiothreitol (DTT)] with gentle agitation for 20 minutes. This was followed by an alkylation step in equilibration 2 (6 M Urea, 0.375 M Tris-HCl pH 8.8, 2 % SDS, 20 % Glycerol, 2.5 % (w/v) Iodoacetamide) with gentle agitation for 20 minutes. The proteins were finally resolved by mass by loading the equilibrated strips in on top of a 12 % SDS-PAGE gel and sealed with 1 % agarose supplemented with a tint of Bromophenol Blue to track migration.

4.2.6 Phosphoproteome Analysis with Pro-Q Diamond stain.

After proteins were resolved according to their mass the gels were used for phosphoproteome analysis. The gels were fixed overnight in a solution containing 50 % (v/v) Methanol and 10 % (v/v) Acetic acid. The following day gels were washed with ultra pure distilled H₂O for 15 minutes (3 times) followed by staining the gels with Pro-Q diamond™ stain (Invitrogen Corp., California) for 90 minutes in the dark. The gels were then destained in 50 mM Sodium acetate (pH 4.0) and 20 % (v/v) Acetonitrile (Sigma-Aldrich Corp., Missouri) for 30 minutes with gentle agitation in the dark at room temperature. Gels were imaged using the Pharos FX™ plus molecular imager (Bio-Rad Laboratories, Inc., California) with the multiplexing application set to detect Pro-Q Diamond stain. Excitation was set at 532 nm (λ_{ex} 532) and emission at 605 nm (λ_{em} 605).



4.2.7 Mass spectrometric analysis.

4.2.7.1 In-gel tryptic digestion.

Spots of interest were excised manually and transferred into sterile microcentrifuge tubes. The gel pieces were washed twice with 50 mM ammonium bicarbonate for 5 minutes each time and a third time for 30 minutes with occasional vortexing. The gel pieces were then destained twice with 50 % (v/v) 50 mM ammonium bicarbonate and 50 % (v/v) Acetonitrile for 30 minutes with occasional vortexing. The gel pieces were dehydrated with 100 μ L of 100 % (v/v) Acetonitrile for 5 minutes, and then completely dried using the Speed Vacuum SC100 (ThermoSavant, , Massachusetts). Proteins were in-gel digested with approximately 120 ng sequencing grade modified trypsin (Promega Corp., Wisconsin) dissolved in 25 mM ammonium bicarbonate for 6 h at 37 °C. The protein digestion was stopped by adding 50 μ L

of 1 % (v/v) trifluoroacetic acid (TFA) and incubating for 2-4 hours at room temperature before storage at 4 °C until further analysis.

4.2.7.2 Protein identification by MALDI-TOF mass spectrometry.

Digested proteins (1 µL) were mixed separately with the same volume of α -cyna-hydroxycinnamic (CHCA) matrix and spotted onto a MALDI target plate for analysis by MALDI-TOF mass spectrometry using a Voyager DE Pro Biospectrometry workstation (Applied Biosystems Inc., California) operated by Dr Ludivine Thomas (King Abdullah University of Science and Technology, Kingdom of Saudi Arabia) to generate a peptide mass fingerprint (PMF). The MALDI-TOF was operated in the positive ion delayed extraction reflector mode for highest resolution and mass accuracy. Peptides were ionized with a 337 nm laser and spectra were acquired at 20 kV acceleration potential with optimized parameters. Close external calibration was employed using the Sequazyme calibration™ mixture II containing angiotensin I, ACTH (1-17 clip), ACTH (18-39 clip) and bovine insulin (Applied Biosystems Inc., California). This calibration method typically provided mass accuracy of 100 to 200 ppm across the mass range 900 to 5000 Da. Peptide spectra of accumulated 1200 shots each were automatically processed for baseline correction, noise removal, and peak de-isotoping. The threshold was manually adjusted between 2 and 8 % base peak intensity. All searches were performed against the National Center for Biotechnology Information (NCBI) and Mass Spectrometry DataBase (MSDB) peptide mass databases using MASCOT (http://www.matrixscience.com/search_form_select.html). Candidate identifications with Molecular weight search (MOWSE) scores higher than 85 were automatically considered as positive assignments. If more than one protein satisfied mentioned threshold criteria, the entry with the highest MOWSE score was assigned.

4.2.7.3 Confirmation of the expression of AtBIKI in Arabidopsis leaves with RT-PCR.

Total RNA was extracted from leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) and RNA was digested with DNase I (Ambion) for 1 hour at 37 °C. Total RNA (1 µg) was reverse transcribed with SuperScript III Reverse Transcriptase (Invitrogen Corp., California) using oligo dT₁₅ primers according to the manufacturer's specifications in a total volume of 20 µL. PCR reaction mixtures contained 1 µL cDNA (or negative RT reaction), 1x PCR coral load buffer (Qiagen, Venlo, Netherlands), 0.1 µM primers, 200 µM dNTPs and 0.2 U Taq DNA polymerase (Qiagen, Venlo, Netherlands) per 20 µL reaction. Gene specific primers were used to amplify AtBIKI, Forward: 5'TTGCCTTGTGGGTTGAAAT3' and Reverse: 5'ATGGGACATGTAACCGGAAA 3'. All reactions were denatured at 94 °C for 3 minutes and then 35 cycles of amplification were performed (60 seconds denaturation at 94 °C, 60 seconds annealing at 52 °C and 60 seconds extension at 72 °C) with a final extension at 72 °C for 10 minutes in a MyCycler thermal Cycler (Bio-Rad Laboratories Inc., California). The quality of cDNA produced was assessed by amplifying cDNA for the *UBQ-10* gene (*A.thaliana*, AT4G05320). After PCR amplification, equal volumes of PCR products were separated in 1.5 % agarose gels, stained with ethidium bromide and visualised under UV light.

4.3 Results.

Previously we demonstrated that two members of the Leucine Rich Repeat Receptor-Like Kinases family, AtPSKR1 (see Chapter 3) and AtBRI1 have GC activity *in vitro* (Kwezi *et al.*, 2007). If the GC activity observed *in vitro* for both these molecules has a biological role in general and a signalling function in particular, then we might also expect to observe it *in vivo*. To test this possibility we isolated protoplasts from Arabidopsis leaves and treated them with the natural ligands for both these receptors and assessed whether they can stimulate cGMP production. Protoplasts have been suggested as an ideal system to carry out investigations into the roles of cyclic nucleotide signalling as they can respond rapidly to extracellular signals (Assmann, 1995). The Brassinosteroid insensitive receptor is ubiquitously expressed (Friedrichsen *et al.*, 2000) and AtPSKR1 is expressed in leaves of higher plants (Kutschmar *et al.*, 2008; Yang *et al.*, 2001). Therefore it is conceivable that the mesophyll protoplasts would have naturally occurring ligand binding sites for both the A-PSK and Epibrassinolide. In the presence of α -PSK and Epibrassinolide but not nPSK, cGMP levels were raised in protoplasts (Figure 4.3.1). This increase in cGMP levels was measured in the absence of any intracellular phosphodiesterase inhibitors and the levels of cGMP that were detected in the protoplasts are consistent with those reported in plants (Pharmawati *et al.*, 1998; Szmids-Jaworska *et al.*, 2008; Wang *et al.*, 2007a). The observed increases in intracellular cGMP levels were time dependent with the highest level reached at five minutes for Epibrassinolide (Figure 4.3.1 A) and 5 minutes to 15 minutes for α -PSK (Figure 4.3.1 B).

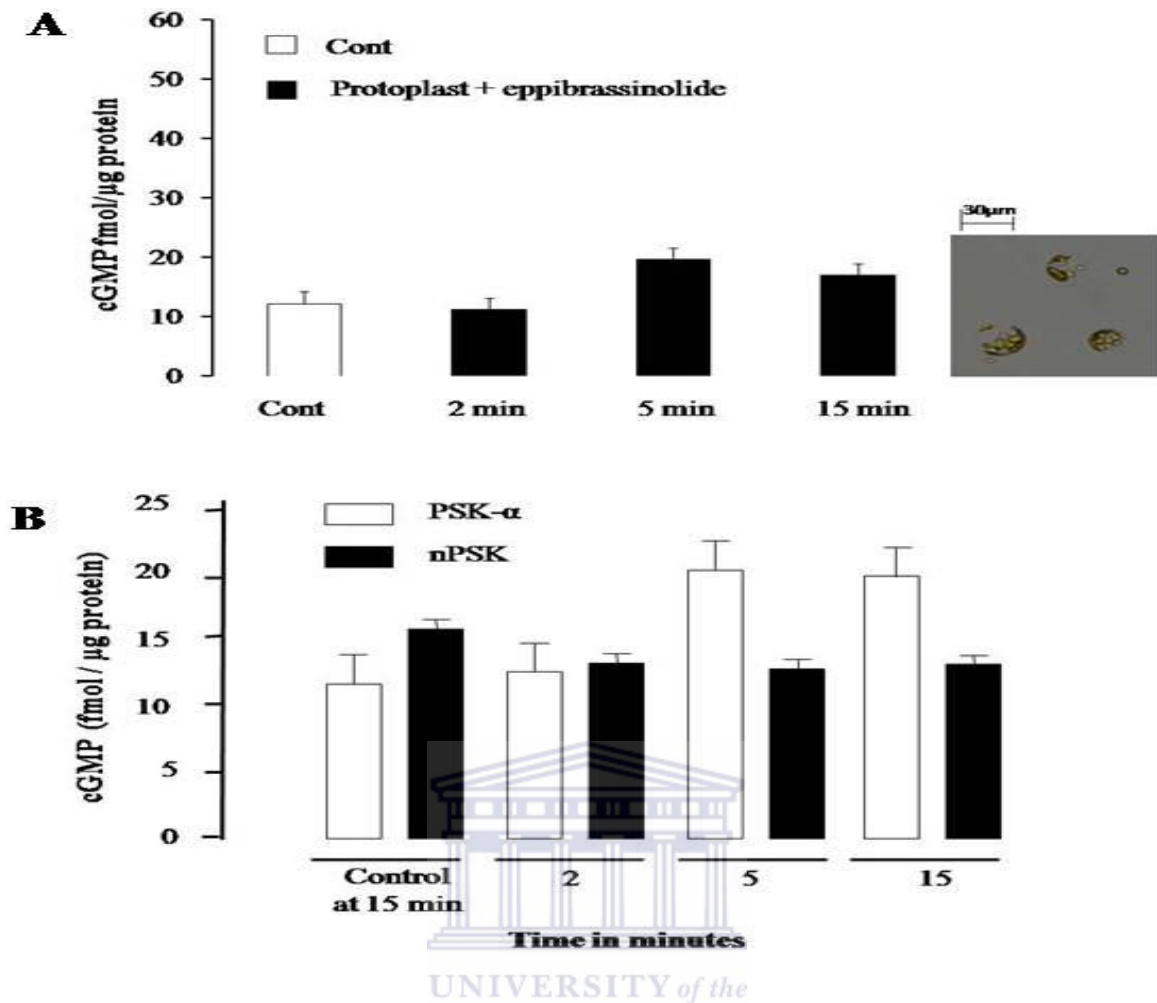
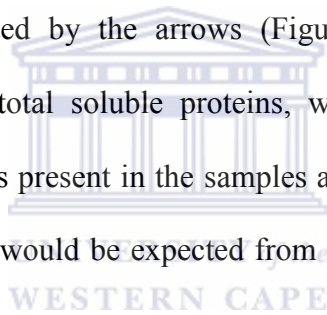


Figure 4.3.1 Demonstration of the *in vivo* GC activity of AtPSKR1 and AtBRI1. (A) Freshly isolated leaf mesophyll protoplasts were treated with 10 μM of an analogue of the natural ligand of the Brassinonosteroid receptor (Epibrassinolide) (Sigma-Aldrich Corp., Missouri) for 2, 5 and 15 minutes. Control were treated with ethanol and the insert shows a picture of the protoplast that were used. (B) Cyclic GMP production following treatment with α -PSK (0.1 μM) or nPSK (0.1 μM) in protoplasts over 15 minutes in the absence of any phosphodiesterase inhibitor. Intracellular cGMP were assessed with the enzyme immunoassay and error bars represent the SEM of the means of three independent and representative assays ($N = 3$).

Cyclic GMP has increasingly become implicated in a large number of plant processes including responses to abiotic stresses such as dehydration and salt (Donaldson *et al.*, 2004), hormone dependent signalling and changes of the transcriptome of *A. thaliana* (Maathuis, 2006). In chapter 3 we have also shown that one of these downstream processes and targets of cGMP are kinases. In order to further determine the effects of cGMP on intracellular kinases and their substrates, we have used a proteomics approach to gain some insight into the phosphoproteome as modulated by cGMP.

Through phosphoproteomics it is possible to quantitatively assess phosphate group as a post-translational modification on individual proteins within complex protein samples (Schreiber *et al.*, 2008). We have used 2-dimensional electrophoresis (2-DE) as it provides an opportunity to profile complex protein samples (Gorg *et al.*, 2004). Firstly, we analyzed the protein on one dimension (separation by mass) from all prepared protein samples so as to analyze for protein sample quantity and preview quality and representation of each of the extractions. To quantitatively profile the phosphoproteome at the first dimension we used Pro-Q[®] Diamond stain, a stain that selectively stains phosphoproteins in polyacrylamide gels (Figure 4.3.2 A). The stain reveals that the extraction procedure is able to maintain proteins in their phosphorylated state. From this we were able to detect changes in the phosphorylation state as indicated by the arrows (Figure 4.3.2 A). To determine the representation of the extracted total soluble proteins, we used a Coomassie blue based detection which stains all proteins present in the samples and from this were able to observe the quality and representation as would be expected from a complex sample as total soluble proteins (Figure 4.3.2 B).



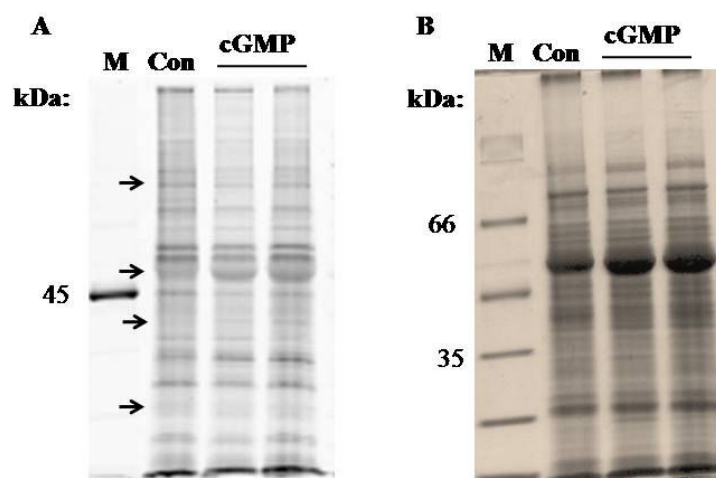


Figure 4.3.2 Demonstration of cGMP mediated changes in the phosphoproteome of *A.thaliana*. (A) Proteins were extracted, from plants treated with 10 μ M, resolved on 12 % SDS-PAGE and stained with Pro-Q[®] Diamond (Invitrogen Corp., California). Controls (Con) were treated with H₂O and, (M) is the molecular weight marker (Fermentas) with a phosphoprotein positive control. (B) A Coomassie blue stained SDS-PAGE of treated plant protein extracts Experiments were done in duplicate and 20 μ g of protein was loaded per well and arrows indicate some of the notable changes in the first dimension.

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Secondly, we have used 2-D analysis as a method to identifying individual proteins, specifically kinases and their targets that are affected by cGMP (Figure 4.3.3). Protein samples were separated by charge over a pH gradient of 4 – 7 on 7 cm IPG strips. The strips allow for separation of one sample at a time i.e. one experimental condition and represented below are the control (Figure 4.3.3 A) and the treatment with cGMP (Figure 4.3.3 B). Changes in phosphorylation are observed as both as a decrease or increase in intensity of the protein spots on SDS-PAGE as indicated by arrows. Out of the observed protein spots stained with Pro-Q[®]CF protein spots, seven were selected for identification by MALDI-TOF and database searches. Spot selection was done on the basis of high abundance and good resolution on the IPG strip pH ranges used. Selected protein spots are numbered 1-7 (Figure 4.3.3 A and B). These protein spots of interest were picked and trypsinised and peptide

digests were analysed using MALDI-TOF. The resultant peptide mass fingerprints generated by MALDI-TOF MS were used in sequence database searching to find their matching protein identities. One of the protein spots was of particular interest as indicated by the red arrow (Figure 4.3.3 A and B) since it is a kinases and identified as the Arabidopsis Botrytis induced kinase (AtBIKI) (Figure 4.3.3 C)

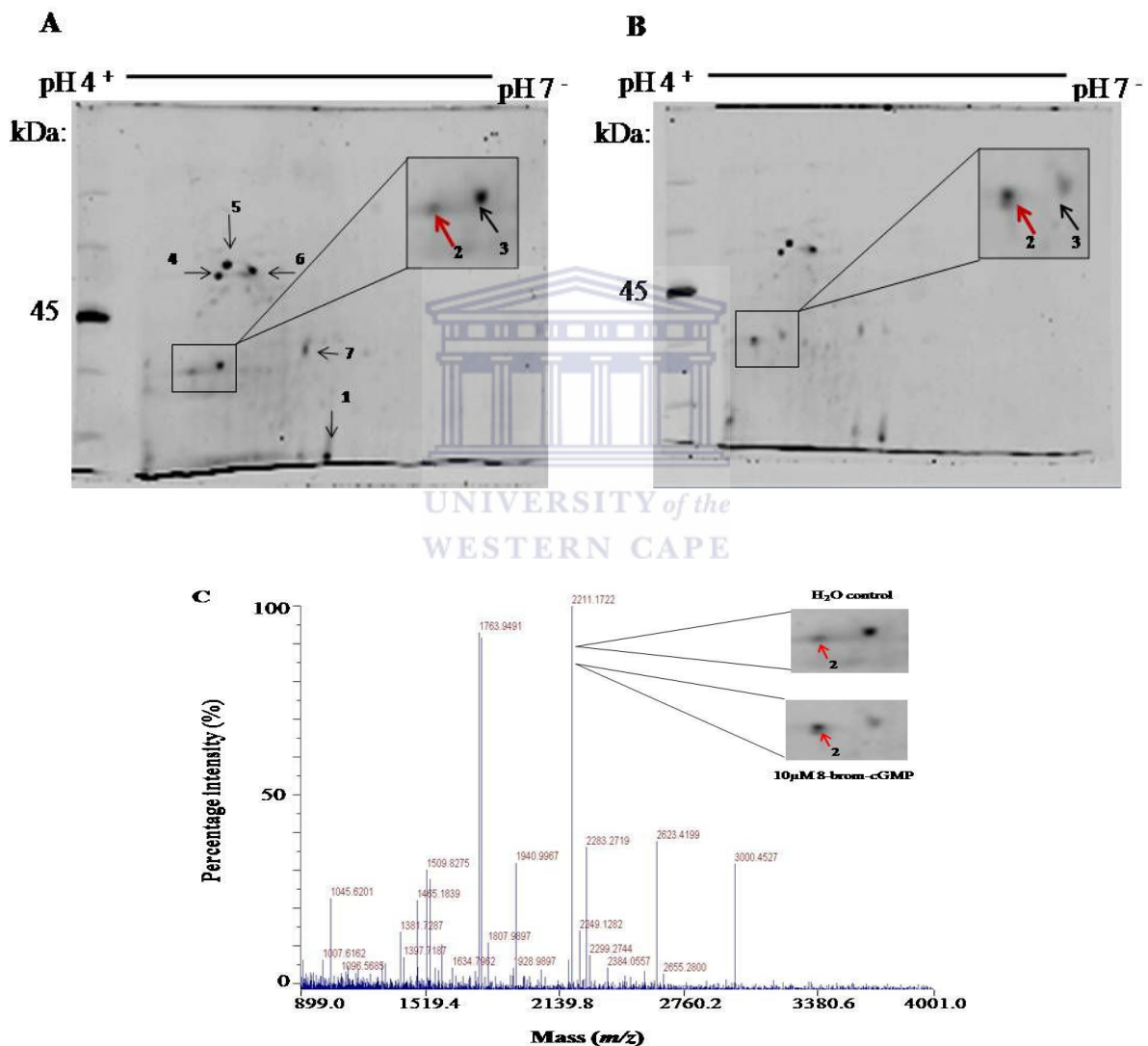


Figure 4.3.3 Two dimensional protein analysis of cGMP induced phosphorylation and MALDI-TOF mass spectrometry identification in *A. thaliana*. (A) Untreated plant protein. (B) Protein extract (100µg) from 10µM 8-bromo-cGMP treated plants were passively absorbed into 7cm IPG (Immobilised pH gradient) strips and focused over a pH gradient of 4-7 followed by resolution by mass on 12 % SDS-PAGE. Arrows indicate notable changes between control and treatment with red indicating the Botrytis induced kinase (AtBIKI, At2g39660). (C) Protein that exhibited changes in the autophosphorylation state were excised from the gel and digested with trypsin to gain their mass fingerprint and identified with MALDI-TOF mass spectrometry. The insert indicates the protein spots

of interest. Mass spectra were obtained for the spot and allowed for the interrogation of databases and thereby identification of protein.

RT-PCR is an efficient way for testing for temporal and spatial “expression of a protein”.

We have therefore used a PCR approach here and extracted total RNA from leaves of the same plants that were used in the phosphoproteomics study and reverse transcribed to produce cDNA. When the cDNA was probed with AtBIKI gene specific primers, the PCR product corresponded to the expected size of AtBIKI (1946 base pairs) (Figure 4.3.4 A). This then confirmed that AtBIKI is expressed in the tissue that was used. Since RNA is highly unstable and is prone to degradation we tested for any degradation by using the cDNA to amplify the Ubiquitin gene which confirmed that the cDNA had remained stable from extraction to use as a template in the RT-PCR. (Figure 4.3.4 B).



Figure 4.3.4 Determination of the expression of AtBIKI in *A. thaliana* leaves using RT-PCR. (A) Total RNA was extracted from 4 week old leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). Total RNA was then reverse transcribed with SuperScript III Reverse Transcriptase (Invitrogen Corp., California). The cDNA was used as a template and gene specific primers were used to amplify AtBIKI with high fidelity Taq DNA polymerase (Qiagen, Venlo, Netherlands) on a MyCycler thermal Cycler (BioRad Laboratories). (B) The quality of cDNA produced was assessed by amplifying cDNA for the *UBQ-10* gene. After PCR amplification, equal volumes of PCR products were separated in 1.5% agarose gels, stained with ethidium bromide and visualised under UV light.

4.4 Discussion.

Receptor like kinases play a critical role in mediation of extracellular signals and a major subgroup of this super family of protein receptor are the leucine-rich repeat receptor kinases (LRRRLKs). These types of receptors perceive signal through their extracellular domains with the consensus residues within the LRR motif providing a structural scaffold for protein-protein interactions whilst the non-consensus residues within LRRs are thought to determine the specificity for the interactions (Kobe and Deisenhofer, 1994). Though some redundancy in the binding of ligands in this type of receptors has been reported where systemin was shown to bind to the tomato homologue of AtBRI1 (Scheer and Ryan, 2002). However, although it binds specifically, Systemin does not activate the BRI1 receptor autophosphorylation cascade (Malinowski *et al.*, 2009). We have previously shown that three members of the family of LRR have GC activity *in vitro*, AtWAKL (Meier *et al.*, 2010), AtBRI1 (Kwezi *et al.*, 2007) and AtPSKR1 (see Chapter 3). To investigate whether this activity is exhibited *in vivo* we have used the natural ligands for two of these receptors, Phtysulfokine and Brassinolide the most biologically active Brassinosteroid (Li and Chory, 1997), to treat protoplast and monitor whether they do affect intracellular cGMP levels.

Plant protoplasts are without cell walls and therefore provide a versatile cell-based experimental system to study environmental and biological cues and monitor and to monitor these within that biological system. When PSK and Epibrassinolide were applied to protoplast, increases in cGMP levels were observed within minutes following application of both α -PSK but not the non-sulphated nPSK and Epibrassinolide but not its control (Figure 4.3.1). The increases in intracellular cGMP levels are of similar orders of magnitude to those reported previously in response to extracellular signals such as Gibberellic acid (Penson *et al.*, 1996) or plant natriuretic peptide (Pharmawati *et al.*, 1998; Pharmawati *et al.*, 2001;

Wang *et al.*, 2007a) and here they have proven both sufficient and essential for a downstream response.

Furthermore, these observed increases in cGMP occur in a time frame that is consistent with activating the GC catalytic function of the AtPSKR1 and AtBRI receptor. A similar time frame would be expected to result in protein phosphorylation from the kinase as well as it is ligand binding that induces these changes and the kinases are also activated. Importantly, cGMP levels are consistently observed to rise in response to α -PSK but not the non-sulphated PSK backbone and this is also observed for Epibrassinolide indicating that cGMP production is part of the Phytosulfokine and Brassinosteroid signalling cascade as was speculated for the Brassinosteroid signalling pathway in (Kwezi *et al.*, 2007).

These early effects are unlikely to have been previously observed as the initial assays used to identify and characterise PSK activity were over considerably longer periods of three to six days where cell growth was assessed (Matsubayashi *et al.*, 1996). Unlike for the Brassinosteroid receptor whose downstream signalling cascade is well characterised, the actual proteins and other components relaying the downstream signalling cascade following binding of α -PSK to AtPSKR1 are not known. Our results implicate cGMP in the early events and so raise the possibility that cGMP dependent proteins as well as kinase substrates form part of the relay network of not only that of AtPSRK1 but also for AtBRI1. The involvement of cyclic nucleotide is a novel and may be one of the ways in which to explain the signal transduction and amplification from the ligand to the nucleus. Communication with the nucleus is relevant since cGMP does effect transcription (Maathuis 2006). Changes in the transcriptome as induced by hormones is know and has been shown in mammalian systems (Beato *et al.*, 1995).

Contrary to plants, the hormones are perceived by intracellular receptors, which act as ligand-dependent transcription factors that regulate gene expression and belong to nuclear receptor super family (Beato *et al.*, 1995; Bishop and Koncz, 2002).

However in plants, hormone perception occurs at the extracellular levels and therefore in case AtPSKR1 and AtBRI1 may be transducing the hormone signal to the nucleus

Cyclic nucleotide have been shown to affect kinase activity *in vitro* with a recent example shown in *Pharbitis nil* (Szmidt-Jaworska *et al.*, 2003). In addition, we have reported (Chapter 3) that this characteristic is also observed in PSKR which has kinase activity. However, cGMP mediated changes at the phosphoproteome (Ficarro *et al.*, 2002) level is still far from established let alone understood. Most cellular processes involve or are regulated by the reversible phosphorylation of proteins on serine and threonine residues and this phosphorylation serves as good modulator of protein function (Hubbard and Cohen, 1993). Phosphoproteomics followed by identification of candidate proteins by Mass spectrometry is a versatile and novel technology for the identification of these phosphorylation targets (Ficarro *et al.*, 2002). To test, but not with the intent to profile, we adopted this strategy in order to determine whether the effects of cGMP on protein kinases and their ability to phosphorylated a substrate *in vitro* are can also be observed *in vivo*. When Arabidopsis plants were treated with a cell permeable form of cGMP (8-bromo-cGMP), changes in the phosphorylation levels of kinases and/or their substrates was observed (Figure 4.3.3 B). The resulting proteins were identified by Mass Spectrometry. Amongst the identified protein spots were Ribulose biphosphate carboxylase, a GTP binding protein, a Botrytis induced kinase (AtBIKI), a serine/threonine-protein kinase and a hypothetical protein.

Both forms of phosphorylation were observed, firstly, promotion of kinase activity, a common trait of previously documented cGMP regulated kinases for example a GC homologue in *Pharbitis nil* (Szmids-Jaworska *et al.*, 2003) and for an LRR AtWAKL 10 (Dr Oziniel Ruzvidzo, personal communication). Secondly, inhibition of phosphorylation was also observed and this is consistent with what was observed for AtPSKR1 *in vitro* kinase activity assessment in which we demonstrated that the ability of the kinase is inhibited by cGMP. These effects of cGMP may vary from protein to protein, in AtPSKR1 it may be the inhibition of *in vitro* kinase activity (see Chapter 3) whilst in AtWAKL10 which is also a LRR it may promote of *in vitro* kinase activity (Dr Oziniel Ruzvidzo, personal communication) and this may be indicative of the complexity of cGMP responses and tell us that the second messenger has many different functions within a signalling cascade. Perhaps the most interesting phosphorylated protein identified is the Botrytis induced kinase (AtBIKI. At2g39660), Figure 4.3.3 C). It is a plasma membrane-localized Ser/thr protein kinase that is a crucial component of host response signalling required to activate the resistance responses to necrotrophic and biotrophic pathogens (Veronese *et al.*, 2006). When an alignment was done against other kinases that we have shown to be affected by cGMP, AtPSKR1 and AtBRI1, we also identified a putative GC catalytic center (Figure 4.4.1 B and C). This further highlights the diversity and importance of cGMP and its pivotal role in stress responses (Donaldson *et al.*, 2004) and suggests that GCs may be involved in the Botrytis signalling cascade. For some of the proteins, their identities could not be assigned which may be due to the phosphorylation dependent change in mass which “masks” the resulting peptide fingerprint after tryptic digestion.

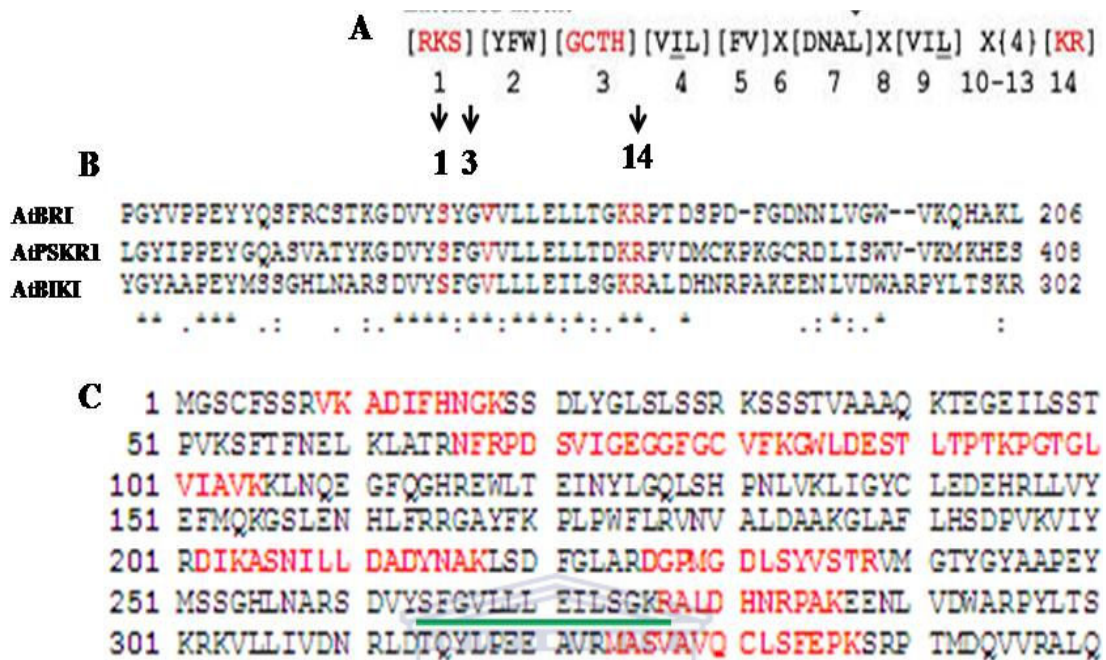


Figure 4.4.1 Clustal W alignment of AtBIKI with AtBRI1 and AtPSKR1. (A) Original guanyl cyclase (GC) search motif, within the square brackets are allowed residues within each position with the X indicating any residues in the 14 amino acid conserved GC motif. (Must tell us what the “red” residues stand for). (B) Clustal W alignment of matched sequence against AtPSKR1, AtBRI1 and AtBIKI. The black arrows depict the start and end of the search motif of the catalytic centre. (C) Protein sequence of AtBIKI in which the sequence underlined in green indicates candidate GC motif and residues in bold red being matched sequences from trypsin digest. Compared to a known GC (AtBRI1) it has the conserved amino acids that are characteristic of a putative GC i.e. position 1, 3 and 14 which are important for hydrogen bonding with the guanine, substrate specificity and stabilization of the transition state respectively.

The Phytolsulfokine and Brassinosteroid receptors amongst other LLRs have identified ligands. The binding domain for Brassinosteroids consists of a 70-amino acid island domain situated between LRR21 and LRR22 in the extracellular domain of AtBRI1 (Tang *et al.*, 2010) and AtPSKR1 a 36-amino acid island domain between the LLR17 and LRR18 (Matsubayashi *et al.*, 2006b; Shinohara *et al.*, 2007).

Our main interest in this study was to elucidate the role of cGMP in the downstream signalling cascade of these LLRs we have therefore used AtPSKR1 and developed a speculative model (Figure 4.4.2).

The model takes into account the novel finding that AtPSKR1 has overlapping dual enzymatic activity that is also observed for AtBRI1 and therefore also speculates the role of cGMP in Brassinosteroid signalling. The model proposes that in its inactive form, AtPSKR1 can be either a monomer or a dimer. Upon binding of α -PSK, AtPSKR1 becomes catalytically active and forms cGMP which modulates cGMP dependent proteins such as various cyclic nucleotide dependent ion channels (Kaplan *et al.*, 2007; Leng *et al.*, 1999). It is possible that cyclic nucleotide activity may require the homodimer to correctly form the GC catalytic site. Alternatively, it is conceivable that cross phosphorylation of AtPSKR1 may enhance dimerisation and lead to formation of cGMP-PSKR1 complexes. These in turn may promote association with receptor associated kinases such as BAK1 an interaction which is not dissimilar to Brassinosteroid signal transduction and forms a key component thereof. What is also interesting is that the GC catalytic center specifically position 1 which is involved in hydrogen bonding with the guanine in cGMP catalysis falls within the kinase domain IX of AtPSKR1. This residue in AtPSKR1 is a Serine and we have shown (Chapter 3) that cGMP inhibits autophosphorylation. It is therefore possible that once the cGMP-AtPSKR1 complex has formed it may serve as a negative feedback mechanism to switch off the cGMP production and may lead to desensitisation of the receptor. Although we have no evidence for the involvement of BAK1 in this system, BAK1 has been shown to be a promiscuous molecule that associates with several LRR RLKs including AtBRI1 and FLS2 (Chinchilla *et al.*, 2009). Once these complexes are formed, further binding of α -PSK leads to PSKR1-BAK1 cross phosphorylation (and perhaps autophosphorylation) and this can then enable the delayed adaptive response to α -PSK which may involve the activation of cGMP

dependent transcriptome. Several previous studies have implicated cGMP and calcium crosstalk in plant cell expansion (Bowler *et al.*, 1994; Donaldson *et al.*, 2004; Pharmawati *et al.*, 2001) which could be supported by the *in vitro* studies where AtPSKR1 GC activity was enhanced in the presence of the another second messenger, cytosolic free calcium (Kwezi *et al.*; under revision).

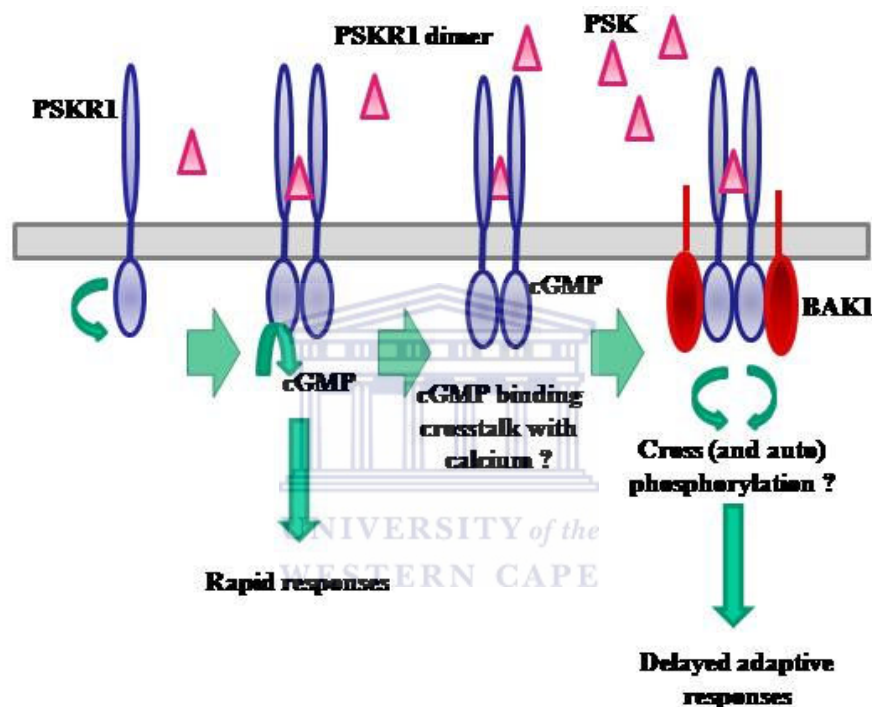


Figure 4.4.2 A Speculative model of the activation of AtPSKR1 by α -PSK and how this may relate to kinase and GC activity. In its inactive form PSKR1 could be either a monomer (pictured) or dimer that may be autophosphorylated. Upon binding of α -PSK, AtPSKR1 (probably in the dimer form as pictured) activates GC activity and this in turn may stimulate cGMP binding to the receptor. Increases in cytoplasmic Ca^{2+} may also stimulate the GC activity and there is likely to be crosstalk between the two messengers. Other rapid responses to cGMP may be initiated as well (e.g. opening of ion channels). The cGMP-PSKR1 complex may in turn promote association with receptor associated kinases such as BAK1. Once these complexes are formed, further binding of α -PSK leads to AtPSKR1-BAK1 cross phosphorylation (and perhaps autophosphorylation) and this in turn activates the delayed adaptive responses to α -PSK (e.g. activation of cGMP dependent transcriptome).

General conclusion and outlook.

Pathogens, drought and salinity are amongst the major negative determinants of plant growth and contribute to crop loss especially in marginal semi-arid agricultural areas (Denby and Gehring, 2005). This is a situation that will inevitably lead to rise in demand and therefore a rise in cost of limited resources. Food security is therefore heavily dependent on the development of crop plants with increased resistance to environmental and pathogenic factors. This continues to call for advances in the area of plant biotechnology, some of which may including overexpression of some genes be able to confer increased tolerance to biotic and abiotic stresses in plant systems. This approach has not only sparked ethical issues because of the transfer of antibiotic genes as selective markers but this approach also may have the down side in that it may cause a metabolic burden that is unprofitable (Meier and Gehring, 2006). To overcome the problems of metabolic imbalance a more rational and systemic approach will be required and bioengineering and may have to concentrate on regulatory genes and/or genes encoding molecules that activate signalling cascades in a stimulus specific way (Denby and Gehring, 2005). Guanylyl cyclases may be a promising target group of genes that hold potential for such approaches for the following three reasons. Firstly, it has been shown that cGMP is a specific second messenger in both salinity and drought responses (Donaldson *et al.*, 2004). Secondly, it has been demonstrated that increasing cytosolic cGMP levels caused by external addition of cell permeant cGMP analogues can increase salinity tolerance in *A. thaliana* by directly affecting sodium up-take in roots (Maathuis 2006). Thirdly, cGMP induced transcripts (Maathuis, 2006) include genes encoding proteins that are directly involved in the maintenance of ion and water homeostasis such as monovalent cation transporters including nonselective ion channels and cation proton antiporters.

In Chapter 2 it was demonstrated that the Brassinosteroid receptor has an active GC domain *in vitro* and subsequently it was shown (Chapter 4) that this activity is also observed *in vivo*. Brassinosteroids are of agricultural interest as they have been shown to promote plant growth (Mitchell, 1972) as well as acceleration (Braun, 1984; Gregory, 1981). The application of Brassinosteroid in corn was shown to increase the ear fresh weight by about 7 % (Lim, 1988) whilst in wheat an increase of 25-33 % and seed weight by 4-37 % was achieved (Takematsu, 1988).

Brassinosteroids have also been shown to enhance resistance to infection by various pathogens (Nakashita *et al.*, 2003). Infection by tobacco mosaic virus (TMV) causes a necrotic lesion as the result of a defence response in *Nicotiana tabacum* cv. Xanthi nc, which possesses the N gene, a gene that confers resistance to TMV (Whitham *et al.*, 1994). It was reported (Nakashita *et al.*, 2003) lesions decrease in size up to about 50 % in response to Brassinosteroid treatment. These effects were shown to not be limited to the treated leaves but also on the distal upper leaves indicating that the effects of Brassinosteroids and their role in disease resistance are not only local but are also systemic (Nakashita *et al.*, 2003). This would suggest that there are mobile signal components in the Brassinosteroid response that amplify and transduce the signal to other parts of the plant. The Brassinosteroid signalling pathway has been shown to transduce downstream signals through a phosphorylation cascade. However this phosphorylation on its own may not be sufficient in regulating the systematic pathogen resistance related roles of Brassinosteroid. There have been studies that have been done to look at Brassinosteroid responsive genes (Hu *et al.*, 2001) and proteins (Deng *et al.*, 2007) and amongst these some are involved in secretion and membrane trafficking (Deng *et al.*, 2007) and signal transduction (Deng *et al.*, 2007; Hu *et al.*, 2001) and they may well be involved in systematic effects of Brassinosteroid.

Here we suggest that cGMP may have a role in the Brassinosteroid response and its role in disease resistance and that this effect includes a transcriptional response and the phosphorylation cascade which is a characteristic of Brassinosteroid signalling (Chapter 4). The phosphorylation cascade may be mediated by cGMP and be reflected in the phosphoproteome. It is also conceivable that cGMP may be involved in transducing the systematic effects of Brassinosteroid as cGMP has been shown to affect membrane channels that in turn could transduce these signals to mobile units within the apoplast which directly or indirectly may initiate a systematic response.

In addition we have also shown that the Phytosulfokine receptor has GC activity both *in vitro* and *in vivo*. Phytosulfokines have been shown to cause strong proliferation in rice (*Oryza sativa* L.) (Matsubayashi *et al.*, 1997) and therefore are also of agronomic interest in accelerating growth. Our findings suggest that cGMP is involved in both Brassinosteroid and Phytosulfokine and that their effects on plant growth may be mediated by cGMP further highlighting the prospects of using GCs as targets for improving crops with agronomic value.

Furthermore, protein kinases play a central role in signalling during pathogen recognition and the subsequent activation of plant defence mechanisms (Romeis, 2001) and for this reason make good candidates as targets for enhancing crop resistance to diseases and environmental stresses. Several protein kinases, especially the family of Arabidopsis receptor-like kinases, have been implicated in osmotic stress responses based on their transcriptional responses to different environmental stimuli (Boudsocq and Lauriere, 2005; Chae *et al.*, 2009).

While the effects of cGMP and its role in plants defence mechanism have been shown, most of the work done demonstrates its involvement in the induction of gene expression but few studies show a direct effect of cGMP on defence related proteins and more specifically those that involve kinase signalling cascades.

Here we also demonstrated (Chapter 4) that direct application of cGMP activates a disease resistance protein (Botrytis induced kinase, AtBIKI). This observation is of interest as it may mean that it would be possible to avert from using vectors to confer disease resistance to crop plants of agronomic interest and in turn use cGMP or derivatives thereof to switch on these responses. On a more speculative note, this could be indicative of the possibility to use cGMP or cGMP derivatives as biological switches to specifically activate the required response as we have shown (Chapter 3) that these cyclic nucleotide dependent changes in phosphorylation are very specific.

To further investigate the effects of cGMP and to use it as a potential molecular switch in specifically turning on responses depending on what plants may be susceptible to under specific conditions, it would be useful to study the interaction of this second messenger with specific targets. This could be done by isolating cGMP responsive protein and doing *in silico* homology modelling and virtual docking of cGMP to potential binding sites so that the specific residues that are involved in binding cGMP can be identified. An alternative approach would be to do nuclear magnetic resonance (NMR) on the protein in the presence of cGMP so that these interactions can be determined empirically. Data gathered from these can then provide a platform to design cGMP derivatives with high affinity to activate and/or modulate the response.

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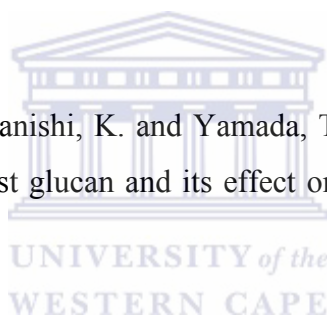
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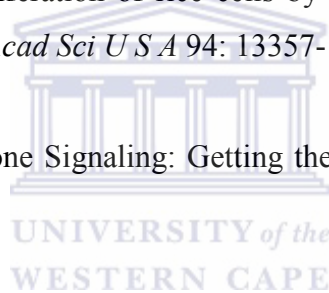
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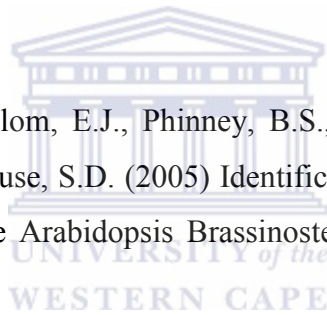
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