EFFECTS OF NITRIC OXIDE ON NOVEL SOYBEAN CYSTATIN GENE EXPRESSION UNDER SALT STRESS IN SOYBEAN

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DECLARATION

I declare that "*The effects of nitric oxide on a novel soybean cystatin gene expression under salt stress in soybean*" is my original own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full Name: Nasiphi Loyola Silulwane **Date…15 November 2012...**

Signed … 

KEYWORDS

Nitric oxide

Salt stress

Plant cystatins

Caspase-like activity

Reactive oxygen species (ROS)

Oxidative damage

Programmed cell death (PCD)

Soybean

Transcription factors

Gene expression

ABSTRACT

Nitric oxide (NO) has been shown to orchestrate multiple defense responses to both abiotic and biotic stress. Importantly, elevation of nitric oxide content in plants by using nitric oxide-generating compounds has been shown to enhance plant tolerance to abiotic stresses such as salt and drought via up-regulation of genes involved in the regulation of plant responses to abiotic stress. In this study, the effect(s) of nitric oxide (generated from 10 μM of the nitric oxide donor DET/NO) on the expression of a novel soybean cystatin gene (Glyma20g08800), lipid peroxidation, caspase-like activity and cell death in salt (150 mM)-stressed soybean leaves, roots and nodules were investigated. Salt treatment resulted in elevated lipid peroxidation, caspase-like activity and increased cell death in organs studied while the observed detrimental effects of salt stress were reversed by NO treatment. Salt stress suppressed the expression of Glyma20g08800 while the levels of expression of Glyma20g08800 returned towards those of unstressed plants when the salt-stressed plants were supplemented with nitric oxide (DETA/NO). Furthermore, promoter sequences of GmCYS1p626 and three of its homologues (Glyma20g08800, Glyma14g04250 and Glyma18g12240) were analyzed for putative abiotic stress and/NO *cis*-regulatory elements based on co-expression analyses using bioinformatics. Several abiotic stress-induced transcription factors (TFs) were identified and were hypothesized to be co-acting either directly or indirectly through additional factors in the regulation of soybean cystatin expression in response to NO and abiotic stress. Taken together, these results highlight the possibility of using NO to drive high levels of expression of cystatins during salt stress and lead to accumulation of the cystatin to levels that are sufficient to inhibit salt stress-induced caspase-like

activity, which will inhibit salt stress-induced cell death and thus enhance the tolerance of the plant to salt stress and possibly tolerance to drought stress as well.

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Contents

ABBREVIATIONS

ABA	abscisic acid
ABRE	ABA-responsive element
APX	ascorbate peroxidase
AtCYS1	*Arabidopsis thaliana* cystatin 1
ATNOA1	*Arabidopsis thaliana* Nitric oxide Associated 1
AtNOS1	*Arabidopsis thaliana* Nitric oxide synthase 1
ATHENA	*Arabidopsis thaliana* expression network analyses
CAT	catalase
cGMP	cyclic guanosine monophosphate
CYPs	cysteine proteases
cADPR	cyclic ADP-ribose
DETA	diethylenetriamine
DETA/NO	2,2' –(hydroxynitrosohydrazone) bisethanimine/ diethylenetriamine
DRE	dehydration-responsive element
GPX	gluatathione peroxidase
GSH	glutathione
MYB	myeloblastosis oncogene
NO	nitric oxide
NOS	nitric oxide synthase
NR	nitrate reductase
PCD	programmed cell death
RNA	ribonucleic acid
ROS	reactive oxygen species

SGC	soluble guanine cyclase
SNP	sodium nitroprusside
SOD	superoxide dismutase
STRING	search tool for the retrieval of interacting genes/proteins
TAIR	the Arabidopsis information resource
TBA	2-thiobarbituric acid
TFs	transcription factors
TFBM	transcription factor binding motif
μM	micromolar
mM	millimolar

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Chapter 1

Literature review

1. Introduction

Abiotic stresses, including soil salinity, are serious threats to the sustainability of crop yields and will become even more prevalent in the coming decades due to the effects of global climate change (Ashmore et al., 2006; Ortiz et al., 2008; Battisti and Naylor, 2009; Feng and Kobayashi, 2009; Fuhrer, 2009; Wassmann et al., 2009a). Developing countries with high population growth, such as Asia and Africa, are at high risk of food shortage due to the effects of salinity (Lobell et al., 2008; Wassmann et al., 2009a).

Exposure to salinity (salt stress) leads to a series of physiological changes such as changes in the photosynthetic gas exchange and assimilate translocation (Martin and Ruiztorres, 1992; Morgan et al., 2004), altered water uptake and evapotranspiration (Rivelli et al., 2002), nutrient uptake and translocation (Huh and Schmidhalter, 2005; Sanchez-Rodriguez et al., 2010), antioxidant reactions (Blokhina et al., 2003; Apel and Hirt, 2004), programmed cell death (Kangasjärvi et al., 2005), altered gene expression and altered enzyme activity (Yamakawa et al., 2007; Guo et al., 2009; Frei et al., 2010b). These changes affect the chemical composition of crops and reduce the quality of agricultural products. Generally, a high concentration of salt hampers water and nutrient uptake (Mahajan and Tuteja, 2005), resulting in inhibited growth and even cell death (Tuteja, 2007). Thus, it is of essence to uncover ways to improve crop tolerance in order to improve crop yield under these stressful conditions.

Agricultural yield losses due to salinity have been well documented and the awareness of this growing impact has led to the research of salt stress with the aim of improving crop tolerance by genetic engineering, identification of novel genes and determination of their pattern of expression in response to abiotic stresses (Kawasaki et al., 2001; Lobell et al., 2008; Ortiz et al., 2008; Wassmann et al., 2009b). Plants respond to soil salinity mostly by manipulating genes which protect and maintain function and structure of cellular components. However, the genetically complex responses to salt stress conditions are more difficult to control and engineer. Proposed strategies to face these challenges include the use of modern molecular biology tools for engineering plants which are tolerant to salt stress, based on the expression of specific stress-related genes (Gill and Tuteja, 2010).

Because salt stress generally induces programmed cell death (PCD) in plants and PCD execution requires changes in gene expression (Desikan et al., 1998), it is important to identify major genes responsible for the execution of cell death and their inhibitors so as to maintain the capacity to regulate this process. Plant cysteinee proteinases have been established as the key regulators of PCD. Cysteine proteases are the most abundant group of endo-proteases which serve important roles in plant physiological processes including growth and development (Grudkowska and Zagdanska, 2004). They are highly induced when plants are exposed to abiotic stress and play essential roles in degradation of proteins denatured by physiological stress and in the activation of specific proteins (Stroeher et al., 1997). Cysteine proteinases respond dramatically to abiotic stresses and their induction in salt stress may be a result of oxidative stress (Bray, 2002). On the other hand, the activity of cysteine proteinases can be inhibited by specific protease inhibitor proteins known as cystatins, which are also induced in response to stress (Solomon et al., 1999).

Plant cystatins modulate the activity of cysteine proteases by interfering with the active site of the target enzyme.

Several studies support the hypothesis that plants employ cystatins to control cysteine protease activity and modulate the cell death processes (Barrett et al., 1998; Watanabe et al., 1991). The overexpression of a cystatin in soybean has been established to block cell death triggered by avirulent pathogens or oxidative stress (Solomon et al., 1999). Moreover, overexpression of AtCYS1 in Arabidopsis cell cultures was shown to suppress cell death induced by avirulent pathogens or oxidative stress and nitrosative stresses (Belengni et al., 2003). These studies support the hypothesis that overexpression of cystatins may inhibit abiotic stress-induced cysteine protease activity and prevent induction of cell death in plants. Thus, cystatin expression may provide efficient mechanisms for improving plant resistance to salt stress.

Scant evidence supporting the notion of overexpression of cystatins to improve salt and drought tolerance in plants exists (Zhang et al 2008) but this has not yet been achieved in crop plants. Although there has been slow progress in introducing salt stress tolerance genes into crop plants, there are a number of reasons for optimism. The use of transgenes to improve the crop tolerance remains an attractive option. It is imperative to address how the tolerance to specific abiotic stresses is evaluated and whether the accomplished tolerance would have any undesired effects on plant development.

Alternatively, nitric oxide (a signaling molecule involved in diverse physiological processes in plants) has been implicated to be involved in plant responses to multiple abiotic and biotic stresses. A role for endogenously synthesized nitric oxide in plant cell death regulation has been

demonstrated by Leach et al (2010) and linked to cysteine protease activity in soybean nodules. Recently, Keyster et al (2011, personal communication, unpublished) discovered that exogenous application of nitric oxide induced the expression of a soybean cystatin GmCYS1P626 (homologous to AtCYS1), which inhibited cysteine protease-like activity in soybean root nodules. This suggests that the use of nitric oxide-generating compounds can enhance plant tolerance to abiotic stresses such as salt and drought via up-regulation of genes involved in the regulation of plant responses to salt and drought stress (Siddiqui et al 2010).

1.2 Nitric oxide in plants

Nitric oxide (NO) is a widespread intracellular and intercellular signaling molecule with diverse physiological functions in plants. Research on the role of NO in plants has indicated that NO participates in various cell processes such as plant growth and development (Gouvea et al., 1997; Leshem et al., 1998), senescence, respiratory metabolism, maturation as well as regulation of plant responses to abiotic and biotic stresses (Durner et al., 1999; Garcia-Mata and Lamattina, 2001; Zottini et al., 2002; Prado et al., 2004; Besson-Bard et la., 2008). In mammals, NO participates in a broad range of functions in neural communication, immune regulation and apoptosis (Schmidt and Walter, 1994). On the other hand, NO has also been implicated in a wide range of human pathologies such as heart disease, tumors and diabetes among others (Corpas et al., 2009).

Several recent reviews on the roles of NO in plants (Meyer et al., 2002; Lamattina et al., 2003; Crawford et al., 2005) indicate that NO may be an important second messenger but it still remains unclear how NO is synthesized in various situations, how its concentrations are regulated and

where exactly NO exerts its effects in various signaling processes (Planchet et al., 2005). Exogenous application of NO to plant cells has provided valuable information on the role of this molecule in the regulation of catalase and ascorbate peroxidase activity (Navarre et al., 2000; del Rio et al., 2004) and wound signaling (Orozco-Cardenas and Ryan, 2002) and cell death (Saviani et al., 2002).

1.2.1 Potential sources of Nitric oxide

Sources of NO in plants have been the subject of much debate. To date, several potential sources of NO have been distinguished, with the physiological role of each source depending on the plant species, type of cells or tissues, external conditions as well as the potential activation of the signaling pathway within the plant (Cueto et al., 1996; Ninnemann and Maier, 1996; Tun et al., 2006; Corpas et al., 2009). In plants, NO is primarily generated by enzymatic or non enzymatic mechanisms (Neil et al., 2003; Wilson et al., 2008; Corpas et al., 2009); the L-arginine and nitrite pathways being the two most distinct pathways. NO production via L-arginine-dependent NO synthase (NOS) activity has been detected in different plant species such as peas (Barasso et al., 1999), soybean (Delledonne et al. 1998), maize (Riberro et al., 1998) and many more other species and has been shown to be similar to mammalian NOS despite the fact that the gene(s) responsible for this plant NOS-like activity have not yet been identified/isolated (Cueto et al. 1996; Simontacchi et al., 2004; Corpas et al., 2009). Different methods have been used to demonstrate the existence of NOS activity in plants and these include the conversion of radiolabelled arginine into radiolebelled L-citrulline (Cueto et al., 1996; Ninnemann and Maier, 1996), measurement of NO production sensitive to NOS inhibitors by fluorometry or chemiluminiscence in crude extracts incubated with L-arginine and all NOS cofactors

(Simontacchi et al., 2004). Furthermore, it appears that NOS-like proteins in higher plants could be different structurally from animal NOS as no homologue of animal NOS has been identified so far in higher plant genomes (Besson-Bard et al., 2009).

A study by Guo et al (2004) isolated a gene (*AtNOS1*) from the *Arabidopsis* genome which was indicated to encode a protein associated with NO synthase activity and to be involved in regulation of growth and hormonal signaling. However, most studies questioned the nature of AtNOS1 due to impaired NOS activity and reduced endogenous NO levels in AtNOA1 mutants (Guo et al. 2003; He et al. 2004; Zeidler et al., 2004). Consequently the possibility of AtNOS1 as a NO source was refuted. However, AtNOS1 involvement in NO biosynthesis and accumulation was suggested to be either indirect or regulatory (Crawford et al. 2006; Zemojtel et al. 2006). AtNOS1was later renamed as AtNOA1 (Crawford et al. 2006).

Another NO producer in plants is nitrate reductase (NR), which uses NO_2^- and NADH as substrates (Yamasaki et al., 1999; Rockel et al., 2002). NR has been reported to produce NO and its derivative; peroxynitrite ($ONOO^-$) *in vitro* and *in vivo*. NR activity is inhibited by sodium azide (a NR inhibitor) (Yamasaki and Sakihama, 2000). Production of NO from NR was reported in several plant species such as cucumber (Haba et al., 2001), sunflower, spinach, maize (Rockel et al., 2002), wheat, orchid, aloe (Xu and Zhao, 2003), tobacco (Rockel et al., 2002), and Arabidopsis (Desikan et al., 2002). However, available information on the generation of NO derived from NR activity came from NR mutants impaired in NO production and there is inadequate information on the direct production of NO in plant stress (Modolo et al., 2005; Corpas et al., 2009). In addition, an enzyme, (NOR), identified only in tobacco roots and localized in the plasma membrane, has been reported to be another endogenous source of NO in

plants (Stohr et al., 2001). Other enzymatic sources of NO shown to generate NO in mammalian systems which are present in plants may also be considered as NO sources in plants and these include cytochrome P450, xanthine oxidoreductase and other hemoproteins (del Rio et al., 2004).

On the other hand, NO may also be formed non-enzymatically in a reaction between nitrogen oxides and plant metabolites or by a chemical reduction of nitrite (NO_2^-) at acidic pH values (Wendehenne et al., 2001; Lamotte et al., 2006). Non–enzymatic reduction of nitrite to NO and nitrate has been demonstrated to occur under specific pH conditions in the apoplast of barley aleurone cells when incubated with additional nitrite (Bethke et al., 2004). Wojtaszek (2000) and del Rio et al. (2004) also reported that carotenoids and light were capable of catalyzing the conversion of NO_2^- to NO. Furthermore, nitrification and denitrification reactions also release NO to the atmosphere and thus may also be considered as alternative sources of NO in plants (Wojtaszek, 2000). Tun et al. (2006) also documented that polyamines such as putrescine, spermidine or spermine synthesized from L-arginine can induce NO production but the mechanism underlying this activity still remains unclear.

1.2.2 Nitric oxide signaling in plants

NO has emerged as a key molecular signal involved in diverse physiological processes in plants which include growth, germination (Beligni and Lamattina, 2000), stomatal closure (Neill et al., 2002), disease resistance (Delledonne et al., 1998), modulation of cell cycle gene expression (Correa-Aragunde et al., 2006), inhibition of certain enzyme activities (Clarke et al., 2000) and responses to pathogens (Wendehenne and Klessig, 1998). In mammalian systems, the signaling pathways of NO are designed as either cGMP (cyclic guanosine monophosphate)-dependent or

independent of cGMP (Wendehenne et al., 2001; Wendehenne et al., 2004). In the cGMP-dependent pathway, NO covalently binds to the heme domain of soluble guanylate cyclase (sGC; a crucial for NO signaling in mammalian systems) and induces its enzymatic activity. Soluble guanylate cyclase activation results in increased production of the second messenger cGMP, which in turn activates cGMP targets which include cGMP-dependent protein kinases, cyclic nucleotide-gated channels and phosphodiesterases (Beck et al., 1999).

A study by Willmott et al. (1996) and Reyes-Harde et al. (1999) established a role for cGMP-dependent protein kinase signaling in intracellular Ca^{2+} mobilization through activation of ryanodine-sensitive calcium channels (RYRs), which is mediated by cyclic ADP ribose (CADPR), a Ca^{2+} second messenger. Similar mechanisms for NO-mediated signaling also appear to co-exist in plants as indicated in the figure below (**Figure 1**). This was first detected and quantified in *Zea mays* (Janistyn, 1983) by mass spectrometry and radio-immune assays. Durner et al. (1998) discovered that treatment of tobacco cell suspensions or leaves with an NO donor (GSNO) induced a transient increase in endogenous cGMP levels. Moreover, under the influence of sGC inhibitors, NO failed to induce activation of expression of the genes encoding phenylalanine ammonia (PAL) and PRL. In Arabidopsis, cGMP synthesis accompanied NO-induced cell death (Clarke et al., 2000), ABA- and NO-induced stomatal closure (Neill et al., 2002a; Bruckdofer, 2005). Also, results by Leshem and Pinchasov (2000) demonstrated the role of cGMP in plant developmental processes where treatment of plants with NO led into the inhibition of ethylene production. This supports the findings that NO may act as an endogenous regulator of plant maturation and senescence in higher plants. However, it still remains unclear

how NO induces increased levels of cGMP as no plant homologues of NO sensitive sGCs have been identified.

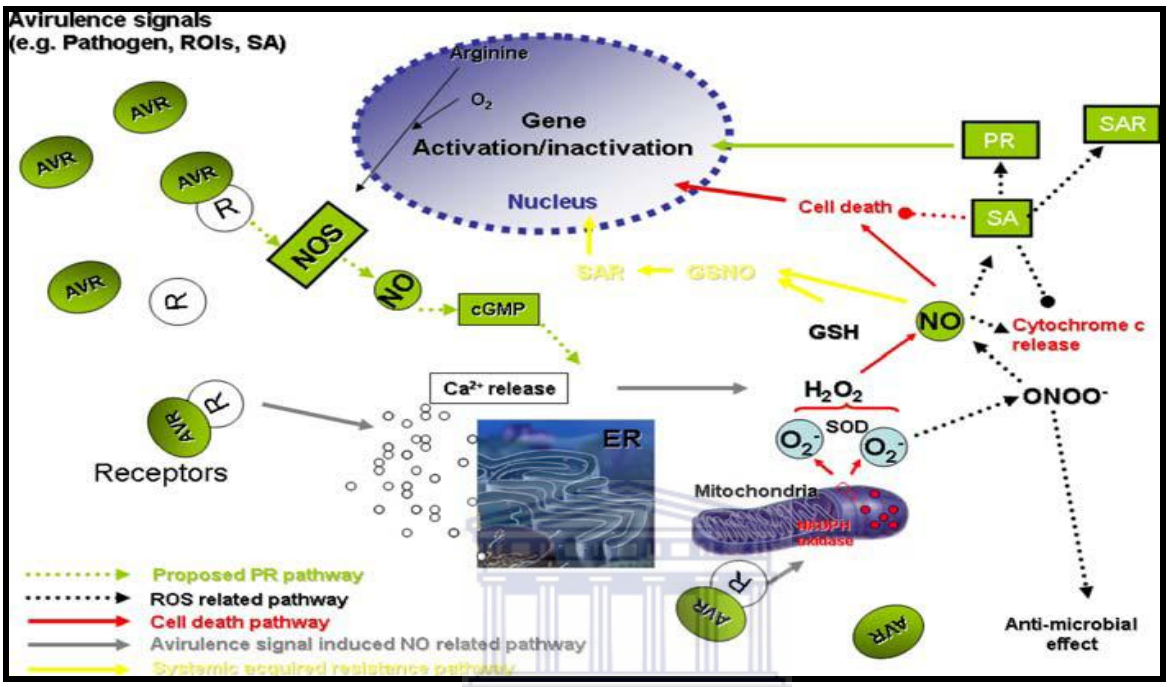

Figure 1 Different mechanisms for NO-mediated signaling in plants (Palavan-Unsal *and* Arisan, 2009).

As mentioned previously, NO may also exert its functions through Ca^{2+} mobilization and cyclic ADP-ribose (cADPR), which acts as a second messenger by stimulating Ca^{2+} release through intracellular ryanodine receptor calcium channels (RYRs). NO may also function through Ca^{2+} mobilization and this is reported in various studies. Studies by Durner et al. (1998) and Klessig et al. (2000) discovered that NO may lead to increased levels of free Ca^{2+} by acting through cGMP and cADPR in order to activate intracellular Ca^{2+}-permeable channels.

In addition to the above mentioned NO targets, NO may also exert its function through mitogen activated protein kinases (MAPKs) (Kumar and Klessig, 2000; Pagnussat et al., 2004; Wilson et al., 2008). Activation of MAPKs may be due to extracellular signals such as cold,

phytohormones, drought, pathogen attack and osmotic stress which lead to the activation of signal transduction pathways resulting in nuclear gene expression (Hirt, 1997). Treatment of Arabidopsis and tobacco leaves with a NO donor induced MAPKs (Clarke et al., 2000) and when tobacco cells were treated with NO donors (GSNO and SNAP) together with salicylic acid (SA), jasmonic acid (JA) and ethylene, SA induced protein kinases only in the presence of NO.

Various studies have also established that treatment of some plant species with NO also induces an increase in endogenous amounts of salicylic acid (SA) (Durner et al., 1998; Huang et al., 2004; Wendehenne et al., 2004). However, it remains uncertain whether the induction of MAPKs by NO occurs either directly or through other messengers (Lamotte et al., 2005). All these studies provide strong evidence that NO can direct several cellular processes either by co-operating directly with other signals or can co-operate with other signaling pathways.

1.2.3 NO in plant growth and development

NO is a compound with hormone-like characteristics functioning in growth and development (Leshem and Haramaty, 1996; Beligni et al., 1997; Beligni and Lamattina, 1999a; Delledonne et al., 2002). NO plays a significant role in leaf expansion, seed germination, de-etiolation, hypocotyl growth and internode growth (Beligni and Lamattina, 2000). However, NO-induced effects on plant growth have been shown to be concentration-dependant. Hufton et al (1996) and Leshem et al. (1997) established that high concentrations of NO (40-80 ppm) inhibited the growth of tomato, lettuce and pea plants, while low concentrations (10-20 ppm) enhanced growth. Takahashi and Yamasaki (2002) also found that NO can also suppress ATP synthesis and electron transport in chloroplasts. In addition, NO generated by NR inhibits photosynthesis

while treatment with NO donors increased chlorophyll levels in lettuce, red cabbage and Arabidopsis (Beligni and Lamattina, 2000). NO also regulates maturation and senescence processes in higher plants. Treatment of pea leaves and *Helianthus annulus* L. cotyledons with NO was established to cause a delay in senescence (Leshem et al., 1998; Selcukcan, 2005). In *N. tabaccum*, antisense modulation of NR induced nitrite accumulation, the release of NO in significant amounts and led to retarded growth (Valderrama et al., 2007; Blume et al., 2009).

Moreover, use of NO donors also inhibits hypocotyl and internode elongation in dark-grown seedlings of Arabidopsis and *Lactuca sativa* (Belinghi and Lamattina, 2002) and enhances de-etiolation and increased levels of chlorophyll content. Treatment of wild type maize with NO inhibited chlorosis that results from iron deficiency. NO also induces iron availabity and thus stimulates chloroplast development (Belinghi and Lamattina, 2002). For example; application of NO to yellow stripe mutants improved iron availability (Graziano et al., 2002). Hung and Kao (2003) also discovered that, in rice leaves, application of NO exerts a protective effect against abscisic acid (ABA)-induced senescence through inhibition of leaf senescence, enhancement of antioxidant enzyme activity, increasing ascorbic acid level and decreasing malondialdehyde content. A recent study by Leach et al (2010) reported that the development of functional soybean nodules requires NO production.

1.3 Abiotic stress

Plants are exposed to various environmental stresses such as salinity, drought, extreme temperatures, oxidative stress and heavy metals. These stresses are major limitations to agricultural production worldwide and they all induce oxidative stress. Because plants lack the

capability of locomotion, they must adapt to environmental changes in other ways. Plants are equipped with complex processes including perception, transduction and transmission of stress stimuli (Turner et al., 2002; Xiong et al., 2001; Kopyra and Gwozdz, 2003). The negative effect of abiotic stress is associated with damage produced by oxidative stress to the cell as a result of a decrease in energy dissipation (Loggini et al., 1999). Oxidative damage occurs due to imbalances between production of ROS (such as the superoxide radical, hydrogen peroxide and the hydroxyl radical) and efficiency of antioxidant defenses (Iturbe-Ormaetxe et al., 1998).

During stress, ROS production can pose a threat to cells, causing plant cell injury and even cell death (Mano, 2002). However, ROS can also act as intracellular signaling molecules to control processes such as PCD, abiotic responses, pathogen defense, development and systemic signaling (Mittler, 2002). Depending on the concentration, ROS can either be toxic or protective. For example, at low concentrations, ROS act as signals for activation of defense responses while higher amounts cause severe cellular injury. In a system where toxicity is acquired due to production of uncontrollable ROS generation, NO may limit the damage caused by ROS generation by acting as a chain breaker (Lipton et al., 1993). Thus, NO is believed to mediate the modulation of ROS and enhance antioxidant defense systems in plants under abiotic stress (Yamasaki et al., 2001; Zottini et al., 2002). NO also alleviates oxidative damage caused by abiotic stresses by eliminating the superoxide anion O_2^- and lipid radical R^- and the activation of antioxidant enzyme activities such as superoxide dismutase (SOD) (Shi et al., 2007)

1.3.1 NO and Abiotic stress

NO production has been commonly observed to be induced rapidly by different types of chemical, mechanical and environmental stresses in a various plant species and to regulate plant responses to abiotic and biotic stresses. Indications that NO regulates plant responses to stresses such as drought, extreme temperatures, salinity, heavy metals and oxidative stress have been well documented in many experiments (Garcia-Mata and Lamattina, 2001; Zhao et al., 2001; Uchida et al., 2002; Kopyra and Gwozdz, 2003; Zhao et al., 2004). NO possesses sufficient biochemical reactivity and acts as a mediator of diverse physiological functions and defense mechanisms (Beligni andLamattina, 1999a, b). Almost all abiotic stresses induce ROS generation (Neill et al., 2002b; Vranova et al., 2002; Arasimovic and Floryszak-Wieczorek, 2007) and there is an increasing list of studies demonstrating that exogenous application of NO, acting together with other signals, enhances stress tolerance in various plant systems (Wang et al., 2004; Zhang et al., 2006; Liu et al., 2005; Tanou et al., 2009; Uchida et al., 2009; Wang et al., 2009). As mentioned previously, NO may act as a chain breaker and limit the damage caused by ROS generation (Lipton et al., 1993) but the combination of NO and ROS is believed to be either toxic or protective to the plant, depending on the metabolic state of the plant and the level of NO. At lower concentrations, NO has been shown to protect plants from oxidative damage by eliminating the superoxide anion O_2^- and lipid radical R^- and activates the antioxidant enzyme activities, especially the activity of SOD. On the other hand, higher amounts of NO induce superoxide production in mitochondria by inhibiting electron flow cytochrome C oxidase (Millar and Day, 1996).

In addition, the antioxidant role of NO in abiotic stress is mainly its ability to maintain the cellular redox homeostasis and to regulate the toxicity of ROS. NO has also been recognized to participate in signaling pathways downstream of jasmonic acid synthesis and upstream of H_2O_2 synthesis and to regulate the expression of some genes involved in tolerance to abiotic stress (Orozco-Cardenas and Ryan, 2002; Wendehenne et al., 2004). There is also a synergistic effect between NO and ROS in ABA biosynthesis (Zhao et al., 2001). Exogenous application of NO in the presence of ROS induces ABA synthesis in response to water deficit (Uchida et al., 2002) and also induces drought tolerance (Garcia-Mata and Lamattina, 2001). In contrast, the presence of a NOS inhibitor and ROS scavengers inhibits ABA accumulation, indicating that NO accumulation may be necessary during ABA-induced stomatal closure (Garcia-Mata and Lamattina, 2002). Furthermore, under salt stress conditions and high temperatures, an increase in NO and NO-derived products can be observed (Leshem, 2001; Valderrama et al., 2007). This effect of NO in different plant species under varying environmental conditions reflects the antioxidant properties of NO, acting by inhibiting excessive ROS accumulation (Neill, 2002b).

1.3.2 Plant tolerance to salt stress

Soil salinity is one of the major abiotic stresses affecting plant productivity worldwide. High salt concentrations impose both ionic stress which is induced by elevated Na^+ and Cl^- concentrations and also induces osmotic stress which lowers water potential and results in loss of cell turgor. These stresses result in nutritional disorders and oxidative or nitrosative stress (Molassiotis et al., 2010; Zhu, 2001). Soil salinity negatively affects the activities of some enzymes involved in nitrate and sulfate assimilation pathway and also leads to decreased energy levels (Siddiqui et al., 2009b). Moreover, most of the damage caused by salt stress is associated with oxidative damage

due to ROS accumulation, which destructs the antioxidant system, thus causing denaturation of functional and structural proteins (Smirnoff, 1998). Plants adapt to salinity stress conditions and exercise specific tolerance mechanisms mostly based on manipulating genes which protect and maintain function and structure of cellular components. The nature of the genetically complex mechanisms of abiotic stress tolerance and the potential detrimental side effects make it more difficult to improve plant salt stress tolerance (Wang et al., 2003).

Plants either become dormant during salt stress or adjust their cellular metabolism to tolerate the salt episode (Yokoi et al., 2002). Experimental evidence shows that NO plays systemic signaling roles which generate defense responses following salt stress in many plant species. NO has been shown to significantly alleviate the oxidative damage caused by salinity (Uchida et al., 2002; Kopyra et al., 2003; Li et al., 2005; Wang et al., 2009). Exogenous application of a NO donor (SNP) protected rice seedlings from oxidative damage (Uchida et al., 2002), enhanced seed germination and growth of lupin (Kopyra and Gwozdz, 2003), cucumber (Fan et al., 2007; Yu-qing et al., 2007) and promoted the dry weight of maize (Zhang et al., 2006b) under salt stress. In addition, treatment of salt-stressed plants with NO resulted in a better balance between carbon and nitrogen metabolism by causing an increase in total soluble protein and by promoting endopeptidase and carboxypeptidase activities (Zhang et al., 2010). An Arabidopsis mutant, *Atnoa1*, with impaired *in vivo* NO synthase (NOS) activity and reduced NO levels exhibits hypersensitivity to salt stress in comparison to wild type plants (Guo et al.,2003; Zhao et al., 2007b). Pretreatment of *Atnoa1* plants with an NO donor (SNP) lessened the oxidative damage of salinity on the mutant (Zhao et al., 2007a), suggesting that NO can mediate plant responses to salinity. NO has also been reported to serve as a signal which induces salt tolerance by

improving the Na^+/K^+ ratio. This process is dependent on H_2O_2-driven increase in plasma membrane H^+-ATPase activities (Zhao et al., 2004; Zhang et al., 2006; Wang et al., 2009). Oxidative damage caused by salinity is accompanied by NO accumulation as a defense response but also causes an increase in the levels of other reactive nitrogen species (RNS), leading to nitrosative stress (Corpas et al., 2007; Valderrama et al., 2007).

A body of evidence shows that exposure of salt-stressed plants to NO may alter their physiology and metabolism, suggesting that NO-derived nitrosative stress events may be involved in salt priming (Kopyra et al., 2003; Li et al., 2005; Tanou et al., 2009). These studies reveal that prior exposure to NO may act as priming agent capable of rendering plants more tolerant to subsequent exposure to salinity (Molassiotis et al., 2010). Even though NO has been shown to elicit ion homeostasis and antioxidant-related defenses under salinity, there is limited knowledge on how NO signaling induces whole-plant salt tolerance. Furthermore, a role of NO has been established in plant cell death regulation and linked to cysteine protease activity (Belinghi et al., 2006; Leach e al., 2010). The use of NO-generating systems has been shown to induce the expression of a number of genes in diverse plant species (Besson-Bard et al 2009), including the expression of a soybean cystatin (Keyster et al 2011, personal communication, unpublished) thus enhancing plant tolerance to abiotic stresses (Siddiqui et al 2010).

1.4 Plant proteases

Proteases are enzymes which perform proteolysis of the peptide bonds between amino acid chains which make up protein molecules. They contain an autoinhibitory prodomain which, when removed, leads to enzyme activation (Bryan, 2002). Most of their activity depends on pH

(indicative of the compartment where they localize) and on the presence of endogenous protease inhibitors or activators (Beynon and Bond, 2000). Proteases are involved in almost every aspect of a plant's life cycle, required for a broad range of genetically programmed and inducible processes, in addition to their roles in nutrient stress, biotic/abiotic stress responses and nutrient mobilization.

Proteases are categorized into two groups of enzymes (exopeptidases and endopeptidases) according to the point at which they break the peptide chain. Exopeptidases cleave peptides bonds on the termini of peptide chains and their classification is done according to their substrate specificity as aminopeptidases (acting at a free N-terminus) and carboxypeptidases that degrade peptides at the C-terminus (Barret, 1994). Endopeptidases act on the interior of peptide chains and their classification is based on the kind of active site residue (cysteine, serine-, aspartic- and metallotypes) (Barret, 1986). Over 550 proteinase sequences which represent all five catalytic types: serine, cysteine, aspartic acid, metallo and threonine (MEROPS, peptidase database, http://merops.sanger.ac.uk/) have been estimated in the *Arabidopsis thaliana* genome (Beers et al., 2004). The Serine proteases comprise the largest class with approximately 200 members while the Cys, aspartic, and metallo protease classes each contain about 100 members (Van der Hoorn and Jones, 2004). Beers et al. (2004) showed that serine, cysteine and aspartic proteinases are required in plant growth and developmental events such as stomatal distribution, embryo development and disease resistance.

1.4.1 Cysteine proteases

Cysteine proteases are a large class of endopeptidases which depend on the free thiol group of a cysteine residue for their activity. Cysteine proteases have been detected in various plant species where they play essential roles in diverse physiological processes such as the development and ripening of fruits (Brady, 1985), proenzyme activation and degradation of defective proteins (Rudenskaya et al., 1998), degradation of storage proteins in germinating seeds (Callis, 1995), control of developmental and pathogen-activated programmed cell death (Lam et al., 1999), stress conditions and response to pathogens (Grudkowska and Zagdan`ska, 2004). Cysteine proteases have been grouped into families and clans on the basis of structural and evolutionary relationship (Rawlings and Barret, 1993). A clan comprises a group of families of sequence identities and similarities (Barret and Rawlings, 2001). The largest clan of cysteine proteases is clan CA, with the papain-like family (C1) being the most studied among all other cysteine proteases (Gruddkowska and Zagdan`ska, 2004). Most plant cysteine proteases with elucidated crystal structures belong to the papain family (Kamphuis et al., 1984; Baker, 1980; Pickersgill et al., 1991; Choi et al., 1999) and usually exhibit acid or neutral pH optima. Papain-type proteinases are synthesized as less active precursors which comprise 38-250 amino acid pro-sequence, an N-terminal signal sequence and a 220-260 amino acid mature enzyme (Grudkowska and Zagdanska, 2004). Their activation occurs by limited intra-or intermolecular proteolysis (Wiederanders, 2003). Most commonly known plant cysteine proteases belong either to the papin (C1) or legumain (C13) families.

The legumains (C13), commonly known as vascular processing enzymes, belong to the asparaginyl-specific subclass of the cysteine endopeptidase family that cleave peptide bonds with Asn

or Asp (less efficiently) in the P1' positions at the C-terminal flank (Becker et al., 1995). Plant legumains are only active at acidic pH and function not only to precede protein processing but also in protein breakdown in the cell wall and vacuole (Muntz et al., 2002). In recent years, several members of cysteine proteases have been identified in plants and these include caspases (family C14) which require an Asp residue adjacent to the cleavage site and a recognition sequence of at least four amino acids N-terminal to the cleavage site (Woltering et al., 2002). Their activity is blocked by specific caspase inhibitors but are resistant to typical cysteine proteinase inhibitors (del Pozo and Lam, 1998; Lam and de pozo, 2000). Other cysteine proteases include the calpains, the calcium-dependent proteinases which require micro- or millimolar concentrations of Ca^{2+} for activity with a highly conserved molecular structure in the catalytic site (family C2), the ubiquitin C-terminal hydrolases (family C12) and ubiquitin-specific proteinases (C19) (Viestra, 2003).

1.4.2 Biological roles of cysteine proteases

Cysteine proteases play essential roles in almost every aspect of plant physiology and development. They are involved in plant growth, senescence and programmed cell death (PCD) and accumulation/remobilization of storage proteins. Furthermore, they participate in signaling pathways as well as in the response to biotic and abiotic stress (Grudkowska and Zagdan`ska, 2004). In most cereals, they account for over 90% of the total degradation activity of prolamins (the main storage proteins of cereals in germination of maize) (de Barros and Larkins, 1994) and wheat (Bottari et al., 1996). In certain germinating dicot seeds, legumain-like (VsPB2 and proteinase B) and papain-like (SH-EP, CPR1,CPR2, CPR4, proteinase A) proteinases were

established to be involved in remobilization of storage proteins (Okamoto and Minamikawa, 1998; Tiederman et al., 2001).

During proteolysis (an irreversible process of polypeptide cleavage with essential physiological roles in several cellular processes, important in confining the cleavage of peptides in space and time) cysteine proteases have been identified to be the major executors of protein degradation in senescing leaves (Guo et al., 2004). Guo et al. (2004) estimated a total of 116 genes to be involved in leaf senescence. According to digital northern estimates from ESTs, 75 of these genes account for almost 38% protein degradation and are associated with the ubiquitin proteolysis pathway while 35 genes are proteinases of which cysteine proteinases account for 57% of total proteolysis. The remaining 5-6% is accounted for by serine, aspartic and other peptidases (Guo et al., 2004). In addition, their study identified eight cysteine proteinase genes to be involved in senescence, of which four proteinases :SAG12 (At5g45480), AALP (At5g60360), Cathepsin B-like proteinase (At4g01610) and cysteine proteinase-like protein (At4g16190) had the most abundant ESTs of 136, 42, 22 and 15 respectively.

Cysteine proteases have also emerged as key enzymes in the regulation of PCD in animal cells (Martin and Green, 1995). However, recent evidence implicates cysteine proteases in the induction of plant PCD (Minami and Fukuda, 1995; Ye and Vamer, 1996). Kono et al (2004) and Maza et al (1999) indicated the involvement of cysteine proteinases in oxidative stress-induced soybean PCD. Cysteine proteinase gene mutant such as *ced-3* prevent normal cell death in nematodes while overexpression of specific cysteine proteinase genes leads to cell death in many cells (Cohen, 1997). The induction of cysteine proteinases was identified in plant systems during xylogenesis in Zinnia (Minami and Fukuda, 1995) during leaf and flower senescence and after

levels of cytokines dropped (Journaire et al., 1996). PCD in the endosperm of germinating castor bean seeds (*Ricinus cummunis*) is associated with a KDEL-tailed 45 kDa papain-like pro-peptidase (CYsEP) accumulation in endoplasmic reticulum-derived structures called ricinosomes. The mature 35 kDa form of CYsEP is released from ricinosomes during cell collapse after mobilization of storage proteins to the developing cotyledons has occurred (Schmid et al., 1999). In addition, homologous KDEL-tailed proteinases have been identified in several senescing tissues such as in white spruce (*Picea glauca*), megagametophyte seeds (He and Kermode, 2003), withering daylily petals and drying seed coats (Gielt andSchmid, 2001). This suggests that a similar mechanism can also occur in other plant species and organs. A papain-like cysteine proteinase (BnCYsP1) in *Brassica napus* is also associated with PCD of the inner integument of the seed coat during early stages of seed development (Wan et al., 2002).

Another form of PCD which occurs during xylogenesis, anther senescence and ovule development has also been associated with induction of a brinjal (*Solanum melongena*) cysteine proteinase SmCP (Xu and Chye, 1999). A study by Hatsugai et al (2004) discovered that a tobacco VPEs (Vascular processing enzyme) sharing several enzymatic properties with caspases is induced during hypersensitive (HR) cell death caused by tobacco mosaic virus. VPEs have also been reported to trigger vacuolar collapse which leads in PCD. During abiotic and biotic stress, papain-like cysteine proteinases and their inhibitors have been shown to be the modulators of PCD (Solomon et al., 1999). Oxidative stress, wounding, pathogen attack, salt stress and drought have all been shown to induce the expression of a set of cysteine proteinases which can be inhibited by ectopic expression of endogenous cysteine proteinase inhibitor genes (Solomon et al., 1999; Belenghi et al., 2003). Levine et al (1996) showed that the cell death process in

cultured soybean cells mediated by H_2O_2 can be blocked by synthetic protease inhibitors such as AEBST and leupeptin. This indicates that plants can control PCD by inducing specific inhibitor genes which regulate the activity of cysteine proteases.

1.5 Plant proteinase inhibitors

Proteinase inhibitors are natural, defense-related proteins present in various plant organs. They have been long known in plants since the work of Kunitz (1945) in a trypsin inhibitor from soybean seeds was isolated and crystallized. However, their function is presently the subject of interest. Proteinase inhibitors have been classified into several families based on extensive homology, topological relationships of disulfide bridges and the localization of the active site (Laskowski and Kato, 1980). Recent studies strongly implicate proteinase inhibitors in the defense mechanisms that plants have developed against pests and pathogens (Ryan, 1991).

Inhibitors of cysteine proteinases are widely distributed in plants and are classified and named after classes of proteinases (serine, cysteine, aspartic and metallo-proteinases) that they inhibit. Cysteine proteinase inhibitors are grouped into four families based on sequence homology, molecular mass of the protein, the number and arrangement of disulfide bonds (Barret, 1987; Turk and Bode, 199; Koiwa et al., 1997). The first family, the stefins, consists of proteins with a molecular mass of about 11 kDa and is devoid of any carbohydrate groups or disulfide bonds (Machleidt et al., 1983; Stato et al., 1990). Members of the second family, the cystatins, have a molecular mass of 13.4-14.4 kDa and about 120-126 amino acids. This family also contains four conserved cysteine residues forming two disulfide bonds (Grzonka et al., 2001). The kininogen family consists of larger glycoproteins of 60-120 kDa. Family four, the phytocystatins, includes

almost all cysteine proteinase inhibitors (PIs described in plants). Phytocystatins are similar to stefins and cystatins, but lack free cysteine residues (Fernandes et al., 1993; Zhao et al., 1996). The expression of these genes is usually limited to specific organs or to particular phases during plant growth, germination (Botella et al., 1996), early leaf senescence (Huang et al., 2001), drought (Waldron et al., 1993), wounding (Botella et al., 1996) and salt stress (Van der Vyver et al., 2003; Pernas et al., 2000). PIs are implicated in plant defense against insects (Botella et al., 1996; Zhao et al., 1996).

1.51 Plant cystatins (phytocystatins)

Cysteine (Cys) proteinase inhibitors are widely distributed in plants, animals and micro-organisms (Rawlings et al., 2008). Plant cystatins have been characterized in both monocots and dicots, including maize (Massonneau et al., 2005), rice (Abe et al., 1987; Kondo et al., 1990), cow pea (Diop et al., 2004), barley (Gaddour et al., 2001), potato (Waldron et al., 1993), tomato (Bolter, 1993) and *Arabidopsis thaliana* (Belenghi et al., 2003). These ubiquitous inhibitors of family C1A (papain-like) Cys proteases habour a Gln-Val-X-Gly motif in the central region of the polypeptide chain, a Pro-Trp (or Leu-Trp) dipeptide motif in the C-terminal region and a conserved Gly residue in the N-terminal region (Barret et al., 1986; Turk and Bode, 1991). Plant cystatins form tight, equimolar complexes with Cys proteases, acting as pseudo-substrates that block access to protein substrates. The cystatin inhibitory mechanism involves a wedge formed by three structural elements (shown in **Figure 2**) which slots into the active site of the target enzyme (Bode et al., 1988; Machleidt et al., 1989; Stubbs et al., 1990).

The first structural element interacts directly with the active site of the target enzyme and consists of a surface hairpin loop which bears the conserved Gln-X-Val-X-Gly motif. The second element consists of a surface hairpin loop with the conserved Pro (Leu)-Trp motif in the C-terminal region and also interacts with the target enzyme active site. The third element consists of a conserved Gly residue in the N-terminal region and does not interact with the active site of the target enzyme but is essential for the binding process and the specificity of the cystatin towards Cys proteases (Machleidt et al., 1989; Turk and Bode, 1991). The functional significance of these three structural elements was first characterized and confirmed in oryzacystatin and soybean cystatin N.

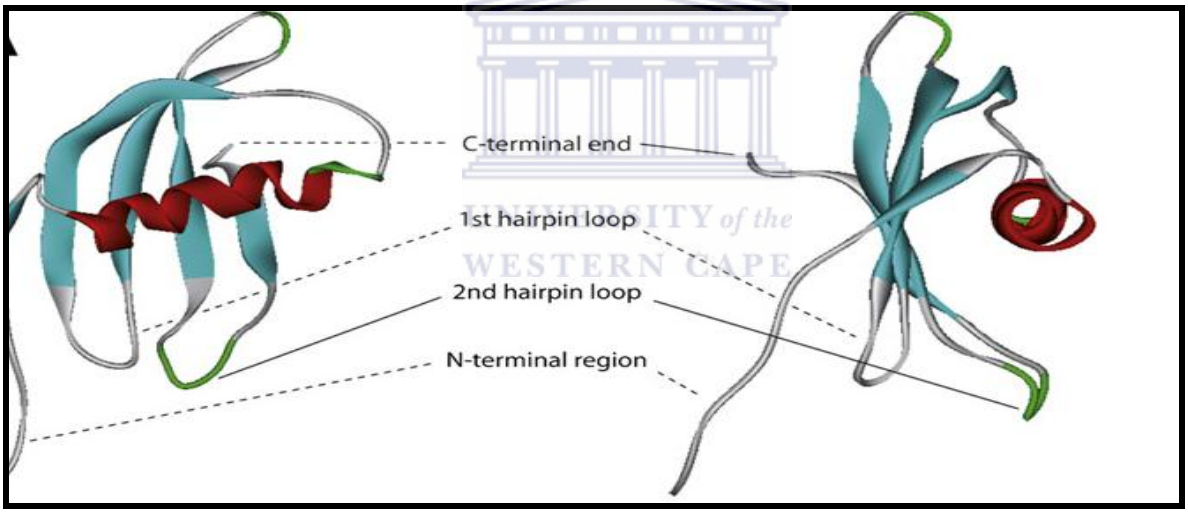

Figure 2 Structural model for the first well characterized plant cystatin, oryzacystatin (Benchabane et al., 2010).

1.5.2 Biological roles of plant cystatins

Several roles have been attributed to cystatins, ranging from the regulation of various endogenous proteolytic processes to the inhibition of exogenous cysteine proteases (Arai and Abe, 2000; Arai et al., 2002). The regulatory roles for inhibitory cystatins have been reported in a variety of physiological processes involving Cys proteases, including the deposition and mobilization of proteins in storage and senescent organs (Benchabane et al., 2010). A relationship between storage protein deposition, cystatin biosynthesis and the down-regulation of Cys proteases in storage organs was established in gene expression studies. The accumulation of cystatin mRNA transcripts in developing seeds of rice, two weeks after flowering and one week before glutelin deposition, also confirmed this correlation. Weeda et al. (2009) also established a connection between high patatin content, a large number of multicystatin transcripts and a low Cys protease activity in protein extracts of potato tubers. Several studies, over the years, suggested multiple complementary roles for different cystatins and Cys protease variants of cereal seeds (Hwang et al., 2009; Martinez et al., 2009).

The cystatin/Cys protease stoichiometric balance has been implicated as a key determinant for the fate of storage proteins in reproductive organs of plants. Deposition of storage proteins occurs after inhibitory cystatins (synthesized in developing seeds or vegetative storage organs) outnumber Cys proteases (positive cystatin/Cys protease balance) (Benchabane et al., 2010). Storage proteins are maintained over dormancy and made available to growing seedlings with an excess of cystatins (high cystatin/Cys protease balance). However, an excess of Cys proteases allows for storage protein processing and mobilization during germination. A low cystatin/Cys protease balance during germination was also reported in transgenic Arabidopsis lines unable to

synthesize a seed endogenous cystatin (AtCYSb) naturally responsive to gibberellins and abscisic acid (Hwang et al., 2009). In addition, seeds expressing AtCYSb under a viral constitutive promoter germinated later as compared to wild type seeds. However, the exact mechanism by which the cystatin/Cys protease balance is modulated in plants is not fully understood.

Plant cystatins have also been implicated to play roles in leaves and other metabolically active organs, controlling Cys proteases involved in various processes. Etienne et al. (2007) reported a down-regulation of cystatin and Ser protease inhibitor-encoding genes in senescent organs, correlated with an up-regulation of Cys and Ser protease activities in germinating seed storage organs. Leaves subjected to adverse growth conditions such as salinity, drought or low temperatures were also observed to show up-regulation of cystatin mRNA transcripts (Zhang et al., 2008), along with the detection of abscisic acid- and dehydration-responsive *cis* regulatory elements in the promoters of some abiotic stress-inducible cystatins. A protective role for cystatins has been suggested for plants grown under unfavorable conditions (Van der Vyver et al., 2003; Zhang et al., 2008), in line with the expression of stress-inducible proteins in leaves of cystatin-expressing plants.

Constitutive expression of specific plant cystatins has been reported to suppress plant PCD by inhibiting the activity of cysteine proteases (Belenghi et al., 2003). PCD triggered directly by an avirulent strain of *Pseudomonas syringae* pv *glycinea* or directly by oxidative stress was inhibited by ectopic expression of cystatin genes which inhibit cysteine protease activity (Solomon et al., 1999). Overexpression of a papain inhibitor in cell cultures of Arabidopsis blocked cell death in response to avirulent bacteria and NO. In tobacco plants, this papain

inhibitor blocked the hypersensitive response induced by avirulent bacteria (Belenghi et al., 2003; Hoorn and Jones, 2004). AtCYS1 expression after treatment with either avirulent pathogens or NO donor blocked cell death triggered by these pathogens or oxidative and nitrosative stresses in Arabidopsis cell suspensions (Belenghi et al., 2003). Synthetic protease inhibitors such as AEBSF and leupeptin can inhibit cell death triggered by H_2O_2 in cultured soybean cells (Levine et al., 1996). Plant cystatins have also been suggested to impact abiotic and biotic stress tolerance in plants (**Table 1;** Benchabane et al., 2010). Enhanced resistance against insect predation has been observed in transgenic rice expressing a maize cystatin (Irie, 1996). Transgenic tobacco and sweet potato have been shown to exhibit resistance against polyviruses (Campos et al., 1999).

Table 1 Examples of Abiotic and biotic stress-inducible cystatins in plants

Plant	Cystatin	Source	Stress treatments	References
Abiotic stress cues				
Amaranth	AhCP1	Stem, root, leaf	Drought, salinity, cold shock , heat shock	Valdes-Rodriguez et al, 2007
Arabidopsis	AtCYSa, AtCYSb	Leaf, cells	Drought, salinity, cold shock, oxidation	Valdes-Rodriguez et al, 2007
Barley	Icy	Leaf, root	Anaerobiosis, cold shock , heat shock	Zhang et al, 2008
Chestnut	CsC	Leaf, root	Salinity, cold shock, heat shock	Gaddour et al, 2001
Cowpea	VuC1	Leaf	Drought, abscisic acid	Pernas et al, 2000
Maize	CC8, CC9	Kernel	Cold shock	Pernas et al, 2000
Sea rocket	CmC	Leaf	Drought, salinity	Diop et al, 2004
Wheat	TaMDC1	Shoot, leaf, root	Cold shock, salinity, drought, abscisic acid	Massonneau et al, 2005
Biotic stress cues				
Chestnut	CsC	Leaf, root	Fungal infection, wounding	Megdiche et al, 2009
Potato	Multicystatin	Leaf	Herbivory	Christova et al, 2006
Soybean	N2, R1	Leaf	Wounding, Methal jasmonate	Pernas et al, 2000
Tomato	SICYS8 (TMC)		Recombinant prosystemin	Bouchard et al, 2003
	SICYS9	Leaf	Methyl jasmonate	Bolter, 1993
	SICYS10		Arachidonic acid	Girard et al, 2007

The expression of two cystatins (AtCYSa and AtCYSb) from Arabidopsis was induced by multiple abiotic stresses such as salt, drought, cold and regulated by abscisic acid treatment. Transgenic Arabidopsis plants overexpressing these two genes had increased tolerance to salt, drought, oxidative tolerance during seed germination and early seedling development (Zhang et al., 2008). Transgenic rice overexpressing *Oryza sativa* chymotrypsin inhibitor-like 1 (OCPI1) had more total proteins than their wild type in response to drought stress. This suggests the possible role of OCPI1 in proteinase regulation (Huang et al., 2007). Tobacco plants overexpressing Oryzacystatin I (OC-I), showed more resistance to chilling stress compared to control plants. Moreover, OC-I expressing lines had low endogenous cysteine proteinase activity, indicating that the presence of OC-I under stressful conditions modifies the physiology and metabolism of plants (Van de Vyver et al. 2003). These studies support the hypothesis that plant cystatins are crucial in plant defense mechanisms and that overexpression of cystatins can improve plant tolerance to abiotic stresses.

1.6 Aim of the study

Experimental evidence shows that NO plays systemic signaling roles which generate defense responses following salt stress in many plant species. The use of NO-generating compounds has been shown to improve plant tolerance to abiotic stresses via up-regulation of genes involved in the regulation of plant responses to salt stress. On the basis of the literature discussed above, the role of NO and salt stress on cystatin expression in soybean leaves, nodules and roots was evaluated. This was achieved by studying expression levels of a novel soybean cystatin (Glyma20g08800) following NO treatment in salt stressed soybean plants. The objective was to

also confirm whether exogenous application of NO in plants regulates the cell death process by inhibiting caspase-like activity.

1.7 References

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Chapter 2

Role of nitric oxide in regulating plant lipid peroxidation, caspase-like activity and cell death processes during salinity stress

2.1 Abstract

Salinity stress induces a hyper-ionic and hyper-osmotic stress which restricts water and nutrient uptake and consequently causing ionic imbalance and toxicity in plants. Accumulation of toxic ions (Na^+ and Cl^-) in plant tissues results in cell death and growth inhibition and severely reduces plant biomass. Several studies have now recognized that exogenous application of nitric oxide (NO) in plants can induce tolerance to salinity in several plant species. However, the pathway by which NO mediates these responses remains only partially understood. Effects of salt treatment at 150 mM NaCl together with a nitric oxide donor (DETA/NO) at 10 µM on changes of plant tissue (roots, nodules and leaves) lipid peroxidation, caspase-like activity and cell death were studied in soybean (*Glycine max*) after 48 hours of treatment. Salt-stressed soybean tissues exhibited higher levels of lipid peroxidation and cell death than the untreated tissues. However, a combination treatment of 150 mM NaCl with 10 µM DETA/NO (a nitric oxide donor) resulted in reduced lipid peroxidation levels and less cell death. Caspase-like activity also increased in all three plant tissues in response to salt treatment when compared to the untreated controls while treatment with a combination of NaCl and DETA/NO resulted in caspase-like cysteine protease activity similar to the untreated samples. These results suggest that NO plays pivotal roles in modulating plant cell death processes.

2.2 Introduction

Soybean is one of the essential crops providing oil and protein in human diet, animal feeds and biodiesel globally. However, its productivity is radically reduced by abiotic stresses such as salinity (Nuccio et al., 1999; Beck et al., 2007), drought (Manavalan et al., 2009) and extreme temperatures (Nouri et al., 2011). The extent of damage induced by salinity stress on soybean growth, nodulation, agronomic traits, seed quality and quantity accentuate the need to develop soybean plants with enhanced salt stress tolerance. High concentrations of salt inflicts both ionic and osmotic stresses on plants as a result of Na^+ and Cl^- ions (Wang et al., 2003; Zhu, 2003), which results into secondary effects and oxidative stress or reactive oxygen species (ROS) (Molassiotis et al., 2010). Most of the damage caused by salt stress is associated with oxidative stress resulting from an increase in (ROS) accumulation (Alscher et al., 1997; Mittler, 2002; Neil et al., 2002). High levels of ROS alter normal cellular metabolism through oxidative damage to lipids, proteins and nucleic acids (Mckersie and Leshem, 1994; Alscher et al., 1997; Imlay, 2003).

Under optimal conditions, ROS act as signals for the activation of antioxidants. These include an antioxidant system consisting of low-molecular weight antioxidants such as ascorbate, α-tocopherol, glutathione and carotenoids as well as antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) (Nakano and Asada, 1981; Zhang and Kirkham, 1994), catalase (CAT) and glutathione peroxidase (GPX) (Mckersie and Leshem, 1994; Noctor and Foyer, 1998). SOD activity scavenges the superoxide radicals and forms H_2O_2 and O_2, while CAT, APX and GPX scavenge the H_2O_2 produced by SOD into H_2O and O_2 (Mckersie and Leshem, 1994; Balley-Serres and Mittler, 2006). APX is

regarded as the most essential plant peroxidase in the detoxification of H_2O_2 (Noctor and Foyer, 1998) and uses ascorbate as an electron donor in the first step of the ascorbate-glutathione cycle. GPX decomposes H_2O_2 by oxidation of glutathione (GSH). Oxidation of GSH produces glutathione disulfide (GSSG) which is converted back into GSH in a reaction catalyzed by GR (Edwards et al., 1990) leading to an increase in the GSH/GSSG ratio required for ascorbate regeneration and activation of several CO_2 fixing enzymes in the chloroplasts (Crawford et al., 2000).

However, abiotic stresses disrupt the activity of these antioxidant enzymes (Dhindsa and Matowe, 1981) and result in excessive accumulation of ROS, leading to severe oxidative damage which triggers caspase-like activity, which in turn may induce plant cell death (Earn-Shaw, 1995; Martin and Green, 1995; Martins et al., 1997; Solomon et al., 1999). The existence of caspases in plants is controversial. Although studies implicate plant cysteine proteolytic enzymes to be associated with PCD (Minami and Fukuda, 1995; D'Silva et al., 1998; Schmid et al., 1999; Solomon et al., 1999), no direct homologues of animal caspase genes have been identified in plants. On the other hand, some specific peptide inhibitors of animal caspases have been shown to affect the development of PCD in plants (Greenberg, 1997; Heath, 2000). del Pozo and Lam (1998) showed that some inhibitors of animal caspase-1 and -3 (Ac-YVAD-cmk and Ac-DEVD-CHO) were able to attenuate bacteria- and *Tobacco mosaic virus* (TMV)-induced HR in tobacco leaves. In addition, other studies on the same set of caspase inhibitors showed reduced levels of cell death induced by isopentyladenosine (Mlejnek and Prochazka, 2002) in tobacco and inhibition of cell death during menadion-induced apoptosis in tobacco protoplasts (Sun et al., 1999).

On the other hand, an emerging number of studies has revealed that nitric oxide (NO) can generate defense responses following salt stress in many plant species (Uchida et al., 2002; Wang et al., 2003; Zhao et al., 2004; Li et al., 2005; Zhang et al., 2006; Liu et al., 2007; Zhao et al., 2007; Tanou et al., 2009; Molassiotis et al., 2010). Exogenous application of NO has been shown to significantly alleviate the oxidative damage caused by salinity (Uchida et al., 2002; Kopyra and Gwozdz, 2003; Fan et al., 2007; Yu-qing et al., 2007; Song et al., 2009), increase total soluble protein by preventing endopeptidase and carboxypeptidase activities in salt-stressed plants (Zheng et al., 2010), decrease the rate of ROS production and lipid peroxidation as indicated by malondialdehyde (MDA) content and induce the activity of ROS scavenging enzymes (Zheng et al., 2009). Nonetheless, most of these studies were performed with leaves and scarce information is available for other plant tissues such as roots and nodules (the first organs exposed directly to salt stress). None of these studies focuses on the role of caspase-like cysteine proteases and NO in salt stress-induced cell death.

On this basis, lipid peroxidation (measured as MDA content), caspase-like activity and cell death were investigated in soybean roots, nodules and leaves following salt and NO treatment, to further establish the role of nitric oxide in modulating caspase-like activity and cell death in plants exposed to salt stress.

2.3 Materials and Methods

2.3.1 Chemicals and Reagents

Soybean seeds were provided by Pannar Seeds (Greytown, South Africa) and unless otherwise stated, all chemicals used were purchased from Sigma-Aldrich and Bio-Rad.

2.3.2 Sterilization of seeds and plant growth

Soybean (*Glycine max* L. Merr. cv. PAN626) seeds were surface-sterilized in 10% bleach and 0.005% Tween-20 for 10 minutes, followed by five washes with sterile distilled water. The seeds were imbibed in sterile distilled water at room temperature for 1 hour to allow for the breakage of the seed coat. Seeds were sown in filtered silica sand (pre-soaked in distilled water) in 15 cm diameter plastic pots. The sand was kept moist during germination by watering with tap water until VC (unifoliolate leaves on the first node unroll in addition to cotyledons) stage. Seeds were allowed to germinate (one plant per pot) on a 25/19°C day/night temperature cycle and 16/8 hours light/dark regime with a photon flux density of 300 μmol photons.m^{-2}.s^{-1} during the day (light) period. At VC stage of vegetative growth, plants were supplied with soybean nitrogen free-nutrient solution [1 mM K_2SO_4, 2 mM $MgSO_4$, 5 mM $CaCl_2$, 1 mM K_2HPO_4/ KH_2PO_4 buffer at pH 7.2, 12.5 μM H_3BO_3, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 2 μM Na_2MoO_4, 0.1 μM $CoSO_4$, 5 μM $MnSO_4$, 100 μM Fe-NaEDTA, 50 μM KCl and 50 mM 4-(2- hydroxethyl)-1-piperazineethanesulfonicacid (HEPES) at pH 7.2]. Plants were then removed from the sand and the root systems were inoculated by immersion in a suspension of *Bradyrhizobium japonicum* (as the commercial inoculant 'Nodulator Peat-based Soybean HiStick 2'(Becker Underwood Ltd, Little Hampton, West Sussex) made up of 5 grams of inoculants mixed with 500 ml of sterile

distilled water. At this stage, plants were returned to the same pots and were watered with 200 ml of soybean nitrogen-free nutrient solution (every two days) until they reached the V3 stage (three fully expanded trifoliolate leaves).

2.3.3 Plant treatments

Treatments of plants were done at V3 stage and included: untreated control, 150 mM NaCl, a combination treatment of 150 mM NaCl and DETA/NO (nitric oxide donor 'diethylenetriamine/nitric oxide adjunct') or DETA (similar to DETA/NO but lacks the NO molecule). Untreated samples were supplied with 200 ml of soybean nitrogen-free nutrient solution at pH 7.2. All other treatments were supplemented in soybean nitrogen-free nutrient solution at pH 7.2.

2.3.4 Evaluation of cell viability

For evaluation of cell death (and plasma membrane integrity), freshly harvested soybean plant tissues were tested after 48 hours of treatment using Evans Blue stain. Plant tissues (roots, nodules and leaves at 100 mg of each tissue per treatment) were stained in 0.25 % Evans Blue for 45 minutes at room temperature. The tissues were then thoroughly washed with sterile distilled water and soaked overnight. Plant tissues were incubated in 1 % SDS at 55°C for 1 hour and the level of Evans Blue uptake was determined by measuring the absorbance of the extract at 600 nm.

2.3.5 Lipid peroxidation levels

Lipid peroxidation (measured as MDA content) was assayed in soybean root, leaf and nodule tissues following salt and NO treatment. Briefly, frozen plant tissues (approximately 100 mg per treatment) were ground into a fine powder in liquid nitrogen and homogenized in 500 µl of 6 % (w/v) trichloroacetic acid (TCA) (Buege and Aust, 1978). The resulting homogenates were centrifuged at 12,000 x *g* for 10 min at room temperature. The supernatant was then collected and mixed with 400 µl of 0.5 % 2-thiobarbituric acid (TBA) diluted in 20 % TCA. Samples were incubated at 95°C for 30 min and the reaction was ended by incubating on ice for 5 min. Samples were centrifuged for a further 10 min (12, 0000 x *g*, room temperature), followed by spectrophotometric measurement of absorbance at 532 nm and 600 nm. The OD_{600} values were subtracted from the MDA-TBA values at 532 nm and the MDA concentration was calculated using the Lambert-Beer law, with an extinction coefficient of 155 $mM^{-1}.cm^{-1}$ (Hodges et al., 1999).

2.3.6 Caspase-like activity

Total protein extracts were also prepared by grinding frozen plant tissue (nodule, leaf, roots) in liquid nitrogen and resuspended in 1 ml of ice-cold buffer [100 mM Tris-HCl (pH 7.2), 2 mM EDTA, 10 % (v/v) glycerol and 10 mM β-mercaptoethanol]. This was followed by centrifugation at 12, 000 x *g* for 10 min at room temperature. Proteolytic activity was assayed by mixing the homogenate with 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by incubation at 37°C for 10 min (Zhang et al., 2008). The absorbance was measured at 405 nm, then followed by addition of the colorimetric caspase substrate N-Acetyl-Asp-Glu-Val-Asp-p-Nitroanilide(DEVD-

pNA) at a final concentration of 0.5 mM and incubation at 37°C for 20 min. Caspase-like protease activity was determined by measuring the absorbance of released p-nitroanilide (pNA) from the colorimetric substrate at 405 nm for every 20 min after incubation at 37°C. This was carried out for approximately 120 min. the proteolytic activity was expressed in $nmol.mg^{-1}.min^{-1}$ using the molar extinction coefficient of 9.6 $mM^{-1}.cm^{-1}$ for p-nitroaniline.

2.4 Results

2.4.1 Exogenously applied NO reduces of lipid peroxidation under salt stress

Salinity leads to oxidative stress due to an increase in ROS production (Alscher et al., 1997; Mitler, 2002; Neil et al., 2002), which alters normal cellular metabolism by causing oxidative damage to lipids, proteins and nucleic acids (McKersie and Leshem, 1994; Imlay, 2003). The indicator of oxidative damage in plants is the product of lipid peroxidation known as malondialdehyde (MDA), which is considered a useful and reliable indicator of oxidative damage to lipids, due to the susceptibility of membranes to attack by reactive oxygen species (Wise, 1995; Hodges *et al.*, 1999). The effects of nitric oxide (NO) and salt stress on lipid peroxidation were studied in leaves, roots and nodules of soybean (*Glycine max*) plants grown under control (nutrient solution) or salt stress (nutrient solution containing NaCl at a final concentration of 150 mM) or salt stress and NO (nutrient solution containing NaCl at a final concentration of 150 mM and DETA/NO at a final concentration of 10 μM) or salt stress and DETA (nutrient solution containing NaCl at a final concentration of 150 mM and DETA at a final concentration of 10 μM) conditions for two days. Lipid peroxidation levels in roots, leaves and nodules were measured as MDA content [**Figure 2.1** (**A**, **B**, **C**)].

Compared to the untreated controls; leaves, roots and nodules all had enhanced lipid peroxidation levels. A significant increase in MDA content (about ± 252 %) was observed in leaves under 150 mM NaCl treatment and approximately, a similar percentage increase (±261%) was observed for a combination treatment of 150 mM NaCl and 10 μM DETA (**Figure 2.1A**). However, the increase observed in response to 150 mM NaCl and 10 μM DETA was reduced by ± 63 % in plants treated with a combination of 150 mM NaCl and 10 μM DETA/NO when compared to plants that were treated with NaCl.

A similar trend was observed with respect to nodule and root lipid peroxidation (**Figure 2.4.1B** and **C**). Salt-treated samples with or without DETA showed a higher increase in lipid peroxidation when compared to untreated controls. Nodules experienced an increase of ± 90 % and ± 69 % for roots. On the other hand, a combination treatment with 150 mM NaCl and 10 μM DETA/NO limited the amount of lipid peroxidation to almost the same level as the untreated samples.

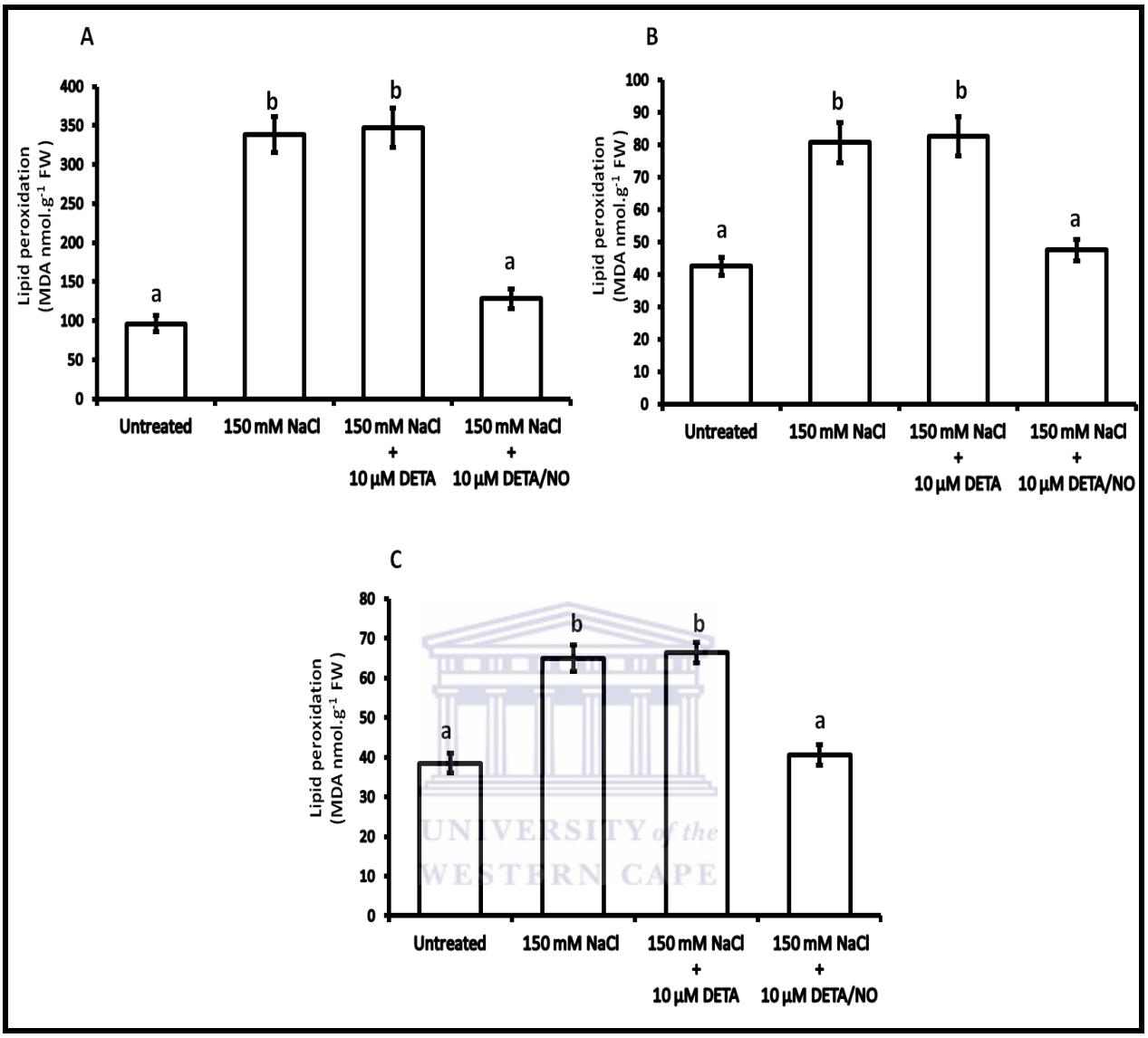

Figure 2.4.1 NO effects on lipid peroxidation in soybean leaves (A), nodules (B) and roots (C) treated with 150 mM NaCl or a combination of 150 mM NaCl plus DETA or DETA/NO. Data are means ± standard error from three independent experiments done in triplicate.

2.4.2 NO and Salt stress alter caspase-like activity in plants

Numerous physiological, biochemical and molecular biology studies on the mechanisms of abiotic stress tolerance of agriculturally important crops have been performed (Yamaguchi-Shinozaki et al., 2002); however, the relationship between caspase-like proteolytic activity and

salt stress in relation to nitric oxide is not well established. The discovery that caspase-like cysteine proteases are the main executers of PCD in plants (Andronis et al., 2010; Wang et al., 2010) and that nitric oxide induces gene expression in response to different abiotic stresses in several plant species (Mollasiotis et al., 2011); led us to investigate if exogenously applied NO can alleviate caspase-like cysteine protease activity in salt-stressed soybean roots, leaves and nodule extracts.

After 48 hrs of treatment with 150 mM NaCl, induction of caspase-like activity by salt stress in leaf, nodule and root increased enormously compared to the caspase-like activity obtained for the untreated controls (**Figure 2.4.2 A**, **B** and **C**). However, the increase was significantly high in salt-treated leaves (±118 %) and nodules (±105 %) than for caspase-like activity observed in salt-stressed roots (±74 %). Meanwhile, exogenous application of NO (as DETA/NO) in salt-stressed plants decreased the activity of caspase-like proteases in leaves (when compared to NaCl-treated leaves) to levels similar to those found in leaves of untreated plants, as shown in **Figure 2.4.2 A**. Nodule and root caspase-like activity also decreased in response to a combination of salt and nitric oxide treatment compared to treatment with salt alone (**Figure 2.4.2 B** and **C**). Furthermore, the same percentage increase observed in caspase-like activity of salt treated samples was also observed in salt and DETA treated samples.

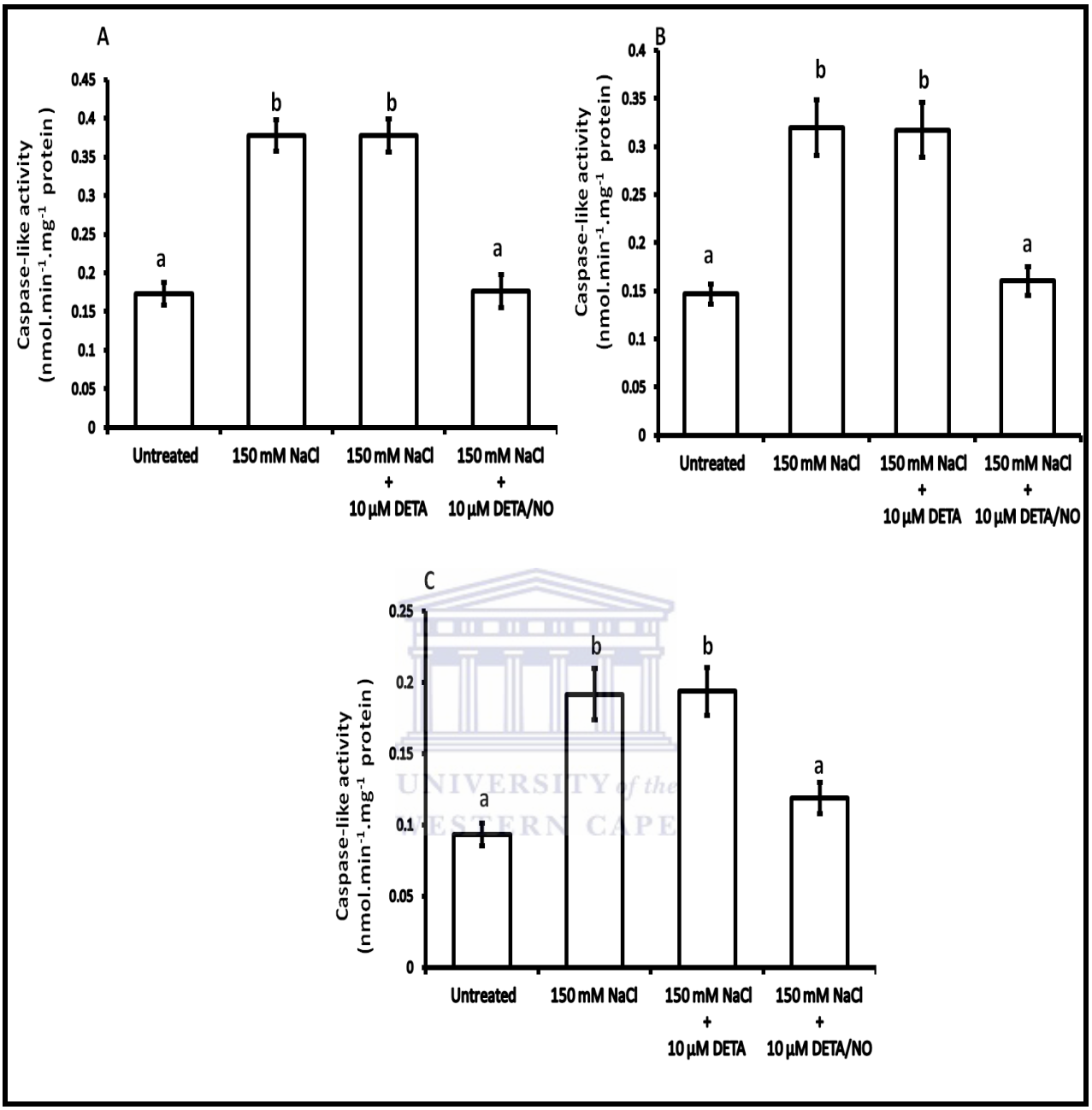

Figure 2.4.2 Salt and NO effects on caspase-like activity in soybean leaf (A), nodule (B) and root (C). Caspase-like enzymatic activity was measured after 2 d of treatment with nutrient solution (untreated), 150 mM NaCl, 150 mM NaCl + 10 µM DETA and 150 mM NaCl + 10 µM DETA/NO. Data are means ± standard error from three independent experiments measured in triplicate**.**

2.4.3 NO alleviates the extent of plant cell death caused by salt stress

Salt stress has been shown to induce both ionic and osmotic stress; leading to restricted plant growth, arrested development and accelerated cell death (Molassiotis et al., 2010; Zhu, 2007). Having discovered that salt stress up-regulates the activity of caspase-like proteases (implicated in PCD execution) and that this increase in caspase-like activity can be decreased by a combination treatment of salt and nitric oxide; this study evaluated if exogenous application of NO can reverse the effects of salt stress-induced cell death in soybean plants. As shown in **Figure 2.4.3**(**A**), leaf cell death was increased by ±262 % in response to treatments of plants with 150 mM NaCl in comparison to untreated controls. The percentages of cell death were about ±187 % and ±356 % for nodules and roots after exposure to salt stress respectively [**Figure 2.4.3.** (**B** and C)]. When treated with salt and DETA/NO, the level of cell death decreased in leaves nodules and roots when compared to cell death in corresponding salt-treated tissues (**Figure 2.4.3 A**, **B** and **C**)

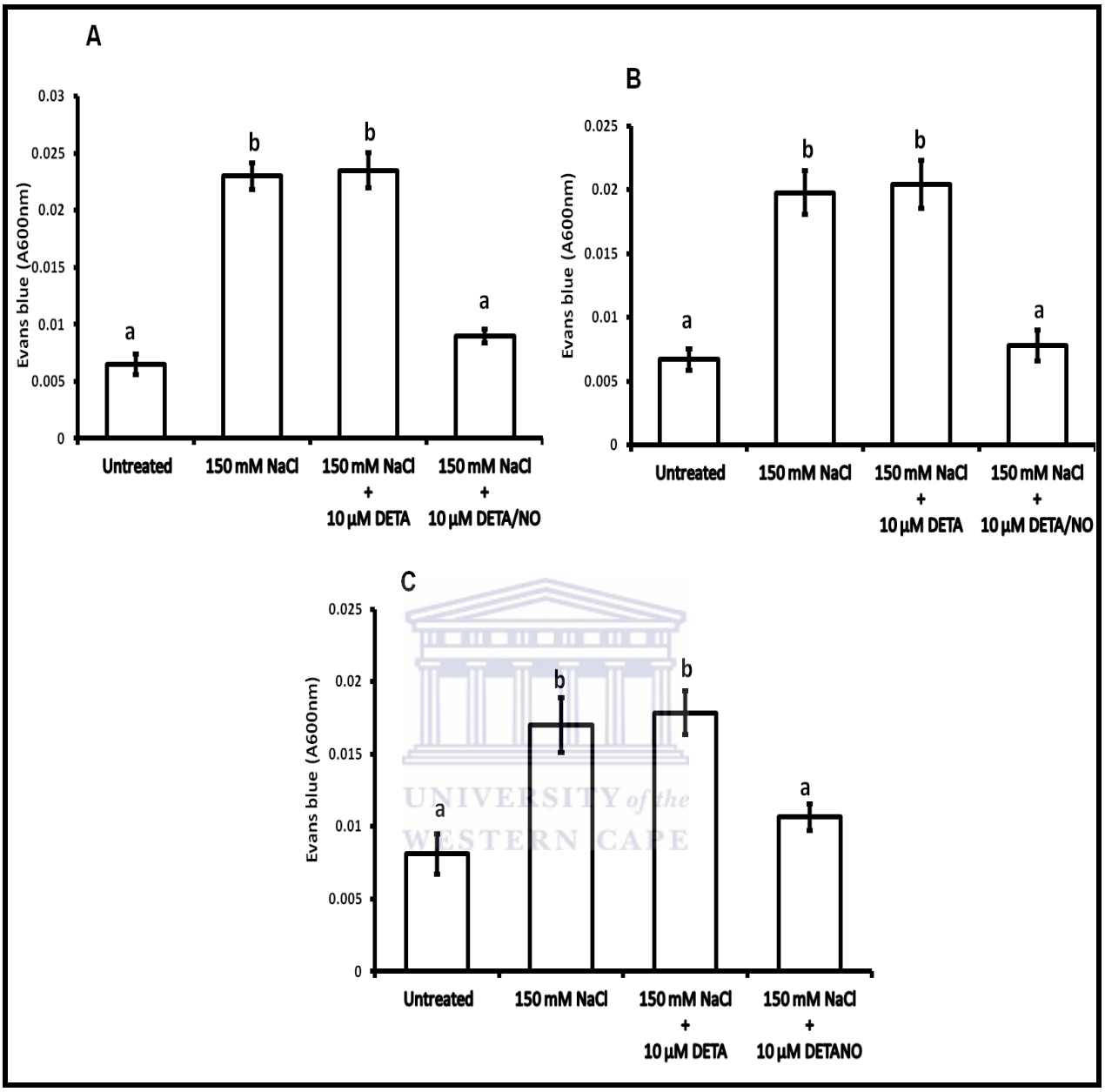

Figure 2.4.3 NO effects on salt stress-induced plant cell death in leaves (**A**), nodules (**B**) and roots (**C**). Evaluation of cell viability was done after 2 d of treatment with nutrient solution (untreated), 150 mM NaCl, 150 mM NaCl + 10 µM DETA and 150 mM NaCl + 10 µM DETA/NO. Data are means ± standard deviation of three independent experiments done in triplicate.

2.5 Discussion

Previous studies have shown that most environmental stresses such as salt, drought, extreme temperatures and air pollutants promote ROS formation in plants and exert oxidative damage which causes damaging effects on cellular macromolecules such as lipids and proteins and may eventually lead to cell death (Sudhakar et al., 2001; Grene, 2002; Laloi et al., 2004; Chinnusany et al., 2005). NaCl is widely used as a source of salt stress which imposes both osmotic and ionic stress to plant cells. NO has been indicated to counteract the effects caused by ROS in response to environmental conditions (Belegni et al., 2002; Shi et al., 2005; Zhao et al., 2007). However, the role of NO in salt stress-induced oxidative damage to lipids, leading to activation of caspase-like cysteine proteases that trigger cell death is not clear. Analysis of the results obtained for lipid peroxidation assessed as the content of MDA showed a significant increase in MDA content after salt treatment in leaves, roots and nodules (**Figure 2.4.1 A**,**B**,**C**) when compared to control plants, while exogenous application of DETA/NO, an NO donor, alleviated the adverse effect of NaCl on MDA concentration. However, the leaf was the most affected part of the plant, thus indicating that leaves are highly susceptible to salt stress-induced oxidative damage than roots and nodules.

Elevated lipid peroxidation in salt-stressed plants has been reported in other studies (Shalata and Tal., 1998; Jaffel et al., 2011; Valentovic et al., 2006). Under salt and other environmental stresses, plant membranes are subject to changes often associated with increases in membrane permeability and integrity loss (Blokhina et al., 2003). A decrease in membrane stability has been suggested to reflect the extent of lipid peroxidation caused by ROS (Sairam et al., 2002). Lipid peroxidation is mostly ascribed to oxidative damage (Zhang and Karkham, 1996) and is

used as an indicator of increased oxidative damage/stress (Halliwel, 1987; Spychalla and Desborough, 1990; Lin and Kaao, 2000). Several studies have reported a protective effect of NO on membrane injury under salt (Zhao *et al.*, 2004), drought (Nasibi and Kalantari, 2009) and heavy metal stress (Singh *et al.*, 2008). It has been suggested that the role of NO in suppression of lipid peroxidation probably is related to reaction of NO with radicals of lipid alcoxyl ($LO^{\cdot}$) and lipidperoxyl ($LOO^{\cdot}$) which suppresses a chain of peroxidation (Beligni and Lamattina, 1999), compatible with results obtained from this experiment on reduction of MDA content by NO treatment in the presence of elevated NaCl concentrations. Furthermore, the defensive role of NO may be related to the suggested function of NO in suppression of ROS (mostly O_2^- and H_2O_2) accumulation (Seregelyes et al., 2003), thus acting as an antioxidant.

Under salt and other various environmental constraints, several cellular proteins undergo conformational changes while others require synthesis (Grudkowska and Zagdan´ska 2004). The drastic change in plants as a result of salt stress induces gene expression including genes encoding proteases (Bray, 2002; Cruz de Carvalho et al., 2001). Caspase-like cysteine proteases play a major role during protein degradation and are reported to constitute the critical point in the PCD pathway of animal cells (Earnshaw, 1995; Martin and Green, 1995; Martins et al., 1997). To date, no functional homologues of animal caspases have been identified in plant cells (Matsumura et al., 2000), yet, a review by Bonneau et al (2008) suggested caspase-like protease activity to also be induced in plants. Furthermore, the involvement of cysteine proteases in total proteolytic activity has been reported to increase drastically in response to abiotic stresses such as salinity and drought in several plant species (Zagdañska and Winievski, 1996).

In support of the role of caspase-like activity in plant PCD, this study showed an increase in the induction of caspase-like cysteine protease under salt treatment. However, the extent was higher in leaves (**Figure 2.3.2A**) than that observed for nodules (**Figure 2.3.2B**) and roots (**Figure 2.3.2C**). This trend observed for caspase-like activity corresponded to that seen for lipid peroxidation and cell death. Increases in plant caspase-like activity have been suggested in tobacco plants (Andronis et al., 2010) and *T. halophila* suspension cultured cells (Wang et al., 2010) in saline conditions. The involvement of Cys protease activity in response to salt stress was also demonstrated in transgenic Arabidopsis plants, in which the expression of Cys protease affected tolerance of plants to salt stress (Chen et al., 2010). These results suggest that salt stress-induced oxidative stress is either directly or indirectly involved in regulating the activity of caspase-like proteases which may be part of the core mechanism of plant PCD, although caspase-like independent PCD exists in several plant systems (Fukuda, 1997; Krzymowska et al., 2007). However, in a case where the salt stressed plants were supplemented with exogenous NO, a reduction in caspase-like activity to levels similar to those observed for untreated plants occurred (**Figure 2.3.3 A**, **B** and **C**).

Induction of cell death (evident from Evans Blue uptake) was also significantly increased by salt stress and this salt stress-induced increase in the extent of cell death was reduced by exogenous NO. These results indicate that salt treatment causes extensive cell death possibly by causing excessive accumulation of ROS, which in turn causes macromolecular peroxidation that triggers caspase-like activity which elicits a form of PCD in plants. Discovering that exogenously applied NO may restrict or limit the extent of salt stress-induced lipid peroxidation, caspase-like activity and cell death; it can be concluded that NO induces plant salt tolerance possibly by enhancing

ROS scavenging systems and regulating salt stress-responsive cysteine proteases or their inhibitors (plant cystatins).

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Chapter 3

Effects of NO on soybean cystatin expression under salt conditions

3.1 Abstract

Studies on plant cystatins with respect to stress have displayed the stress-combating ability of cystatins under *in vitro* experimentation. In soybean, a few groups of plant cystatins with inhibitory activity against cysteine proteases (key regulators of programmed cell death) have been identified and biological functions have been attributed to them. In this study, identification and expression analysis of a novel soybean cystatin (Glyma20g08800) has identified that alterations in NO content regulate the expression of this gene. Given that a number of investigations concerning cystatin expression have mainly focused on seeds and no study has been conducted to link expression of plant cystatins to responses to abiotic stress and NO, this study investigated the respective role(s) of NO in Glyma20g08800 (a novel soybean cystatin gene) expression in response to salt stress in soybean leaves, roots and nodules based on gene-specific expression profiles established by semi-quantitative RT-PCR (semi-qRT-PCR). In response to salt stress, with or without a NO donor (DETA/NO), variations in the expression of a gene encoding Glyma20g08800 were observed. Visual inspection of semi-qRT-PCR amplicons and densitometry analysis showed that salt stress caused a decline in the level of expression of this cystatin while use of a nitric oxide generating compound (DETA/NO) caused an increase in Glyma20g08800 expression under salinity stress. Because cystatins are the main inhibitors of cysteine proteases, the increase observed for the expression of the cystatin (inhibitor of caspase-like activity) Glyma20g08800 in NO-treated samples correlates with the decrease in caspase-like

activity (main executers of PCD) observed in NO-treated samples (Chapter 2) in the presence of salt. The suppression of the salt stress-mediated caspase-like activity and cell death in plants with induced Glyma20g08800 implies that NO induces plant salt tolerance possibly by regulating salt stress-responsive cysteine proteases or their inhibitors (plant cystatins). Increasing the level of expression of cystatins may inhibit abiotic stress-induced caspase-like proteases and inhibit salt stress-induced cell death.

3.2 Introduction

Recently, the involvement of plant cystatins in stress tolerance has been an object of much research. Cystatins are widely distributed in plants, animals and microorganisms (Dubey et al., 2004; Habib and Fazili, 2007; Rawlings et al., 2008) and have been characterized in both monocots and dicots (Massonneau et al., 2005; Belenghi et al., 2003). They are inhibitors of cysteine proteases and are involved in the regulation of protein turnover and play crucial roles in pathogen and insect resistance as well as in abiotic stress responses and PCD. Several cystatins are induced upon exposure to biotic (Leple et al., 1995) and abiotic stresses (Pernas et al., 2000) and may protect plants against invasion by viruses, bacteria, insects, salt and drought as they can inhibit the activity of cysteine proteases from a wide range of organisms.

Role (s) of plant cystatins in plant defense has been shown by transgenic plants overexpressing plant cystatins (Grudkowska and Zagdanska, 2004; Massonneau et al., 2005; Benchabane, 2010). The expression of two cystatins (AtCYSa and AtCYSb) from Arabidopsis was observed to be induced by multiple abiotic stresses such as salt, drought, cold and regulated by abscisic acid treatment (Zhang et al., 2008). Transgenic Arabidopsis plants overexpressing these two genes

had increased tolerance to salt, drought, oxidative stress during seed germination and early seedling development (Zhang et al., 2008). Moreover, overexpression of AtCYS1, an Arabidopsis cysteine proteinase inhibitor gene, blocked cell death activated by either avirulent pathogens or by oxidative and nitric oxide (NO) (Belenghi et al. 2003), while plants overexpressing a rice cysteine proteinase inhibitor gene (Oryza cystatin I) showed resistance against two important potyviruses, tobacco etch virus (TEY) and potato virus (PYY) (Gutie´rrez-Campos et al., 1999). Further evidence confirming the role of soybean cystatins in abiotic or biotic stress responses comes from the fact that the closest homologues of these genes were found to contain *cis*-regulatory elements known to regulate gene expression and confer tolerance under abiotic stresses such as drought, salt, and cold stress (Chapter 3 and evidence from Yamaguchi-Shinozaki and Shinozaki 1994; Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000 in relation to the role of cystatins in plant stress responses). These soybean cystatin genes can also be considered to be targeted by similar *cis*-elements or transcription factors.

To date, there has been recurring amount of interest in the use of plant cystatins in transgenic approaches for the improvement of crop tolerance to both biotic and abiotic stresses (Pernas et al., 2000; Belenghi et al., 2003). However, very limited progress in overexpression of cystatins to improve abiotic (salt or drought) tolerance has been achieved in crop plants. Recent approaches for improving plant abiotic stress tolerance by modulating the expression of cystatins relies on overexpression of the cystatins using the strong CaMV35S promoter, which results in constitutive overexpression of the cystatins (Zhang et al 2008). However, constitutive and continuous inhibition of cysteine proteases by cystatins could have undesired effects on

development and may lead to abnormal growth since cysteine proteases also regulate plant development (Benchabane et al 2010). Furthermore, impaired plant development is known to result in several cases where the CaMV35S promoter is used for overexpression of genes in an attempt to confer abiotic stress tolerance (Ashraf 2010). Therefore, use of inducible promoters that will drive the expression of the cystatins only when the plant is exposed to abiotic stress, rather than using a constitutively active promoter appears to be the best tool for use in engineering plants for abiotic stress tolerance (Ashraf 2010, Mittler and Blumwald 2010).

On the other hand, it has been shown extensively that nitric oxide induces expression of a number of genes in diverse plant species (Besson-Bard et al 2009). Importantly, elevation of nitric oxide content in plants by using nitric oxide-generating compounds has been shown to enhance plant tolerance to abiotic stresses such as salt and drought via up-regulation of genes involved in the regulation of plant responses to salt and drought stress (Siddiqui et al 2010). On this basis, this study evaluated the role of NO and salt stress on cystatin expression in soybean leaves, nodules and roots. This was achieved by studying expression levels of a soybean cystatin gene (Glyma20g08800) following NO treatment in salt-treated soybean plants.

3.3 Materials and Methods

3.3.1 Chemicals and Reagents

Soybean seeds were provided by Pannar Seeds (Greytown, South Africa) and unless otherwise stated, all chemicals used were purchased from Sigma-Aldrich and Bio-Rad.

3.3.2 Sterilization of seeds, Plant growth and treatment

Refer to chapter 2

3.3.4 Glyma20g08800 gene expression studies

Total RNA was extracted using the method described by Gasic et al (2004). Briefly, tissues were ground to powder using a pestle and a mortar under liquid nitrogen and transferred to tubes containing equal volumes of RNA extraction buffer (200 mM Tris–HCl, pH 9, 400 mM KCl, 200 mM sucrose, 35 mM $MgCl_2$, 25 mM EGTA) and phenol/chloroform (24:1, pH 8) and vortexed for 30 s, followed by incubation at 65°C for 10 min. The aqueous phase resulting from a 10 min centrifugation at 16,000 *g* was re-extracted twice with chloroform /isoamylalcohol (24:1). After precipitation with $LiCl_2$, the RNA pellet was washed with 70 % ethanol, precipitated and resuspended in RNAse-free water. The resulting total RNA was treated with RNAse-free DNase for removal of contaminating genomic DNA and then subjected to 1 % agarose gel electrophoresis, followed by image acquisition on the Alpha Image 2200 system (to determine the quality of the isolated RNA). This was then followed by RNA quantification and 1st strand DNA synthesis using reverse transcriptase on 0.1 mg of the total RNA from each sample with the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas). The generated 1st strand cDNA

template (2 µl from each cDNA sample) was used for PCR amplification (RT-PCR) analysis using the following oligonucleotides (10 pmol each):

Glyma20g08800 [forward primer: 5′-AACAGCGTTGAGATCGATGCTC-3′; reverse primer: 5′-ACCTCCTTGAAGTTCAACCATGA-3′]

β-tubulin [forward primer: 5’′- CTGCGAAAGCTTGCAGTGAACC-3’′; reverse primer: 5′-TCTTGCCTCTAAACATGGCTGAGG-3′].

RT-PCR thermal cycling conditions included the following: 2 min at 95°C, followed by 25 cycles of 20 sec at 95°C, 20 sec at 62°C and 30 sec at 72°C and one cycle of 7 min at 72°C catalysed by 1 U of Truestart™ HotStart Taq DNA Polymerase (Fermantas). Aliquots of the PCR reaction were then subjected to 1 % agarose gel electrophoresis, followed by image acquisition on an Alpha Image 2200 system. Densitometry analysis was then performed using Spot Denso tool (AlphaEase FC imaging software, Alpha Innotech Corporation) and individual gels were scored as relatively densitometry values of three independent gels used for expression analysis.

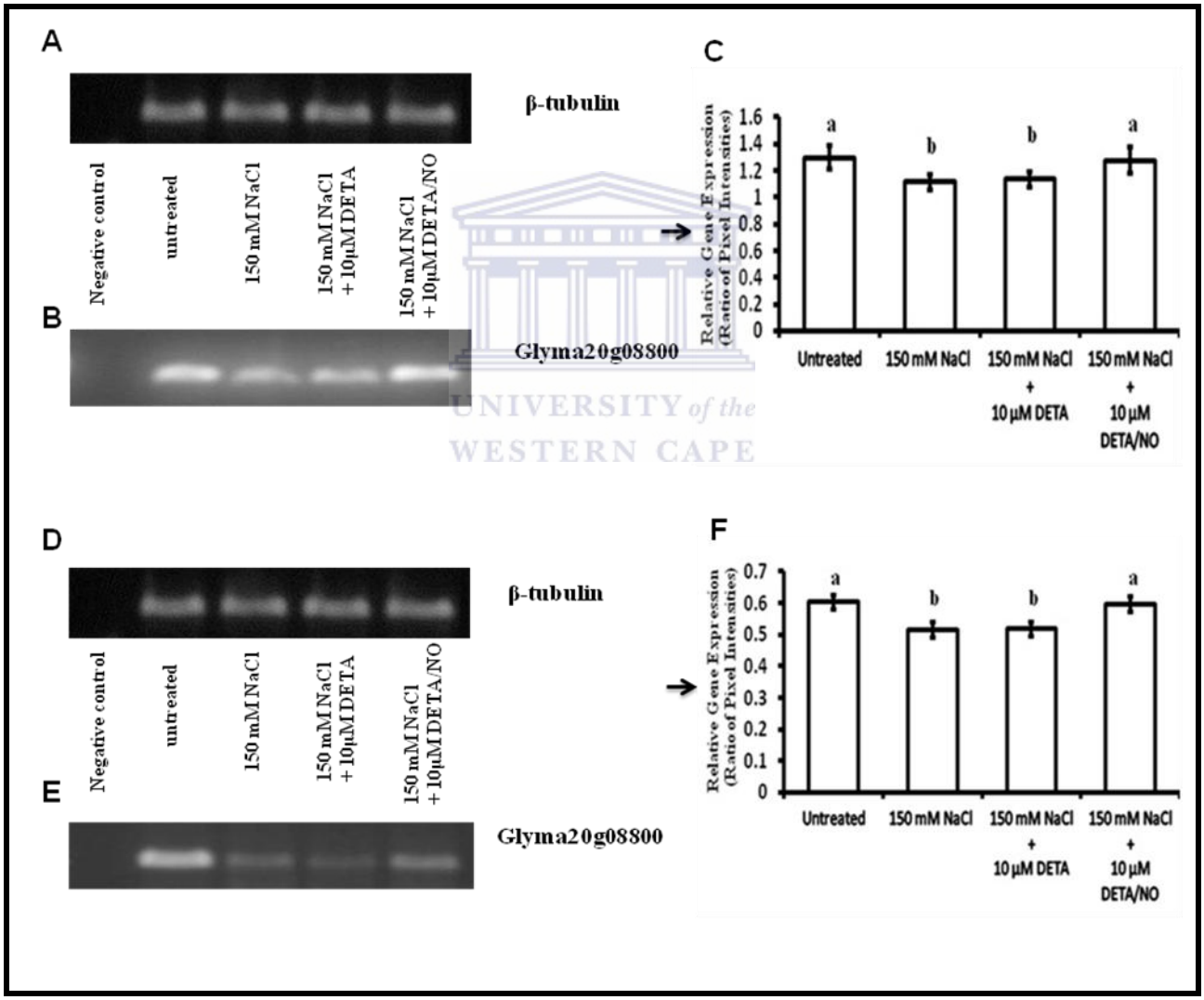
3.4 Results

3.4.1 NO induces Glyma20g08800 gene expression under salinity stress in soybean

A novel role for cystatins as regulators of proteolytic processes and abiotic stress-responses has been proposed (Zhang et al., 2008) and several other studies support the hypothesis that plants employ cysteine protease inhibitors to control cysteine protease activity and modulate the cell death processes (Watanabe et al., 1991; Barrett et al., 1998; Solomon et al., 2009). On the other hand, endogenously synthesized NO has been linked to cysteine-like protease activity in soybean nodules (Leach et al., 2010). It was on this basis that the effects of NO on soybean cystatin gene expression were investigated in response to salt stress.

Gene-specific expression profiles established by semi-quantitative RT-PCR were studied in the leaves (**A**), roots (**D**) and nodules (**G**) of soybean plants submitted to salt stress (150 mM NaCl), salt plus DETA (150 mM NaCl + 10 μM DETA) and salt plus NO donor (150 mM NaCl + 10 μM DETA/NO) for a period of two days. When soybean plants were subjected to salt stress, the transcript levels of Glyma20g08800 significantly decreased in all indicated soybean tissues [**Figure 3.4.1 (A-I)]** compared to control conditions. However, the decrease in Glyma20g08800 expression was reversed by a combination treatment with salt and NO. Both visual inspection of the semi-RT-PCR amplicons and densitometry analyses displays that salt stress with or without DETA suppresses the expression of Glyma20g08800 while use of exogenously applied NO supplemented to the salt-treated plants up-regulates the expression of the gene when compared to the expression observed in salt-treated plants or reverses the decline to levels similar to the controls [**Figure 3.4.1(A-I)].** In leaves, Glyma20g08800 gene expression was induced under

NaCl plus NO treatment compared to salt-treated samples. In roots and nodules, Glyma20g08800 expression was also induced compared to salt treated samples. Furthermore, β-tubulin expression levels remained unchanged in all samples irrespective of the treatments (**Figure 3.4.1B**, **E** and **H**); hence β-tubulin was used as a reference gene to accurately analyze semi-qRT-PCR data for gene expression studies.



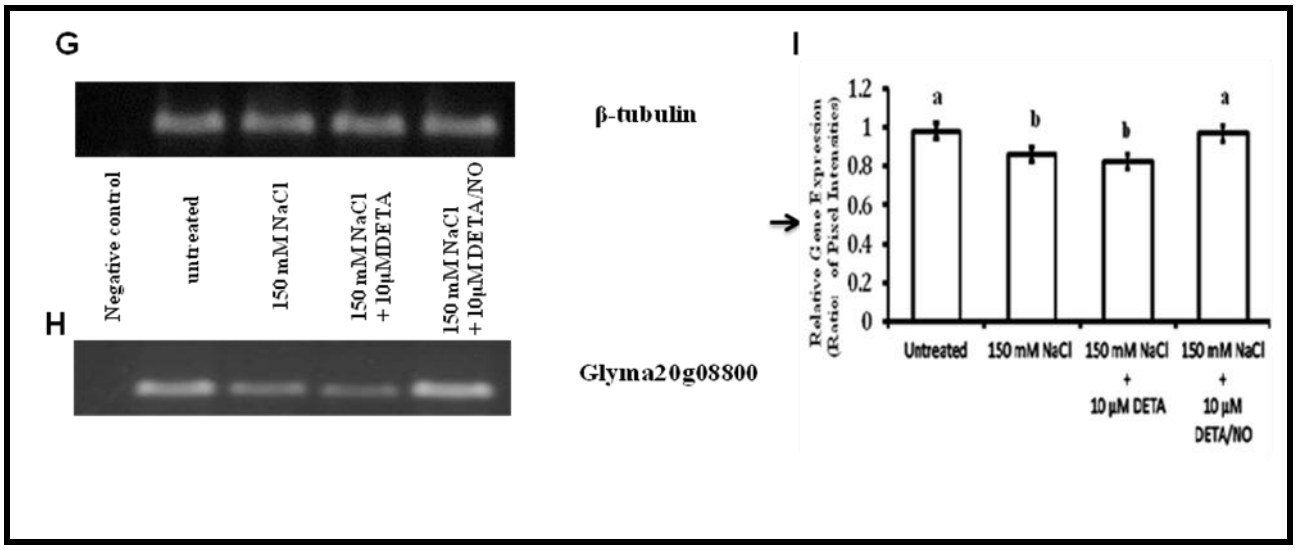


Figure 3.4.1 Expression of Glyma20g08800 cystatin in soybean plants subjected to NO and salt conditions. Total RNA samples were extracted from leaves, roots and nodules grown in normal nutrient solution, followed by treatment with 150 mM NaCl, 150 mM NaCl plus 10 μM DETA and 150 mM NaCl plus μM DETA/NO for two days. Semi-quantitative RT-PCR was performed on indicated tissues using Glyma20g08800 and β-tubulin gene-specific primers described in section 4.3.4. Transcript levels for Glyma20g08800 and β-tubulin in leaves (**A and B**), roots (**D and E**) and nodules (**G and H**) as displayed in agarose (1 %) gel images; (**C, F and I**) relative transcript levels analyzed by densitometry (cystatin relative to β-tubulin).

3.5 Discussion

An increasing volume of research has focused on the involvement of cysteine proteases and their inhibitors in plant responses to wounding and pathogen attack. However, investigations aimed at evaluating the molecular basis and mechanisms of modification of proteolytic activities in response to abiotic stresses such as salinity, drought and extreme temperatures has not been extensively studied in plants in relation to how these are influenced by NO. Only a few cystatins have been described and studied in great detail, mainly in Arabidopsis (Belenghi et al., 2003) and rice (Kondo et al., 1991). Exposure to various environmental conditions leads to the induction of several stress-related genes and accumulation of stress-response/tolerance-related proteins meant to protect plants from damage induced by stress. Many plant cystatins show differential expression when exposed to different abiotic stress conditions (Massonneaua et al., 2005; Pernas et al., 2000).

A few studies have shown the possible involvement of some proteolytic enzymes and their inhibitors in environmental stress responses (Huang et al., 2007; Zhang et al., 2008; Wang et al., 2012). Huang et al. (2007) reported that overexpression of a proteinase inhibitor, *Oryza sativa* chymotrypsin inhibitor-like 1 (OCPI1) improved plant tolerance to abiotic stresses and several other studies have indicated improved resistance to various biotic and abiotic stresses due to overexpression of various proteinase inhibitors (Pernas et al. 1998; Koiwa et al. 2000; Oppert et al. 2003; Yang and Yeh 2005).

Regardless of much interest on the effects of abiotic stress on proteolytic protein expression and involvement of NO in plant signaling pathways, no data on the regulation of cystatins by NO in

salinity stress has been reported. This is the first study indicating that salt stress induces a decline in the level of expression of a soybean cystatin in various major organs while use of nitric oxide-generating compounds (DETA/NO) causes an increase in the level of expression of the cystatin (**Figure 3.4.1**), thus leading to a decrease in caspase-like activity and cell death as observed in Chapter 2. Glyma20g08800 transcripts were found to be induced in leaves, roots and nodules of salt-treated soybean plants following NO treatment (**Figure 3.4.1**). This result is consistent with the findings of Belenghi et al. (2003), who discovered that homologous AtCYS1 (homologous to Glyma20g08800) requires NOS activity and that the use of a NO generating compound (SNP) regulates the expression of this AtCYS1. It is possible that the expression pattern of genes in abiotic/biotic stress responses is related to the systemic signaling roles of NO in plant defense. Such possibilities warrant further studies on the role(s) of NO in stress tolerance.

Our findings constitute a new finding of the physiological function of NO, as results indicated that Glyma20g08800 was responsive to NO in salt-treated leaves, roots and nodules (**Figure 3.4.1**). Therefore, it can be hypothesized that elevation of nitric oxide content in plants by using nitric oxide-generating compounds induces the expression of cystatins at levels high enough to inhibit abiotic stress-induced caspase-like proteases and thereby prevent salt stress-induced cell death. Detailed mechanisms underlying the NO mediated cystatin gene expression in abiotic stress tolerance will be the focus of upcoming studies.

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Chapter 4

Bioinformatics analysis for soybean cystatin genes

4.1 Introduction

Plants are immobile and are frequently exposed to adverse environmental conditions to which they have to adapt and respond to, through not only physiological and biochemical processes but also molecular and cellular processes. This involves different plant genes that can mitigate the effects of stress and induce tolerance (Shinozaki & Shinozaki, 2005). These genes include proteins which function in abiotic stress tolerance mechanisms, such as those involved in protecting macromolecules, antioxidation, signal transduction and those that regulate the expression of genes (Shinozaki & Yamaguchi-Shinozaki, 1997). In addition, abiotic stresses such as high salinity, drought and cold have been indicated in regulation of different plant genes (Thomashow, 1999; Bray et al., 2000; Shinozaki et al., 2003), leading to accumulation of a number of cysteine proteases which constitutes a critical point in plant programmed cell death (PCD) (Solomon et al., 1999; Grudkowska and Zagdan´ska 2004).

Cysteine proteases are involved in various functions like protein degradation and proteolytic activation of specific proteins as a result of physical damage due to stress (Wisnniewski & Zagdanska 2001; Grudkowska and Zagdan´ska, 2004). On the other hand, stress-induced cell damage also leads to the synthesis of proteinase inhibitors known as cystatins, which regulate the activity of cysteine proteinases (Yang and Yeh, 2005). The role of cystatins as cysteine proteinase inhibitors has been implicated in plant defense against pathogens (Belinghi *et al*. 2003), abiotic stress responses and programmed cell death (PCD) (Solomon et al., 1999; Zhang

et al., 2008). A few studies on the effects of overexpression of cystatins in plant defense have been performed (Gutie´rrez-Campos -Campos et al., 1999; Belinghi et al., 2003). However, the mechanisms by which cystatins respond to abiotic stresses and the effect of their over-expression by nitric oxide (NO) on stress-tolerance have not been executed.

One of the main challenges in molecular biology is the understanding of the underlying mechanisms which regulate gene expression under adverse environmental conditions. One of the promising strategies is to identify binding sites (motifs) for transcription factors in promoter regions of the regulated proteins (Palmieri et al., 2008). The activation of stress induced proteins is regulated by certain transcription factors (Hu et al., 2008) which can protect plants from oxidative damage caused by osmotic stress, oxidative stress and ion toxicity amongst many other stresses (Bartels, 2005). It is hypothesized that transcriptional co-regulation induces co-expression of proteins which share some similarities in their regulatory mechanisms. Therefore, co-expressed proteins might contain common motifs which are binding sites for transcription factors involved in the expression of stress-responsive genes (Yamaguchi-Shinozaki & Kazuo Shinozaki, 2005).

To date, several abiotic stress inducible proteins and their physiological functions have been identified and characterized in plants (Kim et al., 2000; He et al., 2002; Lia et al., 2003). A number of different sets of *cis*-acting elements and *trans*-acting factors associated with abiotic-stress responses have also been identified. These include abscisic acid responsive element (ABRE), myelocytomastosis oncogene (MYB/MYC), dehydration responsive elements (DREBs), (Abe et al., 1997; Saibo et al., 2009; Nakashima et al., 2009), basic leucine zipper (bZIP) and WRKY (Yamaguchi-Shinozaki and Shinozaki, 1994; Kizis and Pagés, 2002; Wang

et al., 2007; Qiu & Yu 2009). These transcription factors play crucial roles in inducing multiple stress tolerance generally in both an abscisic acid (ABA)-dependent and -independent manners and through respective *cis*-acting elements and DNA binding domains (Nakashima et al., 2009; Saibo et al., 2009).

These are present in several environmental stress-responsive gene promoters (Yamaguchi-Shinozaki and Shinozaki, 1994; Baker et al., 1994; Stockinger et al., 1997; Liu et al., 1998) and are essential in controlling the expression of several stress-related proteins under salt, drought and extreme temperature stress. Nonetheless, several other transcriptional regulatory systems that are important in regulating plant responses to different stresses have been reported (Bohnert et al., 2001; Seki et al., 2001; Zhu et al., 2001; Agarwal et al., 2006).

Consequently, a precise understanding of gene networks involved in cystatin-induced stress response and functional analysis of *cis*-acting elements within these proteins together with their transcription factors can be targeted in abiotic-stress tolerance studies. More importantly, identification and characterization of stress-responsive transcription factors and their *cis*-acting elements might provide a promising tool for improving the tolerance of crop plants to abiotic stress in general by identifying co-expressed gene that would be important for regulating plant stress tolerance.

The aim of this study was to identify *cis*-acting elements involved in abiotic (salt) and nitric oxide (NO)-mediated gene expression in the promoter regions of four soybean (*Glycine max*) cystatin genes namely; Glyma20g08800, Glyma13g04250, Glyma14g04250 and Glyma18g12240. These regulatory elements are presumed to interact with transcription factors

and act as molecular "switches" ensuring a proper response to environmental stress conditions. The information gleamed from this study will help identify the interactions between signaling pathways, along with novel *cis*-acting elements, in the promoters of soybean cystatin genes and co-regulated proteins.

4.2 Methods

The protein sequences of all four soybean *(Glycine max)* cystatin genes were obtained by using the protein sequence of AtCYS1 that is implicated in PCD and is known to be NO-inducible (Belenghi et al., 2003) as a search query in the BLAST search in (Phytozome; http://phytozome.net)

4.2.1 Identification of protein functional binding proteins/partners

Protein coding sequences of the GmCYS1p626 and three of its homologues (Glyma20g08800, Glyma14g04250 and Glyma18g12240) were used as a query in the Search Tool for the Retrieval of Interacting Genes or proteins [STRING; http://string-db.org) version 9.0] to identify known and putative protein interactions between soybean cystatin proteins and their interacting proteins (Szklarczyk et al., 2011; Jensen et al ., 2009). A raw list of all proteins available in the database which were mostly similar to the query sequences was identified. An *Arabidopsis thaliana* (AtCYSb) gene was indicated to have the highest similarity to all query sequences and was used to produce a gene networks using the following updated parameters: confidence score of 0.700, depth of 4 and interactions of no more than 10. In addition, seven parameters including neighborhood, gene fusion, co-occurrence, experiments, databases, co-expression and text mining. The output results were presented as a graphical network consisting

of the highest scoring interacting proteins. For each interacting protein, a popup window containing important information (3D structure, domains and homology models) about the protein was also provided (Szklarczyk et al., 2011).

4.2.2 Identification of functional domains

Upon obtaining protein-protein association networks, identified proteins were further analyzed for conserved (functional) domains and related function(s) using the Conserved Domains Database (CDD; http://ncbi.nlm.nih.gov/.../cdd_help.shtml) (Marchler-Bauer et al., 2011). CDD resource consists of a compilation of well annotated multiple sequence models for domains and full length proteins which are presented as position-specific score matrices for domain identification in protein sequences via RPS-BLAST. On CDD's web portal, the protein sequences of the four soybean cystatin genes and their co-expressed proteins were used as a query for conserved domain identification and to also provide accessory information imported from other databases. Additionally, the CDD also includes NCBI-curated domains, which utilizes 3D-protein structure information (Marchler-Bauer et al., 2005). This clearly defines domain boundaries, thus providing insights into protein sequence, structure and function relationships.

4.2.3 Promoter content analysis and functional annotation

For this analysis, the transcription factor binding site enrichment tool *Arabidopsis thaliana* expression network analysis (http://bioinformatics2.wsu.edu/.../ATHENA/.../visualize_select.pl) database was used to identify statistically over-represented transcription factor binding sites occurring in promoter regions of the query proteins (O'Connor et al., 2005). Briefly, ten gene

ids (AtCYSb and co-expressed proteins) were used as queries for promoter sequence analyses in ATHENA's visualization tool promoter selection page. A compact display was selected for the visualization of transcription factor binding sites, transcription start sites and predicted CpG islands in the promoter regions of these proteins. The predicted transcription factor binding sites were then shown by color-coded hash marks visible in each of the predicted gene. Furthermore, compact display also provided a transcription factor site name with a link to accessory information about each transcription factor site and sequence. The significance of each transcription factor was provided with a calculated P-value and the number of proteins containing each of the identified transcription factors was also presented for each gene id**.**

4.2.4 Motif discovery using MEME suite

In search for novel signals (motifs) in DNA or protein sequences of selected proteins and their structural, regulatory or biological significance, a Multiple EM for Motif Elicitation (MEME; HTTP://meme.nbcr.net) database was used. MEME allows for the identification of binding sites for the shared transcription factors in the set of promoters or common protein-protein binding domains in promoter sets of selected proteins or genes (Timothy et al., 2006; 2009).

Promoter sequences (200 bp upstream and 100 bp downstream) for AtCYb and co-expressed proteins were extracted from phytozome.net and saved in the FASTA format. These promoter regions were used as an input for motif analyses in MEME. The number of motifs to be identified was set to ten and the sequences used were in DNA format. The MEME results were visualized HTML format and displayed as block diagrams. Each block diagram showed the relative positions of the motifs in each of the query sequences.

The MEME HTML results allowed for access to motif alignment and comparison of motifs discovered in the input sequences with databases of known motifs (Timothy et al., 2009). To compare these motifs, MEME motifs were submitted directly to TOMTOM web tool. TOMTOM searches databases of known motifs to discover matches to motifs discovered by MEME (Gupta et al., 2007). The results are then displayed as LOGOS, together with numeric score and statistical significance of each motif match.

Lastly, MEME also allowed for the search of the functional role of each DNA motif. For this analysis, the GOMO tool was used. GOMO assesses gene ontology (GO) terms associated with each gene by linking gene sequences and GO annotations through the sequence identifier (Boden and Bailey, 2008).

4.3 Results

4.3.1 Protein identification and functional co-expression networks

Cystatin genes are not well characterized in soybean; in this study, protein coding sequences of soybean cystatin gene GmCYS1p626 and three of its homologues (Glyma20g08800, Glyma14g04250 and Glyma18g12240) were used as a query to search for known and putative protein-protein interactions between co-regulated proteins. The protein-protein interaction associations are provided with a confidence score which allows for better coverage, accuracy and accessory information such as protein domains and 3D structures.

An *Arabidopsis thaliana* gene (At3g12490) was identified as a closest homologue (90% similarity) to all four soybean cystatin genes and was used as a driver gene to produce protein interacting networks using STRING database (**Figure 4.3.1.1**). At3g12490 (commonly known as AtCYS6 or AtCYSb) is a cysteine protease inhibitor which occurs as a single cystatin domain-containing protein with no cysteine residues at the N-terminal region and has been implicated in stress responses (Benchabane et al., 2010). STRING was used in order to uncover and annotate functional interactions among proteins closely related with the protein of interest based on direct (physical) and indirect (functional) associations (Mering et al., 2003; Szklarczyk et al., 2011), ranked by estimated confidence. Ten proteins were identified and ranked as the highest interacting partners to At3g12490 based on co-expression data (shown in **Figure 4.3.1.1**). Furthermore, a fully interactive network display allowed for navigation through the combined functional associations and related molecular and biological function within co-regulated proteins.

AtCYS6 functional interaction proteins

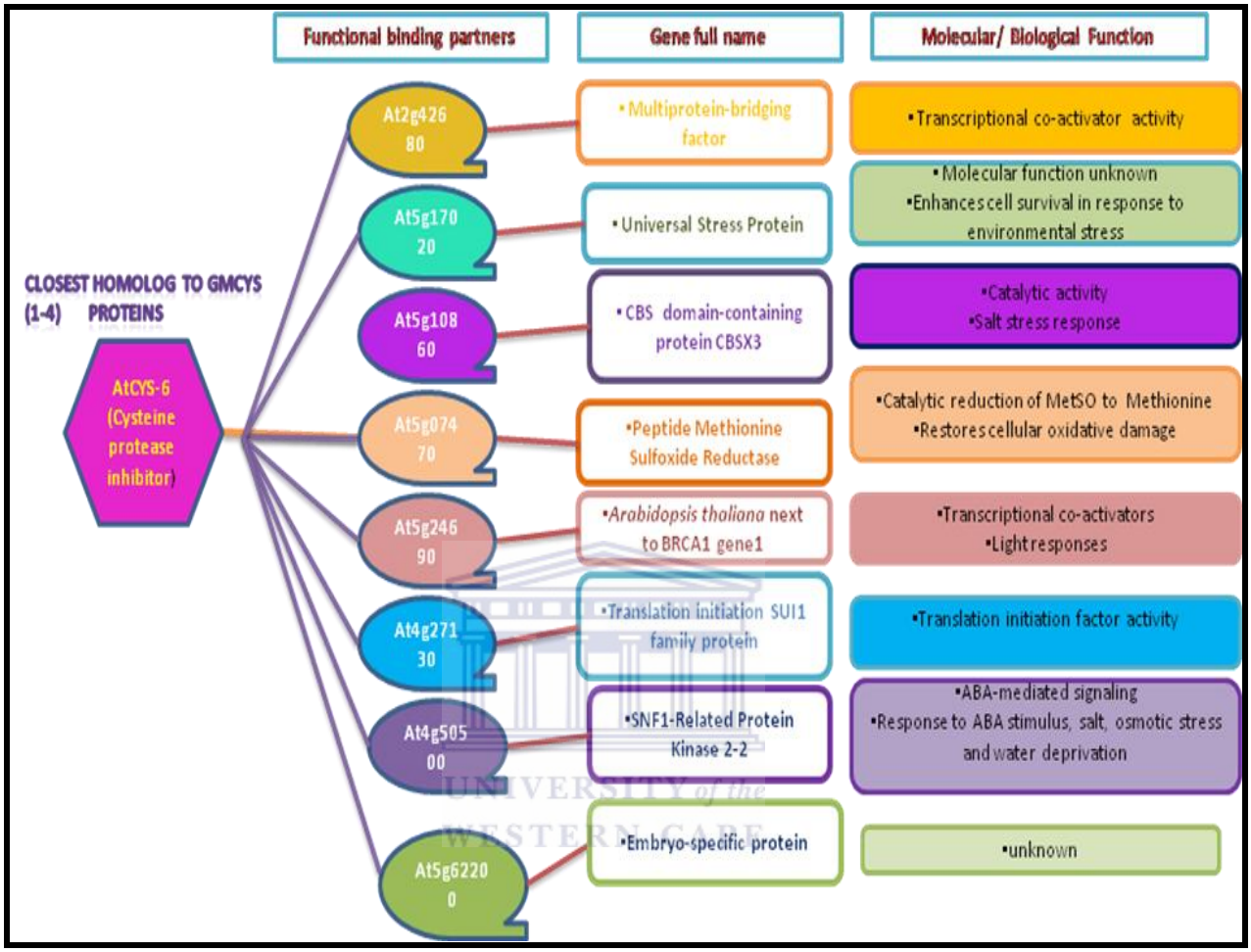


Figure 4.3.1.1 List of *Arabidopsis thaliana* co-regulated proteins with their corresponding functions obtained from STRING database. These proteins were identified as the highest scoring interacting partners of soybean cystatin genes. The identified co-regulated proteins were also searched for curated biological or molecular pathway knowledge.

To further extrapolate the potential functions of the proteins under investigation, protein coding sequences of soybean cystatin gene GmCYS1p626 and three of its homologues (Glyma20g08800, Glyma14g04250 and Glyma18g12240), together with AtCYb and co-expressed proteins were annotated for the presence as well as domain location. Domains are

regarded as distinct functional or structural units of a protein which may exist in various contexts (Marchler-Bauer et al., 2005). Importantly, what is found as an independently folding unit of a polypeptide chain also carries specific functions (Marchler-Bauer et al., 2011). It was then necessary to identify conserved domains within these proteins as an attempt toward obtaining cellular or molecular function of the protein(s) in questions.

Several domains were identified within the four novel soybean cystatin proteins and co-expressed proteins (**Table 4.3.1.1**). All four soybean cystatin proteins contained a conserved cystatin-like domain which is implicated in plant regulatory processes and stress responses (Diop 2004; Massonneau et al., 2005). Moreover, At3g12490 also contained two conserved cystatin-like domains involved in the same processes with those present in soybean cystatin proteins. In addition, most of the domains identified in co-regulated proteins have also been indicated in responses to abiotic or biotic stimulus as indicated below (**Table 4.3.1.1**).

Table 4.3.1.1 Functional domains within four novel soybean cystatins and interacting proteins.

GENE	POSITION	CONSERVED DOMAIN	Potential mechanisms of action
Glyma20g08800 **At3g12490**	15-90 36-126 145-233	**CY (cystatin-like)** **2 CY**	• Essential for the regulatory and protective effects in plants (Megdiche et al., 2009). • Indicated in plant stress responses, pathogen defense and programmed cell death (Belinghi et al., 2009)
At2g42680	85-135	**MBF1 (multi-protein bridging factor-1)**	• Highly conserved transcriptional co-activator (Liu et al., 2003). • Augments the tolerance to heat and osmotic stress (Suzuki et al., 2005)
At3g17020	8-147	**USP_like (universal stress protein)**	• Highly induced in oxidative, temperature and metabolic stresses, ensuring for survival under stressful conditions (Chen and Griffiths, 1999)
At4g24690	8-94	**UBA (ubiquitin associated)**	• One of the major regulators of stress-responsive transcription factors and other regulatory proteins (Lyzenga and Stone, 2011).
At3g500500	29-280	**PKc (Protein kinase)**	• Involved in directing cellular responses to a diverse array of stimuli, such, osmotic stress, heat shock and pro-inflammatory cytokines. • Regulates proliferation, gene expression and differentiation
At5g07470	38-182	**PMSR (peptide methionine sulfoxide reductase)**	• Also known as an oxidative stress repair enzyme. • Referred to as the "last chance" defense mechanism against ROS (Levine et al., 1999; Moskovitz et al., 1998)
At5g10860	57-122 132-189	**2 CBS (cystathionine)**	• Highly induced in response to high salinity, oxidative stress and heavy metal (Singh et al., 2012). • Involved in protein regulation, intracellular ion strength, cytoplasmic targeting, etc (Ignoul & Eggermont, 2005)
At5g07470		**Transcription factor subunit of SRB subcomplex of RNA polymerase 11**	• Involved in DNA transcription and synthesizes precursors of mRNA (Kornberg, 1999; Sims et al., 2004).
At4g27130	25-106	**SUI1/eIF1 (translation/eukaryotic initiation factor)**	• Implicated as a physiological target of salt toxicity in plants (Wyn Jones and Pollard, 1983). • Improves in vivo and in vitro protein synthesis under salt stress conditions. (Rausell et al., 2003).

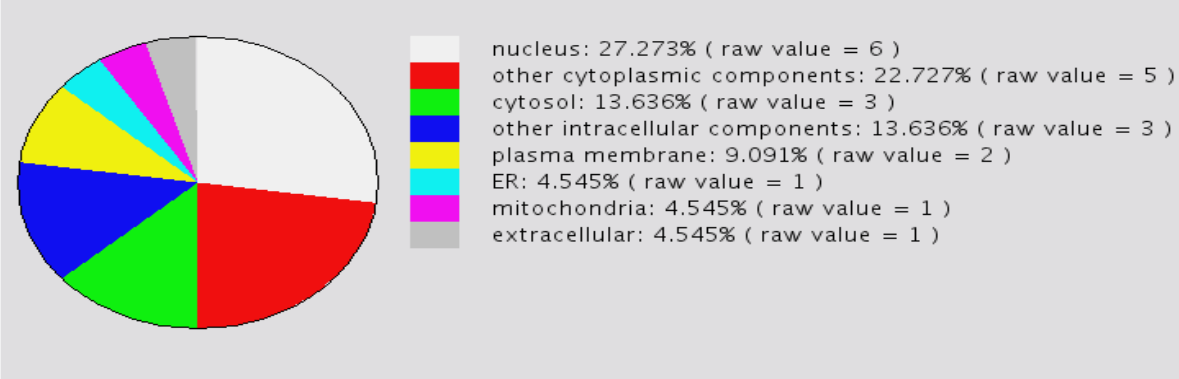
4.3.2 Functional characterization of AtCYb and co-expressed proteins

In order to obtain a clear understanding of the functional relatedness of the four novel soybean cystatin genes and co-expressed proteins, gene ontology (GO) annotation was used. GO terms allowed for functional annotation and enrichment analysis of AtCYb and co-expressed proteins in The Arabidopsis Information Resource (TAIR; http://arabidopsis.org) database (Seung et al., 2003; Poole, 2007). GO takes a list of proteins or genes and identifies gene ontology terms associated with the query proteins, allowing for annotation of gene products to GO terms at varying levels of detail to query for gene products that are involved in similar processes, functions and components.

GO annotations are separated into three groups: cellular component which describes where in the cell a gene acts or what functional complex an enzyme is part of, biological process (biological roles) and molecular function, which classify the function carried out by each gene product (**Figure 4.3.2.1**). GO terms such as nucleus and other cytoplasmic components were enriched in five proteins under the ontology cellular component, while under molecular function; three GO terms such as transferase activity, kinase activity and other binding functions were observed to be the most prevalent (not shown). Moreover, six proteins were identified to be involved in biotic or abiotic stimulus (**Figure 4.3.2.1**). Using these ontologies, one can give meaning to any known gene and define patterns assigned to each gene.

Functional Categorization by annotation

(A)



(B)

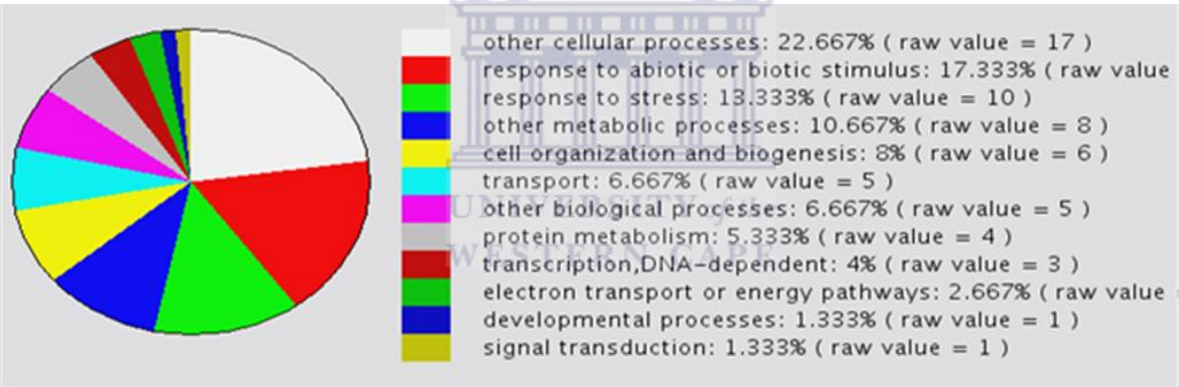


Figure 4.3.2.1 GO term enrichment and functional annotation of AtCYb and co-expressed proteins under the ontologies: celluar component (**A**) and biological process (**B**). The percentage represents the number of annotations to terms in the GOslim category/ total number of annotations to terms in this ontology.

4.3.3 Identification of TFBSs present in promoters of co-expressed proteins

In response to abiotic stresses, several genes are activated at the transcriptional level, and their products are contemplated to provide stress tolerance by the production of vital metabolic proteins and also in regulating the downstream genes (Kavar et al., 2007). Therefore, to better understand the regulatory networks which control gene expression, transcript profiling was a significant tool for the characterization of stress-responsive genes (Palmieri et al., 2008). To meet this challenge, ATHENA database was used to systemically analyze and visualize these promoter regulatory sequences (O'Connor et al., 2005). ATHENA also allowed for the identification of statistically over-represented TF sites occurring in selected subset of promoters and displayed the distribution of TF binding site positions. A list of the TF binding sites identified in the promoter regions of AtCYb and co-regulated proteins and the significance of each TF binding site in the promoter sequences is shown in **Table 4.3.3.1** The majority of the identified TF binding sites have also been implicated in abiotic stress responses.

Table.4.3.3.1 Predicated TFBM identified in AtCYSb and co-expressed proteins

Transcription factor/Motif name	Prom's bound in subset		P-value
MYB1AT	70%	**7**	0.746
TATA-BOX motif	60%	**6**	0.927
Ibox promoter motif	60%	**6**	**0.070**
W-box promoter motif	50%	**5**	**0.079**
CARGCW8GAT	40%	**4**	0.884
Evening element promoter motif	40%	**4**	**0.002**
DRE core motif	40%	**4**	**0.095**
ARF binging site motif	30%	**3**	0.636
MYB4 binding site motif	30%	**3**	0.994
AtMYC2 BS in RD22	30%	**3**	0.603
MYCATERD1	30%	**3**	0.603
BOXII promoter motif	30%	**3**	0.768
T-box promoter motif	30%	**3**	0.910
CDA1ATCAB2	20%	**2**	**0.030**
GAREAT	20%	**2**	0.984
ABRE-like binding site motif	20%	**2**	**0.569**
ATHB5ATCORE	10%	**1**	0.246
E2F binding site motif	10%	**1**	**0.242**
GADOWNAT	10%	**1**	0.649
TGA1 binding site motif	10%	**1**	**0.351**
ATHB2 binding site motif	10%	**1**	0.750
ACGTABREMOTIFA20SEM	10%	**1**	0.932
MYB2AT	10%	**1**	0.163
ATHB1 binding site motif	10%	**1**	0.750
MYB1LEPR	10%	**1**	0.932
GCC-box promoter motif	10%	**1**	0.173
ATHB6 binding site motif	10%	**1**	0.418
UPRMOTIFIAT	10%	**1**	**0.297**
CACCGTMOTIF	10%	**1**	**0.259**

Table 4.3.3.1 Enriched transcription factor (motif) binding sites in promoter sequences of selected proteins. Column one represents the transcription factor name, column two shows the number of proteins containing at least one instance of the TF binding site in the selected subset of proteins. Column three represents the occurrence of the motif in the whole Arabidopsis genome and column four contains the P-value associated with the significance of each motif in the promoter sequence (obtained using a hypergeometric probability distribution).

4.3.4 Motif discovery and analyses

Motif analysis was done to determine binding sites for the shared transcription factors and discover significant motifs in sets of DNA sequences from co-expressed proteins (Timothy et al., 2006; 2009). Ten motifs were identified with MEME search tool and displayed as sequence LOGOS (a visualization tool for motifs). The number identifying the motif and the statistical significance of each motif (E-value) are presented in column one and two respectively. The sequence motifs and the reverse complement strand of each motif are displayed in column three and four. Each of the identified motifs may be present in some or all of a set of input sequences from co-expressed proteins as shown in **Figure 4.3.4.1A**. The order and spacing of non-overlapping matches of the identified motifs in each input sequence is displayed, with the height of each letter indicative of the relative frequency at the given position in the motif.

The input motifs discovered with MEME were compared to databases of known motifs, to determine similarity with known regulatory motifs using TOMTOM web tool. TOMTOM searches databases of known motifs, for matches to motifs discovered by MEME and provide a significant score for each motif match (Gupta et al., 2007). A list of proteins previously identified to contain each of the query motifs is shown in **Figure 4.3.4.1B**, along with motif logos and number of matches. The identified motifs were further analyzed for presumed function using the motif-Gene Ontology (GO) term association tool (GOMO), which searches for GO terms associated with proteins that each DNA motif regulates (Boden and Bailey,, 2008). The number of GO term predications, motif identifier and the top 5 specific predicted GO terms associated with each motif are displayed in **Figure 4.3.4.1C** and are specified by the

following abbreviations: BP for biological process, CC for cellular component and MF for molecular function.

Seven of the ten identified motifs had comparable GO terms associated to them. Four motifs contained common GO terms such as transcription factor activity or structural constituent of ribosome under molecular function. Under cellular component, the GO terms such as nucleus and mitochondrion were the most common. For the category biological process, only three motifs were associated with the same GO term “translation”. The three remaining motifs have no GO term(s) associated to them.

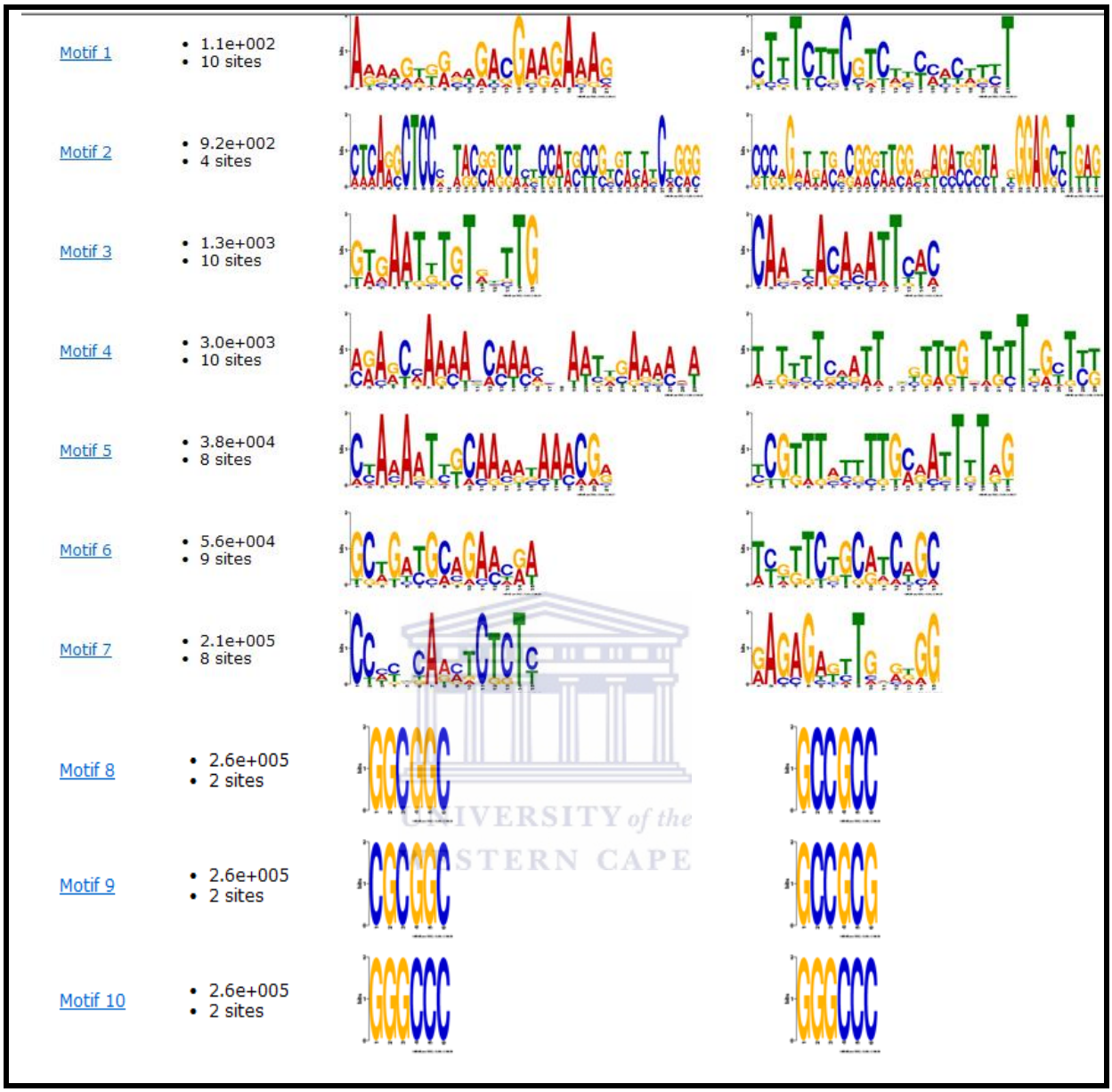
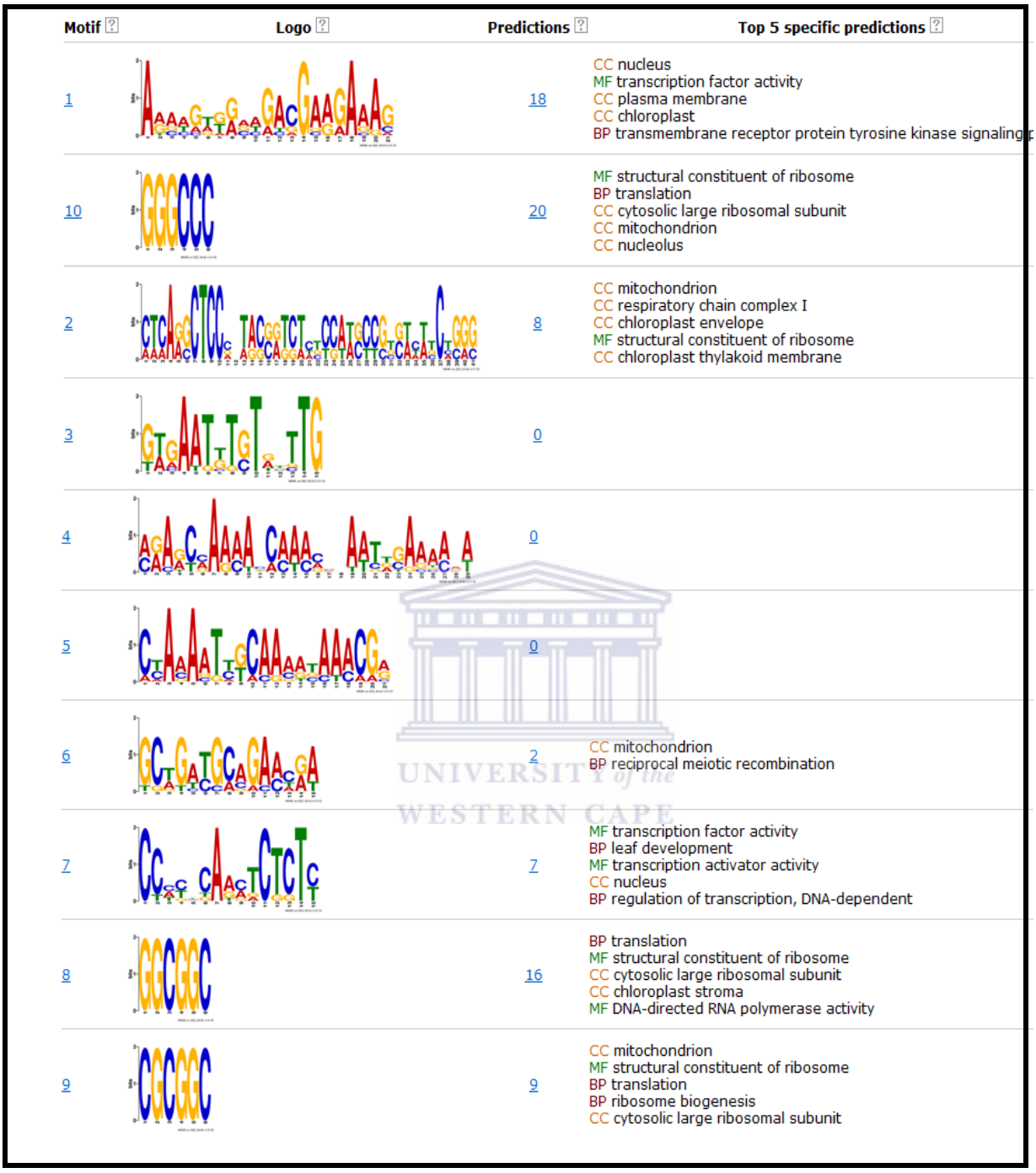


Figure 4.3.4.4.1A List of motifs discovered by MEME in query sequences of co-expressed proteins. Column one shows the motif name, column two shows the calculated E-value which is indicative of the statistical significance of the motif, column three displays the motif "LOGOs" and column four displays the reverse complement strand of the motif.

Figure 4.3.4.1B List of motifs similar to motifs identified in MEME. Column one displays the motif number, column two contains the motif LOGO, column three shows the number of motifs that match with atleast one of the query motifs and column four displays the list of the matched motifs.



Motif	Logo	Predictions	Top 5 specific predictions
1		18	CC nucleus MF transcription factor activity CC plasma membrane CC chloroplast BP transmembrane receptor protein tyrosine kinase signaling
10		20	MF structural constituent of ribosome BP translation CC cytosolic large ribosomal subunit CC mitochondrion CC nucleolus
2		8	CC mitochondrion CC respiratory chain complex I CC chloroplast envelope MF structural constituent of ribosome CC chloroplast thylakoid membrane
3		0	
4		0	
5		0	
6		2	CC mitochondrion BP reciprocal meiotic recombination
7		7	MF transcription factor activity BP leaf development MF transcription activator activity CC nucleus BP regulation of transcription, DNA-dependent
8		16	BP translation MF structural constituent of ribosome CC cytosolic large ribosomal subunit CC chloroplast stroma MF DNA-directed RNA polymerase activity
9		9	CC mitochondrion MF structural constituent of ribosome BP translation BP ribosome biogenesis CC cytosolic large ribosomal subunit

Figure 4.3.4.1C Go terms associated with each motif of the query motifs. Column one displays the motif number, column two contains the motif LOGO, column three shows the number of term predictions and column four displays the top 5 predicted GO terms associated with each motif.

4.4 Discussion

Plant cystatins have recently been implicated to be induced by multiple abiotic stresses such as drought, salt, oxidative and cold stress and are hypothesized to improve tolerance to these stresses (Gutie´rrez-Campos -Campos et al., 1999; Belinghi et al., 2003; Zhang et al., 2008). In light of this, we considered the possibility that the four recently identified novel soybean cystatin gene GmCYS1p626 and three of its homologues (Glyma20g08800, Glyma14g04250 and Glyma18g12240) might also play crucial roles in plant defense mechanisms under abiotic stresses.

As an initial step towards understanding the molecular mechanisms regulating cystatin gene expression in response to these stresses, a bioinformatics analysis of soybean cystatin *cis*-regulatory elements and their roles in mediating abiotic stress responses was carried out. To date, very limited stress-related data for soybean cystatin genes is available which would assist in selection of stress-responsive regulatory elements for functional analysis studies. Therefore, it was essential to identify previously characterized proteins that are (closely related) with the soybean cystatin proteins as an attempt to glean information about these proteins.

Toward this goal, an online web tool (STRING) was used to obtain gene co-expression networks to extract information on the group of proteins that are 'functionally' related or co-regulated with the proteins of interest (Mering et al., 2003; Szklarczyk et al., 2011). AtCYSb, a cysteine proteinase inhibitor implicated in endogenous regulation of protein turnover and defense mechanisms against insects, pathogens and cell death processes (Hwang et al., 2009) was identified as a closest homologue to all four soybean cystatin proteins. Thus, it was used as

a driver protein to perform protein-protein interaction networks. Ten *Arabidopsis thaliana* proteins were identified as the highest ranked functional interacting partners to AtCYb based on co-expression data (**Figure 4.3.1.1**). Most of the identified proteins have been indicated to be involved in signal transduction, transcription and biotic or abiotic stress responses, including salt, drought, oxidative and osmotic stresses (Chen and Griffins, 1999; Levine et al., 1999; Suzuki et al., 2005; Singh et al., 2012). The fact that AtCYb contains two cystatin-like domains (Benchabane et al., 2010) found in soybean cystatin proteins which are known to play a role in plant stress responses and developmental processes, confirms that the identified co-regulated proteins belong to the same group of plant cystatins and might therefore contain similar *cis*-acting elements involved in abiotic (salt) and nitric oxide (NO) gene expression.

To screen these sets of co-regulated proteins for any common function(s), GO annotation and enrichment analyses for the description of the biological process, molecular function and cellular component of gene products was carried out (Poole, 2007). Similar GO terms shared amongst these proteins were identified. GO annotation provided for each category revealed that most of the identified co-regulated proteins were either localized in the nucleus, cytosol or other cytoplasmic or intracellular components. A similar pattern in localization was observed for multicystatins from potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) and rice (*Oryza sativa*) (Madureira et al., 2006; Prins et al., 2008; Nissen et al., 2009), thus indicating the similarity of these co-regulated proteins to well characterized plant cystatins. However, the subcellular localization of most plant cystatins is still unknown. Furthermore, six of the co-regulated proteins namely, (At2g42680, At3g12490, At3g17020, At3g515000, At3g07470 and At5g10860) were indicated to be involved stress responses, either abiotic or biotic as indicated

in **Table 4.3.1.1**. This result confirms that these soybean cystatin genes also play crucial roles in plant stress responses. However, the mechanisms underlying the activation of defense responses by cystatins remain unclear.

A sensible approach to understand the regulatory networks which control gene expression under abiotic stresses was to search for transcription factor binding motifs (TFBM) in promoter regions of co-expressed proteins (Nakashima et al., 2007; 2009). Studies have demonstrated that the expression of stress-induced proteins is mostly regulated by specific transcription factors. These TFs greatly enhance tolerance to various abiotic stresses such as salinity, cold, osmotic, light and drought (Dubouzet et al., 2003; Vannini et al., 2004; Nakashima et al., 2007; Xiang et al., 2008; Hu et al., 2008; Song et al., 2011).

Given that these co-expressed proteins are presumed to share some similarities with the soybean cystatins in their regulatory mechanisms, their promoter regions might contain common TFBM as a result of co-ordinated action of transcription factors. This analysis identified thirty one transcription factors (TFs), with fourteen TFs highly enriched (E-value <1) in the promoter sequences of more than three of the co-expressed proteins (**Table 4.3.3.1**). These included significant TFs which have been previously identified to be responsive to abiotic stresses such as MYB1AT, W-box promoter motif, DRE core motif, MYB4 binding site motif, AtMYC2 BS in RD22 and ABRE-like binding site motif (Abe et al., 1997; Saibo et al., 2009; Nakashima et al., 2009).

Overexpression of these stress-inducible TFs is known to induce better tolerance to salt and freezing. Taken together, these results suggest that the expression of these proteins in abiotic-

stress tolerance is regulated by the same or similar TFs. Since studies have indicated that TFBMs are highly conserved among orthologous and co-regulated proteins (Shannan et al., 2005; Nain et al., 2011) and given the fact that these proteins have been identified to be involved in similar regulatory pathways with soybean cystatin proteins; it can be hypothesized these TFs may be co-acting either directly through physical contact or indirectly through additional factors in the regulation of soybean cystatin expression in stress tolerance.

These observations prompted the search for novel motifs and possible associated transcription factors that can explain the co-regulation among these proteins and their significance in plants with the integration of GO annotation and comparative sequence analysis. This was aimed at identifying enriched motifs, some similarities between known regulatory motifs and their functional roles. Ten highly conserved motifs were identified in DNA (promoter) sequences of co-regulated proteins through MEME. GO term analysis revealed that seven of the ten motifs carry structural, regulatory or biological significance. However, three motifs had no GO terms associated to them, indicating that these might be *de novo* (novel) motifs. It is highly possible that these motifs might be involved in similar regulatory and biological processes.

Finally, integration of stress responsive *cis*-motif(s) annotation and comparative sequence analysis and GO annotations with Arabidopsis (co-regulated proteins) stress responsive TFs allowed for the prediction of soybean cystatin TFs. This was based on the existence of major stress responsive *cis*-elements and associated stress-responsive GO term(s) which have been previously reported (Abe et al., 1997; Saibo et al., 2009; Nakashima et al., 2009). However, more detailed functional studies on abiotic-stress-inducible TFs are necessary for a better

understanding of the complex regulatory gene networks underlying plant cystatin stress responses.

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Summary and Perspectives

Climate change has led to adverse environmental conditions such as prolonged drought, flooding and temperature extremes, which have resulted in salinization (high salt levels caused by either natural processes such as mineral weathering or artificial processes such as irrigation) of soil and have severe negative impact on plant growth. The consequence of these unfavorable conditions in plants is a drastic decrease in crop yield, posing a serious challenge for agricultural production worldwide. This threatens to severely reduce food security for the increasing world population and cause severe economic losses. Nonetheless, unraveling the molecular, physiological and biochemical responses of plants to environmental stress conditions and identification of the plant regulatory pathways responsible for stress adaptation or tolerance still remains a challenge. To date, significant achievements have been made in understanding gene function during abiotic and/or biotic stress conditions. However, current technologies for combating the effects of stress on plants have had limited capacity to improve plant tolerance to abiotic stress. Consequently, the focus should be on developing plant varieties with improved tolerance against these abiotic stress factors and the use of genetic engineering to alter the level of expression of plant genes that confer tolerance to abiotic stress.

In this study, a novel soybean cystatin gene Glyma20g08800 was shown to be up-regulated by application of a chemical that releases nitric oxide (10 μM DETA/NO) into plant tissue but down-regulated by salt stress (150 mM NaCl). This study has also shown elevated caspase-like activity during salt stress which corresponds to suppressed expression of Glyma20g08800 and elevated cell death. Thus, this raises the hypothesis that sufficient expression of

Glyma20g08800 may prevent salt stress-induced cell death by blocking the caspase-like activity. This is supported by the fact that the levels of expression of Glyma20g08800 return towards those of unstressed plants if the stressed plants are supplemented with 10 μM DETA/NO. The level of caspase-like activity and cell death in these DETA/NO supplemented salt-stressed plants returns towards that of unstressed plants. Furthermore, integration of sequence analysis, *cis*-regulatory motifs and GO annotations with the stress-responsive Arabidopsis TFs allowed for prediction of the TFs present in a novel soybean cystatin gene GMCYSP626 and three of its homologues (Glyma20g08800, Glyma14g04250 and Glyma18g12240) based on co-expression analysis. Several well-known abiotic stress-responsive regulatory elements were identified within co-expressed proteins and these were predicted to be the main regulators of cystatin expression in response to abiotic stresses. However, the predicted stress-responsive function of the identified TFs shall be confirmed by experimental studies. More detailed studies on abiotic stress- and NO-inducible TFs within promoter regions of plant cystatins are necessary for better understanding of the complex regulatory gene networks underlying NO and plant cystatin stress responses.

Overall, these results indicate that overexpression of plant cystatins under salt stress conditions may prevent salt stress-induced PCD by inhibiting the activity of caspase-like cysteine proteases (main executers of salt stress-induced PCD), thus suggesting that it is the ratio of caspase-like cysteine proteases ad cystatins which determines a plant response to salt stress. This study also raises the possibility of using nitric oxide-generating compounds to up-regulate the expression of plant cystatins to levels that are sufficient to inhibit salt stress-induced caspase-like activity, which will inhibit salt stress-induced cell death and thus enhance the tolerance of the plant to salt stress and possibly tolerance to drought stress as well.

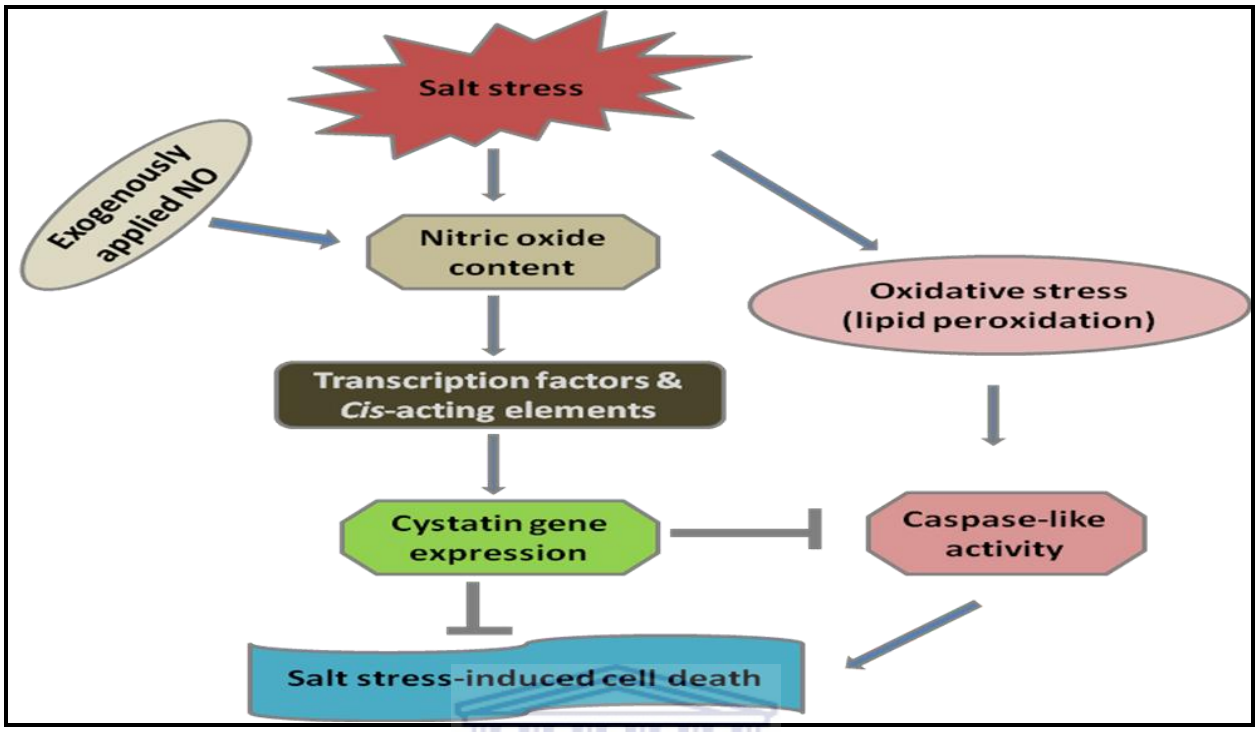

Figure 5.1 A simply model for nitric-oxide induced cystatin expression under salt stress conditions.

Nonetheless, use of genetic engineering to alter nitric oxide synthase and cystatin gene expression whose gene products may confer salt tolerance in plants merits further studies. This includes the use of abiotic stress-inducible promoters to up-regulate the expression of a nitric oxide-synthesizing gene and nitric oxide-inducible promoters to up-regulate the expression of cystatin genes, so that plants containing this genetic alteration produce higher levels of nitric oxide in response to salt stress than plants that do not have the genetic enhancement. This would lay a foundation for studying the salt tolerance of the transgenic plants and transferring this technology to crop plants of importance worldwide.