Identification of regulatory elements mediating responses of SOD and cystatin transcripts to salt stress and nitric oxide in soybean nodules

by
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Declaration

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Abstract

Nitric oxide (NO) has previously been shown to play a vital role in plants that are undergoing oxidative stress arising from abiotic stress. To better understand the role of NO on the antioxidative pathway, the effect of NO on Superoxide Dismutase (SOD) activity was studied during salt stress on soybean nodules. The enzymatic activity of specific MnSOD and FeSOD isoforms increased upon 1 week of exposure of nodules to NO or salt stress, the activity of CuZnSOD isoforms however increased in response to salt stress only. Furthermore, 4 putative FeSOD and MnSOD transcripts were identified and shown to increase in response to NO and salt stress. The promoter sequences of these NO-responsive putative SOD genes were analysed alongside a cystatin (*AtCYS-1*) which is also NO-inducible. Putative NO-responsive *cis*-acting elements as well as abiotic stress-responsive *cis*-acting elements were studied amongst these promoter sequences. The MYCL element and the AtMYB4 binding site were found to occur in all four NO-inducible SOD promoter sequences as well as in the *AtCYS-1* promoter sequence. This suggests that NO acts via MYCL and/or AtMYB4 to up-regulate specific FeSODs and MnSODs, causing an increase in the activity of these SOD isoforms, thus reducing oxidative stress and cell death in soybean nodules. Furthermore, NO may also be up-regulating cystatins to inhibit cysteine proteases, thus preventing the onset of programmed cell death (PCD) and subsequently reducing salt stress-induced cell death.

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Abbreviations

ARE	Antioxidant Response Element
ABRE	Abscisic acid response element
APX	Ascorbate Peroxidase
bHLH	basic-Helix-Loop-Helix
C4H	Cinnamate 4-hydroxylase
CAT	Catalase
CHS	Chalcone synthase
C-PTIO	2-(4-carboxyphenyl)-4,4,5,6-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO)
CRE	cAMP-response element
CREB	CRE-binding protein
DAF-2DA	Diaminofluorescein-2 diacetate
DETA	Diethylenetriamine
DETA/NO	2,2'-(hydroxynitrosohydrazono)bis-ethanimine
DRE	Dehydration response element
EDTA	Ethylenediaminetetraacetic acid
GFP	Green fluorescent protein
GPX	Glutathione peroxidase
GSH	Glutathione
HR	Hypersensitive Response
HSE	Heat shock element
NF-κB	Necrosis Factor-κB
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
OCSE	Octopine synthase element
PCD	Programmed Cell Death
PVP	Polyvinylpyrrolidone
ROS	Reactive Oxygen Species
SA	Salicylic acid

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SDS   Sodium dodecyl sulphate

SOD   Superoxide Dismutase

TBA   Thiobarbituric acid

TCA   Trichloroacetic acid

YFP   Yellow fluorescent protein

Chapter 1: Literature Review

1.1 Importance of Soybean

Glycine max, more commonly known as soybean, is a crop plant with relatively high protein content and is used as food for humans and feed for animals. Other uses for soybeans are in the production of biofuels, crayon, plastic, ink, clothing, cosmetic products and soaps (Ahlors et al., 2009). Soybean forms a symbiotic relationship with the nitrogen fixing rhizobium *Bradyrhizobium japonicum* (Taiz and Zeiger, 2006). This offers a natural way to enrich soils with nitrogen instead of using environment-polluting artificial fertilizers. Inoculation of *G. max* with the *B. japonicum* can improve plant vigour, root biomass, yield and tolerance towards abiotic stresses like salinity stress (Bressano et al., 2010).

1.2 Salt Stress

Approximately 800 million hectares of land in the world are affected by saline soils and with current irrigation systems raising the water table from salt deposits, the area of salt-affected lands is increasing. Almost all plants have a mechanism by which to increase their tolerance towards NaCl although some are better at this than others (Munns and Tester, 2008).

The salinity stress responses from plants can be separated in time as two different stresses, namely osmotic followed by ionic stress. Initially the hyperosmotic saline solution will cause osmotic stress on the roots which the whole plant will need to adapt to. Plants can adapt to osmotic stress by adjusting their own osmotic potential through increasing concentrations of osmolytes like glycine betaine and proline (Munns and Tester, 2008). Plants experiencing salt stress initially start to show reduced growth in terms of slower leaf expansion, rate of new leaf emergence and other similar traits (Munns and Tester, 2008).

The next phase of salt stress is the influx of Na^+ and Cl^- ions, which can cause havoc on cellular function. The Na^+ ions travel through their specific transporters to get into the plant cell as well as through K^+ ion transporters. This penetration of Na^+ ions into the cells forces the plant to have to deal with these excess Na^+ ions, which most plants seem to do by compartmentalizing the Na^+ into vacuoles of the cells in older leaves. These older leaves are then generally senesced and the Na^+ ions are disposed of. It must be noted that in soybean it has been said that the Cl^- ions are more toxic to the plant (Munns and Tester, 2008). The reasoning behind this is that soybean is highly adept at keeping the Na^+ ions out of the plant leaving the high amount of Cl^- ions to be the main issue (Munns and Tester, 2008).

1.3 Salt Stress results in increased ROS content

A broader consequence of salinity stress and abiotic stress in general is the increased generation of oxidants known as reactive oxygen species (ROS) (Mittler et al., 2002). Oxidative stress results if the cells are overwhelmed by these oxidants. The increase in the osmotic potential of the plant cells as well as the accumulation of Na^+ ions inside the cell can disrupt the functioning of the redox active organelles (mitochondria, chloroplasts, peroxisomes) and result in ROS generation (Sandalio et al.,

2008). ROS are also generated under normal conditions but this occurs at a much slower rate than when plants are under abiotic stress. One way ROS are formed is through complex I of the mitochondria, the NADH dehydrogenase normally passes electrons to quinones in the electron transport chain but can also leak electrons to oxygen to yield superoxide. It is estimated that approximately 2% of total oxygen consumed by mitochondria result in ROS through this leakage of electrons to oxygen and approximately 10 to 20 % of electrons that travel through photosystem I in the chloroplast also result in ROS production (Becana et al., 2000; Davies, 2007).

Reactive oxygen species include superoxide, hydrogen peroxide, singlet oxygen and the hydroxyl radical. These compounds and ions are highly reactive. Increased reactive oxygen species generation in plant cells due to salinity stress on the whole plant causes major cell damage such as lipid peroxidation, oxidation of proteins, inhibition of enzymes as well as damage to DNA and RNA (Mittler, 2002). A representation of ROS production and some enzymes involved in ROS scavenging is depicted in Figure 1.1. This damage to the cell is used as a measure to determine the level of oxidative stress experienced by the cells.
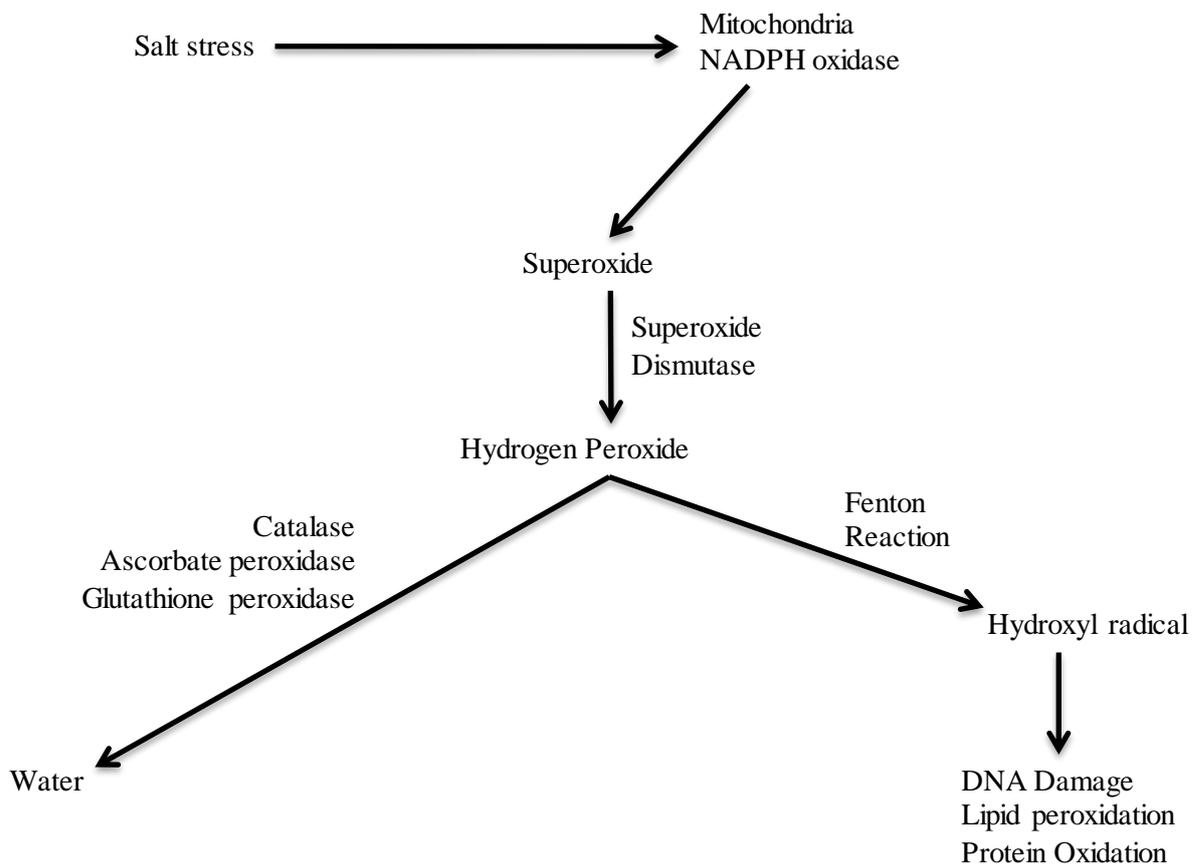
Figure 1.1 Salt stress leading to reactive oxygen species (ROS) production and enzymes involved in scavenging them.

Lipid peroxidation can change the structure and function of the lipid, cell to cell interactions, permeability, fluidity and changes in the ionic environment of the membrane (Grune, 2007). With regard to proteins, ROS can cause oxidation of proteins increasing their hydrophobicity and leading to

fragmentation or denaturation of the protein and thus inactivating enzymes and causing loss of function in proteins. Oxidation of proteins also marks the proteins for degradation by the proteasome. If these oxidised proteins are not degraded and left for further oxidation, they can cross-link with each other to form insoluble aggregates (Grune, 2007). DNA damage caused by ROS, more specifically the hydroxyl radical can result in mutations, altered transcription and initiation of cell death (Davies, 2007).

Generation of ROS in legume nodules has been demonstrated from both the plant and the bacteroid cells. Superoxide radicals and hydrogen peroxide are generated because of the increased respiration rate required to support the nitrogen fixation that occurs in the nodules. Other sources of ROS in the nodule include auto-oxidation of cytosolic leghemoglobin, oxidation of nitrogenase, ferredoxin, hydrogenase and free iron (Fenton reaction) (Becana et al., 2000).

1.4 Reduction of superoxide content by superoxide dismutase

The main areas of superoxide are the mitochondria, chloroplasts, endoplasmic reticulum, peroxisomes, bacteroids and the plasma membrane. The enzymes involved in superoxide generation include xanthine oxidase, membrane bound NADPH oxidase, enzymes in the electron transport chain as well as other enzymes that also contain NADPH oxidase activity. Other sources include oxidation of leghemoglobin in the cytosol, nitrogenase and ferredoxin (Matamoros et al., 2003).

Superoxide dismutase (SOD) catalyses the scavenging of superoxide, resulting in molecular oxygen and hydrogen peroxide. This reaction can occur without the SOD enzyme but has a relatively low reaction rate (1×10^2 $M^{-1}.sec^{-1}$) in comparison to when SOD is present (1.9×10^9 $M^{-1}.sec^{-1}$). By reducing the cellular superoxide content, less damage is conferred to the cell as can be detected by reduced lipid peroxidation. Although reduced superoxide content results in less damage, superoxide dismutase still produce hydrogen peroxide which also can cause cellular damage if not kept in check by cellular systems for scavenging of the hydrogen peroxide. Hydrogen peroxide can break down to OH^- radicals in the presence of free iron and these radicals are possibly the most reactive of all the ROS (Davies 2007).

For hydrogen peroxide scavenging, there is a myriad of systems as reviewed in Becana, 2010; Foyer and Noctor, 2011; Miller et al., 2010. Catalase is one scavenger of hydrogen peroxide but has the lowest affinity for hydrogen peroxide in comparison with other antioxidative enzymes. A second pathway is the Halliwal-Asada pathway, which consists of ascorbate peroxidise, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase. This 2nd pathway is focused on recycling redox scavenging metabolites, namely glutathione and ascorbate. A third mechanism is through peroxiredoxins, which are recycled through glutathione and thioredoxin (Becana et al., 2010; Foyer and Noctor, 2011; Miller et al., 2010).

1.5 ROS signalling and cell death

During growth, highly dividing cells generate relatively high levels of ROS and the growth can be inhibited by the removal of these species (Foreman et al., 2003). ROS production is possibly to mark current proteins for degradation to replace them achieving a new cell phenotype and growth. This demonstrates the necessity of ROS within the plant. In these actively growing areas there will be changes in plant hormone levels. Increased concentrations of hormones like abscisic acid, auxin, jasmonic acid and salicylic acid (SA) within the plant all trigger generation of ROS in the cells, further demonstrating that ROS play important signalling roles within the plant (Neill et al., 2008; Wang JW and Wu JY, 2005).

An increase in hydrogen peroxide is observed during programmed cell death (PCD) and the inhibition of excessive hydrogen peroxide generation can delay the onset of PCD (Delledonne et al., 1998). PCD can be characterised by cell shrinkage, chromatin condensation, DNA fragmentation and selective protein degradation by caspases (Wang et al., 2010).

Caspases are cysteine aspartate cleaving proteases, named this way because of the cysteine residue that occurs in the active site and the aspartate residue that it cleaves after. Caspases occur in the cell as a precursor form that needs to be activated before protease activity begins. Once active the caspases are susceptible to proteins that directly inhibit their activity, namely cystatins (Lazebnik, 2007). This demonstrates how tightly regulated caspases are.

Programmed Cell Death (PCD) also known as apoptosis is the process by which cells die in an organised programmed fashion. During PCD, DNA laddering can be detected. This is due to DNase activity, which needs to be activated by caspases. The DNA ladder looks uniform on a gel when genomic DNA is subjected to gel electrophoresis because of the DNA being cleaved at the nucleosomes every 180 bp. Another characteristic of PCD is nucleosomal degradation (Lazebnik, 2007).

Apoptosis is probably best studied in *Caenorhabditis elegans* and can occur via either one of two pathways. To demonstrate how well this is controlled, every *C. elegans* worm will lose exactly 131 cells during development to a mature worm due to PCD. The number of cells that die can be increased if the worm is exposed to stresses like radiation. The developmental pathway begins with up-regulation of a gene where the resulting protein interacts with a mitochondrial membrane protein. This in turn leads to a release of a caspase activator, caspases being a type of protease. The activation of the protease leads to degradation of proteins within the cell and completion of the cell death program. The stressed PCD pathway similarly increases the transcription of the PCD initiation gene to achieve PCD (Hengartner, 2007). It is likely that plants would also initiate PCD in a similar manner.

1.6. Increased superoxide leads to cell death

With increased superoxide generation and ROS generation, the plant cell may eventually undergo PCD. In the case of nodules, exposure to salt can induce early senescence or programmed cell death (Mittler, 2002).

One source of superoxide within a cell is the NADPH oxidases, which have been well studied with regards to their roles in cell death. The NADPH oxidases are activated during HR or upon exposure to ozone, high light or wounding (Mullineaux and Baker, 2010). The two NADPH oxidase homologs *AtrbohD* and *AtrbohF* are known to contribute the highest amount of superoxide during HR as when mutated the plants show reduced superoxide content. These plants demonstrate reduced cell death but are more susceptible to pathogen infection (Lam, 2008).

In terms of responses to superoxide, a mutant was identified that initiated cell death upon exposure to superoxide and once the cell death was initiated, it spread to neighbouring cells and is named the runaway cell death (rcd) phenotype (Epple et al., 2003). Interestingly rcd phenotype cannot be initiated by hydrogen peroxide in this mutant but is inducible though abiotic stress like cold stress (Huang et al., 2010) as well as biotic stresses (Epple et al., 2003). The gene that was mutated in this mutant that displays the rcd phenotype is *Lesion Simulating Disease resistance1* (*LSD1*) and codes for a zinc finger protein (Dietrich et al., 1997). Studies involving *lsd1*, *atrbohd* and *atrbohf* mutants and combinations thereof lead to the conclusion that NADPH oxidases are negatively regulating LSD1 (Torres et al., 2005). Since salicylic acid can also initiate the rcd phenotype in *lsd1* and up-regulates Cu/Zn SOD, it has been suggested that LSD1 is required for Cu/Zn SOD up-regulation to stop the rcd phenotype (Delledone et al., 2001). Interestingly nitric oxide (NO) is known to influence Cu/Zn SOD through post translational modification and therefore may also play a role in the rcd phenotype (Lindermeyr et al., 2005). Another role for the LSD1 protein is its cytosolic retention of AtBZIP10 which is a positive regulator of cell death (Kaminaka et al., 2006). More often than not hydrogen peroxide has been attributed to PCD but authors often overlook hydrogen peroxides role in inhibition of Cu/Zn SOD which could possibly increase cytosolic superoxide content inducing cell death (Zago et al., 2006).

1.7 Inhibition of cell death by cystatins

During PCD the cell increases cysteine protease activity which can be inhibited by cystatins. Furthermore these cystatins can inhibit PCD through this inhibition of cysteine protease activity. Cystatins are small proteins that specifically inhibit cysteine proteases. Cystatins have been over-expressed in plant cell cultures to successfully reduce cell death when exposed to 5 mM hydrogen peroxide (Solomon 1999). In animal systems cystatins have been separated into three different groups. The first are the stefins, which are approximately 11kDa and contain no disulphide bond and a single cystatin domain. The cystatin domain is conserved through all cystatin groups and contains the active site. The second group are the cystatins which are approximately 15kDa and also contain a single cystatin domain as well as 4 conserved cysteine residues that stabilize the protein. The

kininogens, the last group contain multiple repeated stefin-like domains. The plant cystatins are also sometimes referred to as phytocystatins (Benchabane et al., 2010)

1.8 Salt stress results in increased NO content

Numerous studies have shown that plants experiencing abiotic stress generate relatively high levels of NO. Unfortunately the exact source has not been elucidated yet. Inhibitor studies suggest that the main sources of NO is through Nitric Oxide Synthase (NOS) activity and nitrate reductase and to a lesser extent non-enzymatic reduction of nitrate to NO in the presence of ascorbate (Wang et al., 2010). Since NO can freely diffuse through cells due to its hydrophobic properties, the same level of NO within the abiotic stressed plant can be achieved in a healthy plant through addition of NO donors (Ederli et al., 2009). The different NO donors release different forms of NO, for example SNP releases the nitrosonium anion (NO^+), others release (NO^-) or the radical form ($NO^{\cdot}$). When the concentration of NO is increased the plant demonstrates a higher tolerance towards abiotic stresses as well as increased growth rate, increased chlorophyll content and many other benefits. To date there are numerous hypothesis about how NO achieves this but no definitive explanation has been found (Lamattina et al., 2001).

1.9 Possible role of Nitric Oxide

In trying to understand how NO achieves increased abiotic stress tolerance there are several processes that NO may be directly/indirectly be modifying within the cell. NO can directly scavenge superoxide and form peroxynitrite in a reaction that is faster ($1 \times 10^{10} \, M^{-1}.sec^{-1}$) than the ability of SOD to scavenge superoxide. Peroxynitrite has been shown to be highly toxic to animal cells but plant cells can withstand up to millimolar concentrations before signs of stress appear. This can directly reduce superoxide content but elevated NO in combination with hydrogen peroxide may lead to activation of PCD (Wang et al., 2010).

NO also causes various post-translational modifications, namely s-nitrosylation, s-nitration by peroxynitrate and s-guanylation by 8-nitro-cGMP (Akaike et al., 2010). S-nitrosylation is achieved through a direct reaction of NO with specific cysteine thiols within the protein in the presence of an electron acceptor (Lindermayr and Durner, 2009). Often the cysteine residues that are targeted occur in the active site, modulating protein activity or changing protein-protein interactions (Wang et al., 2010).

Although NO has been shown to lead to PCD, removal of NO by C-PTIO or NOS activity inhibition also leads to PCD and others have shown that addition of low concentration of NO reduces PCD (Leach et al., 2010). These are very confusing results. For NO inhibition of PCD, several post-translational modification targets have been identified in mammalian cells; caspase inactivation, p53 tumour suppresser, FLIP, Bcl-2 and Bcl-x in inhibiting mitochondrial release of cytochrome c leading to ROS production (Wang et al., 2010).

NO can also change the transcript profile of the cell as can be seen from several microarray studies (Palmieri et al., 2008; Parani et al., 2004; Besson-Bard et al., 2009). One manner in which NO may influence gene expression is by a pathway that is well characterised in mammals and consists of Keap1 and Nrf2 activation of the Antioxidant Responsive Elemenet (ARE) *cis*-element. Keap1 binds and maintains the Nrf2 transcription factor in the cytosol where it is degraded but is susceptible to s-guanylation. Once Keap1 is s-guanylated it cannot bind Nrf2, allowing it to translocate to the nucleus. Once Nrf2 is in the nucleus it binds to the ARE element, increasing the transcription of many antioxidant genes like glutathione (GSH) synthesis genes, Glutathione-S-transferase and thioredoxin reductase (Akaike et al., 2010).

One of the genes that NO has been shown to up-regulate is a cystatin gene in soybean cell culture (Solomon 1999). As explained earlier, cystatin up-regulation can lead to PCD inhibition by inhibiting the caspases involved and this inhibition of PCD may result in abiotic stress tolerance.

Addition of NO to soybean plants has been shown to decrease ROS within the nodule as well as increase ascorbate peroxidase (APX) activity, either by increasing the amount of APX protein within the tissue or by modulating activity (Keyster et al., 2011). This leads to the question "Does NO influence other key ROS scavenging enzymes like superoxide dismutase?".

1.10 Aim to study SOD and cystatin during NO-mediated alleviation of stress

Since NO has been shown to improve abiotic stress tolerance in plants, numerous groups have tried to explain this phenomenon and because NO has an effect on almost every level in the cell, this study is focused specifically on key elements that NO influences as can be seen in Figure 1.2. The aim of this project is to analyse the effect of NO on cystatin and superoxide dismutase transcript levels as well as superoxide dismutase enzymatic activity and to identify the NO-responsive *cis*-acting elements (using bioinformatics) causing the changes in gene expression.
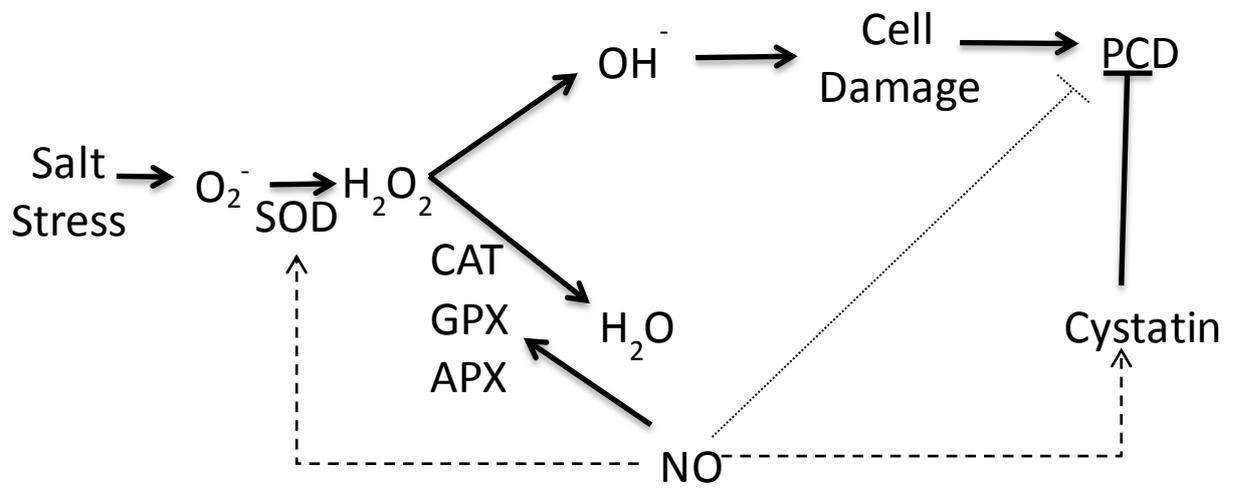
Figure 1.2 Summary of aims of targeting of SOD and cystatin by NO during abiotic stress. Superoxide

Dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), programmed

cell death (PCD).

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Chapter 2: Bioinformatic analysis of soybean superoxide dismutase and cystatin genes

Superoxide dismutase (SOD) and cystatin genes are not well characterised in *Glycine max*. This lead to a bioinformatic approach to identify putative SOD and cystatin genes using the *Glycine max* genomic sequence in combination with known SODs and cystatins previously characterized in *Arabidopsis thaliana*. Since these genes are involved in abiotic stress, their promoter sequences were analysed for abiotic stress *cis*-acting elements as well as elements proposed to respond to nitric oxide. The identified putative *cis*-acting elements can be targeted in subsequent studies to identify those that are truly responsive to nitric oxide (NO).

2.1 Introduction

The 'Central Dogma of Molecular Biology' is a concept that describes the process by which genes are transcribed in to mRNA, mRNA is translated into proteins and the proteins cause some or other phenotypic change to the cell. This process is regulated on various levels; transcriptional, post-transcriptional and post-translational. Regulation occurring at the transcriptional level is regulated by upstream *cis*-acting elements in the promoter of the gene, which determine when and to what extent the transcriptional machinery can be recruited. Currently there are databases consisting of known *cis*-acting elements: Plant *cis*-acting Regulatory DNA Elements (PLACE), TRANSFAC, The Bio-Array Resource for Plant Biology (BAR) and many more (Hampsey et al., 2007).

In mammalian cell cultures, a number of NO-responsive *cis*-acting elements have been identified, including cyclic AMP-response element (CRE), AP1, AP2, Brn-3a, EGR, E2F1 and SP1. The CRE-binding protein (CREB) has been shown to bind the CRE element in the *bcl-2* promoter and can increase bcl-2 transcription. Overexpression of bcl-2 increases cellular resistance to programmed cell death (PCD). S-nitrosylation of fos/jun complex and Necrosis Factor-κB (NF-κB) lead to their activation and s-nitrosylation of HIF-1α increases its DNA binding capacity. NO also induces phosphorylation of p53, promoting its nuclear retention. This demonstrates that NO could possibly influence multiple transcription pathways to alter the cell's phenotype in mammals and perhaps there are similar mechanisms in plants (Dhakshinamoorthy et al., 2007).
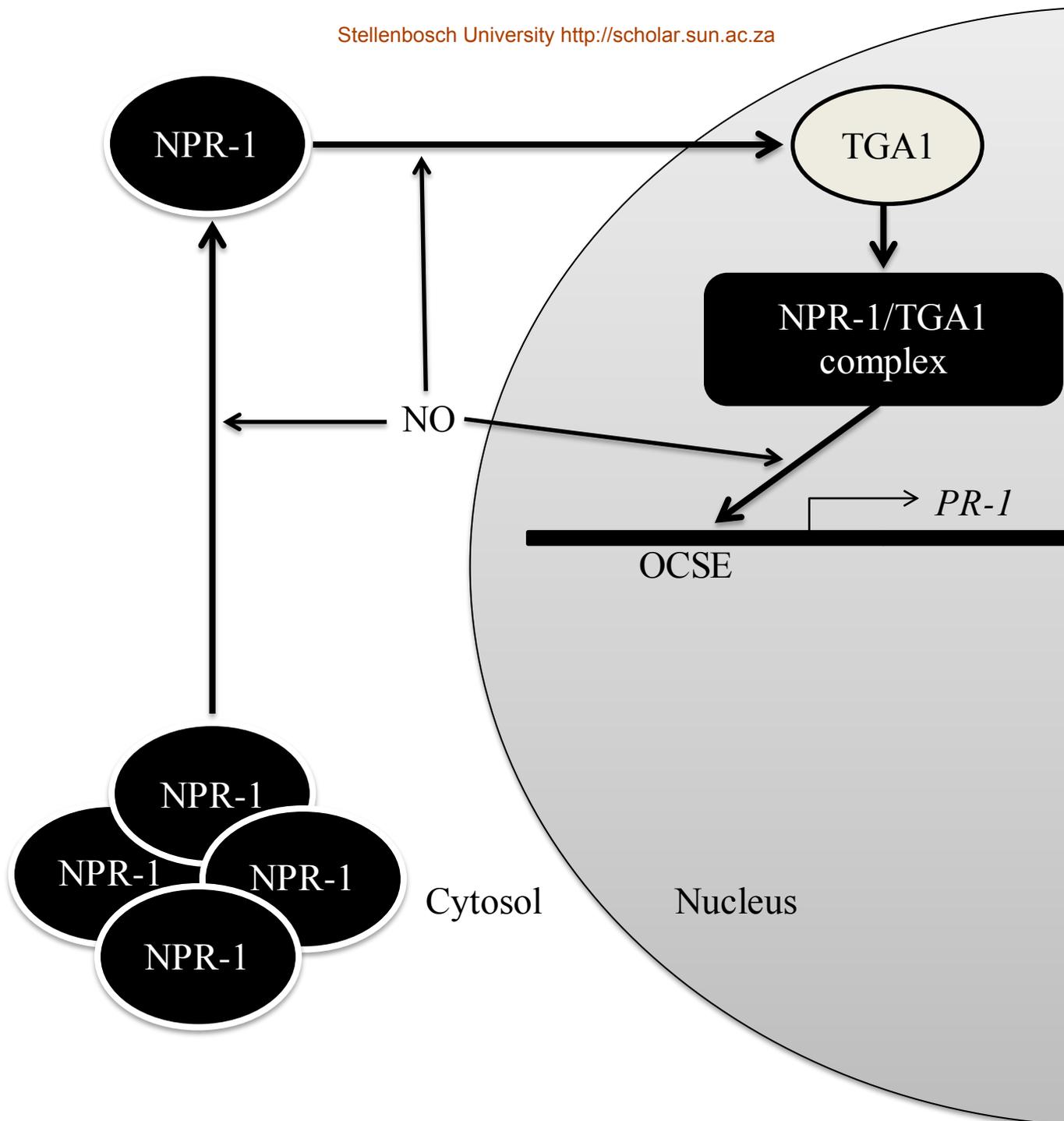
Figure 2.1 Illustration demonstrating NPR1/TGA1 regulation of *pathogen response 1(PR-1)* through the octopine synthase element (OCSE) and nitric oxide (NO) effect on this pathway.

Palmieri et al., 2008 identified putative NO-responsive *cis*-acting elements in plants within the first 500 bp upstream from the transcription start site as well as 100 bp downstream of the transcription start site of the gene from using microarray data. The elements identified were GBOX, octopine synthase element (OCSE), L1BX, MYCL, OPAQ and WRKY. The majority of the transcription factors binding to these elements are bZIP proteins and form hetero/homodimers that generally bind a DNA sequence consisting of the ACGT core sequence. The naming of these elements is based on the sequences bordering the ACGT core (for instance the G-box element is CACGTG). The TGA transcription factors bind to the OCSE to up-regulate gene expression of *Pathogen Response 1 (PR-1)* as in Figure 2.1. TGA transcription factors are also bZIPs. Non-pathogen response 1 (NPR1) resides in the cytosol and

translocates to the nucleus when NO is added to cells, where it can bind TGA1 and activate the expression of *PR-1*. Exogenous application of NO also enhances TGA1 DNA binding ability and both TGA1 and NPR1 can be s-nitrosylated (Lindermeyr et al., 2010). WRKY is also a type of bZIP transcription factor and binds to the w-box TTGACC/T, which is suggested to mediate *NPR1* (Non Pathogen Response 1) gene expression.

Many of these transcription factors and elements play roles in adaptation to abiotic stress and will modulate the transcription of antioxidative genes like SODs as well as genes controlling cell death like cystatins.

2.1.1 Superoxide Dismutase

Superoxide dismutase catalyses the dismutation of superoxide to hydrogen peroxide and oxygen. Several isoforms of SOD exist and are grouped according to the metal bound at their active site, namely FeSOD, MnSOD and CuZnSOD. All three types consist of homotetrameric or homodimeric forms and have been found to exist in both prokaryotes and eukaryotes. In *Arabidopsis thaliana*, three FeSODs, one MnSOD and three CuZnSODs have been identified. Promoters in genes encoding these SODs contain a variety of *cis*-acting elements involved in oxidative stress responses, namely abscisic acid response element (ABRE), NF-κB, Heat Shock Element (HSE) as well as the redox-responsive Y-box (Alscher 2002; Kliebenstein 1998). These elements are also found in hydrogen peroxide scavenging systems that contain enzymes like glutathione-S-transferase and ascorbate peroxidase as the product of SOD is hydrogen peroxide (Fujita et al., 2005; Wang et al., 2004). Hydrogen peroxide has been attributed to inducing PCD through elevating cysteine protease expression and subsequent activity (Solomon et al., 1999).

2.1.2 Cystatins

Cystatins compete with the substrate of family C1A cysteine proteases for the active site and result in reversible inhibition of the cysteine protease activity. Cystatins contain a conserved Gln-X-Val-Gly motif in the central region of the peptide, a conserved Trp amino acid in the C-terminus and a conserved Gly amino acid in the N-terminal region. Plant cystatins have been shown to contain an α-helix in the centre, surrounded by a 5-stranded anti-parallel β-sheet similar to their well characterised animal homologs. Plant cystatins are separated into three families. The first are type 1 cystatins also known as stefins, are approximately 11 kDa, sugar free and contain no disulfide bridges. Type 2 cystatins are approximately 15 kDa, glycosylated and contain 4 conserved cysteine residues for disulfide bridges which stabilize the protein. Type 3 cystatins are also known as Kininogens and contain multiple type 1 cystatin domains repeated next to each other (Benchabane 2010).

Solomon et al., 1999 demonstrated that oxidative stress-induced as well as biotic stress-induced PCD is mediated by activation of cysteine proteases as well as serine proteases. They also showed that inhibition of cysteine proteases and not serine proteases by their respective protein inhibitors could block the stress-induced PCD. More recently Zang et al., 2008 manipulated this mechanism to increase abiotic stress tolerance in *Arabidopsis* successfully by overexpression of cystatin genes. The

AtCYS-1 gene from *Arabidopsis* has been shown to be induced upon exposure to NO (Belenghi et al., 2003).

This has lead to the aim of identifying, *in silico*, putative NO-inducible *cis*-acting elements using the promoter sequences of putative NO-inducible SOD and cystatins. SOD activity in stressed plants has been shown to increase, possibly via increased transcription due to induction of *cis*-acting elements by NO since NO is involved in abiotic stress signalling. Since *AtCYS-1* has previously been shown to be NO-responsive, identification of its homologs in soybean may help identify its possible NO responsive *cis*-elements.

2.2 Methods

The amino acid sequence from *Arabidopsis thaliana* SOD genes in the TAIR database (www.arabidopsis.org), as used by Kliebenstein et al., 1998, were used to find their *Glycine max* homologs by conducting a BLAST search in Phytozome (www.phytozome.net; Schmutz et al., 2010). Cystatins were identified by a similar approach in Phytozome by using the amino acid sequence of the cystatin gene *AtCYS-1* (Belenghi et al., 2003) as a query in the BLAST search. Amino acid sequence alignment and phylogenetic trees were performed using ClustalW2 from EMBL (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Cis-acting elements thought to be involved in plant responses to changes in nitric oxide content in plant tissues were identified on the basis of occurrence of the sequence proposed in previous studies to be the conserved motif in such *cis*-acting elements, as reflected in Table 2.1 below.

Table 2.1 Putative *cis*-elements identified from literature.

Reference	*Cis* element name	*Cis* element sequence
Palmeri et al., 2008	GBOX	CACGTG
Palmeri et al., 2008	OCSE	TGACG
Hartmann et al.,2005	MYCL	CA[ATGC][ATGC]TG
Palmeri et al., 2008	WRKY	TTGAC[CT]
Alscher et al., 2002	HSE	TTC[ATGC][ATGC]GAA
Alscher et al., 2002	Y-BOX	GATTGG
Chen et al., 2002	DRE-like	[AGT][AG]CCGAC[ACGT][AT]
Chen et al., 2002	AtMyb4	A[AC]C[AT]A[AC]C
Chen et al., 2002	ABRE-like	[CGT]ACGTG[GT][AC]

Table 2.1 Proposed *cis*-acting elements with their corresponding sequences obtained from literature. These elements were identified in the putative promoter sequences (the first 1000 base pairs upstream from the transcription start site) of the identified putative SOD and cystatin genes. The amino acid sequences of the SOD and cystatins were also searched for possible target motifs for s-nitrosylation using GPS-SNO (Ren et al., 2010).

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2.3 Results

SOD and cystatin genes in soybean have not been well studied, leading to a necessity to use bioinformatics to identify these genes. In *Arabidopsis*, these genes are well characterised and have been used here to identify their putative homologs. Four homologues of the NO-inducible Type 1 cystatin, AtCYS-1, were identified in Phytozome (Figure 2.2). Cystatins contain a conserved motif as well as two conserved amino acids. The conserved motif consists of Gln-X-Val-X-Gly (Where X is any amino acid), is required to interact with the cysteine protease active site and is found starting at position 55 for AtCYS-1 and 49 for the Soybean homologues. A conserved tryptophan near the C-terminal also enters the active site of the cysteine protease and can be found at position 85 for AtCYS-1 and position 80 for the soybean homologues. The final conserved amino acid is a glycine near the N-terminal, confers inhibition of the cysteine protease and both AtCYS-1 and the soybean homologs contain numerous glycine in the region (Benchabane et al., 2010).

```
Glyma13g04250.1      MA------ALGGNRDVAGSQNSLEIDGLARFAVEEHNKKQNALLEFEKVVSAKQQVVSGT  54
Glyma20g08800.1      MA------ALGGNRDVTGSQNSVEIDALARFAVEEHNKKQNALLEFEKVVTAKQQVVSGT  54
Glyma18g12240.1      MA------MIGGISQVEGSQNSATIDRLAHFAVDEHNKKENAVLEFVKVVNVKQQVVSGM  54
Glyma14g04250.1      MA------ALGGFTDITGAQNSIDIENLARFAVDEHNKKENAVLEFVRVISAKKQVVSGT  54
AT5G12140.1_AtCys-1  MADQQAGTIVGGVRDIDANANDLQVESLARFAVDEHNKNENLTLEYKRLLGAKTQVVAGT  60
                     **          :**   ::  .   *.   ::  **:***:****::*   **: ::: .*  ***:*

Glyma13g04250.1      LYTITLEAKDGGQKKVYEAKVWEKAWLNFKEVQEFKLVGDAPA------  97
Glyma20g08800.1      LYTITLEAKDGGQKKVYEAKVWEKSWLNFKEVQEFKLVGDAPA------  97
Glyma18g12240.1      LYYITLEAKDGEKNKVYETKVWVKPWLDFKEIQEFKLIGDAPYAAPG--  101
Glyma14g04250.1      LYYITLEANDGVTKKVYETKVLEKPWLNIKEVQEFKPITVAVNPLSVTV  103
AT5G12140.1_AtCys-1  MHHLTVEVADGETNKVYEAKVLEKAWENLKQLESFNHLHDV--------  101
                     ::  :*:*.  **   :****:**   *.*  ::*:::.*:  :   .
```

Figure 2.2 ClustalW2 alignment of amino acid sequences of AtCYS-1 and putative homologs from Soybean (genes that start with Glyma).

```
Glyma16g27020.1      ------------------------------------------------------------
AT5G18100.1_CSD3     ------------------------------------------------------------
Glyma19g42890.1      ------------------------------------------------------------
Glyma03g40280.1      ------------------------------------------------------------
AT1G08830.1_CSD1     ------------------------------------------------------------
Glyma12g08650.1      ---------------------------------------------MQLAVAANAVVSP 13
Glyma11g19840.1      ---------------------------------------------MQLAMAANAVVSP 13
Glyma12g30260.1      ---------------------------------------------MQAAIAA----- 7
AT2G28190.1_CSD2     ----------------------------------------MAATNTILAFSSPSRLLI 18
Glyma05g04170.1      MAFLRSIATTAIATIPAALAFSSSSSSSSFPRSSQSPNPQNRLGLVKTLATPPSALHMDHK 60


Glyma16g27020.1      -----------------------------------MEAAKGTVKGVAIIIGDNNIR 21
AT5G18100.1_CSD3     -----------------------------------MEAPRGNLRAVALIAGDNNVR 21
Glyma19g42890.1      -----------------------------------MVKAVAVLGSSEGVT 15
Glyma03g40280.1      -----------------------------------MVKAVAVLGSSEGVT 15
AT1G08830.1_CSD1     -----------------------------------MAKGVAVLNSSEGVT 15
Glyma12g08650.1      SPFRPHPLLRSSFSGVSVK----LTPQSITFSRLKP---LTVFAATKKAVAVLKGTSAVE 66
Glyma11g19840.1      SPFRPQPFLRSSFSGVSVK----LTPQSITLSRSKP---LTVFAATKKAVAVLKGTSAVE 66
Glyma12g30260.1      --MAAHTILFSSFP----------APQSIALP-----------ATTKKAVAILKGNSSVH 44
AT2G28190.1_CSD2     PPSSNPSTLRSSFRGVSLNNNNLHRLQSVSFAVKAPSKALTVVSAAKKAVAVLKGTSDVE 78
Glyma05g04170.1      LSSQPDAVLPELLTEFMVDMKCEGCVNAVKNKLNEINGVKNVEVDLSNQVVRILGSTPVK 120
                                                                   . *. : . :


Glyma16g27020.1      GSLQFLQH----------------------------------PNGTTHVTGRVTG 42
AT5G18100.1_CSD3     GCLQFVQD----------------------------------ISGTTHVTGKISG 42
Glyma19g42890.1      GTIFFTQE----------------------------------GNGPTTVTGSLAG 36
Glyma03g40280.1      GTIHFVQE----------------------------------GSGPTTVTGSLAG 36
AT1G08830.1_CSD1     GTIFFTQE----------------------------------GDGVTTVSGTVSG 36
Glyma12g08650.1      GVATLIQE----------------------------------DDGPTTVSVSITG 87
Glyma11g19840.1      GVATLIQE----------------------------------DDGPTTVSVRITG 87
Glyma12g30260.1      GLVTLTQQQ---------------------------------DNGPTTVTVRVSG 66
AT2G28190.1_CSD2     GVVTLTQD----------------------------------DSGPTTVNVRITG 99
Glyma05g04170.1      TMTEALEQTGRKARLIGQGVPEDFLISAAVSEFKGPDIFGVVRLAQVNMELARIEANFSG 180
                               ::                                 : :   .:*

Glyma16g27020.1      LSQGFHGFHIHAFGDTTNGCNSTVLTSSTPGPHFNPFKKDHGAPSDDKRHAGDLGNIAAG 102
AT5G18100.1_CSD3     LSPGFHGFHIHSFGDTTNGCIST-------GPHFNPLNRVHGPPNEEERHAGDLGNILAG 95
Glyma19g42890.1      LKPGLHGFHVHALGDTTNGCLST-------GAHFNPNNNEHGAPEDENRHAGDLGNVNVG 89
Glyma03g40280.1      LKPGLHGFHVHALGDTTNGCLST-------GSHFNPNNKEHGAPEDENRHAGDLGNVNVG 89
AT1G08830.1_CSD1     LKPGLHGFHVHALGDTTNGCMST-------GPHFNPDGKTHGAPEDANRHAGDLGNITVG 89
Glyma12g08650.1      LTPGLHGFHLHEYGDTTNGCIST-------GAHFNPNNLTHGAPEDEVRHAGDLGNIVAN 140
Glyma11g19840.1      LTPGLHGFHLHEYGDTTNGCIST-------GAHFNPNKLTHGAPEDEVRHAGDLGNIVAN 140
Glyma12g30260.1      LTPGPHGFHLHEFGDITNGCIST-------GPHFNPNKLKHGAPEDKIRHAGDLGNIVAN 119
AT2G28190.1_CSD2     LTPGPHGFHLHEFGDTTNGCIST-------GPHFNPNNMTHGAPEDECRHAGDLGNINAN 152
Glyma05g04170.1      LSPGKHGWSINEFGDLTRGAAST-------GKMFNPVNE-----ENSKEPLGDLGTLEAN 228
                     *. * **: ::   ** *.*. **      *  ***       .:  .  ****.: ..


Glyma16g27020.1      PDGVAEISIRDRQIPLTGVHSIIGRAVVVHADPD--DLGRGGHELSKTTGN-AGARVACG 159
AT5G18100.1_CSD3     SNGVAEILIKDKHIPLSGQYSILGRAVVVHADPD--DLGKGGHKLSKSTGN-AGSRVGCG 152
Glyma19g42890.1      DDGTVSFSITDSQIPLTGPNSIIGRAVVVHADSD--DLGKGGHELSKTTGN-AGGRVACG 146
Glyma03g40280.1      DDGTVSFTITDSQIPLTGPNNIIGRAVVVHADPD--DLGKGGHELSKTTGN-AGGRVACG 146
AT1G08830.1_CSD1     DDGTATFTITDCQIPLTGPNSIVGRAVVVHADPD--DLGKGGHELSLATGN-AGGRVACG 146
Glyma12g08650.1      AEGVAEATIVDNQIPLSGPNSVVGRALVVHELED--DLGKGGHELSLTTGN-AGGRLACG 197
Glyma11g19840.1      AEGVAEATIVDNQIPLSGPNSVVGRALVVHELED--DLGKGGHELSLTTGN-AGGRLACE 197
Glyma12g30260.1      ADGVAEATTVDNQIPLIGPNSVVGRALVVHELED--DLGKGGQELSLSTGN-AGGRLACG 176
AT2G28190.1_CSD2     ADGVAETTIVDNQIPLTGPNSVVGRAFVVHELKD--DLGKGGHELSLTTGN-AGGRLACG 209
Glyma05g04170.1      EKGEAFYSGVKEKLRVAD---LIGRSVVVYATEDKSEHGITAAVIARSAGVGENYKKLCT 285
                     .* .    . :: : .   ::**:.**:   *  : *  .  :: ::*   . : *


Glyma16g27020.1      ----IIGLQSSV------- 167
AT5G18100.1_CSD3     ----IIGLQSSADAKL--- 164
Glyma19g42890.1      ----IIGLQG--------- 152
Glyma03g40280.1      ----IIGLQG--------- 152
AT1G08830.1_CSD1     ----IIGLQG--------- 152
Glyma12g08650.1      ----VVGLTPA-------- 204
Glyma11g19840.1      GEPWCSGKVVPW------- 209
Glyma12g30260.1      ----VVGLSPV-------- 183
AT2G28190.1_CSD2     ----VIGLTPL-------- 216
Glyma05g04170.1      CDGTTIWEATDTDFVTSKV 304
```

Figure 2.3 Amino acid sequence alignment of CuZnSODs, AT1G08830.1 (CSD1), AT2G28190.1 (CSD2), AT5G18100.1 (CSD3) have previously been identified as CuZnSODs where the soybean (where gene names start with 'Glyma') versions are all putative.

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```
Glyma20g33880.1    --------------------------------------------------------GPKVNAK 7
Glyma10g33710.1    -----MAS---LGGLQNLSGINFLIKKE-----------------------GPKVNAK 27
Glyma10g22680.1    -MILVVVA---CSRHRTASSSVFCSEN-------------------------------- 23
Glyma02g09630.1    -MNLLSQS---TAPSTSLSPSCFLPRHPHGSTWFSSGTFKFLKKESRCLRKAGRTKITAK 56
AT4g25100.1_FSD1   -----MA---ASSAVTAN---------------------------------------- 10
AT5g51100.1_FSD2   MMNVAVTA---TPSSLLYSPLLLPSQGPNRRMQWKRNGKRRLG-----TKVAVSGVITAG 52
Glyma20g12510.1    -MASCYFNPIPTSSHLILADLSRQFKIPKLLHR-KKRFDGS-------PRSS---KIAAF 48
AT5g23310.1_FSD3   -MSSCVVT---TSCFYTISDSSIRLKSPKLLNLSNQQRRRS-------LRSRGGLKVEAY 49


Glyma20g33880.1    FELKPPPYPLNGLEPVMSQQTLEFHWGKHHKTYVENLKKQVVGTE-LDGKSLEEIIVTSY 66
Glyma10g33710.1    FELKPPPYPLNGLEPVMSQQTLEFHWGKHHKTYVENLKKQIVGTE-LDGKSLEEIIVTSY 86
Glyma10g22680.1    -----------SLEPIMSEETIEYHWGKHHRTYVDNLNRQIDGTD-LDGNSLEDTMVILY 71
Glyma02g09630.1    FELKPPPYPLSALEPIMSQETLEYHWGKHHRTYVDNLNRQIDGTD-LDGNSLENTIVITY 115
AT4g25100.1_FSD1   YVLKPPPFALDALEPHMSKQTLEFHWGKHHRAYVDNLKKQVLGTE-LEGKPLEHIIHSTY 69
AT5g51100.1_FSD2   FELKPPPYPLDALEPHMSRETLDYHWHKHHKTYVENLKQILGTD-LDALSLEEVVLLSY 111
Glyma20g12510.1    YGLTTPPYELDALEPYMSKRTIDMHWGEYHQNFIEGLNKQLEKDDILYGYTLDELVKVTY 108
AT5g23310.1_FSD3   YGLKTPPYPLDALEPYMSRRTLEVHWGKHHRGYVDNLNKQLGKDDRLYGYTMEELIKATY 109
                      .*** **..*:: ***::*: :::.*::*:    : * . .::. :    *


Glyma20g33880.1    NK----------VQ----VWNHDFFWECMKPGGGGKPSGELLELIERDFGSFVKFLDEFK 112
Glyma10g33710.1    NKGDILPAFNNAAQ----VWNHDFFWECMKPGGGGKPSGELLELIERDFGSFEKFLDEFK 142
Glyma10g22680.1    NKGDILPAFNNAAQACMLAWNHDFFWESMKPGGGGRPSGELLKLIERDFGSLKKFLDEFK 131
Glyma02g09630.1    NKGDILPAFNNAAQ----AWNHDFFWESMKPGGGGRPSGDLLNLIERDFGSFEKFLDEFK 171
AT4g25100.1_FSD1   NNGDLLPAFNNAAQ----AWNHEFFWESMKPGGGGKPSGELLALLERDFTSYEKFYEEFN 125
AT5g51100.1_FSD2   NKGNMLPAFNNAAQ----AWNHEFFWESIQPGGGGKPTGELLRLIERDFGSFEEFLERFK 167
Glyma20g12510.1    NNGNPLPEFNNAAE----VWNHDFFWESMQPGGGDMPKLGLLQQIEKDFGSFTNFREKFI 164
AT5g23310.1_FSD3   NNGNPLPEFNNAAQ----VYNHDFFWESMQPGGGDTPQKGVLEQIDKDFGSFTNFREKFT 165
                   *:            .:    .:**:****.::****. *    :*  :::** *   :* :.*

Glyma20g33880.1    AAAATQFGSGWAWL---------------------------------------------PLLT 130
Glyma10g33710.1    AAAATQFGSGWAWLAYRASKFDGENAANPPSPDEDNKLVVLKSPNAVNPLVWGGYYPLLT 202
Glyma10g22680.1    TAASAQFGSGWAWLAYKESRLDVGNVLNPLQSDEDKKLVVVKTPNAVNPLVWNYYYPLLT 191
Glyma02g09630.1    TAASTQFGSGWAWLAYKESRLDVENAVNPLQSDEDKKLVVVKTPNAVNPLVWNYYHPLLT 231
AT4g25100.1_FSD1   AAAATQFGAGWAWLAYS------------NEKLKVVKTPNAVNPLVLG-SFPLLT 167
AT5g51100.1_FSD2   SAAASNFGSGWTWLAYKANRLDVANAVNPLPKEEDKKLVIVKTPNAVNPLVWD-YSPLLT 226
Glyma20g12510.1    GAALSLFGSGWVWLVLKR--------------EEKRLEIVKTSNAICPIVWD-DIPIIN 208
AT5g23310.1_FSD3   NAALTQFGSGWVWLVLKR--------------EERRLEVVKTSNAINPLVWD-DIPIIC 209
                    **  :  **:**.**                                     *::


Glyma20g33880.1    IDVWEHAYYLDFQNRRPDYISVFMDKLVSWDAVSSRLEQAKALITSA------------- 177
Glyma10g33710.1    IDVWEHAYYLDFQNRRPDYISVFMDKLVSWDAVSSRLEQARL----------------- 244
Glyma10g22680.1    IDVWEHAYFIDFQNQRRDYISVFMDKLVSWDAVSSRLEQAKALIEEREREAERKRRDEE- 250
Glyma02g09630.1    IDVWEHAYFIDFQNQRRDYISVFMDKLVSWDAVSSRLEQAKALIKEREREAERKRREEEE 291
AT4g25100.1_FSD1   IDVWEHAYYLDFQNRRPDYIKTFMTNLVSWEAVSARLEAAKAASA-------------- 212
AT5g51100.1_FSD2   IDTWEHAYYLDFENRRAEYINTFMEKLVSWETVSTRLESAIARAVQREQE----GTETED 282
Glyma20g12510.1    LDLWEHAYYLDYRNDRAKYVDVFMNHLVSWDAAMGRLTRAEAFVNLGEPK---------- 258
AT5g23310.1_FSD3   VDVWEHSYYLDYKNDRAKYINTFLNHLVSWNAAMSRMARAEAFVNLGEPN---------- 259
                   :* ***:*::*:.* * .*:..*: :****::.  *:  *


Glyma20g33880.1    ----------------------
Glyma10g33710.1    ----------------------
Glyma10g22680.1    KPTSSEATPEIFSDSDADLDVE- 272
Glyma02g09630.1    KRTSSEAIPEIYSDGDADLDAE- 313
AT4g25100.1_FSD1   ----------------------
AT5g51100.1_FSD2   EENPDDEVPEVYLDSDIDVSEVD 305
Glyma20g12510.1    -------IPVA----------- 262
AT5g23310.1_FSD3   -------IPIA----------- 263
```

Figure 2.4 Amino acid sequence alignment of FeSODs, AT4g25100.1 (FSD1), AT5g51100.1 (FSD2), AT5g23310.1 (FSD3) have previously been identified as FeSODs where the soybean (genes that start with 'Glyma') are all putative.

```
Glyma04g39930.1    MAARALLTRKTLATVLRNDAKPIIGAGI-AAATHSRGLHVYTLPDLDYDYGALEPAISGE 59
Glyma06g14960.1    MAARALLTRKTLATVLRNDAKPIIGVGITAAATHSRGLHVYTLPDLDYDYGALEPAISGD 60
AT3g10920.1_MSD1   MAIRCVASRKTLAGLKETSSR---------LLRIRGIQTFTLPDLPYDYGALEPAISGE 50
                   ** *.: :***** : ...:: : **:;.:***** ************:

Glyma04g39930.1    IMQLHHQKHHQTYITNFNKALEQLQDAVAKKDSSAVVKLQGAIKFNGGGHVNHSIFWKNL 119
Glyma06g14960.1    IMQLHHQKHHQTYITNYNKALEQLQDAIAKKDSSAVVKLQGAIKFNGGGHVNHSIFWKNL 120
AT3g10920.1_MSD1   IMQIHHQKHHQAYVTNYNNALEQLDQAVNKGDASTVVKLQSAIKFNGGGHVNHSIFWKNL 110
                   ***:*******.*:**:*:*****::*: * *:*:***** .*****************

Glyma04g39930.1    APVREGGGEPPKGSLGWAIDTHFGSFEALVQKVNAEGAALQGSGWVWLGLDKELKRLVVE 179
Glyma06g14960.1    APVREGGGEPPKGSLGWAIDTHFGSFEALIQKVNAEGAALQGSGWVWLGLDKELKRLVVE 180
AT3g10920.1_MSD1   APSSEGGGEPPKGSLGSAIDAHFGSLEGLVKKMSAEGAAVQGSGWVWLGLDKELKKLVVD 170
                   ** ************ ***.****:*.*.:*: ***** *****************:***:

Glyma04g39930.1    TTANQDPLVTKGPNLVPLLGIDVWEHAYYLQYKNVRPDYLKNIWKVINWKYASEVYEKES 239
Glyma06g14960.1    TTANQDPLVTKGPNLVPLIGIDVWEHAYYLQYKNVRPDYLKNIWKVINWKYASEVYEKES 240
AT3g10920.1_MSD1   TTANQDPLVTKGGSLVPLVGIDVWEHAYYLQYKNVRPEYLKNVWKVINWKYASEVYEKEN 230
                   ************ .****:*****************:****:****************.

Glyma04g39930.1    S 240
Glyma06g14960.1    S 241
AT3g10920.1_MSD1   N 231
                   .
```

Figure 2.5 Amino acid sequence alignment of MnSODs, AT3g10920.1 (MSD1) has previously been identified as a MnSOD where the soybean (genes that start with 'Glyma') are both putative.

To identify the homologs of the various SOD isoforms the known SODs from *A. thaliana* were aligned with their most similar homologous genes from *Glycine max* and the sequence alignment can be seen in Figure 2.3, 2.4 and 2.5. Amongst the putative CuZnSOD homologues Glyma05g04170.1 was significantly different to the rest of the SODs although it does contain a CuZnSOD domain at the C-terminal end (Figure 2.3). Glyma05g04170.1 also contains a heavy-metal-associated domain at the N-terminal region. The individual alignments of CSD1 with its closest homologs show high amino acid sequence similarity and the situation is the same for CSD2 and CSD3 excluding Glyma05g04170.1. The FeSOD family is quite diverse as little homology was found at the terminal regions (Figure 2.4). The rest of amino acid sequence in the middle is highly conserved as this is where the FeSOD domain occurs. After the MnSODs were aligned (Figure 2.5) small differences were observed at the N-terminal region from position 15 to 40.
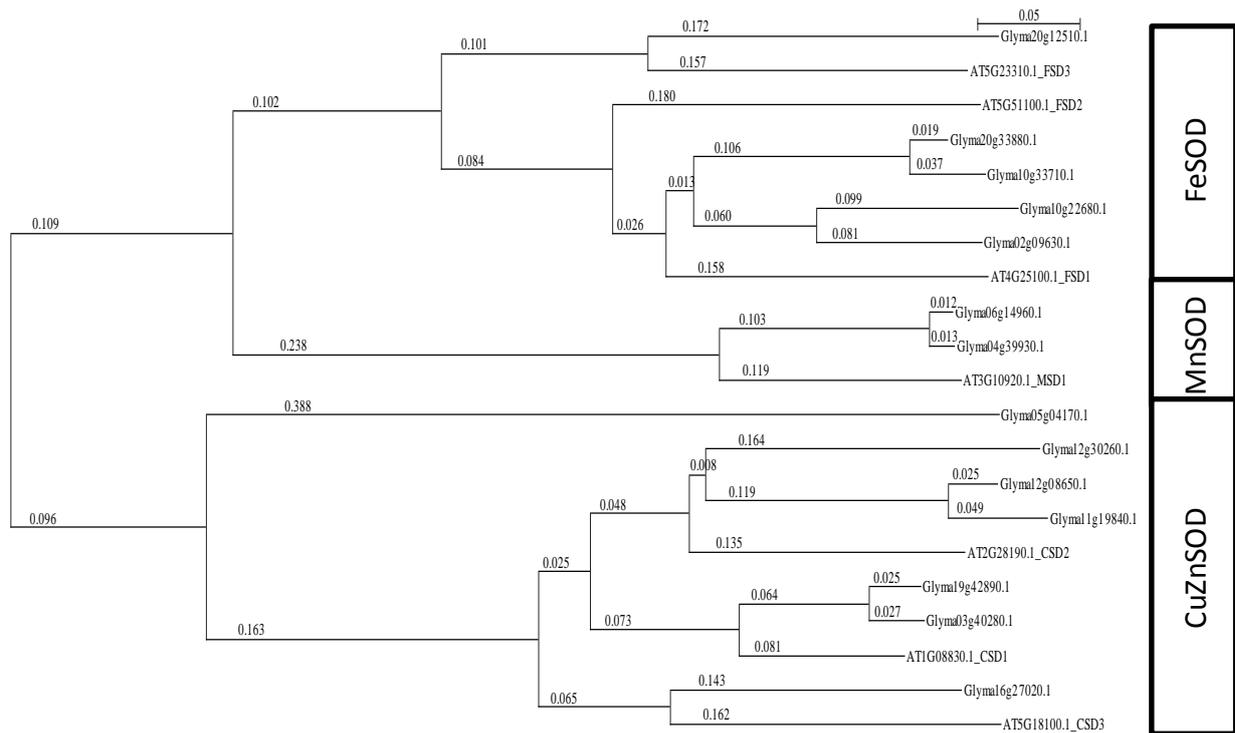
Figure 2.6 Phyologenetic tree of SODs from *Arabidopsis* and putative SODs from *Glycine max*. CuZnSODs AT1G08830.1 (CSD1), AT2G28190.1 (CSD2), AT5G18100.1 (CSD3) MnSODs, AT3g10920.1 (MSD1) (FeSODs, AT4g25100.1 (FSD1), AT5g51100.1 (FSD2), AT5g23310.1 (FSD3). The Soybean homologs are all putative.

The phylogenetic tree in Figure 2.6 neatly divides the various isoforms of SOD. From Glyma20g12510.1 down to AT4g25100.1 are the FeSODs, then from Glyma06g14960.1 to AT3g10920.1 are the MnSODs and the rest below that are the CuZnSODs (as shown by the boxes to the right of Figure 2.6). These various isoforms occur in different organelles of the cell and their expression could be controlled by *cis*-acting elements occurring upstream from the transcription start site. Possible elements responsible for differential gene expression were identified with the focus on abiotic stress and nitric oxide response. These putative elements can be found in Table 2.2 and Table 2.3.

Table 2.2 *Cis*-elements identified in the upstream promoter sequences of various known and putative Cystatin and SOD genes.

	BASE PAIRS UPSTREAM FROM TRANSCRIPTION START SITE									
Gene	1-100	100-200	200-300	300-400	400-500	500-600	600-700	700-800	800-900	900-1000
	CIS-ACTING ELEMENT NAME									
AT5G12140 AtCys-1	HSE x 2	WRKY, AtMyb4, DRE-like	AtMyb4	HSE, ABRE-like x 2 WRKY, Y-BOX		MYCL x 3 Y-Box x 2				MYCL
Glyma20g08800		G-Box, MYCL ,ABRE-like x 2		MYCL	AtMyb4	AtMyb4		AtMyb4		MYCL x 2, Y-box, AtMyb4
Glyma14g04250		G-Box, MYCL x 5			MYCL x 2		MYCL, AtMYB4	OCSE, MYCL, HSE	Y-Box x 2, AtMYB4	AtMYB4
Glyma13g04250	MYCL, WRKY	G-Box, OCSE, MYCL x 3, WRY, ABRE-like x 2	MYCL		OCSE, MYCL				OCSE, WRKY	MYCL, WRKY, HSE
Glyma18g12240	HSE	WRKY	MYCL x 2, AtMyb4 x 2		MYCL			AtMyb4	MYCL	
AT1G08830 CSD1	OCSE, WRKY, ABRE-like	OCSE, Y-Box, AtMyb4	MYCL	AtMyb4		MYCL	DRE-Like, AtMyb4	Y-Box		
Glyma03g40280	OCSE x 2, DRE-Like	WRKY	MYCL x 2, WRKY, HSE, AtMyb4	MYCL x 2, AtMyb4	AtMyb4	MYCL		WRKY	AtMyb4	MYCL, Y-BOX, AtMyb4
Glyma19g42890	OCSE	MYCL, WRKY x 2, AtMyb4	MYCL x 2, HSE	MYCL x 2, WRKY, AtMyb4	MYCL				MYCL, Y-Box	
AT2G28190 CSD2		Y-Box, AtMyb4	WRKY	MYCL	MYCL, WRKY	MYCL x 3		MYCL	MYCL	
Glyma11g19840	AtMyb4 x 4	OCSE, DRE-like			MYCL		OCSE, MYCL, WRKY x 2	Y-Box	AtMyb4 x 2	
Glyma12g08650	OCSE, AtMyb4 x 4	OCSE, AtMyb4	AtMyb4 x 2, ABRE-like			MYCL, ABRE-like			WRKY, AtMyb4	AtMyb4
Glyma12g30260	OCSE x 2, MYCL, WRKY	G-Box, MYCL x 2, AtMyb4	AtMyb4	WRKY			MYCL x 2, AtMyb4 x3	MYCL		MYCL WRKY
AT5G18100 CSD3		MYCL x 2	AtMyb4	HSE, AtMyb4 x2	MYCL	WRKY	WRKY, HSE	MYCL		
Glyma16g27020	MYCL			AtMyb4	MYCL	AtMyb4	MYCL		MYCL x 2, Y-Box	MYCL

Table 2.2 *Cis*-acting elements identified in the upstream promoter sequences of various Cystatin and SOD genes (Continued).

Gene	1-100	100-200	200-300	300-400	400-500	500-600	600-700	700-800	800-900	900-1000
Glyma05g04170	OCSE	MYCL	Y-Box, AtMyb4	AtMyb4	OCSE x 2, MYCL x 2	MYCL, ABRE-like	MYCL x 2, ABRE-like			MYCL x 2
AT3G10920 MSD1	OCSE, MYCL x 2,	OCSE, MYCL, WRKY, HSE, AtMyb4 x 2	MYCL x 3, Y-Box, AtMyb4	AtMyb4 x 2		OCSE, WRKY, HSE, AtMyb4	AtMyb4	OCSE, MYCL x 2, AtMyb4 x 3	AtMyb4	MYCL
Glyma04g39930		MYCL		MYCL		G-Box, MYCL x 2, WRKY, HSE, ABRE-like	MYCL, WRKY, DRE-like	MYCL	MYCL, AtMyb4	AtMyb4 x 2
Glyma06g14960	OCSE		OCSE, MYCL x 2	OCSE	HSE	AtMyb4				MYCL
AT4G25100 FSD1		OCSE			MYCL, AtMyb4 x 4	MYCL x 3, WRKY, AtMyb4	AtMyb4	MYCL x 2, HSE	MYCL	
AT5G51100 FSD2	MYCL, WRKY, Y-Box		WRKY, AtMyb4	WRKY	AtMyb4	HSE	OCSE, MYCL x 3, AtMyb4 x 2	MYCL x 2, WRKY		AtMyb4
Glyma02g09630		OCSE x 3	MYCL	MYCL x 2, ABRE-like		OCSE, AtMyb4		MYCL		MYCL, AtMyb4 x 2
Glyma10g22680	MYCL, Y-Box, AtMyb4, ABRE-like	OCSE	MYCL	MYCL, AtMyb4 x 2, ABRE-like	MYCL, AtMyb4	OCSE	MYCL	WRKY		OCSE, MYCL x 2, DRE-like
Glyma10g33710	MYCL x 2	MYCL		MYCL	WRKY	MYCL			MYCL x 2, Y-Box, AtMyb4	MYCL
Glyma20g33880	MYCL	AtMyb4	AtMyb4		OCSE, MYCL x 2, WRKY	MYCL x 2, WRKY, AtMyb4			AtMyb4 x 2	
AT5G23310 FSD3	MYCL x 2	OCSE, AtMyb4 x 2		ABRE-like			OCSE, MYCL	MYCL, AtMyb4		MYCL, DRE-like
Glyma20g12510	MYCL, WRKY, AtMyb4	OCSE, MYCL	MYCL x 3, WRKY	WRKY	MYCL	MYCL x 2, AtMyb4	OCSE, Y-Box	MYCL, HSE	OCSE, MYCL, WRKY	AtMyb4 x 2

Table 2.3 Possible sites for s-nitrosylation in *Arabidopsis* and putative *Glycine max* SODs.

	Position	Peptide
>AT3G10920.1_MSD1	5	***MAIRCVASRKTL
>AT5G23310.1_FSD3	4	****MSSCVVTTSCF
	10	SCVVTTSCFYTISDS
>Glyma05g04170	85	VDMKCEGCVNAVKNK
>Glyma10g22680	8	MILVVVACSRHRTAS
	20	TASSSVFCSENSLEP
>Glyma20g12510	4	****MASCYFNPIPT

If the SOD genes are not being regulated on a transcriptional level, they could possibly be regulated post-translationally for example by NO leading to s-nitrosylation of cysteine residues. An algorithm was developed for identifying s-nitrosylated proteins and the results of GPS-SNO can be seen in Table 2.4 (Ren et al., 2010). These genes may be regulated both transcriptionally, post-translationally or both.

2.4 Discussion

Since SOD and cystatin genes are not well characterized in *Glycine max*, SOD and cystatin genes from *Arabidopsis* were used to identify their homologs in *Glycine max*. For cystatin, there was a particular interest in AtCYS-1 since it was shown to be up-regulated by addition of NO (Belenghi et al., 2003). AtCYS-1 is a stefin-like cystatin since it contains a single cystatin domain and has no cysteine residues at the N-terminal region (Benchabane et al., 2010). The putative homologs identified in Figure 2.2 show high amino acid sequence similarity except at the terminal ends.

The Copper/Zinc SODs (CuZnSOD) aligned in Figure 2.3 align very well. *Arabidopsis* Cu/ZnSOD1(CSD1) has two highly conserved homologs (Glyma19g42890.1 and Glyma03g40280.1), *Arabidopsis* Cu/ZnSOD3 (CSD3) has one respective homolog (Glyma16g27020.1) and there appear to be three homologs (Glyma12g30260.1, Glyma12g08650.1 and Glyma11g19840.1) of *Arabidopsis* Cu/ZnSOD2 (CSD2). Glyma05g04170.1 is different to the rest of the CuZnSODs because it contains a heavy-metal-associated domain (Pfam: 00403) that occurs in a metallochaperone protein (Portnoy et al., 1999).

The iron SOD (FeSOD) is considered to be the most ancient SOD isoform and is thought to occur in the chloroplast (Alscher et al., 2002). Figure 2.4 demonstrates how diverse the FeSODs are in terms of primary sequence, especially at the ends of the peptide. The phylogenetic tree in Figure 2.6 separates the isoforms as expected since the FeSOD and MnSOD are more closely related out of the three different isoforms found in plants.

The Manganese SODs (MnSOD) in Figure 2.5 show the highest similarity of all the isoforms, possibly due to the essential role they play in the mitochondria. However there are minor differences in the N-terminal region.

In mammalian systems SOD gene expression is controlled by several oxidative responsive transcription factors including Sp1, NF-κB and AP-2, it has also been suggested that Nitric Oxide Synthase (NOS) also plays a role in this system (Miao and Clair, 2009). However, the aforementioned elements have also been found to be NO-responsive *cis*-elements (Dhakshinamoorthy et al., 2007). This suggests that SODs are transcriptionally NO-responsive in mammals and it would not be too farfetched to suggest that a similar mechanism may occur in plants, although the *cis*-acting elements involved may be different. Potential NO-responsive and abiotic stress responsive *cis*-acting elements, based on literature (Palmeri et al., 2008; Chinnusamy et al., 2004; Hartmann et al., 2005; Alscher et al., 2002; Chen et al., 2002), were searched for in the SOD and cystatin promoters (Table 2.2). This is not to say that all genes in Table 2.2 are up-regulated by NO, instead some of them may be NO-responsive because of the roles they play within the cell. Only one gene, *AtCYS-1*, amongst the genes investigated in this study has previously been shown to be up-regulated by exogenously applied NO. *AtCYS-1* contains the DRE-like, Abscisic acid response element-like (ABRE-like) and MYCL *cis*-acting element found in the promoter of the rd22 gene, which is a model abiotic stress responsive gene (Shinozaki and Yamaguchi-Shinozaki, 1997). *AtCYS-1* also contains the MYCL

element which is shared among all the other genes selected but this could be due to the low specificity of only 4 base pairs in the MYCL element.

Since these genes may possibly be post-tranlationally regulated by NO through s-nitrosylation, a computer-based search was done on the cystatin and SOD peptide sequences and only 5 SOD genes were identified to contain possible s-nitrosylation sites, as depicted in Table 2.4. Out of the five genes, four contain the putative s-nitrosylation site near the N-terminal and the one gene that contains an s-nitrosylation site nearer to the centre of the peptide is the gene that contains the unique heavy-metal-associated domain. The four aforementioned genes are MSD1, FSD3 and two putative FeSODs from *Glycine max*.

In conclusion *Glycine max* may contain four stefin-like cysatins that are homologs of AtCYS-1 from *Arabidopsis* and fifteen SODs homologues to previously identified *Arabidopsis* SODs. These genes are possibly up-regulated by NO through NO-responsive *cis*-acting elements presented in Table 2.1 or post-translationaly regulated by NO (Table 2.4). Subsequent studies are required for further identification of the role of NO in gene transcription.

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Chapter 3: Effect of Nitric oxide on Superoxide Dismutase

Nitric oxide (NO) has previously been shown to be beneficial to plants that are undergoing oxidative stress caused by abiotic stress. To better understand the role of NO on the antioxidative pathway, this chapter investigated the effect of NO on superoxide dismutase (SOD) enzymatic activity and gene expression, in which it is shown that NO has differential effects on MnSOD as well as FeSOD, but no response was found for CuZnSOD. Furthermore putative SOD transcripts were identified and shown to increase in response to NO and salt stress. This suggests that during salt stress, the nodule NO content increases and causes SOD transcript levels and enzymatic activity to increase, probably because of increased SOD protein turnover. These results can be used with the bioinformatics from Chapter 2 to identify putative NO cis-acting elements.

3.1 Introduction

Soybean is an economically important crop due to its high protein and lipid content, which soybeans achieve through utilising their symbiotic relationship with *Bradyrhizobium japonicum* (Taiz and Zeiger, 2006). Soybean yield is drastically reduced by salt stress and approximately 800 million hectares of land around the world is affected by salt (Munns and Tester, 2008). Salt stress results in generation of reactive oxygen species (ROS) by disruption of redox active organelles as well as specific enzymes that produce superoxide (Mittler, 2002), as illustrated in Figure 3.1.
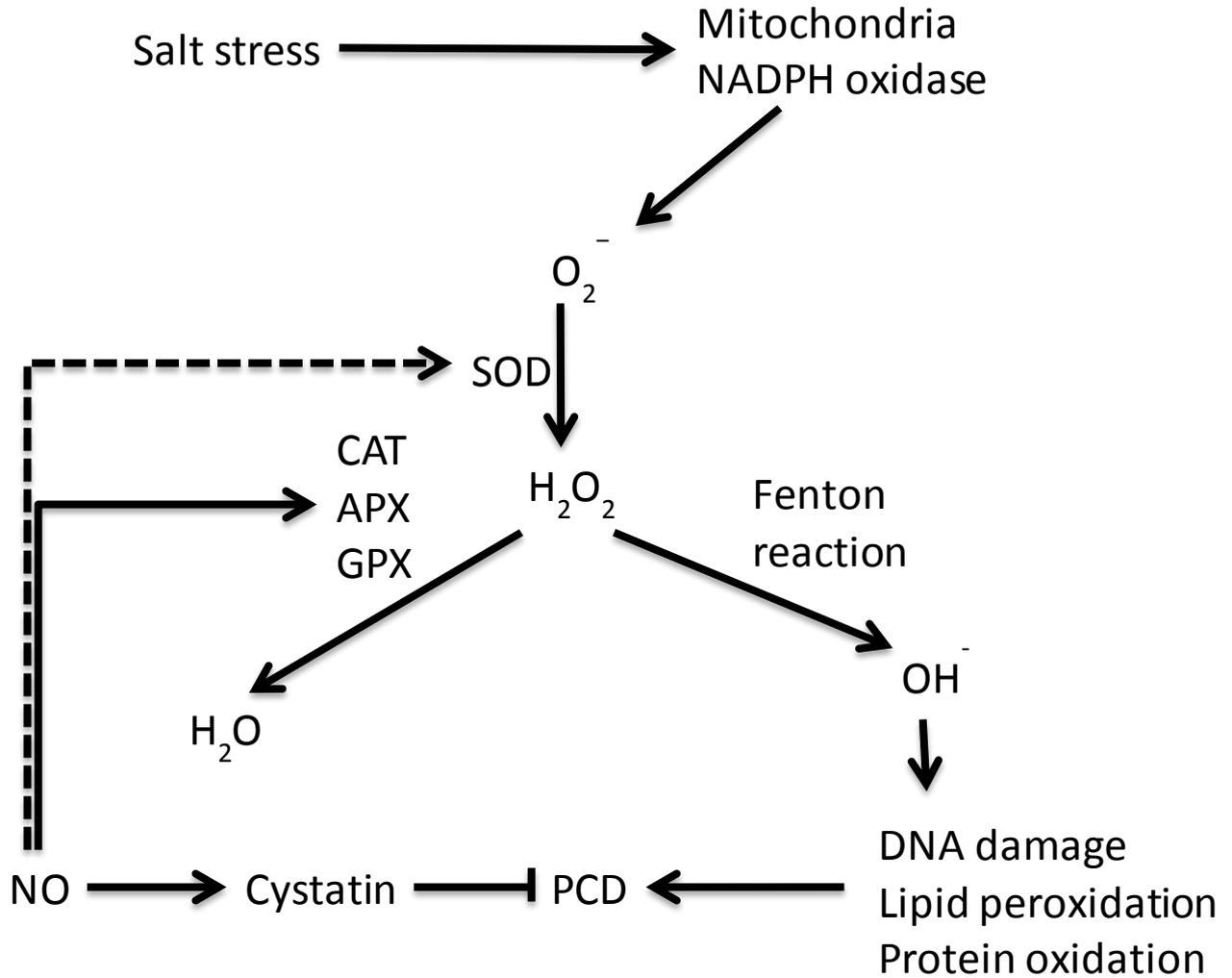
Figure 3.1 Pathway demonstrating reactive oxygen species (ROS) production from salt stress and the subsequent reactions that may occur; including ROS scavenging by superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and ascorbate peroxidase (APX); as well as damage that results in programmed cell death (PCD). Putative targets for nitric oxide (NO) are also illustrated.

Superoxide is a gaseous radical that can be generated by membrane-bound NADPH oxidase, Complex 1 and 3 in the mitochondria, xanthine oxidase and numerous other sources. Superoxide Dismutase (SOD) scavenges these radicals to form hydrogen peroxide and if hydrogen peroxide accumulates in the presence of iron, hydroxyl radicals will be generated through the Fenton reaction. These hydroxyl radicals cause major damage to the cell including DNA damage, lipid peroxidation and protein oxidation and this damage can be used as a biomarker for oxidative stress suffered by plant cells (Davies, 2007).

For protection against oxidative damage, the hydrogen peroxide is scavenged by several different pathways involving antioxidant enzymes such as ascorbate peroxidase, catalase and glutathione peroxidase/peroxiredoxin. The Halliwell-Asada pathway utilizes ascorbate and glutathione as

reductants for the detoxification of hydrogen peroxide (Foyer and Noctor, 2011; Miller et al., 2010; Lazebnik, 2007).

If the oxidative stress becomes overwhelming for the cell, it will initiate programmed cell death (PCD). This initiation involves the activation of proteases, namely caspases, which are cysteine aspartate cleaving proteases. Once activated, the caspases will degrade their respective target proteins within the cell (Wang et al., 2010).

Interestingly, plants experiencing abiotic stress have been shown to accumulate increased levels of a highly reactive signalling molecule Nitric Oxide (NO). When exogenous NO is added to plants experiencing abiotic stress, the extent of cell damage and cell death is reduced (Keyster et al., 2011; Lamattina et al., 2001). Previously NO has been shown to reduce hydrogen peroxide content in stressed soybean nodules by increasing ascorbate peroxidase (APX) activity (Keyster et al., 2011). This project thus aimed to analyse the effect of NO and salt stress on SOD activity (depicted by the dotted line in Figure 3.1).

3.2 Materials and Methods

3.2.1 Plant growth conditions and treatments

Glycine max L. Merr. cv. PAN626 seeds were surface decontaminated using 0.35% sodium hypochlorite with 0.1% tween20 for 10 minutes and washed vigorously 5 times in distilled water. The seeds were then kept at room temperature in distilled water for 1h after which they were coated in *Bradyrhizobium japonicum* peat-based inoculum supplied by Becker Underwood Ltd (United Kingdom). These seeds were then sowed in silica sand (98% SiO_2, Rolfes® Silica, South Africa) that had been pre-soaked in distilled water. Plants were watered with distilled water until the germinated plants reached the VC stage (the cotyledons are fully expanded and the first two leaflets have emerged). Plants were grown in a greenhouse and only plants of the same growth stage were used. The plants were then watered with a nitrogen-free nutrient solution as described in Leach et al., 2010 (1mM K_2SO_4, 2mM $MgSO_4$, 3mM $CaCl_2$, 1mM K_2HPO_4, 25µM H_3BO_3, 2µM $MnSO_4$, 2µM $ZnSO_4$, 2µM $CuSO_4$, 2µM Na_2MoO_4, 0.1µM $CoSO_4$, 50µM Fe-NaEDTA and 10mM 4-(2-hydroxethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.3]. Once plants had grown to the V3 stage (third trifoliate), treatments began and consisted of the aforementioned nitrogen-free nutrient solution containing: nothing (untreated), 150 mM NaCl and 15 mM $CaCl_2$ (Salt-treated), 10 µM 2,2'-(hydroxynitrosohydrazono)bis-ethanimine (DETA/NO)(NO-treated), 10 µM diethylenetriamine (DETA, as a control for NO, i.e. NO control), a combination of Salt-treated and NO-treated and finally a combination of Salt-treated and NO control. All treatments and nitrogen-free nutrient solutions were at pH 7.3. The plants were treated at three day intervals for a week and harvested 24 hours after the last treatment.

3.2.2 Cell Viability

For cell viability, 100 mg of nodule tissue per treatment from each of the treated plants were stained in 0.25% (m/v) Evans Blue for 45 minutes at room temperature. These nodules were then washed

vigorously in distilled water and left overnight. The nodules were then placed in 1% SDS and incubated for 1 hour at 55°C. The supernatant was used to measure Evans Blue released from the nodules, by recording absorbance of the supernatant at 595nm (Leach et al., 2010). Cell viability measurements were done in triplicate.

3.2.3 Lipid Peroxidation

Lipid peroxidation was measured in soybean nodule tissue. For the assay, 100 mg tissue was homogenised in ice-cold 6% (w/v) trichloroacetic acid (TCA) and then centrifuged at 12000 g for 15 minutes at room temperature. The supernatant was then transferred to a new tube, in which an equal volume of 0.5% (w/v) thiobarbituric acid (TBA) in 20% TCA was added. Samples were then boiled at 96°C for 60 minutes and transferred to an ice bath for 10 minutes. Samples were then centrifuged at 12000 g for 5 minutes at room temperature and the absorbance of the supernatant was measured at 532 nm. A blank value was determined using 0.5% (w/v) TBA in 20% TCA and measured at 600 nm. The amount of lipid peroxidation was determined using the extinction coefficient of $155mM^{-1}.cm^{-1}$ (Hodges et al., 1999)

3.2.4 NO content

NO content was determined using diaminofluorescein-2 diacetate (DAF-2DA) using confocal laser scanning microscopy as described in Sandalio et al., 2008 on 150 µM transverse sections of nodule tissue from plants treated as described in 3.2.1.

3.2.5 Protein extraction and quantification

Protein extraction was performed by homogenizing 200 mg of nodule tissue in 1 ml of cold buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 5%(w/v) polyvinylpyrrolidone (PVP) molecular weight = 40,000]. The sample was then centrifuged at 12000 g for 10 minutes and the supernatant used as protein extract (Keyster et al., 2011). The concentrations of the proteins were determined using the RC DC Protein Assay Kit 11 (Bio-Rad Laboratories) as per manufacturer's instructions.

3.2.6 Superoxide dismutase activity in native gels

SOD activity was determined by separating proteins on a 10% native polyacrylamide gel, followed the staining procedure described in Beauchamp and Fridovich, 1971. For this in-gel SOD assay, 100 µg of protein was loaded per well.

3.2.7 RNA extraction and semi-quantitative RT-PCR

RNA extraction was done as per Gasic et al., 2004. The sample RNA was then treated with DNase1 (Fermentas), followed by addition of Ribolock™ RNase inhibitor (Fermentas) as per manufacturer's instructions. RNA concentrations were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA). For reverse transcription, 1µg RNA per sample was

used in the reverse transcriptase step using RevertAid™ Premium Reverse Transcriptase (Fermentas) according to the manufacturer's protocol.

Table 3.1 Gene accesion numbers, primer sequences and annealing temperatures of putative SOD genes studied

Gene Accession number	Forward Primer	Reverse Primer	Annealing Temperature
Glyma02g09630.1	ATG AAT TTG TTA TCG CAA TCC A	TTA CAG CAT TGG AGT CTT CAC	53
Glyma04g39930.1	ATG GCC GCG CGA GCC CTG	GAG CTC TCT TTC TCA TAC ACT T	59
Glyma20g33880.1	ATG GCC TCA TTG GGT GGG TT	TCA TGC ACT GGT AAT TAA AGC	53
Glyma10g33710.1	ATG GCC TCA TTG GGT GGG TT	TTA AAG CCT TGC TTG TTC AAG T	55
Glyma05g04170.1	TGA CGC TGT CTT GCC CGA GT	AGT GTT CCC AGG TCG CCC AG	59
Glyma12g30260.1	CAA TCC ATT GCC CTT CCC GCC	TGG ACT CAA ACC AAC CAC ACC A	57
Glyma16g27020.1	TCC TCT ACC CCA GGT CCT CA	TGA GTT CAT GCC CAC CCC TT	57

For the PCR, TrueStart™ Hot Start *Taq* DA Polymerase (Fermentas) was used. The cycling parameters consisted of the following: initial denaturation of 95°C for 1 min, denaturation at 95°C for 30 s, annealing at various temperatures as in Table 3.1 for 30 s, an extension at 72°C for 1 minute with a final extension of 72°C for 7 min. Primers were designed across a minimum of 1 intron. Analysis of gels was done using Alpha Ease FC version 4.0.0 (Alpha Innotech Corporation) software to quantify band intensities. All 15 SOD genes were attempted to be amplified but only 7 (Table 3.1) showed satisfactory amplification for RT-PCR. The other putative SOD genes not shown either did not amplify or showed non-specific amplification rendering them non usable for semi-quantitative RT-PCR. Primers were also checked for complementation, GC content, GC clamps, BLASTed against the Soybean genome to check for non-specific annealing.

3.3 Results

NO has beneficial properties when added to plants experiencing abiotic stress and has been shown to be an important signalling molecule (Lamattina et al., 2001; Neil et al., 2002). To further understand the role of NO in abiotic stress responses, this thesis investigated the influence of NO in terms of the first line of defence against oxidative stress, i.e. superoxide dismutase, in *Glycine max* nodules under salt stress.
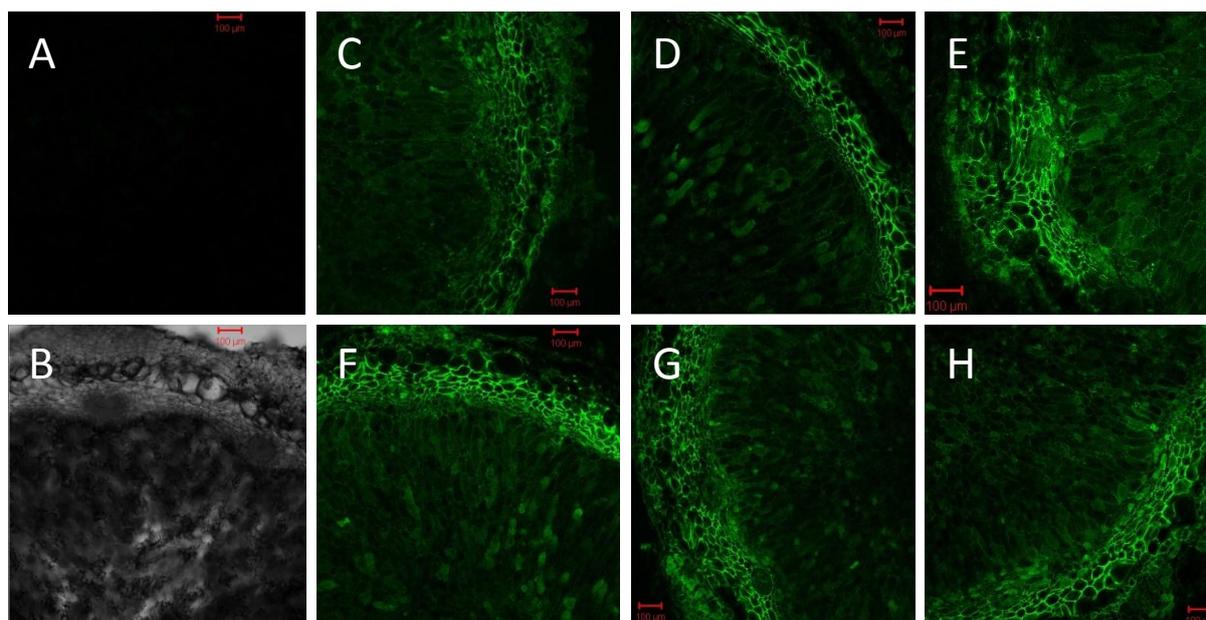
Figure 3.2 Fluorescent microscopy photographs of 150 μM slices of soybean nodules incubated in DAF-2DA to detect NO (green), (A) contains no DAF-2DA, (B) Greyscale image of a nodule, (C) Untreated, (D) 150 mM NaCl, (E) 10μ M DETA/NO, (F) 10μ M DETA/NO + 150 mM NaCl, (G) 10 μM DETA, (H) 10 μM DETA + 150 mM NaCl.

To show that NO is generated by soybean nodules and that the NO donor DETA/NO releases NO that penetrates the nodule tissue, DAF-2DA fluorescence was detected by confocal laser scanning microscopy on *G. max* nodules (Figure 3.2). The salt-treated sample shows increased fluorescence, thus demonstrating that NO is generated in response to elevated NaCl in the cell. The DETA/NO-treated samples also show a similar increase but the DETA-treated samples showed fluorescence similar to that of untreated tissue.
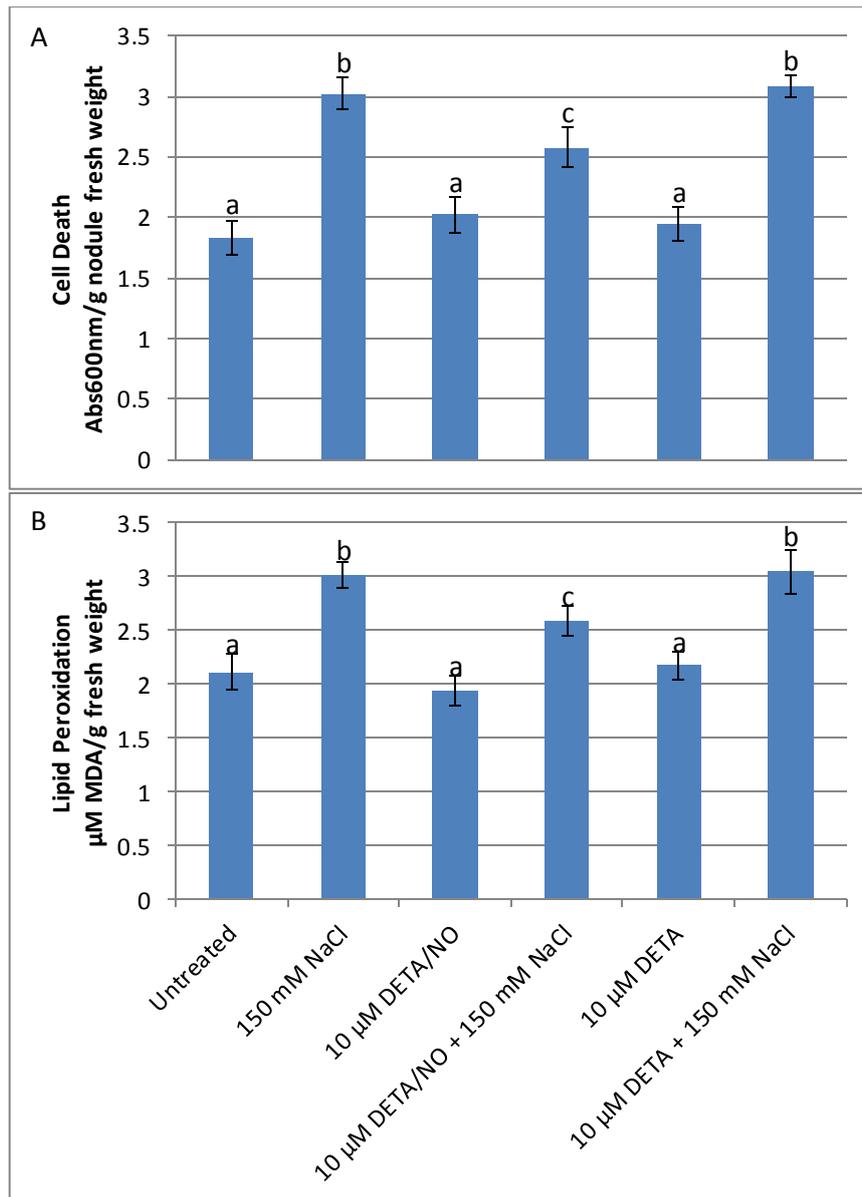
Figure 3.3 Effect of NO and salt stress on (A) cell death measured by evans blue uptake and (B) lipid peroxidation measured by quantification of MDA. Measurements were performed on soybean nodules that were treated for a week at the V3 stage.

Plants experiencing abiotic stress generate reactive oxygen species (ROS) and show increased cell death. In this study cell death was quantified by evans blue uptake (Figure 3.3A). The salt-treated samples showed increased cell death, except where NO was added in combination with salt (NaCl). NO treatment alone did not result in any differences between the untreated and the NO control (DETA) samples. A similar trend was observed with respect to lipid peroxidation (Figure 3.3B), where NO in combination with salt resulted in less damage than either of the other two salt treatments.
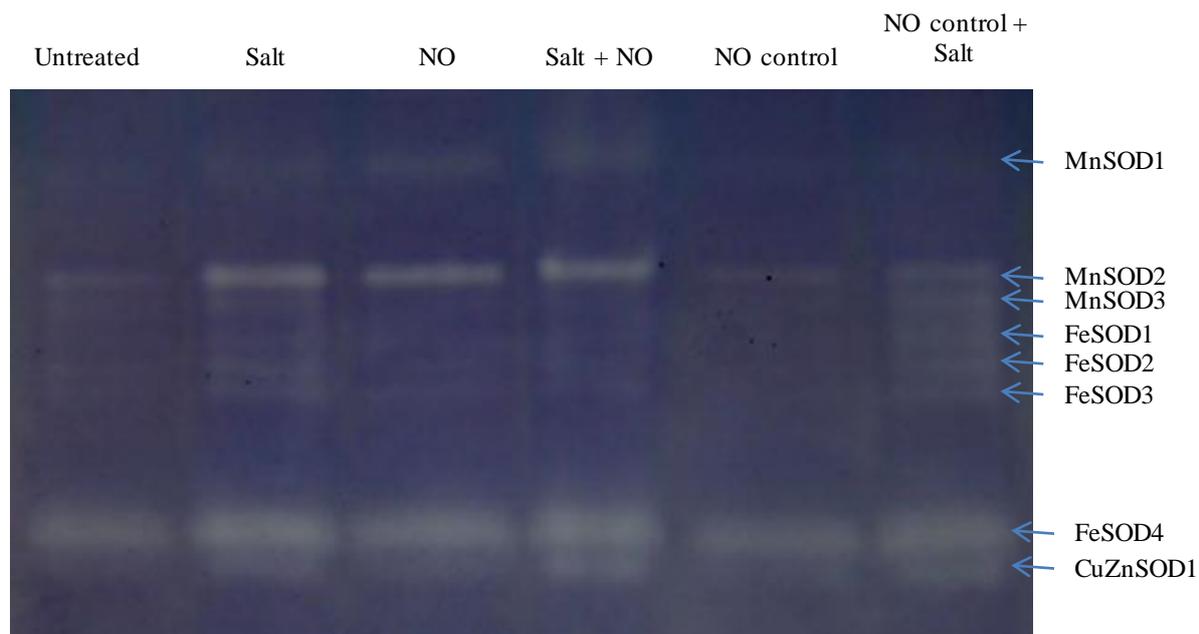
Figure 3.4 SOD native in-gel activity from left to right: Untreated, 150 mM NaCl, 10 μM DETA/NO, 10 μM DETA/NO + 150 mM NaCl, 10 μM DETA, 10 μM DETA + 150 mM NaCl.

Oxidative stress in most cases is the result of electrons leaking to oxygen to form superoxide (Alscher et al., 2002). SOD removes the superoxide enzymatically. Figure 3.4 shows SOD activity from proteins from *G.max* nodules in the treatments described in 3.2.1. The proteins were separated via native PAGE and stained for superoxide dismutase activity. The various SOD isoforms all show differential responses across the various treatments. Inhibitor gels were run at the same time to identify the various isoforms and consisted of KCN and H_2O_2 to identify CuZnSOD and FeSOD respectively. MnSOD is resistant to both KCN and H_2O_2 (Alscher et al., 2002). Isoforms were named according to their position from the top of the gel.
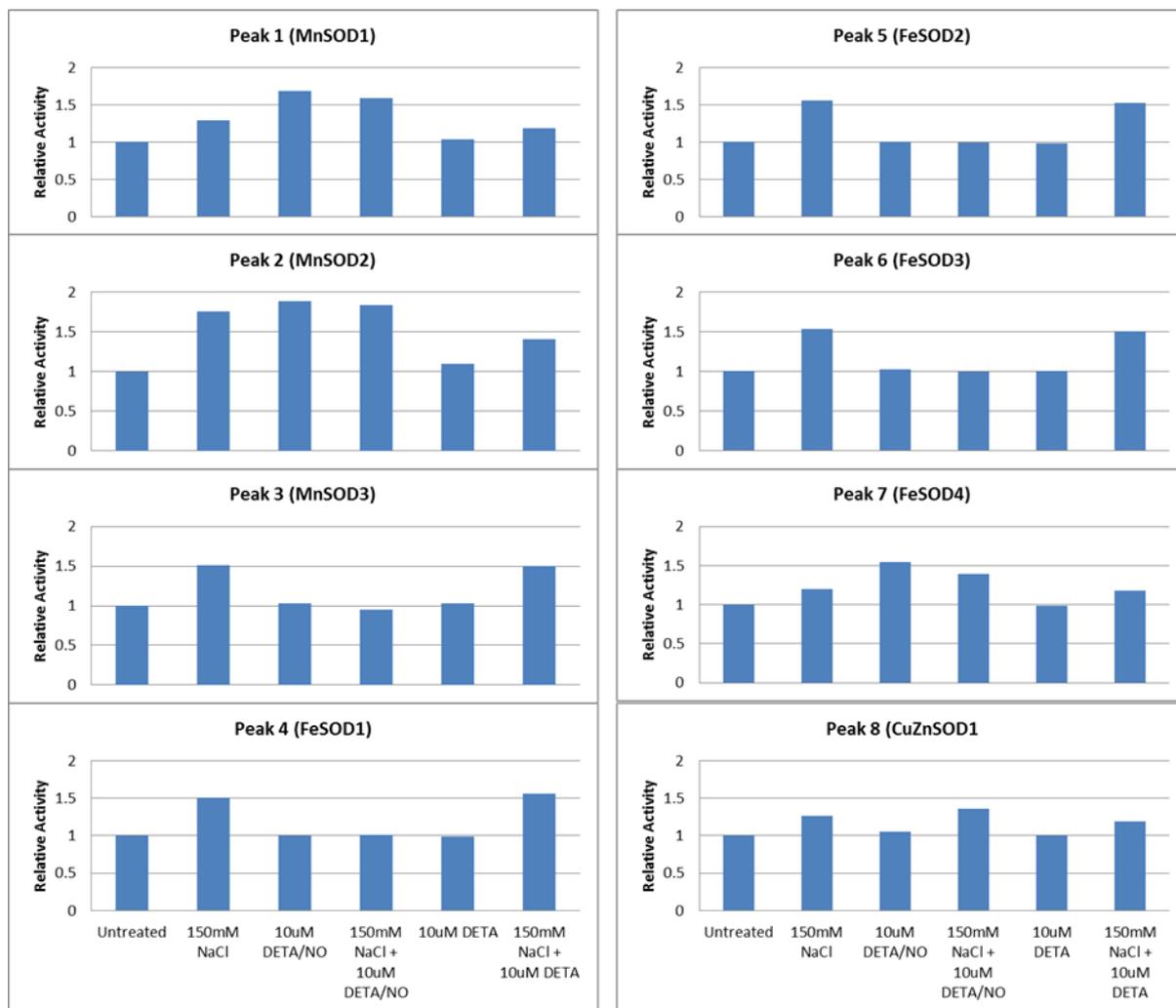
Figure 3.5 Integrated Density Value ratios relative to the untreated value of bands from Figure 3.4 SOD activity gel. The 4 upper most bands are presented on the left and the 4 lower bands from Figure 3.4 are shown on the right. Pixel intensity values were determined using the Alpha Ease FC software.

To better analyse the SOD native PAGE in-gel activity, Alpha Ease FC Software was employed and each band analysed individually. Increased activity in response to NO as well as salt were observed for, MnSOD1, MnSOD2 and FeSOD4 (Figure 3.5). The activity of MnSOD3, FeSOD1, FeSOD2, FeSOD3 all increased in response to salt but when NO was added in combination with salt the activity for these isoforms returned to that of the untreated controls. The CuZnSOD1 showed increased activity in response to salt treatment but showed little/no response, relative to the untreated sample, when NO was added.
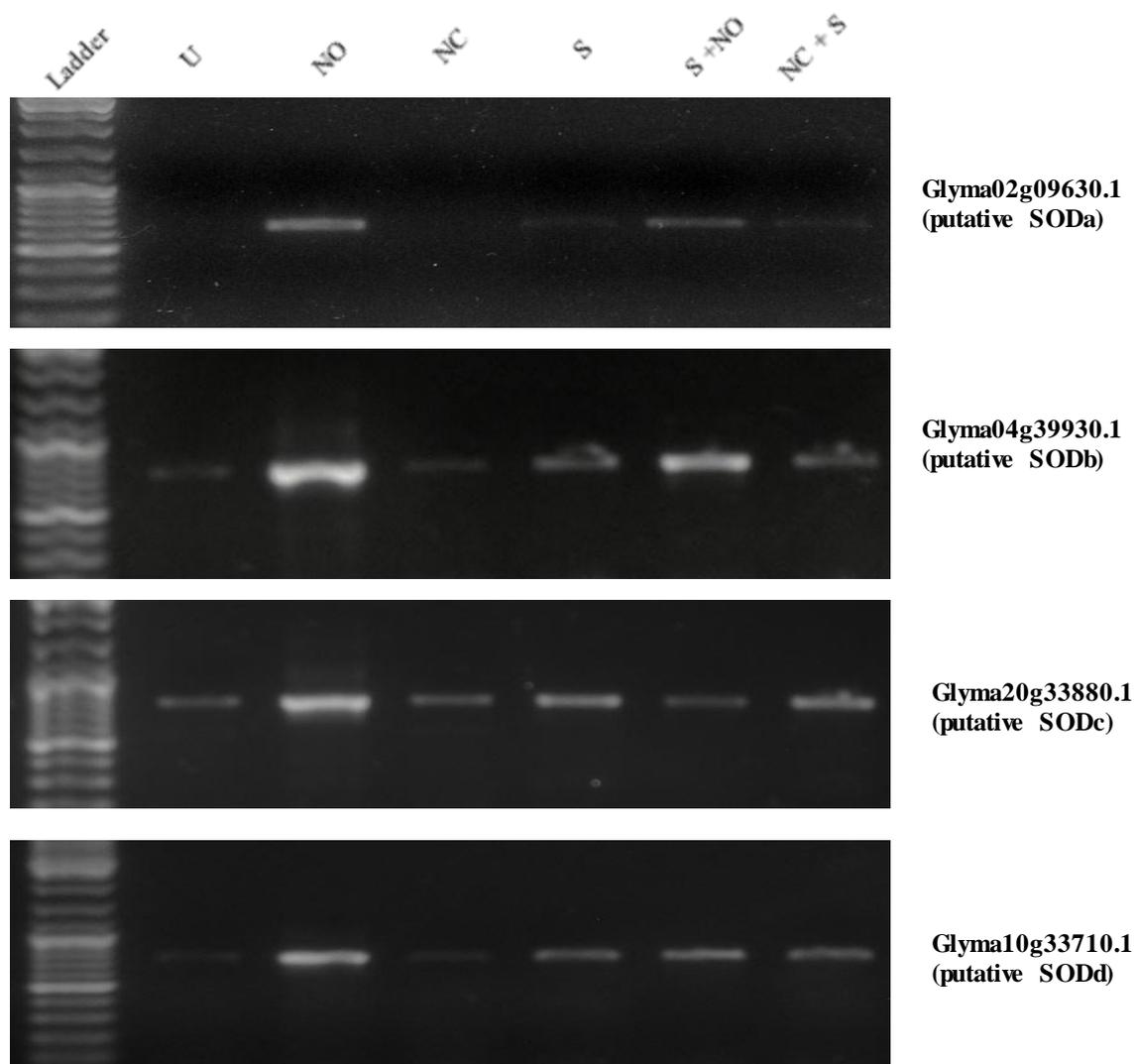
Figure 3.6 Effect of NO and salt on putative SOD transcripts measured by semi-quantitative RT-PCR. Left to right: (U) Untreated, (NO) 10 µM DETA/NO, (NC) 10 µM DETA, (S) 150 mM NaCl, (S+NO) 10 µM DETA/NO + 150 mM NaCl, (NC+S) 10 µM DETA + 150 mM NaCl.
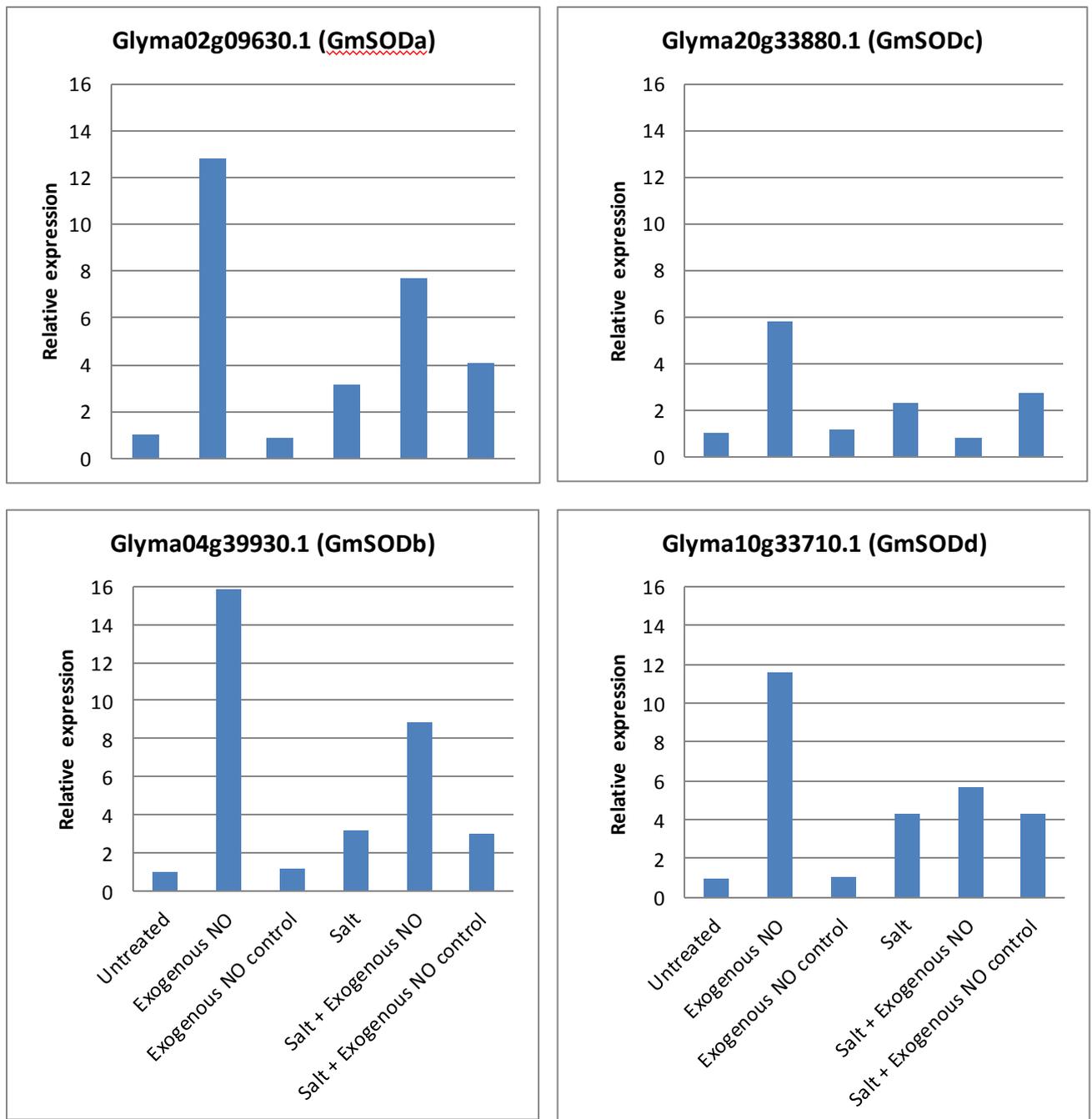
Figure 3.7 Integrated density value (IDV) ratios relative to the untreated transcript bands shown in Figure 3.6.

Semi-qRT-PCR was performed on putative SOD transcripts identified through the use of Bioinformatics. All 4 of these genes (Figure 3.7) were significantly up-regulated by the NO treatment (Figure 3.6). These transcripts showed a smaller increase in response to salt treatment and GmSODa showed no amplification for untreated and NO control (DETA). To better analyse the data observed in Figure 3.6, the bands were quantified via pixel intensity and ratios were generated using the untreated band of each sample as a reference (Figure 3.7). The graphs show that *GmSODb* transcript changed the most and that a combination of salt and NO reduced the transcript increase seen with NO alone. *GmSODc* was NO-inducible but in combination with salt treatment the expression returned to that of

untreated control. There were no differences between the control (untreated) and the NO control (DETA) treatments.
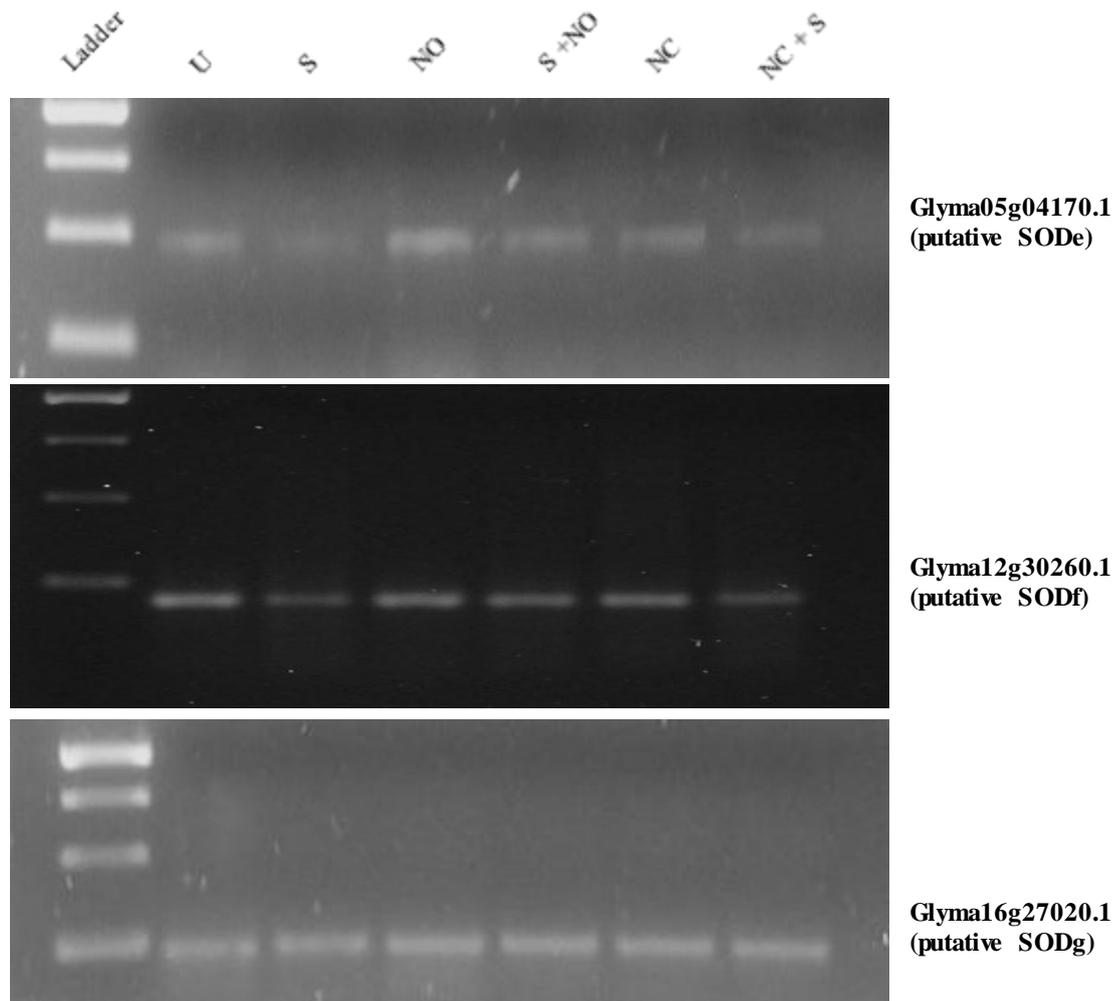
Figure 3.8 Effect of NO and salt on putative SOD transcripts measured by semi-quantitative RT-PCR. Left to right: (U) Untreated, (S) 150 mM NaCl, (NO) 10 μM DETA/NO, (S + NO) 10 μM DETA/NO + 150 mM NaCl, (NC) 10 μM DETA, (NC + S) 10 μM DETA + 150 mM NaCl.

**Glyma05g04170.1 (GmSODe)**

Glyma12g30260.1 (GmSODf)

Glyma16g27020.1 (GmSODg)
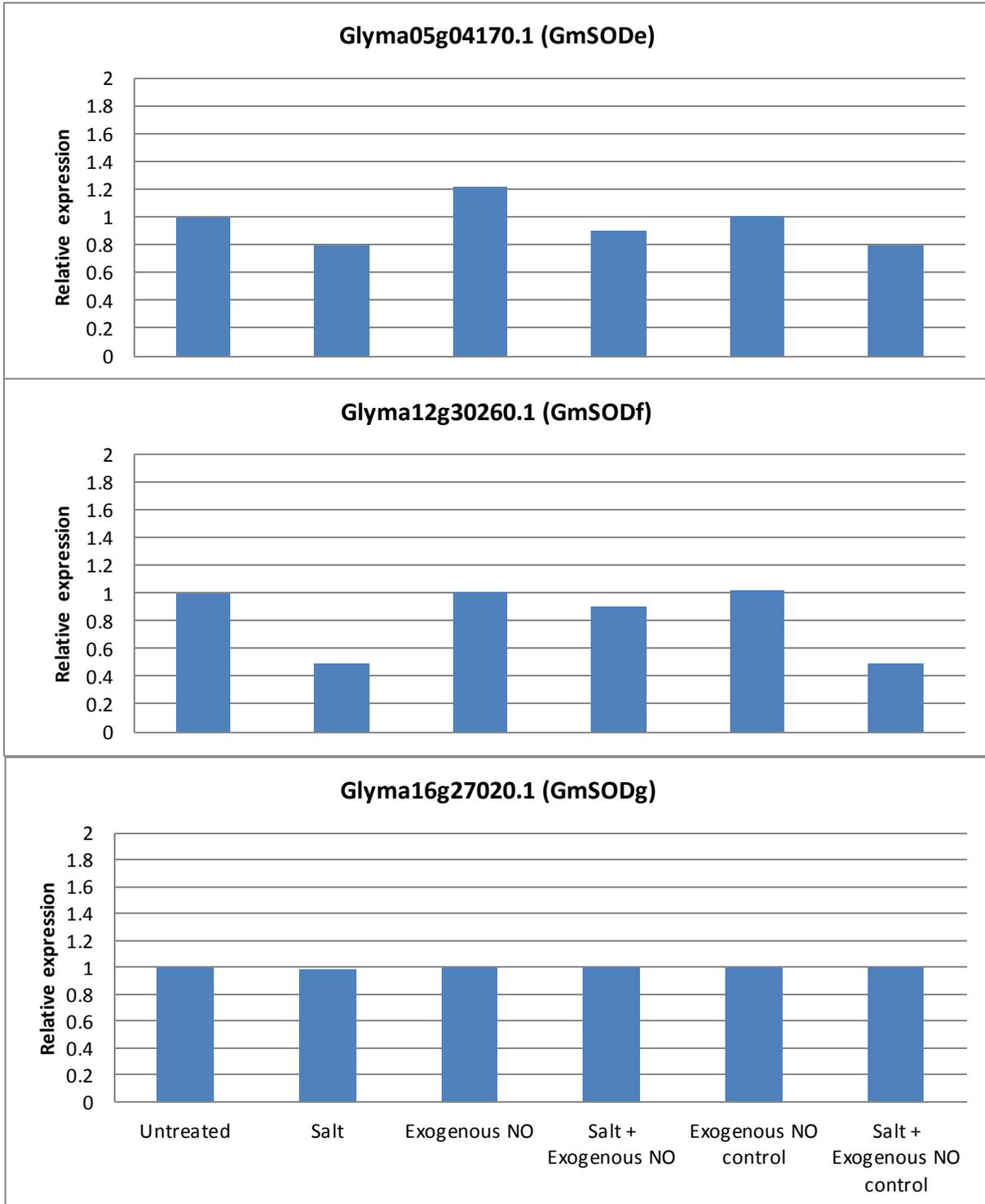
Figure 3.9 IDV ratios relative to the untreated transcript bands shown in Figure 3.8.

Figure 3.8 shows the transcriptional responses of another 3 transcripts to the various treatments, with Figure 3.9 showing the relative transcript ratios. *GmSODe* showed a small increase in transcripts in response to NO treatment whereas salt treatment reduced transcript levels. For *GmSODf*, the decrease in transcript levels caused by salt treatment was rescued by NO. *GmSODg* showed no response to any treatment.

3.4 Discussion

Exogenously applied NO has been shown to be beneficial to plants experiencing abiotic stress and has been shown to increase antioxidative enzyme activity (Lamattina et al., 2001; Keyster et al., 2011). To better understand this phenomenon, the influence of NO on SOD was studied in plants undergoing salt stress.

To show that DETA/NO releases NO *in vivo* and that salt stress in the nodules causes NO production, DAF-2DA fluorescence was detected by confocal laser scanning microscopy. The DETA/NO control (DETA) showed little or no difference from the untreated soybean nodule samples with respect to NO content. The biggest changes in fluorescence were detected in the conductive tissue followed by the cortical tissue. The images show that NO is produced by nodules and is elevated in nodules treated with salt, as well as in nodules treated with DETA/NO.

The effect of salt on the nodule was assessed by the number of dead cells in the tissue. In Figure 3.3A, the plants experiencing high levels of stress are the salt-treated and the ones treated with a combination of both salt and DETA. Where the plants were not treated with salt it is apparent that they are "healthy" and show low levels of cell death.

Interestingly, the plants treated with the combination of NO and salt experienced less cell death than those treated with salt alone, leading to the conclusion that NO is reducing the stress experienced by the soybean nodule. A precursor to PCD onset in response to salt stress is oxidative damage as shown in Figure 3.1 (Alesandrini et al., 2003). Oxidative damage was measured as the amount of lipid peroxidation occurring in the nodules (Figure 3.3A) as peroxidation is a biomarker of oxidative stress. In Figure 3B, the trend is similar to the cell death shown in Figure 3A, suggesting that NO reduces PCD by decreasing the oxidative damage that is experienced by the cells. To better understand this phenomenon, superoxide dismutase activity was studied. SOD catalyses the dismutation of superoxide to hydrogen peroxide and oxygen and is the first step in detoxification of the ROS superoxide. Although it does produce hydrogen peroxide, which is also considered to be a ROS, hydrogen peroxide has a much lower oxidative capacity than superoxide (Alscher et al., 2002). Figure 3.4 shows the changes in the activity of various SOD isoforms in response to NO and salt treatments. The isoforms were named according to their relative position from the top of the gel and also on the basis of sensitivity to their inhibitors.

After close examination of the image in Figure 3.4 using Alpha Ease FC software, graphs were generated according to the intensity of the bands formed and ratios made against the untreated samples. The top two isoforms, MnSOD1 and MnSOD2, showed increases in response to NO and salt treatment. In the case of MnSOD3, NO treatment had no effect on its own but it reduced the salt-induced increase in activity when applied in combination with salt. MnSOD is generally found in the mitochondria of the cell and is resistant to both cyanide and hydrogen peroxide (Alscher et al., 2002).

Close examination of FeSOD1, 2 and 3 shows that NO is also ameliorating salt-induced increases in activity but FeSOD4 shows NO-induced activity. FeSODs are generally found in the chloroplasts and peroxisomes and are inhibited by hydrogen peroxide (Alscher et al., 2002). The lowest band in the gel is the CuZnSOD which shows no response towards NO treatments and is induced by salt stress. CuZnSOD generally occurs in the cytosol but also occurs in the outer membranes of the mitochondria and the chloroplasts. The CuZnSODs are inhibited by both hydrogen peroxide and cyanide (Alscher et al., 2002). NO seems to be selectively increasing and decreasing the activity of MnSODS and FeSODS, respectively. This specific change in SOD activity suggests a focus to increase superoxide scavenging capacity in the mitochondria while decreasing superoxide scavenging in the cytosol and peroxisomes. This strategy may allow a faster change in the cells phenotype as the current proteins in the cytosol will be marked for degradation more easily in oxidising conditions, allowing the new set of proteins to take their place. This should then result in a salt stress-adapted cell.

One mechanism by which NO could be influencing SOD activity is through modulating transcription of the various SOD isoforms. By analysing the *Glycine max* genome, various putative SODs were identified using amino acid sequence similarity to their homologs in *Arabidopsis thaliana*. Once identified, semi-quantitative RT-PCR was performed and the results for the 4 putative SOD genes can be seen in Figure 3.6.

All 4 genes show up-regulation in response to NO and also in response to salt, although NO treatment showed a significantly stronger induction than salt did. The combination of salt with NO treatment resulted in a higher increase in SOD actvity for SODa, b, and d than salt alone but less than that for NO alone. This shows that salt reduces NO induced transcription in a manner similar to that of the MnSOD1, 2 and FeSOD4 activity. This could be due to the Na^+ ions that have accumulated in the cells, interfering with protein function, thus reducing SOD transcription and activity.

For SODc the increase in transcription by salt treatment was completely reversed by the addition of NO. Another interesting observation is that SODa could not be amplified without NO or salt treatment, and since salt treatment increases NO content (Figure 3.2), this gene is perhaps NO-dependent. In conclusion, the beneficial effect of NO during abiotic stress can be attributed to a reduction in oxidative stress as a result of enhanced SOD activity to remove excessive superoxide that would otherwise be generated in response to salt stress in the absence of NO. This can be extended to inhibition of PCD through increasing cystatin transcription based on the putative *cis*-acting elements identified in the *in silico* analysis. The reduction in oxidative stress is partly because of the increased SOD activity. A summary of the proposed mechanism can be seen by the dotted lines in Figure 3.1.

3.5 References

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Chapter 4: Conclusion and Future Prospective

4.1 Conclusion

In this project soybean nodules were studied during salt stress and following treatment with nitric oxide (NO). The salt-stressed nodules showed an increased in NO content as well as increased lipid peroxidation and cell death.

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Nitric Oxide  ◄───────────────────  Salt Stress

     │                                    │
     ▼                                    ▼

Cis-Acting Elements ──► SOD transcription ──► SOD activity ──┤ Oxidative Stress

     │                                                            │
     ▼                                                            ▼

Cystatin Transcription ──┤ Cysteine Protease activity ──►  Cell Death
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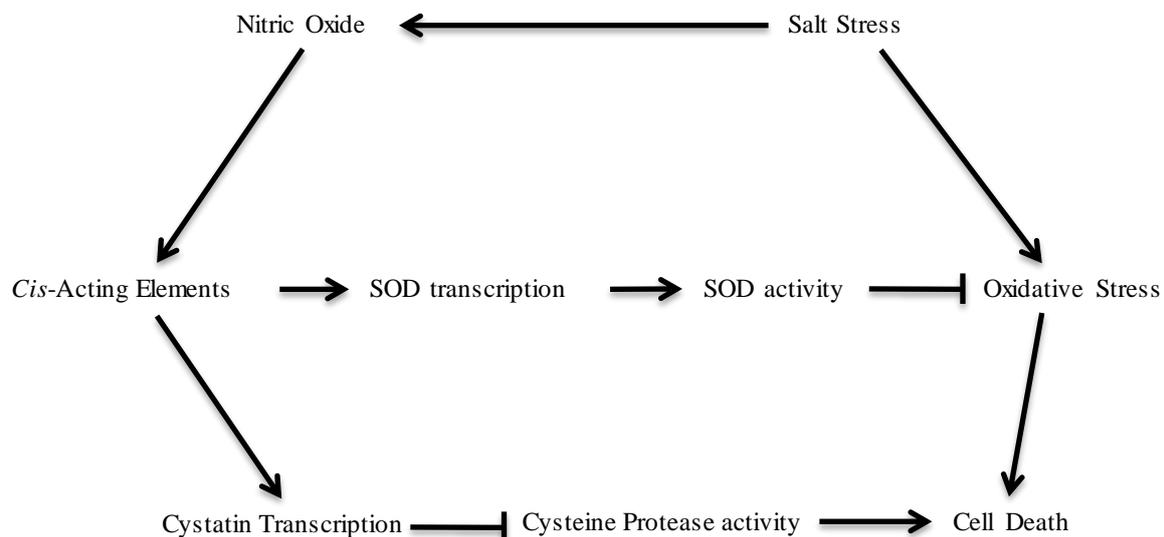
Figure 4.1 Illustration of the mechanism by which NO reduces salt stress-induced oxidative stress and cell death.

When NO was added in combination with salt the nodules fared better than nodules treated with salt alone as they showed reduced lipid peroxidation and cell death. The first antioxidative enzyme in the pathway involved in reducing reactive oxygen species (ROS), i.e. superoxide dismutase (SOD), was studied in the NO- and salt-treated nodules to better understand why there was a reduction in oxidative stress, as indicated by reduced lipid peroxidation. Results showed that NO enhanced SOD activity, specifically the acitvity of MnSOD and FeSOD isoforms. MnSOD is known to exist in the mitochondria and peroxisomes of nodule cells (Alscher et al., 2002; Becana et al., 2000). Rhizobia in the nodules may also contribute SOD isoforms and so it cannot be ruled out that some of the SOD activity seen from the nodule extracts may be from rhizobial SOD. The FeSODs are known to exist in chloroplasts of leaves and have been shown to occur in nodule-chloroplasts (Becana et al., 2000). Mitochondria, peroxisomes and chloroplasts are the major redox-active organelles (Miller et al., 2010; Sandalio et al., 2008) of plant cells, so an increase in SOD activity at these locations can be expected.

Although three isoforms showed increased activity towards NO treatment another four showed that NO treatment reduced any salt induced increase in activity. This could have occurred because these SODs are not NO responsive and the nodules are under reduced oxidative stress relative to salt-treated nodules suggesting that NO did not prevent the salt-induced responses but they were not up-regulated since they were experiencing less stress.

The CuZnSOD did not show a response to NO but did increase in activity during salt-stressed conditions. This suggests that the increase in SOD activity may be due to either the accumulation of the Na^+ ions or the Cl^- ions. In soybean Cl^- ions are considered more toxic than Na^+ ions because of the high efficiency with which soybean cells exclude Na^+ (Munns and Tester, 2008).

The change in SOD activity over a week is most likely due to changes in transcriptional profiles but can also be due to post-transcriptional/translational changes. To better understand transcriptional changes, putative SOD genes were identified. Previously a cystatin from *Arabidopsis* (AtCYS-1) was shown to be up-regulated in response to NO (Belenghi et al., 2003). To try and identify NO responsive *cis*-acting elements soybean homologs of the cystatin were putatively identified and the promoter sequences collected for all the putative soybean SOD and cystatin genes. These promoter sequences were then analysed for abiotic stress and previously suggested NO-responsive *cis*-acting elements. The transcriptome of some of the SOD genes identified were then quantified by semi-quantitative RT-PCR to identify NO responsive. GmSODa, b, c and d all showed a significant increase in transcript levels when the tissue was treated with either NO or salt. The salt-induced up-regulation may be due to the increased NO concentration observed within the salt-treated nodules.

Table 4.1 *Cis*-acting elements relative to the starting position of NO inducible genes

Gene	1-100	100-200	200-300	300-400	400-500	500-600	600-700	700-800	800-900	900-1000
*AT*5G12140 *At*CYS-1	HSE x 2	WRKY, *At*Myb4, DRE-like	*At*Myb4	HSE, ABRE-like x 2	WRKY, Y-BOX	MYCL x 3	Y-Box x 2			MYCL
Glyma02g09630 *Gm*SODa		OCSE x 3	MYCL	MYCL x 2, ABRE-like		OCSE, *At*Myb4		MYCL		MYCL, *At*Myb4 x 2
Glyma04g39930 *Gm*SODb		MYCL		MYCL		G-Box, MYCL x 2, WRKY, HSE, ABRE-like	MYCL, WRKY, DRE-like	MYCL	MYCL, *At*Myb4	*At*Myb4 x 2
Glyma20g33880 *Gm*SODc	MYCL	*At*Myb4	*At*Myb4		OCSE, MYCL x 2, WRKY	MYCL x 2, WRKY, *At*Myb4			*At*Myb4 x 2	
Glyma10g33710 *Gm*SODd	MYCL x 2	MYCL		MYCL	WRKY	MYCL			MYCL x 2, Y-Box, *At*Myb4	MYCL

From the phylogenetic tree analysis GmSODa, b, c, d are similar to AtMSD1 and AtFSD1, which may suggest that soybean MnSODs and FeSODs could be up-regulated by NO and salt, similarly the activity of these isoforms is increased in response to NO and salt, as seen from the native gel SOD activity assays. This suggests that increases in FeSOD and MnSOD activity may be due to augmented transcriptional levels, as depicted in Figure 4.1. These four NO-responsive genes were also scanned for putative s-nitrosylation sites using GPS-SNO (Ren et al., 2011) and none were found suggesting again that the increase in SOD activity is likely due to increases in SOD transcription. The *cis*-acting elements that occur in all 4 NO-responsive SOD genes as well as the NO responsive *AtCYS-1* are the binding sites for AtMYB4 and MYCL as can be seen in Table 4.1.

MYB transcription factors occur in all eukaryotes and vary from one another mainly by the number of MYB domains that allow MYBs to bind DNA. Plants have a larger group of R2-R3-MYBs than any other kingdom (Dubos et al., 2011). AtMYB4 belongs to sub-group 4 of MYBs and is involved in the regulation of anthocyanin/flavonol biosynthesis as well as UV responses.

The binding site for the MYCL is also known as the E-box and is bound by basic-Helix –Loop-Helix (bHLH) transcription factors. The DNA binding domain is the basic domain in the N-terminal region and consists of 13-17 amino acids (Feller et al., 2011).

Abe et al. (1997) identified a 67 bp region in the promoter required for ABA- and drought-induced expression of response to dessication 22 (rd22). They mutated various suspected *cis*-acting elements and found that MYC (bHLH) and MYB elements were required for abiotic stress-inducible expression of rd22. Later they overexpressed the TFs, AtMYB2 and AtMYC2 that bound to the rd22 promoter and found that this increased abiotic stress tolerance and caused the plants to become ABA hypersensitive (Abe et al., 2003). More recently a MYB and MYC from *Gynura bicolor* were studied and found both to be highly induced by methyl jasmonate in the presence of light. Similarly, the majority of the genes involved in anthocyanin biosynthesis were also induced upon exposure to methyl jasmonate and light. It was then demonstrated that the GbMYB1 and GbMYC1 together were able to induce the promoters of two of the anthocyanin biosynthetic genes anthocyanin synthase and dihydroflavonol 4-reductase (Shimizu et al., 2011). The pathway in soybean has been studied in response to NO and cGMP and the majority of the anthocyanin biosynthesis genes showed NO inducibility (Suita et al., 2009). Methyl jasmonate and ABA have been shown to induce NO production in plants (Wang et al., 2005, Neill et al., 2003). This suggests that NO may regulate anthocyanin biosynthesis genes via MYB and MYC and similarly the SOD and cystatin genes as their promoters contain *cis*-acting elements to which MYB and MYC potentially binds.

Numerous studies have also been done on AtMYB4 and interestingly when this gene is mutated in *Arabidopsis* the plants show increased UV-B tolerance whilst the plants become UV-B hypersensitive when AtMYB4 is overexpressed (Jin et al., 2000). Jin et al. (2000) also studied the phenyl propranoid pathway to demonstrate that AtMYB4 is a repressor of this pathway. A mutant was recently found called sensitive to ABA and Drought (*sad2*) and shows up-regulation of cinnamate 4-hydroxylase (*C4H*), chalcone synthase (*CHS*) and *MYB4* (Zhao et al., 2007).Both *C4H* and *CHS* are genes in the phenyl propranoid pathway. *MYB4* was up-regulated whereas previously it was shown to repress *CHS* and *C4H* (Jin et al., 2000). The authors then fused GFP and YFP to MYB4 separately and showed that SAD2 is required for MYB4 translocation to the nucleus where it can repress its own transcription as well as the transcription of genes in the phenyl propranoid pathway (Zhao et al., 2007). AtMYB2 has been shown to be s-nitrosylated and it is thought that the s-nitrosylation removes the ability of AtMYB2 to bind DNA. Bioinformatic work on AtMYB4 identified a putative s-nitrosylation site. Soybeans closest homolog of AtMYB4 based on amino acid sequence similarity is Glyma11g11450 which contains the same s-nitrosylation site. The same was found for SAD2 and its closest soybean homolog Glyma08g29030.
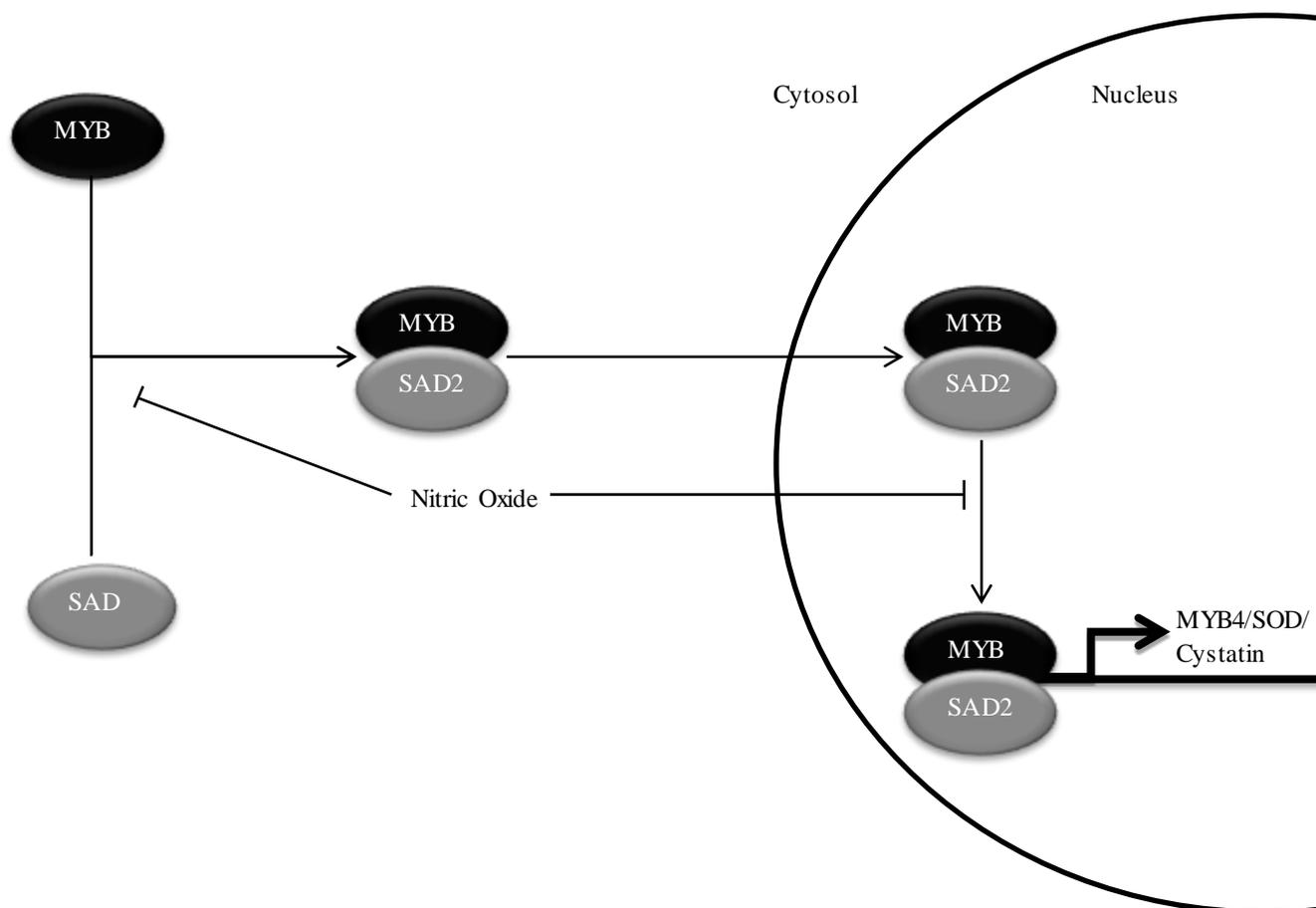
Figure 4.2 Hypothetical model for a possible mechanism by which NO regulates transcription of superoxide dismutase (SOD)/cystatin through MYB4 and SAD2.

With this information a hypothetical model seems to be forming as depicted in Figure 4.2. Under normal conditions MYB4 and SAD2 bind to form a complex that translocates to the nucleus where MYB4 can repress its own transcription as well as that of SOD and cystatin and the phenyl propranoid biosysnthesis genes. When NO is produced or added exogenously it causes MYB4 and SAD2 to become s-nitrosylated perhaps preventing them from binding one another and therefore preventing MYB4 from translocating to the nucleus to regulate transcription. Another possible mechanism could be that the s-nitrosylation of MYB4 prevents it from binding DNA in the nucleus. Either way, the relief of restraint then allows up-regulation of *MYB4* as well as the other target genes. This up-regulation of *MYB4* can continue until such time as the newly translated MYB4 protein is unable to be s-nitrosylated and the cell can return to a "resting sate". This of course is still completely hypothetical and requires much experimentation to be validated.

The other SOD genes that were not NO-inducible also contained the AtMYB4 and MYCL *cis*-acting elements. These genes are possibly under constitutive expression in this organ and the mechanisms regulating their expression will require further study.

 Considering that mammalian systems contain multiple NO-responsive *cis*-acting elements (Dhakshinamoorthy et al. 2007) the same may be true for plants. Some but not all of the SOD and cystatin promoters contain the W-box and/or the octopine synthase element (OCSE) that are

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implicated in NO-mediated gene regulation This suggests that NO may act via the OCSE as suggested by Lindermayr et al. (2010). The W-box was previously suggested to be an NO-responsive *cis*-acting element by Palmeiri et al. (2008).

4.2 Future prospects

Since the change in SOD activity has not been directly attributed to specific SOD genes further evidence is required to confirm that neither s-nitrosylation nor tyrosine nitration affected SOD activity and to show that the change in activity is certainly from an increase in transcription rather than post-translational modification. Identification of the genes corresponding to the bands in the in-gel activity assays to correlate them with the transcriptional changes will further confirm whether the changes seen are at the level of transcription or are post-transcriptional/post-translational.

Identification of a solely NO-inducible SOD gene implies that one can isolate the promoter of the NO-inducible SOD, fuse it to a reporter gene such as luciferase or β-glucuronidase (GUS) and identify the functional NO-inducible *cis*-acting elements.

If a NO inducible *cis*-acting element is confirmed, overexpression of the corresponding transcription factor/s that binds it can be used to see if the SOD genes increase in transcription as well as SOD activity. Since the transcription factor may possibly function in other anti-oxidative mechanisms a plant overexpressing this transcription factor may show increased abiotic stress tolerance which would be of great benefit to commercial agriculture.

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