CHARACTERIZATION OF A NOVEL SOYBEAN CANDIDATE GLUTATHIONE PEROXIDASE/THIOREDOXIN-DEPENDENT PEROXIDASE UNDER SALT STRESS

By

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Thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae

as in the Department of Biotechnology of University of the Western Cape

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ABSTRACT

Characterization of a novel Soybean candidate Glutathione Peroxidase/Thioredoxin-dependent Peroxidase under salt stress

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The production of reactive oxygen species (ROS) is prominent in all aerobic metabolisms including plants. For this reason, the redox homeostasis of the production and scavenging of these intermediates is imperative for growth, development and survival during unfavourable conditions. In this study, a putative glutathione peroxidase gene (*Glyma17g34110*) from *Glycine max* (soybean) was identified and analyzed. The successful characterisation of Glyma17g34110 provided evidence of it being a glutathione peroxidase using glutathione as its preferred electron donor and substrate. Furthermore, it is known that antioxidant enzymes such as GPX exist in various tissues, performing a diverse set of functions. By a bioinformatic analysis of Glyma17g34110 and its promoter region, it was indicated that Glyma17g34110 could be a putative chloroplast protein that could play an important role in photosynthesis.

One of the major factors affecting plant growth and development worldwide is abiotic stresses such as salinity. In the presence of salinity the production of harmful ROS is increased, resulting in detrimental reactions with important biological features (DNA, protein and lipid membranes), leading to cell death. The analysis of *Glyma17g34110* under salt stress revealed that it is a salt sensitive gene and thus, the down-regulation of *Glyma17g34110* could be due to the lack of known defence and response *cis*-acting elements present in the promoter region. Furthermore, it was proven in previous studies that the application of exogenous nitric oxide (NO) increases the activity of antioxidant enzymes. In this thesis it

was observed that the presence of exogenously applied NO increased the expression of *Glyma17g34110* tremendously in all soybean tissues (leaves, roots and nodules) investigated. Studies have found numerous *cis*-acting elements to be NO responsive, however, none of these elements were found in the promoter region upstream of glyma17g34110. This suggests that novel *cis*-acting elements could be present in the promoter region of Glyma17g34110. Thus, increasing the expression of Glyma17g34110 during salinity in the presence of NO, as well as the identification of these novel *cis*-acting elements, could lead to the enhancement of the defence mechanisms against ROS, which could lead to increasing plant tolerance to stress.

November 2012

DECLARATION

I declare that '*Characterization of a novel soybean candidate glutathione peroxidase/thioredoxin-dependent peroxidase under salt stress'* is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Ruqaiyah Adams Signed.....

November 2012

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CONTENTS

LIST OF ABBREVIATIONS

CTAB	cetyl trimethylammonium bromide
DETA	diethylenetriamine
DETA/NO	2,2'-(hydroxynitrosohydrazono)bisethamine
E. coli	*Escherichia coli*
ETC	electron transport chain
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GST	glutathione-S-transferase
H_2O_2	hydrogen peroxide
IPTG	isopropyl β-D-1-thiogalactopyranoside
KI	potassium iodide
LiCl	lithium chloride
MDA	malondialdehyde
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate

NBT	nitro blue tetrazolium
NO	nitric oxide
O_2^-	superoxide
OH^-	hydroxyl radical
PBS	phosphate buffer saline
PCR	polymerase chain reaction
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
TBA	thiobarburic acid
TCA	trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TPX	thioredoxin peroxidase
Trx	thioredoxin

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

LISTS OF TABLES

Chapter 2

Chapter 4

AIMS OF STUDY

The study aimed to investigate the following:

1. Investigate a putative glutathione peroxidase gene (Glyma17g34110) within *Glycine max* by an *in silico* analysis and spatial expression.
2. Determine the effects of exogenously applied nitric oxide on the expression of *Glyma17g34110.*
3. Investigate the antioxidant mechanism with attention to *Glyma17g34110*, reactive oxygen species and cell death in the response to salt stress.
4. Establish whether Glyma17g34110 is a glutathione peroxidase or thioredoxin-dependent peroxidase gene.

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

The world's population is estimated to exceed nine billion people by 2050 (Balch, 2012). Thus, to support the increase in the population worldwide, the production of foods will have to rise tremendously (Foley, 2011). However, adequate food supply is threatened by a decline in crop yield by about 30% each year mainly caused by abiotic and biotic stress. Abiotic stress such as salinity causes a wide range of damage to plants/crops affecting it morphologically, physiologically and biochemically (Anjum *et al*., 2011). Salinity is the increase of salt within the soil causing toxic ion stress due to accumulation of Na^+ and Cl^- ions, as well as osmotic stress, which is characterized as the loss of water within the cell (Hasegawa *et al*., 2000; Manajan & Tuteja, 2005; Munns 2002; Türken & Demiral, 2009). The effects of osmotic and toxic ion accumulation within the plant includes the disruption of ion homeostasis, photosynthesis, respiration, nitrogen fixation, inhibition of plant growth by the limitation of reproductive tissues and eventually cell death (Golldack, Luking, & Yang, 2011; Manaa, 2011; Miller *et al.*, 2010; Paridaa & Das, 2005).

One of the important crops being affected by salinity is soybean. Soybeans are an important crop used as a source of food and income in many developing countries. Over the years, soybean has been considered one of five important grains together with wheat, barley, millet and rice. Many plants and crops like soybean have developed biochemical and molecular mechanisms to cope with the detrimental effects of abiotic stress (Manajan and Tuteja, 2005; Munns, 2002). These mechanisms include the maintenance of ion homeostasis, modification of responses to osmotic stress and the metabolic and structural adaptations. A well known example of the response to osmotic stress is the production of molecules such as reactive

oxygen species (ROS) (Trachootham *et al*., 2008; Türken and Demiral, 2009; Phang *et al.*, 2008).

ROS are highly reactive oxygen intermediates generating from the transfer of 1 or more electrons to an oxygen molecule. These molecules can exist as free radicals, including superoxide radicals (O_2^-), hydroxyl radicals (OH^-) as well as non-radicals such as hydrogen peroxide (H_2O_2), amongst others (Thannickal and Fanberg, 2000; Turrens, 2003). Under normal conditions, ROS are found at low levels within various cellular organelles, for instance the chloroplast, mitochondria and peroxisomes, and are constantly produced as by-products of various metabolic pathways. ROS molecules more importantly serve as signalling molecules for cellular responses to developmental and environmental stimuli (Gill & Tuteja, 2010; Miller *et al*., 2010). Under stress, however, the levels of these molecules increase thus resulting in the reactions with cellular components causing oxidative damage to DNA, proteins, lipids and ultimately resulting in cell death (Guerin *et al*., 2001; Miller *et al*., 2010). Thus, to keep these ROS at low levels, antioxidant mechanisms of enzymatic and non-enzymatic defence systems are present in almost all organelles within the cell. Glutathione peroxidases are one of many important antioxidant enzymes used to scavenge H_2O_2, lipid peroxides and organic hydroperoxides in different sub-cellular organelles (Gill & Tuteja, 2010; Mittler *et al*., 2011; Sharma, 2012). The balance in the redox homeostasis of ROS molecules plays a major role in how well the plants respond and adapt to stress. Thus, to understand the mechanism of a plants response to abiotic and biotic stress, ROS and plant antioxidant mechanisms needs to be studied to enable improved resistance to these stresses (Manajan and Tuteja, 2005; Mandhania, *et al*., 2006; Mittler, 2002).

The chemistry of ROS signalling has made these intermediates distinct to other signalling pathways as the chemical reactivity; mobility and their interactions with cellular compounds

within cells are distinctive (D'Autréaux & Toledano, 2007). The ability of ROS molecules to generate signals specific to a particular stress, factor or to a specific location within the plant is due to the unique chemical identity, localization and many other components of ROS molecules (Foyer & Noctor, 2005a; Karuppanapandian *et al*., 2011; Mittler *et al*., 2011).

1.2 ROS CHEMISTRY

Reactive oxygen species are independent molecules containing one or more unpaired electrons. These molecules are produced from the reduction of molecular oxygen used in metabolic processes within plants and animals. This can occur from either enzymatic catalysis or by electron leakage via various electron transfer pathways (Karuppanapandian *et al*., 2011; Nordberg and Arner, 2001; Thannickal & Fanburg, 2000). Molecular oxygen in its ground state contains two unpaired electrons, thus accounting for its nonreactive state. The photoexcitation of one electron results in the production of toxic singlet oxygen molecules ($^1O_2$). The reduction of this singlet oxygen produces highly reactive, superoxide radicals that are instantaneously dismutased by antioxidant enzymes or by donating an electron to another superoxide in environments with low pH (Arora *et al*., 2002; Bergendi *et al*., 1999; Bhattacharjee, 2005). Both these reactions lead to the formation of hydrogen peroxide (H_2O_2) molecules. Hydrogen peroxide is a stable, non-free radical that is able to diffuse across membranes, unlike other ROS molecules. In the presence of transition metals, such as Fe^{2+} and Cu, these molecules undergo a Fenton reaction producing hydroxyl radicals (OH^-) (Karuppanapandian *et al*., 2011; Nordberg and Arner, 2001 Thannickal & Fanburg, 2000). The reactivity of OH^- with biological compounds causes immense damage that leads to cell death. To prevent the production of OH^- radicals, an NADPH-dependent mechanism (ascorbate-glutathione cycle) of antioxidants (enzymatic and non-enzymatic) are used in the

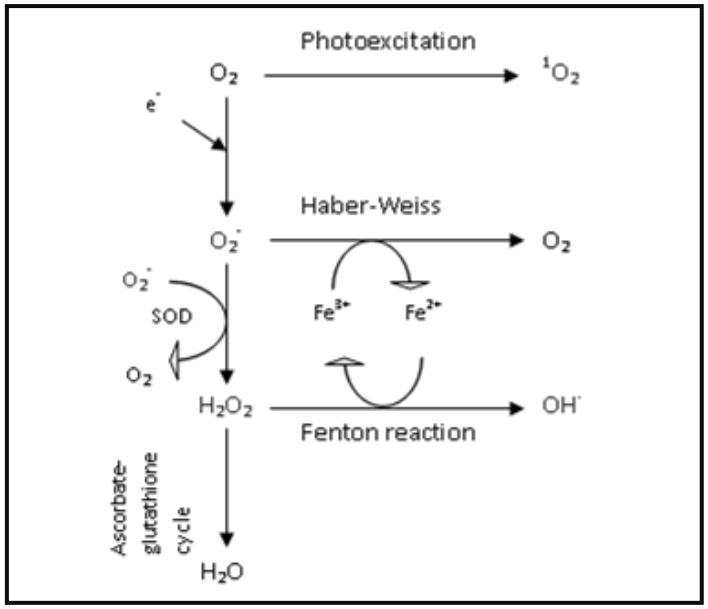

Figure 1.2 The generation and scavenging of ROS molecules from molecular oxygen and the different reactions that take place within a cell. This diagram was adopted from Temple *et al.* (2005).

scavenging of H_2O_2 molecules to water molecules (Bergendi *et al*.,1999; Gill & Tuteja, 2010; Temple *et al*., 2005).

1.2.1 Singlet Oxygen ($^1O_2$)

Singlet oxygen ($^1O_2$) does not form part of the regular ROS intermediates, as it is not produced from the electron transfer of O_2 but during photoexcitation (Bergendi *et al*., 1999). Chlorophyll pigments are responsible for the absorption of light used in the reaction centres photosystem I (PS I) and photosystem II (PS II), as well as in the light harvesting complexes during photosynthesis. The dispersion of energy during the photosynthetic pathway may result in the conversion of the single state chlorophyll into triple state chlorophyll as consequence of an electron transfer (Arora *et al*., 2002; Gill & Tuteja, 2010). The reaction of this triple state chlorophyll with $^3O_2$ results in the formation of $^1O_2$ molecules. During abiotic stress as such as salinity, the generation of $^1O_2$ molecules is favoured (for reasons such as stomatal closure) and therefore resulting in low concentrations of CO_2 within the chloroplast

(Triantaphylides & Havaux, 2009). Singlet oxygen is the excited electronic state of O_2 and acts as a high reductant in reactions with a variety of biological compounds such as nucleic acids, cellular thiols, lipids and secondary metabolites (Krieger-Liszkay, 2004). These toxic molecules cause immense damage to the photosynthetic systems including the reaction centers. Singlet oxygen is relatively short lived, having a half life of 3 µs and is capable of diffusing through considerable distances during that time. It has been noted that the half life of $^1O_2$ increases in H_2O and polar solvents (up to 4 µs and 100 µs respectively) (Bergendi *et al*., 1999). In a recent study by Gill & Tuteja (2010) it has been shown that $^1O_2$ also benefits plants in resistance to pathogens. The production of $^1O_2$ signals the production of antimicrobial metabolites such as phytoalexins. These substances act as photosensitizing molecules, generating phototoxins to kill pathogens. The levels of $^1O_2$ must be kept under control, and different mechanisms exist within the cell to scavenge $^1O_2$ molecules. These are by the reaction of $^1O_2$ specific compounds producing excited complexes. This results in the dispersion of energy or by an oxidation reaction (Arora *et al.*, 2002; Gill & Tuteja, 2010; Krieger-Liszkay, 2004; Triantaphylides & Havaux, 2009).

1.2.2 Superoxide (O_2^-)

Superoxide (O_2^-) molecules result from the partial reduction or a single electron transfer to an oxygen molecule in the electron transport chain (ETC). Ferredoxin attached to photosystem I (PS I) containing an electronegative potential is able to donate electrons to oxygen, reducing them to O_2^- (Karuppanapandian *et al*., 2011). Superoxide is the first reactive oxygen intermediate to be produced and initiates the production of the other molecules such as OH^- and H_2O_2 (Bergendi *et al*., 1999; Guerin *et al*., 2001). It has been said that 1% of O_2 taken up by the plant are reduced to O_2^- molecules in various subcellular locations and pathways (Gill & Tuteja, 2010). Superoxide molecules are unable to diffuse through membranes and are

relatively reactive in hydrophobic conditions within the cells. The half life of O_2^- is moderately short (2-4 μs) due to its immediate scavenging by antioxidant enzymes such as SOD or reactions with metal ions. In the Haber-Weiss reaction, Fe^{2+} is produced by the reduction of Fe^{3+} by O_2^- which undergoes a Fenton reaction with H_2O_2 to give rise to OH^- molecules, as seen in Figure 1.2 (Arora *et al*., 2002; Bergendi *et al*., 1999;).

1.2.3 Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is produced from the dismutation of O_2^- molecules and has a half life of approximately 1 ms, more than that of most intermediates such as O_2^- and $^1O_2$. Hydrogen peroxide is scavenged by antioxidant enzymes within the ascorbate-glutathione cycle (i.e. glutathione peroxidase and ascorbate peroxidase). High concentrations of H_2O_2, are able to inactivate these antioxidant enzymes by reacting with their thiol groups (Gill & Tuteja, 2010). Although the reaction with H_2O_2 causes damage to the cells, it is also produced to play an important role as a regulator in physiological, molecular and biochemical pathways (photosynthesis and program cell death) (Quan *et al*., 2008). Due to its diffusible nature, H_2O_2 acts as a secondary messenger in various signalling pathways for signals generated by ROS, thus resulting in a selective induction of defence genes. Hydrogen peroxide has also been demonstrated to be a component in hormonal signalling (Neill *et al*., 2002). Studies involving abscisic acid (ABA), a hormone in responsible for the opening and closing of the stomata during stress, have demonstrated that H_2O_2 acts as a downstream component in the signalling pathway involving Catalase 1 (an antioxidant enzyme) induction (Guan *et al*., 2000). In addition, many studies have shown that H_2O_2 plays important roles in plants. Hydrogen peroxide aids in the enhancement of the resistance machinery present within plants by the production of phytoalexins, strengthening of the cell walls, the induction of the expression of antioxidant genes, thus restricting the effects of any microbial infection in

plants and killing of any pathogens in the presence of H_2O_2 (Bergendi *et al*., 1999; Quan *et al*., 2008).

1.2.4 Hydroxyl radical (OH^-)

Hydroxyl radicals (OH^-) are highly reactive intermediates produced from O_2^- and H_2O_2 via the Haber-Weiss mechanism. In the presence of chromatin-bound metals, the reactions with OH^- molecules unavoidably results in protein fragmentation and DNA strand breakage (Gill & Tuteja, 2010; Guerin *et al*., 2001). Hydroxyl radicals can be oxidized within the cells by reactions to an organic molecule. Furthermore, OH^- are said to be involved in the formation of hydroxylated substances which are further oxidized by Fe^{2+} (Arora *et al*., 2002).

1.3 ROS SIGNALLING

The conversion of oxygen to water molecules is prominent in all important biological processes, hence ROS molecules are continuously produced. Although ROS molecules can cause immense damage to the plant, they also play a role as key regulators of biological processes (such as development growth and cell cycle) and as signalling molecules (during important pathways and environmental changes) (Bhattacharjee, 2005; Shao *et al*., 2008). Unlike other signalling pathways, ROS signalling is accomplished by a redox homeostasis of the production and scavenging of ROS molecules (Bergendi *et al*., 1999; Foyer & Noctor, 2005b). During a specific stress, signals are transmitted to the ROS signaling network, resulting in a shift in the balance. These signals are cell or compartment specific allowing the necessary proteins, enzymes and defence pathways to identify and execute (Mittler *et al*., 2006). The response to these stresses involves transcriptional changes and in due course cellular reprogramming. This cellular reprogramming suggests the involvement of retrograde signalling. Retrograde signalling involves signals formed by the over-production of ROS molecules and transferred from the organelle to the nucleus where cellular responses are

taken. This may result either in the protection of the plant against stress or the stimulation of programmed cell death (PCD). Retrograde signalling is used in two instances that may occur within the plant: developmental control of biosynthesis of cellular components and in adjustment to environmental and developmental cues (Bhattacharjee, 2005; Miller *et al*., 2010). Previous studies have shown that the importance of retrograde signalling for the response to abiotic stress and thus could be a key factor to engineering of tolerant crops (Mittler *et al*., 2006).

1.4 ROS REGULATION IN DIFFERENT ORGANELLE LOCATIONS

1.4.1 Chloroplast

Chloroplasts are one of the main organelles to produce ROS molecules. Under normal conditions, the reaction centres present in the thylakoid, photosystems I (PS I) and II (PS II) as well as the ETC are major sites for ROS production (Chang *et al*., 2009; Sharma *et al*., 2012). In the ETC, the occurrence of high oxygen concentrations within the chloroplast unavoidably leads to the production of ROS through electron leakage. Under stress, the availability of CO_2 becomes greatly reduced, enhancing the production of ROS. This is caused by the closure of the stomata as well as the presence of excess exposure to light, resulting in the production of O_2^- at PS I (Karuppanapandian *et al*., 2011). Under normal conditions, the electrons are passed through the photosystems where they are taken to reduce NADP to NADPH. However, under abiotic stress, the ETC is overloaded with electrons and these are thus instead taken to ferredoxin, reducing it to superoxide (O_2^-) as seen in Figure 1.4.1 (Asada, 1999; Edreva, 2005). In 1951, Mehler discovered the photoreduction of oxygen (O_2) to H_2O_2 in PS I of the thylakoid (Gill & Tuteja, 2010). It was later learned that O_2 was actually reduced to O_2^- which was subsequently reduced to H_2O_2 and thus ultimately reduced to water. It was there after discovered that Q_A and Q_B provided sites of electron leakage

during the electron transfer to PS II (Sharma *et al*., 2012). The reductions of these ROS are performed by the activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX) respectively. These antioxidant enzymes are the first line defence mechanisms and are bound to the area of the PS I. Antioxidants found in the stroma of the chloroplast, such as GPX, acts as the secondary defence mechanisms (Asada, 2006; Karuppanapandian *et al*., 2011; Miller *et al*., 2010). These mechanisms play a critical role in the ascorbate-glutathione cycle. This cycle prevents the diffusion of H_2O_2 into the stroma where it may interact with other molecules, producing hydroxyl radicals (OH^-) and thus ultimately causing damage to important biological molecules leading to cell death (Asada, 1999). Photosynthesis absorbs carbon dioxide to produce O_2; these O_2 molecules can take up electrons flowing within the photosystems thus producing O_2^-. In the ascorbate-glutathione cycle, O_2^- is scavenged by superoxide dismutase (SOD) to H_2O_2 which is ultimately reduced by APX in the reduction of ascorbate (AsA) to monohydroascorbate (MDA). Monohydroascorbate can be reduced back

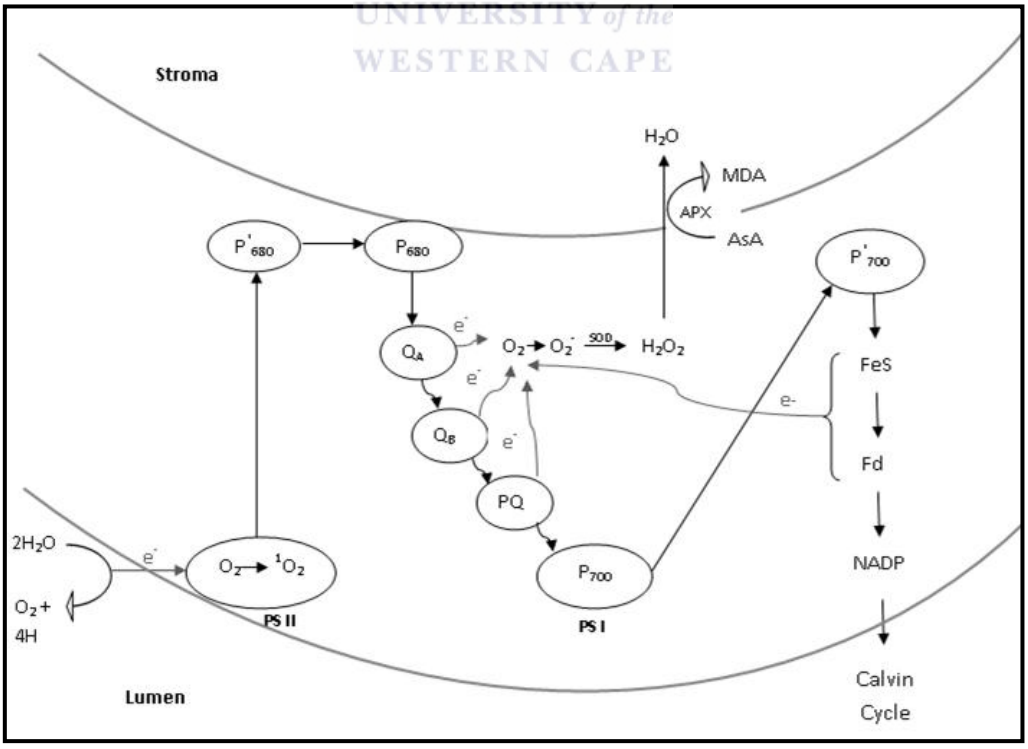

Figure 1.4.1 The non-cyclic photosynthetic pathway and the electron (e^-) leakage in the ETC (Q_A, Q_B, PQ, FeS, Fd) contributing to the production of ROS. Superoxide dismutase is scavenged by superoxide dismutase (SOD) and reduction of H_2O_2 to water in the ascorbate-glutathione cycle. Adopted from Edreva (2005).

to AsA in the presence of ferredoxin, if not, to dehydroascorbate (DHA) catalyzed by dehydroascorbate reductase (DHAR). The conversion of DHA back to AsA is done utilizing GSH as an electron donor. The oxidation-reduction of GSH favours the detoxification of H_2O_2 to water by GPX (Chang *et al*., 2009; Miller *et al.*, 2010). The production of ROS molecules in chloroplasts is affected by not only environmental factors, but also physiological ones. During photosynthesis, the overload of protons present in the membranes restrains the interaction of O_2^- with electron donors in PS I (Asada, 2006). This disables the dispersion of O_2^- out of the membrane, whereas H_2O_2 diffuses easily and rapidly. Through this, the reduction of O_2 is increased, thus the O_2^- mediated cyclic electron flow suppresses the release for the O_2^- out of the membranes, protecting the cells from oxidative damage. In the PS II the oxidation of the electron acceptor produces O_2^-. (Asada, 1999; Karuppanapandian *et al*., 2011).

Previous studies have shown the control of ROS homeostasis in chloroplasts is critical for the survival of transgenic cultivars during drought and salt stress. It was mentioned that the deficiency of antioxidant enzymes in the chloroplasts made these plants more susceptible to abiotic stress (Chang *et al*., 2009).

1.4.2 Mitochondria

A process such as cellular respiration catalyzes the reduction of oxygen for the production of ATP and other important products. Sometimes, partial reduction or electron transfer to O_2 takes place leading to the formation of O_2^- which eventually leads to the development of H_2O_2 and other reactive intermediates (Karuppanapandian *et al*., 2011; Møller, 2001). In the mitochondria, the electron transport chain (mtETC) consists of complexes (complex 1 and III) that play a huge role in the production of ROS and in signalling pathways. These ROS molecules can be produced in different locations within the mitochondria: the matrix, inner as

well as outer membrane (Foyer & Noctor, 2003; Møller, 2001; Sharma *et al*., 2012; Turrens, 2003). As a result of an increase in cellular respiration, the mitochondrial electron transport chain (ETC) transfers electrons directly to the O_2 molecules absorbed, thus causing the formation of O_2^-. Complex I and III are said to be one of the main sources of ROS generation in the mitochondria, this may be due to the ubisemiquinone intermediates situated at these complexes (Mackenzie & McIntosh, 1999; Rhoads *et al.*, 2006; Sharma *et al*., 2012). These complexes were discovered to be the basis of electrons donating to O_2 molecules. The O_2^- radicals produced here are directly scavenged by MnSOD (SOD containing a manganese active site) to H_2O_2 (figure 1.4.2). It was suggested that 1 to 5% of the O_2 taken up for cellular respiration is reduced to form O_2^- molecules under normal conditions, these levels tend to elevate with abiotic stress (Gill & Tuteja, 2010). Excess H_2O_2 produced by the scavenging of O_2^- by SOD undergoes a Fenton reaction with Fe^{2+} to form OH^-. These OH^- molecules diffuse through the mitochondrial membrane, reacting with lipids causing lipid peroxidation and therefore cell death. Glutathione peroxidase plays an important role in the scavenging of these OH^- and membrane lipid peroxides (Rhoads *et al*., 2006; Turrens, 2003; Yang *et al*., 2006). The mitochondria also play a vital role in the adaptation of cells to stress. A study has shown that once the complex I undergoes stress, its signalling pathways initiate the enhanced expression of antioxidant enzymes and thus increases the antioxidant capacity throughout the cell (Foyer & Noctor, 2003; Karuppanapandian *et al*., 2011; Møller, 2001).

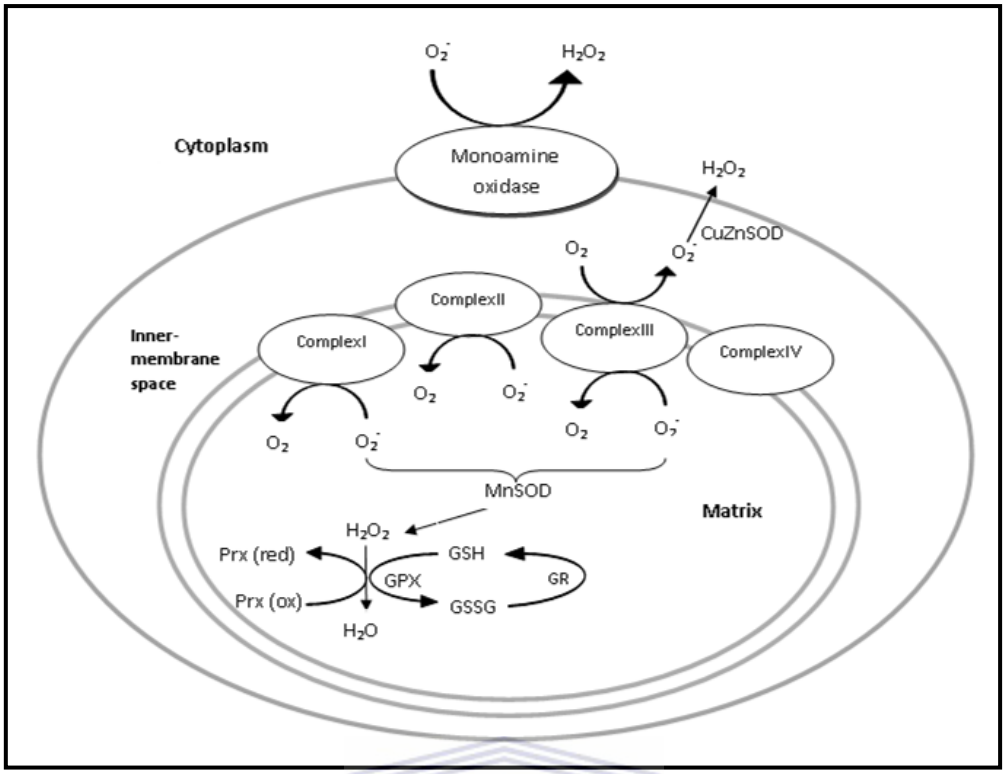

Figure 1.4.2 The production and scavenging of O_2^- to H_2O_2 by SOD within the matrix and inner-membrane space of the mitochondria. The H_2O_2 produced are scavenged in the ascorbate-glutathione cycle by antioxidants such as GPX. Modified from Turrens (2003) and Murphy (2009).

1.4.3 Peroxisomes

Peroxisomes are single lipid bilayer membranes that are one of the major sources of H_2O_2 as well as O_2^- generation within a cell during normal metabolic processes. There are many pathways within various types of peroxisomes found throughout the plant which contribute too much of the H_2O_2 production (Del Rio *et al*., 2002; Karuppanapandian *et al*., 2011; Sharma *et al*., 2012). These include glyoxysomes, specialized peroxisomes, that uses fatty acid β-oxidation in the storage of fatty acids, as well as flavin oxidase reactions, and photorespiratory glycolate oxidase in other parts of the plants to perform necessary functions. These components used by glyoxysomes are additional sources of H_2O_2 production (Del Rio *et al*., 2003; Del Rio *et al*., 2006; Gill & Tuteja, 2010). Under stress conditions, the availability of CO_2 decreases and results in the increase of glycolate production in chloroplasts. During photorespiration in the peroxisomes, the oxidation of the glycolate by

glycolate-oxidase results in majority of the ROS produced in this organelle. Like other organelles, the production of O_2^- occurs within its normal metabolism processes (Corpas *et al*., 2003; Del Rio *et al*., 2002). Nonetheless, two sites within the peroxisomes have been identified as to the main sources of O_2^- production; the organelle matrix, in which xanthine and hypoxanthine are oxidized to form uric acid and in the peroxisomal membranes. The disproportionation of O_2^- is catalyzed by SOD and various pathways generating O_2^- radicals such as the oxidation reaction of xanthine and hypoxanthine by xanthine oxidase as well as through the ETC in the membrane (Figure 1.4.3). The ETC that exists within the peroxisome membrane is composed of three integral polypeptides (Del Rio *et al*., 2006; Miller *et al*., 2010). In recent studies, three membrane polypeptides were identified. These polypeptide membrane proteins included PMP18 (18 kDa), PMP32 (32 kDa) and PMP29 (35 kDa), and were concluded to be of the main O_2^- producers in the peroxisomes (Figure 1.4.3). PMP18 and 32 use NADH as the electron donor in the formation reactions of O_2^-, whereas PMP29, a NADPH-dependent protein, reduces cytochrome c in the peroxisome (Corpas *et al*., 2003 ; Del Rio *et al*., 2003; Del Rio *et al*., 2006; Sharma *et al*., 2012).

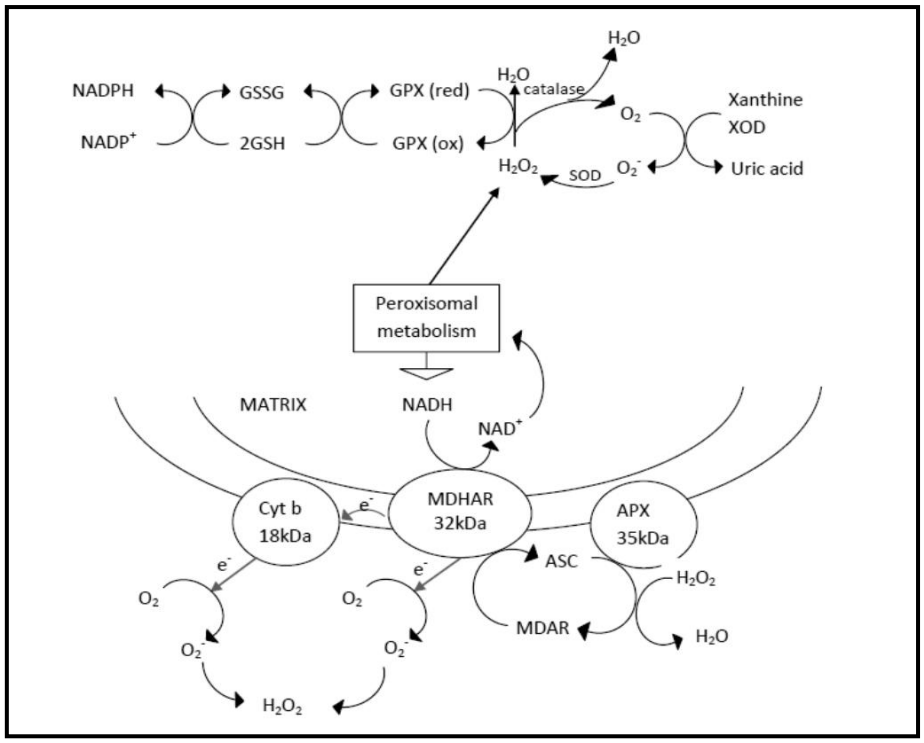

Figure 1.4.3 The production of ROS and its scavenging via glutathione peroxidase in leaf peroxisomes. The glutathione-ascorbate cycle comprises of different enzymatic and non enzymatic antioxidants which are used to scavenge these reactive molecules during peroxisomal metabolism. Adopted from Del Rio *et al*. (2003).

1.5 EFFECTS OF ROS IN ABIOTIC OR BIOTIC STRESS

1.5.1 Lipid Peroxidation

The increase of ROS during abiotic and biotic stress causes immense damage to the cell, leading to cell death. The peroxidation of lipid membranes by ROS deteriorates important membranes, thus resulting in the decrease of membrane fluidity and permeability as well as damaging important membrane proteins, receptors, enzymes and ion channels (Bor *et al*., 2003; Khan & Panda, 2008). The process of lipid peroxidation entails three stages: initiation, progression and termination. The initiation involves metals that start the reactions of lipid peroxidation. These metals, such Fe and Cu, form alkoxy or peroxy radicals in the decomposition of lipid peroxides, stimulating the reactions of the progression and termination stages of lipid peroxidation (Buege & Aust, 1978; Bhattacharjee, 2005). The initiation process entails the extraction of a hydrogen atom by a ROS molecule such as OH^- from a

fatty acyl chain at the centre carbon of a polyunsaturated fatty acid giving rise to a lipid peroxy radical (Bor *et al.*, 2003). The lipid peroxy radical undergoes a further hydrogen atom extraction on adjacent side chains in the polyunsaturated fatty acid in the propagation phase (Gill & Tuteja 2010). The derivatives of these reactions are thereafter broken into various products such as epoxides, hydroperoxides, glycol and aldecydes. Lipid peroxidation leads to the damage of other components of the cell such as DNA and proteins (Bhattacharjee, 2005; Tuteja *et al.*, 2001).

1.5.2 Protein Oxidation

The oxidation of proteins is an irreversible reaction mediated by ROS molecules, resulting in protein modifications, thus changing their activity and making them more vulnerable to attack (Sharma, 2012; Trachootham *et al.*, 2008). In the oxidation process, the amino side chains give rise to a carbonyl derivative; this process is known as carbonylation. These processes have been shown to be more prominent during salinity and drought stress than other abiotic stresses. These ROS molecules often target proteins containing sulfur amino acids and thiol groups (Bartosz, 1997; Gill & Tuteja, 2010). These sulfur-containing proteins can result in either reversible or irreversible oxidation. Reversible reactions include the oxidation of sulfhydral groups, resulting in glutathionylation and cross links, whereas irreversible reactions entails nitrosylation of proteins, thus inactivating or disrupting important processes and components, resulting in cell death (Rhoads *et al.*, 2006; Trachootham *et al*., 2008).

1.5.3 DNA Damage

Spontaneous DNA damage is occurs from the reactivity with ROS molecules and DNA molecules, affecting many components of the cell. This type of damage to the DNA involves base deletions, strand breaks, cross links, the alkylation and oxidation of DNA bases (Gill &

Tuteja, 2010; Rhoads *et al.*, 2006). These damages to the DNA affect growth and development and can result in plant cell death. Hydroperoxides, highly reactive intermediates produced by the reactions of H_2O_2 and OH^- with lipids, amino acids and proteins can cause immense damage to the DNA (Guerin *et al*., 2010). These hydroperoxides are able to oxidize radicals containing a centred oxygen or carbon in the presence of metals such as Cu^+ (Tuteja *et al.*, 2001; Rhoads *et al.*, 2006). Cross linking of DNA bases occurs in reactions between these hydroperoxides and pyrimidine DNA bases. Thus, to protect itself, various recovery mechanisms exist within the plant to repair damage done to the DNA by reactive intermediates and other harmful components. These mechanisms include the direct reversal, thus removing DNA damage using a particular enzyme (Gill & Tuteja, 2010; Tuteja *et al*., 2001).

1.6 ROS SCAVENGING ANTIOXIDANTS, WITH FOCUS ON THE GLUTATHIONE PART OF THE ASCORBATE-GLUTATHIONE CYCLE

Exposure to salinity and drought increases the production of reactive intermediates such as H_2O_2, O_2^-, and more damaging molecules such as OH^-. Thus, to protect plants against these intermediates, a defence mechanism of enzymatic and non-enzymatic antioxidants, present in almost all sub-cellular components within a cell has evolved (Gill & Tuteja, 2010; Miller *et al.*, 2010). Studies have shown that two types of thiol-containing antioxidant enzymes are present within plants and animals (Becana *et al*., 2010; Lee *et al*., 2008). These two groups of antioxidant enzymes share many similarities within their sequences: glutathione and thioredoxin/peroxiredoxins.

1.6.1 Non-enzymatic ROS scavenging

1.6.1.1 Glutathione (GSH)

Glutathione (GSH) to an important non-enzymatic tripeptide used in many processes; both metabolically and physiologically. This tripeptide contains many homologs present throughout the plant. These homologs differ by the replacement of the glycine residue at the carbonyl terminal with another amino acid (Becana *et al*., 2010; Guerin *et al*., 2001). Due to the similarities within these homologs, it was suggested that they all play a similar role within the plant cells (Xiang *et al*., 2001). GSH has been shown to be a powerful reductant and this was suggested to be a result of cysteine residues present at the centre. GSH plays two main physiological functions within plants; the regulation of sulfur uptake by the root and its metabolism and in the defence mechanism against harmful elements. The biochemical functions of GSH are largely due to its thiol group present accounting for its wide range of functions (May *et al*., 1998; Noctor & Foyer, 1998).

In the acclimation of ROS molecules, GSH is able to reduce ROS molecules through the reactivity with its thiol group as well as to oxidize proteins within their cellular components, thus maintaining their proper function (Foyer & Noctor, 2005a). The oxidation – reduction of GSH plays an important part in the redox pathways. GSH exists between two states, oxidized GSH and reduced GSH. The balance of the reduced and oxidized GSH is important in the maintenance of the redox homeostasis used by antioxidant enzymes to aid in the scavenging of ROS in the ascorbate-glutathione cycle. Many studies have shown that increased amount of GSH enhances the antioxidant enzyme activity (Gill & Tuteja, 2010; Guerin *et al*., 2001).

1.6.2 Enzymatic ROS scavenging

1.6.2.1 Glutathione Peroxidase (GPX)

Glutathione peroxidase is a family of isoforms which mainly serves to protect the plant against oxidative damage caused by hydrogen peroxide, organic hydroperoxidase and lipid peroxidases (Beeor-Tzahar *et al*., 1995). These isoforms are found in various tissues within the organism, playing roles specific to the subcellular location and its specific pathway (Wang *et al*., 2012). Phospholipid hydroperoxide glutathione peroxidases (PHGPX) are important antioxidant enzymes as they protect the membrane against oxidative stress. These are membrane-bound proteins that scavenge hydrophospholipids formed in the membranes (Gaber *et al*., 2012; Yang *et al*., 2006). It was discovered that these enzymes differ from the remainder of the GPX family (mostly animal like) as they contain a deletion within their primary structure; this was suggested to be the reason for their affinity for hydrophospholipids instead of H_2O_2. These GPX proteins use electron donors such as GSH to successfully scavenge ROS. In recent studies it was shown that GPX proteins also uses thioredoxin (Trx), as it is a more powerful substrate allowing a more efficient reduction of peroxides (Navrot *et al.*, 2006)

Ramos *et al*. (2009) identified six GPX genes in *Lotus japonicus*. These genes were presumed as GPX-like enzymes as they contained motifs preserved in all plant and animal GPX genes including cysteine residues (CxxC) present at the N-terminus of the catalytic site. These genes were found to originate from different subcellular compartments, and their roles in these compartments were suggested according to their homology. GPX1, 6 and 3 were suggested to play a role in signalling molecules in the mitochondria, chloroplast and cytosol's secretary pathway respectively, due to their poor homology at the N-terminus. The expression of these genes was studied under various responses to factors such as cadmium, aluminum,

nitric oxide and NaCl. Under these conditions, the regulation of these genes varied and it was concluded that GPX 6 played an important role in signalling pathways.

The study of GPX has been conducted extensively in mammals; these GPX proteins contain selenium at the active site, causing them to be highly reactive and efficient antioxidant enzymes in mammals (Tosatto *et al*., 2008). In most plants, the GPX proteins that have been identified so far contain only cysteine residues, thus making their activities lower than in those of animals. In a study of *Chlamydomonas reinhardtii* by Leisinger *et al*. (1999) it was shown that *C. reinhadtii* contained GPX–like genes with similarities to yeast, plant and mammalian genes. The mRNA contained open reading frames with high homology to those of both yeast and plants and a seleno-cys (selenium present at cysteine residue) residue as found in mammalian GPX genes. Genes containing seleno-cys residues in their open reading frames were found to be trameric, whereas those only containing cysteines were monomeric. *C. reinhadtii* was suggested to be a perfect model of GPX study as it contained both GPX genes with seleno-cys and those with cysteine residues at their catalytic sites.

1.6.2.2 Glutathione Reductase (GR)

Glutathione reductase (GR) is an NADPH-dependent oxidoreductase used in the defence mechanisms against ROS molecules. This enzyme can be found in plants and animals and localized in the chloroplast, mitochondria and cytosol. In the ascorbate-glutathione cycle, it catalyzes the oxidation-reduction of an important antioxidant, GSH (Becana *et al*., 2010). This high specific enzyme reduces oxidised glutathione (GSSG) to glutathione (GSH) in the presence of NADPH. Thus, GPX can efficiently catalyze the reduction of peroxides to water molecules. Glutathione reductase is an important enzyme used in many processes of the cellular metabolism, thus it is important for the ratio of GSH: GSSG to remain high thus maintaining cellular redox. Under abiotic stress, if the ratio lowers, the plant suffers from

oxidative stress due to poor performance of antioxidant enzymes as insuficient GSH is present (Gill & Tuteja, 2010; May *et al*., 1998; Miller *et al*., 2010).

1.6.2.3 Thioredoxin and Thioredoxin-dependent peroxidase (TPX)

Thioredoxin-dependent peroxidase (TPX) is of the major enzymatic antioxidants used in the ascorbate-glutathione cycle in the reduction of ROS molecules (Nordberg & Arnér, 2001). Thioredoxin-dependent peroxidase can be found in plants and animals and in various subcellular localizations within a cell (Becana *et al*., 2010). Like glutathione, thioredoxin plays a huge role in many processes and in the defence mechanism against ROS molecules. It is used as a substrate in the reaction catalyzed by TPX or GPX (Dos Santos & Rey, 2006). Studies of thioredoxin have shown that members of this family contain a conserved catalytic site (-Trp-Cys-Gly-Pro-Cys-Lys-) that undergoes reversible oxidation to cysteine disulphide (Trx-S_2) during the reduction of ROS molecules. Oxidized Trx has to be regulated and is thus converted to reduce Trx by an NADPH- dependent Trx reductase. In the chloroplast, Trx is reduced by a ferredoxin-dependent Trx reductase (Arner & Holmgren, 2000; Powis & Montfort, 2001)

1.7 CONCLUSION

The production of ROS is inevitable in any metabolic process. These reactive molecules are present in almost all cellular organelles and play a role as important signalling molecules in the plant as a response to developmental and environmental cues. In the presence of abiotic stress such as salinity, levels of ROS production are amplified. In these cases, as the levels of ROS increase, they cause oxidative damage to the plant by reacting with important biological features such as DNA, protein and lipids, leading to plant death. Thus, plants have developed a mechanism made up of enzymatic and non-enzymatic antioxidants used in the detoxification of these molecules. These antioxidant enzymes scavenge ROS molecules,

however, during stress, as the level of ROS increases, the antioxidant enzyme capacity becomes insufficient and thus the plant cannot be protected resulting in plant death. Therefore, in the future, more studies should be done on the identification of antioxidant genes and the establishment of their roles in the response to drought and salinity. The induction of these ROS scavenging antioxidant enzymes during abiotic stress could result in more tolerant crops.

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CHAPTER 2: SPATIAL EXPRESSION OF GLYMA17G34110 AND THE EFFECTS OF APPLIED EXOGENOUS NO

2.1 ABSTRACT

Glutathione peroxidases (GPX) are one of many antioxidant enzymes found in various organisms such as plants. Although little is known about GPX in plants, it has shown to play multiple roles in mammals. Research has proven that molecules such as nitric oxide (NO) increase the activity of antioxidant enzymes such as GPX resulting in a more effective defence system (by removing harmful H_2O_2), increasing survival during abiotic and biotic stress. Using predictive tools, an *in silico* analysis of a putative *Glycine max* GPX gene (Glyma17g34110) was performed by identifying important characteristic domains and *cis*-acting elements in its promoter region. The gene expression profiles of *Glyma17g34110* were analyzed by semi-qRT PCR to determine the spatial expression in various tissues (leaves, roots and nodules) of soybean as well as the influence of NO on its gene expression. Homology-based bioinformatic studies were done by comparing Glyma17g34110 to GPXs from *Arabidopsis thaliana* (AtGPX) and *Lotus japonicus* (LjGPX). The results showed that Glyma17g34110 is 82% similar to LjGPX6 and that it formed a distinct cluster on a neighbouring joining phylogenetic tree with AtGPX1, AtGPX7 and LjGPX6. From the *in silico* analysis, it was suggested that Glyma17g34110 is a putative chloroplast protein highly expressed within the leaves. The putative promoter region (±1 Kb) upstream of the start site of *Glyma17g34110* was also screened by *in silico* analysis to identify various important regions for regulating *Glyma17g34110*. The study showed that the putative promoter region of *Glyma17g34110* contained various promoter regions involved in light responsiveness and defence as well as regions encoding for plastids (a photosynthetic protein). The semi-qRT PCR expression profiles of *Glyma17g34110* showed it to be highly expressed within the

leaves as compared to roots and nodules thus supporting the *in silico* results. The application of exogenous NO to soybean demonstrated a large increase in the gene expression of *Glyma17g34110* within all tissues (leaves, roots and nodules) examined. *Cis*-acting elements that are responsive to NO were identified in previous studies (Alscher *et al*., 2002; Chen *et al*., 2002; Palmieri *et al*., 2008). In this study, however, none of these *cis*-acting elements identified were present in the promoter region of Glyma17g34110, thus suggesting that novel *cis*-acting elements could be present in *Glyma17g34110* promoter and contributing to *Glyma17g34110* being NO responsive.

2.2 INTRODUCTION

In the late 20th century, nitric oxide (NO) was recognized as 'molecule of the year' by Science magazine for the newly discovered importance that it had in animals. Since then, the interest of nitric oxide in various organisms such as plants has increased tremendously (Ferreira & Cataneo, 2010; Neill *et al*., 2003). Nitric oxide is a gaseous molecule naturally produced within the plant by enzymatic and non-enzymatic mechanisms and contains a diverse set biological functions such as induction of seed germination, regulating maturation and senescence (Beligni & Lamattina, 2001; Popov & Tuan, 2010). It acts as a signal as well as playing a role in various pathways regulating plant growth, development and defence responses. Due to its biological features, NO has been classified as an ideal signalling molecule. Nitric oxide is a small diatomic particle able to diffuse freely through membranes thus activating various processes as well as displaying hydrophobic properties (Durner & Klessig, 1999; Ferreira & Cataneo, 2010; Neill *et al.*, 2003). In contrast to the positive attributes of nitric oxide, excessive nitric oxide can also be harmful to plants (Wodala *et al*., 2010), especially when forming harmful complexes with molecules such as peroxinitrite

($ONOO^-$) nitrogen dioxide (NO_2^-) and dinitrogen trioxide (N_2O_3), also known as reactive nitrogen species (RNS) (Ramos *et al.,* 2009).

Like reactive nitrogen species (RNS), reactive oxygen species (ROS) (O_2^-, H_2O_2 and OH^-) are continuously produced as by-products in plants and act as signalling molecules to environmental and developmental cues (Gill & Tuteja, 2010; Mittler *et al.,* 2011). During abiotic and biotic stress, the accumulation of ROS becomes toxic to the plants, resulting in detrimental reactions with important biological features, leading to cell death and eventually death of the plant. To prevent the accumulation of ROS, plants have evolved antioxidant enzymatic mechanisms such as glutathione peroxidase, ascorbate peroxidase and catalase to regulate the redox homeostasis of these molecules (Chang *et al.,* 2009; Wang & Xu, 2012). It has been shown in previous studies that the activity of these antioxidant enzymes is important for the survival of the plant under abiotic or biotic stress. Stress tolerant plants have demonstrated a higher activity level of antioxidant enzymes in the presence of stress, thus creating an advanced defence against ROS. In contrast, the poor performances of sensitive plants has been linked to the poor defence mechanisms of antioxidant enzymes and their activity which is poorly up-regulated during stress (Asada, 2006; Faltin *et al.,* 2010; Miller *et al.*, 2010).

Thus, researchers have hunted for ways and means to up-regulate antioxidant enzymes to sufficient levels during stress in order to make sensitive plants more tolerant to the stress. Through studies, researchers have shown that NO play important roles in the defence mechanisms by up-regulating the activity of antioxidant enzymes (Ramos *et al.,* 2009). In a study by Murgia *et al*. (2004) it was shown that over-expressing a thylakoid ascorbate peroxidase, increased the resistance to paraquat (herbicide producing O_2^-), but not when exposed to stresses such as metals or high and low temperatures. Thus, during stress, these

transgenic lines would still demonstrate poor performances. However, it was then found that, with the application of exogenous NO (treatment with sodium nitroprusside), these plants showed less damage during abiotic stress. It was concluded that NO played a role in the defence mechanisms against ROS molecules such as H_2O_2 and that it is involved in regulating antioxidant enzymes that scavenge these ROS, lessening the effects of these molecules in plants.

The activities of glutathione peroxidase are important for growth, development and survival of plants. Thus, in this study, a putative glutathione peroxidase gene (Glyma17g34110) was identified and analyzed by bioinformatic analysis of its promoter region and important characteristic domains will be identified and analyzed. The expression profiles of *Glyma17g34110* were examined in different tissues (leaves, roots and nodules) of *Glycine max*, as well as for the effects of exogenously applied nitric oxide on its expression.

2.3 MATERIALS AND METHODS

2.3.1 Bioinformatic analysis

Peptide sequences of Glyma17g34110, 8 GPX genes found in *Arabidopsis thaliana* and 6 in *Lotus japonicus* were analyzed using Phytozome version 7 (www.phytozome.net) and the NCBI databases. These sequences were manipulated and aligned using ClusterW (Thompson *et al.*, 1994) to identify conserved motifs. Shared ancestries amongst these genes were analyzed by a phylogenetic tree constructed on Mega5 (Tamura *et al.*, 2011) via the neighbour joining method. The protein subcellular localization prediction of Glyma17g34110 and its homologues was investigated using online databases such as ChloroP version 1.1 (Emanuelsson *et al.*, 1999; http://www.cbs.dtu.dk/services/ChloroP), TargetP version 1.1 (Emanuelsson *et al.*, 2000; http://www.cbs.dtu.dk/services/TargetP), WoLf PSORT (Horton *et al.*, 2007; http://www.wolfpsort.org) and MultiLoc (Höglund *et al.*, 2006; abi.inf.uni-

tuebingen.de/services/MultiLoc). The domains of this *Glycine max* GPX gene were predicted using Interpro scan version 4.8 (Quevillion *et al*., 2005; http://www.ebi.ac.uk/Tools/pfa/iprscan). An *in silico* analysis of the promoter regions of Glyma17g34110 was performed using the PLANTCARE database (Lescot *et al*., 2002; http://www.oberon.rug.ac.be:8080/PlantCARE).

2.3.2 Plant preparation and growth

Surface sterilization was performed to remove any contaminants present on the seeds. Approximately 100 Soybean (*Glycine max* (L) merr. cv PAN. 626) seeds were washed with 0.35% sodium hypochlorite for 10 minutes thereafter discarding the sodium hypochlorite carefully. The seeds were washed with distilled waster 5 times thereafter soaked in distilled water for 20 minutes and inoculated with *Bradyrhizobium japonicum* (Becker Underwood Ltd). The seeds were grown in filtered silica sand (98% SiO_2, Rolfes® Silica, Brits South Africa) and watered during germination. At the cotyledon (VC) stage, the plants were treated with soya nitrogen-free nutrient solution [1 M $CaCl_2$, 1 M $MgSO_4$, 0.5 M K_2SO_4, 1 M K_2HPO_4, 100 mM FeNa-EDTA, 50 mM H_3BO_3, 10 mM Na_2MoO_4, 2 mM $CuSO_4$, 2 mM $ZnSO_4$, 0.5 mM $CoSO_4$ and 0.5 M 2-(N-Morpholino) ethane-sulfonic acid at pH 6.4] until the V3 stage (3rd nodal stage).

2.3.3 Treatments of plants

Treatments were initiated at the V3 stage (3rd nodal stage) and conducted for 48 hours. Two treatments were applied to the plants to study the effects of exogenously applied nitric oxide on the gene expression of *Glyma17g34110*. Plants were treated with nitrogen-free solution (untreated), nitrogen-free solution containing diethylenetriamine [DETA (10 µM) a control for DETA/NO which lacks the NO moiety] and 2,2'-(hydroxynitrosohydrazono)

bisethanimine [DETA/NO (10 µM), a NO donor]. The plants were harvested, snap-frozen in liquid nitrogen and stored at -80°C until required for further downstream experiments.

2.3.4 Preparation of expression studies of *Glyma17g34110*

2.3.4.1 RNA Extraction

Total RNA was extracted from soybean leaves, roots and nodules from each treatment using a procedure described by Gasic *et al*. (2004). Plant tissues were ground in liquid nitrogen into fine powder and transferred into separate RNAse-free tubes. These samples were mixed with pre-warmed extraction buffer (2% [m/v] CTAB, 2% [m/v] PVP, 100 mM Tris-HCl pH 8, 25 mM EDTA and 2 mM NaCl) containing 2% [v/v] β-Marcaptoethanol. The mixtures were vortexed for 30 seconds followed by an incubation of 2 minutes at 65°C (this step was repeated 5 times). The solutions were centrifuged at 12, 000 X *g* for 10 minutes and the supernatant transferred into new tubes. The supernatant was subsequently mixed with equal volumes of chloroform/isoamylalcohol (24:1) and centrifuged for 8 minutes at maximum speed. Without disrupting the precipitate, the upper (green) layer was transferred into new tubes. The above three steps were repeated, thereafter 2 M LiCl was added and stored overnight at 4°C for RNA precipitation. The following day, solutions were centrifuged for 30 minutes at maximum speed, followed by the discarding of all supernatant. The pellet was washed with 70% [v/v] ethanol and centrifuged for 10 minutes and repeated, thereafter left to dry. The pellet was resuspended in DEPC-treated water together with 20 U Ribolock™ RNAse inhibitor (Fermentas) and stored at -80°C until further use.

2.3.4.2 DNase treatment

Treatment to remove any DNA contamination from extracted RNA was carried out using an RNase-free DNase kit (Fermentas). One microgram of each RNA sample was mixed with 1X

Reaction buffer, 1 U DNAse 1 and made up to a final volume of 20 µl with DEPC-treated water. The solutions were incubated for 30 minutes at 37°C. The respective reactions were terminated by mixing 50 mM EDTA into the solution and incubating for 10 minutes at 65°C. One unit of Ribolock was added to the solution and the samples then stored at -80°C.

2.3.5 Expression studies of *Glyma17g34110*

2.3.5.1 First strand complementary DNA (cDNA) synthesis

First strand cDNA synthesis was performed using a RevertAid™ Reverse Transcriptase kit (Fermentas). Two hundred nanogram of template RNA was used to synthesized cDNA in the following reaction: 20 pmol of the Reverse primer (5'- CGT CTC GAG TCA GGC AGC AAG TAA CTT CTG GAT -3'), 1X reaction buffer, 1 mM dNTPs, 200 U Reverse Transcriptase and made up to a final volume of 20 µl with DEPC treated water. The solution was gently mixed and briefly centrifuged. The reaction was achieved at 60°C for 42 minutes and terminated at 70°C for 10 minutes.

2.3.5.2 Glyma17g34110 gene expression

Standard PCR reactions were applied to amplify *Glyma17g34110* and *β-tubulin* from cDNA using True Start™ Hot Start *Taq* Polymerase (Fermentas). From each sample, cDNA template (1 µl of each) was used for the amplification and mixed with the following reagents: 1X Hot Start *Taq* Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 400 nM each of forward and reverse primer and 1.25 U *Taq* DNA polymerase in a final volume of 50 µl. The following oligonucleotides (10 pmol each) were used for the amplification of *Glyma17g34110*: forward primer (5'- CAC GGA TCC ATG TCC TCC ATG GCT TCC TC -3') and reverse primer (5'- CGT CTC GAG TCA GGC AGC AAG TAA CTT CTG GAT -3') and *β-tubulin* forward primer (5'- CTG CGA AAG CTT GCA GTG AAC C -3') and reverse primer (5'- TCT TGC

CTC TAA ACA TGG CTG AGG -3'). The reactions were performed according to specific parameters, consisting of a single cycle of 95°C for 90 seconds following by 23 cycles of 95°C for 30 seconds, 63°C for 30 seconds, 72°C for 2 minutes and a final cycle of 72°C for 7 minutes. The amplified products were electrophoresed on a 1% [m/v] agarose gel and using Gel Red™ staining. The respective gels were visualised under short UV light and photographed. The densitometry analysis of the agarose gel was analysed using an Alpha Ease FC imaging software (Alpha Innotech Corporation).

2.4. RESULTS

2.4.1 Glyma17g34110 shows homology to the AtGPX and LjGPX families

Peptide sequences of Glyma17g34110 and GPX genes from *Arabidopsis thaliana* and *Lotus japonicus* were obtained from the NCBI and Phytozome databases. These sequences were aligned for bioinformatic analysis using ClustalW alignment software. This analysis showed that all the respective GPX genes contained several characteristic and conserved domains. According to Criqui *et al*. (1992), GPX genes found in plant and animals contained conserved motifs (Figure 2.4.1.1) which include GKVLLI(V/E)NVAS, (E/V)(I/V)L(A/G)FPCNQF(G/L) and WNFxKFLV and a common motif conserved in most GPX genes found in plants, the FTVKD sequence motif. The following residues Cys-120, Cys- 149, Gln-151 and Try-209 (AtGPX1 numbering) were present in all the genes and were proposed in previous studies to be part of the catalytic site (Ramos *et al*., 2009). The presence of Cys-120 indicates that none of the genes are selenium-dependent proteins as found in mammals (Fu *et al*., 2002). From the amino acid sequences obtained and aligned, a phylogenetic tree were constructed to deduce the evolutionary relationships of Glyma17g34110 and GPX genes from *A. thaliana* and *L. japonicus* based on the similarities and differences within their sequences (Figure 2.4.1.2). The phylogenetic tree revealed two

groups, namely 1 and 2, each containing genes of a similar ancestry. In Group 1, Glyma17g34110 was revealed to have a high similarity with LjGPX6 which was supported by a BLASTp search on NCBI and showed an 82% similarity. Furthermore, Glyma17g34110 shares similarity with AtGPX7 of about 78% and AtGPX1 of about 73%. The majority of the differences between Glyma17g34110, AtGPX1, AtGPX7 and LjGPX6 can be seen in the N-terminal region.

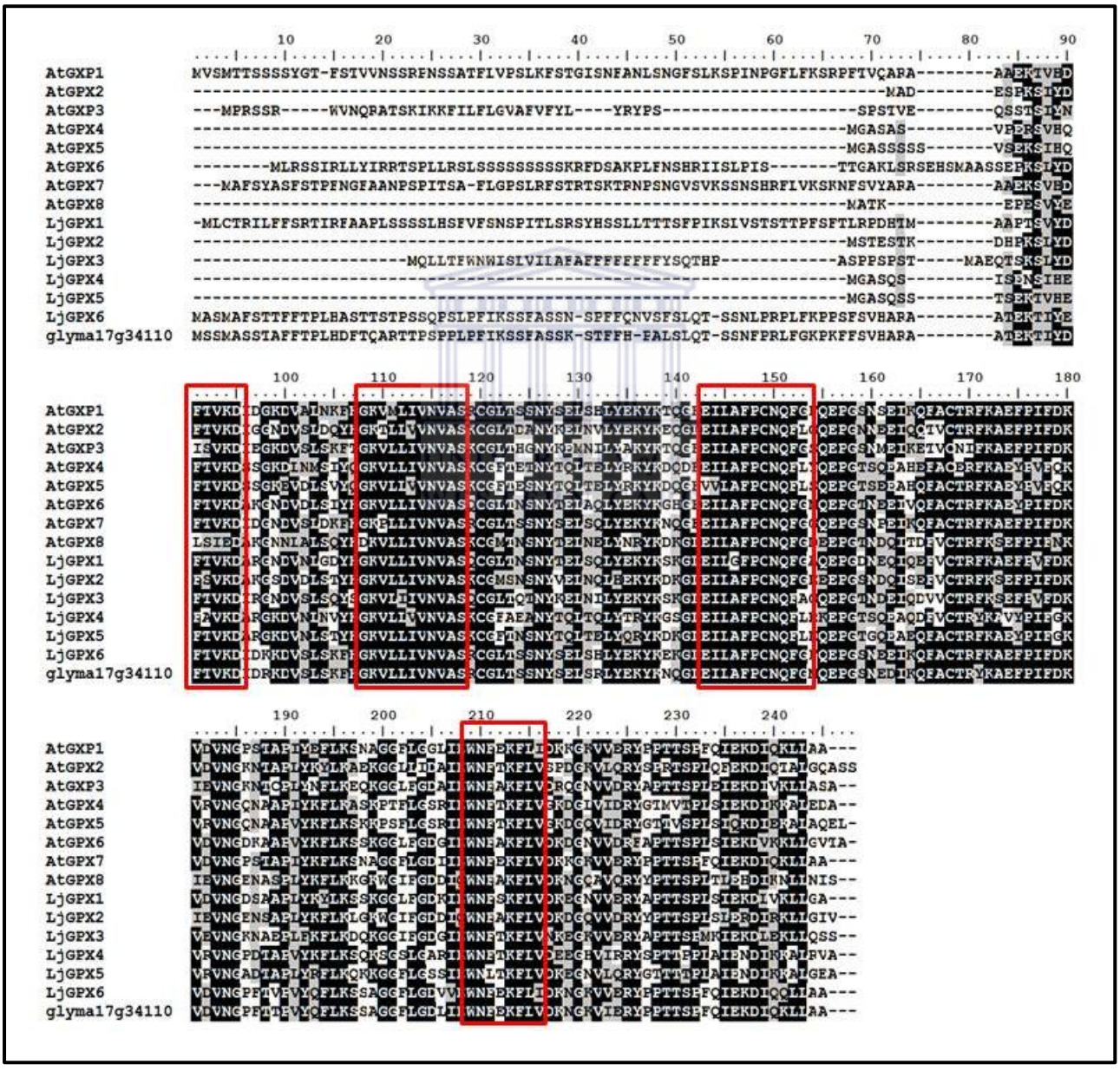

Figure 2.4.1.1 Peptide sequence analysis of Glyma17g34110, the AtGPX and LjGPX families. Peptide sequences of Glyma17g34110, AtGPX and LjGPX were aligned using BioEdit Sequence Alignment Software using ClustalW. Single letter descriptions of amino acids were applied. The black and gray shaded areas indicate the consensus sequences and the conserved motifs found in plant and animal GPX genes are represented by red blocks.

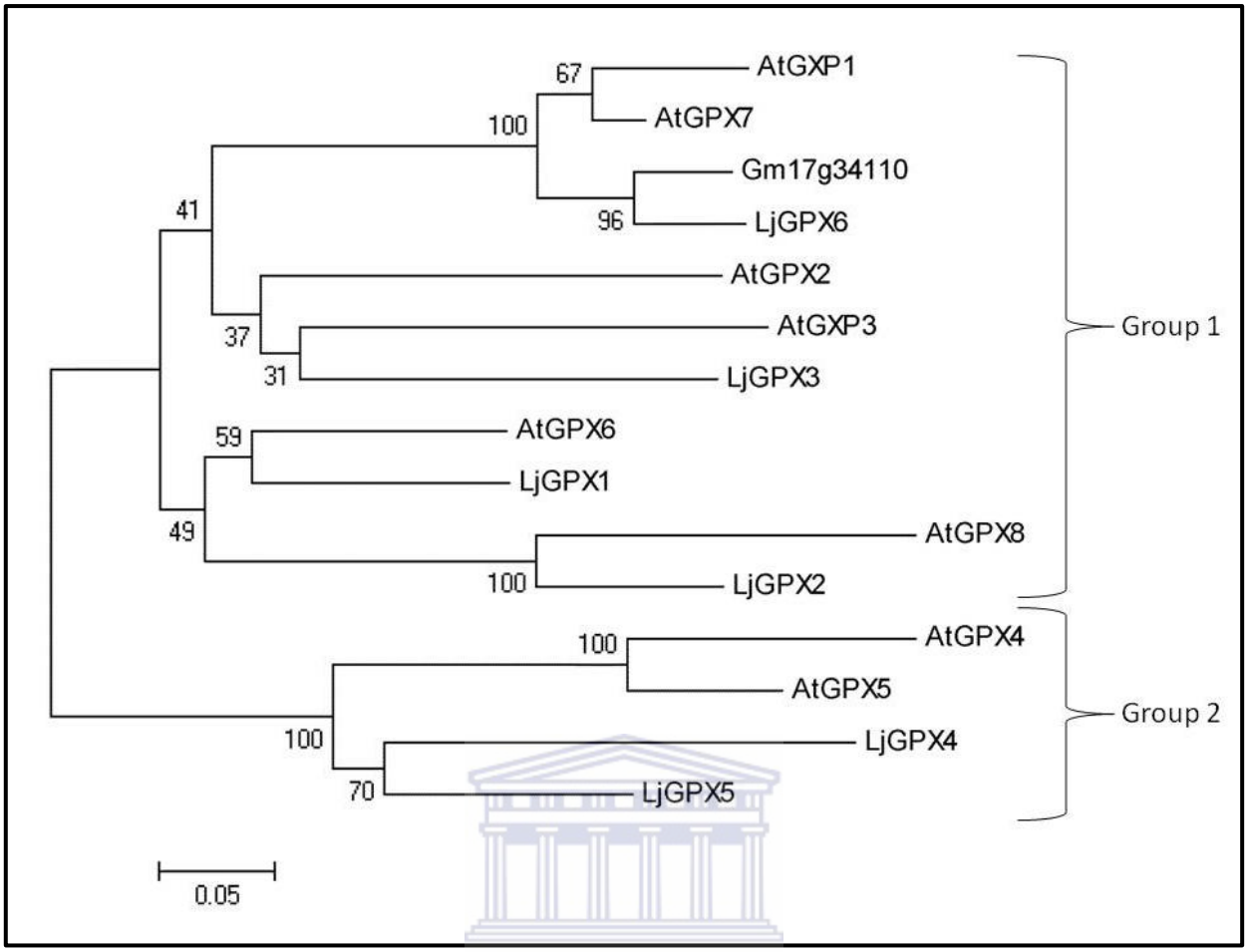

Figure 2.4.1.2 Phylogenetic analysis of Glyma17g34110, the AtGPX and LjGPX families. A phylogenetic tree of the amino acid sequences was constructed using ClusterW via the Neighbour-joining method. The bootstrap value of this analysis was 1000 replicates.

2.4.2 Glyma17g34110 is predicted to play a role in the protection of photosynthesis-induced oxidative damage

Subcellular predictions of Glyma17g34110 and its homologues (LjGPX6, AtGPX1 and AtGPX7) were studied using multiple prediction databases (Table 2.4.2.1). These databases used distinctive parameters and pre-calculated weight factors, thus giving the likelihood of a protein's existence in a specific location within the cell. Glyma17g34110 and its homologues (AtGPX1, AtGPX7 and LjGPX6) were predicted to be found in the chloroplast (Table 2.4.2.1), this result was unanimous in all prediction databases used and was in agreement with previous studies performed (Milla *et al*., 2003; Ramos *et al*., 2009). In addition, these

genes were all found to encode putative plastid proteins; proteins found in the chloroplast to facilitate in photosynthesis.

Table 2.4.2.1: Predicted characteristics of Glyma17g34110 with its homologues

Gene Name	Accession No.	Chromosome No.	Predicted Location	Predicted encoded protein	Putative Homologue
LjGPX6	AFK46172	5	Chloroplast	Plastid	AtGPX7
AtGPX1	NP_180080	2	Chloroplast	Plastid	AtGPX7
AtGPX7	NP_194915	4	Chloroplast	Plastid	AtGPX1
Glyma17g34110	NP_001236504	17	Chloroplast	Plastid	LjGPX6

Therefore, to further understand the regulation and responses of Glyma17g34110, the *cis*-acting elements in the promoter region were analysed using the PlantCare database. From a 1000 bp region upstream of the transcriptional start site (ATG), several *cis*-acting elements were identified, a large portion containing sequences required for light responsiveness (Table 2.4.2.2). These elements were similar to those recognized in AtGPX1 and AtGPX7, as established in previous studies (Milla *et al.*, 2003). The promoters of AtGPX1 and AtGPX7 as well as *Glyma17g34110* were predicted to encode plastid proteins, thus suggesting its role in the plants protection during photosynthesis (Table 2.4.2.2). LjGPX6, however, revealed no elements required for the response to light but contained several elements required for the defence and response to stress (Ramos *et al.*, 2009). TC-rich repeats were found in the promoter regions of both *Glyma17g34110* and LjGPX6 and in addition to elicitor elements (ELI-box3 and Box-W1 respectively), also used in the defence and response to abiotic and biotic stress. Furthermore, two elements were identified used in the growth or developmental stages of soybean, namely two endosperm elements along with a hormone, Gibberellin. Lastly, two unnamed elements were found in the promoter regions of *Glyma17g34110*, an unknown protein binding site and an unknown element.

Table 2.4.2.2: Putative *cis*-acting elements in the promoter of *Glyma17g34110* and its homologues involved in responses to stress

Category	*cis*-Element	Sequence	Promoter
Light	AE-box	AGAAACAA	Glyma17g34110
	Box 4	ATTAAT	Glyma17g34110
	CAAT-box	CAAAT	Glyma17g34110
	CATT -motif	GCATTC	Glyma17g34110
	I-box	GATAAG	AtGPX1
			AtGPX7
		AGATAAGG	Glyma17g34110
	T-box	ACTTTG	AtGPX1
			AtGPX7
			Glyma17g34110
Anaerobiosis	ARE	TGGTTT	Glyma17g34110
		(A/G)GTGACNNNGC	LjGPX6
Defense and stress response	DRE1	ACCGAGA	AtGPX1
	HSE	AAAAAATTTC	LjGPX6
	LTR	CCGAAA	LjGPX6
	MBS	TAACTG	LjGPX6
	TC-rich repeats	ATTTTCTTCA	Glyma17g34110
Elicitor	ELI - box3	AAACAGA	Glyma17g34110
	Box - W1	TTGACC	LjGPX6
Gibberellin	GARE- motif	AAACAGA	Glyma17g34110
Ethylene	ERE	ATTTCAAA	LjGPX6
Salicylic acid	TCA-element	GAGAAGAATA	LjGPX6
Abscisic acid	ABRE	CGCACGTAA	LjGPX6
Endosperm	GCN4-motif	CAAGCCA	Glyma17g34110
	Skn1-motif	GTCAT	Glyma17g34110
Protein binding site	Unnamed 1	GAATTTAATTAA	Glyma17g34110
Unknown	Unnamed 4	CCTC	Glyma17g34110

2.4.3 *Glyma17g34110* spatial analysis shows high expression in leaves

Expression profiles of *Glyma17g34110* were analyzed via the Soybean eFP Browser database (http://bar.utoronto.ca/efpsoybean/cgi-bin/efpWeb.cgi). Using the Bio-Array Resource and transcriptome information available, the expression levels of genes in plants are estimated and shown pictographically. The predicted results for *Glyma17g34110* in *Glycine max* were tabulated in Table 2.4.3. From the database, it was calculated that *Glyma17g34110* is largely expressed in the leaves in comparison to both nodules and roots.

Table 2.4.3: Spatial predictions of *Glyma17g34110* by Soybean eFP Browser

Tissue	Expression level
Leaves	207.03
Nodules	25.26
Roots	17.66

To support the results received from the bioinformatic expression prediction of *Glyma17g34110*, semi-qRT PCR was executed (Figure 2.4.3 A). It can be seen from figure 2.4.3 A that *Glyma17g34110* showed differences in expression levels in each tissue (leaves, roots and nodules) with most abundance in leaves. Furthermore, to validate the results received, a control gene *β-tubulin* showed very little or no change in each tissue examined. Thus, to further evaluate the results received, a comparison of *Glyma17g34110* in the different plant organs were made by normalising its expression levels with respect to that of *β-tubulin* (Figure 2.4.3 B). The expression of *Glyma17g34110* in Figure 2.4.3 B was ±136.3% higher in the leaves than that in the nodules and ±173.5% higher from that in the roots. These results can similarly be seen for AtGPX7 and LjGPX6 which showed a 4-fold and 2-fold increase within the leaves in comparison to the roots respectively (Milla *et al.*, 2003; Ramos *et al.*, 2009).

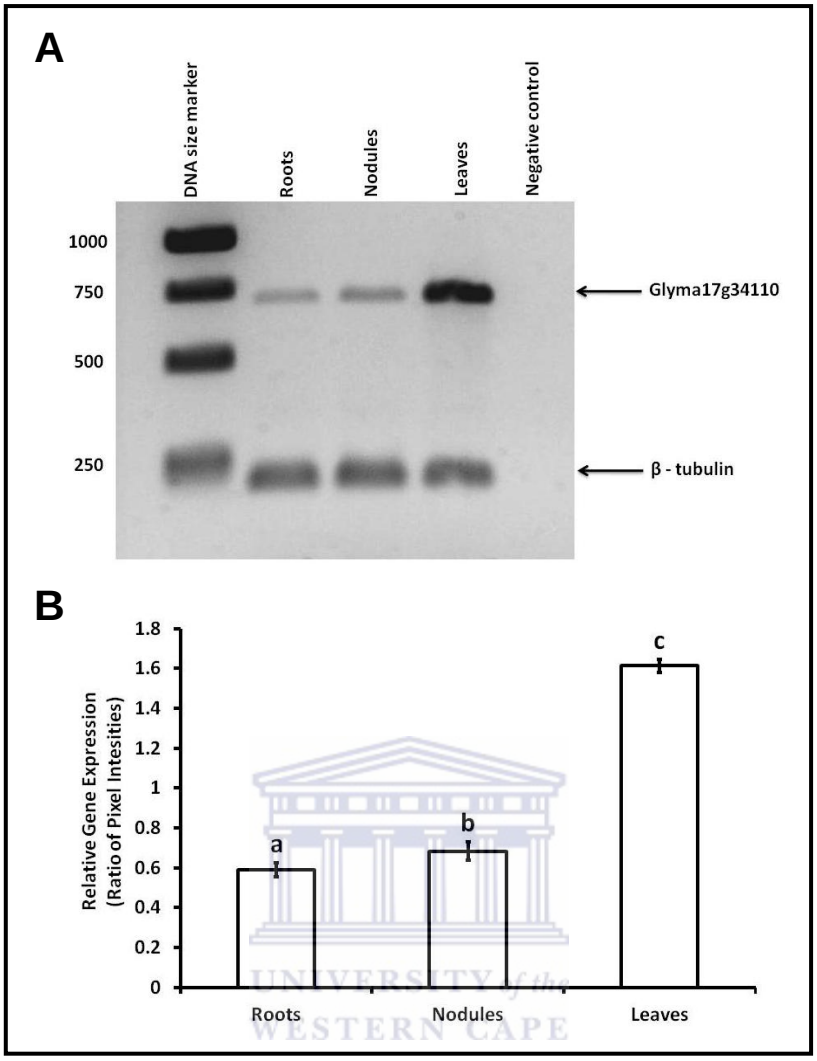

Figure 2.4.3 Expression studies of *Glyma17g34110* in different plant tissues. (A) Spatial expression of *Glyma17g34110* was analyzed by semi-qRT-PCR on untreated soybean organs. A control gene (*β-tubulin*) was used in each experiment. (B) Densitometry analysis of the expression of *Glyma17g34110* relative to the expression of *β-tubulin* within the respective tissues. Error bars (I) represent the mean of the study from three independent experiments.

2.4.4 *Glyma17g34110* is responsive to exogenously applied NO in all plant tissue studied

It was shown in previous studies that NO play a role in the modulation of GPX function in plants (Ramos *et al.*, 2009). Thus, to determine the role of NO and its effects on *Glyma17g34110*, plants were treated with a NO donor, DETA/NO (10 µM). To authenticate that any positive results were due to the presence of NO, a suitable control of similar structure and chemistry, except for the missing NO moiety (DETA [10 µM]), was used. The

expression studies of *Glyma17g34110* in the presence of NO and DETA were assessed in different plant tissues. From the results (Figure 2.4.4 A, B and C) it can be seen that no change in the expression levels were found in the presence of DETA. However, in the presence of DETA/NO, expression of glyma17g34110 was significantly increased due to the presence of NO. *β-tubulin*, on the other hand had no change in expression in all the treatments exposed. The densitometry analysis showed the normalised expression levels of *Glyma17g34110* with respect to those of *β-tubulin* (Figure 2.4.4 D, E and F). From this analysis, *Glyma17g34110* showed an increase in expression of ±21.8% in roots, ±28.2% in nodules and ±18.7% in leaves after exposure to DETA/NO.

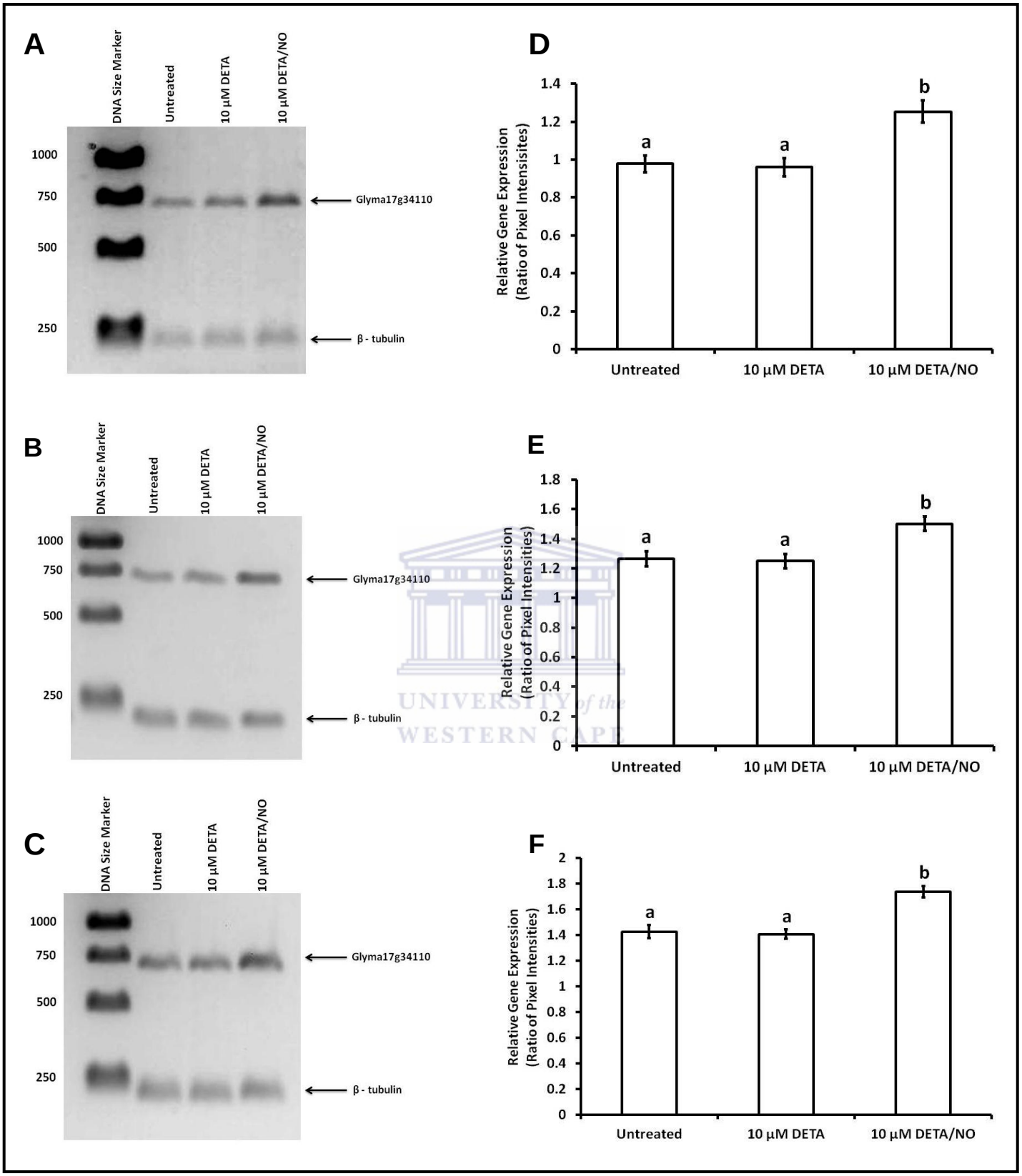

Figure 2.4.4: Expression studies of *Glyma17g34110* after the application of exogenous NO in different plant tissues. Soybean root, nodule and leaves were subjected to semi-qRT PCR to study the effects of 10 µM DETA and 10 µM DETA/NO on *Glyma17g34110*. The expression of *Glyma17g34110* and *β-tubulin* were studied in roots (A), nodules (B) and leaves (C) by gel electrophoresis. Densitometry analysis of the expression of *Glyma17g34110* relative the expression of *β-tubulin* was examined (D, E and F) for each tissue respectively. Error bars (I) represent the mean of the study from three independent experiments.

2.5 DISCUSSION

The study of glutathione peroxidase has been extensively done in mammals, yet little is known about these genes in the plant kingdom. Nonetheless, it has been shown that plant GPX genes contain homologous sequences to animal GPXs, including specific domains which are highly conserved (Criqui *et al.*, 1992). These domains were present in Glyma17g34110 in addition to the 8 characterised GPX genes found in *A. thaliana* and 6 found in *L. japonicus* (Figure 2.4.1). The presence of Cys-120 residue revealed that none of these genes contains selenium at the active site as found in mammalian and *Chlamydomonas reinhatii* GPX genes. It was established that selenium-cysteine containing GPX genes contained a considerably higher activity than those that contained cysteine residues at the active site (Leisinger *et al.*, 1999). Phylogenetic analysis done on the *A. thaliana* and *L. japonicus* GPX families in previous studies (Milla *et al.*, 2003; Ramos *et al.*, 2009) supported the results obtained in this thesis. Glyma17g34110 showed a close relation in sequence homology to LjGPX6, AtGPX7 and AtGPX1. Although most of the sequences of these genes were well conserved, the N-terminal region of each sequence was highly divergent. In previous studies done on GPX genes, it was suggested that the high variability of the N-terminus region in members could possibly be due the presence or absence of signalling transit peptides. Thus, from the sequences present in the N-terminal region of Glyma17g34110 and its homologues, Glyma17g34110 could possibly contain signalling peptides (as suggested by previous studies done on LjGPX6, AtGPX7 and AtGPX1) (Milla *et al.*, 2003; Ramos *et al.*, 2009).

Studies have shown that antioxidant enzymes (like GPX) in higher plants encode different isoforms performing different functions. These isoforms may therefore be differently regulated, based on their specific localization and pathway (Milla *et al.*, 2003; Wang & Xu,

2012). In a previous study done in a single-celled green alga, *Chorella* sp. Nj-18, on two GPX isoforms it was established that both these GPX genes were localized in different subcellular locations and thus contained different features. Nj-18GPX2 carried signalling peptides for secretion and responded differently to stress conditions compared to Nj-18GPX1. It was thereafter hypothesized that these isoforms play different physiological roles within the alga (Wang & Xu, 2012). To further understand the functioning of Glyma17g34110, characteristic predictions were attempted. The results received in this study showed that Glyma17g34110 is a putative chloroplast gene and encodes a photosynthetic protein (plastid) (Table 2.4.1). This supports the spatial expression results which revealed a high level of expression in leaves (which is the location of chloroplast) and low levels of expression in roots and nodules. These results were similarly seen in previous studies and were suggested to therefore play an important role in the protection of the plant against photosynthesis-induced oxidative damage (Milla *et al.*, 2003; Mittler, 2002; Ramos *et al.*, 2009). Interestingly, the majority of the *cis*-acting elements predicted on the promoter regions were those used in responsiveness to light, with one or two used for the defence and signalling to stress. Thus, Glyma17g34110 could contain overlapping functions with LjGPX6 and AtGPX7 but within soybean plants, suggesting Glyma17g34110 could play a role in the defence and signalling response against ROS produced in photosynthesis during normal and abiotic stress conditions.

Nitric oxide has been shown to act as a signalling molecule against oxidative stress. Previous studies have shown that the application of exogenous NO to plants such as soybean increases the level of some antioxidant enzyme (APX) and in turn decreases the level of ROS, such H_2O_2 (Keyster *et al.*, 2011). According to Ramos *et al.*, (2009) the increase in GPX (LjGPX6) levels occurred within the first hour in the presence of NO, suggesting that the application of NO triggered the expression of certain GPX genes. It was further suggested

that NO plays a role in the modulation of the functioning of these genes and thus these genes could possibly play a role in the signalling pathway downstream of NO. Therefore, to determine the effects of exogenously-applied NO on the expression of *Glyma17g34110*, a NO donor, DETA/NO, were applied to soybean plants. DETA with the same concentration as DETA/NO was used in this experiment as a suitable control. Results showed that the expression of *Glyma17g34110* was increased in all the plant tissues tested with the application of NO. Furthermore, it was seen that when treated with DETA alone, no significant change in the expression had occurred in comparison to the untreated plants. Thus, it can be said that the increase in expression was due to the presence of NO and not due to any reactions with DETA. It can be observed that the application of NO enhanced the expression of *Glyma17g34110* and therefore more studies needs to be done to determine whether this GPX play a role in the signalling pathway downstream of NO. In additions, studies have also shown that *cis*-acting elements are responsive to NO and thus could account for the increase in gene expression. Previous studies have identified *cis*-acting elements, namely G-Box, OCSE, MYCL, WRKY, HSE, Y-Box, DRE, AtMyb4 and ABRE, to be involved in the response to NO (Alscher *et al*., 2002; Chen *et al*., 2002; Hartmann *et al*., 2005; Palmieri *et al*., 2008). In this study, however, *Glyma17g34110* had none of these elements present in the promoter region and thus *Glyma17g34110* could contain novel *cis*-acting elements responsive to NO. The identification of these novel *cis*-acting elements could lay the foundation for pathways involved in the tolerance of plants under abiotic or biotic stress. This could aid in a stronger response of antioxidant enzymes such as GPX genes and could possibly be used in the engineering of tolerant crops.

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CHAPTER 3: TEMPORAL EXPRESSION OF GLYMA17G34110 UNDER SALT STRESS

3.1 ABSTRACT

Soil salinity affects a large percentage of crops harvested globally. Due to the accumulation of ions such as Na^+ and Cl^- in saline soil, plants exhibit both toxic and osmotic effects. A common response to these stresses is the over-production of signalling molecules known as ROS. These molecules become toxic to the plant at high levels resulting in detrimental reactions with important biological components (DNA, protein and lipid membrane) leading to cell death. Research has shown that the manipulation of antioxidant enzymes increases the performances of plants under abiotic and biotic stress mainly through the removal of the ROS. This provides an enhanced defence mechanism against harmful molecules (such as ROS). To understand the effects of salinity on soybean and antioxidant enzymes such as a putative glutathione peroxidase, 120 mM NaCl were applied on separate plants for 3 week (long term) and 48 hours (short term). A series of biochemical assays were performed to determine the extent of the damage caused by the increase of ROS during salinity. Expression analysis of a putative glutathione peroxidase gene (*Glyma17g34110*) was analyzed using semi-quantitative reverse transcriptase PCR. In this study under long term exposure to NaCl, it was observed that the level of the ROS molecules such as H_2O_2 had increased significantly and thus resulting in an incline in lipid peroxidation and cell death. The expression of *Glyma17g34110* showed a major decline in response to long term salinity. The decrease in expression of *Glyma17g34110* showed the opposite pattern to the increase in H_2O_2, lipid peroxidation and cell death. This may be due to the lack of defence and responsive elements such as DRE and ABRE in the promoter regions of Glyma17g34110. Thus, it is suggested

that *Glyma17g34110* is salt sensitive and could possibly contribute to the poor performances of soybean during salinity.

3.2 INTRODUCTION

Salinity is one of the major abiotic stresses affecting approximately 50% of crops globally. Salinity is the increase of salt (e.g. NaCl) concentrations within the soil, resulting in both osmotic (cell dehydration) and toxic (accumulation of ions such as Na^+ and Cl^-) effects on the plant (Munns, 2002; Türkan & Demiral, 2009). Na^+ and Cl^- are of the major ions found in high concentration within saline toxic soil. The accumulation of these ions within the plant results in a variety of physiological and biochemical changes. These changes include the inhibition of growth and development, reduction of photosynthesis and respiration and the disruption of nucleic acid metabolism, amongst others (Manaa *et al.*, 2011; Parvaiz & Satyawati, 2008; Rubio *et al.*, 2009).

During abiotic stress such as salinity, the availability of CO_2 becomes limited due to stomatal closure and the reduction of NADPH produced via the Calvin cycle. Under these conditions and continuous exposure to excess light, electrons are transferred to molecular oxygen generating superoxide ions, in a process known as the Mehler's reaction (Gill & Tuteja, 2010; Miller *et al.*, 2010). The generation of superoxide ions instigates the production of more harmful reactive oxygen molecules. The increased production of ROS results in an imbalance of the redox homeostasis of ROS production and ROS scavenging within the cells (Bhattcharjee & Soumen, 2005; Parvaiz & Satyawati, 2008; Trachootham *et al.*, 2008). As ROS accumulates, the defence mechanisms becomes insufficient and thus ROS molecules are able to react with important biological components such as lipid membranes, proteins and DNA. These reactions lead to cell death and thus death of the plant (Miller *et al.*, 2010; Sharma *et al.*, 2012).

Many studies have shown that an increase in antioxidant (both enzymatic and non-enzymatic) levels have enhanced the survival of plants exposed to abiotic or biotic stress. In a study done on the effects of thermal and salt stress on tobacco, it was observed that the growth was inhibited and an increased in lipid peroxidation had occurred (Roxas *et al*., 2000). However, in the transgenic tobacco, the over-expression of glutathione-S-transferase (GST) and glutathione peroxidase (GPX) genes resulted in an increased glutathione-dependent peroxide scavenging, as well as alterations in the glutathione metabolism. This lead to the reduction of oxidative damage when compared to wild type tobacco. Thus, in this study it was shown that the manipulation of antioxidant activity often results in the improved performances of plants under abiotic stress (Lee *et al.*, 2007).

In Chapter 2 in this thesis, Glyma17g34110 was revealed a putative glutathione peroxidase gene exhibiting important roles during photosynthesis, based on bioinformatic analysis. The increase in expression of this gene in the presence of NO could aid in the future generation of stress tolerant crops worldwide. Thus, the aim of this chapter was to determine the effects of long and short term treatments with 120 mM NaCl on soybean, as well as on the gene expression of *Glyma17g34110.*

3.3 MATERIALS AND METHODS

3.3.1 Plant preparation and growth

Surface sterilization was performed to rid the seeds of any contaminants. Approximately 100 Soybean (*Glycine Max* (L) merr. cv PAN. 626) seeds were washed with 0.35% sodium hypochloride for 10 minutes and then thoroughly washed in distilled water. The seeds were saturated in distilled water for one hour. Seeds were grown in a 1:1:1 Compost-Silica sand (98% SiO_2, Rolfes® Silica, Brits South Africa)-potting soil mixture until the V3 stage (3^{rd} nodal stage).

3.3.2 Treatment of plants

Treatments were applied to respective plants: Untreated (0 mM NaCl), 120 mM NaCl (long term) for three weeks and 120 mM NaCl (short term) for 48 hours. The plants were harvested, snap frozen in liquid nitrogen and stored at -80°C for further downstream experiments.

3.3.3 Measurement of H_2O_2

The level of H_2O_2 was measured using a standard protocol by Velikova *et al*. (2000). Plant tissues (approximately 100 mg) were ground into a fine powder in liquid nitrogen and mixed with 400 µl of cold 5% (m/v) TCA. The mixture was centrifuged at 12, 000 X *g* for 30 minutes at 4°C. Of the extract, 50 µl was mixed with 5 mM K_2HPO_4 (pH 5.0) and 0.5 M KI. The samples were incubated at room temperature for 20 minutes, and the absorbance was measured at 390 nm.

3.3.4 Lipid peroxidation evaluation

To evaluate the level of lipid peroxidation, a procedure by Buege & Aust (1978) were applied. Using liquid nitrogen, the leaves were ground into fine powder and transferred into clean test tubes. Five times the volumes of 6% (m/v) Trichloroacetic acid (TCA) was added relative to the leaf material and the samples vortexed and centrifuged for 10 minutes at maximum speed using an Eppendorf bench top centrifuge with fixed angle rotor. The mixture was transferred into clean eppendorf tubes and mixed with 20% (m/v) TCA (containing 5% [m/v] thiobarburic acid [TBA]) and gently vortexed. The solution was then incubated at 90°C for 20 minutes and left to chill on ice for 10 minutes. The solutions were centrifuged at 12, 000 X *g* for 5 minutes, and the absorbance was read at 532 nm and 600 nm using a spectrophotometer.

3.3.5 Cell viability analysis

Cell viability by Evans Blue staining was implemented from a procedure by Sanevas *et al.* (2007). The second youngest leaves (per square centimetre) were sectioned and placed in an eppendorf tube containing 0.25% (m/v) Evans blue and left for 30 minutes. The leaves were washed repeatedly with distilled water. To further remove surface-bound Evans blue, the leaf-water mixture was stored overnight in the dark at room temperature. The leaves were then sliced into small pieces and mixed with 1% SDS for 1 hour to extract all Evans blue taken up by the leaves as an indication of cell death taken place in the pant during respective treatments. The SDS-Evans blue mixture was extracted and the absorbance was taken at 600 nm using a spectrophotometer.

3.3.6 Gene expression analysis of *Glyma17g34110* during salt stress

RNA was extracted from leaves as previously described in Section 2.3.4.1. The total RNA was treated with RNase-free DNase to remove any contaminants. Two hundred nanograms of RNA were thereafter used to synthesize cDNA using RevertAid™ Transcriptase kit (Fermentas) according to the manufacturer's protocol.

The reverse transcription products (1µl of each) of *Glyma17g34110* and *β- tubulin* were used in a polymerase chain reaction using True Start™ Hot Start Taq Polymerase (Fermentas). The following oligonucleotides (10 pmol each) were used for the amplification of *Glyma17g34110*: forward primer (5'- CAC GGA TCC ATG TCC TCC ATG GCT TCC TC -3') and reversed primer (5'- CGT CTC GAG TCA GGC AGC AAG TAA CTT CTG GAT -3') and *β-tubulin* forward primer (5'- CTG CGA AAG CTT GCA GTG AAC C -3') and reversed primer (5'- TCT TGC CTC TAA ACA TGG CTG AGG -3'). The reactions were ran according to the specific parameters consisting of a single cycle of 95°C for 90 seconds following by 23 cycles of 95°C for 30 seconds, 63°C for 30 seconds, 72°C for 2 minutes and

a final cycle of 72°C for 7 minutes. The amplified products were electrophoresed on a 1% agarose gel, stained with Gel Red™ and visualised under short UV light and photographed.

The densitometry analysis of the agarose gel was analysed using the Alpha Ease FC imaging software (Alpha Innotech Corporation).

3.4 RESULTS

3.4.1 Hydrogen peroxide content increases with long term salinity

It is known that in the presences of abiotic or biotic stress, such as high salt concentrations within the soil, the level of ROS in plants increases. The accumulation of ROS results in an increase in cell death due to detrimental reactions occurring with important biological features. Thus, the level of H_2O_2 was measured in soybean shoots treated with 0 mM NaCl (control) and 120 mM NaCl in the short (48 hours) and long (3 weeks) term. The results were analyzed, compared and depicted in Figure 3.4.1 A. The presence of NaCl had no effect on the level of H_2O_2 during short term treatments. Long term treatments, however, showed a significant increase of ±142.05% in the H_2O_2 level in comparison to the untreated control.

3.4.2 Lipid peroxidation increased during long term salinity

The measurement of lipid peroxidation present in soybean shoots was evaluated using a thiobarburic acid assay (TBA). Thiobarburic acid assay can be used to measure the quantity MDA, a product of lipid peroxidation. The results were analyzed and represented in Figure 3.4.1 B from plants treated with 0 mM NaCl (control) and 120 mM NaCl. Long term treatments with 120 mM NaCl caused a major increase in the level of lipid peroxidation (±126.71%) in comparison to the untreated control, whereas short term treatments showed no such increase.

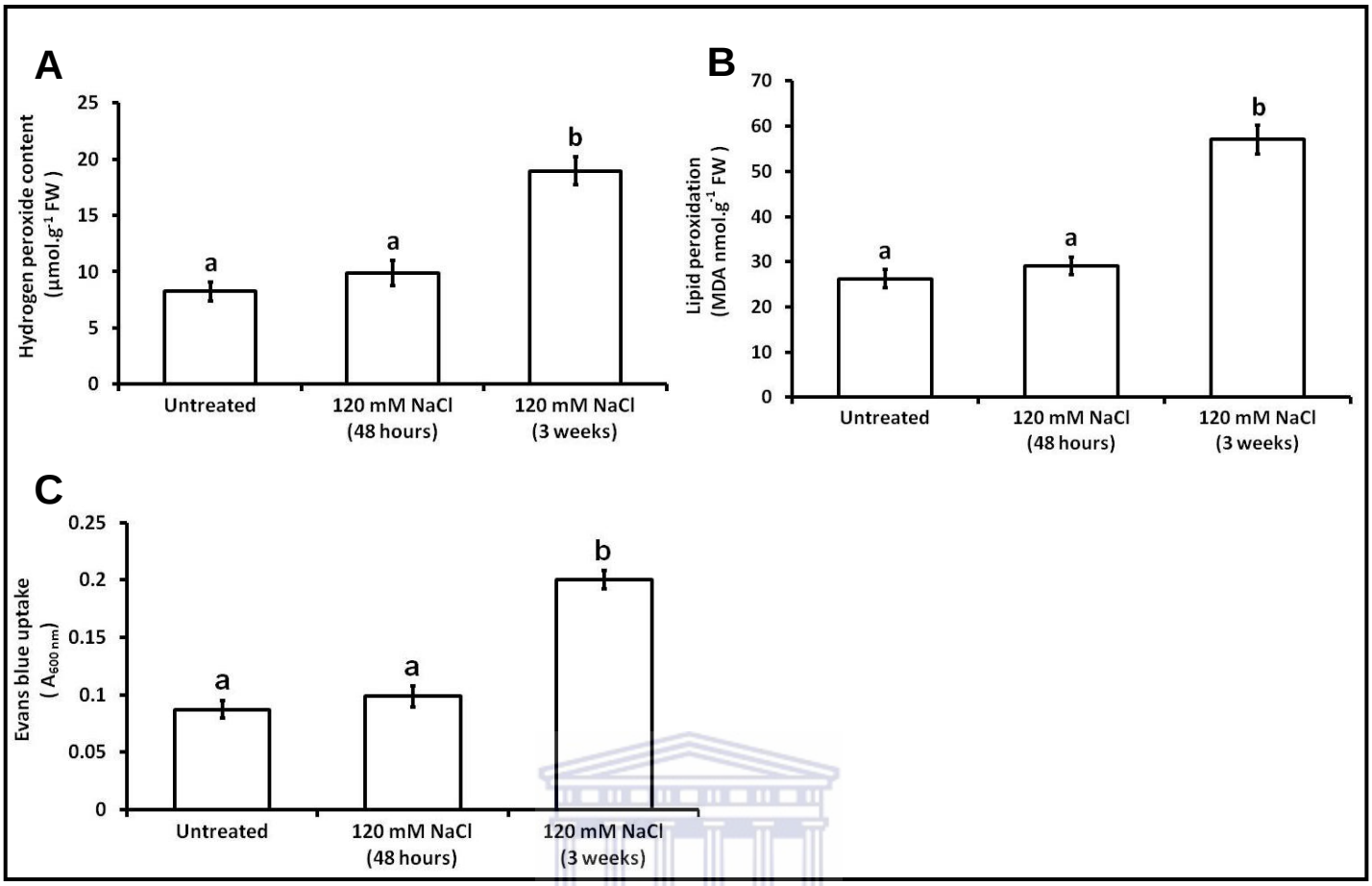

Figure 3.4.1 Changes in hydrogen peroxide content, lipid peroxidation and cell death in soybean shoots in response to salt treatments. Soybean leaf treated with 120 mM NaCl (3 weeks and 48 hours) were examined to measure the hydrogen peroxide content (A), lipid peroxidation via MDA (B) and cell death via Evans blue (C). Error bars (I) represent the mean of the study from three independent experiments.

3.4.3 Cell death induced by long term salinity stress

To investigate the integrity of the plasma membrane, 1 square centimetre of leaf were taken from each plant and evaluated using the Evans Blue assay (Figure 3.4.1 C). Treatments with 0 mM NaCl (control) and 120 mM NaCl had no effect on the short term-treated plants. Nonetheless, visible increases in Evans blue of ±165.5% were demonstrated in the plants exposed to long term treatment with NaCl.

3.4.4 *Glyma17g34110* demonstrates sensitivity under salinity conditions

The gene expression of *Glyma17g34110* was analysed using semi-qRT PCR to determine the effects of short and long term salt stress (Figure 3.4.4 A and B). Short term exposure to 120 mM NaCl resulted in a no change in the expression of *Glyma17g34110* in comparison to the untreated (0 mM NaCl). Nonetheless, a significant reduction in expression was observed in the long term (3 weeks) treatment with NaCl. A control gene, *β-tubulin*, was used in this expression study and showed no change in the expression levels for all treatments. To further study the expression levels, the gene expression of *Glyma17g34110* was normalised with respect to the gene expression of *β-tubulin* in each treatment for densitometry analysis. This analysis can be seen in Figure 3.4.4 B and shows a decrease of ±42.4% compared to untreated plants. Short term treatments showed no change.

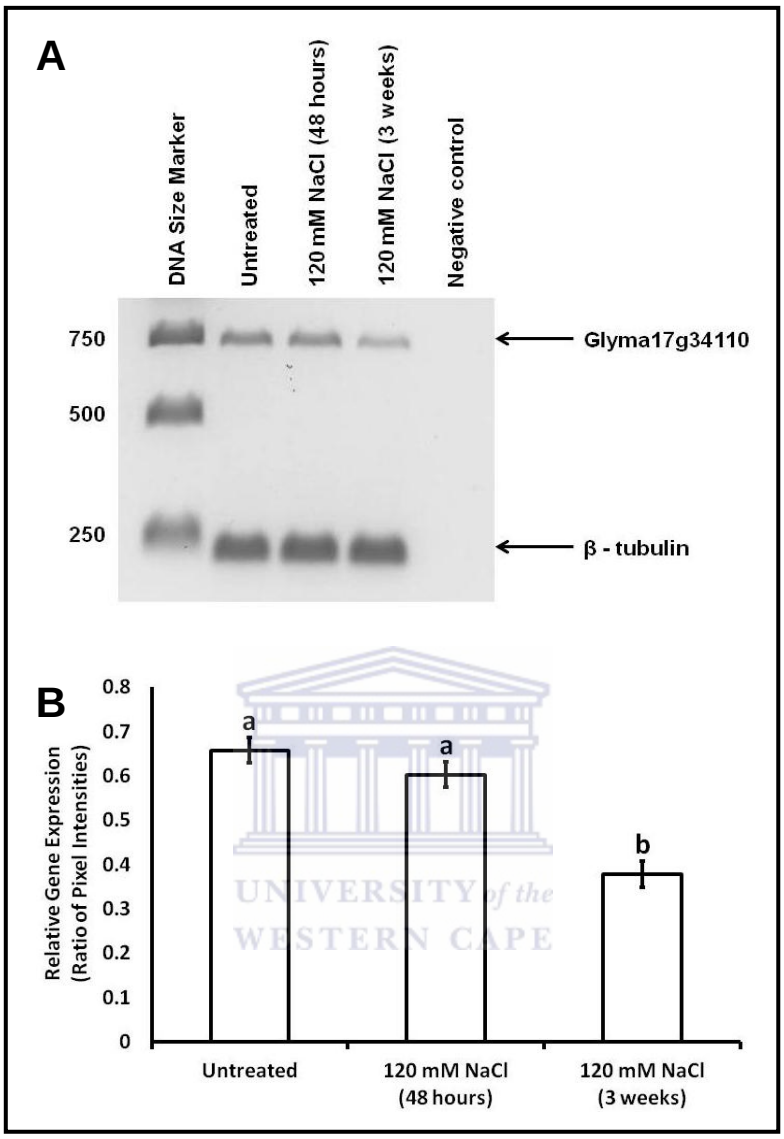

Figure 3.4.4 Expression of *Glyma17g34110* under salt stress. Soybean plants were treated with 120 mM NaCl for 48 hours and 3 weeks. An untreated soybean plant was used as a control. (A) Temporal expression of *Glyma17g34110* was analyzed by semi-qRT PCR on soybean leaves. A control gene (*β-tubulin*) was used in each experiment. (B) Densitometry analysis of the expression of *Glyma17g34110* relative to the expression of *β-tubulin* for each respective treatment. Error bars (I) represent the mean of the study from three independent experiments.

3.5 DISCUSSION

Salt stress causes a variety of disturbances on plant metabolism and growth. The exposure of plants to NaCl results in the accumulation of Na^+ and Cl^- ions within the leaves, leading to a disruption of the water balance, inhibiting growth and the reduction of photosynthesis amongst others (Paridaa & Das, 2005; Phang *et al.*, 2008). To evaluate the damage caused by

salt stress, a series of biochemical assays were performed. Previous studies have shown that salinity increase the level of ROS, inducing oxidative damage and eventually resulting in cell death. One of the by products of ROS production is H_2O_2 (Bor *et al.*, 2003; Dionisio-Sese & Tobita, 1998). It was shown in this study that the level of H_2O_2 increased tremendously under long term salt stress. Furthermore, it has been proven by prior research that the accumulation of H_2O_2 molecules within the cells during abiotic stress such as salinity often results in the peroxidation of unsaturated fatty acids within the membrane (Hernández & Almansa, 2002; Mandhania *et al.*, 2006; Velikova *et al.*, 2000). Malondialdehyde is a common product of lipid peroxidation which can be measured by using the thiobarbaric acid assay. In this study, the level of MDA increased in the same pattern as H_2O_2. Both MDA and H_2O_2 assays showed a significant increase in the presence of long term salt stress. This could be a result of both toxic and osmotic stress exerting on the plant during the long term treatments. Short term treatments had no significant increase in comparison to the untreated plants, thus it is possible that the Na^+ and Cl^- build up was insufficient to cause any notable damage.

Due to the increasing level of lipid peroxidation and ROS molecules such as H_2O_2 caused by salinity stress, the integrity of the cell was determined. To evaluate the level cell death in the plant a non-permeable stain, Evans blue, was used to permeate through the broken membranes. The amount of Evans blue absorbed in these membranes indicates the extent of cell death within the plant (Sanevas *et al.*, 2007). Long term treatments with NaCl showed a high increase in cell death whereas short term revealed no change in comparison to the untreated control, thus, demonstrating similar results to those from of H_2O_2 and MDA assays in the presence of salt stress. The disruption of the membrane lipids due to the interaction of ROS molecules such as H_2O_2 with important biological cellular components (such as cell membranes) resulted in further damage to the plant and eventually caused cell death, similar to results obtained by Hernández & Almansa (2002) and Mandhania *et al*. (2006).

Glutathione peroxidases (GPX) are one of many antioxidant enzymes used in plants against oxidative stress by reducing H_2O_2 to water. In previous studies it has been demonstrated that antioxidant enzymes such as SOD and APX (scavenges H_2O_2) increase at high concentrations of NaCl, thus reducing the effects of salt stress such as lipid peroxidation and cell death (Bor *et al.*, 2003; Gasic *et al.*, 2004; Hernández & Almansa, 2002; Mandhania *et al.*, 2006; Sairam *et al.*, 2005). In Bor *et al*. (2003), 2 types of sugar beet cultivars (cultivated *Beta vulgaris L.* and wild salt-tolerant *Beta maritima L.*) were investigated to widen the knowledge of plant susceptibility to salt stress. It was observed that the wild type contained a lower level of lipid peroxidation and showed higher activities for antioxidant enzymes such as SOD, POX, APOX, CAT and GR for all treatments with salt. It was concluded that the wild type had a better protection mechanism against oxidative damage by enhancing the activities of the antioxidant enzymes. In this thesis, however, *Glyma17g34110* showed a decrease in gene expression in response to NaCl. Short term treatments with NaCl showed no change, whereas long term treatment showed a major decline. This result received were opposite to the increase patterns received for H_2O_2, MDA and cell death. A possible reason for this outcome is the lack of defence and response *cis*-acting elements within the promoter of *Glyma17g34110*, as shown in Chapter 2 of this thesis. Studies have shown that abiotic stress inducible genes contain *cis*-acting elements such as DRE (a dehydration responsive element), ABRE (an abscisic acid responsive element) and MBS (a drought responsive element) (Narusaka *et al.*, 2003; Yamaguchi-Shinozaki and Shinozaki, 1994). The promoter analysis done in chapter 2 revealed only TC-rich repeats as a defence and response element present in the promoter region of *Glyma17g34110*. However, studies have not shown TC-rich repeat elements to be salt responsive (Sun *et al.*, 2010) and the lack of known salt responsive elements could possibly explain the decrease in gene expression under salt (NaCl) stress. *Glyma17g34110* therefore appears to be a salt sensitive antioxidant enzyme, which could

possibly contribute to the poor performances of soybean under salt stress. Further studies of Glyma17g34110 should be done, as genetic engineering of this putative GPX under salinity could possibly result in an increase of GPX activity within soybean, thus enhancing the defence mechanism of plants to salinity and other stresses.

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CHAPTER 4: RECOMBINANT EXPRESSION AND PURIFICATION OF GLYMA17G34110

4.1 ABSTRACT

The study of antioxidant enzymes such as GPX and APX is important to understand the mechanisms that caused susceptibility of a plant to a specific stress. GPX's are isoenzymes performing important roles in growth, development and in the response against the harmful effects of abiotic and biotic stress (scavenging of ROS). In this study a putative GPX gene (Glyma17g34110) was recombinantly expressed as a GST-fusion protein with *E. coli BL21* CodonPLUS cells. It was shown, that the expression of Glyma17g34110 was greatly effected by rare codons. The presence of rare codons such as arginine (AGA, AGG and CGA), proline (CCC) and glycine (GGA) were majorly found in Glyma17g34110 and thus were detrimental to the expression in *E. coli BL21* competent cells. However, the solubility of Glyma17g34110 was affected only 60% when expressed in codon adjusted-bias *E. coli BL21*strains (CodonPLUS). To widen the knowledge of GPX in soybean, the activity of Glyma17g34110 was studied using a GPX In-gel assay. From the assay it was observed that Glyma17g34110 exhibited GPX-like activities. Interestingly, although most GPX enzymes in higher plants are known to use thioredoxin as a substrate, Glyma17g34110 used glutathione as a preferred electron donor and substrate.

4.2 INTRODUCTION

The productions of reactive oxygen species (ROS) in aerobic metabolism are prominent in all organisms such as plants (Gill & Tuteja, 2010). The production of these molecules at high levels results in damaging reactions with cellular components such as DNA, proteins and membrane lipid. In order to protect themselves against these naturally-produced toxic molecules, plants have developed mechanisms of antioxidant enzymes to scavenge these

molecules (Miller *et al.*, 2010; Navrot *et al.*, 2006). In the study presented in Chapter 2 of this thesis, it was shown that Glyma17g34110 (a putative GPX) contained important functions in soybean. Glutathione peroxidases are isoenzymes responsible for the scavenging of hydroperoxides, organic and lipid peroxides to protect the cell from oxidative damage (Gaber *et al.*, 2012). These antioxidant enzymes can be found in various subcellular components within the cell, such as the chloroplast and mitochondria, performing various functions specific to its pathway (Sharma *et al*., 2012). Research has shown that GPX genes found in most plants contains preserved motifs including cysteine residues (Cys-x-x-Cys) present at the N-terminus of the catalytic site. Mammals and lower plants such as *C. reinhadtii* were shown to contain a seleno-cys residue at the active site, thus providing a higher activity than that of most plants (Gaber *et al.*, 2012; Leisinger *et al.*, 1999; Ramos *et al.*, 2009).

Abiotic stress affects a large percentage of crops worldwide. Thus, the generation of stress tolerant crops becomes more important with each year as the world population grows. In the previous chapters of this thesis, the importance of a putative GPX gene (Glyma17g34110) as an antioxidant enzyme has been shown within soybean. Thus, to use Glyma17g34110 in further studies, the characterisation of this antioxidant enzyme is required.

4.3. MATERIALS & METHODS

4.3.1 Bioinformatic analysis of Glyma17g34110

The amino acid sequence of Glyma17g34110 were further analysed to determine any limiting factors such as rare codons present in the sequence. The amino acid sequences obtained from the Phytozome database were analyzed in various prediction databases for any rare codons present in Glyma17g34110. The prediction databases used were Rare Codon Calculator (RaCC) (Carstens & Waesche, 1999; http://nihserver.mbi.ucla.edu) and Rare Codon Caltor (http://people.mbi.ucla.edu/sumchan/caltor.html).

4.3.2 Plant growth

Soybean (*Glycine Max* (L) merr. cv PAN. 626) seeds were sterilized with 0.35% sodium hypochloride for 10 minutes and washed approximately 5 times with sterile distilled water. The seeds were grown in filtered Silica sand (98% SiO_2, Rolfes® Silica, Brits South Africa) and watered during germination. At the VC (cotyledon) stage, the plants were treated with soya nitrogen-free nutrient solution [1 M $CaCl_2$, 1 M $MgSO_4$, 0.5 M K_2SO_4, 1 M K_2HPO_4, 100 mM FeNa EDTA, 50 mM H_3BO_3, 10 mM Na_2MoO_4, 2 mM $CuSO_4$, 2 mM $ZnSO_4$, 0.5 mM $CoSO_4$ and 0.5 M 2-(N-Morpholino)ethane-sulfonic acid at pH 6.4] until the V3 stage (3^{rd} nodal stage). The plants were harvested, snap-frozen in liquid nitrogen and stored at -80°C until required for further downstream experiments.

4.3.3 Amplification of Glyma17g34110

Total RNA was extracted from soybean leaves using a procedure by Gasic *et al*. (2004). From the extracted RNA, cDNA synthesis was achieved using the RevertAid Transcriptase Kit (Fermentas). Two hundred nanograms of template RNA was used to synthesize cDNA in the following reaction: 20 pmol of the Reverse primer (5'- CGT CTC GAG TCA GGC AGC AAG TAA CTT CTG GAT -3'), 1X reaction buffer, 20 mM dNTPs, 200 U Reverse transcriptase and made up to a final volume of 20 µl with DEPC-treated water. The solution was gently mixed and briefly centrifuged. The cDNA synthesis reaction was achieved at 60°C for 42 minutes and terminated by 70°C for 10 minutes.

The reverse transcription products were used in a standard PCR using the following oligonucleotides: forward primer (5'- CAC GGA TCC ATG TCC TCC ATG GCT TCC TC -3') and reversed primer (5'- CGT CTC GAG TCA GGC AGC AAG TAA CTT CTG GAT -3'). Complementary DNA (cDNA) template (1 µl each) was used for the amplification and mixed with the following reagents: 1X *Pfu* DNA Polymerase Buffer, 0.2 mM dNTPs, 100

nM each of forward and reverse primer and 1.25 U *Pfu* DNA polymerase in a final volume of 50 µl. PCR amplification was done according to specific parameters consisting of a single cycle of 95°C for 60 seconds following by 30 cycles of 95°C for 30 seconds, 63°C for 30 seconds, 72°C for 2 minutes and a final cycle of 72°C for 5 minutes. The amplified products were electrophoresed on a 1% agarose gel stained with Gel Red™ and visualised under short UV light and photographed.

4.3.4 Molecular cloning of Glyma17g34110

The amplified product was excised from the gel and ligated using restriction sites of BamHI and XhoI of pET-41a(+) using the restriction digestion and ligation kit (Fermentas) according to the manufacturer's protocol. To screen pET-41a(+) – Glyma17g34110 construct a PCR reaction were performed using an S-TAG forward primer (5'- CGA ACG CCA GCA CAT GGA CAG -3') and Glyma17g34110 reverse primer (5'- CGT CTC GAG TCA GGC AGC AAG TAA CTT CTG GAT -3'). The PCR was performed using the protocol as stated in Section 2.3.5.2 using the following parameters: a single cycle of 95°C for 90 seconds following by 30 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 2 minutes and a final cycle of 72°C for 7 minutes. The amplified products were electrophoresed on a 1% agarose gel, stained using Gel Red™ and visualised under short UV light and photographed.

To ensure Glyma17g34110 have been successfully cloned into the vector, the transformed construct were sent to Central Analytical Facilities at Stellenbosch University for sequencing. The correct constructs were then chemically transformed into *E. coli BL21* CodonPLUS competent cells. The CodonPLUS competent cells were thawed on ice and aliquoted into eppendorf tubes. Ten nanograms of the vector-insert product was added to the competent cells and incubated on ice for 30 minutes. The cells were then heat shock at 42°C for 45 seconds and immediately kept on ice for 2 minutes. Preheated Luria Broth (1% [m/v]

tryptone, 0.5% [m/v] yeast extract, 0.5% [m/v] NaCl, 0.2% [m/v] glucose) were mixed with the solution and incubated at 37˚C for 1 hour shaking at 250 rpms. The mixture was plated on LB agar (1% [m/v] tryptone, 0.5% [m/v] yeast extract, 0.5% [m/v] NaCl, 0.2% [m/v] glucose and 1.2% [m/v] bacteriological agar) plates containing + kanamycin (30 µg/ml) and grown overnight at 37°C.

The colonies were screened using colony PCR to determine if these colonies contained the correct gene. A standard PCR using the following oligonucleotides: S-TAG forward primer (5'- CGA ACG CCA GCA CAT GGA CAG -3') and Glyma17g34110 reverse primer (5'- CGT CTC GAG TCA GGC AGC AAG TAA CTT CTG GAT -3') were used in the screening if the colonies. Colonies were boiled in 2 µl dH_2O at 95°C and this was used as a template used for amplification and mixed with the following reagents: 1X Hot Start *Taq* Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP's, 400 nM each of forward and reverse primer and 2 U Taq DNA polymerase in a final volume of 50 µl. The reactions were run according to the specific parameters consisting of a single cycle of 95°C for 90 seconds following by 30 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 2 minutes and a final cycle of 72°C for 7 minutes. The amplified products were electrophoresed on a 1% agarose gel, stained using Gel Red™ and visualised under short UV light and photographed.

4.3.5 Expression of Glyma17g34110

Single colonies were grown overnight in Luria broth containing kanamycin (30 µg/ml) and 50 µg/ml chloroamphenicol at 37°C. Overnight cultures were diluted 1:50 in Luria broth supplemented with kanamycin (30 µg/ml) and grown at 37°C for 3 hours until an OD_{600} of 0.4. For induction, 0.5 mM IPTG was added to the culture and incubated at 37°C for 4 hours. After induction, the cells were centrifuged for 20 minutes at 12, 000 X *g*. The cell pellet was resuspended in 10X phosphate buffer saline (PBS) pH 7.8 (50 mM K_2PO_4, 400 mM NaCl,

100 mM KCl, 10% [v/v] glycerol, 0.5% [v/v] Triton X-100, 10 mM imidazole and 0.2 mg/ml lysozyme) and left on ice for 20 minutes, inverting the tube every 5 minutes. The cells were flash frozen at -80°C and immediately thawed 5 times at 42°C to ensure that all the cells were lysed. The samples were centrifuged for 10 minutes at 12, 000 X *g*. The supernatant (soluble protein) was transferred to clean tubes. The pellet (insoluble protein) was resuspended in 10X PBS and transferred into clean tubes. The proteins were stored at -20°C until further use.

Soluble and insoluble proteins were analysed on 12% SDS-PAGE (12% resolving - 12% acrylamide-bisacrylamide mix, 0.375 M Tris-HCl pH 8.8, 0.2% [m/v] SDS, 0.2% [m/v] APS and TEMED; 5% stacking - 5% acrylamide-bisacrylamide mix, 0.05 M Tris-HCl pH 6.8, 0.08 % [m/v] SDS, 0.08% [m/v] APS and TEMED) and detected using Coomassie Brilliant blue staining. PageRuler™ prestained protein ladder was used as a reference marker to determine the size of the expressed protein.

4.3.6 Protein purification

Protein purification was performed using Glutathione-sepharose columns (Sigma™). Cell lysates were applied to the glutathione agarose resin and bound at 25°C for 1 hour with gentle agitation. The bound proteins were washed 3 times with 1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH7.3). The target protein was recovered by eluting the protein off the resin with elution buffer (20 mM reduced glutathione in 50 mM Tris-HCl pH 8.0). The purified protein was analyzed on a 12% SDS-PAGE. The purified extracts were dialyzed to remove any GSH present from purification.

4.3.7 Glutathione peroxidase assay

GPX in-gels were carried out for activity of this putative GPX gene in response to GSH. A Native PAGE (10% resolving - 10% acrylamide-bisacrylamide mix, 0.375 M Tris-HCl pH

8.8, 10% [v/v] glycerol, 0.1% [m/v] APS and TEMED; 5% stacking - 5% acrylamide-bisacrylamide mix, 0.05 M Tris-HCl pH 6.8, 0.1% [m/v] APS and TEMED) were performed at 4°C. GPX activity was detected by monitoring gels in 50 mM phosphate buffer pH 7.0 containing 2 mM GSH for 20 minutes. The gel was transferred to 50 mM phosphate buffer pH 7.8 containing 4 mM GSH and 0.09 mM cumene hydroperoxide for 20 minutes. The gel were washed in distilled water and thereafter incubated in 50 mM phosphate buffer pH 7.8 containing 28 mM TEMED and 2.5 mM NBT in the presence of light for ±20 minutes.

4.3.8 Determination of peroxidase activity

To study the activity of Glyma17g34110 using cumene hydroperoxide as a substrate in the presence of GSH, a reaction was set up at 37°C. The following reaction contained 100 mM Tris-HCl pH 8.0, 0.2 mM cumene hydroperoxide, 1 mM GSH, 1 U GR and 0.4 mM NADPH. The reaction was initiated by adding 40 µg protein and doing kinetic measurements at 340 nm. GPX activity was calculated using an extinction coefficient of 6. 22 $mM^{-1}cm^{-1}$ for the degradation of NADPH.

To establish the activity of Glyma17g34110 towards electron donors GSH or thioredoxin, both GPX and TRX assays were performed to determine the reducing substrates in the reduction reactions of cumene hydroperoxide. Glyma17g34110 was assessed in a 37°C reaction containing 100 mM Tris-HCl pH 8.0, 0.5 mM cumene hydroperoxide and 1 mM GSH. For the thioredoxin assay, GSH were replaced with 1 mM thioredoxin and 0.5 mM DTT. Reactions were commenced by adding 40 µg protein and performing kinetic measurements at 353 nm and the activity calculated using an extinction coefficient of 17, 300 for cumene hydroperoxide.

4.4 RESULTS

4.4.1 Expression of Glyma17g34110 limited by rare tRNA‘s present in the amino acid

Due to poor expression of Glyma17g34110 under *E. coli BL21* (DE3) competent cells under various conditions (results not shown), prediction databases were used to analyze for any limiting factors. It was shown in previous studies that the presence of rare codons restricts the expression of the heterologous proteins in *E. coli BL21* competent cells (Carstens *et al.*, 2002). Thus, various prediction databases were used to determine the presence of rare codons in glyma7g34110. The results of the prediction databases were summarized in Table 4.4.1. From Table 4.4.1 it can be seen that 20 rare codons were present in Glyma17g34110, with the majority being for arginine, proline and glycine.

Table 4.4.1 Rare codons predicted in Glyma17g34110

Amino acid	Rare codon	Frequency of occurrence
Arginine	AGG	2
	AGA	3
	CGA	2
Leucine	CTA	2
Isoleucine	ATA	1
Proline	CCC	4
Glycine	GGA	4
	GGG	1
Threonine	ACG	1

4.4.2 Expression with *E.coli BL21* CodonPlus

The isolation of Glyma17g34110 was done by RT-PCR using specific primers. The RT-PCR yielded an amplicon of 705 bp which corresponded to the expected size of the coding region of Glyma17g34110 (Figure 4.4.2 A). Sequence analysis of Glyma17g34110 transformed in to pET-41a(+) showed 100% similarity to the sequence received on the Phytozome database (data not shown). Due to the high number of rare codons present in Glyma17g34110, *E. coli BL21* CodonPlus competent cells were used. BL21-CodonPlus cells are genetically modified to contain extra copies of these codons to accommodate for these heterologous proteins and allow efficient expression (Carstens *et al.*, 2002). Glyma17g34110 was recombinantly expressed as a glutathione S-transferase (GST) fusion protein in pET-41a(+), producing GST-Glyma17g34110 (±53 kDa) (Figure 4.4.2 B). GST-Glyma17g34110 was majorly expressed in inclusion bodies, thus appearing in the insoluble fraction. Various induction time periods, temperature ranges and IPTG concentrations were used in this experiment. The best result, however, was received at 4 hours induction (0.5 mM IPTG) at 37˚C (Figure 4.4.2 B). Upon purification of GST-Glyma17g34110 (Figure 4.4.2 C), the respective band was obtained and used in further experiments.

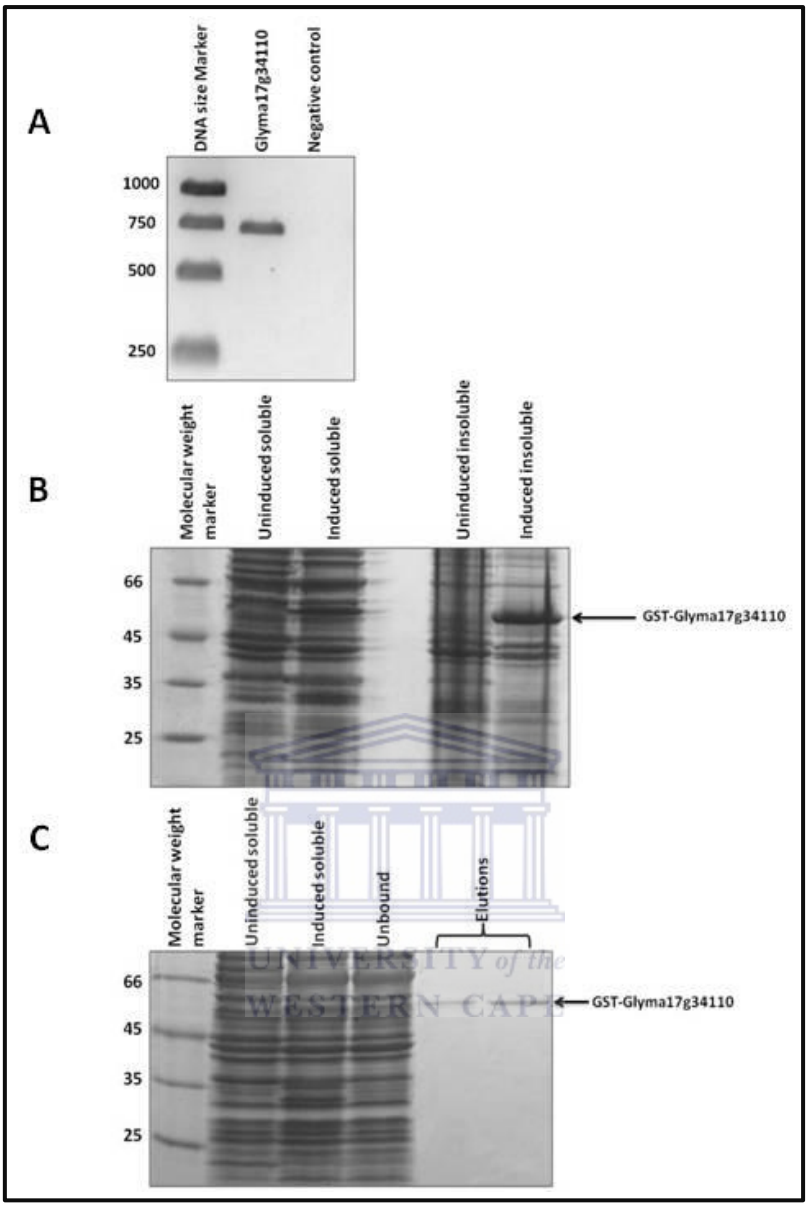

Figure 4.4.2 Cloning, recombinant expression and purification of GST-Glyma17g34110. (A) Isolation of Glyma17g34110 by RT-PCR. (B) Recombinant expression of GST-Glyma17g34110 (± 53 kDa). GST-Glyma17g34110 fusion protein can be seen in the induced soluble as well as insoluble fraction. (C) Purification of GST-Glyma17g34110 via glutathione-sepharose beads.

4.4.3 GST-Glyma17g34110 demostrates GPX activity

The purified GST-Glyma17g34110 fusion protein was tested for GPX activity using a Native PAGE and GPX staining as described by Seckin *et al*. (2010). GST was used in this experiment, with the same concentrations, as a suitable control to determine if any activity received from the GST-Glyma17g34110 was not only from the GST in the construct (Figure 4.4.3.1 A). GST-Glyma17g34110 and GST samples both showed activity in response to the GPX staining. Although GST showed activity, it was not as significant as that received from GST-Glyma17g34110. Further analysis of the in-gel assay was carried out using densitometry analysis. The activity of GST-Glyma17g34110 were analyzed relative to that of the activity demonstrated by GST (Figure 4.4.3.1 B). The activity shown by GST-Glyma17g34110 was 156.31% more than that shown by GST.

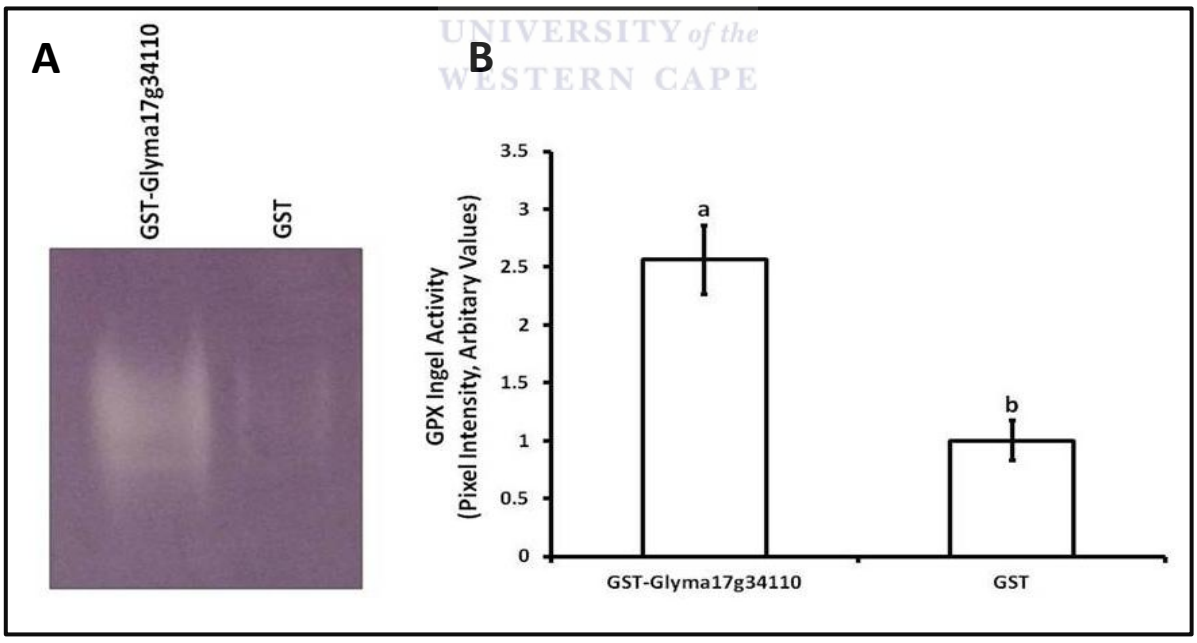

Figure 4.4.3.1 Determination of GPX activity in GST-Glyma17g34110. (A) Purified GST-Glyma17g34110 and GST were analysed for GPX activity in the presence of GSH via a GPX in-gel assay. Lane 1: Purified GST-Glyma17g34110, Lane 2: GST. (B) Densitometry analysis of the activity illustrated by GST-Glyma17g34110 relative to the activity of GST. Error bars (I) represent the mean of the study from three independent experiments.

Enzyme activities of Glyma17g34110 in the presence of cumene hydroperoxide were determined using a series of assays. GST was used as a control to determine whether the scavenging of cumene hydroperoxide was due to Glyma17g34110 and not its fusion partner, GST. Using GSH and thioredoxin as electron donors, the likelihood of Glyma17g34110 being a thiol dependent were deduced (Figure 4.4.3.2 A). GST-Glyma17g34110 scavenged a large percentage of the cumene hydroperoxide present using GSH as an electron donor. GST-Glyma17g34110 revealed a 175.8% increase in activity towards cumene hydroperoxide using GSH as compared to thioredoxin. GST, however, showed little deterioration of the cumene hydroperoxide in the presence of both GSH and thioredoxin. To further study the activity of GST-Glyma17g34110 in the presence of GSH, an assay was performed using a system to regenerate GSH using GR and NADPH (Figure 4.4.3.2 B). In Figure 4.4.3.2 B it can be seen that oxidation of NADPH had taken place thus demonstrating GSH was consumed during the scavenging of cumene hydroperoxide. GST showed little oxidation thus proving that the activity observed was from Glyma17g34110 and not GST.

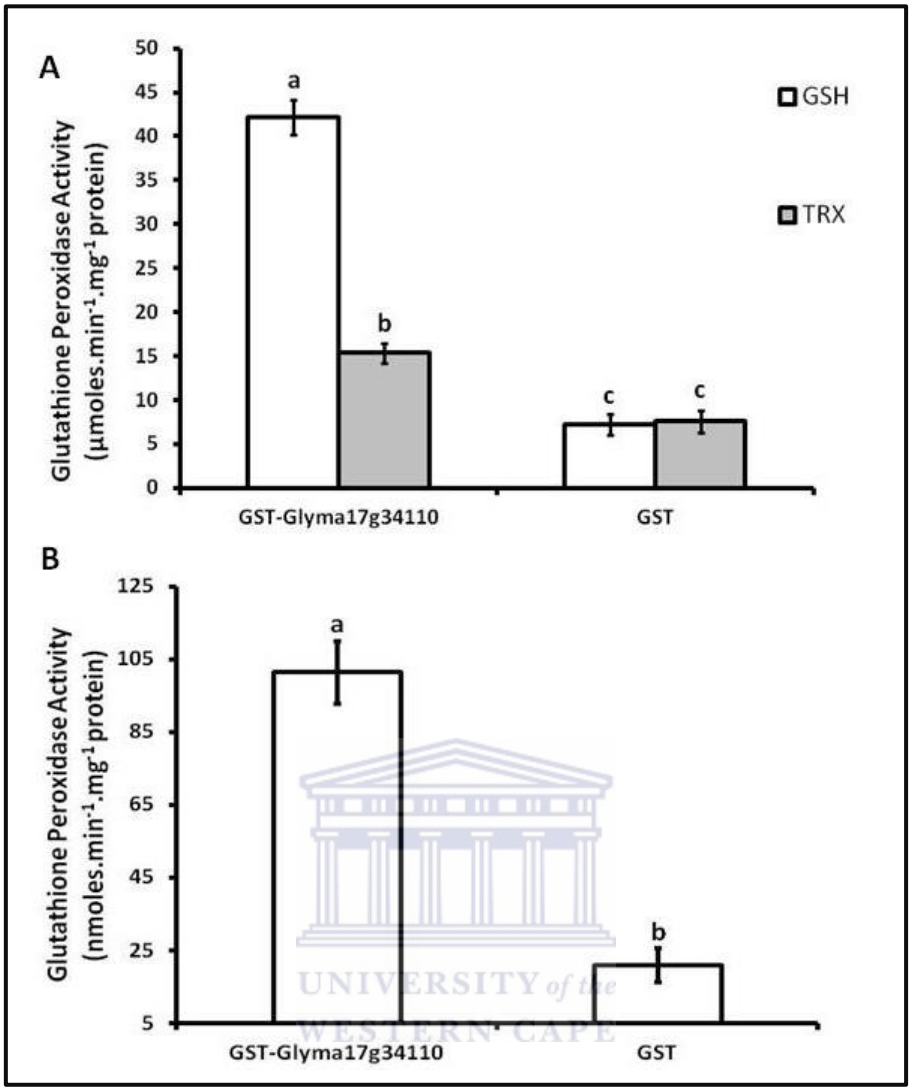

Figure 4.4.3.2 Substrate specificity of recombinant GST-Glyma17g34110. (A) Enzyme activities of GST-Glyma17g34110 and GST towards cumene hydroperoxide using GSH and TRX as substrates. Data represent cumene hydroperoxide content measured throughout the experiment. (B) GST-Glyma17g34110 and GST enzyme activities. Data represent NADPH content measured throughout the reaction.

4.5 DISCUSSION

It was shown in previous studies that the presence of rare codons in transformation constructs restricts the expression of the heterologous proteins in *E. coli BL21* competent cells. Due to the low amounts of tRNA for rare amino acid codons available in cells such as *E. coli*, the translation of heterologous proteins is stalled (Rosano & Ceccarelli, 2009). In this study, the expressions of Glyma17g34110 were unsuccessful in many *E. coli BL21* competent cell strains (data not shown). It was demonstrated that arginine codons such as AGA and AGG

are the rarest codons in *E. coli*, thus affecting the production of the protein and its quality tremendously. It is also believed that codons such as isoleucine (AUA), leucine (CUA) and proline (CCC) affects the expression of proteins as well (Carstens *et al.*, 2002; Trundova & Celer, 2007). The expression of Glyma17g34110 in *E. coli* cells was greatly restricted due to rare codons present in the amino acid sequence. Codons such as arginine (AGA, AGG and CGA), proline (CCC) and glycine (GGA) were majorly found in Glyma17g34110 and therefore suggest a possible reason for no expression. For this reason, *E. coli BL21* CodonPLUS cells were used for expression of Glyma17g34110. CodonPLUS cells are genetically engineered competent cells that contain extra copies of codons such as arginine and proline (Carstens *et al.*, 2002). Thus, the expression of Glyma17g34110 was possible using this strain.

It has been shown that in the presence of rare codons, the solubility of the heterologous proteins is affected. Many studies have shown, although the protein is expressed in modified competent cells, more than 85% of the protein lies in the insoluble protein (i.e. in inclusion bodies) (Rosano & Ceccarelli, 2009; Trundova & Celer, 2007). In this study, recombinant GST-Glyma17g34110 protein were expressed in *E. coli* CodonPLUS cells, however, more than 60% was in inclusion bodies under a variety of conditions tested. In a study by Rosano & Ceccarelli (2009), it was hypothesized and shown that proteins containing approximately 5% of RIL codons (i.e.: AGA/AGG - arginine, ATA - leucine and CTA - isoleucine) are predominantly expressed in the insoluble fractions when expressed with codon bias-adjusted *E. coli* strains. The high percentage of arginine codons contributed to the insolubility of these proteins. It was also shown that the expression of these proteins in other strains were detrimental to expression of the gene. The number of arginine codons (AGG/AGA) contributed to the insolubility of Glyma17g34110.

According to research, *bona fide* GPX enzymes do not exist in plants, but rather those enzymes containing GPX-like activity are thioredoxin peroxidase or peroxiredoxins rather than glutathione peroxidases (Navrot *et al.*, 2006). Many studies have shown that GPX activity exists in plants, playing important roles in the response to abiotic and biotic stress (Chang *et al.*, 2009). In Chapter 2 of this study, Glyma17g34110 was shown to contain sequence homology to GPX genes found in characterized *A. thaliana* and *L. japonicus* and further shown in this section to display GPX activity. To further understand the mechanism of antioxidant enzymes such as GPX, many studies have employed experiments to determine the activity of these genes towards peroxides. In a previous study done on AtGPX8, it was suggested plant GPX enzymes are more specific to thioredoxin as to GSH as they react with the Cys-X-X-Cys motifs present in thioredoxin (Gaber *et al.*, 2012). In this study the activity of GST-Glyma17g34110 towards cumene hydroperoxide were determined in the presence of GSH and thioredoxin. Although research has shown most plant GPX genes prefer thioredoxin as an electron donor, it was observed in this study that GST-Glyma17g34110 prefer GSH for the scavenging of cumene hydroperoxide. To validate the activity received from the protein GST-Gyma17g34110 is from Glyma17g34110 and not the GST in the fusion protein, activity assays were performed using GST as well. Due to the lack of activity in all the assays tested with GST it can be said the activity received were due to Glyma17g34110.

Thus, GST-Glyma17g34110 demonstrates GPX activity using GSH as a preferred electron donor and substrate. The expression of Glyma17g34110 needs to be optimized to obtain a higher concentration of the GST-Glyma17g34110, thus allowing cleavage of the GST and enabling further experiments involving the up-regulation of Glyma17g34110 in cultivars during abiotic stress.

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CONCLUSION AND FUTURE PERSPECTIVES

It has been established that antioxidant enzymes such as glutathione peroxidase are vital for normal growth, development and in the response to the harmful effects (over-production of reactive oxygen species) of abiotic stress. Thus, the aims of this study were to do an *in silico* analysis of a putative glutathione peroxidase gene (Glyma17g34110), analyzing important characteristic domains and its promoter region. To expand on the results received from the bioinformatic analysis, gene expression profiles of *Glyma17g34110* in various soybean tissues (leaves, roots and nodules) were analyzed. The gene expression of *Glyma17g34110* in response to one of the major abiotic stresses affecting soybean (salinity) was also studied. Previous studies have shown that nitric oxide increases the activity of antioxidant enzymes. Thus, nitric oxide was exogenously applied to soybean to analyze the effects on *Glyma17g34110*. Furthermore, to validate the results received from the bioinformatic analysis, the enzyme activity of Glyma17g34110 was characterized.

Bioinformatic analysis revealed that Glyma17g34110 is a putative chloroplast protein with possible roles in the photosynthetic pathway. This was supported by various *cis*-acting elements present in the promoter region involved in light responsiveness. These findings were supported by the high gene expression of *Glyma17g34110* within the leaves (primary location of photosynthetic machinery). To understand how antioxidant enzymes respond to abiotic stress such as salinity, 120 mM NaCl were applied to soybean plants [long term (21 days) and short term (48 hours)]. It was shown that *Glyma17g34110* was down-regulated in the presence of salt. The promoter analysis of *Glyma17g34110* showed that the *Glyma17g34110* promoter contained no known *cis*-acting elements that have been characterized as salt responsive. Thus, this could account for its sensitivity to salt stress. Furthermore, studies have also shown that with the application of nitric oxide, the gene

expressions of antioxidant enzymes increased. In this study, it was shown that with the application of exogenous nitric oxide, the gene expression of *Glyma17g34110* was increased tremendously in all tissue (leaf, roots and nodules) tested. Various *cis*-acting elements responsive to NO were identified in previous studies. However, none of these elements were present in the promoter region of *Glyma17g34110* and thus novel elements could be responsible for the nitric oxide response. Therefore, the identification of this *cis*-acting element and the engineering of Glyma17g34110 under salt stress could result in enhanced activity for an improved defence system. To allow any further studies of Glyma17g34110, the characterisation of Glyma17g34110 as a protein needs to be achieved. In this thesis it was shown that Glyma17g34110 were successfully characterised as a glutathione peroxidase gene using glutathione as an electron donor to remove cumene hydroperoxide as substrate.

Genetic engineering using Glyma17g34110 to transform plants could result in a higher glutathione peroxidase activity within crops, resulting in an enhanced defence mechanism against reactive oxygen species (such as H_2O_2) which are normally produced during salinity.