INVESTIGATION OF THE ROLE OF AtNOGC1, A GUANYLYL CYCLASE PROTEIN IN RESPONSE TO ABIOTIC AND BIOTIC STRESS

By

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DECLARATION

I Mpho Muthevhuli declare that "*Investigation of the role of AtNOGC1, a guanylyl cyclase protein in response to abiotic and biotic stresses*" is my own work and that all sources that I have used or cited have been given and acknowledged by full references and that this work has not previously been submitted to another institution for another degree.

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Dedicated to my late grandfather

Mavhungu Lancelot Muthevhuli "*Vhakoma*"

A farewell letter to you

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ACKNOWLEDGEMENT

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ASBRACT

Title: Investigation of the role of AtNOGC1, a guanylyl cyclase in response to abiotic and biotic stresses.

Agricultural production is one of the most important sectors which provide food for the growing world population which is estimated to reach 9.7 billion by 2050, thus there is a need to produce more food. Climate change, on the other hand, is negatively affecting major global crops such as maize, sorghum, wheat and barley. Environmental factors such as salinity, drought, high temperatures and pathogens affect plant production by oxidatively damaging the physiological processes in plants, leading to plant death. Poor irrigation used to combat drought result in salinasation, which is estimated to affect 50% of arable land by 2050. Plants have developed several mechanisms that protect them against stress and these include overexpression of stress responsive genes and altered signal transduction to change the expression of stress responsive genes, among others. Cyclic 3'5' guanosine monophosphate (cGMP), a second messenger that is synthesised by guanylyl cyclase (GC), transmit signals to various cellular functions in plants during plant development, growth and response to abiotic and biotic stresses. *Arabidopsis thaliana* nitric oxide guanylyl cyclase 1 (AtNOGC1) is a guanylyl cyclase which upon activation by nitric oxide (NO) leads to the production of more cGMP. Cyclic GMP further activates protein kinases, ion gated channels and phosphodiesterase which mediate response to various stresses.

In this project the role of AtNOGC1 was investigated in response to abiotic and biotic stresses through analysis of its evolutionary relationships, promoter, gene expression and functional analysis via the viability assays in *Escherichia coli (E.coli).* Phylogenetic tree, exon-intron structure and conserved motifs were analysed using the Molecular Evolutionary Genetics Analysis (MEGA V.7), Gene Structure Display Server 2.0 (GSDS 2.0), and Multiple Expectation Maximisation for Motif Elicitation (MEME) tools respectively. AtNOGC1's gene

expression was analysed by the Real-Time Quantitative Reverse Transcription Polymerase Reaction (qRT-PCR), whereas functional analysis was carried out using the cell viability (liquid and spot) assays to determine its ability to confer stress tolerance to *E. coli*.

The phylogenetic tree showed the presence of an NOGC1 isoform, which might be due to splicing. Among the different species, AtNOGC1 share a very close relationship with soluble guanylyl cyclase (sGC) from insects indicating that they evolve from the same ancestor. Hence they might share the same function. Analysis of gene structure indicated that the genes from different species have different distribution of exon-intron structure. However AtNOGC1 and the NOGC1-like are closely related. The AtNOGC1 promoter is enriched with cis-regulatory elements that are responsive for development, response to abiotic stress and defence against pathogen attack. These results were supported by the expression analysis, which revealed that AtNOGC1 transcript is induced by salinity, drought, hormones (ABA, MeJA, and SA) and NO treatments, suggesting that AtNOGC1 might be required for protection during stress attack. To demonstrate its ability to protect cells against the effects of stress and oxidative damage, viability (spot and liquid) assays were conducted in *E. coli* cells that were transformed with an empty pET SUMO vector (control cells) and pET SUMO-AtNOGC1 (recombinant cells). Recombinant cells overexpressing AtNOGC1 were able to grow better under different stresses as compared to control cells overexpressing empty vector, thus demonstrating the ability of AtNOGC1 to protect cells against stresses. This study successfully demonstrated that AtNOGC1 shares a common ancestor with sGC from insects and is a multiple stress responsive protein especially during pathogen attacks and has the ability to protect *E.coli* cells against stress damage.

KEYWORDS

Abiotic stress

AtNOGC1

Biotic stress

cGMP

Gene expression

Guanylyl cyclase

Nitric oxide

Promoter

Stress tolerance

ABBREVIATIONS

ABA   Abscisic acid

AC   Adenylyl cyclase

AtBRII   *Arabidopsis thaliana* Brassinosteroid Receptor 1

AtDGK4   *Arabidopsis thaliana* diacylglycerol kinase 4

AtGC1   *Arabidopsis thaliana* guanylate cyclase 1

AtHO1   *Arabidopsis thaliana* heme oxygenase 1

AtNOGC1   *Arabidopsis thaliana* nitric oxide\oxygen binding guanylyl cyclase1

ATP   Adenosine triphosphate

AtPepR1   *Arabidopsis thaliana* Phytosulfokine Receptor 1

AtWAKL10   *Arabidopsis thaliana* Wall Associated Kinase-like 10

cAMP   cyclic 3', 5'Adenosine Monophosphate

Cat#   Catalogue Number

cCMP   cytidine 3', 5'-cyclic monophosphate

cGMP   cyclic 3', 5' Guanosine Monophosphate

cNMP   cyclic Nucleotide Monophosphate

cUMP   cyclic 2', 3' uridine phosphate

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E. coli	*Escherichia coli*
ET	Ethylene
FMO	Flavin-Containing Monooxygenase
GA	Gibberellic acid
GC	Guanylyl cyclase
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
H-NOX	Heme nitric oxide
HNO_2	Nitrous acid
HR	Hypersensitive Response
IAA	Indole-3-acetic acid
IPTG	Isopropyl-D-thiogalactopyranoside
LRR-RLK	Leucine-rich repeat receptor
MAPK	Mitogen-activated protein kinase
MeJA	Methyl Jasmonate
MS	Murashige and Skoog media
NADPH	Nicotinamide Adenine Dinucleotide Phosphate

NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric Oxide Synthase
NR	Nitrate Reductase
SNP	Sodium nitroprussine dehydrate
O_2	Oxygen
OD	Optical Density
PCR	Polymerase Chain Reaction
PDEs	cyclic 3′, 5′nucleotide phosphodiesterase
PEG	Polyethylene glycol
pGC	Particulate guanylate cyclase
PSKR1	Phytosulfokine Receptor 1
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
SA	Salicyclic Acid

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SAR   Systemic Acquired Resistance

SD   Standard Deviation

sGC   soluble Guanylyl Cyclase

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Agriculture is the most important sector that contributes about 2.8% of the world economy and supports to the lives of many people (Alston *et al.*, 2016). There are two different types of the agriculture, namely, crop and animal production. Crop production is arguably the most important sector that both animals and humans depend on for food. However the production of this sector is negatively affected by abiotic and biotic stresses and this is further exacerbated by climate change. Climate change leads to extreme weather that imposes stress on plants (Pereira, 2016). Abiotic and biotic stresses are the most severe factors causing detrimental effects on the plant's life cycle, growth and development by disrupting their metabolic processes (Micco and Aronne, 2012).

Salinity, drought, low and high temperatures are some of the abiotic stresses that mediate severe damage in plants, whereas biotic stresses are induced by pathogens (viral, fungal, bacteria and insects), and mechanical damage caused by the interaction of plants with other living organisms. The most abiotic stresses occur in the semi-arid and arid areas, with drought and salinity stresses being the most frequent stress conditions. It has been estimated that about 50% of the arable land will be affected by salinisation by 2050 (Wang W, 2001), and together, the combination of both abiotic and biotic stresses can reduce crop yield by as much as 50% (Bray, 2000), thus affecting food security.

The United Nations (UN) have reported that the current world population of 7.3 billion is expected to reach 8.5 billion by 2030 and 9.7 billion by 2050 (UN, 2015). High population growth leads to an increased demand for food, and together with the lack of arable land, these factors lead to food insecurity. The global hunger index map shows that comparatively most

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African countries have been affected by hunger to a greater extent (Fig 1.1). Although South Africa is shown to be moderately affected, the projection reported by the Inter-governmental Panel on Climate Change (IPCC) found that crop production will be reduced by 50% in African countries by the year 2020 (IPCC, 2007) which calls for African countries to come up with strategies to manage hunger.
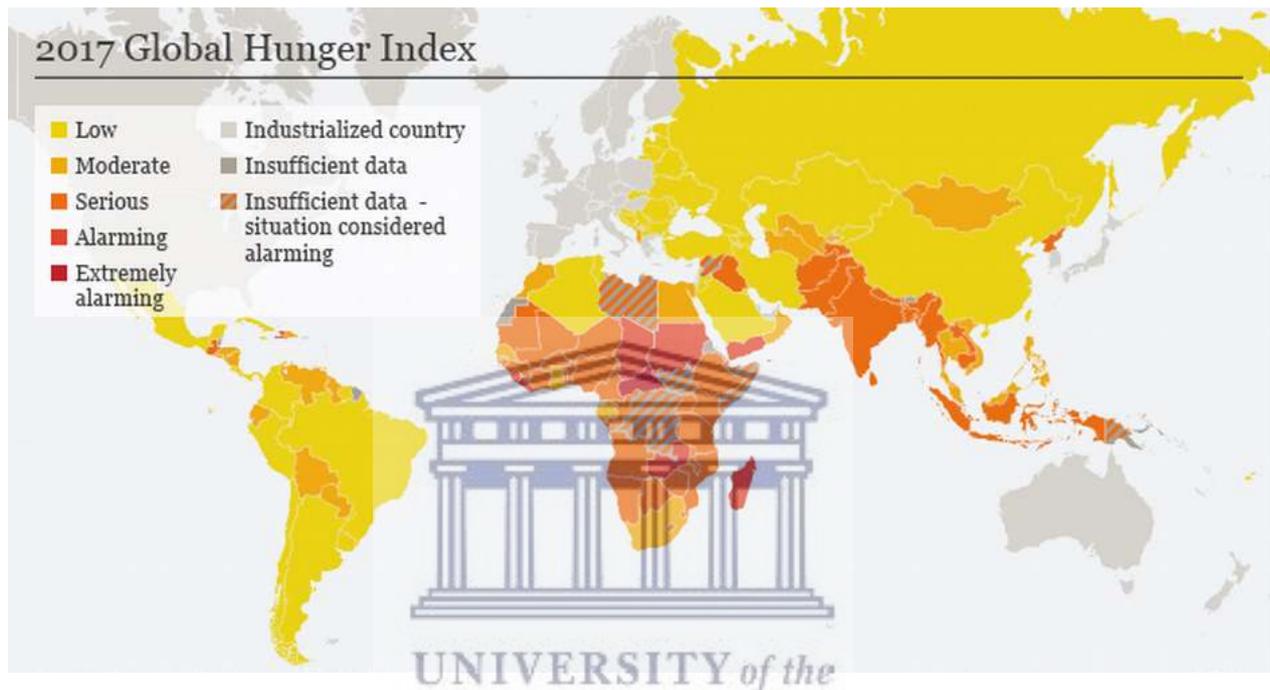
Fig 1.1: Global Hunger Index map. In this map the world hunger is illustrated, which clearly shows that the African continent is the most affected region, with Zambia and central Africa at extreme alarming score as shown in ruby red and South Africa at moderate level shown in light orange (Adapted from Weltungerhilfe, 2017).

Plants usually face the simultaneous effects of both abiotic and biotic stress conditions, which result in the production of potentially toxic or damaging substances that hinder their production, thus reducing crop production (Rejeb *et al*., 2014). Several strategies have been implemented to improve food security. One of the most important plans is the improvement of crop production. This relates primarily to the continuous development of crops, including breeding, screening and selection of existing germplasm, application of osmoprotectants and genetic engineering (Athar and Ashraf, 2009). For one to develop crops that can grow and produce under the harsh conditions, there is a need to understand the effects of stress on plants

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and their mechanism of stress tolerance and these will be discussed in this review with a focus on signalling abiotic and biotic stress responses.

1.2 Abiotic stress

Abiotic stresses are environmental factors that are unfavourable to the growth and development of plants such as drought, salinity, temperature and heavy metals amongst others (Zhu, 2017).

1.2.1 Drought

The stress imposed by drought on plants has resulted on major losses in agricultural production and is considered the most detrimental stress, which affects crop productivity by reducing the available water that is required by plants for their growth and development. A lack of water reduces cell division and expasion, differentiation and nutrient absorption which makes it difficult for plants to complete their life cycle. Lack of energy is caused by stomatal closure that impairs photosynthesis and this in turn causes overreduction of the electron transport chain which produces reactive oxygen species (ROS) that impair enzyme activity (Fathi and Tari, 2016). Drought stress can affect crop yield by inducing phenology which shortens crop growth cycles. Short cycle is triggered by lack of water and results in early maturation of plant crops from the vegetative to mature phase (Desclaux and Roumet, 1996). Thus, if drought occurs at an early stage of plant development crop yield is reduced thus affecting food production (Farooq *et al.*, 2012).

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1.2.2 Salinity

Soil salinisation is increasing due increased use of poor quality water for irrigation and is another factor reducing crop production (Zhang *et al.*, 2010). Salinity stress induces both primary and secondary effects on plants. Primary effects include osmotic and ionic effects which hinder the plant's ability to absorb moisture (Zhang *et al.*, 2010) while secondary effects include nutrient deficiency, ion imbalance and oxidative stress. Primary effects are the first stage of salinity stress and osmotic stress that initiates various changes in physiological processes (secondary effects) such as disruption of membranes, nutrient imbalance, reduction in photosynthetic activity and an increase in ROS. ROS damages the normal metabolism of plant cells which include lipid peroxidation and damage to nucleic acids (Evelin *et al.*, 2009). Excessive salt in plants results in high level of sodium (Na^+) and chloride (CI^-) ions which are absorbed into the cells and cause disorder. Na^+ ions lower potassium (K^+) content by inhibiting the uptake of K^+ ions by cells. Thus affecting growth and development of plants (Gupta and Huang, 2015). The inhibition of K^+ ions leads to K^+ starvation in plants and uptake because Na^+ ions compete with K^+ for binding sites on enzymes, which eventually leads to senescence (Blumwald *et al.*, 2000).

1.2.3 Temperature

1.2.3.1 High temperature

Plants can either be affected by low or high temperature, and as with drought and salinity stresses, extreme temperature results in physiological, morphological and biochemical changes in plants, affecting their production. High temperatures impose heat stress on plants which in combination with other abiotic stresses such drought results in metabolic changes in plants. Different changes have been reported on plants as a results of high temperature, such as reduced root assimilation rates, short life cycle, impaired defence mechanism and decrease in

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production of proteins that are required for survival such as heat shock proteins (Wahid *et al.*, 2007; Barnabás *et al*., 2008; Bita and Gerats, 2013).

1.2.3.2 Low temperature

Normally, most plants survive at temperature ranges between 10° C - 35° C and they can photosythesise and grow as low as 10° C. At temperatures below 10° C, photosynthesis is inhibited for tropical and subtropical plants. There are two major types of low temperature, including freezing and chilling (Oquist *et al.,* 1983). The freezing and chilling of plants damages thylakoid membranes, and thylakoid membranes contain chlorophyll, which absorbs the light used for photosynthesis. Damage to the thylakoid membrane inhibits processes such as the electron transport chain and Adenosine triphosphate (ATP) synthesis, which are essential for photosynthesis. In addition, the stomata get blocked by chilling stress and responds to water deficits in plants is prevented (Simpson and Von Wettstein, 1989; Allen and Ort, 2001).
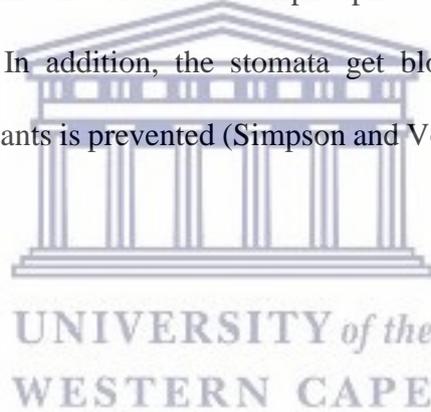
1.2.4 Heavy metals

Plants require certain heavy metals for their growth and development such as iron (Fe), copper (Cu) and Zinc (Zn). Excessive amounts of non-essential elements, including cadmium (Cd), chromium (Cr), nickel (Ni), and lead (Pb) can be toxic to plants and this directly or indirectly affects agricultural crop production (Shin *et al.*, 2013). The direct toxic effects result in oxidative damage which impairs enzymes and damages to cell structure. Indirect toxic effects occur when heavy metals replace the essential elements required for plant development (Assche and Centrum, 1990). A high concentration of heavy metals in the plant reduces absorption and transportation of essential elements which impact growth and development. High levels of Cd have been reported to reduce germination rate, growth and photosynthesis

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of barley and rice respectively and imposed negative effects on plant cell division (Cheng, 2003). Both the direct and indirect toxic effects lead to the inhibition of plant growth and hence plant death (Asati *et al.*, 2016).

1.3 Biotic stress

Biotic stress causes additional challenges in plant crops through changes in plant physiology and defence responses. Biotic stress, is usually caused by pathogens (viruses, fungi, and bacteria) while mechanical wounding is caused by the interaction of plants with other living organisms (Pandey *et al.*, 2017).

1.3.1 Pathogens

Stress imposed by pathogens affects plant development and production by altering primary metabolism (Mcconville, 2014). Microorganisms become virulent as a result of the production of effector molecules, leading to virulence, for example, by inhibiting plant defense mechanisms that prevent the spread of virulent pathogen within plants (Jones and Dangl, 2006). Pathogens alter plant carbohydrate metabolism and use them as their own source of energy and nutrients and this alteration increases the demand for assimilation by plants. Pathogens furthermore, cause chlorosis and necrosis as a result of pre-mature cell death in plants, probably because pathogens and plants compete for carbohydrates and this effect decreases photosynthetic assimalate (Berger *et al*., 2007). Several pathogens of concern to crop plants include *fusarium* species, *tobacco mosaic* and tomato spotted viruses amongst others. Fusarium species affect yield and quality of important crops such as maize by producing mycotoxins including zearalenone (ZON) and deoxynivalenol (DON) that contaminate crops (Czembor *et al*., 2015), whereas viruses cause necrotic local lesion on plants which hinder their growth and production (Scholthof *et al.*, 2011) .

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1.3.2 Wounding

Plants wounding is another biotic factor that imposes negative effects on plant growth and metabolism. Plants are continuously exposed to mechanical damage as a result of injuries caused by insects, herbivores or abiotic stress such as heavy rain and wind. Injuries cause opening that allow pathogen invasion. Upon the entry of pathogens, the wounded tissue provides nutrient to the pathogens as a result of the chemical changes to the damaged area (Gimenez *et al*., 2018). During feeding, insects or herbivores act as carrier of other pathogens such as virus, transmitting them into the plants. Wounding results in ion imbalance and production of ROS which in excess amount can damage the plants or cells (Caverzan *et al.*, 2012).
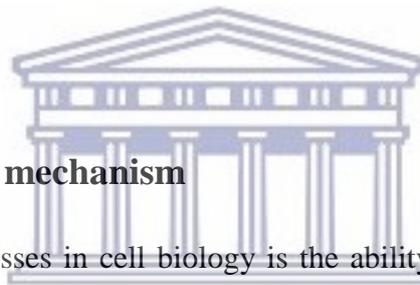
1.4 Signalling as defence mechanism

One of the fundamental processes in cell biology is the ability of cells to interact with and adapt to their environment. This response is mainly achieved by expressing a variety of specific receptors sensitive to the composition of the surrounding environment on the cell surface. The receptors then transmit extracellular signals across membranes and provide the appropriate functional response through the activation of intracellular signalling pathways. In addition, cells have developed complex systems for integrating signals (Uings and Farrow, 2000).

1.4.1 Hormonal responses

During plant response to stress stimuli, signalling pathways are initiated that interact with various hormones including auxins, gibberellins (GA), abscisic acid (ABA), ethylene (ET), jasmonate (JA) and salicylic acid (SA) amongst others (Verma *et al*., 2016). ABA is known to play a key role in abiotic stress responses while SA, JA, and ET are associated with defense responses during pathogen and insect attack (Zhang *et al.*, 1987; Bari and Jones, 2009).

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1.4.1.1 Abscisic acid

The role of ABA in plants has been well studied and it is shown to be associated with the maintenance of water balance by stimulating stomatal closure during salinity and drought conditions. The role of ABA was first observed when wilty tomato *flacca* mutant was deficient in ABA and its phenotype was rescued through exogenous ABA treatment which suggested a role for ABA in water relationships and its application on Xanthium resulted in stomatal closure (Jones and Mansfield, 1970; Tal *et al.,* 1974). Several genes have been reported to be up-regulated by ABA, including zeaxanthin epoxidase and molybdenum cofactor sulfurase genes. Analysis of the promoters of an ABA-inducible genes the revealed multiple cis-regulatory elements such as the well-known ABA-responsive elements (ABREs) and binding sites for basic leucine zipper transcription factors (Zhang *et al.,* 1987; Zhu, 2011). In addition, ABA has been associated with pathogen responses; its effects range from resistance by inhibiting the entry of pathogen via the stomata to increasing susceptibility by interfering with defense responses mediated by other signalling pathways (Ton *et al.,* 2009).

1.4.1.2 Salicylic acid

SA is a plant hormone that has been well studied in the biotic stress response (Vlot *et al.*, 2009). SA protects plant tissues against biotrophic and hemi-trophic pathogens such as *Pseudomonas syringae* and plants increase the accumulation of endogenous SA in necrotic lesions and surrounding tissues following infection with biotrophic pathogens. Accumulation of SA increases the level of pathogenesis related (PR) genes which are diverse and contain antimicrobial activity against pathogens (Loon *et al.*, 2006). At optimal concentration, SA was reported to play a role in abiotic stress responses through its involvement in redox regulation. A high concentration of SA can reduce stress tolerance by disturbing the redox potential and its role in stomatal closure via the production of ROS has also been reported (Dong, 2001).

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1.4.1.3 Jasmonates

Methyl jasmonate (MeJA) and its free-acid 'jasmonic acid' (JA), collectively referred to as jasmonates, are important cellular regulators involved in various processes of development, such as germination of seeds, root growth, fertility, fruit ripening and senescence (Wasternack and Hause, 2002). In addition, these hormones have been associated with activation of defence mechanisms against necrotrophic pathogens and insects, and environmental stress such as salinity and drought (Cheong and Choi, 2003). Accumulation of MeJA in plants, results in the up-regulation of genes involved in jasmonate biosynthesis, cell-wall formation, photosynthesis and stress defences. Regulation of the JA responsive genes are mainly mediated by the MYC2 transcription factor and it's *D*efensin 1.2, marker genes that provides resistance to necrophic pathogens. (Solano *et al.*, 1998).

1.4.2 Nitric oxide

Nitric oxide (NO) is a colourless, gaseous intra and extracellular messenger that is used as a signalling molecule in both plants and animals. NO transmit signals to cell signalling pathways that are known to play a vital role in physiological processes. It is highly diffusible with a low density (4.8×10^{-5} cm^2 s^{-1} in H_2O) and can pass freely through the hydrophilic and lipid phase of the cell membrane. NO has a half-life of about < 6 sec (Bethke, 2004) and it can act as a physiological messenger that can react with other molecules rapidly including superoxide anions, metalloenzyme, and oxygen, which later produce nitrate and nitrite in aqueous environments. It reacts directly with complex metals and other radicals and indirectly with DNA, proteins and lipids as reactive nitrogen species (Wink and Mitchell, 1998). Free radicals are molecules that have unpaired electrons and are thus highly reactive. About three forms of NO are responsible for electron transfer namely the highly reactive (NO˙), the nitrosonium

cation (NO^+) and nitroxyl anion (NO). NO interacts with O_2 to produce NO_2 which is later broken down to form both nitrite and nitrate in the aqueous solution (Cevahir *et al.,* 2007).

1.4.2.1 Nitric oxide synthesis

In animal cells, NO is enzymatically produced by nitric oxide synthases (NOS) in an NADPH-dependent oxidation manner that is well characterised. (Forstermann & Sessa, 2012). In plants, NO is synthesised either enzymatically or non-enzymatically in the cytosol, nucleus, peroxisome matrix and chloroplasts. NO can be produced by several non-enzymatic reactions (Wojtaszek, 2000), under acidic conditions. At low pH, nitrite (NO^-_2) reacts with a proton to form nitrous acid (HNO_2) and two molecules of HNO_2 which can then produce NO and Nitrate Dioxide (NO_2), which is later converted into NO and oxygen (O_2) as shown in equation 1 (Environ *et al.*, 2014).

$$2NO_2 + 2\,H^+ \leftrightarrow 2\,HNO_2 \leftrightarrow NO + NO_2 + H_2O \leftrightarrow \leftrightarrow 2\,NO + \tfrac{1}{2}\ldots\ldots\ldots 1$$

NO is also produced non-enzymatically through the reaction of ascorbate and HNO_2 to produce dehydroascorbic acid and NO under acidic conditions (Bethke, 2004). NO synthesis can also occur via biological nitrification and denitrification, where under aerobic condition ammonia is converted into nitrate (nitrification), N_2O and nitrogen gas under anoxic conditions. NO synthesis can also occur enzymatically through the production of L-citrulline from L-arginine catalysed by NOS-like enzymes. These enzymes are located in the chloroplasts and peroxisomes. Lastly, nitrate reductase (NR) is also known to synthesise NO, from nitrate and arginine. NR catalyses the conversion of nitrate to nitrite in higher plants and produces NO from nitrite using NAD(P)H both *in vitro* and *in vivo* (Cevahir *et al*., 2007). Figure 1.2 shows different pathways for the production of NO (Wojtaszek, 2000)
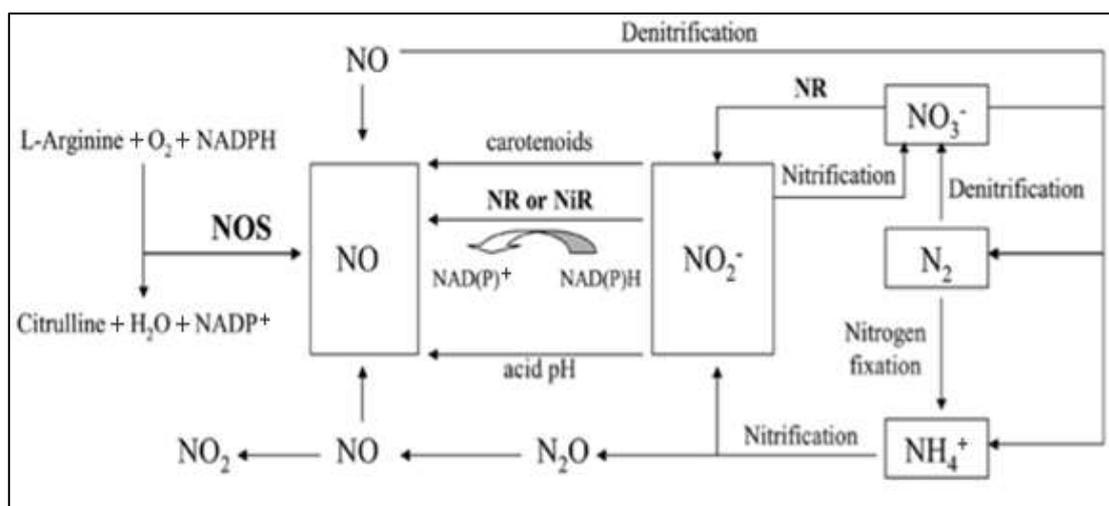
Fig 1.2: Production of NO in plants. The Fig illustrates the possible sources of NO. NO is synthesised by the action of NOS. From L-arginine in a reaction requiring O_2 and NADPH and also from citrulline in an $NADP^+$ dependent reaction. Other sources of NO are produced non-enzymatically from NO_2^- either at acidic pH or using the energy of light absorbed by the presence of carotenoids, and enzymatically is catalysed by NAP (P) H dependent NR or NiR reductase. NO can also produce as a by-product of denitrification and nitrification (Wojtaszek, 2000).

1.4.2.2 Biological roles of nitric oxide in plants

1.4.2.2.1 Plant growth and development

Nitric oxide (NO) is also a signalling molecule that initiates various responses to stimuli. NO functions as a growth regulator during plant development by promoting germination, leaf development, root growth and delaying the final stage of fruit maturation and transmission of signaling molecules. Itinteracts with plant hormones for example by inducing auxin synthesis during root development and influences the mature stage of senescence through ethylene biosynthesis. Exogenous application of an NO donor was reported to induce light dependent germination in *Arabidopsis thaliana* and lettuce (Beligni and Lamattina, 2000; Batak *et al.*, 2018). NO also promotes initiation of root tip expansion on lupine and maize (Kopyra and Gwóz, 2003; Quaggiotti, 2016). In addition NO initiated indole-3-acetic acid (IAA) induction of Mitogen-activated protein kinase (MAPK) cascades, which participate in adventitious root development (Lanteri *et al.*, 2004).

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1.4.2.2.2. The plant defence response against pathogens

In plants, the defence response are classified into an innate and systemic response. The innate response is further divided into specific and non-specific. Non-specific is not well characterised, but it includes constitutive barriers such as morphological and structural barriers, chemical compounds and various enzymes that may be present or produced prior to infection. Innate and induced immune hypersensitive response (HR) is mostly used for pathogen and herbivorous insect attacks. NO causes cell death around the affected area of the leaf, thus preventing pathogens from multiplying. NO also plays an important role in response to pathogen attacks (Ferreira and Cataneo, 2010) by activating the expression of defence genes which leads to resistance required by plant (Romero-Puertas *et al.*, 2004). Another stress which can rise as a result of both abiotic and biotic include mechanical damage through herbivores, wind, or flood. There are several studies which have reported high accumulation of NO during mechanical injury or wounding which was linked with the jasmonic acid defence responses (Huang *et al.*, 2004).
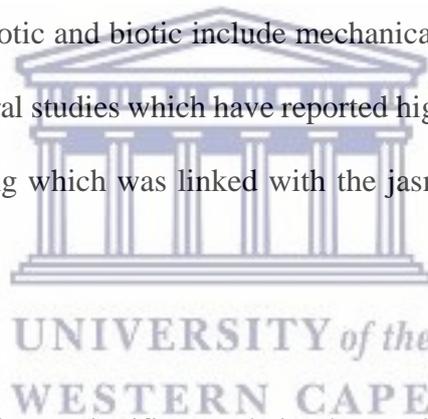
1.4.2.2.3. Abiotic stress

NO as a signalling molecule plays a significant role in plants tolerance to abiotic stresses with the help of other signalling molecules such as cGMP and Ca^{2+} amongst others (Fig 1.3). NO plays a significant role in drought tolerance in plants through the induction of stomatal closure and reduction of oxidative stress. During water deficit NO can interact with other plant hormones including ABA, SA and Auxin. ABA is an important hormone that causes stomatal closure during stress by stimulating the production of NO which in turn initiates stomatal closure (Adimulam *et al*., 2017). Various studies have been performed to show the relationship between drought stress and NO. Based on the study reported by Gar cia-Mata and Lamattina (2001), exogenous application of NO on wheat leaves and seedlings increased stress tolerance.

NO was reported to interact with ROS during abiotic stress and in turn initiates stomatal closure through the synthesis of 8-nitro-cGMP (Joudoi *et al.*, 2013).

NO also plays a crucial role in salt stress tolerance and its biological role was demonstrated in *Arabidopsis thaliana* using *Atnoa1* mutants (Zhao *et al.,* 2007). Exogenous application of a NO donor (S-nitroso-N-acetylpenicillamine) to salinity stressed chickpea was able to mitigate the detrimental effects of salinity stress by regulating the synthesis of osmolytes and antioxidant enzymes (Ahmad *et al.*, 2016). The activity of antioxidant enzymes was also induced in salinity stressed sour orange plants (*Citrus aurantium* L), which were treated with NO (Tanou *et al.*, 2012).
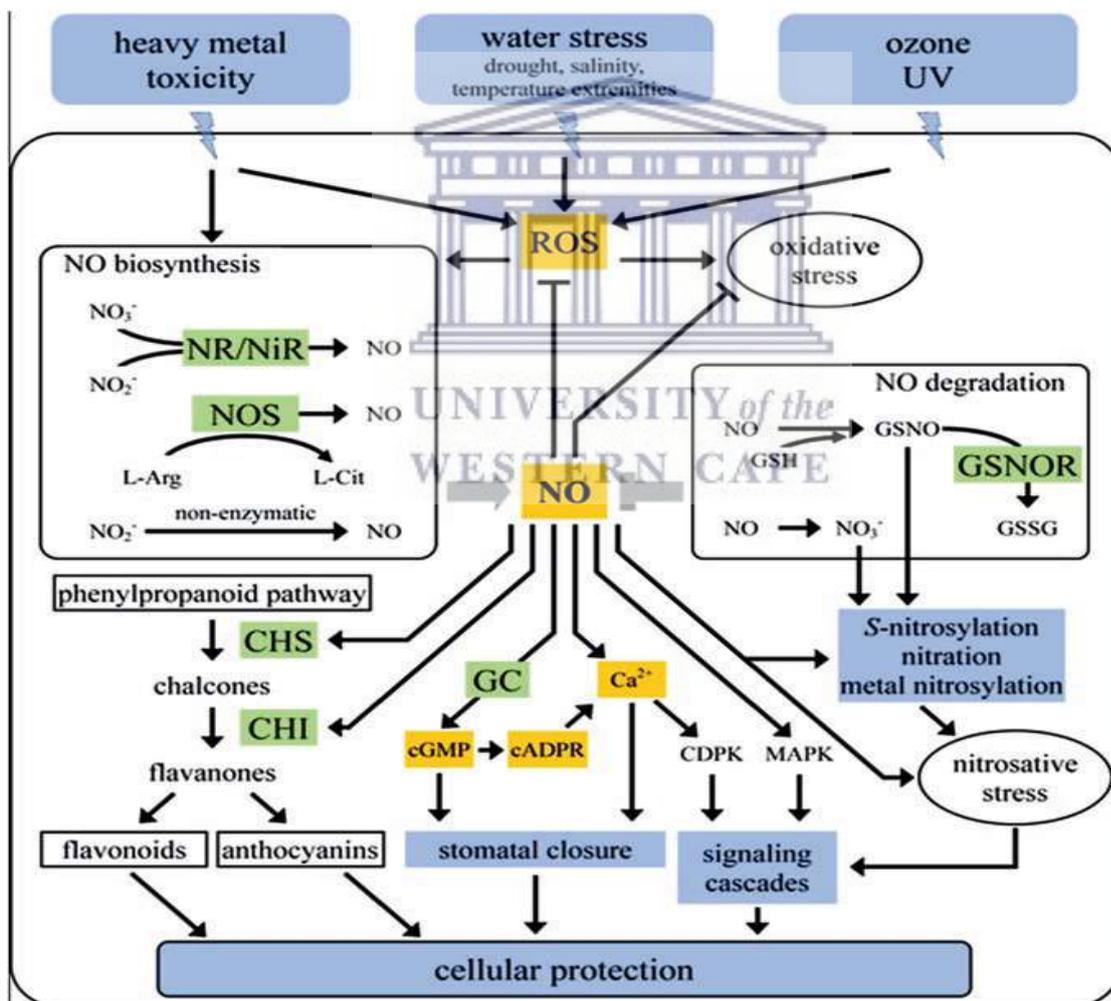
Fig 1.3 Schematic presentation of the relationship between NO and other signalling molecules during abiotic stresses. Via its interaction with other molecules, NO leads to cellular protection, against the effects of abiotic and biotic Stresses (adapted from Farnese, 2016).

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1.5 NO induced downstream signalling pathways during stress responses in plants.

There are different downstream signalling pathways employed by NO in response to environmental stresses. These include cGMP, calcium ion (Ca^{2+}) and MAPKs and their interplay is well demonstrated in Fig 1.3. In both animals and plants, NO regulates Ca^{2+} channels which facilitates Ca^{2+} flux, whereas MAPK cascades are crucial in eukaryotes for transducing the perception of environmental stimuli into internal signalling pathways (Rodriguez *et al.,* 2010). NO also regulates cGMP signalling by inducing the production of cGMP from guanosine triphosphate (GTP) catalysed by guanylyl cyclase enzymes which functions in signal cascade (Denninger and Marletta, 1999).

1.6 Cyclic nucleotides

Cyclic nucleotides (cNMPs) were discovered in the 1950s (Isner, Nu and Maathuis, 2012) and they are some of the most well characterised signalling systems which transport signals to the cytosol in response to environmental or hormonal stimuli. There are two well studied cNMPs identified that act as second messengers, namely 3', 5'-cyclic adenosine monophosphate (cAMP) and cyclic 3'5' guanosine monophosphate (cGMP) (Newton & Smith, 2004). cytidine 3', 5'-cyclic monophosphate (cCMP) and cyclic 2',3' uridine phosphate (cUMP) are less studied (Gomelsky, 2011). The cNMP's are sythesised from nucleotide-5′-triphosphates, in the reaction catalysed by various enzymes including guanylyl cyclases and adenylyl cyclases. Once they have been synthesized and the message transduced, they are metabolized by phosphodiesterases (PDEs) that hydrolyses cyclic nucleotides to nucleotide 5′-monophosphates and a variety of cyclic nucleotide receptor proteins (Linder and Schultz, 2010).

1.6.1 Guanylyl and Adenylyl cyclases

Guanylyl cyclases (GCs) and Adenylyl cyclases (ACs) are enzymes that catalyse the synthesis of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) from guanosine 5' triphosphate (GTP) and adenosine triphosphate (ATP), respectively. Both enzymes play important roles in signalling pathways (Gehring *et al*., 2017). These enzymes are divided into different groups which form the nucleotide cyclase family, these include soluble guanylyl cyclase (sGC), particulate guanylyl cyclase (pGC) and adenylyl cyclase (AC), all which are involved in wide range of signal transduction mediated by cyclic nucleotides (Denninger and Marletta, 1999). Soluble GC is a heterodimeric, hemoprotein that can bind to NO and result in the increased production of cGMP (see Fig 1.4). Unlike sGC, ACs are heterotrimeric G-proteins and monomeric and are activated by a small isoform of diterpene. There are two types of AC's which are either transmembrane helices or intracellular enzymes. Particulate GC is a membrane-bound enzymes and plays a role in the development of the structure and function of sGC. pGC's are characterised by having two domains, extracellular peptide receptor and also an intracellular catalytic domain. Although different, the mechanism of activation of pGC and sGC is known to be the same (John, 1994). Among the three enzymes, sGC is the only conclusively proven receptor for NO which triggers many physiological responses by producing cGMP (Denninger and Marletta, 1999).

1.6.2 cAMP

cAMP is a second messenger and plays the important roles in signalling pathways in both animals and plants. cAMP synthesis occurs when adenylate cyclases (ACs) catalyse the convention of ATP to cAMP. In higher plants cAMP's function is in conclusive because its levels in plants were reported to be far lower than that in animals. Nevertheless, there are data to support a role for cAMP in plants for example, *Vicia faba* which revealed that the outward

potassium (k^+) current increase depend on the intracellular application of cAMP and it was said to occur through cAMP-regulated protein kinase (Li, 1994).

1.6.3 cGMP

Another second messenger is the cGMP that was discovered in the 1960s in both prokaryotes and eukaryotes. In plants it plays an important role in signalling, plant development and responses to abiotic and biotic stress (Gross and Durner, 2016). Similar to NO the role of cGMP in plants is not well demonstrated. Cyclic GMP is synthesised from guanosine triphosphate (GTP) through the action of guanylyl cyclases (GCs) in response to stimuli. It is involved in many cellular responses, including protein kinase activity, cyclic nucleotide-gated ion channels and cGMP regulated cyclic nucleotide phosphodiesterase (Denninger and Marletta, 1999; Gehring *et al.,* 2017).
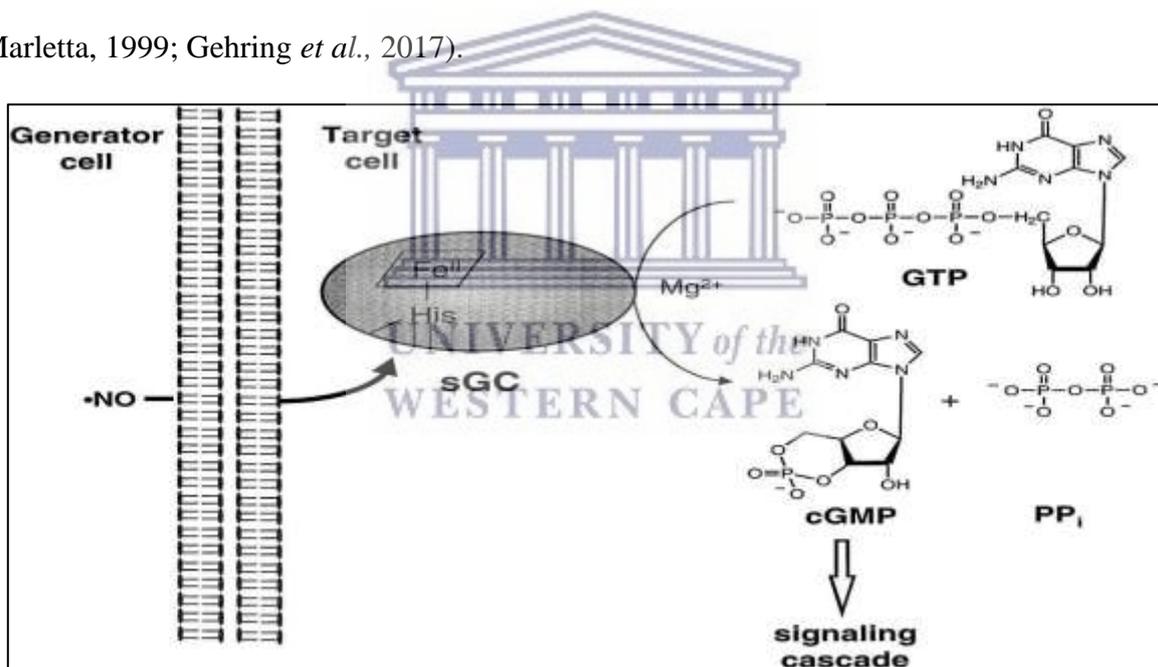
Fig 1.4. Nitric Oxide signalling transduction pathway. NO binds to the sGC heme which leads to cGMP synthesis and to downstream signalling cascade adapted from (Denninger and Marletta, 1999).

1.7 The NO/cGMP signalling pathway

The NO/cGMP pathway was first discovered in the 1980's, and there are several systems that provided concrete evidence that cGMP is a NO signalling intermediate (Neill *et al.,* 2003). This

signalling pathway is well studied in animals, playing major roles in smooth muscle relaxation and blood pressure regulation (Archer *et al.*, 1994; Mergia and Stegbauer, 2016). The pathway is initiated when NO binds to the sGC resulting in the production of high levels of cGMP from guanosine triphosphate (GTP). This increase in cGMP allows the sGC to transport NO the signal to the downstream elements of signalling cascades (see Fig 1.4) that stimulate or initiate various responses. The signal generated can normally be terminated by PDEs enzymes (Denninger and Marletta, 1999).

In plants this pathway is also known to occur and it plays an important role during root development, gene expression regulation, stomatal closure and the defence response against pathogens (Durner *et al.*, 1998; Neill *et al.*, 2008; Xuan *et al.*, 2012). The relationship between NO and cGMP in plants was first reported by Durner *et al* 1998 where *Nicotiana tabacum* was treated with NO donors that triggered the expression of defence genes including phenylalanine ammonia lyase (PAL) and pathogenesis related 1 (Durner *et al*., 1998). The NO-cGMP pathway was also reported to be involved in the development of adventitious roots in cucumber (Xuan *et al.*, 2012). After several studies on the interplay between NO and cGMP in plants, it was concluded that essential NO signalling players also exist in plants such as cGMP, but that metabolism is not really understood (Gross and Durner, 2016).

1.7.1 Identification of enzymes involved in plant cGMP biosynthesis

Towards the search of enzymes that are involved in the synthesis of cGMP in plants, several studies were conducted using motifs searches, which resulted in the identification of proteins that were subsequently demonstrated to have GC's activity *in vitro* (Ludidi and Gehring, 2003; Kwezi *et al.*, 2007, 2011; Meier *et al.*, 2010; Mulaudzi *et al.*, 2011). Several GC enzymes in higher plants were identified based on the strategy that the catalytic centre of the GCs was in part conserved across different kingdoms. Search motifs were designed and tested based on

various functional amino acids present in the GC catalytic sites from animals and lower eukaryotes. About four molecules were identified in higher plants and confirmed to have GC activities, these include the *Arabidopsis thaliana* guanylate cyclase1 (AtGC1) a soluble protein with a GC domain in the N terminus but it does not contain a heme binding domain that is important for NO binding (Ludidi and Gehring, 2003). The *B*rassinosteroid receptor (AtBRII), a leucine-rich repeat receptor like kinase (LRR-RLK) GC identified using the initial search motif [RKS][YFW][GCTH][VIL][FV]X[DNA]X[VIL]X{4}[KR]. It contains a GC domain which is found within the intracellular kinase domain and showed GC functional activity *in vitro* (Kwezi *et al.,* 2007). Wall-associated kinase-like10 (AtWAKL10) was confirmed to contain GC activity and respond to biotic stress (Meier *et al.,* 2010). The phytosulfokine receptor (PSK) is known to stimulate growth of the plant and it has GC activity both *in vitro* and *in vivo* (Kwezi *et al.,* 2011). A novel *Arabidopsis thaliana* nitric oxide binding guanylyl cyclase protein (AtNOGC1) annotated as a flavin-containing monooxygenase (At1g62580) was identified by searching the *Arabidopsis thaliana* sequence database for the key residues in the GC catalytic center and the heme-binding (HNOX), is the only proven protein that have GC activity and has ability to bind NO through the heme binding domain as shown electrochemically (Mulaudzi *et al.*, 2011).

After the discovery and characterisation of AtNOGC1, to date only one study indicated the involvement of a sGC "NOGC" in the plant process of stomatal closure (Joudoi *et al.*, 2013). This study supported that indeed AtNOGC1 is an NO-mediated protein and without its presence ABA cannot activate stomatal closure as shown in Fig 1.5. Thus ABA induces NO production through binding to sGC that mediates the cGMP pathway. cGMP induces 8-nitro-cGMP by reacting with reactive nitrogen species(RNS) that activate SLOW ANION CHANNEL 1(SLAC1).This review chapter indicated the need for research into abiotic and biotic stress responses mediated through the NO/cGMP signalling pathway. But for that to

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occur, a functional characterisation of AtNOGC1 needs to be thoroughly conducted in order to

understand its biological role in plants.
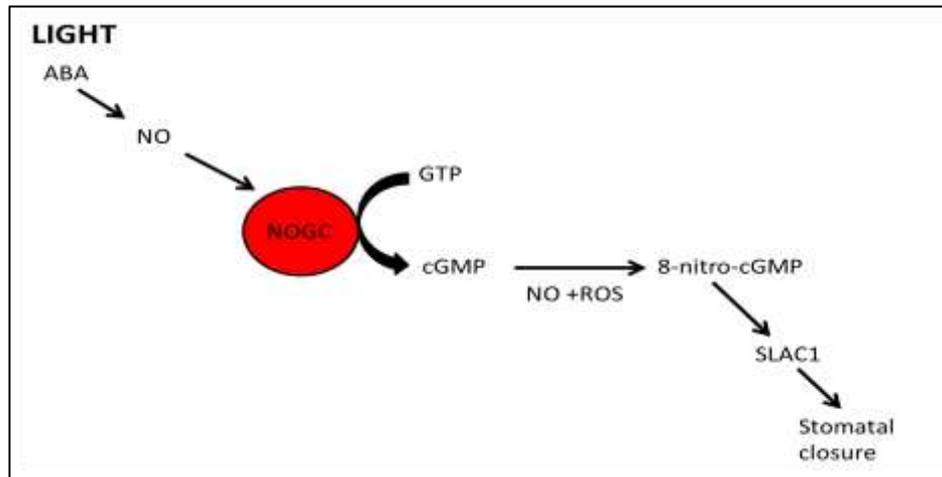
Fig 1.5. Proposed model of stomatal closure under light stress. ABA induces the production of NO which mediate cGMP pathway by binding to sGC. cGMP induces 8-nitro-cGMP by reacting with RNS which activate production of SLAC1, thus activating stomata closure adapted from (Joudoi *et al.,* 2013).

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1.8. Hypothesis and Aim

NO together with cGMP are known to be involved in many metabolic processes involving plant growth, development and stress responses (Domingos *et al.*, 2015). AtNOGC1 is required in the NO/cGMP pathway during plant development, abiotic and biotic stresses. But minimum information regarding its role in plants is available. Although for some years the synthesis of cGMP via the NO activated manner was not yet reported. Hence Mulaudzi *et al.,* (2011) reported the presence of a sGC that has an HNOX motif followed by its role in stomatal closure confirmed *in planta* (Joudoi *et al.*, 2013), thus linking AtNOGC1's role in drought stress. However, the biological role of AtNOGC1 relative to other stress alleviation is not yet elucidated. The aim of this study was to investigate the biological role of AtNOGC1 in response to abiotic and biotic stresses.

1.10 Objectives of the study

- To conduct evolutionary relationships and promoter analysis of AtNOGC1
- To analyse the expression of AtNOGC1 gene in response to abiotic stresses, hormones and NO treatments.
- To study the ability of AtNOGC1 to confer stress tolerance to *Escherichia coli* cells.

CHAPTER 2

CHARACTERISATION OF AtNOGC1 USING AN *IN SILICO* APPROACH

ABSTRACT: AtNOGC1 is the first plant protein containing a guanylyl cyclase (GC) activity and an HNOX motif that senses nitric oxide (NO) with higher affinity than oxygen (O_2) from higher plants. Unlike other soluble guanylyl cyclases (sGC) from mammals, insects and nematodes, which synthesise a 100 fold excess of cyclic 3',5 guanosine triphosphate (cGMP), AtNOGC1 only synthesised 2 fold excess of cGMP upon NO activation. Additionally AtNOGC1 was demonstrated to be involved in stomatal closure, however evolutionary history with other GC's and the cis-regulatory elements associated with this gene have not been reported to date. In this study phylogenetic tree, gene structures, conserved motifs and the cis-regulatory elements of AtNOGC1 were analysed using the Molecular Evolutionary Genetics Analysis (MEGA V.7), Gene Structure Display Server (GSDS), and Multiple Expectation Maximisation for Motif Elicitation (MEME) and PlantCARE tools respectively. Phylogenetic tree analysis revealed that AtNOGC1 has a common ancestor with other GC's from plants, nematode, and mammals, with the closest observed to be an insect sGC "AGAP010398-PA". The tree also showed that AtNOGC1 and NOGC1-like are isoforms, probably due to splicing. The gene structure displayed that exon-intron structures within genes of the same organisms were similarly distributed. The AtNOGC1 promoter sequence is enriched with *cis*-acting regulatory elements required for development, stress response and defence against diseases. These results suggested the potential of further characterising AtNOGC1 towards crop improvement under different stress conditions through generation of transgenic plants.

Keywords: *In silico*, *Cis*-regulatory elements, Phylogenetic analysis, Guanylyl Cyclase, Gene structure

2.1 Introduction

Bioinformatics is an advanced technology that is currently being used in research to analyse biological data. This is as a result of large amount of data generated from genomic and proteomic studies (Raza, 2012). Genomic data is the most essential information used to study plant genetic variation at molecular level. The genome of various plants have been sequenced, including *Arabidopsis thaliana (*Thale cress*), Zea mays* (Maize), *Sorghum bicolor* (Sorghum), and *Oryza sativa* (rice) amongst others (The Arabidopsis Genome Initiative, 2000; Bedell *et al.*, 2005; Yuan, 2005; Pereira, 2016). Bioinformatics has been used in vast biological scientific areas, including transcriptomics, microarray, regulatory sequence and computational proteomics (Rhee and Dickerson, 2006). Computational analysis include gene expression, protein-protein interaction, gene classification and evolutionary history, predictions of structures and functions of unknown genes (Raza, 2012).

Evolutionary relationships provide the foundation to conduct many gene comparison research, by studying the gene or protein sequences of genetically related organisms (Green *et al.*, 2010). The DNA sequence of different organisms has a genetic marker that is used to report the evolutionary relationships and phylogenetic positions. The evolutionary relationship is concluded from phylogenetic analysis and is represented as a tree-like diagram. The tree has estimated pedigree which shows the scale on how organisms are closely related (Waikagul and Thaenkham, 2014). The statistical analysis of molecular history and construction of phylogenetic tree are done by the use of software's such as phylogeny inference package (PHYLIP), MEGA and phylogenetic analysis using parsimony (PAUP) (Swofford, 2002; Ropelewski *et al*, 2010; Kumar *et al*, 2016). However, phylogenetic analysis does not take consideration of all information provided by genomic sequences especially the exon-intron structure and conserved motifs. An exon is a specific region of the nucleic acid sequence referred to as a gene coding region and contains protein synthesis information. Between exons

are introns which do not code for proteins and they are removed by RNA splicing. The exon-intron structure is generally known to be conserved in homologous organisms, therefore comparison of the exon-intron position on the sequence can also provide clarity on the evolutionary history (Pavesi *et al.*, 2008).

During the plant's life cycle, its growth and development are controlled by expression of genes which mediate various responses by activating signalling pathways that activate proteins required for plant development. The expression of genes under different conditions is controlled and regulated by promoters. Plant promoters are categorised in groups as constitutive, inducible and tissue-specific promoters. Constitutive promoters induce the expression of genes during the development of transgenic plants, while inducible promoter initiate gene expression in response to stress. Tissue-specific related promoters initiate gene expression based on the type of tissue (Saranya and Kanchana, 2016).

The AtNOGC1 gene as discussed in chapter 1, is the first nitric oxide binding guanylyl cyclase protein from a higher plant "*Arabidopsis thaliana*". It was identified using both the GC and heme nitric oxide/oxygen-binding domain (H-NOX) search motifs. The protein was annotated as a flavin-containing monooxygenase, and this was confirmed electrochemically and biochemically (Mulaudzi *et al.*, 2011). Studies on evolutionary history between the AtNOGC1 and other GC's related proteins together with its promoter sequence analysis have not yet been reported to date. In this study we report for the first time the evolutionary history of AtNOGC1 and identified *cis*-regulatory elements responsible for AtNOGC1's gene expression.

2.2 Material and Methods

2.2.1 Evolutionary relationship of AtNOGC1

To study the evolutional relationship of AtNOGC1 protein, the phylogenetic analysis was performed. The analysis was performed by comparing GC's from different species including mammals, nematodes, insects and plants. Accession numbers of GC's from different species were selected from publicly available data. Sequences were retrieved from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) in FASTA format. Blast search using AtNOGC1 (NP_176446.1) as query sequence was also performed and only Flavin-containing monooxygenase protein families with high similarity to AtNOGC1 were selected (93% - 82%). Multiple sequence alignment of selected protein sequences was performed using ClustalW (Thompson, Higgins and Gibson, 1994). The Molecular Evolutionary Genetics Analysis (MEGA V.7) was used for phylogenetic and molecular evolution analysis. The evolutionary history was interfered using the Neighbor-joining method and the distance was measured using poisson correction method (Kumar *et al.*, 2016).

2.2.2. Analysis of gene structure and motifs

The gene structure of GC's from mammals, nematodes, insects, lower (chlamydomonas) and higher (Arabidopsis) plants and a few selected Flavin - containing monooxygenase proteins were analysed using the Gene Structure Display Server 2.0 (GSDS 2.0*)* (*http://gsds.cbi.pku.edu.cn/*). To determine for the presence of exons and introns, the corresponding genomic and coding sequences were submitted to the GSDS server (Hu *et al.*, 2015). To analyse conserved motifs, the Multiple Expectation Maximisation for Motif Elicitation (MEME) was used with parameter settings: maximum number of motifs, 10; and maximum width, 100 (Bailey *et al.*, 2009)

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2.2.3 Analysis of AtNOGC1 promoter sequence

2.2.3.1 Germination of Arabidopsis thaliana

Arabidopsis thaliana Columbia-0 ecotype seeds were collected from Dr Lara Donaldson, University of Cape Town (UCT). Seeds were surface sterilised in 95% ethanol for 5 min and rinsed with autoclaved double distilled water (ddH$_2$0). Surface sterilisation was further performed using freshly prepared bleach solution (20% bleach and 0.1% tween) for 5 min followed by rinsing with autoclaved ddH$_2$O$_2$. Seeds were then incubated at 4°C to synchronise germination for 24 hrs. Seeds were then germinated on half strength Murashige and Skoog (MS) media containing 3% sucrose and 1% plant tissue culture agar at pH 5.7. The germination was incubated in the growth room at 22°C under standard controlled light conditions (16 hrs light, 8 hrs dark cycle; 100-150 μmol.m^{-2}.s^{-1}) for 18 days. Seedlings were constantly monitored for any irregularities and contamination.

2.2.3.2 Genomic DNA isolation

Genomic DNA was isolated from 100 mg 18 days old *Arabidopsis thaliana* seedling using the plant genomic DNA extraction mini kit (Cat# FAPGK 001-1, Favorgen Biotech Corp, Ping-Ting, Taiwan) following the manufacture's protocols. The presence of the DNA was confirmed by analysing on a 1% agarose gel and quantification using the Nanodrop™ 2000c Spectrophotometer (Thermo Scientific, USA).

2.2.3.3 Polymerase Chain Reaction (PCR) amplification

To isolate AtNOGC1 promoter (pAtNOGC1), approximately 1.3 Kb promoter region up stream of the AtNOGC1 sequence containing 200 bp sequences overlapping AtNOGC1 sequence was amplified from the genomic DNA using Polymerase Chain Reaction (PCR). The PCR was conducted in a total reaction volume of 25 µL containing 12.5 µL 2x Dream Taq Hot Start green master mix (Cat# EP1713, Thermo scientific, USA), 0.5 µL (10 µM) of each

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forward (5'CTGTTGATACATATGTTTGGCTTTTTGATTCTTTTATTGG 3') and reverse (5'TTCAGAATTGTCGACCCTCACGGCGGAGCTCTC 3') primers and 1 µg of template genomic DNA. The following PCR conditions were used: 95°C initial denaturation for 2 min (1x cycle), 35 cycles of : 95°C denaturation for 30 sec, 60°C annealing for 1 min, 72°C extension for 1 min. Final extension at 72°C for 10 min followed by cooling at 4°C. The PCR product was analysed on a 1% agarose gel and viewed using the ENDURO™ GDS Gel Documentation System (Labnet international, USA, Edison).

2.2.3.4 The pAtNOGC1 purification and DNA sequencing

The PCR products were excised from the agarose gel under a UV trans-illuminator lamp and purified using the Gene JET Gel Extraction Kit (Cat# K0691, Thermo scientific, USA) following the manufacture's protocol. The purified DNA product was quantified using NanoDrop^{TM} 2000c Spectrophotometers (Thermo Scientific, USA) and sent to Inqaba Biotechnical Industries (Pty) Ltd (South Africa, Pretoria) for sequencing. Sequencing was carried out using gene specific primers to obtain the full length nucleotide sequence.

2.2.3.5 Insilico analysis of AtNOGC1 promoter

The pAtNOGC1 sequence, which is the sequence upstream of the AtNOGC1 locus number "Atlg62580" was retrieved from The Arabidopsis Information Resource (TAIR) center (Lamesch *et al.*, 2012) and used as an input for the publicity available plant cis-regulatory elements (PlantCARE) tool (Lescot, 2002). The same sequence was used to design forward and reverse primers and amplified using PCR as described in section 2.2.3.3. The amplified and sequenced pAtNOGC1 was also analysed on PlantCARE.

2.2.4 Agarose gel electrophoresis

DNA was analysed by electrophoresis on 1% agarose gels. The agarose gel was prepared by adding 1 g of SeaKem® LE Agarose to 100 ml 1X TBE buffer and boiled using microwave

followed by pouring into a gel casting tray. About 1 µL GelRed® Nucleic Acid Gel Stain (Cat# S11494, Thermo scientific, USA) was added to the loading buffer and 2 µL of the loading buffer was mixed with 10 µL of the sample DNA and loaded onto the gel. The DNA ladder (Cat# A610141, Ampliqon, Denmark) was loaded on the first lane of the gel. The agarose gel was analysed using the ENDURO™ GDS Gel Documentation System (Labnet international, USA, Edison).

2.3 Results

In order to demonstrate the biological role of AtNOGC1 in plants, characterisation of its molecular evolutionary relationships, gene structure (exon-intron), motifs and promoter analysis were conducted.

2.3. 1. Phylogenetic analysis

The analysis were done by searching the literature and database to retrieve protein sequences that contain GC activity and proteins from the Flavin-containing monooxygenases family specifically those with high sequence similarities (93% - 82%) to AtNOGC1. About 28 protein sequences were retrieved from NCBI database using protein accession numbers, with about 5 protein sequences from each group (mammals, nematodes, insects, and higher plants) whereas only 3 protein sequences from lower plants (Chlamydomonas) that contain GC activity were used. In addition about 5 protein sequences from the Flavin-containing monooxygenases family were also retrieved from BLASTP, due to their highest sequence similarity with AtNOGC1(82% - 93%). The retrieved protein sequences were aligned using ClustalW followed by construction of the phylogenetic tree. The phylogenetic tree was constructed based on 28 protein sequences as shown in Fig 2.1. The multiple sequence alignment revealed no conserved motif in all protein sequences (Fig 6.1, See appendix I). The phylogenetic tree revealed that AtNOGC1 evolved from the same ancestral origin with Flavin-containing monooxygenase (FMO) and GC proteins presented in this study (Fig 2.1). Looking at the pattern of the branching, AtNOGC1 shared a closely common ancestral origin with organisms in branch E (Insects) and D (GC in higher plants). It was also shown that AtNOGC1 share the same branch (branch F) with AtGC1 and AgapAGAP010398 "an uncharacterised protein" from plants and insects respectively. There was also a close correlation between AtNOGC1

and the uncharacterized "NOGC- like" protein with approximately 93% similarities.
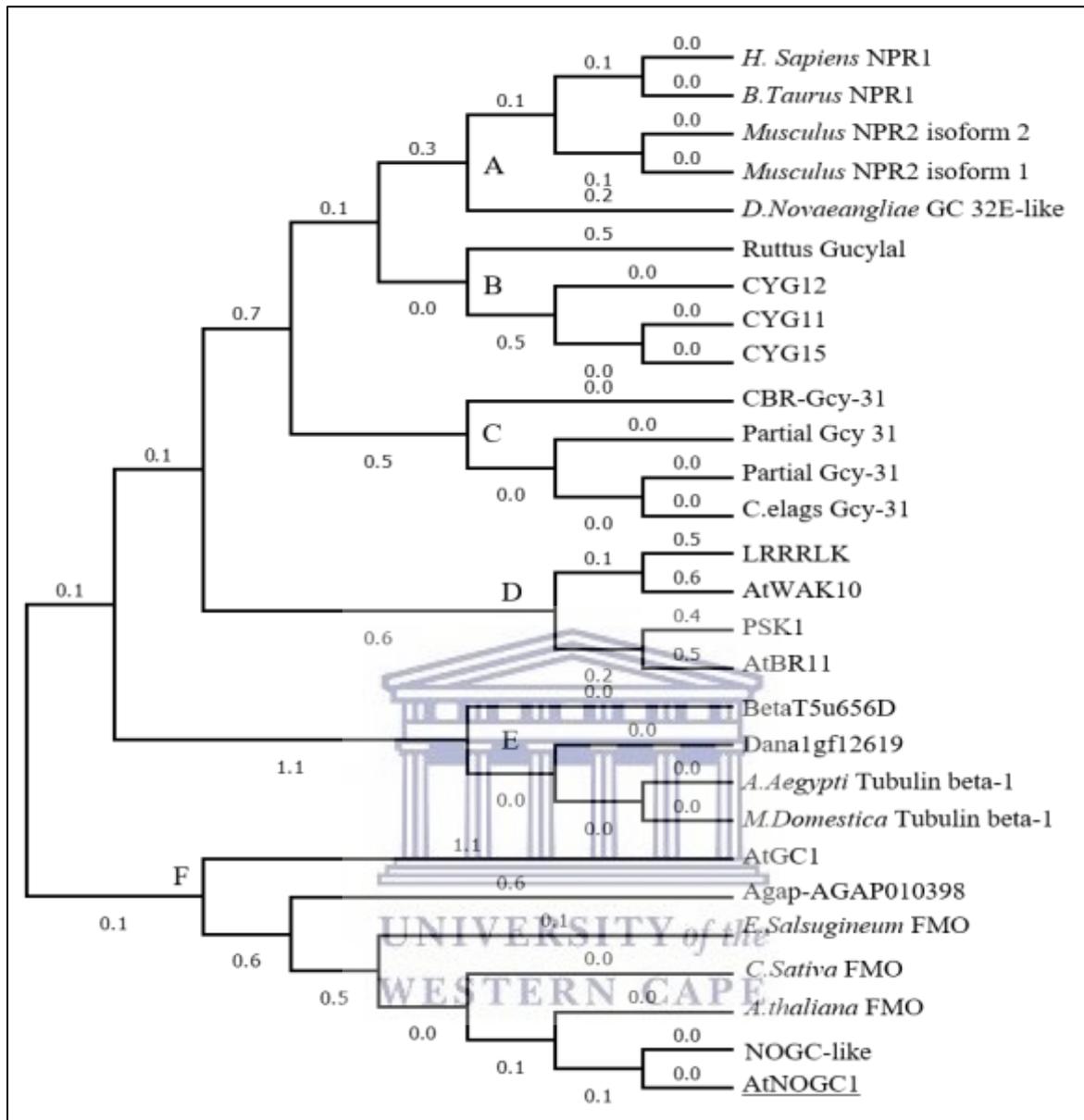
Fig 2.1 Phylogenetic analysis of AtNOGC1 protein with other GC and FMOs as reported from MEGA V7. The inferred phylogeny tree was derived from the protein amino acid sequences aligned by ClustalW using Neighbor-joining method and the distance was done by poisson correction method. (A) *H. sapanies* (NP_00089.3*), B. taurus* (NP_001179680.1), *Musculus* NPR2 isoform 2 (NP_001342395.1), *Musculus* NPR2 isoform 1 (NP_001179680.1*), D. noveangliae* (XP_015431590.1), (B) R. gucy1a1 (AA41206.1), CYG12 (XP_001700847), CYG11 (XP_001700546.1), CYG15 (XP_001701038.1), (C) CBR-Gcy-31(XP_002643872.1), Partial Gcy31 (NP_001024890.1), Partial Gcy31 (NP_001317860.1), C. elegas (NP_001024888.1), (D) LRRLK (NP_001236710.2), AtWAK10 (NP_178086.1), PSK1 (NP_178330.1) AtBR11 (NP_178330.1), (E) BetaT5ub56D (NP_523795.2), Dana1gf12619 (XP_001958879.1), *A. aegypti* Tubulin beta1 (XP_001656025.1), *M. domestica* Tubulin beta1 (XP_00587425.1), (F) AtGC1 (AAM51559.1), AGAP010398-PA (XP_311551.4), *E. salgugineum* FMO (XP_024005062.1), *C. sativa* FMO (XP_010418250.1), *A. thaliana* (NP_176523.4), NOGC-like (NP_001321123.1), AtNOGC1 (NP_176446.1).

2.3.2 Exon-intron structure and motifs analysis

To analyse the exon-intron structures of the AtNOGC1 gene, GC's proteins from mammals, nematodes, insects and plants including a few selected Flavin-containing monooxygenases (FMO's) were analysed by submitting coding sequences and their corresponding genomic DNA sequences to the GSDS online tool (Fig 2.2). GSDS revealed that out of 28 enquiries only 3 (*C. sativa*, AtBRII, and PSKR1) lack introns and the rest of genes have several exons and introns. The analysis also revealed that these genes have different exon-intron structures including their position and size but genes from the same organisms have similar number of exons and introns. About 4 genes from the FMO's family including AtNOGC1 have 7 exons and 6 introns, however the FMO from *C. sativa* only have 1 exon. Genes such as AtBRII, LRR-RLK and AtWAK10 from plants have a few number of exons and introns, nevertheless AtGC1 have 9 exons. CYG and GCY genes from nematodes and Chlamydomonas have high number of exons ranging from 10 to 14. In addition betaT5ub56d and Agap-AGAp0101398 from insects have 2 exons, but other genes from mammals including *musculus* NPR2, *B. taurus* and *H. sapiens* NPR1 were reported to have the highest number of exons.

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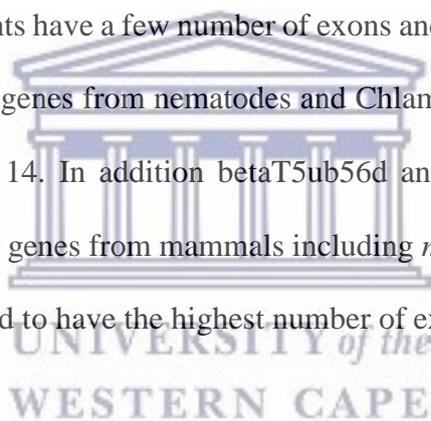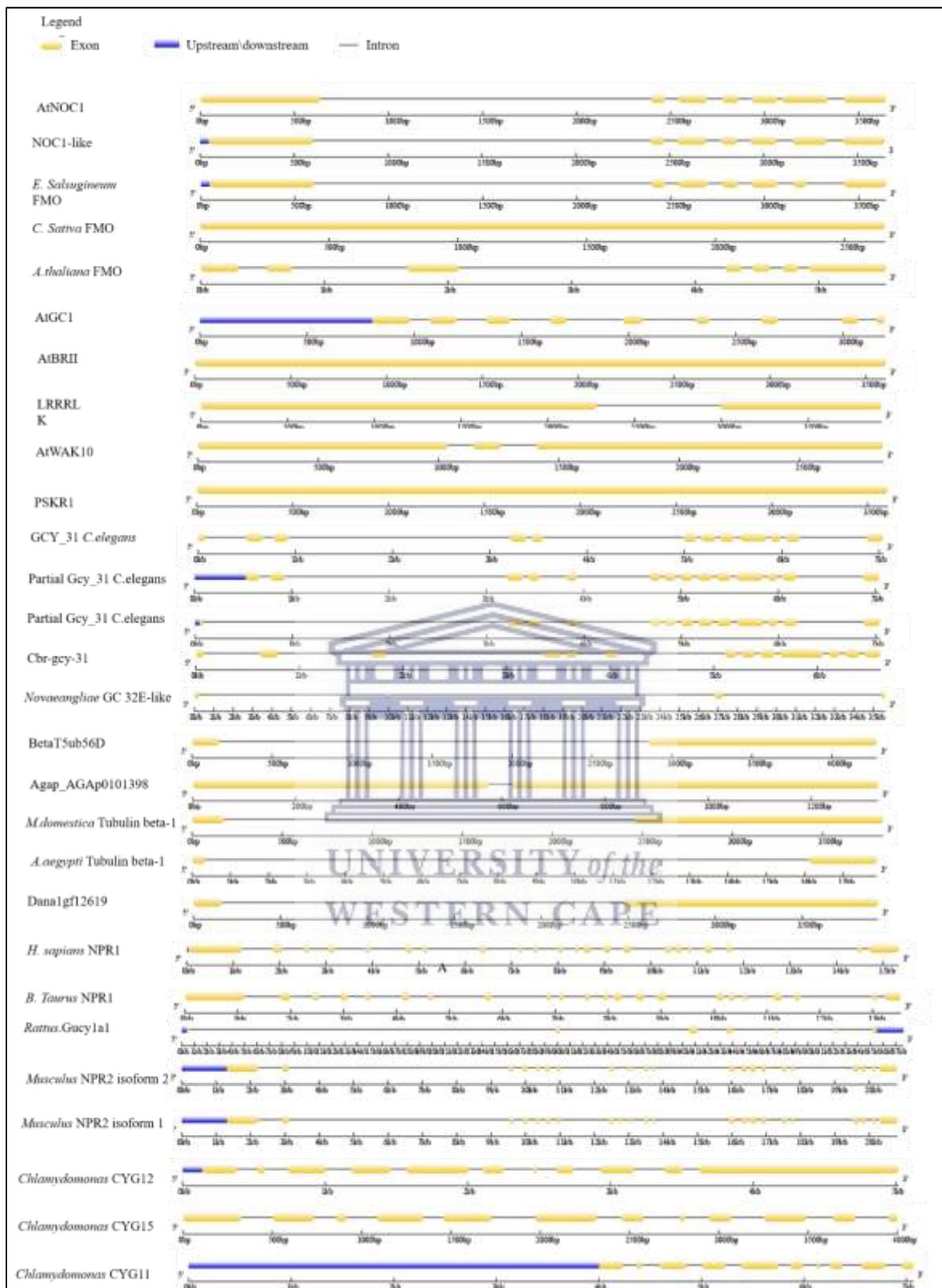
Fig 2.2. Exon-intron structure of GC and FMOs proteins. The genomic and coding region on the sequences were visualised using the Gene Structure Display Server. The accession numbers of the genes used in this analysis were the same as those represented in figure 2.1.

http://etd.uwc.ac.za/

Table 2.1: Summary of exon and intron gene structure numbers

Groups	Protein	#Exons	#Introns
Flavin monooxygenase(FMO) from *A. thaliana*	AtNOGC1	7	6
	NOC1-like	7	6
	Eutre FMO	7	6
	Sativa FMO	1	0
	NAK (Loc104702394)	7	6
Identified GC's in plants (*A.thaliana*)	AtGC1	9	8
	AtBRII	1	0
	LRRLK	2	1
	AtWAK10	3	2
	PSKR1	1	0
Nematodes	GCY_31 *C.elegans*	12	11
	Partial Gcy_31 *C.elegans*	14	13
	Partial Gcy_31 *C.elegans*	13	12
	Cbr-gcy-31	13	12
	LOC107187907-gcy31	4	3
Insects	BetaT5ub56D	2	1
	Agap_AGAp0101398	2	1
	M.domestica Tubulin beta-1	2	1
	A.aegypti Tubulin beta-1	2	1
	Dana1gf12619	2	1
Mammalian's	*H. sapians* NPR1	22	21
	B. Taurus NPR1	22	21
	Rattus.Gucy1a1	9	8
	Muscullus NPR2	21	22
	Varient 1 NPR2	22	21
Chlamydomonas	*Chlamydomonas* CYG12	11	10
	Chlamydomonas CYG15	12	11
	Chlamydomonas CYG11	10	11

http://etd.uwc.ac.za/

As shown in Fig 2.3 the MEME online tool was used to analyse conserved motifs within AtNOGC1, FMO's and GC's from different organisms. A maximum of 10 motifs were analysed within the protein sequences. Motif analysis revealed that AtGC1, AtBRII, LRR-RLK and AtWAK10 did not contain any conserved motif. In this study only motif 5, which encodes for K-oxygenase super family domain was identified and in AtNOGC1, other FMO's , PSKR1 and Agap_AGAp0101398 protein sequences. Motif 2 and 8 were almost conserved across all proteins except in BetaT5ub56D, Tubulin beta-1, and Dana1gf12619 protein sequences but they did not encode for any domain. Other motifs were only conserved in specific members including motif 7 which was only conserved in FMOs, mammals and chlamydomonas.
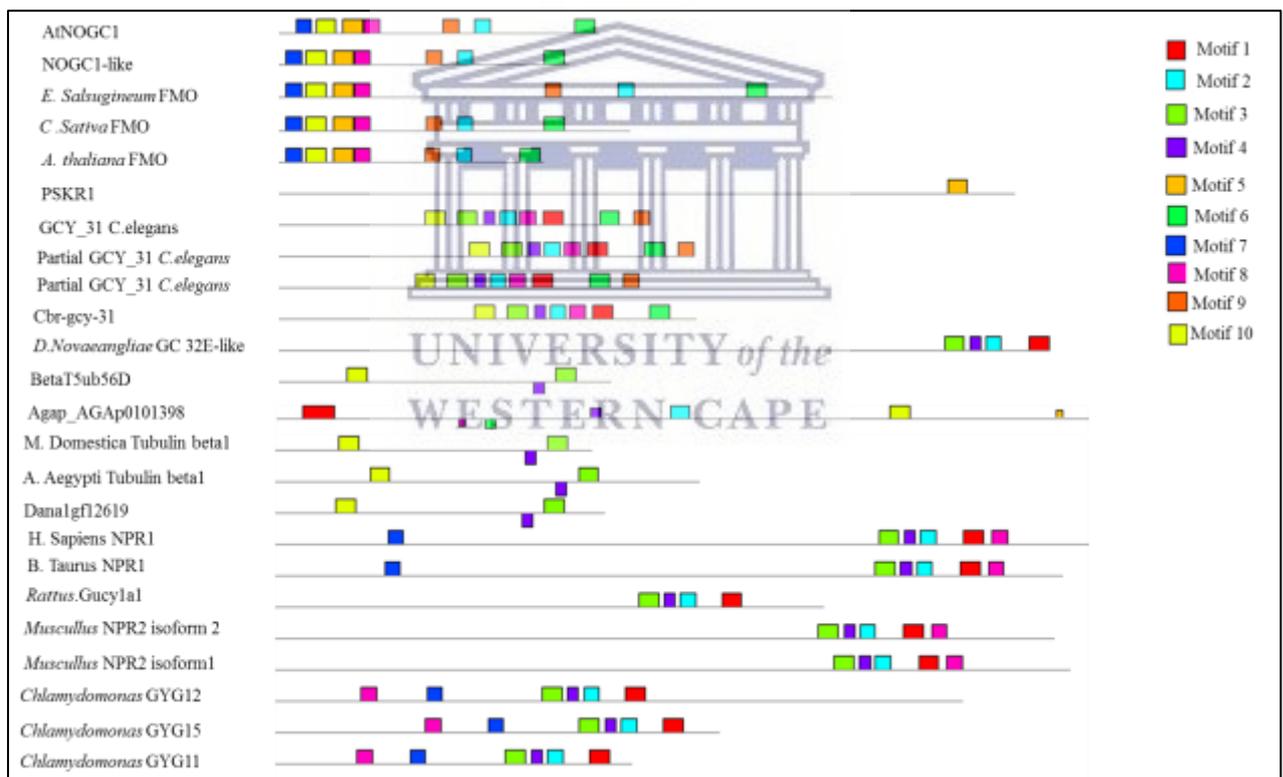
Fig 2.3 Representation of 10 conserved motifs between AtNOGC1, plants FMOs, GC's from Chlamydomonas, nematodes, insects and mammals. Conserved motifs were analysed using MEME online tool on 28 protein sequences using the following parameters, maximum 10 motifs and 100 length. The colour blocks represent different motifs identified and their positions.

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2.3.2 Isolation of AtNOGC1 promoter and cis-regulatory elements analysis

Based on the study that was recently conducted demonstrating the role of AtNOGC1 in ABA and NO-induced stomatal closure (Joudoi *et al.*, 2013), in addition to its role in sensing NO (Mulaudzi *et al.*, 2011), to date, not much has been reported about the functional role of AtNOGC1 especially its response to stress. In this study approximately 1.5 Kb fragments that include 200 bp sequence upstream of the promoter was isolated from *Arabidopsis thaliana* seedlings and successfully amplified using PCR as shown in lane 3 by the band at approximately 1.5 Kb in Fig 2.4.
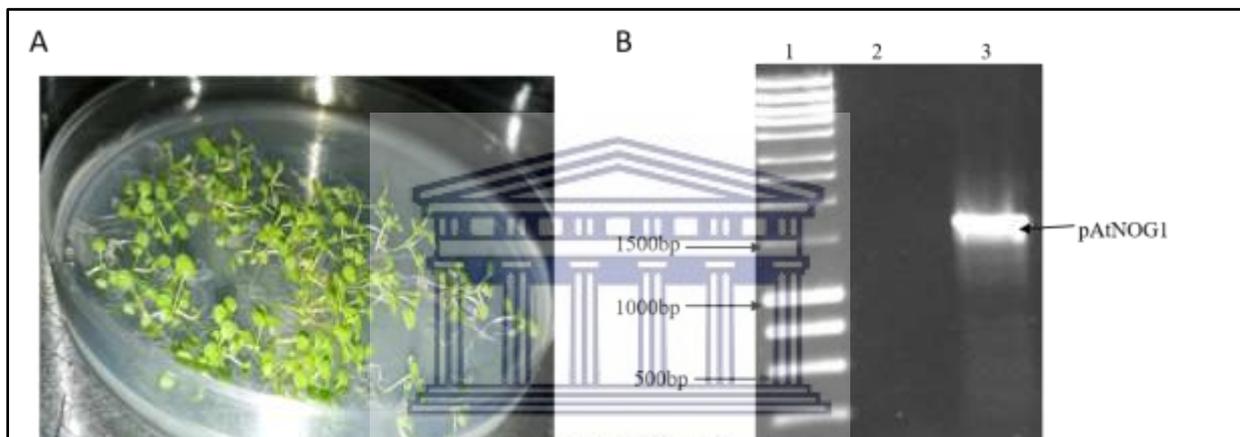
Fig 2.4 Germination of *A. thaliana* seedlings and analysis of the AtNOGC1 promoter. (A) 18 days *A. thaliana* seedlings growing on the MS medium and (B) 1% agarose gel showing PCR amplication of pAtNOGC1. Lane 2: negative control, Lane 3: pAtNOGC1 at ~ 1.5 Kb, M: 10 kb DNA ladder.

The isolated fragment was sent for sequencing and successfully aligned with the AtNOGC1 promoter sequence from TAIR showing 98% similarity using the nucleotide BLAST (Fig 6.2Appendix I). Using PlantCARE online tool, both the raw and experimental pAtNOGC1 sequences were analysed for the presence of cis-regulatory elements. About 16 cis-regulatory elements were identified and are summarised in table 2.2, but major investigation indicates that pAtNOGC1 is induced in response to light, drought, pathogen and during plant development, as indicated by the presence of cis-regulatory elements such as 3-AF1, ABRE, MBS, Box-W1, TCA, TGACG-motif and P-box.

Table 2.2: The detected Cis-regulatory elements from AtNOGC1 promoter with their position and corresponding functions.

Site Name	Sequence	Function
3-AF1 binding site	AAGAGATA	Light responsive element.
ABRE	GACACGTA	Cis-acting element involved in the abscisic acid responsiveness.
ARE	TGGTTT	Cis-acting regulatory element essential for the anaerobic induction.
Box 4	ATTAAT	Part of a conserved DNA module involved in light responsiveness.
Box I	TTTCAAA	Light responsive element.
Box II	AGTCGTGG	Part of a light responsive element.
Box-W1	TTGACC	Fungal elicitor responsive element.
CGTCA-motif	CGTCA	Cis-acting regulatory element involved in the Methyl-Jasmonate (MeJA)-responsiveness.
ERE	ATTTCAAA	Ethylene-responsive element.
GATA-motif	AAGATAAG	Part of a light responsive element.
MBS	CGGTCA	Myeloblastosis (MYB) Binding site.
P-box	CCTTTTG	Gibberellin-responsive element.
TC-rich repeats	GTTTTCTTA	Cis-acting element involved in defense and stress responsiveness.
TCA-element	CAGAAAAG	Cis-acting element involved in salicylic acid responsiveness.
TGACG-motif	TGACG	Cis-acting regulatory element involved in the MeJA-responsiveness.

2.4 Discussions

Since the discovery of GC's in plants, AtNOGC1 is currently the only GC with the HNOX motif that binds NO with higher affinity than O_2 (Mulaudzi *et al.,* 2011). In another study done to prove the existence of NO dependent GC protein in plants, AtNOGC1 was shown to be involved in stomatal closure in an NO-dependent manner (Joudoi *et al.*, 2013). To emphasise more on the role of sGC in planta, over expression of rat sGC in *A. thaliana* resulted in increased cGMP levels and improved the response to pathogen treatment (Hussain *et al.*, 2016). Thus elucidation of the biological role of AtNOGC1 in response to stress is important and may be beneficial towards crop improvement.

2.4.1 Phylogenetic analysis

The existence of the GC activity in plants is reported to be low as compared to animal, which has raised questions on its physiological relevance in plants (Ashton, 2011). The evolutionary history, gene structure and motifs analysis of the latest identified plant GC (AtNOGC1) in comparison to other organisms was studied using the MEGA V.7, GSDS and MEME tools respectively. In addition, promoter analysis was also investigated in this study using the PlantCARE online server.

Phylogenetic analysis revealed that AtNOGC1 has evolved from the same common ancestors with all GCs analysed in this study (Fig 2.1), as evidenced by AtNOGC1 sharing the same branch with other GC from mammals, nematodes, insects, lower (chlamydomonas) and higher plants (Arabidopsis). The relationship might suggest that these proteins are from closely related ancestral origin and may have the same activity with some similar functions although belonging to different family groups. Analysis also revealed that AtNOGC1 and NOGC1-like proteins are paralogous isoforms since they share about 93% sequence similarities and diverge within the same species. The reference protein sequence NOGC-like (NP_001321123) was confirmed by searching on NCBI and is currently annotated as a Flavin-containing monooxygenase (FMO

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GS-OX-like) protein with the same gene name as AtNOGC1. Both proteins are from the same *A. thaliana* chromosome 1 (NC_003070.9), thus the paralogous isoform of AtNOGC1 maybe as a results of alternate splicing that arise from cellular mechanism removing all the introns and combining all the exons. A eukaryotic cell can produce different proteins from one gene by combining the exons in different arrangements (Nilsen and Graveley, 2010). Apart from the isoform identified, phylogenetic tree revealed that AtNOGC1 also share a close relationship (88% similarities) with the partial sGC protein AGAP010398-PA from *Anopheles gambiae*, suggesting an evolutionary history with insects.

2.4.2 Gene structure and conserved motifs

Evolutionary history does not consider all information from the sequence such as exon-intron structures. Analysis of exon-intron structure provides clarity in unresolved phylogenetic relationships (Pavesi *et al.*, 2008). In this study, the exon-intron structure of AtNOGC1 in comparison with other genes was analysed by submitting their coding and corresponding genomic sequences to GSDS for structure and motifs analysis. Similar distribution of exon-intron structure within genes of the same organisms was observed suggesting the similar role that these genes might play. Motif 5 was only conserved in AtNOGC1 and in the uncharacterised "AGAP010398-PA" protein from *Anopheles gambiae*. These results support the phylogenetic tree analysis which showed that AtNOGC1 share a close relationship with AGAP01398-PA. The shared motif between AtNOGC1 and AGAP01398-PA may suggest similar function during binding of dinucleotides such as Flavin adenine dinucleotide (FAD), Nicotinamide adenine dinucleotide (NAD) and NADP (Hanukoglu, 2015). In addition, other motifs including 2 and 3 were conserved across most proteins. Although they did not encode for any characterised domain, it might suggest their similarities. The comparison between the two protein AtNOGC1 and NOGC-like as in Fig 2.2, indicated that they have the same exon-intron distribution and same number, but the first exon on NOGC-like protein started at a

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different size of approximately 100 bp. These findings suggest the need to characterise NOGC-like proteins in order to understand their similarities, towards the identification of other novel GC's in higher plants.

2.4.3 Cis-regulatory elements

Analysis of cis-regulatory elements of AtNOGC1 gene provided insights toward elucidating and undestanding its function in plants. *In silico* promoter sequence analysis performed in this study revealed the presence of well known cis-regulatory elements which play vital roles in plant stress defense and development.

For example, light responsive elements (3-AFI, BoxI, BoxII, Box4 and GATA-motif) which plays a significant role in determining the characteristics of light responsive promoter in plants were identified (Chattopadhyay *et al.*, 1998). AtNOGC1 promoter is enriched with ABRE elements which are responsible for abscisic acid responsiveness. Since ABA is a plant regulatory hormone that is involved in regulating many physiological processes such as stomatal closure thus providing adaptation of plants to stress (Sah *et al.*, 2016).These support the role of AtNOGC1 in stomatal closure as demonstrated previously (Joudoi *et al.*, 2013). These cis-regulatory elements suggest that the ABA pathway in response to stress drives the expression of AtNOGC1 during stomatal closure. The presence of MeJA responsiveness elements (CGTCA and TGACG-motif) also suggest the role of AtNOGC1 during jasmonic acid responses to regulate growth, development and responses to abiotic and biotic stress (Munemasa *et al.*, 2007). The presence of MYB site which is responsible for driving the expression of plant genes during dehydration also revealed that AtNOGC1 might be required during drought stress (Milena *et al.*, 2014). The presence of fungal elicitor (Box-WI) and salicylic acid (Box-W1 and TCA-element) elements also suggest that AtNOGC1's expression might be elevated during pathogen attacks and other stress responses (Vlot *et al.,* 2009). In addition, the presence of gibberenllin-responsive (P-box) elements which regulate key aspect

for plant growth and development may also suggest the involvement of AtNOGC1 during plant growth and development (Daviere and Achard, 2013). In general these results suggest a role of AtNOGC1 in stress response, growth and development.

The phylogenetic, exon-intron structure and motifs analysis of AtNOGC1 provided an insight into understanding its function and evolutionary history. The phylogenetic analysis revealed that AtNOGC1 shares the common ancestors with mammals, insects, nematodes, chlamydomonas and other plant GC's from higher plants, although their exon-intron structures are different. AtNOGC1 share a close relationship and K-oxygenase super family motif with the AGAP010398-PA protein from insects. These results also indicated that AtNOGC1 is the multiple stress inducible gene with about 16 cis-regulatory elements that are known to influence responses to abiotic and biotic stresses and hence plant growth and development. Therefore it remains important to characterise AtNOGC1 gene in response to abiotic and biotic stresses and demonstrate its role in plant growth and development.
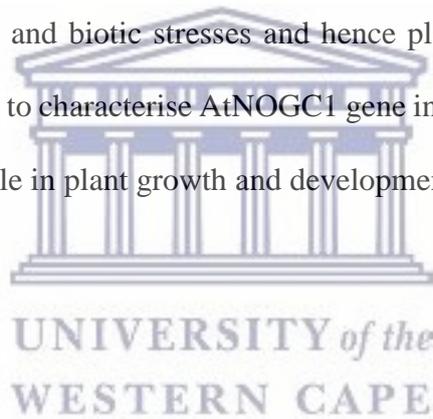
CHAPTER 3

Expression patterns of AtNOGC1 gene in response to salinity, drought stress, hormonal and nitric oxide treatment.

Abstract: Multiple stresses, both abiotic and biotic result in oxidative stress which damages the plant's physiological and metabolic processes. However plants use several mechanisms to reduce the effects of stress and adapt to those harsh conditions. Cellular signalling through the action of guanylyl cyclases (GC) is one of the most important systems known to mediate physiological and metabolic processes. However in plants its mechanism is not well understood. Cyclic 3', 5' guanosine monophosphate (cGMP), a second messenger that is synthesised by GC's is involved in cellular signalling growth, development and response to abiotic and biotic stresses. In this study the expression pattern of AtNOGC1 was analysed in response to salinity, drought, hormone and nitric oxide (NO) treatments, on *Arabidopsis thaliana* tissues using the Real-Time Quantitative Reverse Transcription Polymerase Reaction (qRT-PCR). Expression pattern analysis revealed that AtNOGC1 is expressed in both roots and shoots, but its expression is highly elevated under several stress treatments. The highest level of expression was observed when seedlings were treated with both abscisic acid and methyl jasmonate (BA+MeJA), followed by salicylic acid (SA), ABA, NO and then abiotic stress treatment. The increased expression of AtNOGC1 gene in response to several stresses suggesting that it might be required for a protective role when plants are attacked by multiple stresses through the NO/cGMP pathway.

Keywords: AtNOGC1, abiotic and biotic stress, expression pattern, hormones, signalling, and tolerance.

3.1 Introduction

Plants are constantly attacked by abiotic and biotic stresses simultaneously, which severely affect their growth and production (Pandey *et al.*, 2015). These stresses include, drought, salinity, extreme temperature and heavy metals, and they affect physiological and metabolic processes in plants through the overproduction of reactive oxygen species (ROS) resulting in oxidative damage to cells. Due to the results of stress, plants have developed various protective mechanisms against the effects of stress and some are mediated by secondary messengers, signalling cascades and chemical responses (Huber and Bauerle, 2016). These mechanisms activate ion channels, protein kinases, ion gated channels, plant hormones, signalling molecules such as the regulation of ROS and reactive nitrogen species (RNS) during stress. Phytohormones such as SA, ABA and MeJA amongst others are activated and minimise the effects caused by stress (Rejeb *et al.*, 2014) though inducing the expression of numerous stress responsive-genes. The response of ABA is necessary to maintain water balance during drought and salinity stress through stomatal closure, whereas SA and MeJA play a role during pathogenic attack (Jones and Mansfield, 1970; Bari and Jones, 2009; Vlot *et al.,* 2009). MeJA is also related to plant developmental stages such as germination and growth (Wasternack and Hause, 2002).

RNS including nitric oxide (NO) and peroxynitrite (ONOO^{-}), amongst them NO is the most widely studied (Squadrito and Pryor, 1998). NO is an important secondary messenger that influences many physiological processes in plants including development, germination, responses to abiotic and biotic stresses (Krasylenko *et al.,* 2010). NO is a well-known for its role in activating the soluble guanylyl cyclase (sGC) which leads to an increased production of Cyclic 3', 5' guanosine monophosphate (cGMP) from GTP. cGMP is an important signalling molecule and a second messenger that controls several cellular functions in prokaryotes and eukaryotes. In plants cGMP signalling pathways are involved in many cellular responses

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including light transduction, plant development, defense and hormone responses. In higher plants cGMP was associated with the role in stomatal opening and closure in the presence of NO. In plants the recent identification of NO-dependent guanylyl cyclase (GC) candidate, AtNOGC1 (Mulaudzi *et al.*, 2011) has paved a way in understanding the mechanisms between NO and cGMP in plants. Some of the developments include the role of AtNOGC1 in stomatal closure (Joudoi *et al.*, 2013). However, characterisation of this gene in response to several stresses has not been demonstrated. In this chapter, the expression of AtNOGC1 in response to salinity, drought, hormone and NO treatments was demonstrated.

3.2 Material and Methods

The expression pattern of AtNOGC1 was determined in *Arabidopsis thaliana* seedlings that were treated with salt, mannitol, hormones (SA, ABA, MeJA) and NO using the Quantitative Real- Time Polymerase Chain Reaction (qRT-PCR). Sodium chloride (NaCl), forms part of the main salts in the soil and for drought treatment, mannitol which induces water stress was used to mimic droughts stress. ABA was used since AtNOGC1 was associated with ABA induced stomatal closure, while SA and MeJA were used to mimic biotic stresses.

3.2.1 Germination and treatment of *Arabidopsis thaliana*

The germination and growth conditions were carried out as described in chapter 2, section 2.2.

3.2.1.1 Stress treatments

Eighteen day (18) old *A. thaliana* seedlings were transferred into a newly prepared half strength MS media supplemented with 250 mM NaCl and 300 mM mannitol followed by harvesting the root and shoot materials at different time intervals of 0, 3, 12 and 24 hrs.

3.2.1.2 Hormone treatments

Hormonal treatment was done by transferring 18 day old seedlings to the half strength MS media supplemented with 10 µM ABA, 10 µM MeJA and 0.5 µM SA. The effect of hormonal combination was studied by transferring seedlings into the MS media that was supplemented with 25 µM ABA, 300 µM SA and 100 µM MeJA as it was previously described (Axelos *et al*.,1992) and treated at different time points of 0, 3, 12 and 24 hrs.

3.2.1.3 NO treatment

About 50 µM of sodium nitroprusside dehydrate (SNP) (Cat# 13451, Sigma Aldrich, SA) was added to the MS media and 18 day old seedlings were exposed to the SNP treatment for 24 hrs.

3.2.1.4 Preservation of plant material

After treatment, all plant tissues (roots and shoots) were quickly frozen using liquid nitrogen and stored at -80°C until needed.

3.2.2 RNA isolation and cDNA synthesis

Total RNA was isolated from both treated and untreated (control) Arabidopsis tissues using the FavorPrep™ Plant Total RNA Purification Mini Kit (Cat# FAPRK001-1, Favorgen Biotech Corp, Ping-Ting, Taiwan) and about 1 µg of total RNA was complementary DNA (cDNA) synthesised using the superscript IV reverse transcriptase kit (Cat# 18090050, Invitrogen, SA). Quantification of both the RNA and cDNA was done using the Nanodrop 2000c spectrophotometer (Thermo Scientific™, USA). All products were used according to the manufacturer's instructions.

3.2.4 Transcript analysis of AtNOGC1 under different stress conditions

To study the expression pattern of AtNOGC1 transcript, qRT-PCR was performed on the LightCycler® 480 Real time PCR Instrument II using the LightCycler® 480 SYBR Green I Master kit (Roche Diagnostics, South Africa) according to the manufacturer's protocol. The reaction volume was adjusted to 10 µL containing 1 µL cDNA (diluted 1:10), 5 µL SYBR green mix, 0.2 µL of each 10 µM forward and reverse primer (0.2 µM final concentration) and RNase free water to a final volume of 10 µL. Three biological and technical replicates were performed on each treatment and expression levels were normalised using ACTIN2 and SAND as reference genes. Primers were designed using Primer 3 (V.0.4.0) online tool (Koressaar and Remm, 2007) and primer information is shown in table 3.1. PCR cycling conditions set on the LightCycler® 480 are listed in table 3.2.

Table 3.1 List of primers used for qRT-PCR analysis

Primer	Forward sequence	Reverse sequence	Accession number
AtNOGC1	GGCCACTCTGTCGTTGTTTT	TTGTGGATGATGGTTCGGA	NM_001334040.1
ACTIN2	GAAATCACAGCACTTGCACC	AAGCCTTTGATCTTGAGAGC	NM_00133858.1
SAND	CAGACAAGGCGATGGCGGATA	GCTTTCTCTCAAGGGTTTCTGGGT	NM_128399.4

Table 3.2 The PCR cycling conditions

Program Name	Target (°C)	Hold	Cycles
Pre-incubation	95 °C	10 min	1X
Amplification	95 °C	10 sec	
AtNOGC1 and SAND	55 °C	10 sec	
ACTIN2	57 °C	10 sec	45X
	72 °C	20 sec	
Melting	(Default conditions)		1X
Cooling	(Default conditions)		1X

3.2.5 Data analysis

All the values were reported on the bar graph, which represents an average of the mean from the three independent biological replicates. To check for any significant differences between the mean of samples, student *t*-test was performed between the control samples (0 hr.) and treated samples and the *p*-values were calculated by GraphPad Prism (available online at http://www.graphpad.com). The *p*-value of less than 0.05 ($p < 0.05$) was regarded as

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significant. In order to understand how much AtNOGC1 gene was up-regulated or down-regulated under stresses, as compared to the control. Fold change ($\log_2$) ratio was calculated between the control and treated samples. The $\log_2$ of down-regulated genes had values between 0 and 1, whereas overexpressed genes had a value of 1 and above.

3.3 Results

In order to study the expression pattern of AtNOGC1 under different stress conditions (salinity, drought, hormonal and NO), transcript analysis were conducted using the qRT-PCR. The transcriptional analysis were done in tissue specific to include roots and shoots at different time points, fold changes and the p-value were calculated to understand the significance in the data (appendix table 6.1).

3.3.1 AtNOGC1 expression pattern analysis

After treatment of the 18 day old seedlings, RNA was successfully isolated and reverse transcribed into cDNA. The threshold cycle (Ct) of all three technical replicates was generated automatically by the LightCycler® 480.SW 1.5.1 software. The quantity of each experimental DNA samples was extrapolated from standard curves generated by Microsoft Office Excel 2013 from gene specific PCR correlation coefficient (R^2) from 7 fold series dilution of cDNA. Expression pattern of AtNOGC1 under normal condition and after treatments was analysed and graphically represented by the graphpad. Under control conditions, AtNOGC1 was expressed in both the root and shoot tissues as shown in Fig 3.1.
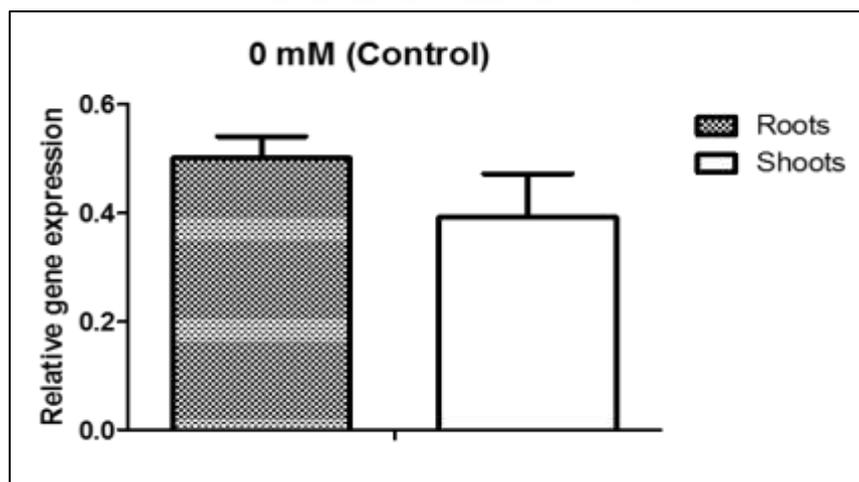
Fig 3.1 Expression pattern of AtNOGC1 transcript. **Expression in the roots and shoots under normal conditions**. ACTIN2 and SAND were used as reference genes to normalise the data. Error bars represent the standard deviation (SD) calculated from three biological replicates.

3.3.1.1 Expression pattern of AtNOGC1 in response to abiotic stresses

To determine whether abiotic stress induces AtNOGC1 gene, the transcriptional responses were analysed from seedlings that were treated with 250 mM NaCl and 300 mM mannitol for salinity and drought stress respectively. Expression of AtNOGC1 on both salinity and drought treated plants was either upregulated or downregulated differently in both roots and shoots at different time points (Fig 3.2). The expression of AtNOGC1 significantly increased at 3 hrs in the roots of 250 mM NaCl treated seedlings as indicated by a $\log_2 = 1.82$ ($p = 0.005$) (Fig 3.2. A), followed by a significant decrease at 3 hrs in the shoots ($\log_2=0.43; p = 0.0106$), both tissues at 12 hrs ($p < 0.0010; 0.0010$) and the roots at 24 hrs ($p < 0.0010; 0.0093$). Overall statistically significant difference was observed in mannitol treated plants. However shoots at 3 hrs and roots at 12 and 24 hrs roots there was an increase in the AtNOGC1 transcript as indicated by $\log_2 = 1.92, 0.2270$ and 0.08 ($p > 0.05$) respectively (Fig 3.2. B).
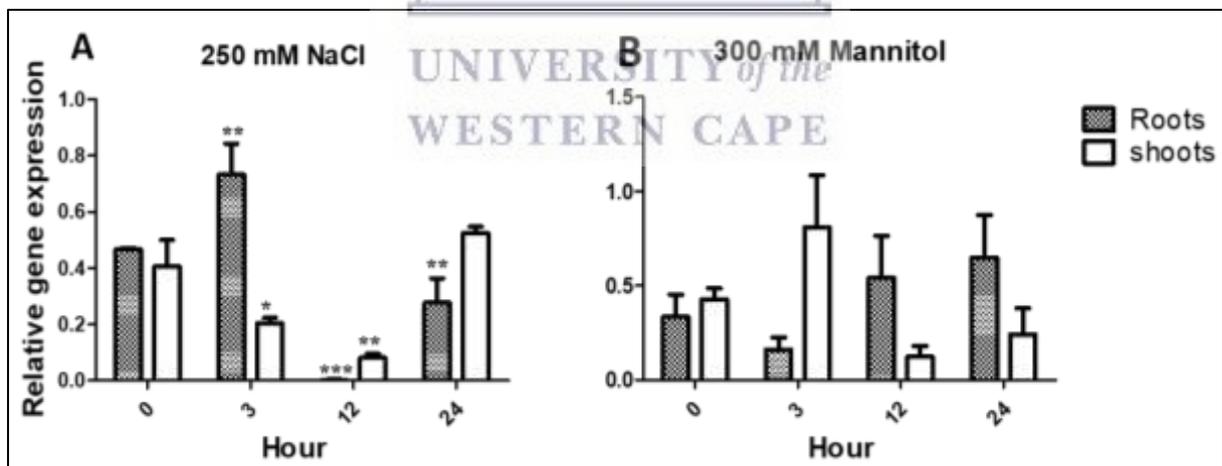
Fig 3.2. Expression pattern of AtNOGC1 transcript in the roots and shoots under salinity and drought induced treatments at different time points (0, 3, 12, 12 hrs). (A) Salinity stress induced by 250 mM NaCl, (B) Drought stress induced by 300 mM Mannitol at different time points. ACTIN2 and SAND were used as reference genes to normalise the data. Error bars represent the SD calculated from three biological replicates and significance differences between control and treated samples were determined using the *t*-test shown as*** = $p \le 0.0001$, ** = $p \le 0.001$ and * = $p \le 0.01$.

3.3.1.2 Expression pattern of AtNOGC1 in response to hormonal treatments

To determine whether hormones induce the expression of AtNOGC1 gene, the transcriptional responses was analysed from seedlings that were treated with 10 µM ABA, 10 µM MeJA and 0.5 µM SA (Fig 3.3). Upon treatment of *A. thaliana* seedlings with 10 µM ABA, AtNOGC1 transcript levels increased significantly from $\log_2 = 0.5$ to $\log_2 = 2.5$ and 2.0 at 3 hrs ($p < 0.0001$) in the roots and shoots respectively, followed by down regulation at 12 and 24 hrs ($p < 0.0001$). Significant decrease in AtNOGC1 transcript was observed at 3 hrs roots ($p = 0.0004$) and 24 hrs ($p = 0.0003$) shoots upon treatment with MeJA, however $\log_2 = 1.82$ showed a slight increase in the expression at 12 hrs shoots. A significant increase in AtNOGC1 expression was observed when seedlings were treated with 0.5 µM SA, at 3 hrs as indicated by a change in $\log_2 = 0.5$ to approximately 3 ($p = 0.004$; $p < 0.0001$), followed by a significant down regulation at 12 hrs ($p < 0.0001$; $p = 0.0004$) and finally a significant increase at 24 hrs ($p = 0.0194$; $p = 0.0009$) in both tissues. The influence of the hormone combination on AtNOGC1 gene expression was also analysed (25 µM ABA and 300 µM SA, and 25 µM ABA and 100 µM MeJA) (Fig 3.4). ABA + MeJA resulted in the down regulation of AtNOGC1 at 3 hrs roots, 12 hrs and 24 hrs shoots. However a significant increase was observed for the shoots ($p = 0.0003$) and roots ($p = 0.0475$) at 12 and 24 hrs respectively as shown by a $\log_2 = 0.5$ which change to 7.5 expression level. But ABA+SA resulted in a significant increase in expression at 3 hr roots and 12 hrs from $\log2 = 0.5$ to 2 ($p = 0.0004$), but slight decrease at 24 hrs in both tissues.

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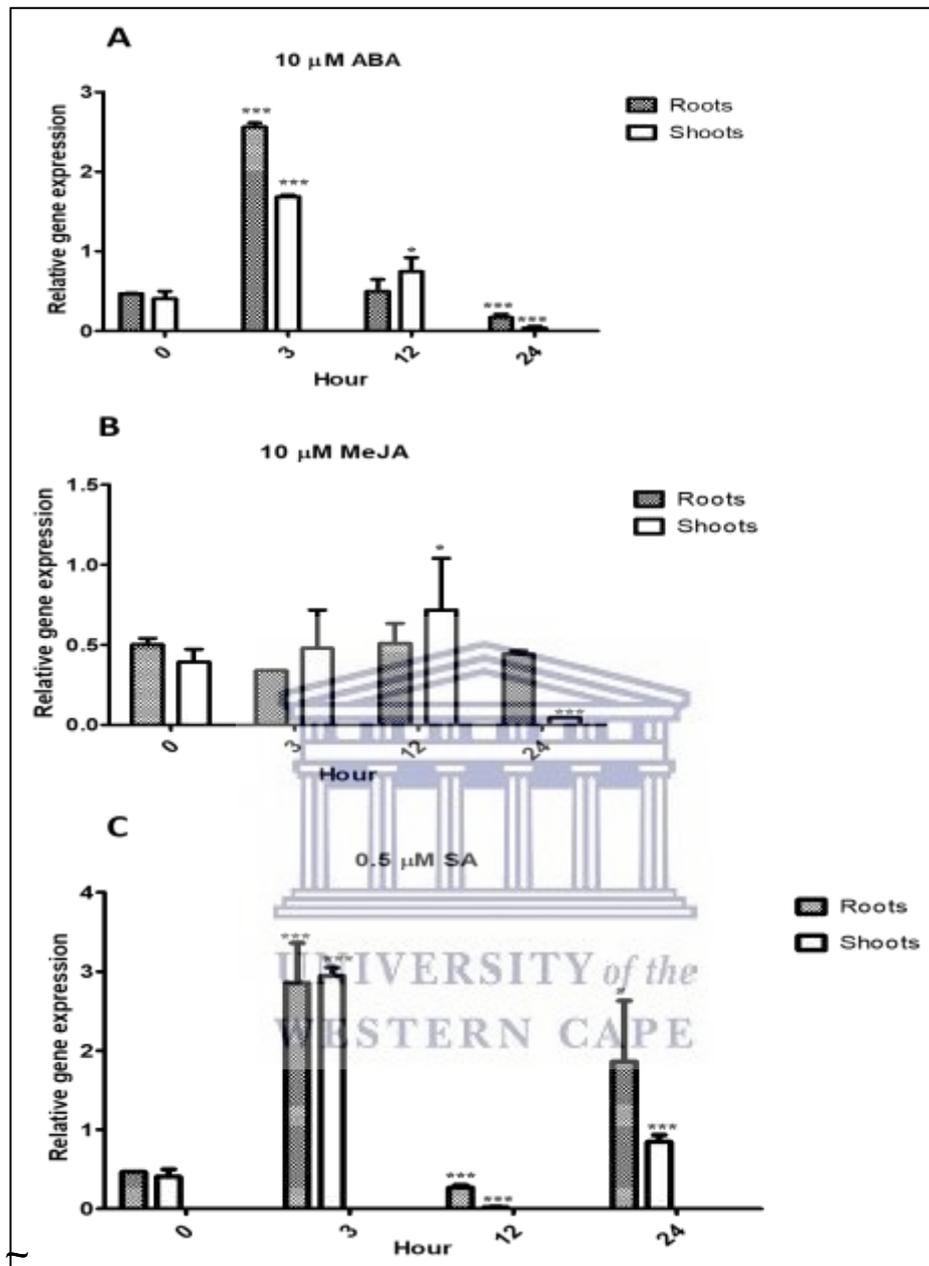
Fig 3.3. Expression pattern of AtNOGC1 transcript in the roots and shoots under different hormonal treatments at different time point (0, 3, 12, 12 hrs). (A) 10 µM ABA, (B) 10 µM MeJA, (C) 0.5 µM SA treatment of *A. thaliana* seedlings at different time points respectively. ACTIN2 and SAND were used as references genes to normalise the data. Error bars represent the SD calculated from three biological replicates and significance differences between control and treated samples were determined using *t*-test shown as *** = $p \leq$ 0.0001, ** = $p \leq 0.001$, and * = $p \leq 0.01$.
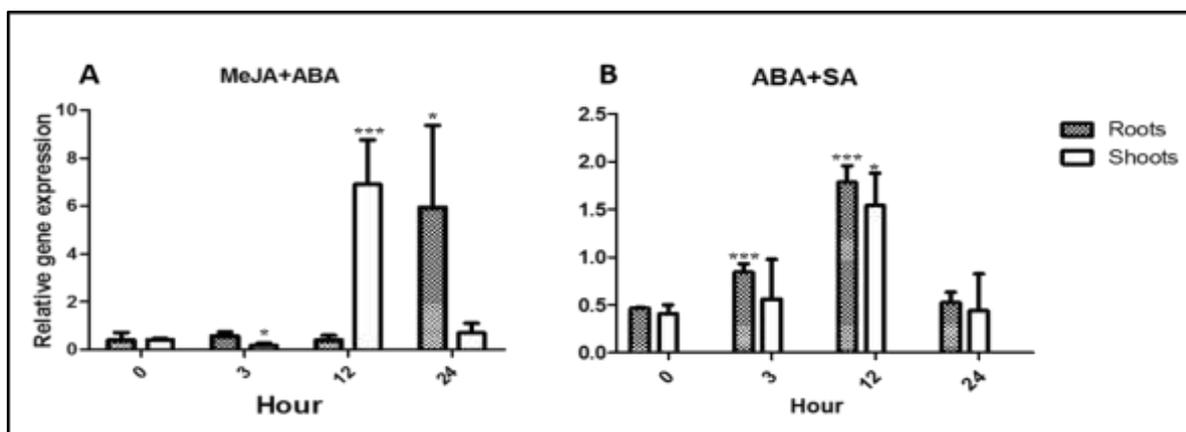
Fig 3.4. Expression pattern of AtNOGC1 transcript in the roots and shoots under different hormone combination at different time points (0, 3, 12, 12 hrs). (A) MeJA+ABA (100 µM and 25 µM), (B) ABA+SA (25 µM and 300 µM) at indicated time respectively. ACTIN2 and SAND were used as reference genes to normalise the data. Error bars represent the SD calculated from three biological replicates and significance differences between control and treated samples were determined using *t*-test shown as *** = $p \leq 0.0001$, ** = $p \leq 0.001$, and * = $p \leq 0.01$.

3.3.1.3 Expression pattern of AtNOGC1 in response to NO treatment

In order to study the expression pattern of AtNOGC1 in response to NO, 50 µM SNP was exogenously applied to MS media as the NO donor. The transcriptional analysis was done on 50 µM SNP treated seedlings on roots and shoots. A significant increase on the roots was observed with $\log_2 = \sim 8.87$ ($p = 0.0003$) as compared to the control and down-regulation on the shoots (Fig 3.5).
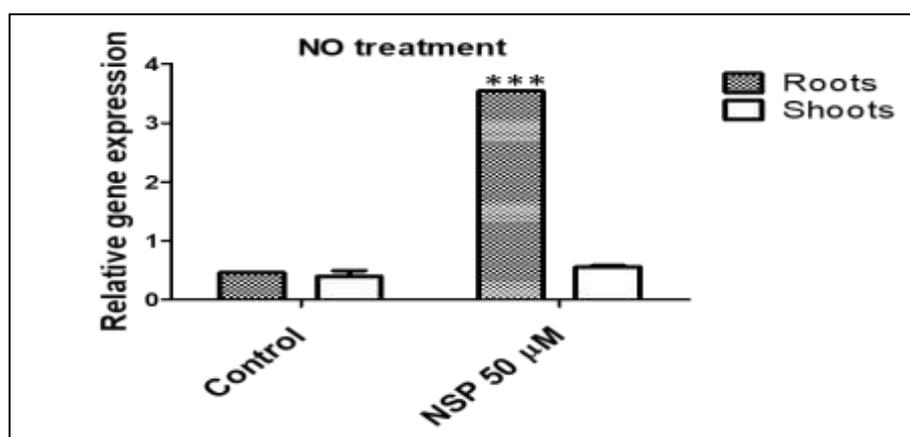
**Fig 3.5. Expression pattern of AtNOGC1 transcript in the roots and shoots under NO treatments**. ACTIN2 and SAND were used as reference genes to normalise the data. Error bars represent the SD calculated from three biological replicates and significance differences between control and treated samples were determined using *t*-test shown *** = $p \leq 0.0001$, ** = $p \leq 0.001$, and * = $p \leq 0.01$.

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3.4 Discussions

The NO/cGMP pathway in plants transmits a signal to the elements of signalling cascades, which activates various processes such as protein kinases, cyclic nucleotide channels and activation of phosphodiesterases. This pathway plays the major roles during stomatal closure, root development and gene expression regulation (Durner et al., 1998; Neill *et al.*, 2008; Xuan *et al.*, 2012; Joudi *et al*., 2013). AtNOGC1 is an enzyme that has a GC activity and synthesise high levels of cGMP from GTP upon activating by NO as known with soluble GC's, additionally AtNOGC1 has the ability to promote stomatal closure in an NO dependent manner (Joudoi *et al.*, 2013). As the NO/cGMP pathway is involved in various physiological processes including plant developments and stress responses. It is therefore important to study the expression of the genes that are related to this pathway in response to abiotic and biotic stresses. In this study the transcriptional pattern of AtNOGC1 was studied in response to abiotic (salinity and drought), hormone and NO treatments. Whereas, NaCl and mannitol were used to induce salinity and drought stress in plants respectively. Various plant hormones play the major roles in plant development and response to stresses. Thus in this study only stress related hormones were used especially biotic stress related including ABA, MeJA and SA. The expression patterns were analysed by looking at the fold change (log2) between control and treated samples.

3.4.1 Effects of abiotic stresses on the expression pattern of AtNOGC1

Salinity and drought stress induce osmotic and ionic stresses which result in oxidative damage, but there are several genes that play roles in signalling networks that induce stress tolerance through minimising oxidative damage (Neto *et.al.*, 2004; Parihar e*t al.*, 2015). AtNOGC1 a novel gene that was characterised in this study to determine its ability to minimise oxidative damage caused by stress in *Arabidopsis thaliana.* At the entire tissue level, cGMP has been associated with reducing Na^+ influx in several species. Thus relieves salt stress, while direct

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cellular measurement of cGMP showed a rapid increase in cellular cGMP following salt and osmotic stress (Donaldson *et.al.*, 2004; Maathuis *et al.,* 2001). In this study, the expression analysis of AtNOGC1 revealed that it was expressed in both tissues under normal conditions, but was slightly induced at 3 hrs in the roots (log2 = 1.82) and 24 hrs in the shoots (log2 = 1.3) that were treated with NaCl. A significant decrease in expression pattern was observed at 12 hrs for the NaCl treated roots and shoots (Fig 3.1 A). Early up-regulation in AtNOGC1 expression pattern in roots may suggest AtNOGC1's involvement in controlling ion absorption and transport to shoots, thus regulating homeostasis and water status of the root ion. While gene activity changes over time, with increased expression in the shoots at 24 hrs suggesting its role in preventing Na^+ toxicity during turgor maintenance (Munns, 2005).

Mannitol lowers the water potential of the medium, inhibiting water absorption by plants (Claeys *et al.,* 2014). In this study, AtNOGC1 was associated with maintaining water potential of *A. thaliana* seedlings that were treated with mannitol as shown by its early induction in the shoots after 3 hrs (log2 = 1.92) and down-regulation in the roots. Up-regulation of AtNOGC1 in shoots might be due to the maintenance of water since this is the most sensitive indicator for stress and water maintenance through stomatal closure (Munns, 2005). This study support the involvement of AtNOGC1 during stomatal closure (Joudoi *et al.*, 2013), thus maintaining water during drought stress. Although no statistically significant difference was observed between the control and experiment ($p > 0.05$), this could be because of the outlier number that results in larger error bars (Cumming *et al.,* 2007). Taken together, these results are in agreement with the promoter analysis which showed the presence of abiotic stress responsive cis-regulatory elements including myeloblastosis (MBY) binding site and TC-rich repeats. Up-regulation of AtNOGC1 during NaCl and mannitol treated samples suggest the protective role played by AtNOGC1 in response to salinity and drought stresses.

3.4.2 Interaction of AtNOGC1 and hormones

ABA is a multifunctional phytohormone that regulates various responses in plant growth, development and abiotic stress responses (Bücker *et al.*, 2017). The second messenger cGMP is involved in the ABA pathway which initiate stomatal closure during stress responses (Dubovskaya *et al.*, 2011). The functional relationship between NO production and ABA during drought stress was reported by various studies indicating that ABA stimulates the high production of NO (Adimulam *et al.*, 2017; Freschi, 2013; Santisree *et al.*, 2015) .Hence regulate NO-stomatal closure through AtNOGC1 (Joudoi *et al.*, 2013). In this study, the exogenous application of ABA significantly induced the expression pattern of AtNOGC1 in both roots and shoots as indicated by log2 ratio of 6.4 and 3.6 respectively at 3 hrs ($p < 0.01$) (Fig 3.2 B). A decrease in the expression pattern of AtNOGC1 was observed at 12 and 24 hrs in the ABA treated seedlings for both tissues. These results correlate with the study done on the rice stress-responsive protein phosphate 2C (PP2C) [OsPP108] when seedlings were treated with ABA (Singh *et al.*, 2015). Thus indicating that the interaction between AtNOGC1 and ABA is required at early stages of plant growth and during stomatal closure for protection against drought stress. These results are in accordance with the previous study (Chapter 2) which showed the presence of ABRE element in the AtNOGC1 promoter suggesting a role for ABA in regulating AtNOGC1's expression.

MeJA is another important hormone that regulates the expression of various genes that are involved in plant growth, development, abiotic and biotic stresses responses (Creelman and Mullet, 1997), especially during necrotrophic pathogen and insects responses (Verma, Ravindran and Kumar, 2016). Based on the study reported by Isner *et al.,* 2012, MeJA induces a change in the concentration of cytoplasmic cGMP levels (Isner *et al.*, 2012). AtNOGC1 promoter analysis indicated the presence of MeJA cis-regulatory element, suggesting the role of AtNOGC1 in necrotrophic pathogen and insects responses. In this study the exogenous

application of MeJA had a slight effect on the expression pattern of AtNOGC1 with the highest expression pattern at 12 hrs in shoots as indicated by a log2 ratio of 1.85. Although the fold change was not statistically significant ($p > 5$) across different time points, AtNOGC1 might be required during the MeJA/cGMP pathway (Cheong and Choi, 2003; Hossain *et al.*, 2014).

SA plays a critical role in activating plant defence responses following a pathogen attack (Verma, Ravindran and Kumar, 2016). Highly significant increase in the expression pattern of AtNOGC1 was recorded in both shoots and roots at 3 hrs with log2 = 7.12 and 6.41 ($p < 0.01$) respectively upon treatment of *A. thaliana* with SA (Fig 3.2) and a significant decreased in the expression was shown at 12 hrs ($p < 0.01$) and slightly decreased at 24 hrs for both tissues. These results correlate with the promoter analysis which revealed the presence of SA, fungal elicitor and defense responsive elements including TCA, Box-W1 and TC-rich repeats suggesting the important role of AtNOGC1 during pathogen attack. Additionally, the interaction between AtNOGC1 and SA is required to activate downstream processes at an early stage of pathogen attack as evidenced by the highest expression at 3 hr for both tissues.
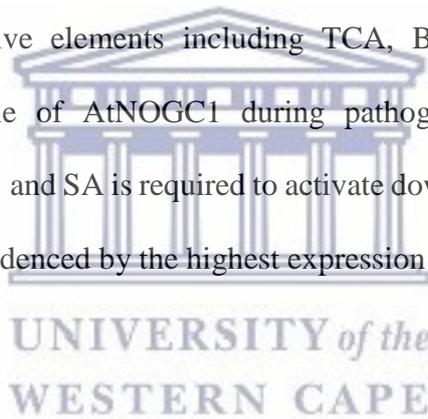
3.4.3 The effects of combined hormones on the transcript levels of AtNOGC1

The response of plants to drought stress depends on highly controlled signal transduction pathways involving the interaction of multiple hormones. This complex crosstalk can result in physiological changes that can confer stress tolerance. ABA is the main mediator of these physiological changes through the regulation of stomatal closure, while other hormones like JA appear to regulate a small subset of plant responses to drought by regulating ABA biosynthesis (de Ollas and Dodd, 2016). To study the crosstalks between ABA+MeJA and AtNOGC1, the AtNOGC1 gene expression pattern was determined in the *A. thaliana* seedlings treated with ABA+MeJA. The expression of AtNOGC1 was significantly ($p < 0.01$) induced by the exogenous application of ABA+MeJA with the highest log2 ratio of ~ 17 in both shoots

and roots (Fig 3.3) at 12 hrs and 24 hrs respectively, with no change observed at 3 hr. These results suggest that both ABA and MeJA are required to mediate protection after longer exposure to stress through the NO/cGMP pathway (Munemasa *et al.*, 2007).

The response of plants to pathogen infection depends on three phytohormones including, SA, JA, and ethylene (ET), and the defence is achieved in an antagonistic manner through a complex network that involves multiple hormones (Jiang *et al.*, 2010). Upon treatment of *A. thaliana* with a combination of ABA+SA, the expression pattern of AtNOGC1 was reduced at 3 hrs and 24 hrs (Figs 3.2 A and B). As observed in section 3.4.2, both ABA and SA when applied individually, had a positive effect on the expression pattern of AtNOGC1, which is different from the combination. These results revealed an antagonistic effect that a combination of ABA+SA at high concentration reduced the expression of AtNOGC1 from log2 = 7 and 4.65 to log2 = 2 and 1 at 3 hrs and 24 hrs respectively. These results correlate with the study done by Jiang *et al.,*(2010) on the ability of ABA to compromise the expression of WRKY45 and OsNPR1 which are components of the SA pathways (Jiang *et al.*, 2010). Interestingly, a significant ($p< 0.01$) increase in the expression of AtNOGC1 at 12 hrs was observed as compared to single hormone treatment, suggesting the positive effect that ABA may have on the expression of SA-responsive genes. Thus suggesting a different role that ABA has on the expression of defence related genes, depending upon the infection time or stage and tissue (Mauch-Mani and Mauch, 2005; Melotto *et al.*, 2006). The results also indicate that both ABA+SA are required to activate the protective role of AtNOGC1 in the roots and shoots at a longer stress exposure.

3.4.4 Exogenous application of NO induces AtNOGC1 expression

NO participates in the great number of plant signalling pathways that mediate stress responses including salinity, drought, high temperature, pathogen attack and physiological processes including germination, growth and development. Exogenous application of NO donor induces

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the endogenous level of NO (Pereira *et al.*, 2011). NO alleviate the effects of oxidative stress caused by abiotic stresses including salinity, drought, and high temperature (Zhao *et al.*, 2007; Lu *et al.*, 2009; Tanou *et al.*, 2012; Parankusam *et al.*, 2017). NO rescue plants by inducing the expression of various genes that are necessary for development and defence (Grün *et al.*, 2006). In this study, the exogenous application of NO donor (SNP) on the *A. thaliana* seedlings significantly increased the expression of AtNOGC1 especially on the roots with a log2 = 7.71 ($p < 0.01$), suggesting the protective role played by AtNOGC1 during plant development and stress responses, via NO to activate the NO/cGMP pathway.

In conclusion, this study indicated that there is a relationship between AtNOGC1 expression pattern and various stresses, suggesting a protective role of AtNOGC1 and its involvement in growth and development in plants. An up-regulation and down-regulation of AtNOGC1 gene were observed in response to all the treatments in a time-dependent manner suggesting that AtNOGC1 is required to act at different stages of the plant upon exposure to stress. Highest expression pattern was recorded when *A. thaliana* was treated with MeJA+ABA with log2 ratio of ~ 17 in both tissues followed by NO, SA, ABA, NaCl and then mannitol treatments and most of the different time points had a log2 of above 1. Small error bars in almost all the treatments were observed representing that the values of the experimental triplicates are not too different, although in some cases such as in mannitol and a combination of MeJA and ABA treatments large error bars were observed at different time points especially at 3 hrs shoots and 24 hrs roots. Gene expression at different times was statistically significantly up-regulated and down-regulated ($p < 0.01$; $p < 0.05$) which shows the reliability of these predictions but mannitol treatment showed no significance due to high variances between triplicates. These results together revealed the multifunction role played by AtNOGC1 during salinity, drought, pathogen and plant development especially during pathogen attack and stomatal closure.

CHAPTER 4

AtNOGC1 CONFERS STRESS TOLERANCE TO *E.COLI* CELLS

ABSTRACT: AtNOGC1 is the first plant guanylyl cyclase (GC) to be identified as a nitric oxide sensing protein. Unlike other GC's from higher plants which synthesise cGMP from GTP, AtNOGC1 when activated by NO it synthesises excess cGMP (~2 fold). This makes it special since NO and cGMP are known to mediate various physiological and metabolic processes in plants including growth, development and response to abiotic and biotic stresses. Biologically AtNOGC1 has been confirmed to be involved in stomatal closure, however its biological role to confer stress tolerance has not yet been demonstrated. This study is the first to demonstrate that a plant GC have a protective role against stress and this was elucidated using *E. coli* viability assays under abiotic stresses. Protein expression indicated that the recombinant AtNOGC1 was overexpressed in *E. coli* under IPTG induction as confirmed by a band at an expected size of 67.7 kDa, which was only observed in the induced *E. coli* cells overexpressing recombinant vector. Overexpression of AtNOGC1 improved the growth of *E. coli* under salinity, oxidative and dehydration stress as compared to control cells. Enhancement in the growth of recombinant cells (*E. coli* overexpressing AtNOGC1) indicated that tolerance to stress was conferred suggesting a protective role for AtNOGC1 against stress.

Keywords: AtNOGC1, *Escherichia coli*, viability assays, stress, tolerance.

4.1 Introduction

Climate change has led to the occurrence of abiotic and biotic stresses. These stresses affect plant growth and development, thus affecting food security globally. Since plants are prone to environmental stresses, they have developed different mechanisms to protect themselves against these stresses. When plants are stressed, ion channels, kinase cascades and signalling molecules are activated including the production of ROS and NO (Rejeb *et al*., 2014). Plants activate similar cell signalling pathways and cellular responses, including upregulation of different stress-responsive proteins such as heat shock proteins and ROS scavenging enzymes, amongst others. There are several plant proteins which have been identified and characterised to have GC activity, these include AtGC1, AtBRII, PSK1, LRR-RLK and AtWAKL10 (Ludidi and Gehring, 2003; Kwezi *et al.*, 2007, 2011; Meier *et al.*, 2010), but none of them can synthesise cGMP in a NO-dependent manner. AtNOGC1 is the first protein to be identified to have a GC's activity and an HNOX motif that binds NO (Mulaudzi *et al.*, 2011). NO plays an important role during root development, stomata closure, pathogen and stress responses. In animals the NO/cGMP pathway is well established, but in plants there are still a lot of gaps including its protective role against stress (Gross and Durner, 2016).

Protein expression in *Escherichia coli (E. coli)* is one of the mostl preferred and widely used method to produce recombinant proteins from both plants and animals amongst other systems such as yeast, filamentous fungi, algae, mammalian and insects (Rosano and Ceccarelli, 2014). This is because the *E. coli* system is inexpensive, easily genetically manipulated, it has good growing kinetics doubling every 20 minutes and reaches the stationary phase in few hours (Sezonov *et al*., 2007). In addition, the T7 RNA polymerase system is one of the most popular approaches to produce recombinant proteins in *E. coli*. This is due to the small size (17 bp) that is easy to manipulate (Company, 2013). The gene of interest is either cloned in the expression vector upstream of the T7 promoter or placed under the control of the heat inducible promoter

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(Tabor, 2001). The expression plasmids are normally chosen based on promoters, selection markers, and combinations of replicons, multiple cloning sites and removal strategies of the fusion protein (Rosano and Ceccarelli, 2014). The exogenous DNA that is cloned into expression vectors such as the commonly used pET series is followed by the production of the recombinant protein in *E. coli* using isopropyl-D-thio galactopyranoside (IPTG) inducible promoter. Promoters of expression usually depend on the RNA of the host cells or foreign polymerase from bacteriophage T7 (Rosano and Ceccarelli, 2014).

They are various *E. coli* protein expression strains that are used, including BL21 (DE3), BL21 (DE3) pLysS, BL21-AI, SI, Rosetta, C4i and BL21 Codon Plus amongst others. BL21 (DE3) is the mostly used during expression screening for the expression of very toxic proteins and the T7 promoter is recognised by RNA polymerase of this strain, BL21 (DE3) pLysS is used to express both toxic and non-toxic proteins. BL21 Codon Plus is a modified strain with high protein expression levels which eliminates codon bias (Carstens *et al.*, 2001). This strain is derived from Agilent's high performace BL21-Gold and rescues expression of proteins from organism containing AT- or GC-rich genomes and contain extra copies of minor tRNAs (argU, ileY) and tRNA (Kleber-Janke and Becker, 2000).

The use of *E. coli* model to functionally characterise plant genes has been widely applied and well reported (Yadav *et al.*, 2014b; Rajan *et al.*, 2015b). This model operates based on the knowledge that bacteria rapidly multiply when growing under favourable conditions and lose their ability when subjected to high toxicity due to environmental stresses such as high concentration of NaCl, H_2O_2, mannitol and high temperature (Pletnev *et al.*, 2015). This study was performed to report on the protective role of AtNOGC1 in response to stress when heterologously expressed in *E. coli* BL21 Codon Plus cells that were subjected to different stress conditions. This was achieved through the analysis of the growth patterns of *E.coli* using viability assays.

Cell viability refers to cells, tissues, and organs that are capable of being alive before or after the change of event (Pegg, 1989). Assays are employed to study the viability of different cells under normal and stressed conditions. In functional characterisation, *E. coli* cells without stress-responsive gene are compared with the cells overexpressing the stress responsive protein. There are various methods used to analyse the viability of cells with or without the stress responsive proteins, these includes colony count, spot and liquid assays (Marathe *et al.*, 2018). The viability of cells can be measured by counting the number of colonies formed after incubation that are plotted as percentage of Colony Forming Units (CFUs) (Brugger *et al.*, 2012). Spot assay is a qualitative form of data representation that is represented as a spot, whereas liquid assay is a quantitative method that is represented as a growth curve in which the growth of culture is monitored by measuring the absorbance (OD) at 600 nm in a time dependent manner (Yilmaz, 2013).

These assays allow researchers to conduct functional characterisation of the protein using convenient and efficient *E.coli* system. Heterologous expression of Stress-responsive protein from *salvia miltiorrhiza* (SmUSP) and *late embryonesis* of Cassava (MeLEA3) in *E. coli* was able to enhance tolerance to salt and heat stress as compared to control cells (Barros et al., 2015; Xiao-fan Wang *et al.*, 2017). The Ras-related protein (AIRab7) gene from *aeluropus lagopoides* was able to improve growth of cells when the growth medium was supplemented with NaCl, KCl and mannitol (Rajan *et al.*, 2015a). The *triticum aestivum* late embryogenesis abundant (WRAB18) containing BL21 cells showed better growth under drought, salinity, heat, and cold as compared to control cells (Xiaoyu Wang *et al.*, 2017).

4.2 Materials and Method

4.2.1 Preparation of the AtNOGC1 construct

An AtNOGC1 expression construct, pET SUMO-AtNOGC1 which was synthesised from previous study was used (Mulaudzi., 2011). Empty Champion™ pET SUMO vector was self-religated using the DNA ligation kit (Cat # K1422, Thermo Scientific™, USA) and served as a control throughout the study. The pET SUMO vector (control cells) and pET SUMO-AtNOGC1 (recombinant cells) were used to transform BL21 Codon Plus competent cells. About 2 µL of the DNA was added into 1.5 mL eppendorf tube with 50 µL of BL21 Codon Plus competent cells and incubated on ice for 30 min. Cells were heat shocked at 42°C for 45 seconds using the dry bath (Dry Bath plus, Lasec, SA) followed by incubation on ice for 2 min. About 450 µL of nutrient broth (Cat# NUB20500, Biolab, Hungary) was added to the mixture, followed by incubation for an hour at 37°C with shaking. About 100 µL of transformed cells was plated on the nutrient agar plate containing 50 µg/ml kanamycin for antibiotic selection and incubated overnight at 37°C.

4.2.2 Expression of the recombinant protein

A single colony was taken from the plate containing cells that are transformed with pET SUMO vector only (control cells) and pET SUMO-AtNOGC1 (recombinant cells) and used to inoculate 2 ml nutrient broth (supplemented with 50 µg/ml kanamycin and 0.4% glucose) and grown at 37°C with shaking overnight at 180 rpm (Innova 4000, New Brunswick Scientific). The following morning the cultures were scaled up to 20 ml with the same nutrient constituents and allowed to grow until OD_{600} 0.5. About 2 mM IPTG was added to both cultures and further grown at 30°C for 2.5 hrs as previously described (Mulaudzi *et al.*, 2011). The expressed recombinant protein alongside with vector cells only were confirmed by analysing on the SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Brunelle and Green, 2014). The SDS-PAGE gels were prepared as shown in Table 4.1

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Table 4.1 Preparation of SDS-page gel electrophoresis

Components	12% SDS-PAGE separating gel	6% Staking gel
Acrylamide 30 %	2.4 ml	0.75 ml
1.5 M Tris pH 8.8	2 ml	1.25 ml
10 % SDS	80 µL	50 µL
10 % APS	80 µL	50 µL
dH$_2$O	3.4 ml	2.9 ml
TEMED	8 µL	5 µL

4.2.3 Assays for stress tolerance of *E. coli* transformed with AtNOGC1

Spot and liquid culture assays were conducted to investigate the role of AtNOGC1 on the growth of *E. coli* cells under stress conditions. Proteins were expressed as described in section 4.2.2. After induction, the induced cultures were re-adjusted to an OD$_{600}$ of 0.5. Spot assay was performed by further diluting the 0.5 OD$_{600}$ culture to 10^{-1}, 10^{-2} and 10^{-3} fold. About 10 µL from each dilution including undiluted (10^0) was spotted on the nutrient agar plates containing 2 mM IPTG, 50 µg/ml kanamycin and supplemented with NaCl (400 mM, 500 mM and 600 mM) for salinity stress and H$_2$O$_2$ (0.4 mM, 0.6 mM and 0.8 mM) for oxidative stress analysis. Plates were incubated at 37°C for 16 hrs. Liquid assay was conducted by taking 800 µL of the culture into a newly prepared 25 ml nutrient broth (control culture) with 2 mM IPTG, 50 µg/ml kanamycin and the medium was supplemented with NaCl and H$_2$O$_2$ (stress culture) at the same concentration as that used for the spot assay. The culture was also added to a nutrient broth that was supplemented with 10%, 15%, and 20% Polyethylene glycol 6000 (PEG6000) to induce drought stress. For liquid assay both bacterial suspensions (control and recombinant cells) were grown at 30°C at 180 rpm incubator shaker (Innova 4000, New Brunswick Scientific). Both cultures were harvested after every 2 hrs for 24 hrs to measure OD$_{600}$ using a spectrophotometer

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(Helios Epsilon, Thermo Scientific™). Each experiment was independently repeated three times and three technical replicates included (Rajan *et al*., 2015).
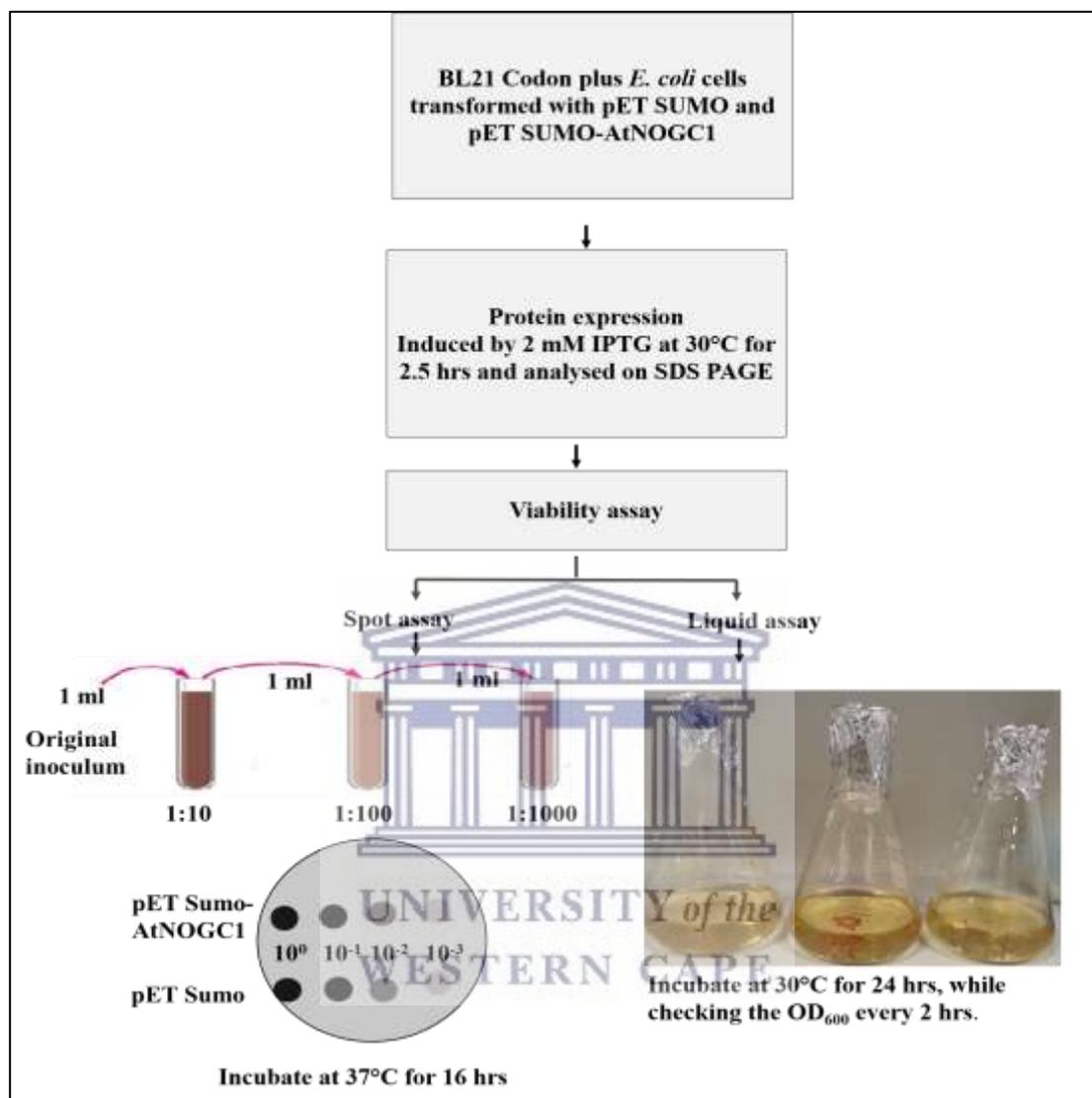
Fig 4.1 Workflow diagram representing how *E. coli* viability assays were performed. pET SUMO vector (control) and pET SUMO-AtNOGC1 were separately transformed into BL21 Codon Plus cells, followed by protein expression. The presence of the protein was confirmed by SDS PAGE. Viability assays: For spot assay 10 µL from each dilution was spotted on the nutrient agar plate supplemented with 2 mM IPTG, while for liquid assay 800 µL of the 0.5 OD_{600} culture was aliquoted into 25 ml of nutrient broth supplemented with different stress inducers, 2 mM IPTG and 50 µg/ml kanamycin.

4.2.4 Statistical analysis

All values reported on the growth measurements of *E. coli* on the liquid culture represent an average of the mean from the three independent biological replicates and error bars represent the SD calculated from three biological replicates.

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4.3 RESULTS

In order to study the ability of AtNOGC1 to confer stress tolerance to *E. coli* cells, viability assays including qualitative (spot assay) and quantitative (liquid assay) analysis were conducted. This was monitored by comparing the growth attributes of recombinant *E. coli* cells against control cells. The presence of the recombinant AtNOGC1 was first determined by expressing prior to stress treatments.

4.3.1 Analysis of protein expression

The recombinant (pET SUMO-AtNOGC1) and the empty (pET SUMO) vector were transformed into BL21 Codon Plus cells and expression was induced by the addition of 2 mM IPTG as described in section 4.2.2. AtNOGC1 is a 56.7 kDa protein which when overexpressed in *E. coli* using the pET SUMO vector system yielded a recombinant protein with a size of 67.7 kDa. The induced fraction revealed the presence of recombinant AtNOGC1 (Fig 4.2, lane 4), which was not present in the uninduced (lane 3) and the control fractions (lane 1 and 2). In order to verify if the control cells are not expressing AtNOGC1, cells were also induced with IPTG (see lane 2) and the vector was confirmed to be empty.
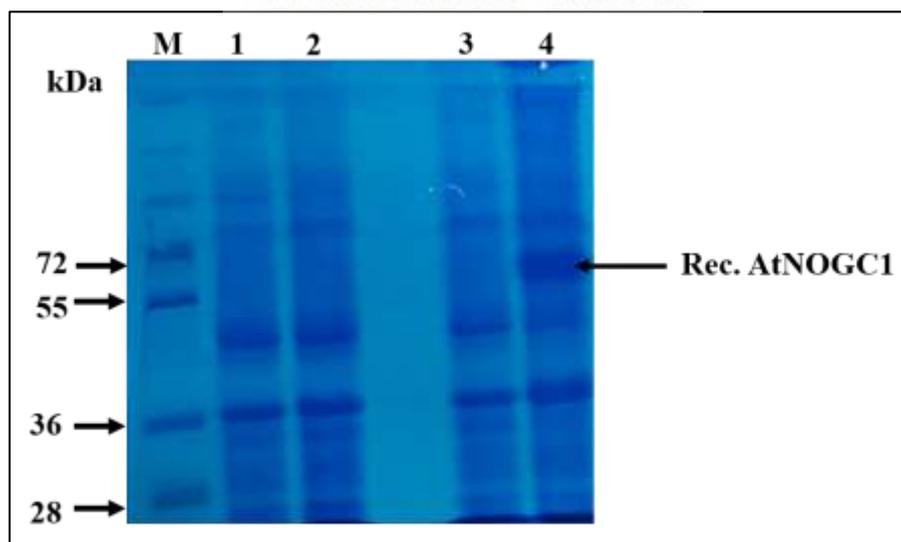
Fig 4.2 SDS-PAGE showing the expression analysis of the recombinant AtNOGC1 protein and the control plasmid. Lane 1 and 2 represent un-induced and induced control cells respectively. Lane 3 represent un-induced recombinant cells and lane 4 represent induced recombinant cells with the expected size of ~67.7 kDa.

4.3.2 Cell Viability Assay

The viability of *E. coli* cells under different stress conditions including salt, oxidative and dehydration was analysed using the spot and liquid assays for qualitative and quantitative analysis. Upon successful expression of the AtNOGC1 protein in the BL21 *E. coli* recombinant cells (pET SUMO-AtNOGC1) and control cells (pET SUMO) their growth was assayed on the solid and liquid media containing different stress inducers. The viability of both cells was compared against each other by observing the growth enhancement and the change in absorbance (OD_{600}) measured in a time dependent manner.

In order to study the ability of AtNOGC1 to confer stress tolerance to *E. coli* cells, AtNOGC1 was successfully expressed in *E. coli* by inducing with IPTG for 2.5 hours (Fig 4.2) as described in section 4.2.2. To elucidate the ability of AtNOGC1 in conferring stress tolerance to *E. coli* cells, the recombinant and control cells were analysed using the spot assay and liquid assays. Both the recombinant and control cells showed the same growth pattern under normal conditions (Fig 4.3).
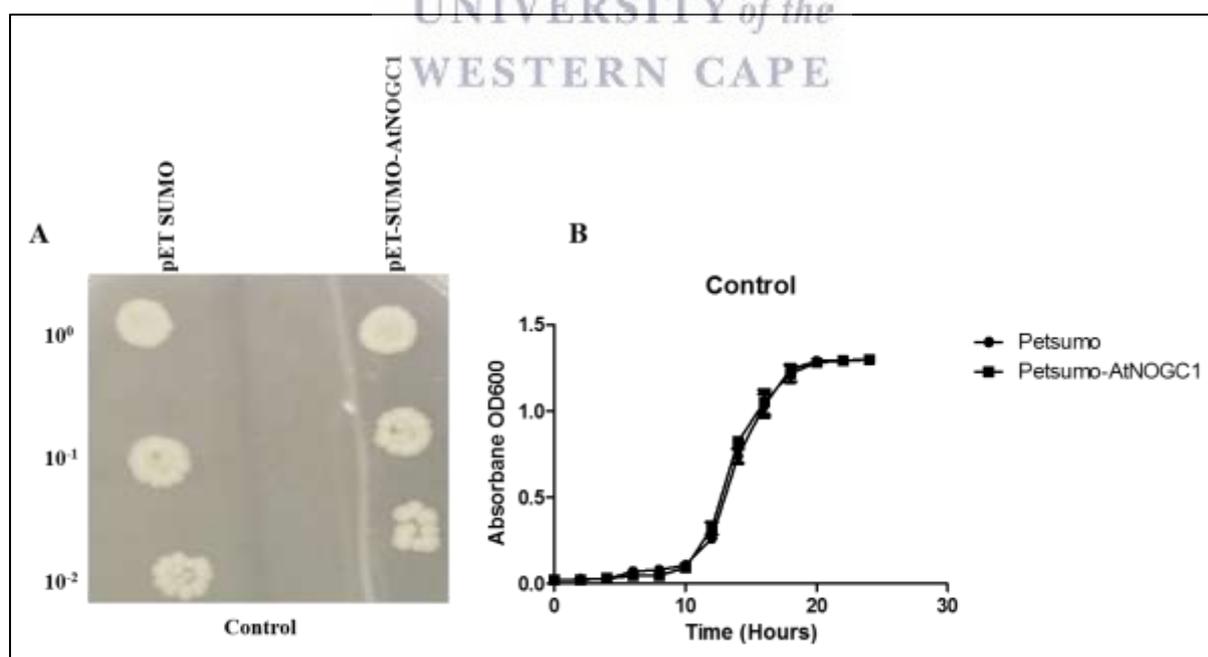
Fig 4.3. Growth analysis of recombinant *E. coli* expressing vector only and *E. coli* expressing AtNOGC1 under normal condition. Spot (A) and liquid (B) assay of pET SUMO and pET SUMO-AtNOGC1/BL21 Codon Plus cells without stress.

The effect of salt stress on the growth of *E. coli* was monitored by growing cells in different NaCl concentrations of 400 mM, 500 mM and 600 mM. As shown in Fig 4.4 A, B, C the growth of recombinant cells is different when compared to control cells. Recombinant cells displayed better growth and number of colonies as observed in 10^0 (undiluted), 10^{-1} and 10^{-2} (diluted) cultures. A more enhanced growth pattern was displayed in the liquid assay (Fig 4.4 D, E and F), with the recombinant cells growing better than the control cells. All the liquid assay analysis indicate that statically the recombinant cells have better growth trends than the control cells. A huge difference is observed in the 600 mM NaCl treated cells (Fig 4.4 F).
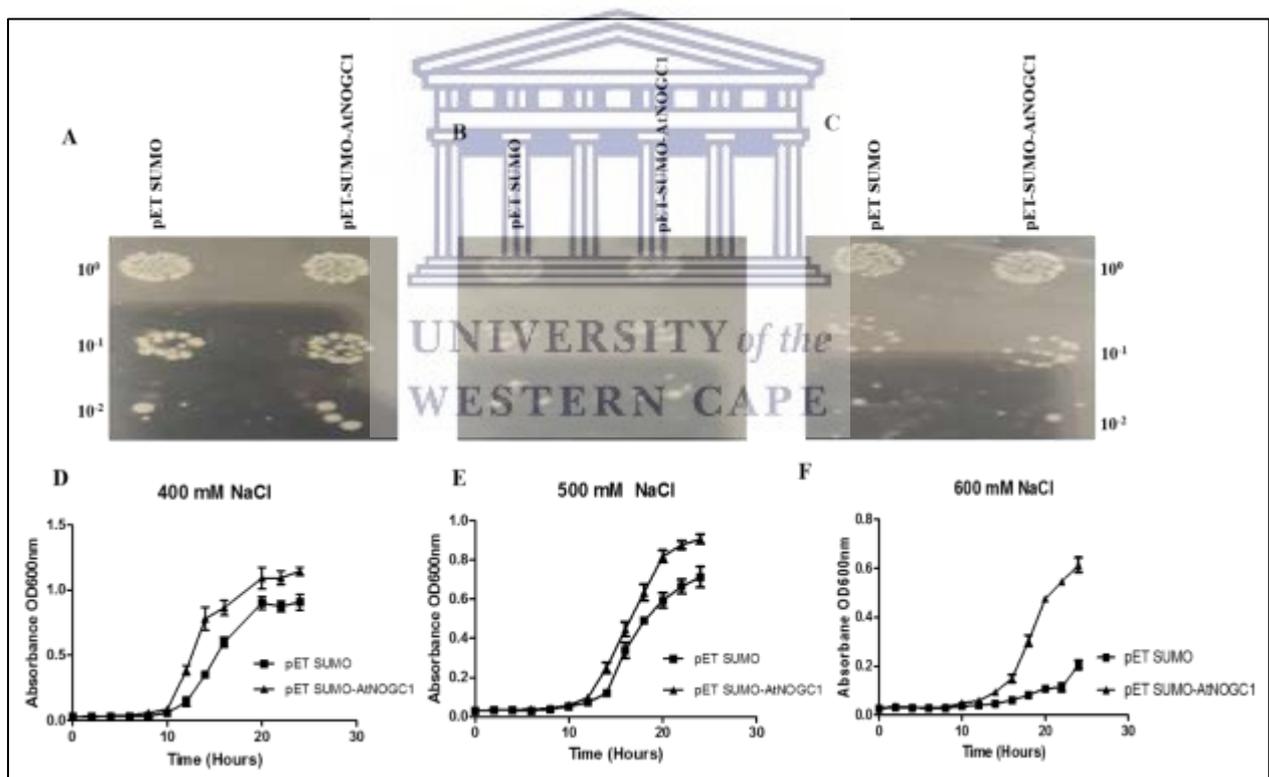
Fig 4.4 Growth analysis of recombinant *E. coli* expressing vector only (pET SUMO) and *E. coli* expressing AtNOGC1 (pET SUMO-AtNOGC1) under salt stress. *E. coli* cells cultured in nutrient medium supplemented with 400 mM (A, D), 500 mM (B, E), 600 mM (C, F) NaCl for spot and liquid assays respectively.

Under oxidative stress using H_2O_2 as the inducer, cells followed the same growth pattern, as observed under salt where recombinant cells displayed better growth than control cells (Fig

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4.5). Growth patterns for both assays indicated that H_2O_2 hinders *E. coli* growth as observed by the spot and liquid assays (Fig 4.5 B, C, D, E, and F) with almost no growth for the control cells in the 0.8 mM H_2O_2 culture.

Under dehydration stress, which was induced by adding PEG, the same pattern of growth was observed. Spot assay was attempted, but due to the inability of PEG to solidify in the media liquid assay were only conducted. Recombinant cells displayed better growth under PEG stress as compared to control cells with the growth at 20% PEG more reduced to OD_{600} of 0.3 (Fig 4.5 A, B, C).
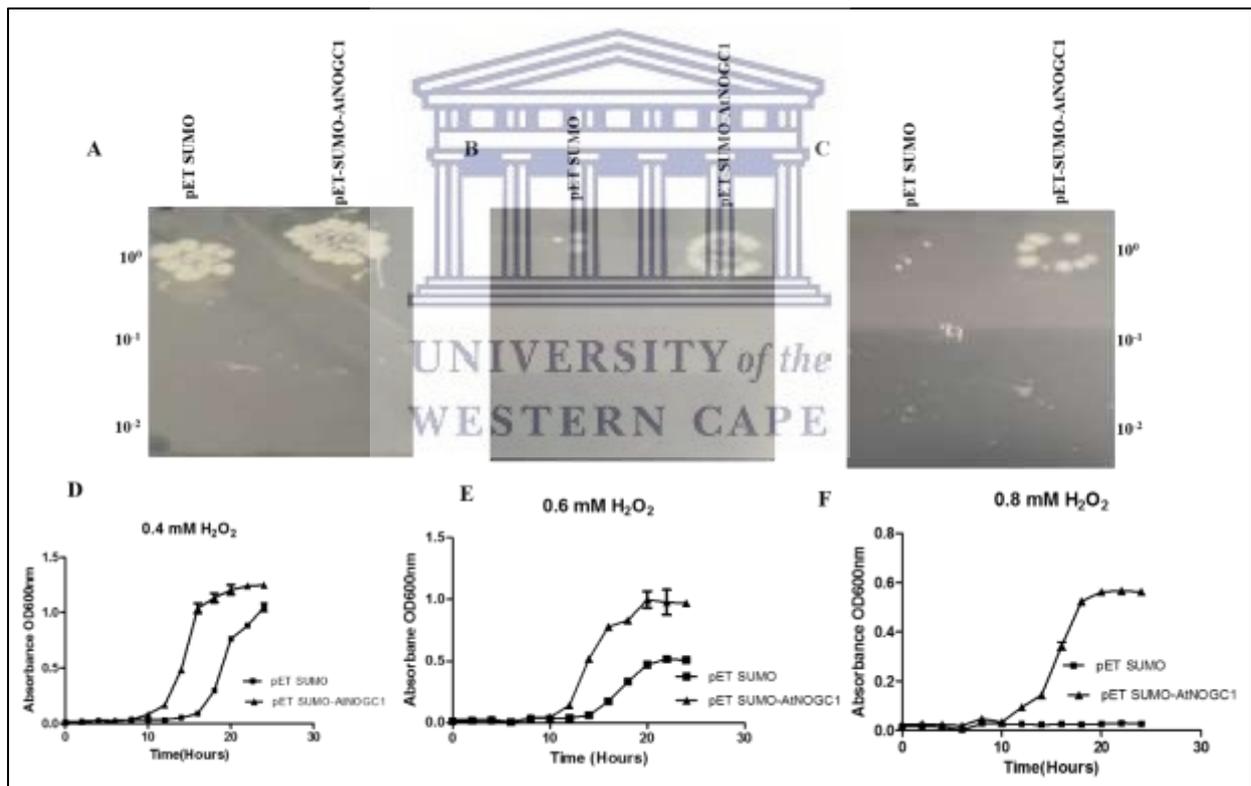
Fig 4.5 Growth analysis of recombinant *E. coli* expressing vector only (pET SUMO) and *E. coli* expressing AtNOGC1 (pET SUMO-AtNOGC1) under oxidative stress. *E. coli* cells cultured in nutrient medium supplemented with 0.4 mM (A, D), 0.6 mM (B, E), 0.8 mM (C, F) H_2O_2 for spot and liquid assays respectively

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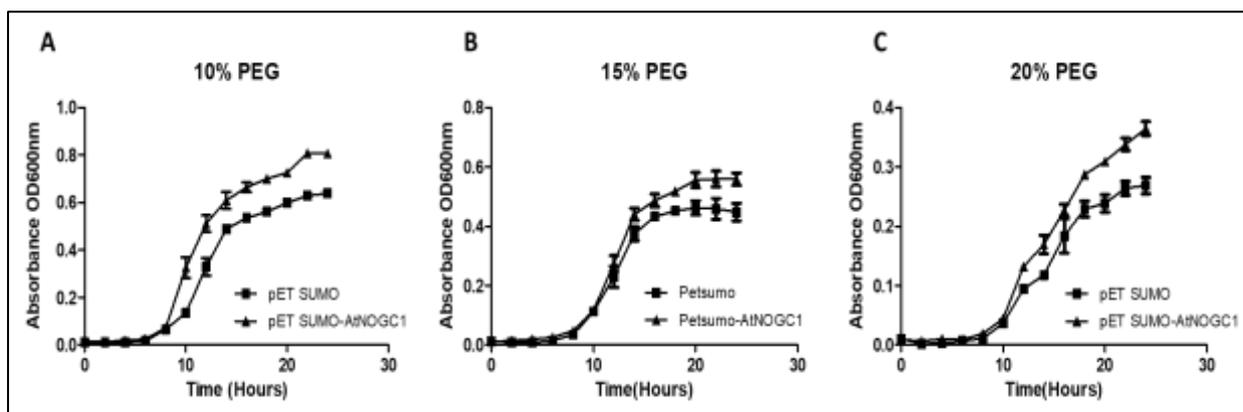
Fig 4.6: Growth analysis of recombinant *E. coli* expressing vector only (pET SUMO) and *E. coli* expressing AtNOGC1 (pET SUMO-AtNOGC1) under dehydration or drought stress. *E. coli* cells cultured in nutrient medium supplemented with 10%, 15% and 20% PEG600 for only liquid assays.

4.4 DISCUSSION

Environmental stress imposes negative effects on living organisms including plants, animals and bacteria. Such stress could be in a form of salinity, drought, extreme temperature and heavy metals amongst others. Organisms have to develop different mechanisms to survive under these stresses. Stress leads to an imbalanced production of certain metabolites including the overproduction of reactive oxygen species, causing oxidative damage to cells (Caverzan *et al.*, 2012). These stresses in turn affects the plant's ability to absorb important minerals and nutrients, thus leading to impaired growth and development and hence death. However, plants use different mechanisms to survive these stresses, including overexpression of stress responsive proteins such as those involved in scavenging ROS, heat shock proteins among others and this is dependent on the type of stress imposed on them (Grene, 2002; Scarpeci *et al.*, 2008; Caverzan *et al.*, 2012). Since plants respond differently to various stresses and are constantly attacked by a combination of stresses (Rejeb *et al*., 2014), identification and functional characterisation of novel stress genes is important towards improving plants growth under several stress.

In this study, the protective role of AtNOGC1 was studied by performing cell viability assays on *E. coli* cells, taking advantage that prokaryotic systems are convenient to work with (Li *et al.*, 2005). Recombinant *E. coli* cells (pET SUMO-AtNOGC1) and control cells *(*pET SUMO) showed similar growth patterns under normal conditions. However, recombinant *E. coli* cells showed enhanced growth when subjected to NaCl, H_2O_2 and PEG. Similar to our study, several studies also demonstrated that heterologous expression of the plant stress-responsive genes enhance growth of *E. coli* cells under stress (Tan *et al.*, 2013; Yadav *et al.*, 2014b; Rajan *et al.*, 2015a). Spot assay is a qualitative way of assaying *E. coli* growth and as seen in Fig 4.4 (A-C), recombinant cells showed more growth (as shown by colonies) as compared to the control cells under NaCl stress although the difference observed between recombinant and control cells

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was not significant. This could probably be due to the fact that the stress imposed was not enough to inhibit the growth of both cells. Liquid assay represents a quantitative analysis of growth and as seen in Fig 4.4 (D-F), recombinant cells displayed more enhanced growth as compared to control cells. Even though for 400 mM and 500 mM there were no major differences. Both recombinant and control cells cultured in 600 mM NaCl had a long lag phase, due to the severity of the stress. Thus the growth was delayed since bacterial cells were trying to adjust in the stress environment. However, from 12 hrs, there was an increase in the growth of the recombinant cells as compared to the control cells. NaCl treatment clearly indicated that AtNOGCC1 has the ability to confer tolerance of salt stress to cells. These results are consistent with several studies done on the protective role of plant genes including vesicle trafficking gene isolated from salt excreting halophyte *Aeluropus lagopoides* (AlRab7), the stress responsive protein from *Salvia mitiorrhiza* (SmUSP), Late embryonesis of cassava (MeLEA3) and novel gene *Salicornia brachiate* salt-inducible-2 (SbSI-2). All these showed that recombinant cells displayed more colonies and enhanced growth under NaCl medium than the control cells (Yadav *et al.*, 2014a; Barros *et al.*, 2015; Rajan *et al.*, 2015b; Wang *et al.*, 2017).

Oxidative stress is a component of many abiotic and biotic stresses in plants and they result in DNA, proteins and lipids damage (Krishnamurthy and Rathinasabapathi, 2013). Exposure of cells to H_2O_2 supplemented medium showed differences with the stress more severe on the control cells than recombinant cells as observed on both spot and liquid assays (Fig 4.5). Recombinant cells showed better cell size, the number of colonies and good growth rate. At 0.8 mM H_2O_2, in the control cells growth was greatly slowed with recombinant cells showing enhanced growth. Based on the spot assay, H_2O_2 stress was severe to the point that no growth was observed for both the recombinant and control cells for the diluted cultures. In the liquid assays, the lag phase for all the concentration was increased for up to 10 hrs and 12-14 hrs for recombinant and control cells respectively as compared to *E. coli* cells grown under normal

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conditions which had a lag phase of up to 8 hrs. For the 0.8 mM H_2O_2, control cells remained at a lag phase for the entire duration of growth. These results are consistent with the spot assay only 3 colonies observed in the undiluted culture ($10°$). Based on the liquid assay recombinant cells were able to grow up to OD_{600} 0.5 for 18 hrs and reached a stationary phase. As compared to the control cells (cultured under normal conditions) (Fig 4.3) cells also reached a stationary phase at ±18 hrs, this may be due to nutrients depletion. These results suggest that AtNOGC1 was able to confer tolerance to *E. coli* cells under oxidative stress.

To further the understanding on the protective role of AtNOGC1 gene, PEG6000 was added to the medium to induce dehydration stress. PEG was chosen for this treatment since it reduces the water potential of the medium and reduces the water and nutrients absorption by cells (Lawlor, 1969). Several studies showed the ability of recombinant *E. coli* to have increased tolerance to PEG stress. These include the stress tolerant dirigent protein (DIR) from sugarcane, a novel *Salicornia brachiate* salt-inducible-2 (SbSI-2) protein from a halophyte and inositol 1, 3, 4-trisphosphate 5/6 kinase-2 (GmITPK2) from *Glycine max (L.) Merr* (Chaurasia *et al*., 2008; Yadav *et al.*, 2014a; Marathe *et al.*, 2018). In this study, recombinant cells showed an enhanced growth as compared to control cells under PEG treatment but no significant difference was observed (Fig 4.6). Surprisingly, all concentrations (10%, 15%, and 20%) showed to have reduced lag phase (Fig 4.6), which is the same with the untreated culture (Fig 4.3). At low PEG concentrations (10%), the absorbance continued to double over time, but at the end, the absorbance was reduced. PEG decreases the homogeneous solution by increasing the particle forming polymer viscosity in the solution and decreases the bacteria forming. Diffraction of dissolved polymers are also measured when reading the absorbance (Plisko *et al.*, 2016) which explains the short lag phase. The lowest absorbance was observed at 24 hrs ~ 0.35 and ~ 2.6 for the recombinant and control cells respectively, showing the severe effect caused by PEG on the cells, resulting in an early stationary phase. As observed from the liquid

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assays, the recombinant cells displayed a more enhanced growth pattern than control cells, thus based on these results AtNOGC1 was able to confer tolerance to *E. coli* under osmotic or dehydration stress.

In both spot and liquid assays, an increase in the concentration of NaCl, H_2O_2 and PEG treatments served as evidence that AtNOGC1 plays a role in conferring stress tolerance against salinity, oxidative and dehydration stress. As the concentration of stress increased, there was a decrease in growth as displayed by small size and less number of colonies (spot assay) and an increased lag phase (liquid assay). Different stresses result in similar responses, which is why in this study, cells cultured under salt, oxidative and dehydration stresses displayed similar patterns of growth. One of the major similarity in stress response include, increased lag phase and decrease in the absorbance reading from $OD_{600} = 1.4$ for cells cultured under normal condition s to 0.7(recombinant cells) and 0.1 (control cells) for cells stressed with the highest salt concentration (600 mM), thus representing hindrance in growth. Whereas under oxidative stress the lowest absorbance for the highest H_2O_2 (0.8 mM) concentration was 0.6 and 0.0 for recombinant and control cells respectively. In the presence of 20% PEG, which was the highest concentration the absorbance decreased to 0.4 and 0.3 for recombinant and control cells respectively. Looking at the growth patterns, PEG indicate the most severe stress, followed by H_2O_2, and NaCl. However AtNOGC1 is a better protector of *E. coli* cells under oxidative stress, where the control displayed long lag phase for the entire 24 hrs of growth. Due to the presence of AtNOGC1 protein in *E. coli*, cells were able to survive the effect of stress imposed on them, thus suggesting the protective role of AtNOGC1 against salt, oxidative and dehydration stress in plants.

CHAPTER 5

Conclusion and future prospects

Food insecurity has become a global crisis following the global decrease of the world economy. This problem has led to high food prices due to high maintenance of crops on farms and the import of foods from other countries. According to the 2004 Food and Agriculture Organization (FAO) report on global food insecurity, more than 814 million people are undernourished in developing countries (FAO, 2004). Salinity, drought and pathogen are one of the factors that cause severe effects on plants. These factors usually occur in semi - arid and arid areas, mainly in developing countries. It has been estimated that by 2050, about 50% of the world's arable land will be affected by salinasation. In addition, the world's population is projected to rise to 9.7 billion by 2050, increasing the global demand for resources and food (FAO, 2004). Therefore, appropriate strategies to tackle food insecurity issues such as the development of stress-resistant crops need to be implemented.

Plant signalling is one of the key mechanisms by which plants respond to environmental stimuli, including secondary messengers, signalling cascades, and chemical reactions. Cyclic guanosine monophosphate (cGMP) is a second messenger that plays an important role in signalling during plant development as well as in response to abiotic and biotic stresses. Several studies have shown that cGMP is a NO signalling intermediate, and it is produced in excess amounts upon activation of guanylyl cyclase (GC) by NO thus triggering various physiological changes. In plants, various enzymes involved in the cGMP pathway have been identified to have the GC's activity. These include AtGC1, AtBRII, LRRRLK, AtWAK10 and PSK, but none of them contain the heme motif that is important for NO binding. AtNOGC1 is the first identified enzyme with GC activity that has HNOX binding motif, which bind NO with high affinity than O_2. Following this discovery, AtNOGC1 was also confirmed to be involved in

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stomatal closure (Joudoi *et al*., 2013), however its role in response to other stress has not been reported.

In this study *in silico*, gene expression and viability assays were used to demonstrate the functional role of AtNOGC1 in response to various stresses. *In silico* characterisations played an important role in the evolutionary relationship analysis of AtNOGC1 and other proteins. Phylogenetic tree revealed that AtNOGC1 has similar evolutionary history with other GC's from different organisms, especially the uncharacterised Agap-AGAP010398-PA from *Anopheles gambiae* among other sGCs with a sequence similarity of 88%. Another interesting finding was that AtNOGC1 has an isoform, which is also annotated as NOGC1 suggesting possible splicing. Gene structure analysis revealed that the distribution of exon-intron structures in various proteins was different. However only proteins from the same species have the similar distributions. These findings provided a new knowledge into genetically relatedness of AtNOGC1 with insects (*Anopheles gambiae*) protein. The successfully isolated AtNOGC1 promoter from *Arabidopsis thaliana* and the analysis of its cis-regulatory elements was important in order to understand its possible functional role in plants in response to certain stimuli. The promoter sequence analysis of AtNOGC1 revealed the presence of cis-regulatory elements that are important for plant development and defence related including elements responsive against light, fungus, pathogens and abiotic stresses. Therefore these results demonstrated the important role that AtNOGC1 might play during plant development, abiotic and biotic stress responses.

In order to analyse AtNOGC1 gene expression under various stimuli including a selected abiotic stress, hormones and NO treatments transcriptional pattern was analysed, which revealed that AtNOGC1 is induced by multiple stresses. The transcript level of AtNOGC1 was dependent on time, and tissue specific in most treatments. AtNOGC1 was required at an early response as observed by high expression at 3 hr treatment for most stresses. The gene was up

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regulated upon treatment with salt, mannitol, hormones and NO, which demonstrated the biological role of AtNOGC1 during development, abiotic and biotic stresses response in plants. Most importantly the role in pathogenic responses and plant development was evident as shown by its high expression profile when the plants were treated with SA, ABA and a combination of ABA + MeJA and SA + MeJA. The gene expression data on the crosstalk between combined hormones and AtNOGC1, brought some interesting insights with regard to understanding plant stress response mechanisms. The transcriptional analysis results were also in agreement with the promoter analysis outcomes, which indicated the presence of plant development and stress responsive elements. NO initiates various responses to stimuli during plant development, defence and abiotic stress responses thus the upregulation of AtNOGC1 in the presence of NO may also suggest its involvement in NO pathways. The response of AtNOGC1 to these treatments may suggest its novel role during plant development and stress responses via the NO/cGMP pathway.

Gene expression analysis indicated that AtNOGC1 is responsive to different stress elicitors, therefore it was necessary to demonstrate whether it can confer stress tolerance to cells. Thus characterisation of AtNOGC1 in *E. coli* cells elucidated its protective role during salinity, oxidative and dehydration stresses using spot and liquid assays. Results indicated that the presence of AtNOGC1 in stressed cells provided protective effect as shown by enhanced growth, number and size of colonies as compared to the control cells, however the mechanism behind its protective role remains elusive. Although the protective role of AtNOGC1 was demonstrated on *E. coli* cells, these results suggest that AtNOGC1 might have the ability to play a protective role in stressed plants.

In this study the role of AtNOGC1 was successfully investigated and demonstrated. AtNOGC1 share close common ancestors and gene structure with FMO's and GC's from insects. It is therefore recommended to further characterise these FMO's for the presence of GC activity

and HNOX motifs towards identification of more NO binding GC proteins in higher plants. It is also important to conduct comprehensive comparative studies between AtNOGC1 and insects GC's since among other soluble GC's the Agap-AGAP010398 GC from *Anopheles gambiae* was the only one that formed the branch within the FMO's. According to promoter and transcriptional analysis, AtNOGC1 is more enriched with pathogenic and stomatal closure responsive elements amongst others, which was confirmed by high levels of gene expression when plants were treated with ABA and SA. This is true since SA pathway, has a downstream marker gene PR1, which is a pathogen induced gene. This is one area that has not received attention with AtNOGC1, since Joudoi *et al.*, 2013 demonstrated its role in stomatal closure. Therefore, it is recommended to put more emphasis on studying the role of AtNOGC1 during pathogenic responses. Although the protective roles of AtNOGC1 in conferring stress tolerance to *E. coli* cells were performed. Therefore it will be important to overexpress AtNOGC1 in *A. thaliana* to further elucidate its protective role in plants. It is also important to develop transgenic plants that are mutant in AtNOGC1 through T-DNA genotyping, thus in order to phenotype both transgenic lines against the wild type for stress tolerance.
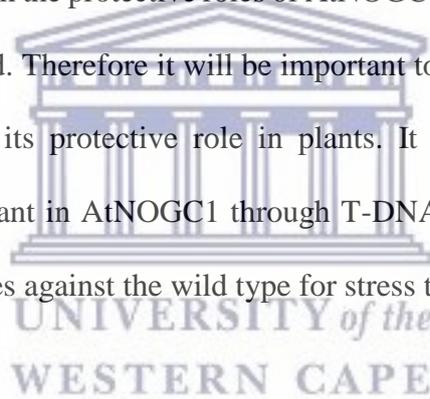
6. Appendices

Appendix I Sequence alignments

```
M57405.1                             --------------------------------------------
 ----
XM_001700795.1
TGGCGATTGCAGCACGTGATCTAATGTGACGGCATAGCTAATGGAATATC
XM_001700986.1                       --------------------------------------------
----
XM_001700794.1                       --------------------------------------------
----
NM_001355466.1                       --------------------------------------------
----
NM_173788.4                          --------------------------------------------
----
NM_000906.3                          --------------------------------------------
----
NM_001192751.1                       --------------------------------------------
----
XM_015576104.1                       --------------------------------------------
----
XM_002643826.1                       --------------------------------------------
----
NM_001029717.1                       --------------------------------------------
----
NM_001330893.1                       --------------------------------------------
----
NM_001029719.1                       --------------------------------------------
----
NM_126282.3                          -------------------A------------------------
----
NM_106617.4                          CATAGTTTACTTCT--TT--------------------------
----
AY118140.1                           --------------------------------------------
----
NM_001249781.2                       -ATCGCATGTGCCA--TAA-------------------------
--AC
NM_079071.3                          --------------------------------------------
----
XM_001958843.2                       --------------------------------------------
----
XM_005187368.3                       --------------------------------------------
----
XM_001655975.2                       --------------------------------------------
----
NM_120100.3                          TGGGGAAT------------------------------------
----
XM_311551.4                          --------------------------------------------
----
NW_006256414.1:c1645606-1642949      --------------------------------------------
----
XM_024149294.1                       --------------------------------------------
----
NM_105013.4                          --------------------------------------------
----
NM_104936.2                          --------------------------------------------
----
NM_001334039.1                       --------------------------------------------
----
```

Fig 6.1: Multiple sequence alignment for GC's from different species including mammals, nematodes, insects and plants and also FMO's protein. The alignment was done using clastalW.

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Sequence ID: Query_115451Length: 1230Number of Matches: 1

Related Information

Alignment statistics for match #1

Score	Expect	Identities	Gaps
654 bits(354)	0.0	363/371(98%)	0/371(0%)

```
Query  29   aaraaTTTGACCGTTCCTCACATGGTTCTGCATCAAGTAGATTATACTAGAGATCAGGCC  88
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57   AAGAATTTGACCGTTCCTCACATGGTTCTGCATCAAGTAGATTATACTAGAGATCAGGCC  116

Query  89   CCGCTTAGAGGGAGTGCTCGTCCMCAttttttttCCAAATGATTTTAAGCTCCTAAGTTTT  148
            ||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||
Sbjct  117  CCGCTTAGAGGGAGTGCTCGTCCACATTTTTTTCCAAATGATTTTAAGCTCCTAAGTTTT  176

Query  149  TGATATTTTTGTAATAAAGGTCCTAAATTTTAATATTCTGATGATGAGACTGTAATTAGA  208
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  177  TGATATTTTTGTAATAAAGGTCCTAAATTTTAATATTCTGATGATGAGACTGTAATTAGA  236

Query  209  TCAAGTATAGTGATAAAGGCCACSATTGTTCAAATGAAAATTTTGCATTTAACGTTTGKG  268
            ||||||||||||||||||| |||||||||||||||||||||||||||||||||||||| |
Sbjct  237  TCAAGTATAGTGATAAAGGCCACGATTGTTCAAATGAAAATTTTGCATTTAACGTTTGTG  296

Query  269  KAGAATTATAAAATAATCTCAGTTTTCTTTATACAATTTTCAGGATGCTAACGTAAATGA  328
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  297  TAGAATTATAAAATAATCTCAGTTTTCTTTATACAATTTTCAGGATGCTAACGTAAATGA  356

Query  329  AAAAATAATAATAAGGaaaaaaaTGTAAAGGCCACGACTGCtttttttttttttttttt  388
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357  AAAAATAATAATAAGGAAAAAAATGTAAAGGCCACGACTGCTTTTTTTTTTTTTTTTTTT  416

Query  389  kktwaaaattt  399
             | ||||||
Sbjct  417  GCTTAAAATTT  427
```

Fig 6. 2:AtNOGC1 promoter Sequence alignment between public available sequence from TAIR and isolated sequence from *Arabidopsis thaliana*. The alignment was done using nucleotide BLAST.

Table 6.1: Calculated fold change and _p_-values from gene expression cp values

250mm NaCl

Hours	Foldchange(Log2)	P-Value
3	Roots= 1.82	Roots= 0.0054
	Shoots= 0.43	Shoots= 0.0106
12	Roots= 0.05	Roots= 0.0001
	Shoots= 0.2	Shoots= 0.0010
24	Roots= 0.67	Roots= 0.0093
	Shoots= 1.3	Shoots= no significance

ABA

Hours	Foldchange(Log2)	P-Value
3	Roots= 6.4	Roots= 0.0001
	Shoots= 3.6	Shoots= 0.0001
12	Roots= 1.22	Roots= 0.7352
	Shoots= 1.6	Shoots= 0.0269
24	Roots= 0.4	Roots= 0.0001
	Shoots= 0.06	Shoots= 0.0005

300 mM Mannitol

Hours	Foldchange(Log2)	P-Value
3	Roots= 0.4	Roots= 0.0694
	Shoots= 1.92	Shoots= 0.0934
12	Roots= 1.6	Roots= 0.2270
	Shoots= 0.2	Shoots= 0.1295
24	Roots= 1.94	Roots= 0.0808
	Shoots= 0.7	Shoots= 0.3369

MeJa

Hours	Foldchange(Log2)	P-Value
3	Roots= 0.6	Roots= 0.0004
	Shoots= 1.2	Shoots= 0.5794
12	Roots= 1.0	Roots= 0.9134
	Shoots= 1.85	Shoots= 0.1421
24	Roots= 1.13	Roots= 0.0717
	Shoots= 0.11	Shoots= 0.0003

SA

Hours	Foldchange(Log2)	P-Value
3	Roots= 7.12	Roots= 0.0002
	Shoots= 6.41	Shoots= 0.0001
12	Roots= 0.6	Roots= 0.0001
	Shoots= 0.04	Shoots= 0.0004
24	Roots= 4.65	Roots= 0.0194
	Shoots= 2.125	Shoots= 0.0009

MeJa+ABA

Hours	Foldchange(Log2)	P-Value
3	Roots= 1.401	Roots= 0.4821
	Shoots= 0.43	Shoots= 0.0031
12	Roots= 0.95	Roots= 0.9866
	Shoots= 16.80	Shoots= 0.0003
24	Roots= 14	Roots= 0.0475
	Shoots= 1.6s	Shoots= 0.2980

SA+ABA

Hours	Foldchange(Log2)	P-Value
3	Roots= 2	Roots= 0.0004
	Shoots= 1.4	Shoots= 0.5484
12	Roots= 3.6	Roots= 0.0001
	Shoots= 3.2	Shoots= 0.0502
24	Roots= 1.3	Roots= 0.3632
	Shoots= 1.07	Shoots= 0.8804

NO

Foldchange(Log2)	P-Value
Roots= 8.87	Roots= 0.0003
Shoots= 5.20	Shoots= 0.2428

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