CHARACTERISATION OF A NOVEL SOYBEAN CANDIDATE GLUTATHIONE PEROXIDASE/THIOREDOXIN-DEPENDENT PEROXIDASE IN SOYBEAN EXPOSED TO OSMOTIC/DROUGHT STRESS

By

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A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae (Biotechnology) in the Department of Biotechnology, University of the Western Cape

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KEYWORDS

Antioxidant enzymes

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Soybean

Substrates specify.

Thioredoxin-dependent peroxidase

ABSTRACT

Drought stress is a major contributor to reduced soybean crop yield and quality, this can however be mitigated by the plant's antioxidant defence mechanisms. One group of antioxidant enzymes that are active in these defence mechanisms are glutathione peroxidases (GPXs). GPXs are antioxidant proteins which are able to reduce H_2O_2, a toxic reactive oxygen species which accumulates under stress conditions. This study aims at isolating the protein encoded by Glyma01g42840 and determining if it has Phospholipid hydroperoxidase glutathione peroxidase (PHGPX) and/or Thioredoxin dependent peroxidase (TRX-PX) activity as well as assaying the effect of Drought stress on the expression of this putative GPX . This will be accomplished by molecular cloning, sequencing as well as the expression of the isolated protein to assay it enzymatic activity. It was found that the enzyme encoded by Glyma01g42840 is able to use glutathione and thioredoxin as electron donors for the detoxification peroxides, however enzymatic activity is more efficient when using glutathione as an electron donor. In conclusion it was found that glyma01g42840 encodes an enzyme which is able to utilise more than one electron donor and as glutathione produces the greatest amount of enzymatic activity it can be said that glyma01g42840 encodes a GPX.

DECLARATION

I declare that "*CHARACTERISATION OF A NOVEL SOYBEAN CANDIDATE GLUTATHIONE PEROXIDASE/THIOREDOXIN-DEPENDENT PEROXIDASE IN SOYBEAN EXPOSED TO OSMOTIC/DROUGHT STRESS*" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name..... Date.....

Signed.....

ACKNOWLEDGMENTS

I would like to thank my principal investigator **Prof. Ndiko Ludidi** for all the advice and demonstration, what I have learnt from you will be invaluable to the furthering of my career. To my co-supervisor **Dr Marshall Keyster** without you I would not have accomplished half of what I did this year, thank you for being my mentor and friend, you helped me through many depressions over failed experiments. Finally thank you to **Mr Ashwil Klein**, your support and friendship is second in value only to the technical advice you provided during my time of struggle.

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LIST OF ABBREVIATIONS

APX	Ascorbate peroxidase
APS	Ammonium persulphate
ATP	Adenine triphosphate
BLAST	Basic local alignment search tool
bp	Base pairs
CAT	Catalyse
CTAB	Cetyl-trimethylammonium bromide
DEPC	Diethylpyrocarbonate
DTT	Dithiotheritol
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
GPX	Glutathione peroxidase
GR	Glutathione reductase
GmPHGPX	*Glycine max* Phospholipid hydroperoxide glutathione peroxidase
GSH	Glutathione
GST	Glutathione-*S*-Transferase
HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kiloDalton
LB	Luria broth
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NTB	Nitrotetrazolium blue
OD	Optical density
OH^-	Hydroxyl anion
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
PHGPX	Phospholipid hydroperoxide glutathione peroxidase
PsPHGPX	*Pisum sativum* phospholipid hydroperoxide glutathione peroxidase
PS1	Photosystem 1
PS2	Photosystem 2
PVP	Polyvinylpyrrolidone

rpm	Revolutions per minute
ROS	Reactive oxygen species
RWC	Relative water content
SDS	Sodium dodecyl sulfate
SOS	Superoxide dismutase
TBE	Tris Boric acid Ethylenediaminetetraacetic acid
TEMED	Tetramethylethylene-diamine
TRX	Thioredoxin – dependent peroxidase
UV light	Ultra violet light

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OBJECTIVES OF THE STUDY

The thesis was aimed at achieving the following objectives:

- Isolation of a gene encoding a putative glutathione/thioredoxin-dependent peroxidase from soybean
- Expression of the protein encoded by soybean
- glutathione/thioredoxin-dependent peroxidase gene
- Assaying the enzymatic activity of the glutathione/thioredoxin-dependent peroxidase
- Determining the spatial and temporal expression patterns of the gene encoding the glutathione/thioredoxin-dependent peroxidase
- Determining the effect of drought on the expression and activity of the glutathione/thioredoxin-dependent peroxidase

CHAPTER 1:

LITERATURE REVIEW

1.1 INTRODUCTION

Isaac Newton's law of motion states that for every action there is an equal and opposite reaction, this does not only apply to physical science but to biology as well. When oxygen was introduced into the enviroment approximately 2.7 billion years ago by evolving photosynthetic organisms, the equal and opposite action was the evolution of reactive oxygen species (Halliwell, 2006). Subsequently the equilibrium between the generation and detoxification of Reactive oxygen species (ROS) that is required to prevent oxidative stress is another application of Newton's law in biology. As the name implies, ROS are oxygen radicals or oxygen containing molecules that are able to react with macromolecules and cause changes to DNA, proteins and membrane lipids (Foyer *et al.*, 2005). In order to protect themselves against oxidative damage, plants have evolved a number of enzymatic and non-enzymatic antioxidant systems which prevent the accumulation of ROS and maintain them at low concentrations where they can be used as signalling molecules in a variety of growth, development and defence processes. In the subsequent section, the chemistry of ROS as well as their production in various organelles and their function as signalling molecules will be discussed and an overview of plant antioxidant mechanisms used to maintain ROS equilibrium will be given with focus on the characteristics identifying glutathione peroxidases.

1.2 REACTIVE OXYGEN SPECIES

Oxygen constitutes approximately 21% the earth's atmospheric gases, making it available for use by plants animals and other organisms. As explained by Gill and Tuteja (2010) oxygen molecules have the potential to damage the organisms that rely on it to survive; the destructive properties of oxygen are attributed to the molecules having two unpaired electrons that have the same spin quantum number. As a result of this spin restriction, oxygen is able to accept a single electron at a time, which causes the formation of reactive oxygen species (ROS) (del Río *et al.*, 2006). There are several reactive oxygen species which from under varying conditions and affect plants in different ways. These reactive oxygen species include superoxide radicals ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), Hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). These ROS are continuously produced as by-products of regular plant metabolism and are localized to different cellular components including the chloroplast, mitochondria and the peroxisome (Navrot *et al.*, 2007).

One of the major sites for ROS synthesis in plants is the thylakoid membrane-bound primary electron acceptor in the chloroplast; here superoxide radicals are continuously produced during photosynthesis as a result of the partial reduction or energy transfer to oxygen (Gill*, et al.*, 2010). Superoxide radicals are usually the first ROS produced and may trigger the generation of other ROS including, $^{\cdot}OH$ and $^1O_2$, which results in peroxidative damage to membrane lipids and a general weakening of cellular structure. The reaction of superoxide radicals with iron (Fe^{3+}) produces reduced iron (Fe^{2+}) which can reduce H_2O_2 to O_2^- and OH (Gill*, et al.*, 2010). According to Krieger-Liszkay (2005), singlet oxygen is another ROS that is generated during photosynthesis but its formation is not a result of electron transfer to

oxygen but rather insufficient energy dissipation during photosynthesis, which leads to the formation of the chlorophyll triplet state. The chlorophyll triplet state is a lower energy state which has a longer half-life and can react with $^3O_2$ to generate $^1O_2$, which has been proven to severely damage both photosystem I (PSI) and photosystem II (PSII) and the photosynthetic apparatus in general. Singlet oxygen is considerably damaging to plants because of its ability to oxidize a wide range of biological molecules and react with proteins, nucleic acids and lipids. Singlet oxygen is also thought to be the ROS that is responsible for light-mediated loss of PSI activity which contributes to the triggering of cell death (Krieger-Liszkay *et al.*, 2008).

Generated as a result of the univalent reduction of oxygen, H_2O_2 is a moderately reactive oxygen species with a longer half-life (1 ms) relative to other ROS (2-4 µs). Unlike $^1O_2$ which targets membrane lipids, H_2O_2 causes oxidative damage, when present at high concentrations, by inactivating enzymes via the oxidation of the thiol groups as explained by Tewari *et.al* (2006). Aside from its destructive capabilities, H_2O_2 also acts as a signalling molecule when it is present at homeostatically favoured concentrations and is a regulator of various physiological processes including senescence, photorespiration and photosynthesis. The use of H_2O_2 as a signalling and regulatory molecule can be attributed to its relatively long half-life and high permeability across membranes (Gill *et al.*, 2010; Quan *et al.,* 2008). The most reactive ROS are hydroxyl radicals ($^{\cdot}OH$) which can be generated from O_2^- and H_2O_2 by the Fenton reaction in the presence of iron (Gill et al., 2012). Hydroxyl radicals are the most destructive ROS as they can react with all biological molecules and there is no characterized enzymatic mechanism that can be used to keep hydroxyl radical concentrations in a steady state equilibrium, as a result increased concentrations of hydroxyl radicals always result in cell death (Gill*, et al.*, 2010).

1.3 THE PRODUCTION OF REACTIVE OXYGEN SPECIES IN VARIOUS ORGANELLES

Three organelles have been identified in plants to be the main sources of ROS generation. These are the chloroplast, the peroxisome and the mitochondria (Sharma *et.al*, 2012). As a result of this, photosynthetic plants are prone to oxidative damage because of the presence of the chloroplasts in their cells.

1.3.1 ROS PRODUCTION IN THE CHLOROPLAST

Photosynthesis is the process by which plants capture light energy from the sun and use it to produce triose phosphate and release oxygen. Photosynthesis occurs in the chloroplast which is believed to have evolved from prokaryotic organisms similar to cyanobacteria. Martin *et.al* (2002) explains that these prokaryotic organisms were assimilated into eukaryotic organisms by endosymbiosis. The chloroplast is structured to allow for optimal light harvesting as it contains the thylakoid membrane system which is highly organised and houses all the components required for the capturing of light (Pfannschmidt, 2003). Due to the nature of the chloroplast's function, $^1O_2$ is generated as a natural by-product of photosynthesis and it is produced by PS II even in conditions of low light. There are various routes for singlet oxygen production in the chloroplast, as depicted in Figure 1.3.1. Singlet oxygen can be produced during photosynthesis when oxygen accepts electrons that have been passed through the photosystem (Gill*, et al.*, 2010), alternatively when the electron transport chain becomes overloaded the flow of electrons may be partially diverted from ferredoxin to oxygen (Rinalducci *et al.*, 2008). Electron leakage at the acceptor side of PS II is another means of singlet oxygen generation, here electrons that have leaked from the PS II are accepted by oxygen and singlet oxygen is produced. Superoxide radicals are also

generated in the chloroplast, this however only occurs under conditions of high light intensity and insufficient CO_2 supply (Triantaphylidès *et al.*, 2008). The generation of superoxide radicals occurs via the Mehler reaction, which is described by Makino, Miyake and Yokota (2002) as the photoreduction of O_2 at PS I. The disproportionation of the superoxide anion (O_2^-) by superoxide dismutase then leads to the generation of H_2O_2 and O_2. The generated H_2O_2 is then detoxified to produce H_2O by the anti-oxidant activity of ascorbate peroxidase (Asada, 1999). Although the chloroplast is a significant site for ROS production under stress conditions, certain plants have evolved mechanisms to use this to their advantage by linking chloroplast-generated ROS to the hypersensitive response as explained by Mur *et.al* (2008).

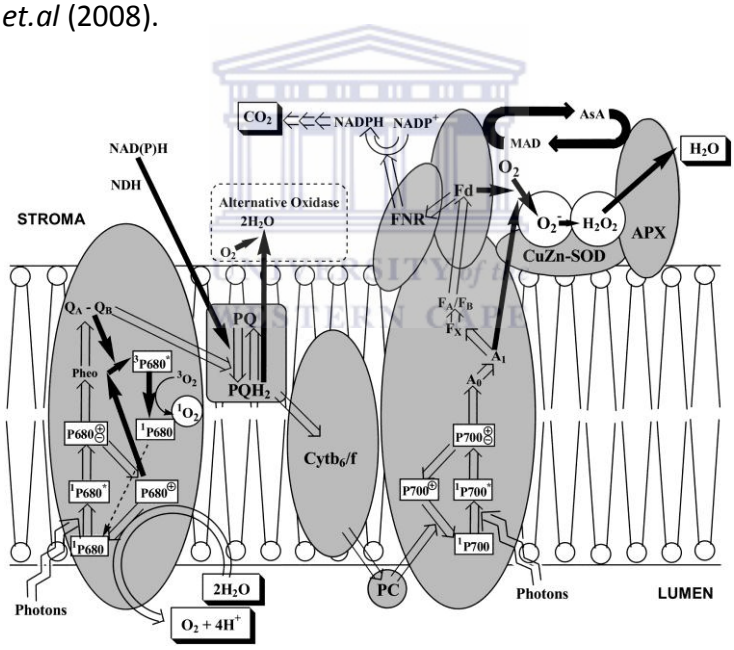

Figure 1.3.1: The production of $^1O_2$ and O_2^- in the chloroplast. This figure shows the production of $^1O_2$ at PS II and O_2^- at PS I. The white arrows indicate the flow of electrons and excitation of Chl reaction centers under normal light intensity, with all the electrons generated being used for the fixation of CO_2. Under conditions where light intensity exceeds photosynthetic capacity, the electron flow is diverted resulting in the production of ROS (indicated by the black arrows). The top right corner shows the dismutation of superoxide to hydrogen peroxide by CuZn superoxide dismutase and the detoxification of hydrogen peroxide into water by ascorbate peroxidase (figure adapted from Asada 2006).

1.3.2 ROS PRODUCTION IN THE PEROXISOME

Peroxisomes are small, spherical subcellular organelles that are enveloped by a single lipid bilayer membrane. These microbodies have oxidative metabolism which produces O_2^- as a natural by-product. These organelles function in the degradation of branched amino acids via photomorphogenesis, the biosynthesis of jasmonic acid and auxin, which are plant hormones as well as the production of glycine betaine (Gill *et al.,* 2010; Hu, 2007).

Corpas *et al*. (2001) explain that there are two main sites for ROS production in the peroxisome, the first of these sites is the organelle matrix where xanthine oxidases are responsible for the generation of superoxide radicals. The superoxide produced in the organelle matrix then becomes the precursor for the generation of H_2O_2 and O_2 by superoxide dismutase which scavenges the superoxide. The H_2O_2 can then be removed from the system by catalase under conditions of steady state equilibrium. The second site of ROS generation in the peroxisome is the peroxisomal membrane which houses an NAD(P)H-dependent O_2^- production site in the form of a small electron transport chain (ETC) that uses oxygen as an electron acceptor. The direct production of H_2O_2 also occurs at the peroxisome, here this ROS is generated via the photorespiratory glycolate oxidase reaction which is the fixation of carbon to be used in the oxygenation of ribulose-1,5-bisphosphate (Hofmann, 2011; Willekens *et al.*, 1997). Peroxisomal H_2O_2 generation may also occur as a result of fatty acid β-oxidation, which is the metabolism of triacylglycerols composed of mainly long chain fatty acid and are found mostly in storage organs such as endosperms and cotyledons. H_2O_2 generation by fatty acid β-oxidation occurs mainly in glyoxysomes, these are specialized peroxisomes dedicated to the degradation of fatty acid (Hayashi *et al*., 2002).

The pathway described here is shown in Figure 1.3.2. The increase in ROS concentration beyond antioxidant capacity leads to oxidative damage and eventually cell death. Here, antioxidant capacity refers to the ability of the cellular antioxidant system to efficiently scavenge ROS to levels that are homeostaticlly favoured to maintain the optimal redox status required for normal signalling and metabolism. However, it has been shown that O_2^- and H_2O_2 generated at low concentrations may act as signalling molecules which induce the hypersensitive response and induce controlled cell death when plants are attacked by pathogens (Grant *et al.*, 2000; McDowell *et al.*, 2000).

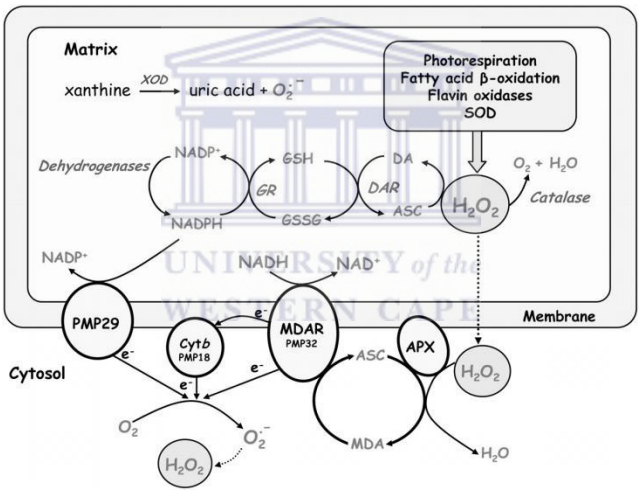

Figure 1.3.2: The production of superoxide radicals and the functioning of antioxidant systems in leaf peroxisomes. The production of ROS occurs at two sites in the peroxisome, the first site is in the organelle's matrix where the oxidation of xanthine by xanthine oxidase (XOD) liberates uric acid and O_2^-. The second site of ROS production is in the peroxisomal membrane where a small ETC composed of three membrane polypeptides namely PMP29, Cytochrome b and monodehydroascorbate reductase (MDAR) leads to production of O_2^-. (Figure adapted from del Rio *et.al* 2006)

1.3.3 ROS PRODUCTION IN PLANT MITOCHONDRIA

Plant mitochondria are the organelles responsible for the production of energy in plants cells, in order to perform this function they contain specific ETC components and drive processes such as respiration. The mitochondria in plants are considered to be a major site for ROS generation as the ETC in the mitochondria contains electrons with enough free energy to facilitate the reduction of oxygen (Rhoads *et al.*, 2006). Due to the presence of the electron transport chain, ROS are produced in mitochondria under normal respiratory conditions. However, this ROS production becomes aggravated under biotic and abiotic stress conditions and O_2^- is produced at mitochondrial complex I and III. Once O_2^- is generated it is then disproportionated by SOD to H_2O_2. This ROS production pathway is shown in Figure 1.3.3. It is estimated the between 1-5% of oxygen consumed by the mitochondria results in the production of H_2O_2 (Moller, 2001). The accumulation of H_2O_2 is especially dangerous as these molecules can react with reduced Fe^{2+} and Cu^+ and generate the highly reactive $OH^{\cdot}$. Due to the uncharged nature of H_2O_2 molecules, they are able to travel across membranes, leading to the peroxidation of the polyunsaturated fatty acids (PUFA) that make up the mitochondrial and cell membranes. The peroxidation of the membrane PUFA ultimately leads to the formation of cytotoxic lipid aldehydes, alkenals and hydroxyalkenals, these lipid peroxidation products then go on to react with proteins, nucleic acids and other lipids, which causes cellular damage (Almeras *et al.*, 2003). Due to the effect of abiotic stress on plant cell bioenergentics, plant mitochondria use a system of energy dissipation to mitigate the generation of ROS. The use of an energy dissipation system in plant mitochondria was confirmed in a study done by Pastore *et.al* (2007), which showed

that the dissipation of energy by durum wheat mitochondria lowered the levels of ROS production in the organelle under abiotic stress conditions.

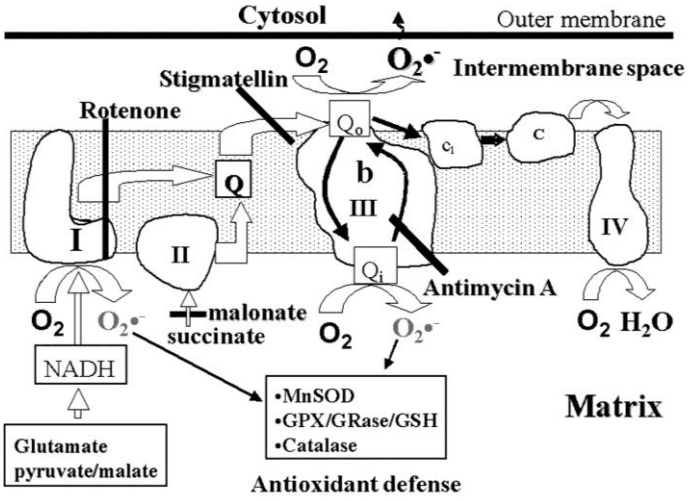

Figure 1.3.3: Depiction of the mitochondrial electron transport chain and ROS producing site. There are three sites for O_2^- production along the mitochondrial ETC, which include the site at complex I and the Q_1 site at complex III, which release ROS into the mitochondrial matrix where they are detoxified by manganese superoxide dismutase (MnSOD), Glutathione peroxidase (GPX) and catalyse (CAT). The alternate site for O_2^- generation is the Q_0 site on complex III, wherein O_2^- produced at this site is released away from the matrix antioxidant defences and is more likely to leave the mitochondria (figure adapted from Chen *et.al* 2003).

1.4 THE DAMAGING EFFECTS OF ROS ON CELLULAR STRUCTURE AND MACROMOLECULES

1.4.1 LIPID PEROXIDATION (LPO)

It has been established that various abiotic stresses causes plants to over-produce ROS, which are known to attack PUFA and result in lipid peroxidation (LPO). This phenomenon is described by Gill and Tuteja (2010) as the most damaging process that occurs in living organisms. The process of LPO occurs in three distinctive stages: initiation, propagation and termination. The initiation of LPO occurs when a hydrogen atom is abstracted from a carbon in an unsaturated fatty acid, this occurs when the unsaturated fatty acid is attacked by O_2^-, H_2O_2 or $OH^{\cdot}$. When LPO is initiated in an aerobic environment, the fatty acid at the carbon

centred lipid radical ($L^{\cdot}$) will have oxygen added to it, forming a lipid peroxyl radical ($LOO^{\cdot}$). The $LOO^{\cdot}$ then causes the process of LPO to move to the next stage, which is referred to as propagation. During this state the $LOO^{\cdot}$ abstracts hydrogen atoms from vicinal unsaturated fatty acid and starts a peroxidation chain reaction leading to the formation of lipid hydroperoxides (LOOH). Once the LOOH molecules are formed, they can be easily decomposed into several types of reactive molecules which include lipid alkoxyl radicals, aldehydes, alkanes, lipid epoxides and alcohols. The final stage of LPO is referred to as termination. In this stage fatty, acid dimers as well as peroxide-bridged dimers are formed (Davies, 2000). Once LPO is complete, the affected membrane exhibits reduced fluidity, which makes it easier for phospholipids to move from one half of the bilayer to the other. Peroxidised membranes are also more permeable and substances that are not allowed to cross the membrane under normal conditions would now be able to move across the membrane freely. LPO is not only extremely damaging due to its effects on membranes but also because it leads to the production of aldehydes such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), which damage DNA and proteins by forming conjugates with the molecules (Gill*, et al.*, 2010).

1.4.2 PROTEIN OXIDATION

As described by Ghezzi and Bonetto (2003), the induction of covalent protein modification by reactive oxygen species or the by-products of oxidative stress is termed protein oxidation. Protein oxidation occurs when a number of amino acids in a protein are oxidised, giving rise to free carbonyl groups, which leads to the inhibition of or altered enzyme activity. Particular amino acids; which include Arg, His, Lys, Pro, Thr and Trp; are usually targets for protein oxidation, which causes the proteins to become more susceptible to

proteolytic attack (Moller *et al.*, 2007). Alternatively Hancock *et.al* (2006) suggest that amino acids that contain sulphur and thiol groups, such as Cys and Met, are likely targets for reactive oxygen species in particular $^1O_2$ and $OH^{\cdot}$. The action of ROS on Cys causes the animo acid to lose a hydrogen atom and form a thiyl radical. These thiyl radicals then react with membrane lipids and result in *cis-trans* isomerization of the lipids (Ferreri *et al.*, 2005). In the case of Met, the reaction with ROS leads to the addition of oxygen to the amino acid and the formation of methionine sulphoxide derivatives (Gill*, et al.*, 2010). It has be observed that protein oxidation occurs shortly after ROS concentrations have exceeded antioxidant capacity and causes a decrease in cellular ATP production and subsequently cell death (Stadtman *et al.*, 2000).

1.4.3 DNA DAMAGE

In spite of the stability of the plant genome, it is still susceptible to the effects of genotoxic stress induced by biotic and abiotic stresses (Tuteja *et.al*, 2009). The damage suffered by DNA as a result of the activity of reactive species is referred to as spontaneous DNA damage. The reactive molecules responsible for the damage of DNA are $OH^{\cdot}$ and $^1O_2$. The majority of DNA damage can be attributed to the reaction of $OH^{\cdot}$ with DNA, as it reacts with the purine and pyrimidine bases as well as the deoxyribose backbone, although $^1O_2$ is not as reactive with DNA it still causes damage by reacting with guanine (Gill*, et al.*, 2010). ROS usually damage DNA by causing base deletion and modifications (alkylation and oxidation) as well as causing the formation of pyrimidine dimers, cross-links and strand breaks (N. Tuteja *et al.*, 2001). Damage to DNA by ROS has seriously detrimental effects on plants as it causes a reduction in protein synthesis, damage to the cell membrane as well as

photosynthetic proteins resulting in an overall retardation of plant growth and development (Britt, 1999).

1.5 REACTIVE OXYGEN SPECIES AS SIGNALLING MOLECULES

Signalling molecules are chemicals which transmit information between cells in multicellular organisms. In order for a molecule to perform this function, it must be small, with a relatively long half-life and it must be diffusible (Pitzschke *et al.*, 2006). A prime example of a ROS that acts as a signalling molecule is H_2O_2, which functions in cell-to-cell signalling due to its ability to cross plant membranes. Reactive oxygen species are able to control the activity of certain transcription factors; by doing this, these molecules are able to regulate the expression of certain genes. In addition to regulating gene expression, ROS may also generate oxidation products which may act as secondary messengers. This is seen in the alteration of gene expression by the action of biologically active lipid peroxidation products that arise from the attack of polyunsaturated fatty acids by ROS (Grether-Beck *et al.*, 2000). Although the activity of ROS as signalling molecules are not completely understood, the distinct mode of ROS signalling have been identified and will be discussed below.

1.5.1 REACTIVE OXYGEN SPECIES AND STOMATAL SIGNALLING

As explained by McAinsh (1996), H_2O_2 is an essential signalling molecule in the closure of stomata induced by abscisic acid (ABA). Under conditions of dehydration the accumulation of abscisic acid occurs, this triggers several adaptive responses including the closure of the stomata. As shown by Pei *et.al* (2000), dehydration is perceived by a receptor which is unknown and this triggers the synthesis of ABA in the guard cells. The increased levels of ABA then cause increased levels of H_2O_2 to be generated. The increased levels of H_2O_2 in

turn activate Ca^{2+} channels present in the plasma membrane of the stomatal guard cells, the increase Ca^{2+} concentrations in the cytoplasm of the guard cells then result in closure. In a study completed by Bright *et.al* (2006), the relationship between ABA-induced H_2O_2 production, nitric oxide (NO) and stomatal closure was shown in *Arabidopsis thaliana*. The study suggested that both NO and H_2O_2 are required to achieve stomatal closure in *A. thaliana*. This was proven by the use of nitrate reductase (NR) mutants *nia 1* and *nia 2*. Nitrate reductase was shown by genetic data to be the main source of NO generation in guard cells during ABA induced H_2O_2 synthesis. In both mutants (which have severely reduced NR activity) the ABA-mediated generation of H_2O_2 did not induce NO generation and as a result stomatal closure failed to occur (Wang *et al*, 2010). In an independent study carried out by Zhang *et.al* (2001) H_2O_2 was shown to be an important signalling molecule in the ABA mediated closure of stomatal guard cells in *Vicia faba* (faba bean). Here, the addition of exogenous catalase (CAT), which is a scavenger of H_2O_2, prevents the induction of stomatal closure by ABA. This indicates that H_2O_2 is required for the ABA-mediated closure of the stomata.

1.5.2 REACTIVE OXYGEN SPECIES SIGNALLING IN ROOT HAIR DEVELOPMENT

Root hairs are thin hair-like outgrowths found on trichoblasts (hair forming epidermal cells) of plant roots. These tubular structures assist the plant in the uptake of water and nutrients as well as aiding in anchoring the plant in the soil (Šamaj *et al.*, 2004). The development of root hairs can be divided into three phases: in the first phase, or bulge formation, is the establishment of trichoblast cell polarity and site selection for bulging occurs, the nucleus then migrates to the centre of the trichoblast and then to the base of the developing root

hair. Once this occurs, localized acidification, thinning and loosening of the cell wall results in bulge formation. Once the bulge is formed, the second phase (referred to as elongation) comes into effect. Here, initial growth at the tip is slow at first but gradually increases to rapid sustained elongation. During the elongation phase, the organisation of the polarized cytoplasm occurs as well as the exocytosis of the cell wall and membrane constituents at the growing tip. The elongation phase ends when the nucleus migrates into the growing hair and vacuolation is complete. Once the elongation of the root hair is complete, the hair matures and enters into the final phase of development i.e. maturation (Carol *et al.*, 2002). In a study by Foreman *et.al* (2003), *Arabidopsis root hair defective 2* (rhd2) mutants were used to show that the elongation of root hairs are dependent on ROS signalling. The rhd2 mutants are able to form bulges but not mature root hairs and this is attributed to a defective Ca^{2+} uptake and NADPH oxidase. It was shown that in wild type plants an increase in ROS concentration is localized at the growing tip while the concentrations of ROS in the rhd2 mutant root hair tips were much lower. Furthermore when NADPH oxidase was inhibited by the addition of diphenylene iodonium (DPI), root hair development in wild type plants was stunted. When the rhd2 mutants were treated with ROS, there was a partial suppression of the root hair mutant phenotype due to stimulation of the hyperpolarization-activated Ca^{2+} channel (predominant Ca^{2+} acquisition system in roots) in the plasma membrane of trichoblasts. These finding (Foreman *et.al*, 2003) are indicative of the role of ROS produced by NADPH oxidase as a signalling molecules to regulate the elongation of root hairs by activating Ca^{2+} uptake.

1.5.3 REACTIVE OXYGEN SPECIES AS SIGNALLING MOLECULES UNDER BIOTIC STRESS

Reactive oxygen species are not only used as signalling molecules during regular plant function and development, but also as a defence mechanism against pathogen infection. As explained by Grant and Loake (2000), plants produce a biphasic oxidative burst in response to pathogen infection. It is believed that membrane-bound NADPH oxidase, which is the same enzyme responsible for ROS production in root hair development, is responsible for the generation of ROS during pathogen attack. According to Baker and Orlandi (1995), the first oxidative burst resulting from pathogen attack exhibits a rapid increase in H_2O_2 concentration, while the second burst occurs more slowly but H_2O_2 is produced over a longer period of time. During compatible pathogen interaction, only the first oxidative burst occurs and the pathogen is able to infect the host. However, during incompatible pathogen interactions the second oxidative burst establishes resistance to the pathogen (Grant*, et al.*, 2000). This system of biphasic oxidative burst as a defence against pathogen attack is seen in Arabidopsis leaves where the induction of oxidative burst in parts of the plant distal to the site of attack results in a systemic acquisition of immunity due to the expression of defence related genes (Alvarez *et al.*, 1998). In order for the plant to use ROS as signalling molecules in pathogen defence, the plant needs to produce ROS at increased concentrations as well as down-regulate the expression of antioxidant enzymes. During biotic stress (pathogen attack), salicylic acid and nitric oxide signal the down-regulation of antioxidant enzymes such as APX and CAT, which allows H_2O_2 concentrations to increase to sufficient levels to induce programed cell death (PCD), which is thought to be induced in an attempt to inhibit the spread of disease from the point of infection (Klessig *et al.*, 2000; Pitzschke, *et al.*, 2006). It was shown in a study carried out by Mittler *et.al* (1999) that tobacco with

reduced APX and CAT activity exhibited superior PCD when exposed to low bacterial doses. The relationship between ROS and induction of PCD was further illustrated by Tiwari *et al.* (2002) who showed that Arabidopsis knock-out mutants that lack functional rboh genes have reduced ROS generation and this influenced PCD during bacterial infection. It can therefore be concluded that ROS are important signalling molecules in plant defence against pathogen infection and that antioxidant capacity is the limiting factor in the induction of PCD by reactive oxygen species.

1.6 PLANT ANTIOXIDANT SYSTEMS USED TO MAINTAIN ROS EQUILIBRIUM

In the preceding sections, it has been argued that ROS have a dual role in plants, where ROS act as important signalling molecules in the growth and development of plants under steady state conditions but the production of ROS exceeds antioxidant capacity when plants suffer from biotic or abiotic stress and their toxic properties take effect. Due to their dual role, the levels of ROS are strictly controlled by a network of antioxidant enzymes which cooperate to systematically detoxify ROS when the concentration exceeds a predefined threshold (Miller *et al*., 2008). The ascorbate-glutathione cycle plays an important role in the detoxification ROS (Miller *et al*., 2008). In this cycle, superoxide dismutase (SOD) disproportionates O_2^- into H_2O_2, which in turn is disproportionated by ascorbate peroxidase (APX) into H_2O and O_2. In order to provide a steady supply of ascorbate to the peroxidase, ascorbate is regenerated form monodehydroascorbate (the product of the ROS detoxification reaction) by monodehydroascorbate reductase through a NAD(P)H-dependent reaction. When monodehydroascorbate is not reduced back into ascorbate rapidly enough, it tends to spontaneously disproportionate into ascorbate and dehydroascorbate. The

dehydroascorbate in then reduced to ascorbate by dehydroascorbate reductase, this is accomplished by using reduced glutathione generated from the reduction of oxidised glutathione (glutathione disulphide) by glutathione reductase in a NAD(P)H-dependent reaction (Inzé *et al.*, 1995). Mittler *et.al* (2004) explains that the H_2O_2 generated as a result of the scavenging of O_2^- by SOD may also be scavenged by glutathione peroxidase (GPX), which uses two molecules of glutathione to disproportionate H_2O_2 and forms glutathione disulphide (GSSG). The GSSG is then reduced back into 2 molecules of glutathione by the NAD(P)H-dependent action of glutathione reductase. Along with SOD, APX and GPX other enzymatic antioxidants include: catalyse (CAT) and guaiacol peroxidase (GPOX) which are scavengers of H_2O_2, as well as Glutathione reductase (GR), Monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) which do not directly detoxify ROS but play a supporting by regenerating the substrates required for detoxification to occur (Gill*, et al.*, 2010). Plants also have non-enzymatic systems for reducing damage as a result of elevated ROS levels. Ascorbate (vitamin C) and glutathione are two of the most abundant non-enzymatic antioxidants present in plants and are found in their reduced form in all plant tissues and are concentrated in cellular compartments like the chloroplast and the mitochondria (Jimenez *et al.*, 1998; Smirnoff, 2007). Ascorbate and glutathione are important in the detoxification of ROS as they are the substrates used by ascorbate peroxidase and glutathione peroxidase respectively. The antioxidant system described here is represented diagrammatically in Figure 1.6. Other non-enzymatic antioxidants include proline, which according to Chen and Dickman (2005), is a potent antioxidant and can potentially inhibit PCD. α-Tocopherol (vitamin E) is a lipid soluble non-enzymatic antioxidant which is a scavenger of ROS and lipid radicals (Hollander-Czytko *et al.*, 2005).

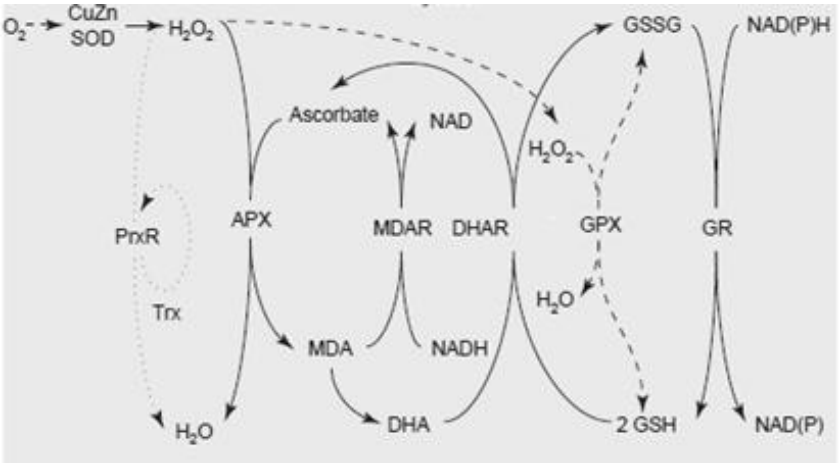

Figure 1.6: Detoxification of reactive oxygen species in plants via the ascorbate-glutathione cycle. CuZn SOD disproportionates O_2^- that is produced as a part of regular plant metabolism and produces H_2O_2. The H_2O_2 can then be detoxified by three different pathways: As indicated by the dotted lines, peroxiredoxin is a scavenger of H_2O_2 and uses thioredoxin as a substrate to catalyse the detoxification of H_2O_2. Another pathway that results in the scavenging of H_2O_2 is the glutathione peroxidase (GPX) pathway indicated by the broken lines; here GPX uses two molecules of glutathione (GSH) to detoxify H_2O_2 and produces oxidised glutathione/glutathione disulphide (GSSG). Another pathway for the detoxification of H_2O_2 is catalysed by ascorbate peroxidase (APX) using ascorbate as a substrate and liberating H_2O from H_2O_2. In order to keep the supply for ascorbate and glutathione constant, a network of recycling enzymes are employed: ascorbate is regenerated from monodehydroascorbate (denoted as MDA in the figure) by the action of monodehydroascorbate reductase (MDAR), when this does not occur rapidly enough MDA spontaneously disproportionates into ascorbate and dehydroascorbate (DHA), which is recycled back into ascorbate by dehydroascorbate reductase (DHAR) which accomplishes this by oxidising 2 GSH into GSSG. The level of GSH is kept constant for DHAR and GPX by glutathione reductase (GR) which reduces the GSSG back into two molecules of glutathione. Figure adapted and modified from Mittler *et al*. (2004).

1.7 GLUTATHIONE PEROXIDASES (GPX)

As explained by Noctor *et.al* (2002) glutathione peroxidases are a large and diverse family of isozymes that are capable of utilizing glutathione to detoxify H_2O_2 and lipid hydroperoxides, as a result of this they help defend plants against oxidative stress. The GPX family of enzymes have been identified in the cytosol, chloroplast, mitochondria and endoplasmic reticulum of plants such as Arabidopsis where they have been designated AtGPX1-AtGPX7 (Dayer *et al*., 2008; Herbette *et al*., 2007). Through the phylogenetic analysis of the genes that encode GPXs, three main groups have been identified: group 1 which contains human GPX1 and GPX2, group 2 containing human GPX3, GPX5 and GPX6 and group 3 which is referred to as the phospholipid hydroperoxide glutathione peroxidases (PHGPXs). The

PHGPX group contains human GPX4 as well as GPXs from other organisms including plants (Herbette *et al.*, 2007). The defining difference between PHGPXs and GPXs is the presence of the selenocystine in the active site of GPXs which, according to Stadtman (1996), is essential for the functioning of selenoprotiens. It has been suggested by Merchant *et.al* (2007) that non-seleno GPXs have lost their selenocystines as a result of evolution. As the concentration of atmospheric oxygen increase, the easily oxidized selenocystines became disadvantages leading to the selection of non-seleno GPXs during the evolution of terrestrial plants.

The activity of PHGPXs can be attributed to the three strictly conserved amino acids in the catalytic site; which are cystine, glutamine and tryptophan (Maiorino et al., 1995). The catalytic activity of PHGPXs is initiated by the oxidation of the catalytic cystine into sulfenic acid. The sulfenic acid then forms a disulphide with a second cysteine on the protein, this disulphide is then reduced and liberates GSSG (Navrot *et al*., 2006). In a study carried out by Navrot *et al.* (2006), it is shown that the GPXs isolated from poplar utilise only reduced thioredoxin (TRX) in their regeneration and that the proteins do not react with glutathione. Due to this it was suggested that a fifth class of peroxiredoxins be established. These findings have been corroborated by numerous studies, which have shown that PHGPX use thioredoxin as an electron donor rather than glutathione; as a result of this, most non-seleno GPXs are functionally TRX-dependent peroxidase although they show high sequence homology with selenoGPXs (Dayer *et al.*, 2008). Due to their ability to reduce H_2O_2 and organic hydroperoxides, the main function of GPXs is to protect the cell form oxidative stress caused by the accumulation reactive oxygen species during normal plant metabolism and under environmental stress. As a result of their ability to scavenge lipid hydroperoxides,

it was suggested by Gaber *et al*. (2001) that PHGPXs protect the cell by defending the cells against lipid peroxidation and subsequently maintaining membrane integrity.

1.8 CONCLUSION

Various biotic and abiotic stresses contribute to an increased production of ROS in plants, which leads to oxidative stress and ultimately cell death. ROS also play an important function as signalling molecules in various growth and defence process in plants in the absence of stress. As a result of this dual function of ROS, plants have evolved a number of enzymatic and non-enzymatic mechanisms to maintain the equilibrium between ROS generation and detoxification. Under stress conditions when this equilibrium shifts toward excessive generation of ROS, one of the mechanisms to defend plants against oxidative stress is the expression of genes encoding antioxidant enzymes. These antioxidant enzymes include PHGPXs, which detoxify lipid hydroperoxides and aid in the maintenance of membrane integrity. The expression of antioxidant enzymes is not always sufficient to protect plants and as a result crop yields and quality may be drastically reduced. This can, however, be mitigated by using genetic engineering to up-regulate of antioxidant genes. The production of plants that are able to survive the increasingly harsh environmental conditions will be a valuable tool in improving the prospects for food security for future generations.

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CHAPTER 2:

CHARACTERISATION OF A PUTATIVE GLUTATHIONE/THIOREDOXIN DEPENDENT PEROXIDASE FROM *GLYCINE MAX*

2.1 ABSTRACT

Legumes are an important crop in agriculture and industry. However, the yield and quality of legumes (e.g. soybeans) are usually poor due to the conditions they are grown under. In an attempt to aid in improvement of soybean crop productivity, the following chapter will focus on identification of a glutathione peroxidase (GPX) from soybean. Antioxidant enzymes such as GPX help to protect plants against oxidative stress, which aids in maintaining crop quality under stress conditions. Identification of putative GPX/thioredoxin-dependent peroxidases was accomplished using a pea GPX sequence as a query in a BLAST search, sequence specific primers were then used to isolate the homologous gene (Glyma01g42840) from soybean and, using molecule cloning techniques, the gene of interest was inserted into *E. coli* BL21 and expressed as a recombinant protein. The expressed protein was then purified and used in various spectrophotometric and in-gel enzymatic assays to determine and measure GPX activity. The expressed protein shows GPX activity, as well as being able to utilise ascorbate and thioredoxin to scavenge H_2O_2 albeit at a lower level when compared to activity obtained using glutathione as an electron donor for peroxide scavenging.

2.2 INTRODUCTION

Leguminous plants are part of the family *Fabaceae,* which formally is commonly referred to as *Leguminosae.* Legumes are easily identified by their distinctive pods (Zohary *et al.*, 1994) as well as their unusual flower structure and the ability of 88% of the species to enter into a symbiotic relationship with *Rhizobia* (nitrogen fixing bacteria) that inhabit nodules formed on the roots of legumes (Bauer, 1981; De Faria *et al.*, 1989). As a result of this relationship with *Rhizobia*, legumes preform an important environmental function by replenishing nitrogen reserves in the soil for use by legumes and other organisms. In 2002, legumes constituted 27% of global crop production, of which grain legumes (beans, peas, chick peas, pigeon peas, cow peas, lentils and soybean) alone constituted 33% of the required dietary protein for humans (Graham *et al.*, 2003). Legumes also provide approximately 35% of global processed vegetable oil and are used as animal feeds in the chicken and pork industries (Graham *et al.*, 2003). Soybeans, which are the legume representative used in this study, have a similar nutritional value as meat, making it an important part of the vegetarian and vegan diets (Agroudy *et al.*, 2011). As reported by Friedman and Brandon (2001), soybeans have homeopathic properties that cause a reduction in cholesterol, aid in the prevention of cancer and obesity as well as mitigating the contraction of kidney and liver diseases. Although soybeans are such a beneficial crop, they are usually grown toward the end of the growth season, this is done so that nitrogen reserves in the soil maybe replenished for the next growth season of other crop species. As a result of this practice, soybeans are grown in soil with a low water and nutrient content and high salt content, producing low crop yields that are of poor quality (Graham*, et al.*, 2003).

The yield and quality of soybean can be improved by better understanding the antioxidant machinery involved in protecting the plants from oxidative stress when grown under stress-inducing conditions. Glutathione peroxidases (GPX) are antioxidant enzymes involved in the maintenance of cell integrity by detoxifying lipid hydroperoxides (Gaber*, et al.*, 2001). It was on this bases that GPX are was studied here. This chapter aimed to determine whether Glyma01g42840, isolated from soybean, is a GPX or a thioredoxin-dependent peroxidase.

2.2 MATERIALS AND METHODS

2.2.1 IDENTIFICATION OF PUTATIVE PHGPX FROM SOYBEAN

The Glyma01g42840 sequence was obtained from the phytozome (http://www.phytozome.org) database using Basic Local Alignment Tool (BLAST) with PsPHGPX (Pea phospholipid hydroperoxide glutathione peroxidase) as the query sequence. The molecular weight of the protein was predicted by using the compute pI/Mw tool found on the Expasy website (http://web.expasy.org/compute_pi/). Perdictions were also made on the localization of Glyma01g42840, this was carried out using the TargetP topology prediction tool available from http://www.cbs.dtu.dk/services/TargetP/. The amino acid sequence of Glyma01g42840, *Pisum sativum* PHGPX and *Homo sapiens* GPX4 (a phospholipid hydroperoxide glutathione peroxidase from humans) were then aligned using Geneious (version 5.5.2).

2.2.2 GROWTH OF *GLYCINE MAX*

The surface-sterilization of soybean seeds (*Glycine max* L. merr.cv. PAN626) was carried out by imbibing the seeds in 0.35% (v/v) sodium hypochlorite for 10 minutes. The seeds were then washed with sterile distilled water five times. Subsequent to surface-sterilization, the seeds were inoculated with *Bradyrhizobium japonicum*, which was supplied as HiStick2 Soybean inoculant (Becker Underwood Ltd. West Sussex, United Kingdom). Upon completion of inoculation the seeds were sown in plastic pots (the pot diameter was 15 cm) containing 1 L of pre-soaked (in distilled H_2O) filtered silica sand (98% SiO_2, Rolfes® Silica, Brits, South Africa). During germination the sand was kept moist by regular watering with distilled water. Once the plants entered the VC stage they were supplied with nutrient

solution [1 mM K_2SO_4, 2 mM $MgSO_4$, 3 mM $CaCl_2$, 1 mM K_2HPO_4 buffer at pH 7.3, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZNSO_4$, 2 µM $CuSO_4$, 2 µM Na_2MoO_4, 0.1 µM $CoSO_4$, 50 µM Fe-NaEDTA and 10 mM 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3] until they reached the V3 stage. Plants that were similar in height and physiological stage were then selected and used for RNA extractions.

2.2.3 Molecular cloning and sequencing of Glyma01g42840

2.2.3.A RNA EXTRACTION

RNA extraction buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl, 25 mM EDTA, 2 M NaCl) was prepared in DEPC-treated water.

Prior to extraction, RNA extraction buffer was added to a 2 mL microcentrifuge tube and preheated to 65°C. The leaf material was prepared for RNA extraction by flash freezing it in liquid nitrogen. The frozen material was then ground to a fine powder using a mortar and pestle. While grinding, liquid nitrogen was periodically added to ensure that the sample remains frozen. The powder was transferred to a pre-chilled 2 mL centrifuge tube and homogenised with 1.2 mL of the pre-heated RNA extraction buffer and 1.6% β-mercaptoethanol. The sample was then gently mixed for 30 seconds and incubated for 2 minutes at 65°C. The sample was centrifuged for 10 minutes at 12 000 *x g*. The supernatant was then transferred into a clean 2 mL centrifuge tube and an equal volume of chloroform/isoamylalcohol (24:1) was added. The sample was centrifuged at 12 000 *x g* for 8 minutes and the aqueous layer of the sample was transferred into a clean 2 mL microcentrifuge tube (this was done without disturbing the bottom layer) and the volume was recorded. To the aqueous layer, $LiCl_2$ was added to a final concentration of 2 M, after

which the sample was incubated at 4°C for 16 hours. After incubation, the sample was centrifuged at 12 000 *x g* for 10 minutes and the supernatant discarded. The RNA pellet was washed twice with 70% ethanol and the excess ethanol was evaporated by air-drying in a laminar flow after removal with a pipette. The washed pellet was then re-suspended in 50 µl DEPC treated water. DNase treatment of the extracted RNA samples was performed by adding the following reagents into a nuclease free tube: 1 µg of RNA, 1X reaction buffer (Fermentas), 1 µl of RNase-free DNase 1 (Ferementas). The reaction was made up to final volume of 10 µl using DEPC-treated water. The reaction was allowed to proceed by incubation at 37°C for 30 minutes, after which 1 µl of 50 mM EDTA was added to the reaction, which was further incubated at 65°C for 10 minutes to inactivate DNase. Prior to storage, 1 µl of RiboLock™ RNase Inhibitor (Fermentas) was added to the sample. The sample was spectrophotometrically analysed for quantity and quality and used in first strand cDNA synthesis or stored at -20°C for later use.

2.2.3.B FIRST STAND CDNA SYNTHESIS USING EXTRACTED RNA

First stand cDNA was synthesised from 400 ng of template RNA (total RNA) in the presence of 20 pmol of gene-specific reverse primer (5′-GTC CTC GAG TCA TCG ATC TAG CAG CTT CTT-3′) using RevertAid™ Reverse Transcriptase (Fermentas) according the manufacturer's instructions at 42°C for 60 minutes. After incubation the reaction was terminated by incubating the reaction at 70°C for 10 minutes. The cDNA produced was then used in PCR or stored at -20°C for later use.

2.2.3.C PCR AMPLIFICATION OF GLYMA01G42840

Polymerase chain reaction (PCR) was used to amplify Glyma01g42840 using 2 µl of previously generated cDNA as a DNA template. The reaction also contained 200 µM of dNTPs, 600 nM of gene-specific forward primer (5′-GCA GGA TCC ATG GCT AGC CAA TCA AAC ACT AAA TCA-3′) and 600 nM of gene-specific reverse primer (5′-GTC CTC GAG TCA TCG ATC TAG CAG CTT CTT-3′). This was mixed with 10X Expand High Fidelity™ Reaction Buffer containing $MgCl_2$ (Roche) to a final concentration of 1X and 2.6 Units of Expand High Fidelity™ Enzyme Mix (Roche). The reaction was made up to a final volume of 50 µl with distilled water. The reaction was carried out using to the following parameters: Initial denaturation was carried out at 95°C for 2 minutes, this was followed by 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 63°C for 30 seconds and extension at 72°C for 2 minutes. Once the 30 cycles were completed, final extension occurred at 72°C for 7 minutes. After the PCR amplification, the PCR product was size fractionated on a 1% agarose gel. The gel was visualized by mixing the sample with DNA loading dye that contained 100x GelRed™ (Biotium) and exposure to short wave UV light using an AlphaEaseFC UV gel visualization system.

2.2.3.D PURIFICATION OF PCR PRODUCT

The PCR product was excised from the gel under long-wavelength UV light in the shortest possible time using a sterile blade/scalpel and purified using the GeneJet™ Gel Purification Kit (Fermentas) according to the manufactures instructions. The PCR product was cloned into the BamHI and XhoI sites of pET-41a(+/-) (Invitrogen), since the forward primer was designed to contain a BamHI restriction site and the reverse primer was designed to contain a XhoI site, using standard procedures.

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2.2.4 EXPRESSION OF GLYMA01G42840

2.2.4.A TRANSFORMATION OF PET-41A (+/-) – GLYMA01G42840 CONSTRUCT INTO *E. COLI* BL21

Luria Bertani (LB) medium was pre-heated at 37°C, while Eppendorf tubes were chilled on ice and *E. coli* BL21 AI™ (Invitrogen) competent cells were thawed on ice. A 100 µl aliquot of competent cells was transferred to the chilled tubes and 10 ng of the construct was added to the competent cells. The cells were then incubated on ice for 30 minutes, after which they were heat-shocked at 42°C for 45 seconds. Immediately after heat shock, the cells were incubated on ice for 2 minutes and 250 µl of LB medium were added and the cells were incubated at 37°C for 10 minutes without shaking. A further 50 minutes of incubation was carried out at 37°C with shaking at 250 rpm. Once the incubation was complete, the cells were aseptically spread onto LB plates containing 30 µg/mL kanamycin and incubated overnight at 37°C.

2.2.4.B CONFIRMATION OF PET-41A(+/-) - GLYMA01G42840 IN *E. COLI* BL21

Colony PCR was performed on transformed *E. coli* BL21 AI™ (Invitrogen) by picking half of a colony from an LB agar plate containing 30 µg/mL kanamycin and inoculating 2 µl of distilled water with the colony, which was then boiled at 95°C for 5 minutes in a thermal cycler to lyse the cells and release the DNA. This was used as the template DNA in the PCR. The colony PCR was set up by mixing the 2 µl of template DNA with PCR components based on the use of TrueStart™ Taq DNA Polymerase (Fermentas) using a protocol supplied by the manufacturer. The reaction was carried out using to the following parameters: Initial denaturation was carried out at 95°C for 2 minutes, this was followed by 30 cycles of

denaturation at 94°C for 30 seconds, primer annealing at 63°C of 30 seconds and extension at 72°C for 2 minutes. Once the 30 cycles were completed, final extension occurred at 72°C for 7 minutes. The PCR product was stained with GelRed (Biotium) and size-fractionated on a 1% agarose gel, then visualized on an AlphaEaseFC UV visualization system.

2.2.4.C EXPRESSION OF RECOMBINANT CONSTRUCT IN *E. COLI* BL21

E.coli BL21 AI™ (Invitrogen) containing the construct (Glyma01g42840 cloned into pET-41a) was used to inoculate 3000 mL of LB containing 30 µg/mL kanamycin and incubated at 37°C with shaking at 250 rpm until an OD_{600} of 0.4 was reached. The culture was then split into 2900 mL and 100 mL aliquots, to the 2900 mL aliquot isopropyl β-D-1-thiogalactopyranoside (IPTG) and L-arabinose was added to a final concentration of 1 mM and 0.2% respectively. The other 100 mL aliquot and the 2900 mL aliquot were incubated at 25°C with shaking at 250 rpm overnight at 25°C. The samples were transferred to centrifuge tubes and the cells pelleted by centrifugation at 4000 *x g* for 10 minutes, after which the LB medium was discarded and the pellet re-suspended in 7 mL of phosphate buffered saline (PBS) lysis buffer (1.4 mM NaCl, 2.7 mM KCl, 4.3 mM K_2HPO_4, 1.8 mM KH_2PO_4, 0.2 mg/mL lysozyme, 1% Triton X-100 and 10% glycerol at pH 7.2). The samples were flash-frozen in liquid nitrogen and immediately thawed at 42°C for 1-2 minutes or until the entire sample was liquid. This freeze/thaw cycle was repeated six times after which the samples were centrifuged for 10 minutes at 4000 *x g* and the supernatant transferred into a clean 50 mL tube to be used as the soluble protein fraction. The pellet was re-suspended in 7 mL PBS lysis buffer and was used as the insoluble protein fraction. The samples were then run on a 12% SDS polyacrylamide resolving gel, after having been stacked in a 5% SDS polyacrylamide stacking gel. Once electrophoresis of the gel was complete, it was removed from the plates

and washed in hot (80°C) water for 5 minutes, after which it was stained in for 30 minutes in hot (60°C) Commassie Brilliant Blue R250 stain and destained overnight. The gel was then digitally captured using a digital camera.

2.2.4.D PURIFICATION OF CRUDE PROTEIN EXTRACT FROM *E. COLI* BL 21

The crude protein extracted from the *E. coli* BL21 AI™ cells was purified using glutathione-agarose packed columns (Sigma-Aldrich) according to manufactures instructions. In order to determine which elution contained the protein, the samples were electrophoresed on a 12% SDS polyacrylamide separating gel after having been stacked in a 5% SDS polyacrylamide stacking gel. Once electrophoresis of the gel was complete, it was removed from the plates and washed in hot (80°C) water for 5 minutes, after which it was stained in for 30 minutes in hot (60°C) Commassie Brilliant Blue R250 stain and distained overnight after which the gel was digitally photographed.

2.2.5 THROMBIN CLEAVAGE OF GLYMA01G42840-GST FUSION PROTEIN

The following procedure was carried out in order to cleave Glyma01g42840-GST fusion protein. The thrombin-agarose resin (Sigma-Aldrich) was thoroughly mixed into a homogenous slurry, then 200 µl of the slurry was transferred into a clean microcentrifuge tube and was centrifuged at 1500 *x g* for 10 minutes. Once centrifugation of the slurry was complete, the supernatant was removed and the thrombin-agarose was re-suspended in 1000 µl of 1X cleavage buffer (Sigma-Aldrich), then the centrifugation and re-suspension were repeated. After the beads were thoroughly washed, they were re-suspended in 200 µl of 10X cleavage buffer (Sigma-Aldrich) and 1 mL of the fusion protein was added. The

cleavage reaction was then incubated at room temperature with agitation for 6 hours, after which it was incubated at 4°C overnight. The cleaved protein was recovered by transferring the cleavage reaction solution into a glutathione agarose column, where the resin was allowed to settle and the eluent was drained and collected. The resin was then washed in 1 mL of 1X cleavage buffer (Sigma-Aldrich) and the eluent was collected and combined with the first elution. The efficiency of the cleavage reaction was then analysed by means of a SDS-PAGE gel.

2.2.6 SPECTROPHOTOMETRIC GPX ACTIVITY ASSAY

Glutathione peroxidase activity was determined spectrophotometrically by measuring the oxidation of NADH based on absorbance readings obtained at 340 nm. A reaction buffer was prepared which contained 100 mM Tris/HCl at pH 7.5, 5 mM EDTA, 0.2 mM NADH, 3 mM GSH and 2 Units of glutathione reductase (GR). The reaction mixture was made up of 60 µg of the protein of interest, 1 mM of the substrate and was made up to a final volume with the reaction buffer. A negative control was carried out in parallel to the GPX activity assay, in the control reaction the protein of interest was substituted with water. The substrates used for this reaction were cumene hydroperoxide or hydrogen peroxide respectively. The reaction was initiated by the addition of the substrate and was allowed to continue for 10 minutes, with the NADH levels being measured at 1 minute intervals. The spectrophotometric data and the extinction coefficient of NADH (6220 $M^{-1}.cm^{-1}$) was then used to determine the activity of the protein. Once the data was obtained the control data was subtracted from the experimental data as to eliminate background activity.

2.2.7 MEASURING ENZYMATIC ACTIVITY SPECTROPHOTOMETRICALLY

The activity of Glyma01g42840 was measured using three different electron donors to determine the preferred substrate for the enzyme: glutathione, ascorbate and thioredoxin. The reaction using ascorbate as an electron donor was set up as follows: 15 µg of the sample protein, 50 mM potassium phosphate (pH 7.0), 1.0 mM ascorbate (ASA) and 1.0 mM H_2O_2 in a final volume of 200 µl. A negative control was set up in the same way as the experiment; however the protein sample was substituted with water. The rate of H_2O_2 consumption was measured by taking absorbance readings at 240 nm over a period of ten minutes and the molecular extinction coefficient of 35 $M^{-1}.cm^{-1}$ was used to calculate the specific activity using ascorbate as an electron donor. The specific activity while using glutathione or thioredoxin was determined in two separate reactions which were set up by mixing 15 µg of the sample protein, reaction buffer (100 mM Tris/HCl at pH 7.5, 5 mM EDTA and 1 mM glutathione or 1 mM thioredoxin (TRX) respectively and 1.0 mM cumene hydroperoxide (CHP). Negative controls were set up for the individual experiments in the same way that the experiments were set up, however water was used as a substitute for the protein sample. The consumption of the cumene hydroperoxide was measured by taking absorbance readings at 240 nm for ten minutes and the molecular extinction coefficient of 35 $M^{-1}.cm^{-1}$ was used to calculate the activity using glutathione and thioredoxin respectively as an electron donors.

2.2.8 GLUTATHIONE PEROXIDASE ACTIVITY ASSAY

For in-gel activity assays, a native polyacrylamide gel was set up as a 10% polyacrylamide separating gel and a 5% polyacrylamide stacking gel without any SDS. The gel was then

loaded with 5µg-20µg of Glyma01g42840, 20µg of Glyma01g42840 boiled at 95°C for 5 minutes, 20 µg of glutathione (GST) as well as 20 µg of boiled GST. The gel was then electrophoresed at 4°C, after electrophoresis the gel was incubated in 1X native page running buffer containing 2 mM GSH for 20 minutes. After this, the primary stain which was made up of 50 mM potassium phosphate (KPO_4) at pH 7.0 and 13 mM GSH was applied, then the gel was stained for 20 minutes in this solution after which the solution was discarded and 50 mL of the secondary stain added. The secondary stain was composed of 50 mM KPO_4 at pH 7.8, 30 mM GSH and 0.002% cumene hydroperoxide. The gel was stained for a further 20 minutes after which the secondary stain was replaced by 50 mL of the tertiary stain made up of 50 mM KPO_4, 0.04% TEMED and 0.5 mM of nitrotetrazolium blue (NTB). The gel was stained for 20 minutes after which the stain was discarded to prevent over-staining of the gel. After staining was complete, the gel was digitally photographed.

2.2.9 ASCORBATE PEROXIDASE ACTIVITY ASSAY

A native polyacrylamide gel was set up as a 10% polyacrylamide separating gel and a 5% polyacrylamide stacking gel without any SDS. The gel was then loaded with 5µg-20µg of Glyma01g42840, 20µg of Glyma01g42840 boiled at 95°C for 5 minutes, 20 µg of GST as well as 20 µg of boiled GST. The gel was then electrophoresed at 4°C, after electrophoresis the gel was incubated in 1X native page running buffer containing 2 mM Ascorbic acid (ASA) for 20 minutes. After incubation, 50 mL of the primary stain which was made up of 50 mM potassium phosphate (KPO_4) at pH 7.0 and 22 mM ASA was applied. The gel was stained for 20 minutes in this solution, after which it was discarded and 50 mL of the secondary stain was added. The secondary stain was composed of 50 mM KPO_4 at pH 7.8, 46 mM ASA and 0.002% cumene hydroperoxide. The gel was stained for a further 20 minutes, after which

the secondary stain was replaced by 50 mL of the tertiary stain made up of 50 mM KPO_4, 0.04% TEMED and 0.5 mM of nitrotetrazolium blue (NTB). The gel was stained for 20 minutes, after which the stain was discarded to prevent over-staining of the gel. After staining was complete, the gel was digitally photographed.

2.2.10 STATISTICAL ANALYSIS

The statistical validity of all the data was tested by means of a One-way analysis of variance (ANOVA) and the Tukey-Kramer test at 5% level of significance was completed to compare the means using GraphPad Prism 5.03 software.

2.3. RESULTS

2.3.1. PROTEIN DOMAINS OF GLYMA01G42840

The amino acid sequence obtained from a BLAST search using PsPHGPX as a query was used in the Phytozome database. Analysis of the domain structure of the identified protein (Glyma01g42840) shows two overlapping domains (in the same sequence), a redoxin domain located from position 4-161 composed of 157 peptides and a glutathione peroxidase domain located at potion 10-118 composed of 108 peptides (Figure 2.3.1). The molecular weight of the protein was predicted using the Expasy compute pI/Mw tool and it was found to be 18.5 kDa. Using the TargetP topology predictor it was shown that Glyma01g42840 is targeted to the cytosol.

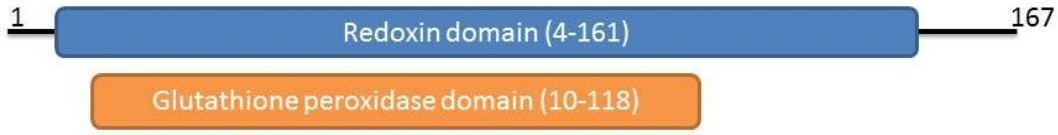

Figure 2.3.1 A: Domain structure of Glyma01g42840. The domain structure above is a representation of Glyma01g42840; two domains are showed which suggests the ability of the protein to use either thioredoxin or glutathione as an electron donor. The redoxin domain is located at position 4-161 and is composed of 157 peptides, while the glutathione domain is 108 peptides long and is located at position 10-118.

```
### targetp v1.1 prediction results ###################################
Number of query sequences:  1
Cleavage site predictions not included.
Using PLANT networks.

Name                    Len     cTP    mTP     SP  other  Loc  RC
-----------------------------------------------------------------------
Sequence                167   0.067  0.184  0.114  0.887    _    2
-----------------------------------------------------------------------
cutoff                        0.000  0.000  0.000  0.000
```

Figure 2.3.1 B: Predicted localization of Glyma01g42840. Using the TargetP topology predictor it was shown that Glyma01g42840 maybe localized in the cytosol of the cell as indicated by the _ symbol in the Loc column.

2.3.2. COMPARISON OF SEQUENCE HOMOLOGY

The protein sequence of Glyma01g42840 was determined and this was compared to the sequences for two previously characterised phospholipid hydroperoxide glutathione peroxidases, namely human GPX4 and the PHGPX form *Pisum sativum* (pea). The three sequences were aligned and conserved regions were identified. The region marked in blue shows the conserved region implicated as the active site of the proteins while the area marked in red shows the domain interaction interface (Figure 2.3.2). It was found that the sequence of Glyma01g42830 shared 61.7% identity with the pea sequence and 52.1% similarity with that of human GPX4.

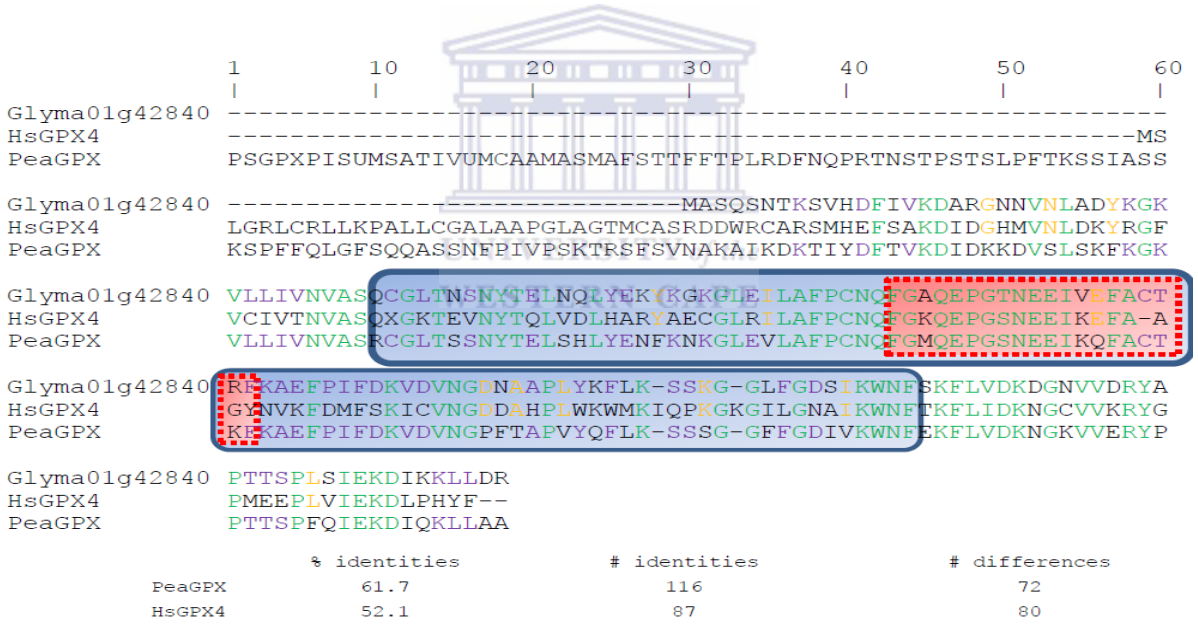

Figure 2.3.2: Comparison of sequence homology between Glyma01g24840, human GPX4 (HsGPX4) and Pea PHGPX (PeaGPX). The figure above is a representation of an alignment, depicting the conserved domains shared, between Glyma01g42804 isolated from soybean and the characterised phospholipid hydroperoxide glutathione peroxidases isolated from human and pea. The regions demarcated in blue represent the conserved active site in the three enzymes, while the region demarcated in red show the conserved proteins that make up the domain interaction interfaces of the examined proteins. The peptides indicated in green are present in all three sequences while the peptides that are conserved between the pea and soybean proteins are indicated in purple and those conserved between soybean and human GPXs are indicated in yellow.

2.3.3 MOLECULAR CLONING OF GLYMA01G42840

PCR amplification using Glyma01g42840 gene-specific primers produced a 504 bp amplicon (Figure 2.3.3 A), this size corresponded to the *in silico* analysis of the coding region of Glyma01g42840. The amplicon was excised from the agarose gel and purified after which the gene was cloned into pET41a by restriction endonuclease digestion followed by ligation using T4 DNA ligase. The recombinant plasmid was transformed into *E. coli* BL21 AI™ cells and grown on selective media; ten positive colonies were selected and used as DNA template for a colony PCR (Figure 2.3.3.B). The colony PCR proved that the recombinant plasmid contained the gene of interest and the absence of a band in the negative control lane which contains the product of a PCR reaction in which water was added instead of template DNA shows that the positive result is not due to contamination.

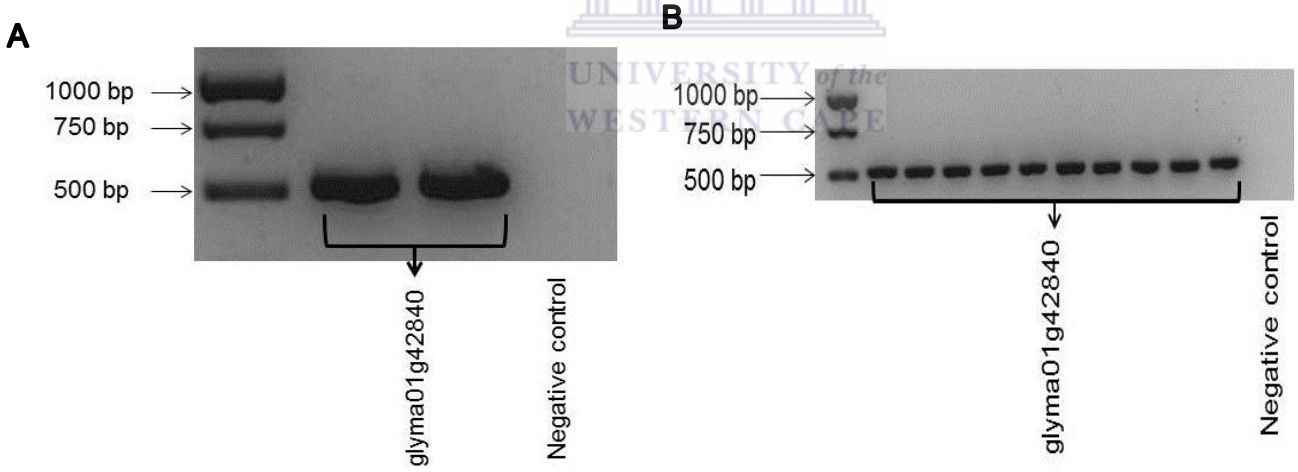

Figure 2.3.3: Molecular cloning of Glyma01g42840. (A) PCR amplification of Glyma01g42840. Lane 1 contains a 1kb molecular weight marker, lanes 2 and 3 contain the 504 bp Glyma01g42840 PCR product and lane 4 shows the negative control. (B) Colony PCR performed using DNA from colonies that tested positive for the Glyma01g42840-pET41a construct. Lane 1 contains a 1 kb molecular weight marker, lanes 2-11 contain Glyma01g42840 amplified from the positive colonies and lane 12 indicates the negative control.

2.3.4. SDS-PAGE ELECTROPHORESIS OF GLYMA01G42840 RECOMBINANT PROTEIN, PURIFICATION AND CLEAVAGE

In order to express the recombinant protein, positive colonies were selected and used to produce overnight cultures. These overnight cultures were then used as starter culture for protein expression, the protein was extracted from the cells and the soluble fractions of both uninduced and induced expressions were electrophoresed on a SDS-PAGE gel. The protein was identified as a ± 40 kDa recombinant protein which consisted of GST and Glyma01g42840 (Figure 2.3.4, lane 3). The crude protein extract was purified using a column containing glutathione agarose beads and the purified protein was electrophoresed (Figure 2.3.4, lane 4). The purified protein was then cleaved using thrombin in order to remove the GST from the protein and electrophoresed, this resulted in a band of ± 18.5 kDa (Figure 2.3.4, lane 5).

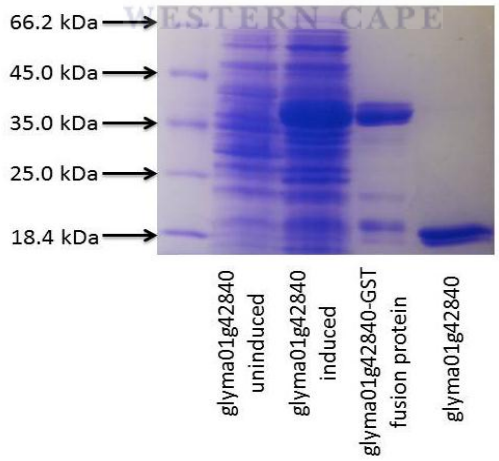

Figure2.3.4: SDS-PAGE electrophoresis of Glyma01g42840 expression, purification and cleavage. Electrophoresis of Glyma01g42840 at various stages of protein isolation. Lane 1 contains a protein molecular weight marker, while lanes 2-5 contain uninduced crude protein extract, induced crude protein extract, purified Glyma11g023630-GST fusion protein and thrombin-cleaved Glyma01g42840 respectively.

2.3.5. DETERMINING THE ABILITY OF GLYMA01G42840 TO ACT AS A GLUTATHIONE PEROXIDASE

In order to determine whether Glyma01g42840 encodes a glutathione peroxidase, an enzymatic assay was carried out which simulates the breakdown of phospholipid hydroperoxides by GPX. For the purpose of this assay, the activity of the enzyme was measured by monitoring the consumption of NADH by the system and it was found that when scavenging cumene hydroperoxide (phospholipid hydroperoxide) the activity of the enzyme was ± 180 µmol.min^{-1}.mg^{-1} of protein, while the activity of the protein decreased to ± 120 µmol.min^{-1}.mg^{-1} of protein when hydrogen peroxide is scavenged (Figure 2.3.5).

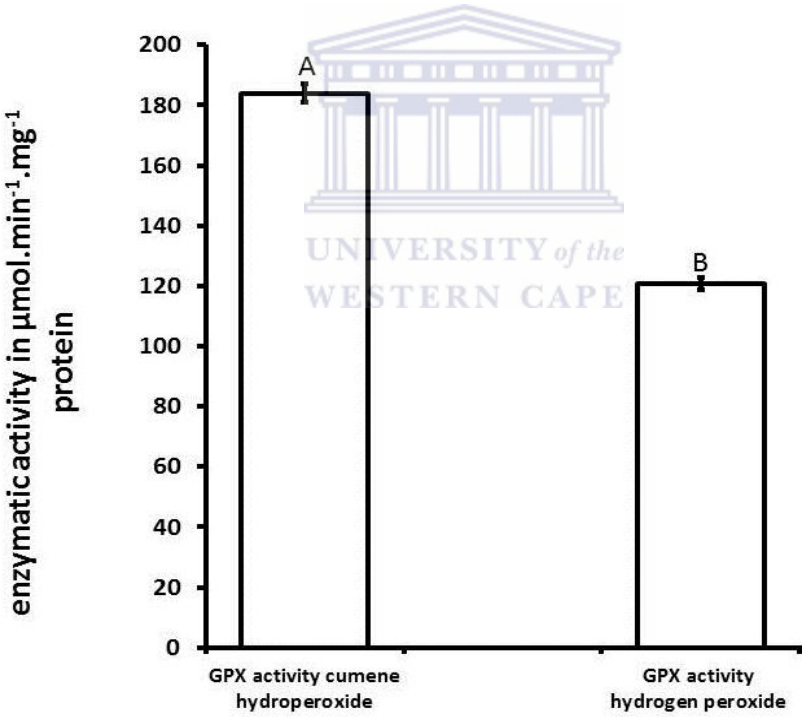

Figure 2.3.5: Determining the ability of Glyma01g42840 to act like a GPX. Enzymatic activity of Glyma01g42840 when scavenging cumene hydroperoxide and hydrogen peroxide respectively. When comparing the enzymatic activity on the two substrates, it was noted that there was a decrease in activity when comparing the scavenging of cumene hydroperoxide with hydrogen peroxide. Data are means ± standard errors from three independent experiments, varying letters above the error bars indicate that the enzymatic activity differ statically

2.3.6. MEASURING THE ACTIVITY OF GLYMA01G42840 SPECTROPHOTOMETRICALLY

The ability of Glyma01g42840 to scavenge H_2O_2 and CHP using ascorbate, glutathione and thioredoxin was measured spectrophotometrically and the activity was determined. Figure 2.3.6 show the enzymatic activity and indicates that, using TRX as an electron donor, Glyma01g42840 is able to scavenge 19.5 µmol.min^{-1}.mg^{-1} protein for CHP, while 34.7 µmol.min^{-1}.mg^{-1} protein for H_2O_2 is scavenged using ASA as an electron donor and 118.2 µmol.min^{-1}.mg^{-1} protein of CHP is scavenged when GSH in used as an electron donor. The figure shows an increase in activity when ASA is used instead of TRX and an even higher increase when using GSH instead of TRX or ASA respectively.

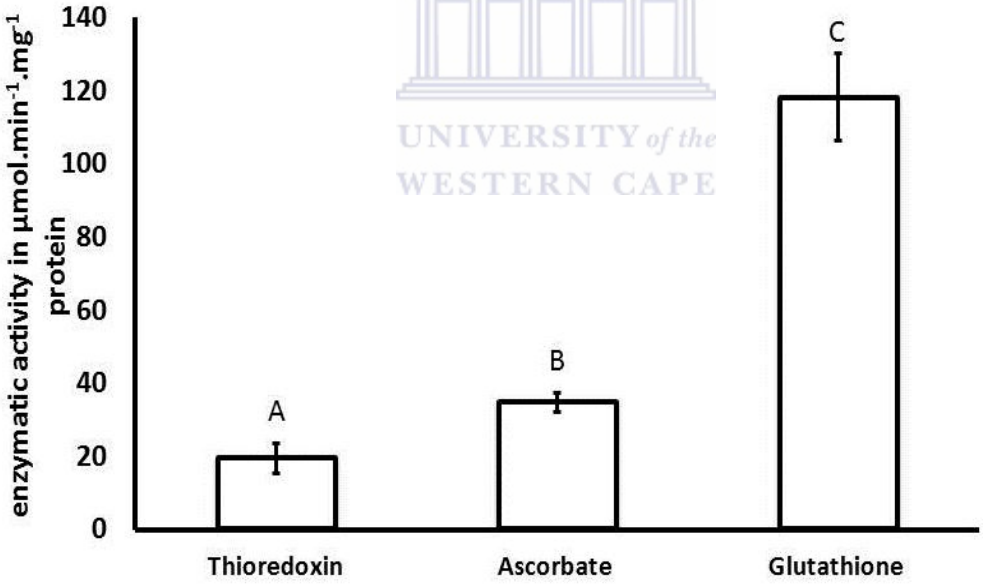

Figure 2.3.6: Measuring the specific activity of Glyma01g42840 spectrophotometrically. The figure above shows the enzymatic activity and indicates that using TRX as an electron donor Glyma01g42840 is able to scavenge 19.5 µmol.min^{-1}.mg^{-1} protein of CHP, while 34.7 µmol.min^{-1}.mg^{-1} protein of H_2O_2 is scavenged using ASA as an electron donor and 118.2 µmol.min^{-1}.mg^{-1} protein of CHP is scavenged when GSH in used as an electron donor, varying letters above the error bars indicate that the enzymatic activity differ statically.

2.3.7. GLUTATHIONE AND ASCORBATE IN-GEL ACTIVITY ASSAYS.

The ability of Glyma01g42840 to scavenge H_2O_2 was tested using an in-gel GPX activity assay. Various concentrations of Glyma01g42840 along with a boiled sample as well as GST and a boiled sample of GST were electrophoresed in this in-gel assay. As seen in Figure 2.3.6 A, the increase in enzymatic activity is directly proportional to the amount of protein loaded in each lane, the boiled Glyma01g42840 lost the lower band which all the native state samples exhibited. Although boiling resulted in a different banding pattern the boiled protein still exhibited activity. In an attempt to confirm that the top band was not GST, purified GST was also loaded and although it was able to scavenge some H_2O_2 the activity was not as pronounced as that of Glyma01g42840, yet boiling of the GST resulted in a total loss of GST activity. In order to confirm that Glyma01g42840 is a glutathione peroxidase, the glutathione was replaced with ascorbate. This ascorbate reaction with Glyma01g42840 however still resulted in enzymatic activity (Figure 2.3.6. B). The activity pattern that resulted from the ascorbate-based activity assay differed from the glutathione-based assay in that both the upper and lower bands were drastically reduced and the GST had no activity. The lack of GST activity in the ascorbate activity assay is further evidence that the activity seen in the glutathione activity assay was solely from Glyma01g42840.

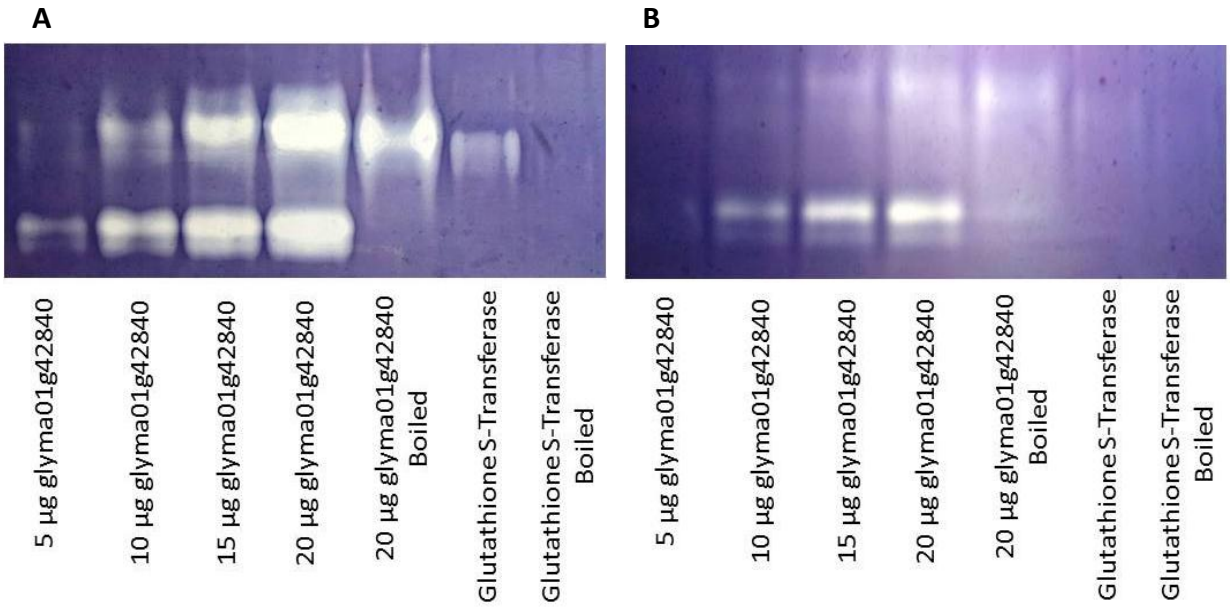

Figure 2.3.7: Glutathione and Ascorbate activity assays. (A) H_2O_2 scavenging capabilities of Glyma01g42840 using glutathione as a co-factor. Lanes 1-4 contains Glyma01g42840 ranging in concentration from 5 µg to 20 µg respectively, while lane 5 contains a 20 µg sample of Glyma01g42840 and lanes 6 and 7 contain GST and boiled GST both at 20 µ, respectively. (B) H_2O_2 scavenging capabilities of Glyma01g42840 using ascorbic acid as a co-factor. Lanes 1-4 contains Glyma01g42840 ranging in concentration from 5 µg to 20 µg respectively, while lane 5 contains a 20 µg sample of Glyma01g42840 and lanes 6 and 7 contain GST and boiled GST, both at 20 µg, respectively.

2.4. DISCUSSION

The *in silico* analysis of Glyma01g42840 suggested that the protein is able to utilize both thioredoxin and glutathione as electron donors. The utilization of numerous electron donors is common in the peroxidase family of enzymes and is also seen in horseradish peroxidases which are able to use a variety of aromatic electron donors to reduce H_2O_2 (Henriksen *et al.*, 1998). The ability of peroxidases to use various electron donors is dependent on the accessibility of the active site to the electron donors; in the case of the horseradish peroxidase the channel to the active site allows the entry of different aromatic molecules. To ensure that the gene isolated from soybean was in fact Glyma01g42840 the gene was sequenced and the nucleotide sequence was then used to determine the protein sequence which in turn was compared with the protein sequences of characterised PHGPXs from *Homo sapiens* and *Pisum sativum* as shown in Figure 2.3.2. It was found that the protein encoded by Glyma01g42840 shared several conserved peptides in the active site domain and interface interaction domains. However, as explained by Dayer *et al.* (2008) sequence homology alone does not dictate functionality as many non-seleno glutathione peroxidase in spite of the sequence homology often are functionally thioredoxin (TRX) dependent peroxidase. In an attempt to relate similarities in sequence homology with the function of Glyma01g42840, the gene was cloned into *E*. *coli* BL21 AI™ and expressed. The recombinant protein was cleaved using thrombin to remove the glutathione S-transferase, resulting in a protein of approximately 18.4 kDa as shown in figure 2.3.4.The purified protein was then assayed to determine its ability to scavenge both phospholipid hydroperoxides and hydrogen peroxide. The assays indicated that Glyma01g42840 is able to scavenge both phospholipid hydroperoxide as well as hydrogen peroxide although the activity using the

latter as a substrate is less efficient, similar results were seen in a study completed by Herbette *et al*. (2002) who isolated GPX from tomatoes and sunflowers, which were able to utilize both glutathione as well as thioredoxin to scavenge H_2O_2. The purified protein was then used to determine the activity of the enzyme using either TRX or GSH as electron donor, as indicated by the domain view of the protein provided in Figure 2.3.1. ASA was used as an electron donor as well to determine the ability of Glyma01g42840 to use various electron donors. Upon analysis of the spectrophotometric data it was found that GSH is the most effective electron donor as its use resulted in the highest GPX activity. The use of thioredoxin as the electron donor yielded the lowest GPX activity (decreased by approximately 5-fold compared to the GPX activity obtained when glutathione is used). On the other hand, GPX activity using ASA as the electron donor yielded activity that is lower (decreased by approximately 2.3-fold) than the activity obtained when using glutathione but this ASA-based GPX activity was still higher (increased by almost 2-fold) than when thioredoxin is used as electron donor. This suggests that Glyma01g42840 is a GPX that preferentially uses glutathione as electron donor but can also use ASA (and thus displays ascorbate peroxidase activity) and thioredoxin (and thus displays thioredoxin peroxidase activity) as electron donors. The spectrophotometric activity assays utilised H_2O_2 as a substrate when ASA was used as the electron donor, as peroxide scavenging when using ASA as the electron donor work more efficiently. In the case of Glutathione and thioredoxin as electron donors peroxide scavenging is more efficient when CHP is used as the substrate. In an attempt to allow the enzymatic activity to be compared between reactions that were performing at optimal levels the ASA assay was completed using H_2O_2 as the substrate, while the GPX and TRx assays were completed using CHP as the substrate. The enzymatic activity determined spectrophotometrically was then confirmed using in-gel activity assay.

Both the in-gel assays using GSH and ASA as an electron donor corresponded to spectrophotometric assays, however TRX usage yielded no activity on the gels. From the in-gel assays, it was observed that two zones of activity are present and this can be attributed to the generation of a truncated protein during cleavage. Truncation of the protein however is unlikely as thrombin recognises LVDKDG as its cut site and this sequence of peptides are only present between the GST tag and the protein and not in the Glyma01g42840 sequence itself. Another possible reason for the two zones of activity may be that some of the purified protein still had some GST that was not cleaved/not removed from the protein during thrombin cleavage/purification. However, this theory was disproven by boiling the protein, which resulted in no activity from purified GST whereas the boiled Glyma01g42840 protein sample retained activity of the upper band (at the same position as the GST) but losses activity of the lower band (Figure 2.3.6 A and B). This loss of activity in the lower band lead to the theory that the upper band may be the protein in oligomeric form, with quartenary structure folding that might possibly confer heat stability, while the lower band may be a monomer with tertiary structure folding that is heat-sensitive. The inactivation of the lower band is indicative of the heat sensitivity of GPX as seen in word carried out by Candell and Tappel (1983) that showed that GPXs lose the activity when subjected to temperatures of 100 °C for 5 minutes. The presence of the two bands as a result of a monomeric form or oligomeric form is possible as GPXs are able to function as monomeric or tetrameric structures (Epp *et al.*, 1983; Sunde *et al.*, 1980), however this will have to be studied in more detail for Glyma01g42480 in future.

2.5. CONCLUSION

From the data obtained in this study, it can be concluded that Glyma01g42840 has higher enzymatic activity when utilising glutathione as an electron donor in comparison with using thioredoxin or ascorbate. As a result of its preference for glutathione as an electron donor it can be concluded that Glyma01g42840 is most likely a GPX and not a thioredoxin-dependent peroxidase. It was also found that the protein encoded by glyma01g42840 has some levels of heat stability, the molecular basis for this however has not been determined and as such future work involves determining the factors that contribute to this phenomenon.

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CHAPTER 3

THE EFFECT OF DROUGHT STRESS ON GLYMA01G42840

3.1 ABSTRACT

Drought stress has, had a major effect on crop yield and quality, as such it is important to understand how plants react when grown under drought stress conditions. In this chapter plants were grown under limited water supply and thus the relative water content (RWC) of the soil in which the plants were grown has be determined to ensure that the plants were grown under drought conditions. The spatial expression patterns of Glyma01g42840 was studied and the expression of Glyma01g42840 was determined in the leaves of the plants in response to drought. In an attempt to assay the degree of oxidative stress suffered by the plants oxidative stress markers such as cellular H_2O_2 content, lipid peroxidation as well as the degree of cell death was measured. From the RWC data obtained it has been confirmed that the plants were grown under drought conditions this was corroborated by the high cellular concentrations of both H_2O_2 and MDA (product of lipid peroxidation) as well as the high degree of cell death. It was also confirmed that the relative expression of Glyma01g42840 was statistically similar in leaves and roots, with expression being highest after three weeks of drought stress.

3.2 INTRODUCTION

Understanding the spatial and temporal expression patterns of a protein is important in understanding the role that the protein plays in the cell. When one considers temporal expression which is defined as the developmental stage or condition under which genes/proteins are expressed, the function of antioxidant enzymes suggest that they would be expressed at high levels when high concentrations of ROS are produced as in cases of abiotic stress such as drought, high soil salinity and high light intensity (Gill, 2010; Oliver, 2001). As previously mentioned, ROS generation is increased under conditions of abiotic stress. One such ROS is H_2O_2 as explained by Rasmusson *et al.* (2004). The increased levels of H_2O_2 subsequently lead to the peroxidation of membrane lipids, which, as stated by Garg *et al.* (2009), is the extremely damaging to living cells and results in the production of polyunsaturated precursors such as ketones and malondialdehyde (MDA). These polyunsaturated precursors can be used to measure the extent of lipid peroxidation suffered by cells (Schmedes *et al.*, 1989). Lipid peroxidation increases the fluidity of cell membranes thus increasing its permeability to substances which are usually unable to cross the membrane as well as damaging membrane proteins and inactivating membrane receptors and enzymes (Moller*, et al.*, 2007). The effects of lipid peroxidation on the cell eventually initiate programmed cell death and cause the plant to suffer necrosis, which reduces crop quality (Jabs, 1999). The objectives of this chapter are to determine if a subset of the plants grown under drought conditions have altered Glyma01g42840 expression paterns and to determine in which organs Glyma01g42840 is expressed as under normal conditions. Furthermore, the aim of the chapter was also to determine the extent of oxidative stress by measuring the concentration of H_2O_2 under untreated, osmotic stress

and drought conditions and determining level of lipid peroxidation by measuring MDA content and the extent of cell death under these conditions. Finally, the chapter also aims to investigated the effect of these treatments on the activity of GPX and APX in total protein extracts from plants exposed to drought/osmotic stress.

3.3 METHODS AND MATERIALS

3.2.1 GROWTH OF *GLYCINE MAX*

Plants were grown in the same manner as described in chapter 2 section 2.2.1, except that a 1:2 mixture of filtered silica sand:sterile potting soil was used as growth medium and tap water was used for watering the plants instead of nutrient solution. Drought was imposed by withholding water for three weeks and osmotic stress was imposed by applying 240 mM sorbitol every 24 hours for a total period of 48 hours.

3.2.2 DETERMINING THE RELATIVE WATER CONTENT OF THE SOIL IN WHICH THE PLANTS WERE GROWN

The relative water content (RWC) of the soil (the 1:2 mixture of sand and potting soil as described above) in which the plants were grown was determined immediately after the plants were harvested. For the purpose of this study the RWC of the untreated soil and the 3 week drought treated soil was determined, by firstly weighing 200 mL of the soil once the plants had been removed. The soil was then saturated with water by running tap water into the soil until the pot over flowed; the water was then allowed to drain from the soil by gravity for 15 minutes. Once again 200 mL of the soil was weighed, the soil was then dried in an 80^{o}C oven for 7 days after which the soil was again weighed and the data was used to determine the RWC of the soil using the following equation:

$$RWC\ in\ \% = [\frac{Hm-Dm}{Sm-Dm}] \times 100$$

Where Hm = the mass of the soil immediately after harvesting the plants

Sm = the mass of the soil after it was saturated with water

Dm = the mass of the soil after drying for 7 days at 80^{o} C

3.2.3 RNA EXTRACTION AND DNASE TREATMENT

RNA extraction was done from roots, nodules and leaves as described in section 2.2.3 A; using untreated, drought-treated and sorbitol-treated plants.

3.2.4 FIRST STAND CDNA SYNTHESIS USING EXTRACTED RNA

First stand cDNA was synthesised from leaf, root and nodule RNA from untreated, drought-treated and sorbitol-treated plants as described in section 2.2.3 B. The cDNA produced was then used in PCR or stored at -20^{o}C.

3.2.5 PCR AMPLIFICATION OF GLYMA01G42840 AND β-TUBILIN FROM LEAVES, ROOTS AND NODULES

PCR was used to amplify Glyma01g42840 using 200 ng of previously generated cDNA as a DNA template. The reaction contained 0.2 mM of dNTPs, 400 nM of gene-specific forward primer (5′-ATG GCT AGC CAA TCA AAC ACT AAA TC-3′) and 400 nM of gene specific reverse primer (5′-TCA TCG ATC TAG CAG CTT CTT-3′). Amplification was done using 1.25 U of TrueStart™ Hot Start Taq Polymerase (Fermentas) in the presence of 1.5 mM $MgCl_2$ according to the manufacturer's instructions. A similar PCR reaction was also set up in order to amplify β-tubilin from the leaves, roots and nodules. The reagents and concentrations used were the same as above with the exception of the primers, which were specific for β-

tubilin, being forward primer (5′-CTGCGAAAGCTTGCAGTGAACC-3′) and reverse primer (5′-TCTTGCCTCTAAACATGGCTGAGG-3′). The reaction was carried out using to the following parameters: Initial denaturation was carried out at 95°C for 2 minutes, this was preceded by 25 cycles of denaturation at 94°C for 30 seconds, primer annealing at 56°C for 30 seconds (Glyma01g42840) / 60°C for 30 seconds (β-tubilin) and extension at 72°C for 2 minutes. Once the 25 cycles were complete final extension occurred at 72°C for 7 minutes. In order to determine whether 25 cycles falls within the PCR exponential phase, PCR reactions were also set up with 15, 20 and 30 cycles. After the PCR amplification, the PCR product was size fractionated on a 1% agarose gel (0.5 g of agarose and 50 mL of 1X TBE). The gel was visualized by mixing the samples with DNA loading dye that contained GelRed (Biotium) and exposure to short wave UV light using an AlphaEaseFC (Alpha Innotech Corporation) UV gel visualization system.

3.2.6 HYDROGEN PEROXIDE CONTENT ASSAY

Plant tissue was flash-frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The powdered tissue was weighted and 100 mg of the tissue was homogenised with 400 µl of 5% (w/v) TCA that had been pre-chilled to 4°C. The solution was then centrifuged at 12 000 *x g* for 30 minutes at 4°C, after centrifugation 50 µl of the TCA extract was transferred to a clean tube. A solution of 50 µl TCA extract, 50 µl of KH_2PO_4 and 100 µl of KI was made up and this was loaded into a 96 well plate in triplicate, a standard curve was also set up with H_2O_2 concentrations ranging from 0 nM to 2500 nM. The plate was then incubated at room temperature for 20 minutes after which the absorbance was measured at 390 nm.

3.2.7 LIPID PEROXIDATION ASSAY

Leaf tissue from untreated, short-term drought (osmotic stress induced by 240 mM sorbitol over a period of 48 hours) treated and long-term drought (plants not watered for 3 weeks)-treated plants was ground to a fine powder by flash-freezing in liquid nitrogen and grinding with a mortar and pestle. Prior to grinding the leaf tissue, clean, empty microcentrifuge tubes were weighed, to these microcentrifuge tubes the ground tissue was added and the tubes were weighed again in order to determine the weight of the ground tissue. To the ground tissue, 6% TCA was added in a 5 times weight:volume ratio. The solution was then centrifuged at 12 000 *x g* for 10 minutes. After centrifugation, 200 µl of supernatant was removed from the tube and was replaced by 400 µl of a solution containing 20% TCA and 0.5% TBA. The solutions were briefly vortexed to ensure proper mixing and were then boiled at 90°C for 20 minutes. After boiling, the solutions were incubated on ice for 10 minutes and then centrifuged at 12 000 *x g* for 5 minutes. After centrifugation, 200 µl aliquots of the supernatant in the microcentrifuge tubes were transferred to a 96 well plate and the absorbance was measured at 532 nm and 600 nm, the data obtained was then used to construct a graph which measures the degree of lipid peroxidation suffered by the cells in the leaves.

3.2.8 CELL DEATH ASSAY

A fully expanded leaf taken from the second youngest trifoliolate of leaves was selected from untreated, short-term drought (240 mM sorbitol) and long-term drought-treated plants (deprived of water for 3 weeks) and a 1 cm^2 section was cut from each leaf. The sections where then placed in 0.25% (w/v) of Evans Blue dye and incubated in the dark for 30 minutes, after the incubation the dye was removed and the tissue was flushed with distilled in order to remove excess dye. The tissue was then incubated in distilled water overnight. The tissue was then added to a clean 1.5 mL microcentrifuge tube containing 1% SDS and incubated at 55°C for 1 hour. After incubation, 200 µl aliquots of the supernatant in the microcentrifuge tubes were transferred to a 96 well plate and the absorbance was measured at 600 nm, the data obtained was then used to construct a graph which measures cell death in relation to Evans Blue up take.

3.2.9 ANTIOXIDANT ENZYMATIC ACTIVITY ASSAYS

Total protein was extracted from the leaves of untreated, 240 mM sorbitol-treated and drought-treated plants. This was done by flash-freezing the leaves with liquid nitrogen and grinding them into a fine powder. The leaf powder was homogenised with plant protein extraction buffer [40 mM Potassium Phosphate (K_2PO_4) at pH 7.4, 1 mM Ethylenediaminetetraacetic acid (EDTA) and 5% polyvinylpyrrolidone (PVP)] in a 1:5 w/v ratio. The solution was then centrifuged at 4°C for 30 minutes after which the supernatant was transferred to a clean tube. The protein concentration was determined using the Bradford assay as explained by Kruger (2002). Polyacrylamide gels were set up as 10% separating non-denaturing polyacrylamide gels and 5% stacking non-denaturing polyacrylamide gels devoid of any SDS. The gels were then loaded with 80 µg of total

protein extracted from the untreated, 240 mM sorbitol-treated and drought treated leaves. The gels were then electrophoresed at 4°C, after electrophoresis the gels were incubated in 1X native page running buffer containing 2 mM GSH for GPX activity staining and 2 mM ASA for APX activity staining, the incubation was carried out over a 20 minute period. After incubation, the primary stain which was made up of 50 mM potassium phosphate (KPO_4) at pH 7.0 and 13 mM GSH (GPX stain) or 2 mM ASA (APX staining) in 50 mL of water was applied, the gels were stained for 20 minutes in this solution after which it was discarded and the secondary stain added. The secondary stain was composed of 50 mM KPO_4 at pH 7.8, 30 mM GSH and 0.002% cumene hydroperoxide (GPX staining) or 4 mM ASA and 2 mM H_2O_2 (APX staining) in 50 mL of water. The gels were stained for a further 20 minutes after which the secondary stain was replaced by the tertiary stain made up of 50 mM KPO_4, 0.04% TEMED and 0.5 mM of nitrotetrazolium blue (NTB) in 50 mL of water for both the GPX and APX stains. The gels were stained for 20 minutes after which the stain was discarded to prevent over-staining of the gels, after staining was complete, the gels were digitally photographed. Densitometry was performed, using the Spot Denso tool of the AlphaEaseFC Software (Alpha Innotech Corporation), to analyse the pixel intensities of each band in the gels. The pixel intensities were expressed as arbitrary units.

3.2.10 STATISTICAL ANALYSIS

The statistical validity of all the data was tested by means of a One-way analysis of variance (ANOVA) and the Tukey-Kramer test at 5% level of significance was completed to compare the means using GraphPad Prism 5.03 software.

3.3 RESULTS

3.3.1 DETERMINING THE RELATIVE WATER CONTENT OF THE SOIL IN WHICH THE PLANTS WERE GROWN

The RWC of the soil in which the untreated and 3 weeks drought-treated plants was measured as it is important to define the desiccation state of the soil to determine if the untreated plants had enough water while the drought-treated plants had a deficit of water. It was found that there was a 1.48-fold lower soil RWC in the drought-treated soil than the untreated soil (untreated soil had a RWC of 86.25% while the 3 weeks drought-treated soil had a RWC of 58.25%).

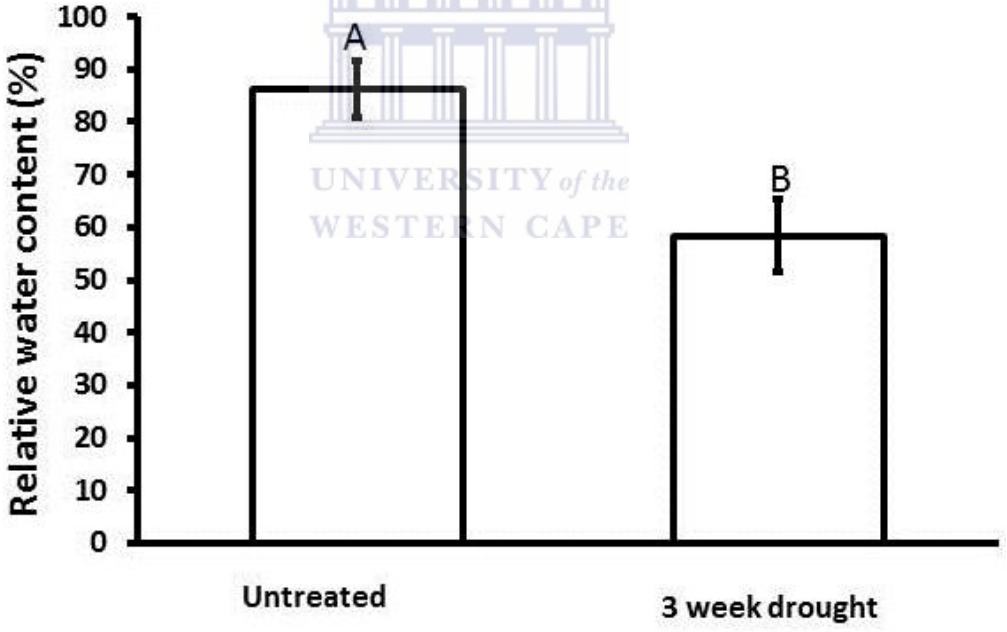

Figure 3.3.1: The relative water content of the soil in which the untreated and 3 week drought treated plants were grown. From the data obtained, it was determined that the RWC of the untreated soil was 86.25% while the RWC of the soil that was deprived of water for 3 weeks was 58.39%. Data are means ± standard errors from three independent experiments consisting of six different plants per treatment. Varying letters above the error bars indicate that the samples are not statistically similar.

3.3.2 SPATIAL EXPRESSION PATTERNS OF GLYMA01G42840

Figure 3.3.1 shows the relative expression levels of Glyma01g42840 in relation to β-tubulin expression from soybean roots, nodules and leaves respectively after a 25 cycle PCR reaction. It was observed that 25 cycles fell in the PCRs exponential phase as 15 and 20 cycles produced bands that were faint and indistinguishable while 30 cycles produced bands that all look similar. It was determined from Figure 3.3.1 that soybean roots and leaves express statistically similar levels of Glyma01g42840, whereas nodules express this gene at levels 0.25-fold lower than those in leaves and roots.

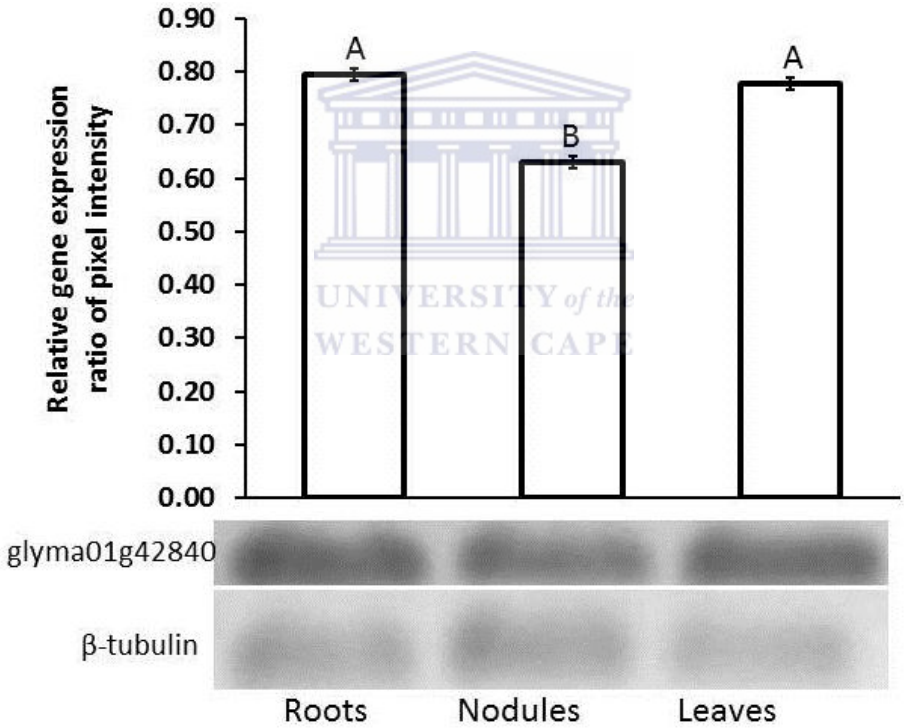

Figure 3.3.2: Spatial expression of Glyma01g42840 relative to β-tubilin in soybean roots, nodules and leaves. The graph depicts the pixel intensity of the bands produced by the semi-quantitative PCR amplification of Glyma01g42840 form roots, nodules and leaves relative to β-tubulin in these tissues. The expression of Glyma01g42840 is statistically similar in the leaves and roots whereas it is lower in the nodules than in leaves and roots. Data are means ± standard errors from three independent experiments. Varying letters above the error bars indicate that the samples are not statistically similar and the same letters indicate statistical similarity.

3.3.3 TEMPORAL EXPRESSION PATTERNS OF GLYMA01G42840 UNDER DROUGHT TREATMENTS

In an attempt to elucidate the temporal expression patterns of Glyma01g42840, RNA was extracted from the leaves of plants that were grown under normal conditions (untreated), and plants that were grown in water deficit for 3 weeks (long-term drought). The extracted RNA was used to synthesize cDNA which was then used as a template in RT-PCR. The amplified products were electrophoresed and the resulting agarose gel was used to determine the relative gene expression of Glym01g42840 (Figure 3.3.2). This figure shows that the relative gene expression of glyma01g42840 increase when plants are exposed to long-term drought conditions

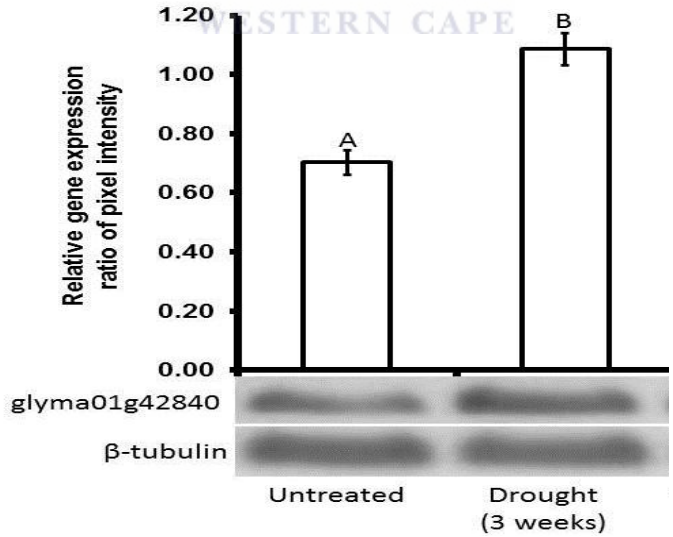

Figure 3.3.3: Temporal expression of Glyma01g42840 relative to β-tubilin in soybean leaves under drought and osmotic stress. The graph depicts the pixel intensity of the bands produced by the PCR amplification of Glyma01g42840 form untreated plants and plants that were deprived of water for three weeks relative to β-tubulin in the leaves of soybean plants. Data are means ± standard errors from two independent experiments. Varying letters above the error bars indicate that the samples are not statistically similar.

3.3.4 DETERMINING H_2O_2 CONCENTRATION IN SOYBEAN LEAVES UNDER VARIOUS DROUGHT CONDITIONS

Towards the determination of the degree of oxidative stress suffered by the plant during drought stress, the concentration of H_2O_2 in the leaves was measured. This was done because high concentrations of H_2O_2 are an indication of oxidative stress. Figure 3.3.3 represents of H_2O_2 concentrations in soybean leaves grown under normal conditions and as well as under long-term drought conditions. From the data obtained it was determined that there is a 1.98-fold increase in the hydrogen peroxide concentration when comparing leaves from untreated plants with that of leaves from plants grown under long-term drought conditions.

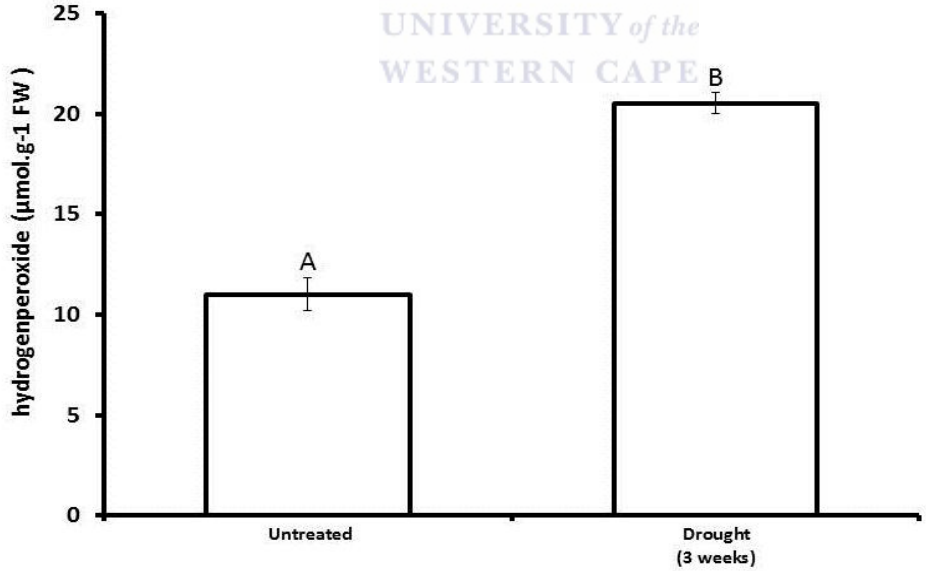

Figure 3.3.4: Hydrogen peroxide content of soybean leaves under various drought treatments. The figure exhibits the difference in the concentration of hydrogen peroxide in soybean leaves under normal growth conditions and long-term drought conditions. Data are means ± standard errors from three independent experiments. Varying letters above the error bars indicate that the samples are not statistically similar.

3.3.5 DETERMINING THE DEGREE OF LIPID PEROXIDATION SUFFERED BY SOYBEAN LEAVES UNDER DROUGHT CONDITIONS

Lipid peroxidation occurs as a result of overproduction of H_2O_2 under abiotic stress, as such it is a secondary indicator of oxidative stress. The degree of lipid peroxidation is measured here by determining the concentration of malondialdehyde (MDA), which is a product that is produced as a result of the peroxidation of membrane lipids. Figure 3.3.4 shows that there is a ± 0.26-fold increase in the concentration of MDA in the drought treated plant in comparison to the untreated plant. The trend in MDA concentration follows the trend in H_2O_2 concentration as shown in Figure 3.3.4.

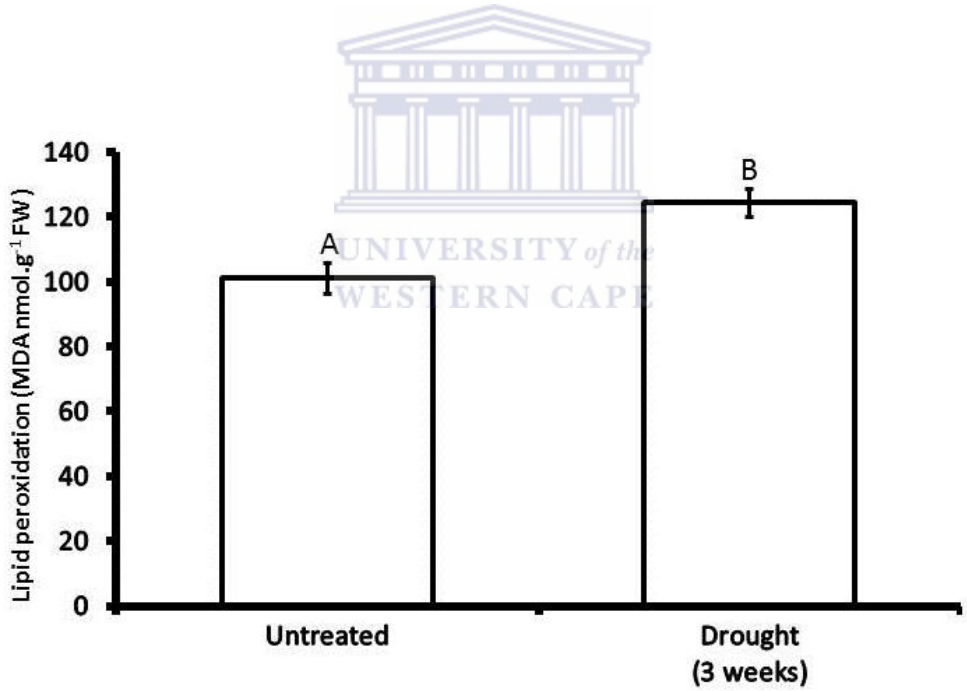

Figure 3.3.5: Degree of lipid peroxidation in soybean leaves under various drought treatments. The figure above illustrates the degree of lipid peroxidation suffered by soybean leaves under drought conditions. The figure shows that the degree of lipid peroxidation in drought treated leaves is higher than that of untreated leaves. Data are means ± standard errors from three independent experiments. Varying letters above the error bars indicate that the samples are not statistically similar.

3.3.6 DETERMINING THE LEVEL OF CELL DEATH SUFFERED BY SOYBEAN PLANTS UNDER DROUGHT CONDITIONS

In an effort to better understand the damaging effects of drought and osmotic stress on soybean, plants were deprived of water for three weeks and as a control plants were also left untreated. Figure 3.3.5 shows that plants deprived of water for three weeks exhibit a 1.1-fold increase in cell death when compared to untreated plants.

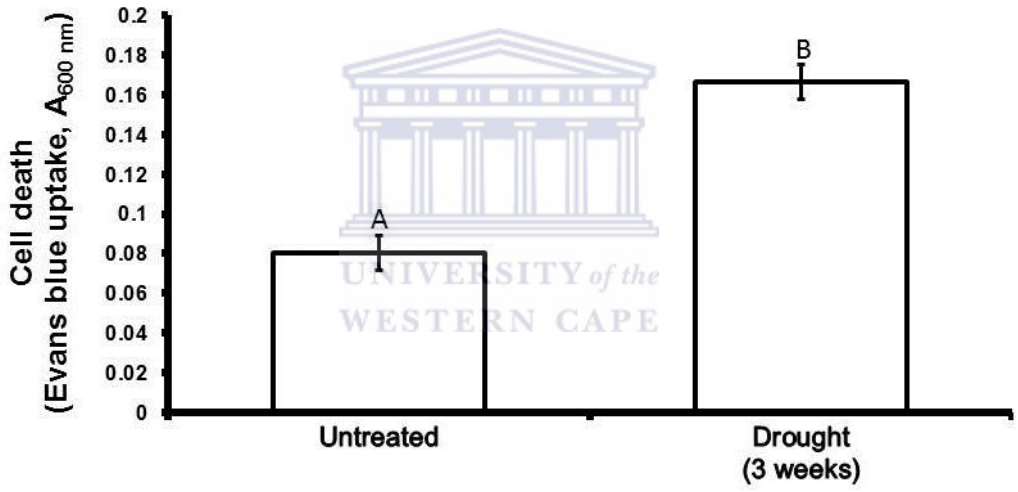

Figure 3.3.6: Measure of cell death via Evans Blue uptake in soybean leaves. Evans Blue uptake in soybean leaves. The untreated plants show low levels of Evans blue uptake while the plants which were deprived of water for 3 weeks exhibits a significant level of cell death. Data are means ± standard errors from three independent experiments. Varying letters above the error bars indicate that the samples are not statistically similar.

3.3.7 THE EFFECT OF LONG TERM DROUGHT STRESS ON GPX AND APX ENZYMATIC ACTIVITY IN SOYBEAN LEAVES

Understanding the effects of long-term drought on the enzymatic activity of drought-responsive antioxidant genes is important in evaluating the contribution that the gene makes to the defence of the plant against abiotic stress. In Figure 3.3.6, lane 1 contains total protein extracted from an untreated plant and lane 2 contains total protein extracted from plants that has been exposed to drought for 3 weeks. The gel images on the left exhibit the relative activity of GPX and APX isolated from soybean leaves when H_2O_2 is used as a substrate, while the gel images on the right exhibit the relative activity of GPX and APX isolated from soybean leaves when cumene hydroperoxide is used as a substrate. The GPX isoforms are labelled 1-9 and the APX isoforms are labelled 1-2 which are the expected numbers of GPX and APX isoforms present in soybean leaves. The data obtained from the gel images were used to provide monetary values to the relative activity of the total protein extracts by measuring the densitometry of each isoform. This data is shown in table 3.3.1 A and B in the case of the GPX isoforms when using H2O2 and cumene hydroperoxide as substrates respectively provides an indication of the relative activity of the GPX isoforms. The relative activity of the APX isoforms are shown in table 3.3.1 C and D with H2O2 and cumene hydroperoxide respectively being used as substrates.

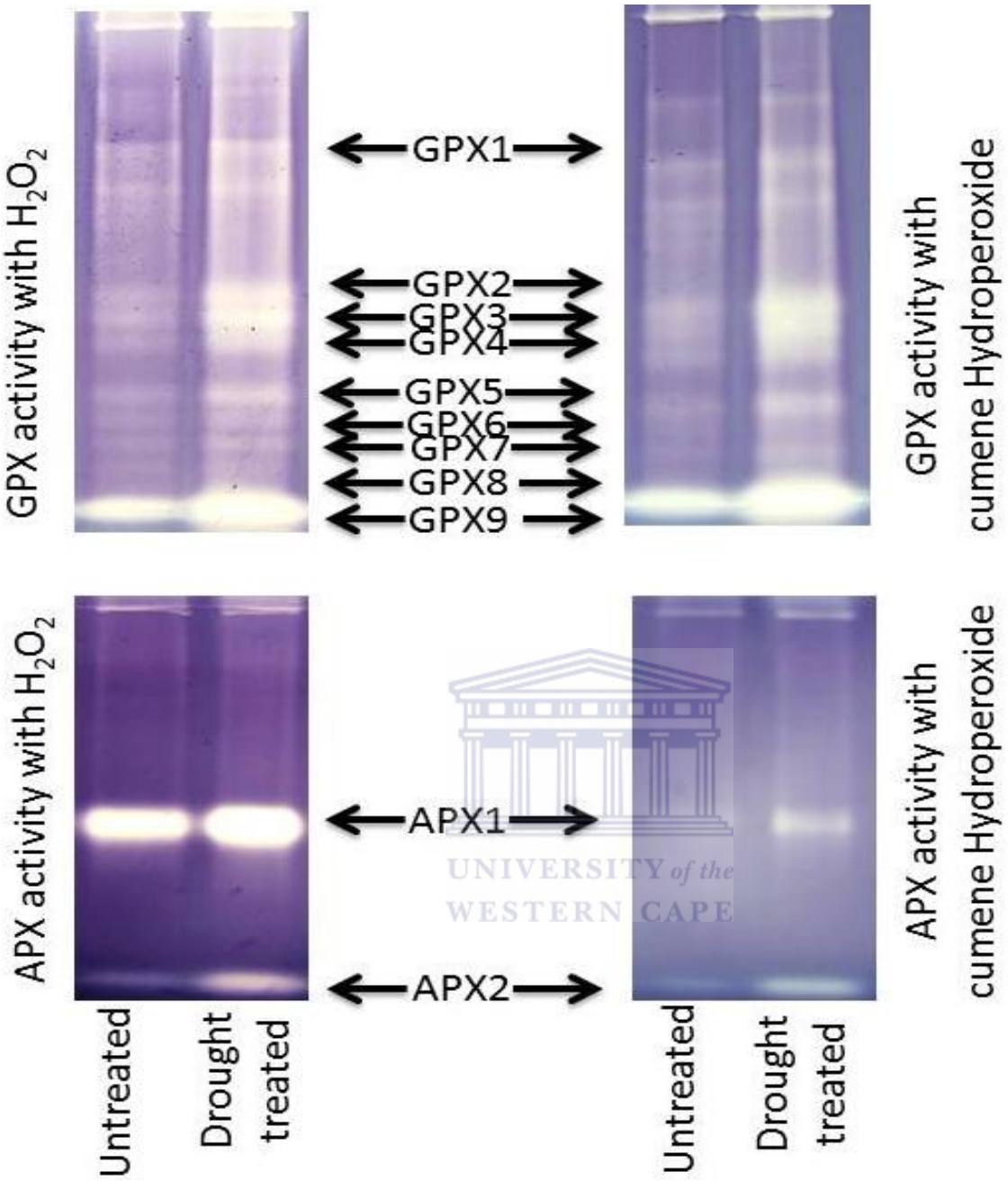

Figure 3.3.7: The effect of short and long-term drought on the enzymatic activity of glutathione peroxidase and ascorbate peroxidase in the leaves of soybean plants. In all of the lanes above the untreated total protein extract samples are loaded into lane 1 and the three week drought treatment total protein extract samples are loaded into lane 2. The gel images one the left show GPX and APX activity respectively when H_2O_2 is used as a substrate and the gel images show the GPX and APX activity respectively when cumene hydroperoxide is used as a substrate.

Table 3.3.1 A and B: Relative GPX activity of the total protein extracted from soybean leaves exposed to various drought treatments using hydrogen peroxide and cumene hydroperoxide, respectively, as substrates

Relative activity of GPX using H_2O_2 as a substrate			
A	Untreated	Drought (3 weeks)	increase/decrease untreated vs. drought
GPX 1	1.39± 0.076[a]	2.13± 0.113c	increase
GPX 2	1.51± 0.104[a]	2.00± 0.134c	increase
GPX 3	1.49± 0.086[a]	2.52± 0.189c	increase
GPX 4	1.45± 0.101a	1.81± 0.134c	increase
GPX 5	1.18± 0.062a	1.82± 0.121c	increase
GPX 6	1.08± 0.061a	1.56± 0.092c	increase
GPX 7	1.05± 0.077a	1.50± 0.109c	increase
GPX 8	3.36± 0.174a	4.73± 0.264c	increase
GPX 9	1.32± 0.074a	2.44± 0.112c	increase

Relative activity of GPX using Cumene Hydroperoxide as a substrate			
B	Untreated	Drought (3 weeks)	increase/decrease untreated vs. drought
GPX 1	1.92± 0.113[a]	3.10±0.201[c]	increase
GPX 2	1.87± 0.125[a]	2.65± 0.180[c]	increase
GPX 3	1.74± 0.123[a]	2.35± 0.124[c]	increase
GPX 4	1.73± 0.110[a]	2.29± 0.157[c]	increase
GPX 5	1.71± 0.101[a]	1.77± 0.095[a]	no change
GPX 6	1.70± 0.091[a]	1.84± 0.137[a]	no change
GPX 7	5.26± 0.294[a]	7.21± 0.497[c]	increase
GPX 8	1.40± 0.082[a]	1.97± 0.116[c]	increase
GPX 9	4.74± 0.346[a]	5.06± 0.359[a]	no change

* Values marked with the same letter are statistically similar

Table 3.3.1 C and D: Relative APX activity of the total protein extracted from soybean leaves exposed to various drought treatments using hydrogen peroxide and cumene hydroperoxide respectively as substrates

Relative activity of APX using H_2O_2 as a substrate			
C	Untreated	Drought (3 weeks)	increase/decrease untreated vs. drought
APX 1	14.36± 0.718[a]	16.10± 0.843[b]	increase
APX 2	1.29± 0.076[a]	5.38± 0.392[b]	increase

Relative activity of APX using Cumene Hydroperoxide as a substrate			
D	Untreated	Drought (3 weeks)	increase/decrease untreated vs. drought
APX 1	2.76± 0.190[a]	4.10± 0.237[b]	increase
APX 2	2.11± 0.141[a]	2.48± 0.171[c]	increase

* Values marked with the same letter are statistically similar

3.4 DISCUSSION

As previously stated, the objective of this study was to study the effects of drought on the expression of Glyma01g42840. It was thus important to firstly establish whether or not the plants were actually exposed to drought conditions. This was accomplished by comparing the relative water content of untreated (regularly watered) soil with that of soil that was deprived of water for 3 weeks, the data obtained in this experiment showed that the RWC of the untreated soil was 86.25% while the RWC of the water deprived soil was 58.25%. This reduction in water availability to the plants that were grown under conditions with a moisture deficit would be sufficient to cause water deficit on the plants, which according to Warrick (1975) is the definition of drought. Once it was confirmed that the plants were grown under drought conditions, further experimentation was carried out.

Determining the relative expression patterns of Glyma01g42840 was accomplished by extracting RNA from the leaves, roots and nodules and it was observed that the expression of the Glyma01g42840 is similar in the leaves and roots of the plant whereas the relative expression in the nodules is lower than that in the leaves and roots. These results were corroborated by the spatial patterns generated using the soybean eFP browser (http://bar.utoronto.ca/efpsoybean/cgi-bin/efpWeb.cgi). Once the relative gene expression in the different organs was determined, the temporal expression of Glyma01g42840 was evaluated in the leaves in response to water deficit. The leaves were chosen as the focus organ for the measurement of temporal expression patterns as they are the ones that would show visible physical indicators of drought stress such as wilting (Carrow, 1996), which are easy to assess visually. It was found that the expression of Glyma01g42840 is up-regulated under drought conditions in comparison with normal growth conditions.

Although the expression of Glyma01g42840 is up-regulated during drought stress, the plants were still suffering damage as a result of oxidative damage. As such, it can be proposed that the degree of oxidative stress was too high for the enzyme encoded by Glyma01g42840 to have a significant effect in protecting the plants. As a result of this it was important to determine the degree of oxidative stress that was suffered by the plants. To do this, the concentration of malondialdehyde (MDA) as well as the degree of lipid peroxidation and cell death was measured, as these are indicators of oxidative stress. There was a significant difference between the untreated plants and the drought treated plants, exhibiting a large increase in H_2O_2 concentration, which may be responsible for the surge in Glyma01g42840 expression during the 3 week drought treatment as H_2O_2 is a known signalling molecule that causes the up-regulation in the expression of stress-defence genes (Hung *et al.*, 2005), which would explain both the increased level of Glyma01g42840 expression and the increased concentration of H_2O_2 during the 3 week drought period. Following determination of the H_2O_2 content, the levels of lipid peroxidation were determined and it was found to have a similar pattern to that of the H_2O_2 concentration, with the untreated plants had a low degrees of lipid peroxidation and the 3 week drought treatment plants having elevated lipid peroxidation. As H_2O_2 as well as O_2^- and OH are able to initiate a lipid peroxidation reaction (Gill*, et al.*, 2010), it was expected that the increased levels of H_2O_2 would result in a high degree of lipid peroxidation. As lipid peroxidation leads to cell death, a similar trend in the data was observed when the degree of cell death was measured. The untreated plants had a lower level of cell death in comparison with the plants that were exposed to drought for 3 weeks, which exhibited a pronounced increase in cell death. As a result of the drastic increase of H_2O_2 concentration as well as the degree of lipid peroxidation and cell

death it is evident that the plants that were exposed to drought stress of 3 weeks are suffering significant oxidative damage.

In an attempt to elucidate the effect of drought stress on the enzymatic activity of antioxidant enzymes in soybean leaves, protein extracts from leaves of plants exposed to va drought conditions were electrophoresed on native polyacrylamide gels and then stained for GPX and APX activity respectively and nine GPX isoforms were identified while two APX isoforms were identified. The data obtained was then analysed using AlphaEaseFC imaging software and it was found that when cumene hydroperoxide (a lipid hydroperoxide) is used as a substrate during GPX staining, there are increases in the activity of the enzymes with exception of isoforms 5 and 6 which remain similar to the untreated samples. When the total protein extract was stained for GPX activity using H_2O_2 as a substrate the enzymatic activity of the untreated samples was lower than that of the drought treated samples. Here the effect of the H_2O_2 as a signalling molecule may have been strong enough to up-regulated the activity of GPX. In the case of isoforms 8 and 10, the activities of these isoforms are much higher in comparison with the other isoforms. A similar pattern is observed when looking at the APX activity of the total protein using both cumene hydroperoxide and H_2O_2 as substrates, here however only to isoforms are identified. In the case of the 3 week water deficit samples, the antioxidant activity is higher in the 3 week drought treatment than in the untreated samples. The fact that the different staining methods yielded different isoform patterns is an indication of the specificity of the in-gel activity staining methods, as a result of this these gels help verify that the activity gels in chapter 2 are accurate representations of the activity of Glyma01g42840 when using ascorbate and glutathione as electron donors in the scavenging of H_2O_2/lipid hydroperoxides.

3.5 CONCLUSION

Spatial and temporal expression pattern analysis of Glyma01g42840 showed that the gene is expressed differentially in the various plant tissues and in up-regulated by drought stress. It was also shown that plants grown under drought conditions suffer oxidative stress as the H_2O_2 content in the cells and the degree of lipid peroxidation as well as the degree of cell death (which are known indicators of oxidative stress) all increase in the drought samples when compared to the untreated samples. It was also shown that the enzymatic activities of several APX and GPX isoforms are up-regulated in response to drought but the increased antioxidant enzyme activity is not sufficient to mitigate the effects of the increased production of ROS under the stress conditions because the plants showed increased H_2O_2 contents together with increased lipid peroxidation and increased cell death. As such, future work entails transferring Glyma01g42840 into plants where its expression will be placed under the control of a promoter which is strongly induced by drought, in doing this the production of the enzyme will be increased and the tolerance of the plants against drought should theoretically be increased. This should assist towards development of cultivars with improved crop yield and quality during conditions of drought.

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FUTURE PROSPECTS AND OUTLOOKS

This study has established that Glyma01g42840 is a glutathione peroxidase encoded by a gene for which the expression is up-regulated by drought. However, the finding showing that drought-induced expression of the gene is insufficient to prevent lipid peroxidation implies that it may be necessary to increase the expression of the gene to levels much higher than those induced by drought in the wild type soybean plants. This would entail the expression of the gene in soybean under the control of a promoter that is strongly induced by drought and examine the physiological effects this would have on the accumulation of reactive oxygen species, lipid peroxidation and survival of the plant under drought stress. Furthermore localisation studies would be useful in proving information on the organelles in which the protein is localized, since this may determine the extent to which the protein offers protection against oxidative stress. Tertiary and quaternary structural determinations and how these relate to the functioning of the protein would provide crucial information relevant to the engineering of the protein for better efficiency in scavenging H_2O_2 and other peroxides. Hence, further investigation into the structure, function and physiological role of Glyma01g42840 could in the future lead to the production of a soybean cultivars with improved tolerance to drought, which would impact positively on food security through improved yield under limited water resources.