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ROLE OF *S*-NITROSOGLUTATHIONE REDUCTASE AND NUCLEOREDOXINS IN REDOX-MEDIATED PLANT DEFENCE

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Abstract

Redox reactions are an essential part of the cell's metabolism, differentiation, and responses to the prevailing environmental conditions. In plants, dramatic changes in cellular redox status are observed upon exposure to environmental stresses, including pathogen attack. These changes affect the oxidative status of reactive cysteine thiols in regulatory proteins. To control oxidative protein modifications, plant cells employ the antioxidant enzymes *S*-nitrosoglutathione Reductase 1 (GSNOR1) and members of the Thioredoxin (TRX) superfamily. Immune signalling by the hormone salicylic acid (SA) is particularly dependent on the activity of these enzymes. SA is synthesized in response to challenge by plant pathogens for the establishment of local and systemic immunity. SA accumulation is regulated by cellular levels of *S*-nitrosoglutathione (GSNO), a redox molecule capable of *S*-nitrosylating proteins (*i.e.*, covalent attachment of nitric oxide to cysteines). GSNOR1 is thought to regulate cellular GSNO and global *S*-nitrosylation levels, but it is unknown how GSNOR1 regulates SA biosynthesis. Furthermore, SA recruits the activities of selected TRX enzymes that act as ubiquitous thiol reductases to counteract cysteine oxidation of SA-responsive regulatory proteins, thereby modulating their activities. However, it is unclear how SA controls nuclear redox processes involved in SA-responsive gene activation. Here we show that GSNOR1 regulates SA accumulation by regulating the expression of SA biosynthetic genes and their transcriptional activators. Moreover, we describe Nucleoredoxins (NRX) that represent novel, potentially nuclear localized members of the TRX superfamily. Mutant *nrx1* plants displayed enhanced disease resistance, which was associated with enhanced expression of genes involved in synthesis of salicylic acid. Unlike classical TRX,

NRX enzymes contain multiple active sites, suggesting they may exhibit significant reductase or remodelling activities. Indeed, insulin turbidity assays indicated that NRX proteins show an unusual form of disulphide reduction activity. Taken together, the data presented in this thesis demonstrate that GSNOR1 and NRX enzymes play critical roles in regulating synthesis of and signalling by SA in plant immunity.

Declaration

The work presented in this thesis was completed by the author Rumana Keyani unless otherwise stated and has not been submitted for a degree at this or any other institution

This dissertation is dedicated to the three most important people in my life:

To My

Loving Parents

Without their knowledge, wisdom, and guidance, I would not have the goals I strive towards and be the best to reach my dreams!

To my teacher Professor Dr. Irfan ul Haque, who encouraged and sometimes pushed me to give my best, and helped me to become the human being and researcher I am.

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Abbreviations

ATP	Adenosine Triphosphate
AUX	Auxin
ABA	Abscisic acid
Avr	Avirulence
AvrB	Avirulence B
AvrPto	*P. syrinage* pv. Tomato Effector protein
AvrRpm1	*P. syringae* Avirulence Resistance to *P. syringae pv maculicola I*
AvrRpt2	Resistance to *Pseudomonas syringae* P. V. tomato 2
bp	Base pair
cDNA	Complementary DNA
CA	carbonic anhydrase
CBP60g	Camodulin binding protein
Cys	Cysteine
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
EDS1	Enhanced Disease Susceptibility 1
EDS5	Enhanced Disease Susceptibility 5
EER	Avriulence Motif (RXLR-EER)
Efr	*A. thaliana* EF-Tu Receptor
EF-Tu	Elongation Factor Thermo Unstable

ER	Endoplasmic Reticulum
ETI	Effector-triggered Immunity
ETS	Effector-triggered Susceptability
F	Phenylalanine (Phe)
Flg22	*P. syringae* Flagellin 22
FLS2	*A. thaliana* FLAGELLIN SENSITIVE 2
G	Glycine (Gly)
GFP	Green fluorescent protein
GSNOR1	S-nitrosoglutathione 1
GUS	Glucuronidases
H	Histidine (His)
HP	Hydroperoxide lyase
Hpi	Hours post-infiltration
HR	Hypersensitive response
IAA	Indole-3-acetic acid
ICS1	Isochorismate synthase 1
IPTG	Isopropyl-ß-D-thiogalactopyranoside
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK4	Interleukin-1 receptor-associated kinase 4
ISR	Induced systemic resistance
JA	Jasmonatic Acid
K	Lysine (Lys)
LB	Lysogeny broth
LOX	Lipoxygenase
LRR	Leucine Rich Repeat

LRR-RK	Leucine Rich Repeat-receptor kinase
MAMP	Microbe-associated Molecular Pattern
MAPK	Mitogen-activated Protein Kinase
MCS	Multiple cloning site
MeJA	Methyl jasmonate
mRNA	messenger RNA
MTI	MAMP-triggered Immunity
N	Amine-terminal; N-terminal
NADP(H)	Nicotine adenine dinucleotide phosphate (reduced form)
NahG	Bacterial transgene encoding Salicylcate hydroxylase
NB	Nucleotide Binding
NBD	Nucleotide Binding Domain
NB-LRR	Nucleotide Binding-Leucine Rich Repeat
NBS	Nucleotide Binding Site
NDR1	*A. thaliana* NONRACE-SPECIFIC DISEASE RESISTANCE 1
NPR1	NONEXPRESSOR OF PR GENES, also known as NIM1 and SAI1
NRX1	Nucleoredoxin 1
NRX2	Nucleoredoxin 2
NTRA	Thioredoxin reductase A
OD	Optical density
PAD4	Phytoalexin deficient 4
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated Molecular Pattern
PBS1	avrPphB Susceptible 1
PBS3	avrPphB Susceptible 3
PCD	Programmed Cell Death

PCR	Polymerase Chain Reaction
PCWDEs	Plant cell wall-degrading enzymes
PEG	Polyethylene glycol
PR	Pathogenesis-related
PRR	Protein Recognition Receptor
Psm	*Psudomonas syringae pv maculicola*
R	Resistance gene
REDOX	Reduction oxidation
RIN4	RPM1 interacting protein 4
RLKs	Receptor-like protein kinases
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPS5	Ribosomal protein S 5
RT-PCR	Reverse transcriptase- polymerase chain reaction
S	Serine (Ser)
SA	Salicylic acid
SABP 3	SA binding protein 3
SAR	Systemic Acquired Resistance
SARD1	Systemic Acquired Resistance deficient 1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SID2	SA induction deficient 2
SOB	Super Optimal Broth
SOC	Super Optimal broth with Catabolite repression

TIR	Toll and Interleukin-1 receptor
TMV	Tobacco mosaic virus
TRX5	Thioredoxin 5
WRKY transcription Factors	Proteins with a Conserved WRKY domain
WT	Wild type

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Chapter 1

Introduction

Chapter 1

1. Introduction

Plants are exploited as a source of food and shelter by a wide range of attackers, including viruses, bacteria, fungi, oomycetes, nematodes, insects and even other plants. In response plants have evolved sophisticated defences that are either constitutive or inducible. Although this renders plants resistant to most pathogens in their environment, some pathogens are highly specialized and able to circumvent these defences. Consequently, the development of novel resistant crops is critical to agricultural advances, thereby avoiding heavy crop losses due to different diseases caused by various attackers (Agrios, 2005).

Plants combat pathogen and insect attack with primary defences that exist of physical and chemical barriers. Primary defences include the cuticle, plant cell wall, and presence of toxic secondary metabolites. If this first line of defence fails, plants can induce further secondary defences, including strengthening of cell walls with the polysaccharide callose and enhanced production of antimicrobial compounds (e.g. phytoalexins, antimicrobial proteins, digestive inhibitors). Thus, resistance against microbes and insects can be mediated through defences that are constitutively present, or through defence mechanisms that are induced only after pathogen attack (Van Loon, 2000; Dicke and Van Poecke, 2002). A broad spectrum of inducible plant defences can be employed to limit further pathogen growth and for this line of defence, plants have evolved sophisticated strategies to recognise their attacker and to translate this perception into an effective immune response (Fig. 1.1; Jones and

Dangl, 2006). Induced defence responses are regulated by a network of interconnecting signal transduction pathways in which the hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play key roles (Glazebrook 2005; Pieterse and Van Loon 1999; Reymond and Farmer 1998; Thomma et al. 2001). SA, JA, and ET accumulate in response to pathogen infection or damage caused by insect feeding, resulting in the activation of distinct sets of defence-related genes (Glazebrook et al. 2003; Reymond et al. 2004; Schenk et al. 2000). These defence hormones generate attacker-specific signatures in gene expression profiles to prioritize defences over normal cellular functions. While SA responses are predominantly effective against biotrophic pathogens (i.e. pathogens that feed on live host cells), JA/ET responses are largely active against insects and pathogens with a necrotrophic lifestyle (i.e. pathogens that kill the host cell to feed) (Glazebrook 2005; De Vos et al., 2005). The crosstalk between these signalling pathways, in such cases, renders the plant to prioritize the one defence over the other. Pathogen infections also affect the hormonal pathways involved in plant growth and development. Many pathogens have developed mechanisms as a virulence strategy, to tap into these hormonal signalling networks to interfere with host defence (Spoel and Dong, 2008). In reaction, crosstalk may be used by the host as a direct defence mechanism against pathogen-induced perturbation of hormone signalling. A noticeable feature of various pathogens is their ability to change plant hormone signalling and takeover host hormonal crosstalk mechanisms as a virulence strategy (Spoel and Dong, 2008) and in this context; pathogens produce different phytotoxins to hijack the host machinery. Among them coronatine (COR) plays very important role. Coronatine is a phytotoxin produced by *Pseudomonas syringae* (Bender et al., 1999) and resembles

JA derivatives, including JA-isoleucine (Staswick, 2008). COR has been reported to affect JA homeostasis as it induces cellular and physiological modifications in plants that are similar to those caused by methyl-JA (MeJA) (Spoel and Dong, 2008). Using COR-deficient *P. syringae* mutants and plants impaired in JA signalling, several research groups have suggested that *P. syringae* employs COR to mimic JA signalling and thereby suppresses SA-mediated defence through antagonistic crosstalk (Koornneef and Pieterse, 2008).

Besides these three major defence hormones, other plant hormones (e.g. abscisic acid, brassinosteroids, auxins, gibberellins and oxylipins) have also been reported to play roles in plant defence (Jameson, 2000; Farmer et al., 2003; Krishna, 2003; Thaler and Bostock, 2004; Mauch-Mani and Mauch, 2005), but are beyond the scope of this thesis.

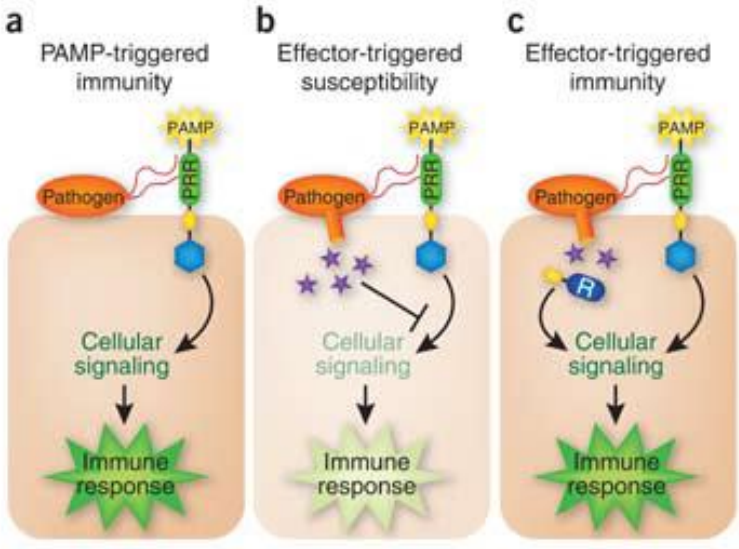


Figure 1-1 Schematic representation of the plant immune system.

(a) Upon pathogen attack, PAMPs activate pattern-recognition receptors (PRRs) in the host, resulting in a downstream signalling cascade that leads to PTI.

(b) Virulent pathogens have acquired effectors (purple stars) that suppress PTI, resulting in effector triggered susceptibility (ETS).

(c) In turn, plants have acquired R proteins that recognize these pathogen-specific effectors, resulting in a secondary immune response called ETI. *This figure has been taken from Jones and Dangle, 2006.*

1.1. Non-Host Resistance

Plants are attacked by a diverse range of pathogens, most of which are unable to infect them. Resistance of an entire plant species against all strains of a pathogen that is able to infect other plants is known as non-host resistance (Heath, 2000). Non-host resistance is based on discrimination between self and non-self. Recognition of

microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) by the plant's array of pattern recognition receptors leads to the development of pattern-triggered-immunity (PTI) (Fig. 1.1a) (Zipfel, 2009; Chisholm et al., 2006; Bent and Mackey, 2007). Classical examples of PAMPs are fundamental molecules, such as bacterial flagellin, elongation factor Tu (EF-Tu), peptidoglycan, and lipopolysaccharides, oomycete glucans, and fungal chitin (Ayers et al., 1976; Felix et al.1993, 1999; Dow et al., 2000; Gust et al., 2007; Erbs et al., 2008; Zipfel et al., 2006). Flagellin, the globular protein that forms filaments in bacterial flagellum, has been particularly well studied in plant PTI (Felix et al., 1999; Che et al., 2000). Plants have been shown to respond to flg22, a 22 amino acid peptide spanning the most conserved part of the N-terminus of flagellin (Felix et al., 1999). In previous work, a genetic approach was used to screen for mutants affected in the perception of the flg22 epitope in *Arabidopsis thaliana*. Several of these *fls* (for flagellin sensing) mutants mapped to the *FLS2* gene (Gomez Gomez and Boller, 2000). Plants carrying mutations in *FLS2* show impaired binding of flg22, lack cellular responses to flagellin, and exhibit enhanced susceptibility to bacterial infection (Bauer et al., 2001; Zipfel et al., 2004). Similarly, mutation of the *Arabidopsis* pattern recognition receptor EFR, which recognizes EF-Tu or the elf18 peptide corresponding to the conserved 18 N-terminal residues of EF-Tu, results in susceptibility to bacterial pathogen infection (Zipfel et al., 2006).

1.2. Host Resistance

Pathogens that circumvent non-host resistance can potentially activate another surveillance system which is based on specific rather than general immunity and is known as host resistance. Plants are attacked by a diverse range of pathogens, most of which are unable to infect them. The success of pathogen to infect a host plant depends on how fast the plant recognizes the pathogen and activates the appropriate defence response (Van Wees and Glazebrook 2003).

In addition to PTI, plants can respond to pathogen effectors by a process called effector-triggered immunity (ETI) (Fig. 1.1c) (Zhu et al., 2010). As opposed to PAMPs that are molecular motifs common to many pathogens, effectors are pathogen-secreted proteins that hijack host cell functions (van der Hoorn and Kamoun 2008). Despite potentially being recognized by the host, effectors have persisted throughout pathogen evolution due to their contribution to pathogen fitness and ability to suppress PTI. It is likely that all pathogenic microbes contain effectors that suppress PTI, but the best examples come from phytopathogenic Gram-negative bacterial pathogens that acquired a type III secretion system (TTSS) through either horizontal gene transfer or adaptation of the flagellar apparatus; the evolution of the TTSS enabled bacteria to directly deliver effector proteins into plant cells to suppress PTI (Chisholm et al., 2006). Bacterial pathogens of animals are known to secrete only a limited number of effectors into host cells. But plant pathogens, such as *Pseudomonas syringae* pathovars, can secrete 20 to 30 effectors during infection (Chang et al., 2005).

ETI is induced by a specific recognition of pathogen effectors by *Resistance* (*R*) genes of the host plant. Effector recognition leads to rapid and efficient defence responses, including a programmed cell death response known as the hypersensitive response (HR) (Zhu et al., 2010). The HR confines the growth and establishment of pathogens by depriving them of nutrients and shares similarity with apoptosis in animals (Mur et al., 2008). The mechanistic commonalities between HR and progammed cell death in animals include membrane dysfunction, vacuolization of cytoplasm, chromatin condensation, and endonucleolytic cleavage of DNA (Zaninotto et al., 2006).

Despite the presence of countless different pathogen effectors, plant genomes contain only a limited number of R proteins to detect these effectors (Shao et al., 2003). To explain this discrepancy the 'guard hypothesis' was put forward, which states that R proteins guard the host targets of pathogen effectors instead of physically interacting with them and that R proteins are activated by the modification of these targets by effectors. Activation of R proteins then induces ETI in the host (Van der Biezen and Jones, 1998; Dangl and Jones, 2001; Hunter, 2005; Mukhtar et a., 2011). For example, AvrPphB is an avirulence protein from *P. syringae* and belongs to a family of cysteine proteases that cleave PBS1 serine/threonine kinase protein of the host. The R protein RPS5 acts as a guard for PBS1 and recognizes AvrPphB-mediated cleavage of PBS1, resulting in RPS5 activation and initiation of downstream ETI responses (Swiderski and Innes 2001; Warren et al., 1999). Other examples of these recognized effector targets or 'guardees' are tomato RCR3 and Pto and *Arabidopsis* RIN4 and PBS1 (Jones and Dangl, 2006).

1.4. Systemic Acquired Resistance

In addition to PTI and ETI, which restrict the local growth of the pathogen, plants have secondary defence mechanisms that provides pathogen resistance in the uninfected parts of the plant (Ross, 1961). This disease resistance mechanism, known as systemic acquired resistance (SAR), is long lasting and has a broad spectrum of effectiveness. SAR is characterized by the local and systemic accumulation of salicylic acid (SA) and it is associated with the secretion of pathogenesis-related (PR) proteins (Kessmann et al., 1994; Ryals et al., 1996; Sticher et al., 1997; Uknes et al., 1992; Ward et al., 1991). Plants impaired in the accumulation of SA fail to establish SAR and this can be supported by the fact that plants expressing the *NahG* transgene fail to accumulate SA and do not establish SAR (Gaffney et al., 1993; Delaney et al., 1995), because *NahG* encodes salicylate hydroxylase, which converts SA into inactive catechol that is not an active SAR inducer (Gaffney et al., 1993). Further, *SID*2 mutations reduce SA levels and are impaired in SAR (Wang et al., 2009), as *SID*2 encodes isochorismate synthase, an enzyme that catalyses the conversion of chorismate to isochorismate, an immediate precursor of SA (Wildermuth et al., 2001). Accordingly, exogenous application of SA also induces SAR in several plant species (Gaffney et al., 1993, Ryals et al., 1996, Van Loon and Antoniw, 1982).

1.5. Salicylic acid signalling in plant immunity

SA is a key signalling molecule involved in the activation of SAR (Durner et al., 1997). In Arabidopsis, biosynthesis of SA in response to pathogen infection requires the genes *SID2*/*ICS1* and *EDS5*. SA production is significantly reduced in *sid2* plants, suggesting that the majority of SA in Arabidopsis is produced from isochorismate instead of the alternative phenylalanine pathway (Coquoz et al., 1998, Leon et al., 1995, Mauch-Mani and Slusarenko 1996, Ribnicky et al., 1998, Wildermuth et al., 2001, Yalpani et al., 1993). Upon pathogen infection, SA synthesis is in part induced via up-regulation of the expression of *ICS1*. The two DNA binding proteins (transcription factors) SAR Deficient 1 (SARD1) and CBP60g (calmodulin binding protein 60 g) are key regulators of the induction of *ICS1* and SA synthesis. Both proteins are recruited to the promoter of *ICS1* in response to pathogen infection, suggesting that they control SA synthesis by regulating *ICS1* at the transcriptional level (Zhang et al., 2010).

EDS5 is also required for SA synthesis in response to pathogen attack (Nawrath and Metraux, 1999). It encodes a MATE family transporter that may be involved in transport of intermediates for SA biosynthesis (Nawrath et al., 2002). In expression-profiling experiments the effect of *eds5* is very similar to that of *sid2*, consistent with the idea that *EDS5* may be required for SA biosynthesis (Glazebrook et al., 2003). Pathogen-induced expression of *EDS5* requires *EDS1* and *PAD4* that encode lipase-like proteins with catalytic motifs (Falk et al., 1999; Jirage et al., 1999; Nawrath et al., 2002). In SA signalling, *EDS1* and *PAD4* function upstream of

pathogen-induced SA accumulation but their expression can be feed-forward up regulated by the exogenous applications of SA (Rustérucci et al., 2001).

1.6. Reactive oxygen and Nitrogen species and Plant Disease Resistance

One of the initial biochemical changes associated with activation of PTI and ETI is an oxidative burst producing reactive oxygen species (ROS) such as superoxide (O_2^-). Superoxide is highly unstable so it is readily converted to hydrogen peroxide (H_2O_2) either non-enzymatically or by the action of superoxide dismutase (Wojtaszek, 1997; Lamb and Dixon, 1997; Bolwell, 1999; Grant and Loake 2000). NADPH oxidase, a cell wall peroxidase, diamine and polyamine type enzymes are the different sources of production of ROS (Grant and Loake 2000), which include superoxide ($O_2^{\cdot-}$), hydroxyl (HO^-) and peroxyl radicals (ROO^-), hydrogen peroxide (H_2O_2), singlet oxygen (O_2) and hypochlorous acid/hypochlorite ($HOCl/^-OCl$) (Halliwell and Gutteridge, 2007; Smith, 2004).

Reactive nitrogen species (RNS), such as nitric oxide (NO) and its higher oxides, are another class of chemically reactive species that are involved in cell signalling during a variety of physiological and pathological processes (Bove and van der Vliet, 2006). NO is an important signalling molecule in plant disease resistance as scavenging of NO or inhibition of its production compromise immunity (Delledonne et al., 1998; Durner et al., 1998). Simple structure and high diffusivity make NO an ideal signalling molecule in species from every biological kingdom (Arasimowicz and Floryszak-Wieczorek, 2007; Hong *et al*., 2007). The initiative to

uncover various roles of NO in plants is a recent one, while its importance to animal biology including in respiration, apoptosis, gene expression, cell motility and blood flow, was recognized quite early. NO plays an important role in many cellular processes of plants such as respiration, programmed cell death, seed germination, flowering and stomatal closure (Lamattina et al., 2003; Neill *et al*., 2003 ; Delledonne, 2005; Lamotte et al., 2005). Several potential NO sources may be distinguished in plants, with the physiological role of each probably depending on the species, type of tissue or cells, and external conditions or signalling cues (Neill et al., 2003). NO is generated by either constitutively expressed or induced NO synthases (NOSs) and NO can also possibly be produced from higher nitrogen oxides (such as NO_2^-) that are derived from exogenous or endogenous sources (Benhar et al., 2009).

NO readily reacts with superoxide (O_2^-) to produce peroxynitrite ($ONOO^-$) (Clarke *et al*., 2000; Zaninotto *et al.*, 2006). The balanced production of NO and H_2O_2 during attempted pathogen infection triggers cell death (Wendehenne *et al.*, 2004). Interestingly there are number of systems where NO even acts as protectant against ROS (Squadrito and Pryor, 1995). NO also plays a vital role in abiotic stresses and its exogenous application improves plants tolerance against drought and cold (Siddiqui et al, 2010). Moreover, exposure to low levels of NO helps in reducing the destructive effects of heavy metals. (Arasimowicz and Floryszak-Wieczorek, 2007).

ROS and RNS are cytotoxic and signal through cysteine modifications (Fig. 1.2) and due to their highly reactive nature and thus must be tightly controlled (Grant and Loake 2000). Abundant small-molecule redox couples in the cell are thought to buffer ROS and RNS and include NAD(P)/NAD(P)H, oxidized and reduced glutathione (GSH/GSSG) and oxidized and reduced ascorbate (Spoel and Loake 2011). In addition to direct modification by ROS/RNS, redox-sensitive proteins are thought to be modified by changes in the ratio of these small-molecule redox couples. These redox-based protein modifications must be reversible to ensure transient signalling. Oxidative cysteine (Cys) modifications are integral to a variety of redox-based cellular signalling mechanisms. These residues possess a low-pKa sulfhydryl group, promoting susceptibility to oxidation (Meng et al., 2002). Reactive Cys may be subject to an assortment of redox-based post-translational modifications, including formation of *S*-nitrosothiols (SNO), sulphenic acid (SOH), disulphide (S–S), *S*-glutathionylation (S–SG), sulphinic acid (SO_2H) and irreversible sulphonic acid (SO_3H) (Spadaro et al., 2010). *S*-nitrosylation, the addition of an NO moiety to a reactive Cys thiol, is quickly emerging as a key regulatory feature during the establishment of plant disease resistance. Deployment of the biotin-switch technique (Stamler et al., 2001) has recently lead to the identification of metabolic, structural and regulatory proteins that are specifically *S*-nitrosylated during the plant defence response (Lindermayr et al., 2005; Romero-Puertas et al., 2007; Wang et al., 2009).

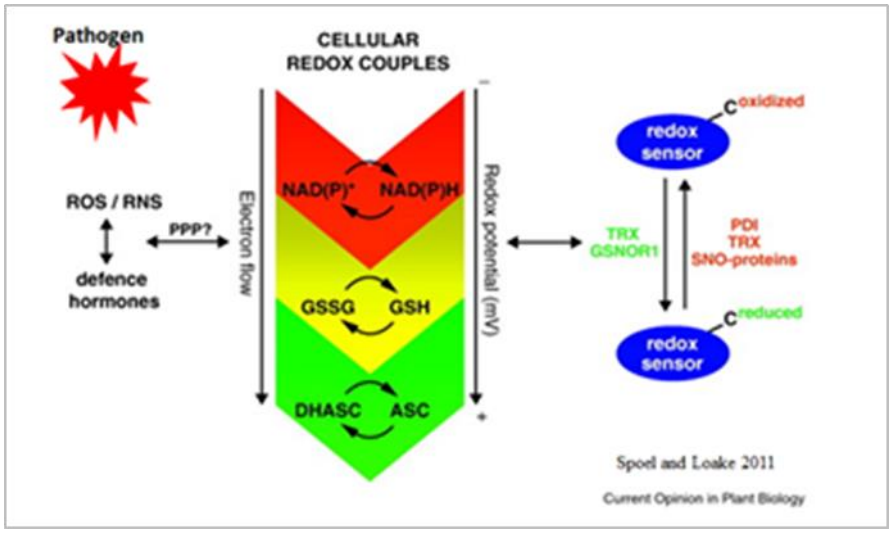


Figure 1-2 ROS/RNS signal through cysteine modifications.

Reactive oxygen and nitrogen species (ROS/RNS) as well as defence hormones accumulate following pathogen challenge. Changes in ROS/RNS are detected by the cellular redox couples $NAD(P)^+$/NAD(P)H, oxidized/reduced glutathione (GSSG/GSH), and dehydroascorbate/ascorbate (DHASC/ASC) in order of increasing redox potential. Changes in these cellular redox couples are ultimately sensed by the reactive cysteines of redox sensor proteins that reversibly switch between oxidized and reduced states. Their reduction is catalysed by TRX and GSNOR, while their oxidation is facilitated by PDI, TRX and selected SNO-proteins. See main text for details. *This figure has been reproduced from Jones and Dangle, 2006.*

1.7. Role of GSNOR and cognate *S*-Nitrosylation in Plant Disease Resistance

The reaction of NO with the antioxidant glutathione (GSH) results in the formation of *S*-nitrosoglutathione (GSNO), which acts as mobile reservoir of NO bioactivity; *S*-nitrosylation is emerging as a prototypic redox-based, post-translational modification (Wang et al. 2006; Yun et al. 2011). *S*-nitrosylation is involved in the regulation of protein functions; for example *S*-nitrosylation of SA-binding protein (SABP) 3 resulted in inhibition of its carbonic anhydrase (CA) activity and ultimately compromised *R* gene-mediated disease resistance (Wang et al., 2006). Protein *S*-nitrosylation is a non-enzymatic and relatively spontaneous event. Therefore, removal of NO from cysteine thiols in proteins, also known as denitrosylation, has emerged as an important regulatory mechanism that is catalysed by dedicated enzymes (Benhar et al., 2009). For example, the cysteine protease, caspase-3 (required for programmed cell death) in mammals is inactivated by *S*-nitrosylation (Melino et al. 1997), while it is reactivated through denitrosylation (Benhar et al., 2008). In this context, two main enzyme systems have emerged as important reductases/denitrosylases, namely the *S*-nitrosoglutathione Reductase (GSNOR) system consisting of GSH and GSNOR and a second system consisting of thioredoxin (TRX) and NADPH-dependent TRX Reductase (NTR) (Benhar et al., 2009). GSNOR was initially purified from *E. coli*, yeast, and mouse macrophages, all of which efficiently turned over GSNO (Liu et al. 2001). Recently this enzyme has also been identified in plants. *Arabidopsis thaliana* GSNOR1 (AtGSNOR1) turns over GSNO and is thought to be the major regulator of protein *S*-nitrosylation (Feechan et al., 2005). Compared to wild type, elevated levels of SNO were found in

the T-DNA knock out mutant *atgsnor1-3*. Conversely, the *AtGSNOR1* overexpresser mutant *atgsnor1-1* displayed reduced SNO levels. Accumulation of excessive SNO is associated with susceptibility, as the *atgsnor1-3* mutant was compromised in all forms of disease resistance (Feechan et al., 2005; Rusterucci et al., 2007; Loake and Grant, 2007; Yun et al., 2011). Molecular analysis revealed that increased *S*-nitrosylation result in the reduction of both SA biosynthesis and signalling (Feechan, 2005; Arasimowicz and Floryszak-Wieczorek, 2007). AtGSNOR1 also plays an important role in the HR since *atgsnor1-3* mutants have been reported to display enhanced HR as compared to wild-type plants when challenged with avirulent *Pst DC3000* expressing *avrRps4* or *avrB*, demonstrating that upon pathogen infection, total SNOs administered by AtGSNOR1 positively regulate the development of cell death even in the presence of reduced SA, a known cell death agonist. However, while SNOs surprisingly promote cell death formation, this cell death is ineffective in limiting pathogen growth, indicating defects in regulation of the HR (Yun et al., 2011).

1.8. Role of Thioredoxins in plant defence

Besides GSNOR1, other reductases have been identified to play important roles in protein denitrosylation in animals. TRX has been reported to act as a protein denitrosylase by directly removing SNO groups from cysteines using the reducing power of NADPH (Benhar et al., 2009). In plants, TRX have been shown to be involved in limiting oxidative damage by preventing protein oxidation (Vieira Dos Santos and Rey, 2006). Plant TRX proteins have been extensively studied and

disulphide reduction is most conspicuously catalysed by these enzymes (Meyer et al., 1999). TRX is a small (12 kDa), multifunctional protein with a redox active dithiol/disulphide active site, frequently consisting of WCxPC (with x = G or P) where reversible oxidation occurs through the transfer of reducing equivalents from the catalytic site cysteine residues to a disulphide target (Meyer et al., 1999). TRX reduce disulphide bonds in many different target enzymes present in all sub-cellular compartments and are involved in many biochemical reactions (Fig. 1.3). Classic TRX contain a highly organized, globular structure composed of five β-sheet strands enclosed by four α-helices (Holmgren, 1985). Although the amino acid sequence among TRX enzymes from different organismal kingdoms can vary greatly, structural studies indicate their tertiary structure is highly conserved (Meyer et al., 2009).

TRX was initially discovered in *E. coli* where it was believed to function as a hydrogen donor to ribonucleotide reductase, an essential enzyme that converts ribonucleotides to deoxyribonucleotides, which are the building blocks of DNA in the cell (Laurent, Moore & Reichard, 1964). TRX in *E. coli* also perform various other functions namely roles in sulphate assimilation, phage assembly and a general function as a disulphide oxidoreductase (Holmgren, 1985). The latter function is certainly now biochemically well documented and relies on the property that the TRX active site shows an exceptional reactivity (*i.e.* 10,000-fold better than dithiothreitol, the best chemical reductant at the same pH) (Holmgren, 1979).

The activity of plant TRX is recycled by two distinctive redox systems using different reducing agents (Spoel and Van Ooijen, 2013). Chloroplast TRX members utilize ferredoxin and ferredoxin reductase in light-mediated reactions that target enzymes of the reductive pentose phosphate or Calvin cycle (Buchanan et al., 1994). Like animal and *E. coli* TRX, plant cytosolic TRX-*h* enzymes utilize NADPH-dependent TRX Reductase A (NTRA) to recycle their reductive capacity (Fig. 1.3). The TRX-*h* system comprised of TRX3/5 and NTRA was recently shown to be involved in NPR1-mediated plant immune responses. NPR1 is a redox sensitive protein that functions as a global coactivator of defence gene expression. Oxidative thiol modifications were shown to play an important role in modulating the activity of NPR1 (Dong, 2004). Before pathogen attack, conserved cysteines in NPR1 form intermolecular disulphide bonds, resulting in the formation of a cytosolic NPR1 oligomer. This renders NPR1 transcriptionally inactive as it is excluded from the nucleus. Following pathogen attack, SA induced redox changes that partly convert NPR1 from oligomer to monomer, which is translocated to the nucleus where it binds to TGA transcription factors and activates *PR* gene expression (Mou et al., 2003; Spoel et al., 2010). SA-induced cellular reduction also breaks down intermolecular disulphide bonds in TGA1 and TGA4, allowing them to form a transcriptionally active complex with NPR1 in the nucleus (Despres et al., 2003). Importantly, the SA-induced NPR1 oligomer-to-monomer reaction is catalysed by TRX5, a highly pathogen-inducible gene (Laloi et al., 2004). Consequently, deletion of *TRX5* or *NTR* isoform A (NTRA) genes results in reduced expression of NPR1-dependent immune genes and failure to induce SAR (Tada et al., 2008). Accordingly, TRX have been reported to control gene expression by modulating the oxidative state

of various transcription factors (Hirota et al., 2000), highlighting the importance of these enzymes in nuclear signalling.

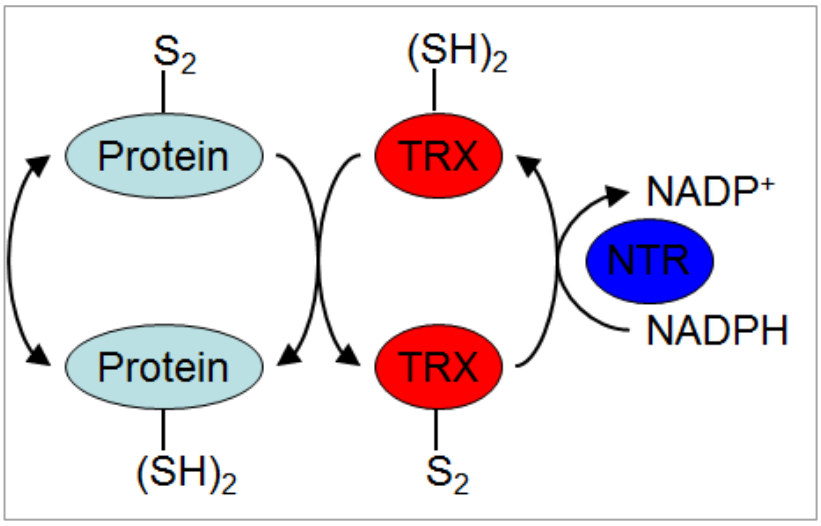


Figure 1-3 Disulphide bond reduction by the TRX system.

Disulphide bonds [S_2] in target proteins are reduced to free thiols [$(SH)_2$] by the action of reduced TRX. Consequently, TRX becomes oxidized but is reactivated by NTR using the reducing power of NADPH.

1.9. Nucleoredoxins are unique TRX family members

Nucleoredoxins (NRX) that represents novel, potentially nuclear localized members of the TRX superfamily were first identified by Kurooka et al., 1997 and have been reported to possess oxidoreductase activity against insulin (Kurooka et al., 1997). NRXs are evolutionarily conserved from invertebrates to vertebrates. NRXs orthologues are found in rats, dogs, red monkey and also in nonmamalian vertebrates that include clawed frog, chicken and zebrafish and are considered to be the novel members of TRX family because unlike TRX, NRXs are composed of multiple TRX-domain modules (Funato and Miki, 2007), so they might target different substrates. Maize NRX has been reported to be composed of three TRX-like modules arranged as direct repeats of the classic TRX domain and the first and third modules contain the amino acid sequence WCPPC which indicates the potential for TRX oxidoreductase activity, and insulin reduction assays indicate that at least the third module possesses TRX enzymatic activity (Laughner et al., 1998). Maize NRX was named on the basis of structural and sequence similarities with mouse NRX (Kurooka et al., 1997). *Arabidopsis* NRX1 and NRX2 have been identified but their function remains completely unknown. *Arabidopsis* NRX1 has also been reported to have three TRX modules (Funato and Miki, 2007) with the first and third containing active site cysteines in the respective motifs WCGPC and WCPPC, both of which are also found in the TRX-*h* subfamily TRX-*h* (Fig.1.4). Moreover, NRX2 also contains two domains with active sites reminiscent of the TRX-*h* subfamily (Fig. 1.4). Besides, it has also been reported that in the case of *Crithidia fasciculate* TryX2 (tryparedoxin 2), the wild-type form (WCPPC) and its TRX-mimicking mutant form

(WCGPC) showed similar reducing activity against insulin and TryX peroxidase (Steinert et al., 200). This result proposes that the WCPPC and WCGPC motifs do not have such a prominent difference, at least in their reducing activities. Thus, NRX proteins may perform functions similar to TRX-*h* but in the nucleus, which currently is a black box with regard to regulation of protein oxidation/reduction events. Because many nuclear defence regulators are active in their reduced states (*e.g.* NPR1 and TGA1/4) (Mou et al., 2003; Tada et al., 2008; Spoel et al., 2009), the action of potentially nuclear localized NRX proteins may be indispensable for appropriate regulation of plant immune responses. It has been reported that in case of Maize NRX, the presence within the nucleus of a multiple-TRX modular protein with a putative Zn finger offers intriguing possibilities for the regulation of transcription factors by alteration of their redox state and the number of transcription factors known are known to be influenced by changes in their own redox state (Laughner et al., 1998).

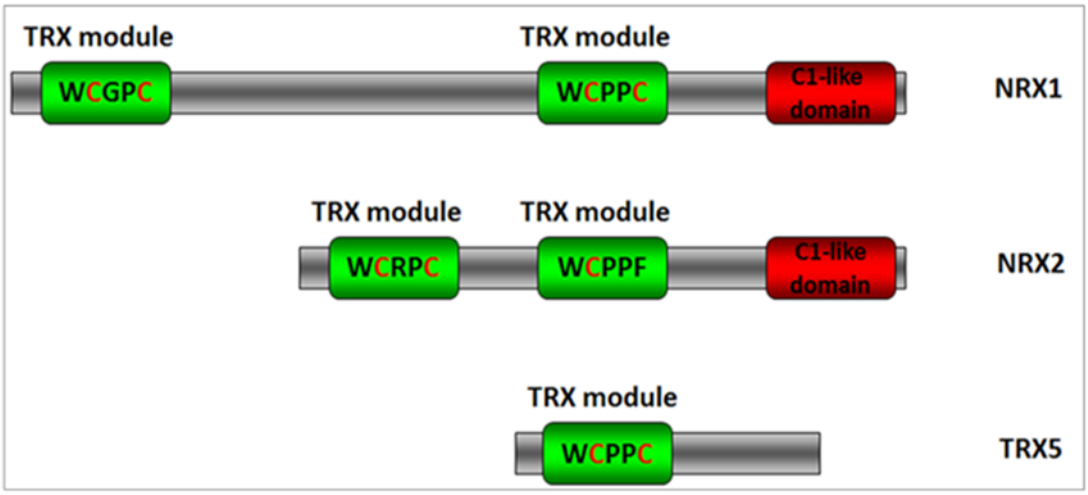


Figure 1-4 Structural comparisons of NRX1, NRX2 and TRX5.

NRX1 and NRX2 have two TRX modules with active site cysteines, while TRX5 has only one. NRX proteins also have C1-like domains (missing in TRX5) that may be involved in binding zinc or lipids.

1.10. Aims of the project

Oxidative stress causes an imbalance in the production of ROS that can cause oxidative damage to signalling proteins. To counteract this oxidative damage, plants have evolved the above described enzymes and antioxidants to detoxify the cellular environment. In this study we focused on two important categories of enzymes that may serve as antioxidants, namely GSNOR1 and NRX enzymes.

In this context, we first hypothesized that elevated GSNO levels suppress accumulation of SA by inhibition of SA biosynthesis genes. So in Chapter 3, we checked the expression of SA biosynthesis genes including *ICS1*, *EDS5* and *PAD4* in wild type Col-0 and mutant *gsnor1-3* plants. We found that the pathogen-induced expression of these genes was suppressed in *gsnor1-3* knock-out mutants. Surprisingly, suppression only occurred in compatible plant-pathogen interactions and was mediated by suppression of *SARD1* and *CBP60g* expression, two key transcription activators of SA biosynthetic genes. Several hypotheses for why SA accumulation is low in the presence of high levels of *S*-nitrosylation are discussed: (1) *S*-nitrosylation suppresses expression of the above mentioned genes that encode enzymes for SA synthesis; (2) *S*-nitrosylation could increase the mRNA turnover of genes encoding enzymes of SA synthesis; and (3) SA biosynthesis enzymes or their transcriptional activators are post-translationally modified, blunting their activity.

In Chapter 4, we hypothesized that like classical TRXs, the novel nuclear members of the TRX superfamily, NRXs are also involved in the immunity.

Therefore, we characterized NRX1 and NRX2, and show these enzymes play a central role in defence signalling. We particularly studied the role of *NRX1* in regulating transcriptional events during the immune response. NRX1 was found to suppress the basal expression of SA biosynthesis genes. Crosses between an *nrx1* knock-out mutant and SA deficient mutants showed that this activity required SA, indicating NRX1 is part of a regulatory feedback loop in SA signalling.

Classical TRX is involved in disulphide bond reduction, so we hypothesized that NRX enzymes would perform the same enzymatic activity. In Chapter 5 we found that both NRX1 and NRX2 are potent disulphide reductases, albeit to a lesser extent than conventional TRX5 protein. *In vitro* disulphide reducing activity of NRX1 was concentration dependent and required both active sites. Surprisingly, NRX1 activity could not be recycled with either reduced glutathione or NADPH/NTRA, the two major cellular reducing systems utilized by the TRX superfamily, indicating NRX1 exhibits novel oxidoreductase features that are distinct from conventional TRX proteins. Cysteine mutations in the two active sites demonstrated that both active sites are capable of disulphide reduction and that they may negatively regulate each other.

Taken together, the presented bioassays, genetic and biochemical analyses demonstrate that the two redox enzymes GSNOR1 and NRX1 play fundamental roles in SA-dependent plant disease resistance.

Chapter 2

Material and methods

Chapter 2

2. Material and methods

2.1. Liquid *P. syringae* cultures for plant inoculation

A relatively generous amount of inoculum of *Pseudomonas syringae* pv. *maculicola* (*Psm*) strain ES4326 was picked up from the stock plate and was inoculated with 5ml of King's broth (KB) medium (20 g/l Protease peptone, 10 ml/l glycerol, 1.5 g/l K_2HPO_4, 15 g/l agar) or LB medium (10 g/l Tryptone, 5 g/l Yeast Extract, 10 g/l NaCl) supplemented with 100 mg/l streptomycin and incubated in the 28 degree shaker for 24 hours. The cultures were quite dense after overnight incubation (OD_{600}=1.0 or higher)). The following day cells were pelleted by brief centrifugation and re-suspended in an appropriate volume of 10 mM $MgSO_4$ to OD_{600} = 0.002 - 0.0002 (the concentration of the bacterial suspension was adjusted according to the purpose of the experiment and inoculation techniques used) expresses as cfu (colony forming units).

2.1.2. Plant disease resistance assay

All the genotypes for this study were grown as six per pot at 20° C in a pathogen free chambers under long day conditions (16 light/8h darkness). The potting medium consisted of peat moss, vermiculite and sand with 4:1:1 respectively. Four weeks old Col-0, *nrx1*, *nrx2* and *npr1* were sprayed with 0.5 mM SA solution

and water (as control). After 24 hours, plants were inoculated with *Psm* ES4326 suspension (OD_{600}= 0.0015) in 10 mM $MgSO_4$ on the abaxial side of the leaf using a 1 ml syringe (Cao *et al.,* 1994). Three leaves per plant and six plants per line were inoculated and examined for bacterial growth 3 days post inoculation. Bacterial growth was assessed by punching out a leaf disc which was subsequently ground in 10 mM $MgSO_4$. Serial dilutions were then plated onto LB plates supplemented with 100 mg/l streptomycin, 10 mM $MgSO_4$ and 100 mg/l cycloheximide.

2.2. Nucleic acid related Methods

2.2.1. Extraction of *Arabidopsis thaliana* genomic DNA

Fresh leaf samples were collected in 1.5 ml micro-centrifuge tubes and put in a container with liquid nitrogen for the total genomic DNA extraction. The leaves were ground using 300 µl CTAB buffer and metal beads. Following grinding, the extract was incubated at 65^{o}C in a water bath for 10 minutes. The extract was then allowed to cool. A total of 300 µl of chloroform was then added to the extract and mixed by thorough vortexing. The samples were centrifuged for 2 min at 15,000 rpm to separate the phases. The aqueous phase (supernatant) was then transferred to a new eppendorf tube containing an equal volume of (~300 µl) isopropanol. The samples were mixed well by inverting the tubes and centrifuged again for 5 min at 15,000 rpm to pellet the DNA. Supernatant was discarded and pellet was washed with 70% ice cold ethanol and centrifuged at 15,000 rpm for 2 min. Finally, the dry pellet was dissolved in 50-100 µl H_2O or TE buffer after carefully removing ethanol.

2.2.2. RNA Extraction

RNA extraction was either performed using RNeasy Plant Mini Kit (QIAGEN) according to manufacturer's instructions or through LiCl method described below.

Tissues were finely grounded in liquid nitrogen and 0.5 ml pre-warmed RNA extraction buffer (100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% SDS) and 0.5 ml phenol:chloroform: isoamylalcohol mixture (25:24:1) were added after which samples were vortexed vigorously. Samples were then centrifuged at maximum speed for 5 min at 4ºC. The aqueous phase (upper phase) was transferred to a new tube and 0.5 ml cold chloroform: isoamylalcohol (24:1) was added. The samples were again centrifuged (maximum speed for 5 min at 4ºC) after vigorous vortexing. A total of 1/3 volume (~150 µl) of 8 M LiCl was added to the aqueous phase and was incubated overnight at 4ºC. The next day, centrifugation was carried out at a maximum speed for 15 min at 4ºC. A small clear pellet of total RNA was visible. The supernatant was carefully removed without disturbing the pellet. Then the RNA pellets were washed gently with ice-cold (-20ºC) 0.5 ml of 70% ethanol. It was centrifuged again at maximum speed for 1 minute at 4ºC to make sure the RNA pellet stayed in place. The supernatant was discarded without disturbing the RNA pellet. The centrifugation step (1 min at 4ºC) was repeated to remove any remaining 70% ethanol and RNA pellet was dried in the Speed Vacuum for 1 min without heating. A total of 400 µl ddH_2O (autoclaved) was added to the pellet and kept on ice

for 30-60 minutes to rehydrate the RNA pellet. The RNA pellet was dissolved by vigorously pipetting the solution up and down. Then 40 µl 3M NaAc pH 5.2 (autoclaved) and 1 ml of ice-cold (-20ºC) 96% ethanol was added to this. The samples were then inverted to mix and incubated at least 60 minutes at -20ºC and centrifuged again at maximum speed for 15 min at 4ºC, visualizing a gel-like pellet of pure RNA. The supernatant was removed and the RNA pellet washed gently with ice-cold (-20ºC) 0.5 ml of 70% ethanol. Centrifugation was carried out at 13000 rpm speed for 1 min at 4ºC to make sure the RNA pellet stayed in place. The supernatant was discarded without disturbing the RNA pellet and the centrifugation step was repeated (1 minute at 4ºC) to remove any remaining 70% ethanol. Finally the RNA pellet was dried in the Speed Vacuum for 1 min without heating. Then 25 µl ddH_2O (autoclaved) was added and RNA pellet allowed to rehydrate for 30 min before resuspension. RNA quality and concentrations were measured on a nanodrop.

2.2.3. cDNA synthesis

cDNA synthesis was carried out either with a Superscript III Reverse Transcriptase (Invitrogen) kit or with an Omniscript RT (QIAgen) kit according to the manufacturer's instructions. In this study, RNA was first denatured at 65°C for 10 min. Then master mix containing all the reagents was added in the denatured RNA along with the measured quantity of water and the reaction mixture was incubated first at 37°C for 1 hour and then at 72°C for 5 min (to stop the reaction).

2.2.4. Reverse transcriptase PCR (RT-PCR)

The cDNA obtained from Col-0, *gsnor1-3*, *nrx1*, *nrx*2, *nrx1nrx*2 and *npr*1 was used as template in PCR amplification using primers listed in Appendix 1. The following components were set up in a PCR tube: cDNA product, 1X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 1X Crimson Taq polymerase (New England Biolabs), 10 µM forward and reverse primers. The PCR was performed with 30 cycles: 30 seconds denaturation at 94°C, 30 seconds annealing at 54-62°C, and 30 seconds extension at 72°C for homemade Taq and 68°C for Crimson Taq. The amplified products were analyzed by electrophoresis on 1.2% agarose gel along with 100 bp DNA ladder.

2.2.5. Quantitative PCR (q-PCR)

Quantitative PCR (q-PCR) is a method used to detect relative or absolute gene expression level. The qPCR machine detects the fluorescence and software calculates thresh hold cycle (Ct) values from the intensity of the fluorescence. cDNA sample was further diluted in a number of different dilutions to set a standard curve in order to generate a CT value. SYBR Green (Sigma) was used as a fluorescent probe. The following components were set in especially designed PCR strips: cDNA dilutions, 1X SYBR Green, 3 µM forward and reverse primers. Rotor gene software was used for complete analysis of the results generated by the q-PCR machine. The primers designed for q-PCR are listed in Table 1.

Table 1 Primers used in qPCR analysis

Gene ID	Forward primer	Reverse primer	Primer length
UBQ10	TGACAACGTGAAGGCCAAGATCC	ATACCTCCACGCAGACGCAACAC	23/23
NRX1	GCGAACAGGTGAAAGTTGAC	CTGTGGAGTAAACCGCTGAC	20/20

2.3. Genotyping of the crosses

Seeds were harvested, sown and stratified in the cold for 2-3 days. A total of 36 plants per cross were used for extracting total genomic DNA. The crosses were then analyzed using their respective primers given in Appendix 2. Genomic DNA (1 µl), 1X Crimson Taq buffer, 10 mM dNTPs, primer 1(10 µM), primer 2(10 µM), water. The PCR was performed with 22-30 cycles of denaturation: 30 seconds at 94°C; annealing: 30 seconds at 54-62°C; and extension 30 seconds at 68°C. The *pad4* and *npr1* mutations are single base pair changes that were analyzed by cleaved amplified polymorphic sequence analysis using the restriction enzymes *BSmF1* and *NlaIII* (New England Biolabs).

2.4. Overview of cloning into pET28a vector and sequencing

DNA fragments obtained by PCR using phusion DNA polymerase were ligated in the pET28a vector system [Promega, Fitchburg, USA]. A reaction mixture containing 4.2 µl of gel extracted PCR product, 1 µl pET28a Vector and 0.8 µl T4 fast ligase within an appropriate buffer were incubated at RT for an hour. Subsequently 10 ul of the ligation mixture was transformed into 100 µl of competent XL-10 competent *E. coli* cells. The transformed cells were plated onto LB medium containing kanamycin50 (50 mg/l) for the detection of integrated insert into pET28a vector. A few colonies were picked under sterile conditions and grown over-night in 5 ml LB medium with the kanamycin50. Plasmid DNA was extracted using the QIAGEN mini kit following the manufacturer's instructions. Plasmid DNA and the

T7 forward and reverse sequencing primers were submitted for sequencing in individual reactions. Sequencing was performed by the Gene-pool genomics facility at the University of Edinburgh. Primers used for cloning of genes used in in this study are listed in appendix 3.

2.4.1. Preparation and transformation of E. coli competent cells

A fresh colony of an *E. coli* strain was used to inoculate a 5 ml LB medium culture grown at 37^{o}C overnight in the presence of the appropriate antibiotic. This was used to inoculate a 500 ml LB medium culture which was grown at 37^{o}C to an OD_{600} of 0.4-0.5. The cells were pre-chilled by swirling the culture flask for 1-2 min in an ice bath and subsequently centrifuged at 13000 rpm for 10 min at 4^{o}C. The cells were resuspended in 50 ml of ice cold TSS buffer [LB-medium supplemented with 10 % (v/v) PEG 3350, 5% (v/v) DMSO, 1 M MgSO4, pH 6.5] and snap frozen in liquid nitrogen in appropriate aliquots. The cells were stored at 80^{o}C until used for transformation.

For transformation of plasmid DNA, a 100 µl competent cell aliquot was thawed on ice. A total of 10 µl plasmid DNA (ligation) was added in competent cells and incubated for 15-30 min on ice. The cells were heat shocked by incubating them for 30 sec at 42^{o}C. This was followed by immediate incubation on ice for a further 2 min. A total of 900 µl of pre-warmed (sterile) SOC-medium [SOB {(2% w/v tryptone (20 g), 0.5% w/v Yeast extract (5 g), 8.56mM NaCl (0.5 g), or 10mM NaCl (0.584 g), 2.5mM KCl (0.186 g), ddH_2O to 1000 ml, 10mM $MgCl_2$ (anhydrous 0.952

g) or 10mM $MgSO_4$ (heptahydrate 2.408 g)}] +20mM Glucose) was then subsequently added to the cells which were then incubated for 1 hour at 37^{o}C shaker. An aliquot of 250-300 µl was spread onto LB-medium containing the appropriate antibiotic within a Petri-dish, which was incubated up-side down overnight at 37^{o}C (Walhout et al., 2000).

2.5. *In vivo* Protein synthesis

2.5.1. Production of recombinant His_6-tagged protein

2.5.1.1. Protein production

The host lab of Dr. Steven Spoel provided wild-type and active-site mutant NRX1 and NRX2 cloned into the *NdeI* and *EcoRI* sites of the pET28a vector. This vector was subsequently transformed into competent BL21 (DE3) cells. From a -80°C glycerol stock, BL21 (DE3) cells carrying pET28a/His_6-protein on LB +Kan50 was spread on plates supplemented with 50 mg/l kanamycin. The cells were grown overnight at 37°C. A colony was then picked and inoculated into 5 ml LB supplemented with 50 mg/l kanamycin. The culture was grown overnight at 37°C and 3ml added to 100 ml fresh LB supplemented with 50 mg/l kanamycin. The colony was grown for no more than 2 hours at 37°C with shaking before sterile-filtered IPTG was added to 1 mM. Cultures were then allowed to grow at 37°C for ~5 hours. The cells were harvested by centrifugation (~6,500 rpm for 15 minutes at

room temperature). The liquid LB media was poured off and excess liquid was removed by blotting paper. The pellet was stored at -20°C for short-term storage.

2.5.1.2. Protein extraction and purification

The bacterial pellet was re-suspended in 5 ml of extraction buffer [1X Bugbuster reagent (Novagen), 50 mM potassium phosphate (pH 7.4), 300 mM NaCl, 10 mM imidazole, 10 mM 2-mercaptoethanol]. Further 1µl/ml benzonase nuclease (Novagen) and 1x proteinase inhibitor was added to this. The cell suspension was incubated on a rotating mixer at a slow setting for 20 min at RT. Then the insoluble cell debris was removed by centrifugation at 16,000 *g* (4000 rpm) for 20 min at 4°C. The supernatants were combined in a fresh tube and kept on ice.

Plastic columns were packed with 0.5 ml of cobalt resin. The storage buffer was allowed to drain by gravity flow and the column was equilibrated with 2 resin-bed volumes (1 ml) of extraction buffer. The protein extract was applied to the column and the flow-through was collected and reapplied to the column. The columns were washed three times with 2 volumes (1 ml) of wash buffer (same as extraction buffer but without Bugbuster reagent and 2-mercaptoethanol) and as soon as the wash buffer was completely drained, the columns were eluted three times with 2 volumes (1 ml) of elution buffer (50 mM potassium phosphate pH 7.4, 300 mM NaCl, 500 mM imidazole). Each elution was then collected in a 1.5 ml eppendorf and placed on ice until further analysis. Proteins were analysed through SDS PAGE and

the gel was stained with Coomassie for at least 4 hours. The gel was finally destained in sequential steps to visualize protein

2.5.1.3. Testing expression and solubility of recombinant proteins

A total of 150 µl of pre-culture was transferred to two fresh vials of 5 ml LB + Kan50. 1 mM Sterile-filtered IPTG was added to one vial (+IPTG sample) and nothing was added to the second vial (-IPTG sample). Continued growth at 37°C was allowed for ~5 hours. A total of 1.5 ml of each vial was then transferred to an eppendorf tube. The cells were harvested by brief centrifugation: ~13,000 rpm for 1 min at RT. This was the ***first set*** of samples used in this study. The remaining 1.5ml culture was centrifuged to get the ***second set*** of samples, which was the 'total' sample used in this study. The liquid LB media was then poured off and excess liquid was removed using blotting paper. The bacterial pellets from the ***first*** and ***second*** set of samples were resuspended in 75 µl of extraction buffer containing 1µl/ml benzonase nuclease. The cell suspension was incubated on a rotating mixer for 20 minutes at RT. For the ***first set*** of samples, centrifuged was carried out at 16,000 *g* for 20 minutes at 4°C. Then 75 µl of supernatant was then transferred to a new tube and kept on ice. This was our 'soluble' sample. Any remaining supernatant was subsequently removed, so that only the pellet was left. The pellet was resuspended in 75 µl of fresh extraction buffer. This was our 'insoluble' sample. The total, insoluble and soluble samples were then analysed through SDS PAGE.

2.6. *In vitro* protein synthesis

The *in vitro* synthesis of proteins in cell-free extracts is an important tool for molecular biologists and has a variety of applications, including the rapid identification of gene products (e.g., proteomics), localization of mutations through synthesis of truncated gene products, protein folding studies, and incorporation of modified or unnatural amino acids for functional studies. The use of in vitro translation systems can have advantages over in vivo gene expression when the over-expressed product is toxic to the host cell, when the product is insoluble or forms inclusion bodies, or when the protein undergoes rapid proteolytic degradation by intracellular proteases.

2.6.1. Wheat germ extracts method

Wheat germ extract is a very efficient method for *in vitro* protein expression. This extract has low background incorporation due to its low level of endogenous mRNA. Wheat germ lysate efficiently translates exogenous RNA from a variety of different organisms, from viruses and yeast to higher plants and mammals. The wheat germ extract is recommended for translation of RNA containing small fragments of double-stranded RNA or oxidized thiols, which are inhibitory to the rabbit reticulocyte lysate. Both reticulocyte and wheat germ extracts translate RNA isolated from cells and tissue or those generated by *in vitro* transcription.

The wheat germ extract method used in this study for *in vitro* protein expression was adapted from Prof. Yasuomi Tada's protocols (Tada et al., unpublished). The protocol is divided into three steps; template DNA preparation, transcription and translation. The primers used for *in vitro* protein synthesis are shown in Table 2.

Table 2 Primers used in invitro protein synthesis

Gene ID	Forward primer	Reverse primer	Primer length
CBP60g At5g26920	ccagcagggaggtactatgaagattcggaacg	ccttatggccggatccaagagctctttttttttttattacaagccttccct	33/52
SARD1 At1g73805	ccagcagggaggtactatggcagggaagagg	ccttatggccggatccaagagctctttttttttttattagaaagggtttatatg	31/55

A. Template DNA preparation

Template DNA was prepared in two steps. In 1st round of PCR, linker sequences were added to the gene of interest. In the second round of PCR, by the aid of linker sequences Flag tag, T7 promoter and other desired sequences were added to the gene of interest. The PCR conditions used are given in Table 3 and 4

B. Transcription

The next step was to transcribe the double stranded DNA into single stranded mRNA *in vitro*. The 25 µl reaction mixture contains 10x transcription buffer, NTPs (2.5 mM), RNase inhibitor, 0.1 M DTT, 1x T7 RNA polymerase and second round PCR product. The reaction was incubated at 37°C for 3 hours. Later on, 1ul of the sample was run on agarose gel to check the mRNA quality and quantity. 4M ammonium acetate and 100% ethanol was added to the reaction mixture and kept at -20°C for 1 hour after careful mixing followed by centrifugation at 15,000 rpm for 20 min at 4°C. The supernatant was removed carefully. The pellet was then dried and dissolved in DEPC water.

Table 3 1st Round PCR (*in vitro* protein synthesis)

1st Round PCR			PCR conditions	
1.	10X reaction buffer	5ul	94°C	5min
2.	dNTPs mixture (2mM)	5ul	98°C	10sec
3.	$MgSO_4$ (25mM)	3ul	55°C	30sec @ 30cycle
4.	1st forward primer (10uM)	1.5ul	68°C	2min30sec
5.	1st reverse primer (10uM)	1.5ul	72°C	2min
6.	Plasmid	1.25ng	4°C	forever
7.	KOD polymerase	1ul		
	ddH2O	Xul		
	TOTAL	50ul		

Table 4 2nd Round PCR conditions for the generation of template DNA (invitro protein synthesis)

2nd Round PCR			PCR conditions	
1.	5X reaction buffer	5ul	98°C	1min
2.	dNTPs mixture (2mM)	5ul	98°C	10sec
3.	$MgSO_{4\ (25mM)}$	3ul	60°C	1min @ 10cycle
4.	SpT7u primer (10uM)	1.5ul	68°C	2min30sec
5.	SpT7dFLAG (100nM)	1.5ul	98°C	10sec
6.	Dereverse (100nM)	1.5ul	60°C	15sec @ 30cycle
7.	1st round PCR product	1ul	68°C	2min30sec
8.	KOD polymerase	1ul	72°C	2min
9.	ddH_2O	30.5ul	4°C	forever
	TOTAL	50ul		

C. Translation

The final step was translation of mRNA into protein. The translation mixture consisted of wheat germ extract, amino acid mix and mRNA. The mixture was incubated at 16^{o}C overnight followed by centrifugation at 15,000 rpm for 10 min at 4^{o}C. The supernatant was collected and stored at -20^{o}C.

2.7. Western Blot Analysis

In this method protein was separated by 10% SDS-PAGE at 120 V for 1 hour. Blotting was carried out using a mini trans-blot cell (Bio-Rad) in transfer buffer (25 mM tris, 200 mM glycine and 20% methanol) at 80 V and 4^{o}C for 3-4 hours. Proteins were then transferred onto a cellulose membrane (GE health care). The membrane was then blocked with 5% (w/v) skimmed milk powder in phosphate buffered saline (PBS) and 0.1% tween 20 (PBS-T) for 1 hour at room temperature. This step was done to block the nonspecific binding sites on the membrane. The blocked membrane was then incubated with primary antibody overnight at 4^{o}C, followed by three washes with PBS-T. Then the blot was incubated with secondary antibody for 1 hour at room temperature followed by three washes with PBS-T. The membrane was then washed with PBS only. Protein was detected by a Pierce ECL western blotting detection system (GE healthcare) according to the manufacturer's instructions and exposed to light-sensitive CL exosure film (Thermo Scientific).

2.8. Insulin Turbidity Assay

Recombinant full-length wild-type and mutant NRX proteins were assayed for enzymatic activity using an insulin turbidity assay described previously (Holmgren, 1979). Briefly, the 100 µl reaction volume contained 100 mM potassium phosphate (pH 7.0), 2 mM EDTA, 130 µM insulin and either 2 µM TRX5 or 2 – 8 µM NRX protein. The assay was initiated by the addition of either 0.3 mM DTT or 1 mM GSH or 0.2 µM NTRA and 1 mM NADPH. Measurements were performed at 650 nm at 2 min intervals in an Infinite® 200 NanoQuant (TECAN). Three replicates per reaction were used.

Chapter 3

GSNOR1 regulates expression of salicylic acid biosynthesis genes

3. GSNOR1 regulates expression of salicylic acid biosynthesis genes

Salicylic acid (SA) plays a central role in plant disease resistance. SA is synthesized in response to challenge by plant pathogens for the establishment of local and systemic acquired resistance (SAR) (Loake and Grant, 2007). NPR1 and TGA1 are key redox-controlled transcriptional regulators of SAR (Lindermayr et al., 2005). NPR1 functions as a global regulator of defence gene expression, modulating directly or indirectly approximately 10% of the *Arabidopsis* genome (Wang et al., 2006). The TGA transcription factors belong to the group of bZIP factors with TGACG motif (Lindermayr et al., 2010) and they bind to elements of the *PR1* promoter (Despres et al., 2000; Zhou et al., 2000; Fobert and Despres, 2005). It has been found that oxidative thiol modifications play an important role in regulating NPR1 activity and selected TGA factors (Fan and Dong, 2002; Mou et al., 2003; Tada et al., 2008; Spoel et al., 2009). In resting cells NPR1 exists as a disulphide-linked oligomer in the cytoplasm. Upon pathogen challenge, SA levels increase and change the cellular redox state, leading to reduction of disulphide bonds in NPR1 (Tada et al., 2008). Reduction of the NPR1 oligomer releases monomer that translocates to the nucleus where these bind to TGA transcription factors and activate the expression of a battery of pathogenesis-related (*PR*) genes (Kinkema et al., 2000). In unchallenged cells, there are intramolecular disulphide bonds in TGA1 and TGA4 that prevent interaction of these TGAs with NPR1 but following pathogen attack, SA-induced cellular reduction breakdowns these disulphide bonds allowing

these TGAs to bind to NPR1, thus making a transcriptionally active complex with NPR1 in the nucleus (Despres et al., 2003).

SA accumulation is regulated by cellular levels of *S*-nitrosoglutathione (GSNO), a redox molecule capable of *S*-nitrosylating proteins (*i.e.*, covalent attachment of nitric oxide to cysteines). GSNO Reductase 1 (GSNOR1) is thought to regulate cellular GSNO and global *S*-nitrosylation levels (Feechan et al., 2005). Mutations in *AtGSNOR1*, an *Arabidopsis thaliana* GSNOR, influence cellular SNO levels both under basal conditions and during attempted microbial ingress (Wang et al., 2006). In the absence of functional *AtGSNOR1*, effector-triggered immunity, basal resistance and NHR are all compromised (Feechan et al., 2005) as SA synthesis and signalling are suppressed in the *atgsnor1-3* mutant; this revealed the requirement of AtGSNOR1 in signalling via SA (Wang et al., 2006). However, how GSNOR1 controls SA accumulation remains unknown.

Pathogen infections induce SA synthesis through up-regulating the expression of *ICS1*, which encodes a key enzyme in SA production. Both SARD1 and CBP60g belong to a plant specific DNA binding protein family and are key transcriptional activators of *ICS1* gene transcription and associated SA synthesis as they are recruited to the *ICS1* promoter upon pathogen infection (Zhang et al., 2010). These proteins specifically bind to DNA motif GAAATTT which is overrepresented in promoters of CBP60g/SARD1 dependent genes and it is highly overrepresented in *ICS1* (Lin Wang et al., 2011).

It has been shown that the *SARD1* mutation leads to compromised basal defence, *cbp60g* mutants show enhanced susceptibility to virulent *Psm ES4326* and *Pto* DC3000 (Wang et al., 2011) while both local and systemic resistance were blocked in the double mutant *cbp60g sard1* preventing their binding to *ICS1* promoter (Zhang et al., 2010). Further SA levels and expression of *PR1* and *ICS1* was largely reduced in this double mutant and expression profiling placed the CBP60g/SARD1 between the PAD4/EDS1 and SA nodes in the defence signalling network, indicating that both CBP60g and SARD1 also affect defence reactions in addition to SA biosynthesis (Lin Wang et al., 2011).

Along with *ICS1*, there are certain other genes that are involved in SA synthesis and signalling that include *EDS5*, *PAD4* (described in chapter 1) and *PBS3*. *PBS3* encodes a GH3 adenylase family protein and mutations in *PBS3* cause enhanced susceptibility to *P. syringae* and SA levels are reduced during some pathogen responses (Jagadeeswaran et al., 2007; Lee et al., 2007; Nobuta et al., 2007).

In this chapter, we investigated if elevated GSNO levels suppress accumulation of SA by inhibition of SA biosynthesis genes. Indeed, we show that the pathogen-induced expression of several genes that are implicated in SA synthesis was suppressed in *gsnor1* mutants. Surprisingly, suppression only occurred in compatible plant-pathogen interaction and is likely mediated by suppression of a SARD1 and CBP60g function.

Results

3.1. GSNO alters the timing of expression of SA synthesis genes during an incompatible interaction

To test if elevated levels of GSNO affect the expression of genes involved in SA synthesis, wild-type Col-0 and *gsnor1-3* plants were infiltrated with avirulent *Pst* DC3000/avrRpt-2 by pressure infiltration. Samples were then harvested at 0, 12, 24 and 48 hours, after which RNA was extracted and converted into cDNA. RT-PCR was performed to analyse the expression of SA synthesis and signalling genes. While the wild type expressed the SA biosynthesis-related genes *ICS1*, *EDS5*, *PAD4*, and *PBS3* at 24 hours post inoculation, the *gsnor1-3* mutant displayed similar expression levels of these genes but already at 12 hours of infection (Fig. 3.1). Moreover, expression of *PBS3* exhibited non-host resistance and it sustained throughout the infection (Fig. 3.1). As reported previously, expression of the downstream SA-responsive *PR-1* gene was only slightly lower in *gsnor1-3* compared to the wild type. These results suggest that during incompatible plant-pathogen interactions the timing of expression of SA synthesis genes is altered but not the level of expression.

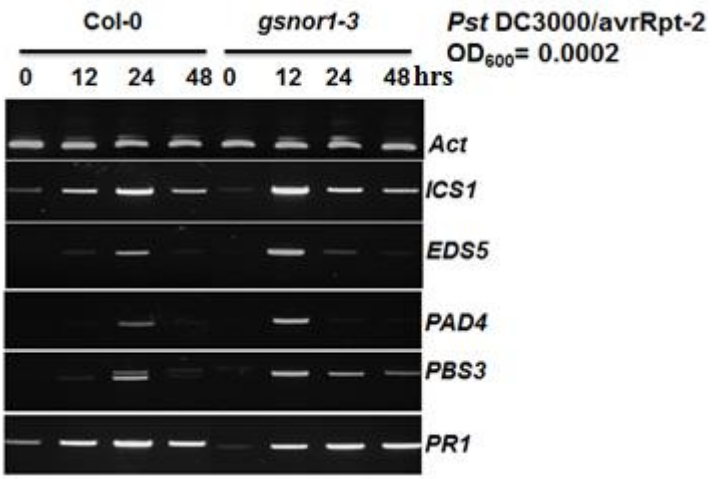


Figure 3-1 SA synthesis gene expression in wild-type Col-0 and mutant *gsnor1-3* plants during incompatible interaction.

Plants were infiltrated with *Pst* DC3000/avrRpt-2 at OD_{600}= 0.0002 with 1 mm needleless syringe and tissue harvested for analysis at 0, 12, 24 and 48 hours post inoculation. Expression of SA biosynthesis-related genes *ICS1*, *EDS5*, *PAD4*, *PBS3*, and SA-responsive *PR-1* was analysed by RT-PCR. Constitutively expressed *Actin* (*Act*) indicated equal loading. Experiments were repeated twice with similar results.

3.2. GSNO suppresses expression of SA synthesis genes during compatible interaction

To explore if GSNO also affects SA synthesis genes during a compatible plant-pathogen interaction, we performed the same experiment with virulent *Pst* DC3000. Wild-type Col-0 and *gsnor1-3* plants were infected with *Pst* DC3000 at a concentration of OD_{600}= 0.0002. Figure 3.2 shows that all the SA synthesis genes and SA-responsive *PR-1* were strongly induced by infection in Col-0 plants. However, induction of both the SA synthesis genes and *PR-1* gene were strongly reduced in *gsnor1-3* plants (Fig 3.2). Thus, elevated endogenous GSNO levels suppressed expression of SA biosynthetic genes during compatible plant-pathogen interaction.

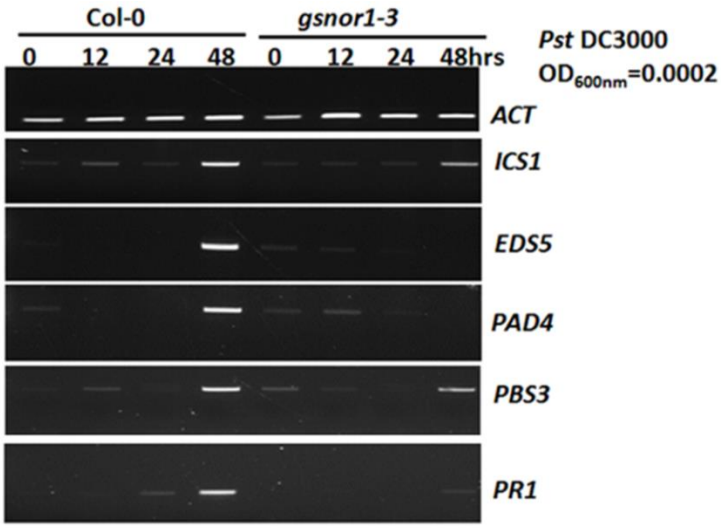


Figure 3-2 SA synthesis gene expression in wild-type Col-0 and mutant *gsnor1-3* plants during compatible interaction.

Wild-type Col-0 and *gsnor1-3* were infiltrated with *Pst* DC3000 at OD_{600}= 0.0002 with 1 mm needleless syringe and tissue harvested for analysis at 0, 12, 24 and 48 hours post inoculation. Expression of SA biosynthesis-related genes *ICS1*, *EDS5*, *PAD4*, *PBS3*, and SA-responsive *PR-1* was analysed by RT-PCR. Constitutively expressed *ACT* indicated equal loading. Experiments were repeated twice with similar results.

3.3. *SARD1* & *CBP60g* gene expression is down regulated in *gsnor1-3* mutants

We then looked at the factors that are involved in the induction of *ICS1* and chose to analyse the expression of *CBP60g* and *SARD1*. Infection with *Pst* DC3000 strongly induced gene expression of *CBP60g* and *SARD1* in wild-type Col-0 plants and was associated with expression of *ICS1* (Fig. 3.3). In contrast, expression of these genes was strongly reduced in the *gsnor1-3* mutant, suggesting that decreased expression of *ICS1* in *gsnor1-3* might be due to suppression of *CBP60g* and *SARD1*.

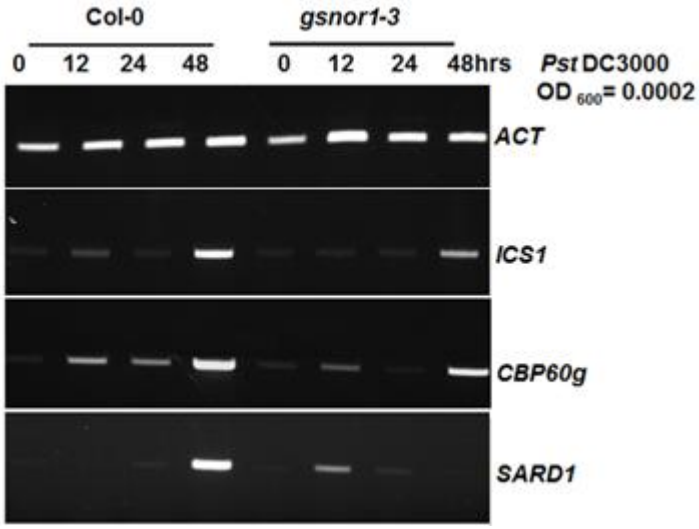


Figure 3-3*CBP60g* and *SARD1* are down regulated in *gsnor1-3* mutant.

Wild-type Col-0 and *gsnor1-3* were infiltrated with *Pst* DC3000 at OD_{600}= 0.0002 with 1 mm needleless syringe and tissue harvested for analysis at 0, 12, 24 and 48 hours post inoculation. Expression of *ICS1* and its transcriptional regulators *CBP60g* and *SARD1* was analysed by RT-PCR. Constitutively expressed *ACT* indicated equal loading. Experiments were repeated twice with similar results.

3.4. CBP60g and SARD1 control transcriptional regulation of other SA defence genes

It has been reported that DNA motif GAAATTT bound by CBP60g and SARD1 in *ICS1* promoter also occurs in the promoters of other CBP60g and SARD1 dependent genes. Therefore CBP60g and SARD1 probably directly control the expression of defence genes in addition to *ICS1* and perhaps CBP60g and SARD1 also bind related sequences in the promoters of genes whose expression was down-regulated in the *cbp60g sard1* mutant (Lin Wang et al., 2011). But the names of the CBP60g and SARD1 dependent defence genes have not been clearly assessed. Therefore, we analysed the occurrence of CBP60g and SARD1 binding motifs and related sequence in the promoters of *PAD4, EDS5*, *PBS3* and *PR1* and found the occurrence of GAAATTT and related sequence GAAATT in the promoters of *EDS5, PBS3* and *PR1* (Fig. 3.4). Hence we suggest that in addition to *ICS1*, CBP60g and SARD1 may directly regulate the expression of *EDS5, PBS3* and *PR1* through binding to GAAATTT and its related sequence in the promoters of these genes. But this motif is not present in the *PAD4* gene may be due to the reason that *PAD4* lies upstream of CBP60g and SARD1 in SA signalling node (Lin Wang et al., 2011).

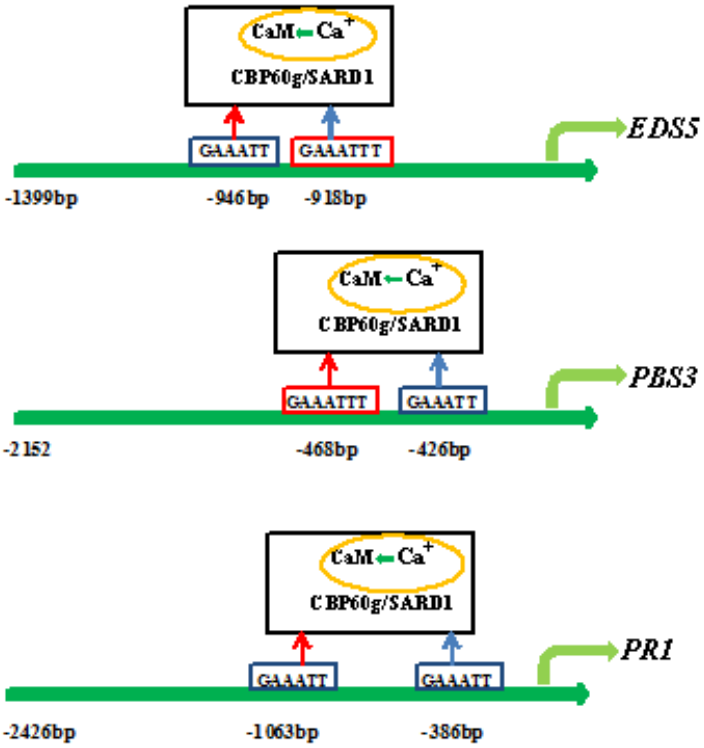


Figure 3-4CBP60g and SARD1 are possibly transcriptional regulators of other defence genes.

Promoters of *EDS5*, *PBS3* and *PR1* indicating CBP60g/SARD1 potential binding motifs. Positions of binding motifs on the promoters are also shown. The sequence motif in red box shows the main binding motif while its related sequence is shown in blue box.

3.5. Testing expression and solubility of CBP60g and SARD1 Proteins

Recombinant CBP60g and SARD1 with His_6-Tag were expressed in *E. coli* and tested for the solubility. Both CBP60g and SARD1 were excised from SDS-PAGE. The solubility test demonstrated that CBP60g was insoluble (Fig. 3.5) with His_6-Tag while SARD1 could not be expressed *in vivo*.

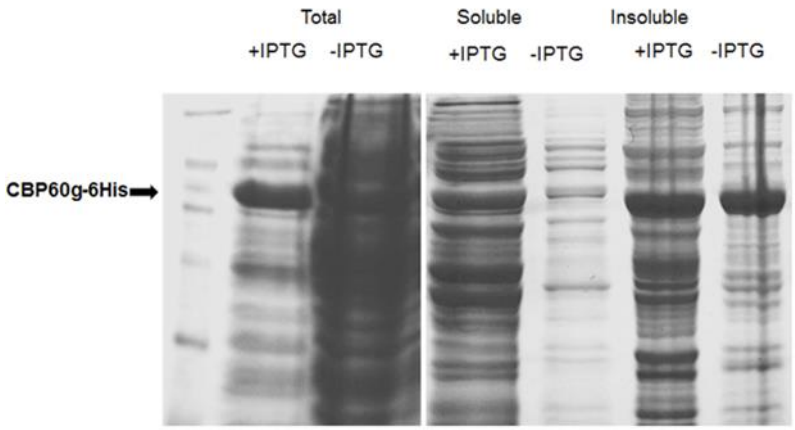


Figure 3-5 Expression and solubility test of recombinant CBP60g through SDS-PAGE.

Figure shows that CBP60g is insoluble with His-Tag. 0.1 mM IPTG was used to induce CBP60g expression in E. coli using BL21 (DE3) strain with incubation conditions of 37^{o}C for 5 hours.

3.6. *In vitro* protein synthesis of CBP60g and SARD1

In order to investigate the role of CBP60g and SARD1, they first need to be synthesized. Both CBP60g and SARD1 were not expressed in *E. coli*. Therefore, both proteins were synthesized in cell free protein synthesis system (Fig. 3.6) using wheat germ extract method (Tada et al., unpublished).

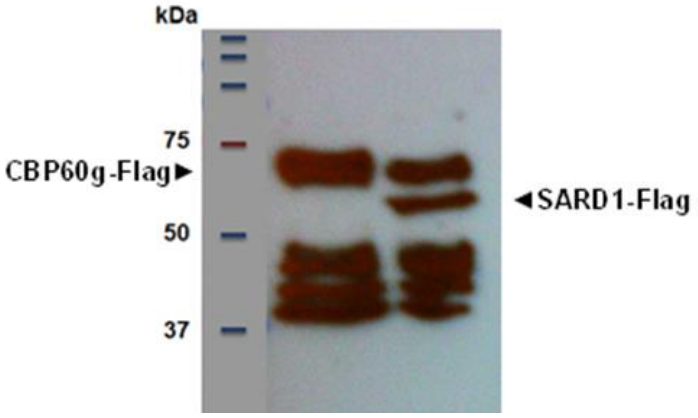


Figure 3-6 Western blot of synthesized (invitro) FLAG-tagged CBP60g (~65kDa) and SARD1 (~55kDa) proteins using wheat germ cell-free system.

The primary antibody against FLAG-tagged proteins was anti-FLAG in 1 in 5000 dilution. The secondary antibody against the primary antibody was anti-mouse (IgG) in 1 in 1000 dilution. The exposure time was 10 seconds.

3.7. Discussion

Our findings in this study suggested that GSNO alters the timing of expression of SA synthesis genes during incompatible interaction. The early expression of SA synthesis genes early during the avirulent challenge of *gsnor1-3* plants may be due to more rapid and stronger onset of cell death in this mutant as reported by Yun et al., 2011 that *gsnor1-3* shows an eminent increase in cell death in response to avirulent pathogen. While during a compatible interaction, high levels of GSNO inhibited SA synthesis through transcriptional suppression. These results are consistent with the findings that AtGSNOR1 positively regulates the signalling network controlled by the plant immune system activator, salicylic acid (Feechan et al., 2005). It has also been demonstrated that accumulation of *PR1* mRNA, which marks the expression of SA-dependent genes (Uknes et al., 1992), was substantial in wild-type plants but was reduced and delayed in the *atgsnor1-3* line in response to virulent *Pst*DC3000 (Feechan et al., 2005). The induced expression of *ICS1* upon pathogen infection in wild-type Col-0 is consistent with the finding that pathogen infections induce SA synthesis through up-regulating the expression of *ICS1*, which encodes a key enzyme in SA production (Zhang et al., 2010). Moreover, pathogen-induced suppression of SA synthesis and signalling genes may be explained by a role of JA mimics during compatible host-pathogen interaction. It has been reported that the JA-mimicking phytotoxin coronatine, which is produced by virulent *Pseudomonas syringae* bacteria, promotes virulence by suppressing efficient SA-dependent defences in *Arabidopsis* and tomato (*Solanum lycopersicum*) (Brooks et al., 2005; Zheng et al., 2012).

Our data show that during a compatible interaction the expression of *CBP60g* and *SARD1* is suppressed in *gsnor1-3* mutants. Based on the findings that both SARD1 and CBP60g are key regulators of *ICS1* induction and SA synthesis, and that both proteins are recruited to the promoter of *ICS1* in response to pathogen infection (Zhang et al., 2010), our findings suggest that the reduced expression of *ICS1* in *gsnor1-3* may be due to suppression of *CBP60g* and *SARD1*. Moreover, the promoter analysis of other defence genes suggested that the reduced expression of other defence genes, *EDS5*, *PBS3* and *PR1* may also be due to the reduced expression of CBP60g and SARD1 since we found CBP60g/SARD1 binding motif GAAATTT and its related sequence GAAATT in the promoters of these genes. It has been reported that CBP60g and SARD1 affect the expression of a subset of *PAD4*/*EDS1*-dependent genes through direct binding to GAAATTT motif in the promoters of these genes (Lin Wang et al., 2011). But the absence of this motif in the *PAD4* promoter might explain the position of *PAD4* gene upstream of CBP60g/SARD1 in SA signalling node as reported by (Lin Wang et al., 2011).

GSNO is known to inhibit the activity of signalling proteins through protein *S*-nitrosylation. Both SARD1 and CBP60g contain 4 and 11 cysteines, respectively. Therefore, it is possible that these proteins become *S*-nitrosylated which may prevent binding to the promoter of *ICS1*. It has been reported that the affinity of transcription factors to bind DNA can be posttranslationally changed by phosphorylation or redox-dependent modifications; for example a group of plant homeodomain transcription factors containing conserved active cysteines (Tron et al., 2002) form intermolecular disulphide bonds in the oxidized state preventing DNA binding activity while their

binding ability is enhanced under reducing conditions (Lindermayr et al., 2010). Moreover, *S*-nitrosylation of *Arabidopsis* MYB2 transcription factor at cysteine 53 results in reduced DNA binding affinity (Serpa et al., 2007). *S*-nitrosylation of AtSABP3 provides another example where, in this case, binding to SA is reduced due to *S*-nitrosylation (Wang et al. 2009).

Furthermore to perform an *S*-nitrosylation assay with CBP60g and SARD1, both proteins need to be synthesized. But it was found to be difficult to express both CBP60g and SARD1 in *E. coli* (pGEX vector) and it is probably due to the fact that these proteins interfere with *E. coli* physiology. However, we managed to express CBP60g in pET28a vector with His_6-Tag but it was insoluble (Fig 3.5A). In contrast, they were successfully synthesized by wheat germ based cell-free protein synthesis system (Fig. 3.5B) (Tada et al., unpublished). This *in vitro* system is an efficient and cost-effective way to produce enough protein efficiently.

Chapter 4

Characterization of Nucleoredoxin Genes Encoding Novel Proteins Related to Thioredoxin

Chapter 4

4. Characterization of Nucleoredoxin Genes Encoding Novel Proteins Related to Thioredoxin

Classic functional roles associated with TRXs include thiol redox regulation of enzymes. In chloroplasts TRX activity is recycled by ferredoxin (FDX) and ferredoxin-thioredoxin reductase (FTR) in light-mediated reactions that target enzymes of the reductive pentose phosphate or Calvin cycle (Buchanan et al., 1994). Like animal and *E. coli* TRX, however, plant cytosolic TRX-h enzymes utilize NADPH as reducing power with the aid of NADPH-dependent TRX Reductase (NTR). Traditional and more recent proteomic approaches have resulted in the discovery of over 500 different TRX target proteins from oxygenic photosynthetic organisms with a wide range of cellular functions (Montrichard et al., 2009). Recent reports continue to elucidate the role of TRX in the regulation of transcription factors by changing the state of critical regulatory Cys residues. In animals TRX-1 is responsible for promoting the binding activities or nuclear import of the transcriptional regulators Sp1, glucocorticoid and estrogen receptors, and NF-κB (Lukosz et al., 2010) In plants, cytosolic TRX-h3 and TRX-h5 were shown to regulate reduction and nuclear import of the immune transcription coactivator NPR1 (Tada et al., 2008). Although TRXs lack a recognized nuclear-localization signal, they can occasionally be translocated from the cytoplasm to the nucleus in response to stress (Hirota et al., 1997; Nakamura et al., 1997; Serrato and Cejudo, 2003).

Nonetheless, it remains unclear how nuclear redox events are regulated and if dedicated nuclear oxidoreductases are involved.

A conspicuous class of potentially nuclear oxidoreductases are the nucleoredoxins (NRX). NRX proteins are found in both animals and plants, and exhibit a curious structure. In contrast to conventional TRX, NRX proteins contain three regions that resemble typical structural domains found in members of the TRX superfamily. In animals two of these folds are TRX-like and the third is more related to protein disulphide isomerase (PDI), while in plants all three domains are more related to structural folds found in typical TRX. Moreover, whereas animal NRX contains only one typical Cys-X-X-Cys active site, plant NRX proteins contain two complete or partial active sites (Funato and Miki, 2007). Plant NRX proteins also contain a C-terminal DC1 domain with similarity to a zinc finger-binding domain (Laughner et al., 1998). Thus, these proteins have the potential to display novel redox roles in the plant nucleus.

In animals NRX activity has recently been associated with regulation of gene expression in cell development and immunity (Funato and Miki, 2007). (Laughner et al., 1998) reported that maize NRX is highly enriched in the nucleus and in its recombinant form possessed limited TRX enzymatic activity. In this chapter we studied the role of *Arabidopsis* NRX proteins in regulating transcriptional events during the immune response. We show that NRX proteins play a central role in SA-dependent immune signalling by regulating the expression of SA biosynthesis genes.

Results

4.1. NRX (nucleoredoxin) knockout and expression analysis

This novel class of Thioredoxins has not previously been explored, therefore Fig. 4.1 displays the sequence alignment of *Arabidopsis* NRX1 and NRX2 proteins. Both NRX1 and NRX2 have two TRX-like active sites and they also contain a DC1 domain. Proteins show 41% similarity at the amino acid level.

Figure 4-1 Amino acid alignment of NRX1 and NRX2.

NRX1 and NRX2 showing amino acid sequence similarity, indicating TRX-like modules and DC1 domains. The proteins share 41% similarity.

We first analysed the expression of *NRX* genes. Because nuclear redox signalling is key to immune responses, we infected plants with the pathogen *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326. RT-PCR analysis showed that both *NRX1* and *NRX2* genes were induced by pathogen infection (Fig. 4.2). Whereas *NRX2* expression was induced transiently at 12 hours post infection, expression of *NRX1* was induced maximally at 24 hours and was sustained throughout the infection.

Next we obtained SALK T-DNA insertion lines for *NRX1* (SALK_113401) and *NRX2* (SALK_021735) (Fig. 4.3). These mutant *nrx1* and *nrx2* plants failed to express the respective genes after pathogen infection, indicating that these mutants are true gene knock outs (Fig. 4.2). Interestingly, *NRX1* was constitutively expressed in the *nrx2* mutant, plus it was slightly up-regulated early during the *Psm* infection but not as strongly induced as wild-type. *NRX2* expression was still induced in wild-type plants but not as strongly as *NRX1*. *NRX2* was also induced by *Psm* in *nrx1* mutant.

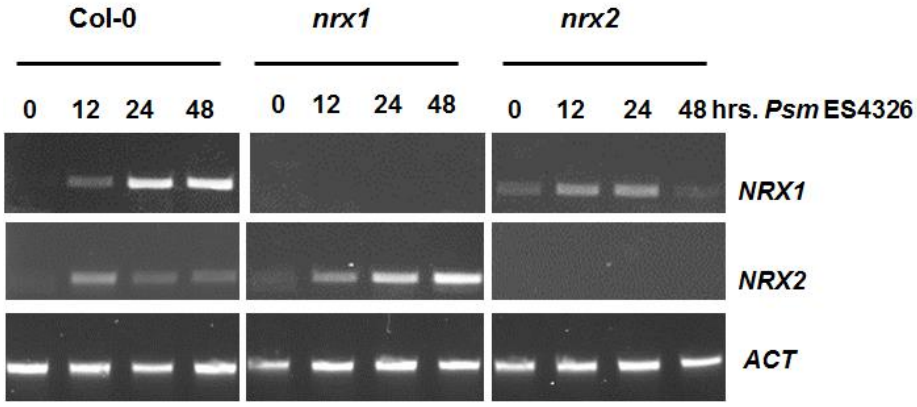
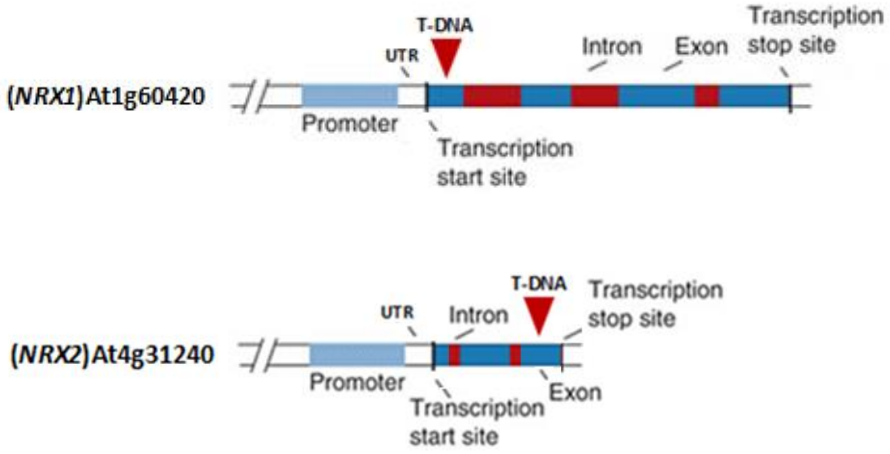


Figure 4-2 Pathogen-induced gene expression of *NRX1* and *NRX2*.

NRX1 and *NRX2* gene expression in Col-0 versus *nrx1* and *nrx2* knock out mutants post infection with *Psm* ES4326 at four time points (*i.e.* 0, 12, 24 and 48 hours) at OD_{600} = 0.0002. Constitutively expressed *Actin* (*ACT*) indicated equal loading. These experiments were repeated twice with similar results.

A



B

Figure 4-3 Position of T-DNA insertion in *NRX1* and *NRX2* and their respective phenotypes.

(A) Gene models and position of T-DNA insertion in *NRX1* and *NRX2* genes. Transcription start and stop sites, exons, introns, UTR and promoters are indicated. (B) Phenotypes of wild-type Col-0, *nrx1* and *nrx2* mutants, indicating there are no apparent morphological phenotypes.

4.2. NRX1 modulates disease resistance against *Psm* ES4326

The pathogen-inducible expression pattern of *NRX* genes prompted us to investigate if these genes are involved in conferring disease resistance. To that end we assessed immunity of wild-type Col-0, single *nrx1* and *nrx2* mutants, as well as a double *nrx1 nrx2* mutant created by crossing the single mutants. Enhanced disease susceptibility (EDS) assay with low doses of *Psm* ES4326 (OD_{600}= 0.0002) did not show enhanced susceptibility (data not shown). Therefore, we performed an enhanced disease resistance (EDR) assay to examine if *NRX* genes were involved in immunity. We pre-treated plants with 0.5mM SA and then infected with a high dosage of *Psm* (OD_{600}= 0.0015). We found that control-treated *nrx1* plants showed constitutive resistance similar to SA-treated wild-type plants and SA could not induce further resistance (Fig 4.4). In contrast, *nrx*2 plants did not show any obvious phenotype. Moreover, the single *nrx1* and double *nrx1 nrx2* mutants displayed almost the same phenotype (Fig 4.4), suggesting that *NRX1* plays a more important role in immunity than *NRX2*.

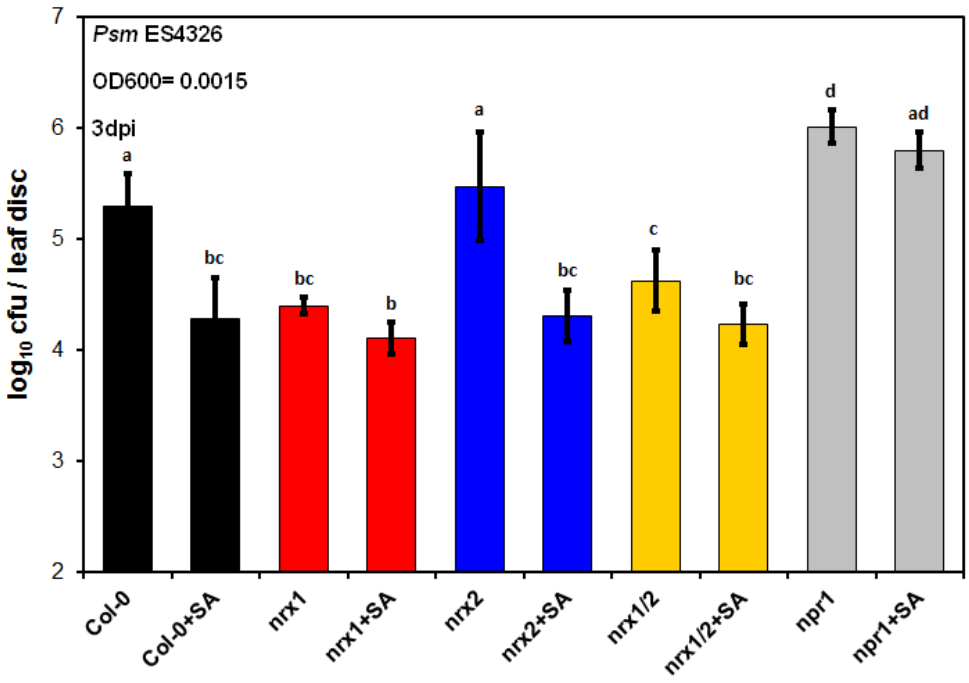


Figure 4-4 Mutant *nrx1* plants exhibit constitutive disease resistance.

Wild-type Col-0, *nrx1*, *nrx2*, double *nrx1 nrx2*, and *npr1* were control-treated or treated with 0.5 mM SA for 24 hours followed by infection with *Psm* ES4326 at OD600= 0.0015. Three days post inoculation (dpi) the growth of *Psm* ES4326 was determined as colony forming units (cfu) per leaf disc. Error bars represent statistical 95% confidence limits (n=8). Bars annotated with the same letter were not significantly different (Tukey-Kramer ANOVA test; $\alpha = 0.05$, $n = 8$).

The data in this figure were contributed by Dr. Steven Spoel.

4.3. Mutant *nrx1* plants exhibit constitutive SA signalling

As *nrx1*mutants showed constitutive disease resistance, we analysed defence gene expression in these plants. Wild-type Col-0 and *nrx1* mutants were inoculated with *Psm* ES4326 by pressure infiltration and tissue harvested after 0, 12 and 24 hours. RT-PCR was performed on these samples to examine the expression of SA biosynthesis and downstream SA-responsive defence genes. Infection by *Psm* ES4326 resulted in the induction of all genes tested in both Col-0 and *nrx1* plants (Fig. 4.5). Compared to Col-0, however, we found that the SA biosynthesis genes *ICS1*, *EDS5*, and *PAD4* were constitutively expressed in *nrx1* mutants even without pathogen infection (Fig 4.5). Similarly, the SA-responsive *PR-1* was also constitutively expressed in *nrx1* mutants. These data indicate that *NRX1* is a negative regulator of defence genes. We also analysed defence gene expression in *nrx2* mutants but did not find any differences relative to wild-type (data not shown).

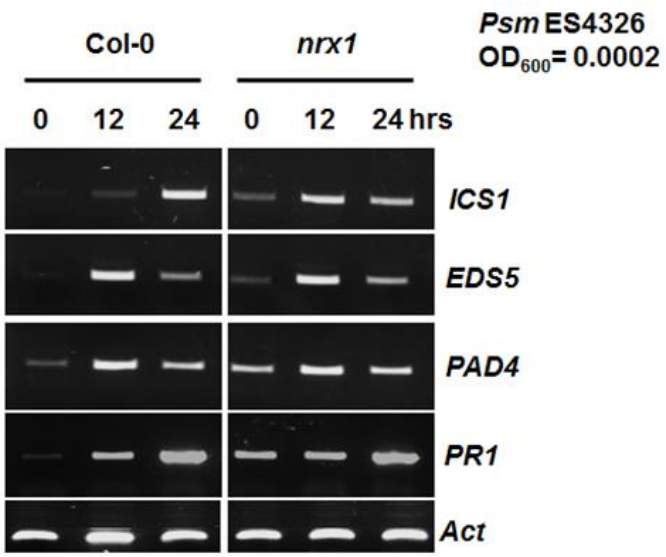


Figure 4-5 Pathogen-induced expression of SA biosynthesis and downstream SA-responsive genes in *nrx1* mutants.

Col-0 and *nrx1* plants were infected with *Psm* ES4326 at OD600 = 0.0002 and expression of the SA biosynthesis *ICS1*, *EDS5*, *PAD4*, and SA-responsive *PR-1* genes determined by RT-PCR. Constitutively expressed *Actin* (*ACT*) indicated equal loading. These experiments were repeated twice with similar results.

4.4. SA dependency of constitutive immune gene expression in *nrx1* plants

To explore if constitutive immune gene expression in *nrx1* mutants is dependent on SA, we performed crosses with genotypes that are impaired in SA accumulation. First, we made crosses with SA-degrading transgenic NahG plants that accumulate virtually no SA, and with mutant *pad4* plants that have basal levels of SA but fail to accumulate SA upon pathogen infection (Gaffney et al., 1993; Delaney et al., 1994; Delaney et al., 1995. We then analysed basal expression levels of SA biosynthesis and SA-responsive genes in the resulting *nrx1* NahG and *nrx1 pad4* plants. Figure 4.6 shows that compared to transgenic NahG and mutant *pad4* plants, *nrx1* single mutants displayed enhanced basal expression of all genes tested. Interestingly, *nrx1* NahG plants exhibited significantly higher expression levels as compare to NahG, suggesting that SA may not be required for constitutive gene expression in *nrx1* mutants (Fig. 4.6A). Surprisingly, however, *PAD4* and *EDS5* were strongly down regulated and *ICS1* and *PR1* were moderately down regulated in *pad4* single mutants and even in the *nrx1 pad4* double mutant, relative to *nrx1* single mutant, at almost similar level, suggesting that NRX1-mediated suppression of immune genes requires *PAD4* or accumulation of SA (Fig. 4.6B).

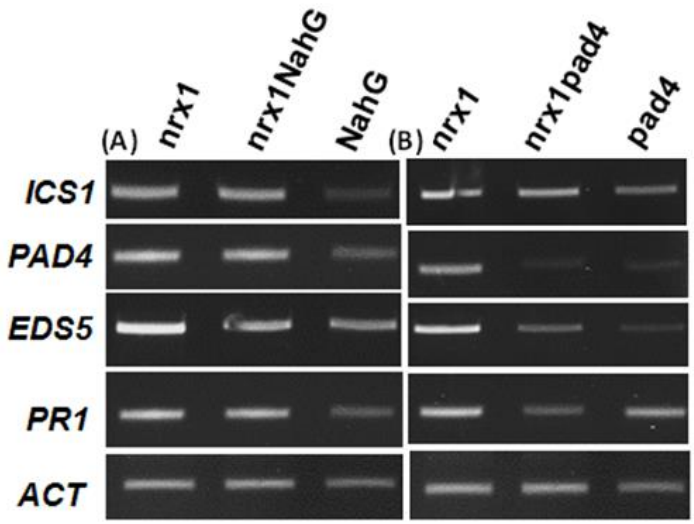


Figure 4-6 Basal expression of immune genes in *nrx1*NahG and *nrx1pad4* plants. RT-PCR results showing basal expression of the SA biosynthesis *ICS1*, *EDS5*, *PAD4*, and SA-responsive *PR-1* genes in *nrx1*, *nrx1* NahG (A), and *nrx1 pad4* (B) mutants. Constitutively expressed *Actin* (*ACT*) indicated equal loading. These experiments were repeated twice with similar results.

4.5. Disease resistance of *nrx1* is differentially affected by NahG and *pad4*

To understand better the discrepancy between effects of NahG and *pad4* genetic backgrounds on *nrx1*, we analysed *nrx1 pad4* and *nrx1* NahG double mutants in a disease bioassay. The plants were inoculated with *Psm* ES4326 and the number of bacterial colonies per leaf disc counted after 5 days. As expected, the data established that pathogen growth was dramatically higher in NahG and *pad4* plants compared with the *nrx1* mutant (Fig. 4.7). No significant difference in bacterial growth was detected between *pad4* and *nrx1pad4* mutants (Fig. 4.7), consistent with the immune gene expression data in Fig. 4.6B. However, *nrx1* NahG plants exhibited significantly less pathogen growth compared to NahG plants (Fig. 4.7). Taken together, these data suggest that phenotypic effects of *nrx1* are dependent on *PAD4* but not NahG.

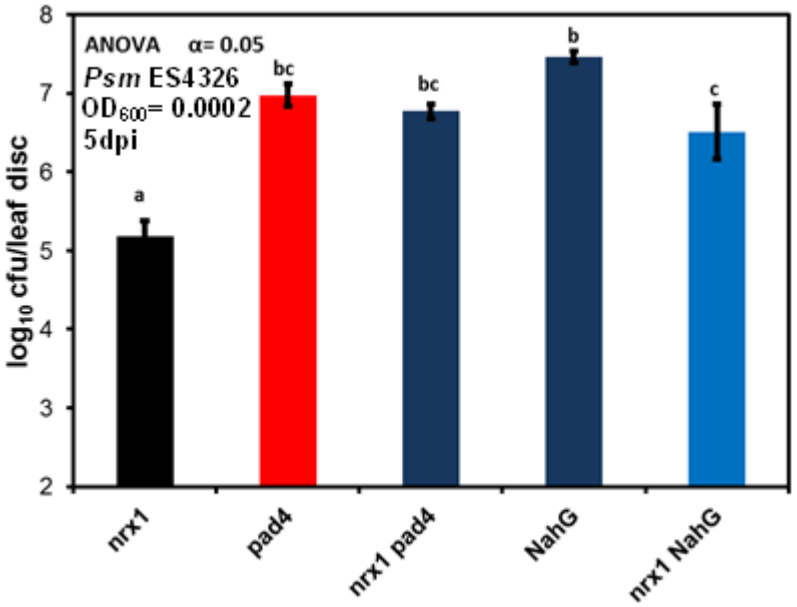


Figure 4-7 Disease resistance of *nrx1* depends on *PAD4* but not NahG.

Plants were infected with *Psm* ES4326 (OD_{600}=0.0002) and five days post inoculation (5dpi) pathogen growth was determined as colony forming units (cfu) per leaf disc. Standard error is shown. Bars annotated with the same letter were not significantly different (Tukey-Kramer ANOVA test; α = 0.05, n = 8). Experiments were repeated three times with similar results.

4.6. Disease resistance of *nrx1* is dependent on SA

Break down of SA by the NahG transgene produces catechol as a degradation product (Van Wees and Glazebrook 2003.), which may explain the differential effects of NahG and *pad4* on the *nrx1* mutant phenotypes. Therefore, we utilized another mutant, *ics1*, defective in the main pathogen-inducible SA biosynthesis pathway (Wildermuth et al., 2002.). Mutant *nrx1* plants were crossed with *ics1* mutants and the resulting *nrx1 ics1* double mutants were inoculated with *Psm* ES4326. As expected, *ics1* mutants were highly susceptible to infection, but no significant difference in pathogen growth was detected between *ics1* and *nrx1 ics1* mutants (Fig. 4.8). These data confirm that disease resistance phenotypes of the *nrx1* mutant are dependent on SA.

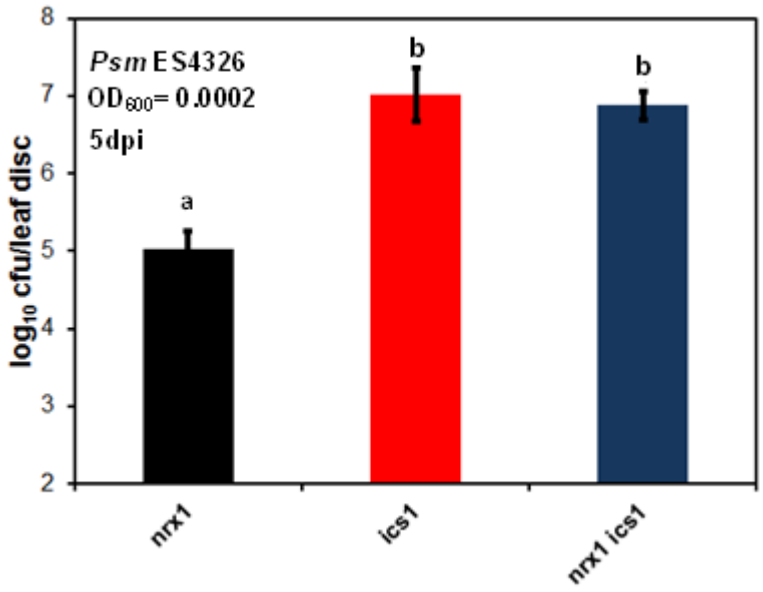


Figure 4-8 Disease resistance of *nrx1* depends on *ICS1*.

Plants were infected with *Psm* ES4326 (OD_{600}=0.0002) and five days post inoculation (5dpi) pathogen growth was determined as colony forming units (cfu) per leaf disc. Error bars represent statistical 95% confidence limits (n = 8). Bars annotated with the same letter were not significantly different (Tukey-Kramer ANOVA test; $\alpha = 0.05$, n = 8). Experiments were repeated three times with similar results.

4.7 The NahG transgene may induce stress responses in plants

It was previously reported that SA degradation by the NahG transgene may induce oxidative stress in plants. *NRX1* encodes a potential oxidoreductase that is expressed upon exposure to various oxidative stress-inducing conditions (BAR expression browser, data not shown). Thus, we analysed expression of the *NRX1* gene in NahG plants. Figure 4.9 shows that compared to wild-type Col-0 plants, *NRX1* was expressed approximately three-fold higher in NahG plants. By contrast, *NRX1* gene expression was normal in *pad4* mutants. These data suggest that as opposed to SA biosynthesis mutants, the NahG transgene may induce oxidative stress in plants.

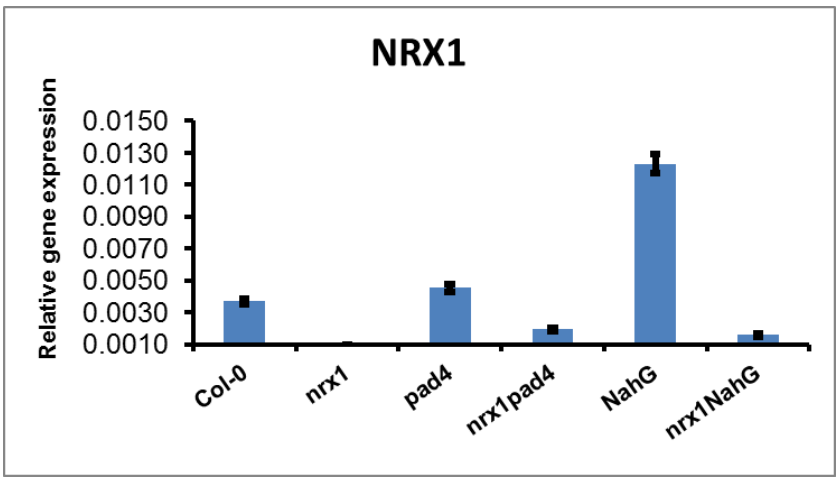


Figure 4-9 *NRX1* gene expression in NahG and *pad4* genetic backgrounds.

Relative *NRX1* gene expression was analysed by qPCR in the indicated genotypes. Data was normalized against constitutively expressed *Ubiquitin* (*UBQ).* Error bars represent the standard deviation of three technical replicates. Experiments were repeated twice with similar results.

4.8. Discussion

In this chapter, analysis of *nrx1* and *nrx2* mutants established that there were no transcripts for *NRX1* and *NRX2* genes respectively, confirming the complete knock out of both genes (Fig. 4.2). Data also indicated that both *NRX1* and *NRX2* genes were inducible by pathogen infection. Moreover, the induction of *NRX2* in *nrx1* mutant suggested that there is some redundancy among both *NRX1* and *NRX2* i.e. plants induce *NRX2* expression in the absence of *NRX1* and vice versa to compensate the defence induced by the *NRX*.

Though there were no apparent morphological or developmental phenotypes associated with *nrx1* (Fig. 4.3B) as inducible defence responses are expected to be costly and in the absence of a pathogen, their constitutive expression is expected to exert negative effect on fitness; for instance, the *cpr* mutants (exhibiting constitutive activation of SAR) were shown to have reduced fitness as compare to wild-type (Heidel et al., 2004). The findings from gene expression and infection assays indicated that SA signalling was constitutively switched on in *nrx1* mutants (Figs. 4.4 & 4.5). The findings demonstrate that *NRX1* inhibited the defence genes *ICS1*, *PAD4*, *EDS5* and SA marker gene *PR-1*. This inhibitory roles for *Arabidopsis NRX1* in gene transcription are consistent with results on mouse *NRX* by Funato et al., (2006). These authors showed that mouse NRX inhibits Dishevelled, a key adaptor protein of the Wnt–β-catenin signalling pathway for early development and stem cell maintenance.

We also reported that *NRX1* gene is induced by pathogen infection in wild-type plants. Because *NRX1* genetically opposed SA signalling, this suggests that NRX1 functions to limit the activation of SA-dependent immunity, perhaps due to the fact that high levels of SA can become toxic to plants. It has been reported by (Heidel et al., 2004) that elevated SA levels are associated with fitness costs. These authors performed a field experiment with two sets of *Arabidopsis* genotypes: one group that was blocked in SA-inducible defences and another group that constitutively expressed SA-inducible defences. Both classes of genotypes were negatively affected in growth and seed set, suggesting that plant fitness reaches an optimum at a certain intermediate level of resistance that balances the costs of fitness and defence. Indeed, it was confirmed in a later study that activation of SA signalling has high fitness costs (van Hulten et al., 2006). Hence, *NRX1* may be part of an SA-induced negative feedback loop that prevents excessive activation of immune responses.

Furthermore, we constructed crosses of *nrx1* with SA deficient mutants (NahG, *pad4*, *ics1*) to investigate if resistance of *nrx1* is SA dependent. The *ics1* and *pad4* mutations reduce SA levels (Wang et al., 2009). *ICS1*, *PAD4* and NahG are described in chapter 1 (Introduction). Analysis of gene expression and disease resistance in *nrx1 pad4* and *nrx1 ics1* double mutants revealed that *nrx1*-mediated resistance was SA dependent, while analysis of *nrx1* NahG plants suggested it was not. A direct effect of the degradation of SA by NahG is the production of catechol (Yamamoto et al., 1965). Catechol production might be the reason why NahG plants behaved different from *sid2* and *pad4* genotypes that also accumulate low levels of

SA. It has been reported that NahG plants exert stronger effects (susceptibility) to the virulent pathogens *P. syringae* than *sid2* and *pad4* plants (Nawrath and Metraux, 1999) An earlier study by (Van Wees and Glazebrook, 2003) also indicated that *Arabidopsis* NahG converts SA to catechol which further leads to the production of H_2O_2 and the generation of H_2O_2 causes oxidative damage (Schweigert et al., 2001) which might be the possible reason of strong NRX1 induction in NahG plants (Fig. 4.9) to prevent the plants from oxidative damage. Therefore, it has also been demonstrated that NahG plants not only exert their effects on SA accumulation but also have some additional uncharacterized effects on disease resistance (Van Wees and Glazebrook, 2003). The role of catechol can be further investigated by the application of catechol solution on wild-type Col-0 plants. Moreover, it has been reported that following H_2O_2 production, an efficient antioxidant system including catalases, glutathione S-transferases, and ascorbate peroxidase, is activated to protect the plants from oxidative damage caused by H_2O_2 (Dat et al., 2000) explaining the reason why resistance in *nrx1* is not eliminated. In conclusion we could say that NRX proteins play an important role in SA-dependent immune signalling by regulating the expression of SA biosynthesis genes.

Chapter 5

Redox properties of Nucleoredoxins

Chapter 5

5. Redox properties of Nucleoredoxins

Redox regulation is a central element in adjusting plant metabolism, development, and responses to the prevailing environmental conditions. TRX enzymes are ubiquitous disulphide reductases that regulate the redox status of target proteins and play a fundamental role in plant tolerance of oxidative stress. Plants have an extended TRX system consisting of several TRX isoforms localized in different sub-cellular compartments such as the chloroplast, mitochondria, and cytosol, but their role in the nucleus remains poorly understood (Gelhaye et al., 2005). Components of the cytosolic TRX system were previously reported to translocate to the nucleus. In wheat, NTR is found in the nucleus of cells from the developing seed that are exposed to oxidative stress (Pulido et al., 2009). Similarly, TRX *h*-type (cytoplasmic) enzymes co-localize to the nucleus of cells from oxidative stressed wheat seeds (Serrato et al., 2001; Serrato and Cejudo, 2003). However, the role of these TRX proteins remains largely unknown. Moreover, the TRX-like NRX proteins described in the previous chapter are also predicted to localize to the nucleus, but it is unclear if these proteins function like TRX.

The CxxC/S motif in TRX family members is essential for the reduction of inter- and intramolecular disulphide bonds and other forms of oxidized cysteines in target substrates (Fomenko and Gladyshev, 2003; Gelhaye et al., 2004; Rouhier et al., 2004). NRX1 being unique member of TRX family containing three TRX modules (detail is in chapter 1) might be target different substrates from TRXs. The

first active site cysteine is present at the N-terminal end of an a helix (TRX conserved domain) resulting in a permanent dipole that lowers the pKa of this cysteine (Kortemme et al., 1996)**.** This first active site cysteine with low pKa attacks the disulphide bonds in the target proteins resulting in a mixed disulphide bond between TRX and the target protein, then the second active site cysteine reduces that mixed disulphide bond releasing the reduced target and the oxidized TRX (Spoel and van Oijen 2012). Changing one of the amino acids of the active site by site-directed mutagenesis disturbs the functional properties of these thiol-disulphide oxidoreductases (Chivers et al., 1997; Mössner et al., 2000).

In this chapter, we investigated the oxidoreductase activity of NRX1. We show that NRX1 is a potent disulphide reductase, albeit to a lesser extent than conventional TRX5 protein. *In vitro* disulphide reducing activity of NRX1 was concentration dependent and required both active sites. Surprisingly, NRX1 activity could not be recycled with either reduced glutathione or NADPH/NTRA, the two major cellular reducing systems utilized by the TRX superfamily. These findings demonstrate that NRX proteins exhibit novel oxidoreductase features that are distinct from conventional TRX proteins.

Results

5.1. NRX1 and NRX2 have different physical properties

To study their activities, recombinant NRX1 and NRX2 with His_6-Tag were purified using cobalt resins and their purity and solubility examined by SDS-PAGE. Figure 5.1A shows that recombinant His_6-NRX1 was readily produced and purified to high purity. In contrast, recombinant His_6-NRX2 was produced but was nearly completely insoluble, preventing its native purification (Fig. 5.1B). Consequently, this chapter is largely focussed on the redox properties of NRX1. These findings suggest that while NRX1 and NRX2 share high sequence homology (Fig. 4.1, Chapter 4), they have different physical properties.

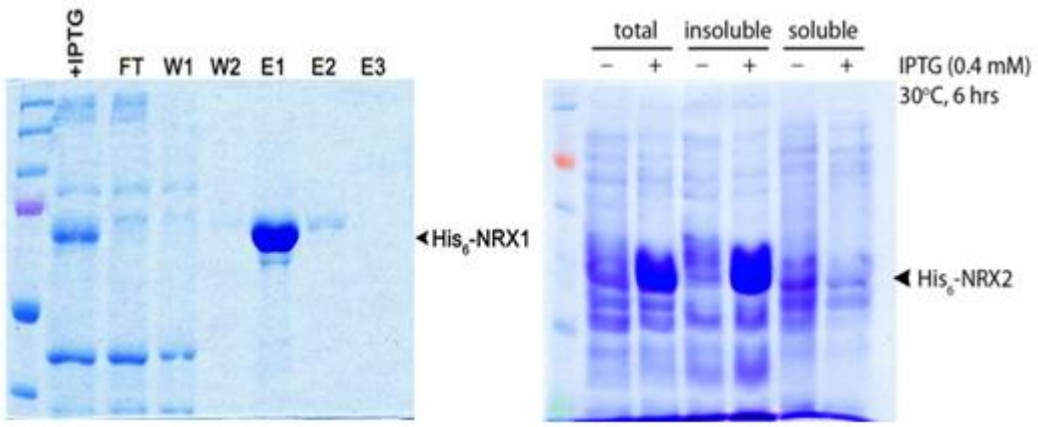


Figure 5-1 Production and purification of recombinant NRX proteins.

Recombinant His_6-NRX1 and His_6-NRX2 protein purification (FT, flow through; W, wash; E, elution) and solubility test showing both proteins have different physical properties, *i.e.* His_6-NRX1 is highly soluble while His_6-NRX2 is insoluble. 1 mM IPTG was used to induce His_6-NRX1 expression with an incubation temperature of 37^{o}C for 5 hours, while His_6-NRX2 was induced with 0.4 mM IPTG at 30^{o}C for 6 hours.

5.2. NRX1 displays disulphide reduction activity

Recombinant full lentgh His_6-NRX1 was assayed for disulphide reducing capicity using an insulin turbidity assay. Insulin was used as the target protein as it contains two intermolecular disulphide bridges. When insulin is reduced, the reduced monomers precipitate with a characteristic absorbance of 650 nm. Recombinant His_6-Recombinant His_6-TRX5 was included as a positive control and a low concentration of dithiothreitol (DTT; 0.33 mM) was used to recycle enzyme activities. The low concentration of DTT alone was insufficient to reduce insulin (Fig 5.2). In contrast, addition of 2 μM His_6-TRX5 to the reaction mixture demonstrated significant disulphide reducing activity as expected. Finally the assay was performed using His_6-NRX1, which demonstrated a very low level of activity at 2 μM concentration (Fig. 5.2), indicating that NRX21 can reduce disulphide bonds but its activity was not as high as TRX5.

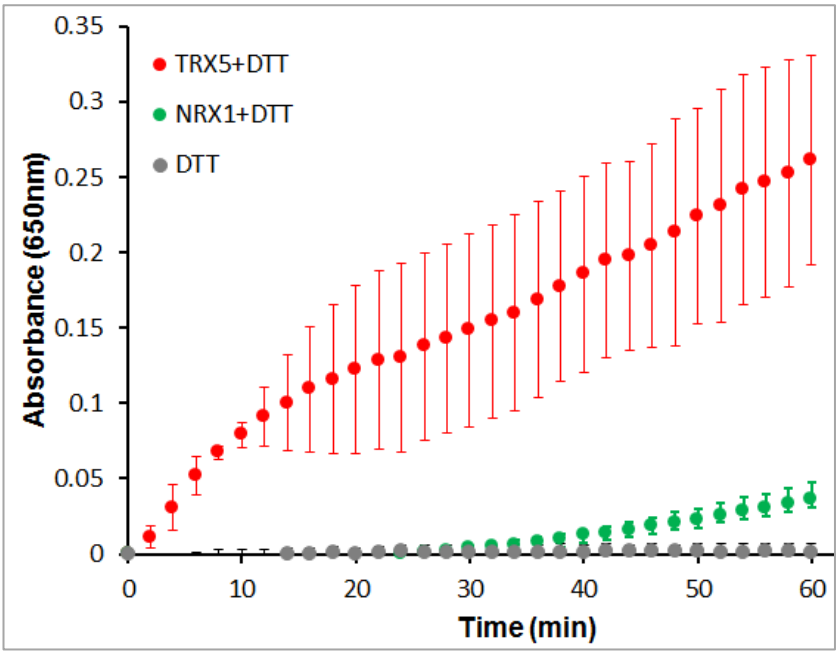


Figure 5-2 Disulphide reduction activity of NRX1.

Disulphide reduction activities of His_6-NRX1 (2 µM) and His_6-TRX5 (2 µM) were measured for 60 minutes in an insulin turbidity assay. The reducing agent DTT (0.33 mM) was used to recycle enzyme activities. Insulin reduction was observed by measuring its precipitation at 650 nm.

5.3. NRX1 displays concentration-dependent disulphide reduction activity

Since addition of 2 µM His_6-NRX1 did not display insulin precipitation as efficient as TRX5, therefore, we proceeded with the assay using increased His_6-NRX1 concentrations and observed disulphide reducing activity above background at 4 µM (Fig. 5.3). His_6-TRX5 accelerated the reaction more dramatically than the His_6-NRX1 protein. Like TRX5, NRX1 was thus able to reduce disulphide bond in insulin, but it was not as active. Therefore, we used even higher concentrations of His_6-NRX1 to find out how much is required to achieve the same activity as 2µM His_6-TRX5. A concentration of 8 µM His_6-NRX1 shortened lag time and increased the reaction rate to similar levels as observed with 2 µM His_6-TRX5. These data show that NRX1 activity is dose-dependent and that approximately quadruple the amount of NRX1 is required to give the same activity as TRX5.

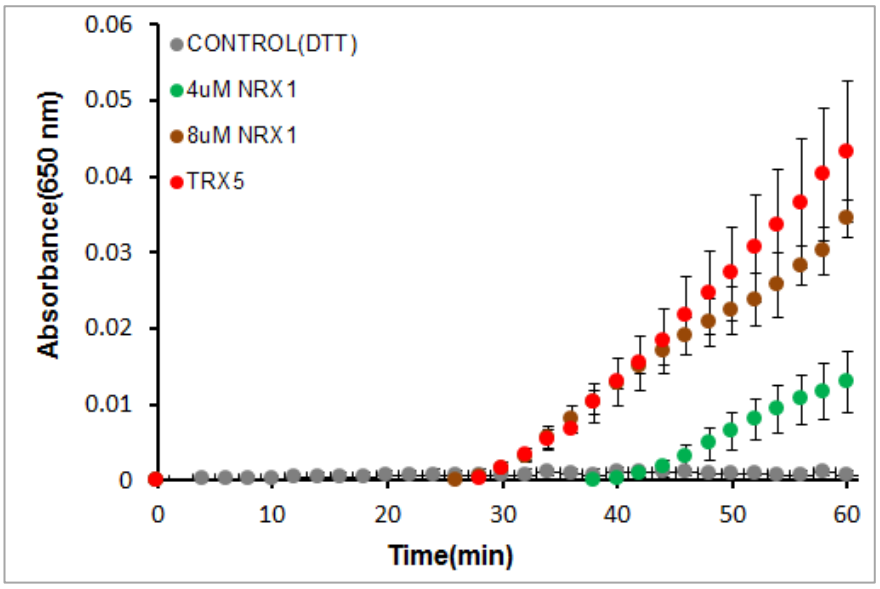


Figure 5-3 Concentration-dependent disulphide reduction activity of NRX1.

Disulphide reduction activities of indicated concentrations of His_6-NRX1 and His_6-TRX5 were measured for 60 minutes in an insulin turbidity assay. The reducing agent DTT (0.33 mM) was used to recycle enzyme activities. Insulin reduction was observed by measuring its precipitation at 650 nm. Error bars indicate standard deviation (n = 3).

5.4. NRX2 also displays disulphide reduction activity

Though His_6-NRX2 was largely insoluble, we managed to get a small amount of soluble protein after purification and performed a standard insulin turbidity assay on it. The reduction of insulin was determined with 2 μM His_6-NRX2 protein. His_6-TRX5 was used as positive control while DTT alone as the negative control. As expected, DTT alone did not show any activity, while His_6-TRX5 exhibited significant activity at 2 μM concentration (Fig. 5.4). Finally the assay was performed using His_6-NRX2, which demonstrated a very low level of activity at 2 μM concentration (Fig. 5.4), indicating that NRX2 can also reduce disulphide bonds but like NRX1, its activity was not as high as TRX5.

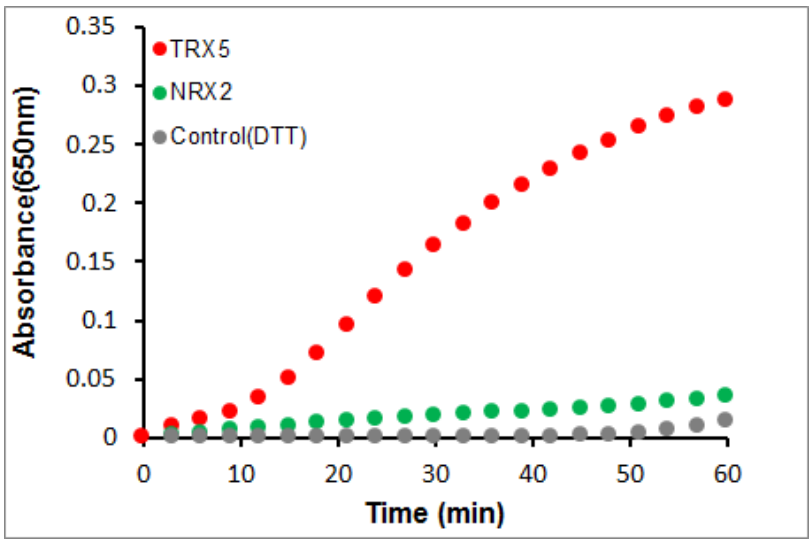


Figure 5-4 Disulphide reduction activity of NRX2.

Disulphide reduction activities of His_6-NRX2 (2 µM) and His_6-TRX5 (2 µM) were measured for 60 minutes in an insulin turbidity assay. The reducing agent DTT (0.33 mM) was used to recycle enzyme activities. Insulin reduction was observed by measuring its precipitation at 650 nm.

5.5. Activity of NRX2 is enhanced by increased DTT concentration

The reduction activity of His_6-NRX2 was not as good as even NRX1. Therefore, we decided to increase the concentration of the reductant DTT to 1 mM to see if we could improve its activity. His_6-TRX5 (2 µM) showed drastic increase in activity with 1 mM DTT concentration while His_6-NRX2 (2 µM) also showed significant increase in activity with this DTT concentration (Fig. 5.5) indicating that the reduction activity of His_6-NRX2 can be improved at high DTT concentration.

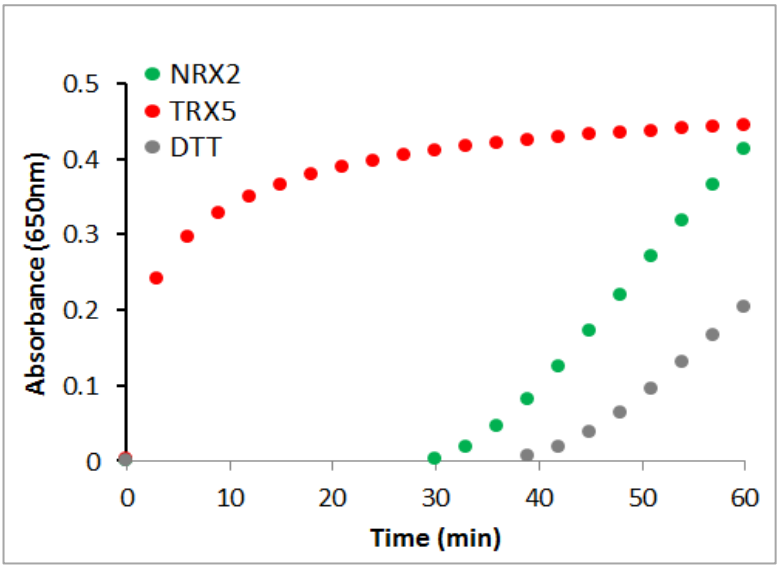


Figure 5-5 Disulphide reduction activity of NRX2 is improved with high DTT concentration.

Disulphide reduction activities of His_6-NRX2 (2 µM) and His_6-TRX5 (2 µM) were measured for 60 minutes in an insulin turbidity assay. The reducing agent DTT (1 mM) was used to recycle enzyme activities. Insulin reduction was observed by measuring its precipitation at 650 nm.

5.6. Activity of NRX1 is not enhanced by addition of zinc

NRX1 has previously been reported to contain a C-terminal DC1 domain with similarity to a zinc finger-binding domain (Laughner et al., 1998). We decided to add zinc in insulin turbidity assays to find out if activity of His_6-NRX1 could be improved. The rate of precipitation of reduced insulin by His_6-NRX1 was compared to reactions catalysed by His_6-TRX5 as positive control or DTT alone as negative control. The activity of His_6-NRX1 was the same before and after addition of zinc (Fig. 5.6), indicating zinc did not affect the activity of NRX1.

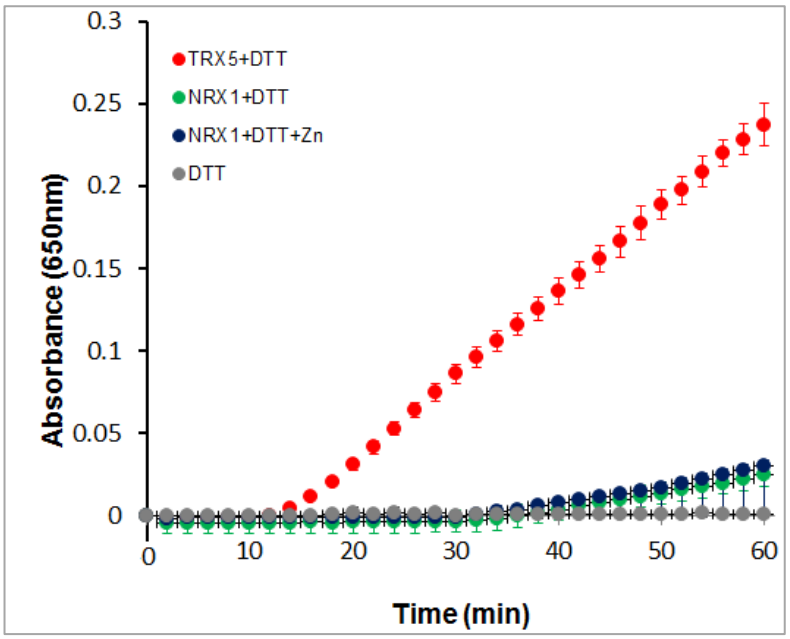


Figure 5-6 Effect of zinc on the activity of NRX1.

Disulphide reduction activities of His_6-NRX1 (2uM) and His_6-TRX5 (2 uM) were measured for 60 minutes in an insulin turbidity assay in the absence (-Zn) or presence (+Zn) of 25 µM zinc. The reducing agent DTT (0.33 mM) was used to recycle enzyme activities. Insulin reduction was observed by measuring its precipitation at 650 nm. Error bars indicate standard deviation (n = 3).

5.7. NRX1 activity is not recycled by NTRA or glutathione

TRX enzymes are specifically reduced by several NTR proteins in difference cellular compartments (Buchanan et al., 1994). NRX1 has active sites that are very similar to active sites found in TRX proteins, so we examined if NTRA is capable of recycling NRX1 activity. Because the cytosolic *A* isoform of NTR has been reported to localize to the plant nucleus during stress (Pulido et al., 2009), we performed the insulin turbidity assay with NTRA and NADPH as the reducing agent instead of DTT. Activity of the positive control, His_6-TRX5, yielded a steep slope, indicating as expected that TRX5 is recycled by NTRA (Fig. 5.7). However, NTRA did not stimulate activity of His_6-NRX1, suggesting that unlike TRX5, NRX1 is not recycled by NTRA.

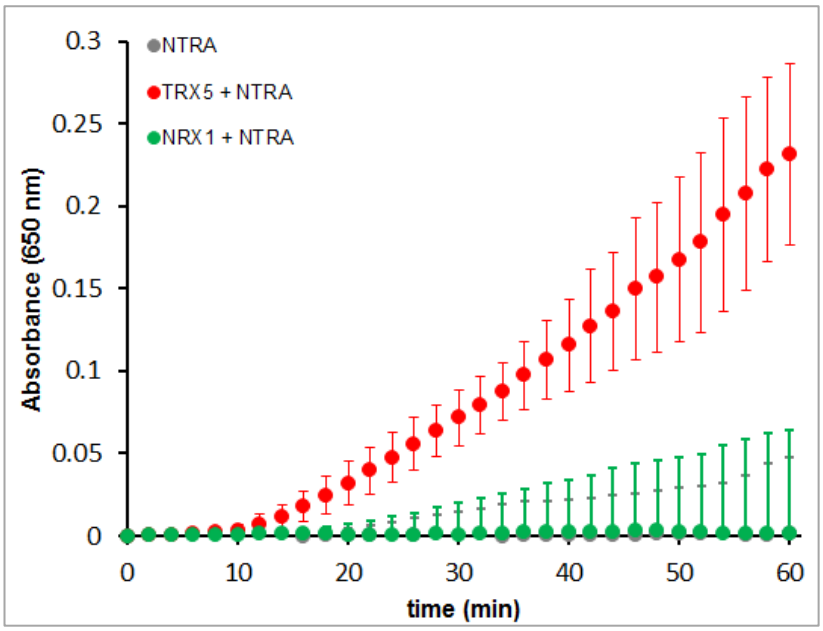


Figure 5-7 NTRA does not recycle the activity of NRX1.

Disulphide reduction activities of His_6-NRX1 (2 uM) and His_6-TRX5 (2 uM) were measured for 60 minutes in an insulin turbidity assay in the presence of 0.2 µM His_6-NTRA and 1 mM NADPH. Insulin reduction was observed by measuring its precipitation at 650 nm. Error bars indicate standard deviation (n = 3).

The presence of two TRX-like active sites in NRX1 is reminiscent of active sites found in protein disulphide isomerases (PDI) whose cysteine-modifying activities are recycled by glutathione. Therefore, we performed the insulin turbidity assay with reduced glutathione (GSH) as the reductant to explore if NRX1 behaves like PDI. We used 1 mM GSH with 4 μM His_6-NRX1 but NRX1 still did not show any activity, indicating that it was not recycled by GSH, while the positive control, His_6-TRX5, showed normal activity with DTT (Fig 5.8).

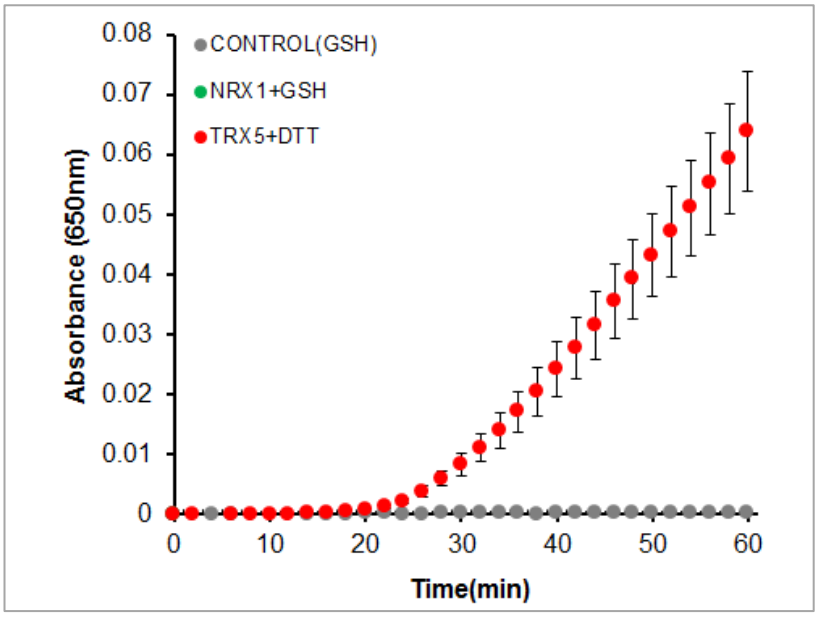


Figure 5-8 NRX1 activity is not recycled by glutathione.

Disulphide reduction activities of His_6-NRX1 (4 µM) and His_6-TRX5 (2 µM) were measured for 60 minutes in an insulin turbidity assay in the presence of 1 mM GSH or 0.33 mM DTT. Insulin reduction was observed by measuring its precipitation at 650 nm. Error bars indicate standard deviation (n = 3).

5.8. Both active sites of NRX1 participate in the disulphide reduction reaction

To test the requirement for the redox activity of NRX1, Cys55 and Cys58 from the first active site, Cys375 and Cys378 from the second active site and Cys55/58 and Cys-375/378 from both active sites together were mutated to Ser residues by site directed mutagenesis. Expression of the resulting mutant proteins was verified by SDS-PAGE after transformation into *E. coli* BL21 (DE3) cells (data not shown). After confirmation by SDS-PAGE, proteins with cysteine mutations were purified and utilized in the insulin turbidity assay to understand the role of individual active sites in disulphide bond reduction. Incubation with DTT alone had no effect on oxidized insulin, while 4 µM of wild-type His_6-NRX1 reduced significant amounts of insulin during 90 minutes of incubation (Fig. 5.9). Strikingly, the His_6-nrx1(C55/58/375/378S) protein in which both active sites are mutated did not show any activity, indicating that one or both active sites are necessary for disulphide reduction activity of NRX1 (Fig. 5.9). Mutation of either active site alone reduced activity, with His_6-nrx1(C55/58S) having a much larger effect than His_6-nrx1(C375/378S), suggesting that the first active site is predominantly important for disulphide reduction. These findings suggest it is plausible that the two active sites cooperate during disulphide reduction. To explore this possibility we mixed the His_6-nrx1(C55/58S) and His_6-nrx1(C375/378S) mutant proteins together and assessed their ability to reduce insulin. Surprisingly, this yielded a very low activity comparable to that of the His_6-nrx1(C55/58S) mutant protein alone, suggesting that the two active sites of NRX1 may negatively interact with each other.

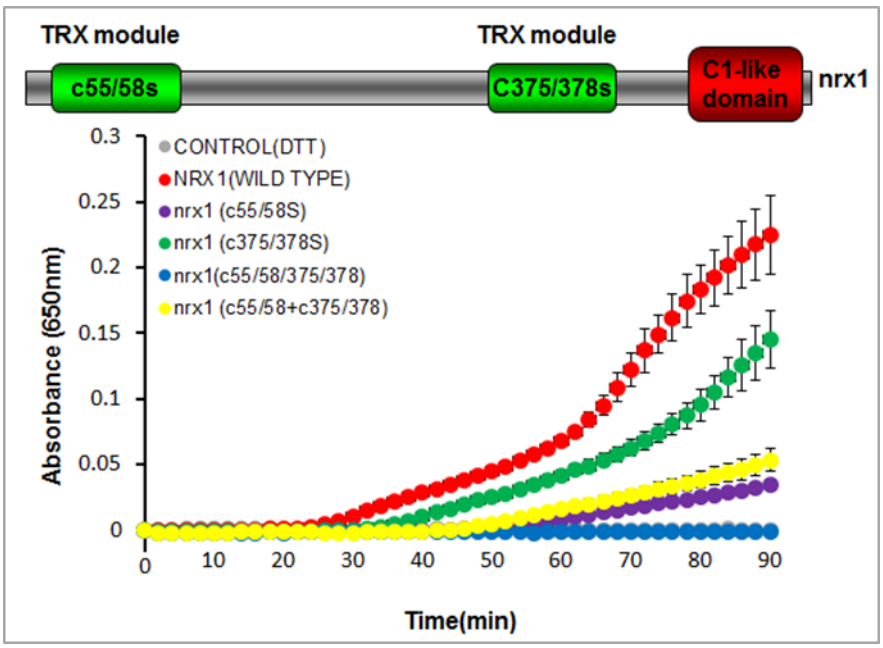


Figure 5-9 The active sites of NRX1 are required for disulphide reduction.

Disulphide reduction activities of indicated wild-type and mutant His_6-NRX1 proteins (4 µM) were measured for 90 minutes in an insulin turbidity assay. The reducing agent DTT (0.33 mM) was used to recycle enzyme activities. Insulin reduction was observed by measuring its precipitation at 650 nm. Error bars indicate standard deviation (n = 3). Upper panel shows modular structure of NRX1, indicating the position of mutated Cys residues. Experiment was repeated three times with similar results.

5.9. Discussion

SDS-PAGE analysis and solubility testing of recombinant His_6-NRX1 and His_6-NRX2 proteins indicated that the two proteins have different physical properties (Fig. 5.1) despite 41% sequence homology at amino acid level. However, amino acid alignment indicated that there is a big portion in NRX1 that is missing in NRX2 (Fig. 4.1 in Chapter 4), so there might be something in that sequence which renders NRX1 soluble.

Laughner et al., (1998) reported the isolation from maize of a cDNA encoding a novel, multiple TRX-domain protein called NRX that was highly localized to the nucleus and its recombinant form possessed TRX enzymatic activity. Our insulin turbidity assays with recombinant *Arabidopsis thaliana* NRX1 and NRX2 indicated that these proteins are also involved in reduction of the disulphide bonds (Figs. 5.2 & 5.4). However, the activity of NRX1 was not as pronounced as for the typical TRX family member TRX5. (Laughner et al., 1998) and Funato and Miki (2007) reported that the carboxy terminus of the NRX proteins contain a putative zinc finger binding domain. Therefore, we included zinc in the NRX1 reduction assay in the hope that it could improve activity, but this was not the case (Fig. 5.6). Recently, Colón-González and Kazanietz, (2006) published that C1 domains in PKCs have the characteristic motif HX12-CX2CXnCX2CX4HX2CX7C, where H is histidine, C is cysteine, X is any other amino acid, and n is 13 or 14. Sequence analysis of NRX1 and NRX2 indicated that the zinc finger binding domain

may instead be a C1-like domain that binds lipids (Fig. 4.1 in chapter 4). The function of this domain remains unclear.

A reducing system is required to maintain the pool of reduced TRX in the cell. In the cytosol, this function is performed by NTRA, which has also been shown to localize to the nucleus (Florencio et al., 1988; Pulido et al., 2009). Surprisingly, insulin turbidity assays with NTRA demonstrated that NRX1 does not behave like a typical TRX, as this reducing system was unable to recycle NRX1 activity (Fig. 5.7). The presence of two active sites in NRX proteins is reminiscent of PDI, another member of the TRX family whose activity is recycled by glutathione. However, glutathione was also unable to recycle NRX1 activity (Fig. 5.8), suggesting that NRX1 activity is regulated by an unusual cellular reducing system. The identity of this system remains unknown.

In TRXs the two Cys residues in the active site are responsible for their disulphide reducing activities. When these active cysteines are in the reduced form, the first Cys in the active site can form a mixed disulphide with the target protein. This intermolecular disulphide bond is quickly reduced by the second Cys of the active site, resulting in release of the reduced target protein and formation of an oxidized TRX with a disulphide bond between the two active-site cysteine residues (Kallis and Holmgren, 1980). But in NRX1 there are two active sites with two cysteines each (Fig 1.4). Cysteines were therefore mutated in the two active sites to explore their role in the reduction of the disulphide bonds. Our data indicate that the active sites are necessary for disulphide reduction, as no activity was observed in a

double active site mutant (Fig 5.9). Single active site mutants showed that the first active site is predominantly active. However, by combining single active site mutants into the same reaction we found that the active sites may also cooperate during disulphide reduction (Fig.5.9). Maize NRX also contains three TRX like modules with first and second containing active cysteines and at least the third TRX like module in insulin turbidity assay has been reported to possess oxidoreductase activity (Laughner et al., 1998). Maize NRX with a truncated third module has also been reported to possess higher TRX activity compare to full length NRX during insulin turbidity assay (Laughner et al., 1998). Although these authors truncated the whole module instead of the active cysteines, their results are consistent with our findings at the intermolecular level that the two active sites might cooperate during reduction but they may not have an additive function. However, the mixing of the two active sites indicated that the active sites may undergo an intermolecular interaction that could be negative or competitive and this opposed the findings by Laughner et al., 1998.

Chapter 6

General discussion

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General Discussion

Plants are under constant threat of diseases by different pathogens. It has formerly been shown that the defence signalling compound SA plays a central role in plant disease resistance. SA is synthesized by plants in response to challenge by a diverse range of plant pathogens and is essential to the establishment of both local and systemic-acquired resistance as its application induces accumulation of PR proteins and mutations leading to either reduced SA production or impaired SA perception enhances susceptibility to avirulent and virulent pathogens (Loake and Grant, 2007). There are two pathways for SA biosynthesis: one is through *ICS1* and the other is through PAL (Wildermuth et al., 2001; Mauch-Mani and Slusarenko, 1996. SA accumulation is regulated by cellular levels of GSNO, a redox molecule capable of *S*-nitrosylating proteins.

S-nitrosylation plays an important role in plant disease resistance (Feechan et al., 2005). Molecular analysis revealed that increased *S*-nitrosylation result in the reduction of both SA biosynthesis and signalling. In plants, the enzyme GSNOR1 regulates global *S*-nitrosylation and is a key player is plant disease resistance. In *Arabidopsis* elevated levels of SNO are found in a T-DNA knock out mutant (*atgsnor1-3*) as compare to wild type. Accumulation of SNO is associated with disease susceptibility, as the *atgsnor1-3* mutant was compromised in all forms of resistance (Feechan et al., 2005; Rusterucci et al., 2007; Loake and Grant, 2007; Yun et al., 2011).

Two reducing systems, consisting of GSNOR and TRX are thought to regulate global *S*-nitrosylation levels, how these enzymes control SA accumulation in plant immunity remains unknown. In this study, we focussed on GSNOR1 and the novel members of TRXs superfamily, the NRXs.

In Chapter 3 we observed a key difference in SA synthesis gene expression during compatible and incompatible interaction among the wild type and *gsnor1-3* mutants that GSNO alters the timing of expression of SA synthesis genes during incompatible interaction and the expression of these genes early during the infection in *gsnor1-3* may be due to more rapid and stronger onset of cell death in this mutant. It has been reported that *gsnor1-3* plants show an increase in cell death in response to avirulent pathogen attack (Yun et al., 2011).

High levels of GSNO inhibited SA synthesis through transcriptional suppression of SA biosynthesis genes during a compatible plant-pathogen interaction. It has also been demonstrated that AtGSNOR1 positively regulates the signalling network downstream of SA and a decrease in the concentration of SNOs promotes protection against microbial infection (Feechan et al., 2005). We also found that the pathogen-induced expression of *ICS1* was suppressed in *gsnor1-3* knock-out mutants and *ICS1* down regulation most likely causes the low levels of SA in these mutants. Surprisingly, suppression only occurred in compatible plant-pathogen interactions and was mediated by suppression of SARD1 and CBP60g, two key transcription activators of SA biosynthetic genes (Zhang et al., 2010). Therefore, we speculated that the reduced expression of *ICS1* in *gsnor1-3* might be due to the

reduced expression of *CBP60g* and *SARD1*. Additionally, *CBP60g* and *SARD1* contain many cysteine residues that may be redox sensitive, so these proteins might be *S*-nitrosylated in *gsnor1-3*, preventing their binding to the promoter of *ICS1*. This would be a particularly interesting to test in future because *S*-nitrosylation of protein thiols inhibits the DNA binding activities of the transcription factors NF-$_k$B (nuclear factor kappa-B) (Marshall and Stamler, 2001), hepatocyte nuclear factor-4 (Vossen and Erard, 2002), heterogeneous nuclear ribonucleoprotein A/B (Gao et al., 2004), p53 (Schonhoff et al., 2002), and hypoxia-inducible factor-1 (Palmer et al., 200). To that end we already successfully synthesized SARD1 and CBP60g proteins using a wheat-germ based cell free protein synthesis system (Fig 3.4B) (Tada et al., unpublished) to perform an *S*-nitrosylation assay. Cell-free protein synthesis can produce large peptides at the same or higher speed, and as accurately as ones synthesized by *in vivo* translation (Kurland 1982; Pavlov and Ehrenberg 1996). Moreover, this system can produce proteins that have a significant negative impact on the physiology of host cells. This cell free protein synthesis system, which is prepared from wheat embryos, is highly efficient and robust (Madin et al., 2000). Wheat germ based cell free system has several advantages such as low cost, capacity to produce large proteins and easy availability in large amounts. Furthermore, it is more suitable for synthesizing eukaryotic proteins, as it is a eukaryotic system.

In Chapter 4 and 5 we investigated the role of a novel class of redox enzymes, the nucleoredoxins which are included in thioredoxin superfamily as mentioned above. We first analysed the expression of *NRX* genes in *nrx1* and *nrx2* T-DNA insertion mutants. The data established that both *NRX* genes are inducible by

pathogen challenge but both mutants do not show any morphological or developmental phenotypes.

Constitutive expression of defence genes, including *ICS1*, *PAD4*, *EDS5*, and *PR-1*, was observed in *nrx1* mutants, suggesting NRX1 negatively regulates SA signalling. Accordingly, *nrx1* mutants displayed constitute disease resistance to a similar level as SA-treated wild-type plants. These findings are consistent with a study on mouse *NRX*, in that mouse NRX inhibits Dishevelled protein in Wnt–*β*-catenin signalling (Funato et al., 2010), a signalling pathway essential for early animal development and stem cell maintenance and the misregulation of Wnt/*β*-catenin signalling leads to tumorigenesis (Clevers, 2006; Moon et al., 2004; Reya and Clevers, 2005. Interestingly, NRX interacts with Dishevelled in a redox-dependent manner since the interaction between recombinant Dvl and NRX proteins has been reported to be strengthened by treatment with DTT and is weakened by treatment with H_2O_2 and this is supported by the fact that the intracellular colocalization of Dvl and NRX is suppressed by H_2O_2 and the Wnt/*β*-catenin pathway is upregulated by H_2O_2 (Funato and Miki, 2010). When cells are challenged with H_2O_2, NRX may form an intermolecular disulphide bond and the resulting conformational change allows the dissociation of Dvl from NRX. Moreover, NRX was shown to negatively regulate Toll-like Receptor 4 signalling; NRX enhances the negative effect of Fli-I (identified as an interacting partner of NRX) upon lipopolysaccharide-induced activation of NF-$_k$B through the Toll-like receptor 4/MyD88 pathway i.e. NRX forms a link between MyD88 [myeloid differentiation primary response gene (88)] and Fli-I to mediate negative regulation of the Toll-like

receptor 4/MyD88 pathway (Hayashi et al., 2010). So NRX aids binding of Fli-I to MyD88 by interacting with Fli-I as Fli-I suppresses the TLR4 signalling. Fli-I possesses a leucine-rich repeat in the N terminus and a gasolin-like domain in the C terminus and has been reported as major interacting partner of NRX (Hayashi et al., 2010) and in the nucleus, Fli-I acts as a cofactor in the nuclear receptor complex and facilitates the transcriptional activation of nuclear receptors such as the estrogen receptor and androgen receptor (Jeong et al., 2009; Lee et al., 2004). MyD88 is an important adaptor protein for the Toll-like receptor (TLR) signalling pathway and is essential to innate immunity and inflammation (Kawai and Akira, 2006; O'Neill and Bowie, 2007). TLR4 recognizes lipopolysaccharide (LPS) and recruits IRAK1 and IRAK4 through MyD88, resulting in the activation of transcription factor NF-$_k$B (Hayashi et al., 2010). No difference in the expression of defence genes observed in *nrx2* mutant (data not shown).

We also observed pathogen-induced expression of *NRX1* in wild-type plants, implying that NRX1 may function to limit prolonged activation of defence, as high levels of SA are thought to be toxic to plants. This is supported by findings reported by (Heidel et al., 2004) who performed a field experiment with two sets of *Arabidopsis* genotypes: one group that was blocked in SA-inducible defences (*npr1*) and another group that constitutively expressed SA-inducible defences (*cpr1* and *cpr6*): both classes of genotypes were negatively affected in growth and seed set, suggesting that plant fitness reaches an optimum at a certain intermediate level of resistance that balances fitness and defence. Heidel et al., (2004) argued that the loss of fitness in *cpr1* and *cpr6* is not from pleiotropy but rather from the constitutive

activation of the SAR pathway itself. It has also been reported that induced resistance protects plants against a wide spectrum of diseases; however, it can also entail costs due to the allocation of resources or toxicity of defensive products (van Hulten et al., 2006). So there may be a loop i.e. once defence is activated it is later curtailed by the activation of NRX1 in the wild type plants.

The constitutive expression of SA synthesis and signalling genes in *nrx1* mutants urged us to investigate whether *nrx1* resistance is SA dependent. Therefore, we constructed crosses of *nrx1* and the SA deficient mutants *NahG*, *pad4* and *ics1*, as well as the SA signalling *npr1* mutant. Homozygous *nrx1NahG*, *nrx1pad4* and *nrx1sid2* were generated successfully. However, the *nrx1* x *npr1* cross failed, probably because both genes are closely linked on the same chromosome. Analysis of gene expression in *nrx1pad4* double mutant indicated that constitutive defence gene expression in absence of functional *NRX1* depends on SA. Accordingly, pathogenicity assays on *nrx1pad4* and *nrx1 ics1* double mutants indicated that resistance of *nrx*1 is SA dependent.

Surprisingly, *nrx*1*Nah*G analysis suggested that *nrx1*-mediated resistance is SA independent. However, a direct effect of the degradation of SA by *NahG* is the production of catechol (Yamamoto et al., 1965). A study by (Van Wees and Glazebrook, 2003) indicated that the loss of non-host resistance of *Arabidopsis NahG* to *Pseudomonas syringae* pv. *phaseolicola* is not due to absence of SA but due to the degradation products of salicylic acid. H_2O_2 is also produced during catechol production (Schweigert et al., 2001). So H_2O_2 might be the reason why *NahG* plants

behave differently than other genotypes that accumulate similarly low levels of SA and H_2O_2 may also explain the strong *NRX1* gene induction in NahG plants (Fig. 4.8), which may provide protection against oxidative damage. In conclusion we believe that the resistance of *nrx1* plants is SA dependent.

In Chapter 5 we further explored the role of NRX1 protein as a potential tetrathiol reductase. Production and purification of recombinant NRX proteins demonstrated that NRX1 is highly soluble while NRX2 is insoluble. TRX activity assay showed that like TRX5, both NRX1 and NRX2 are involved in the reduction of the disulphide bond. Maize NRX having multiple TRX-domain modules is also reported to possess TRX enzymatic activity in its recombinant form (Laughner et al., 1998). Moreover, we observed that NRX1 is recycled by neither NTRA and NAD(P)H (TRX reducing system) nor GSH (reducing agent for PDI) suggesting NRX1 behaves different from both TRXs and PDI. Some other antioxidants have been reported to be reduced by TRXs namely peroxiredoxins (PRXs) and sulfiredoxins (SRX) (Spoel and Van Ooijen, 2013). Therefore, we speculate that NRX1 is also possibly recycled by TRXs. Though TRX *h* proteins have been reported to be evenly distributed in the cytosol, but the highest levels of thioredoxins *h* have also been detected in the nucleus upon stress (Serrato et al., 2001; Serrato and Cejudo, 2003). In this context, how NRX1 activity is recycled will be an intriguing analysis in the near future.

Finally, mutating cysteines among the two active sites in NRX1 demonstrated that the two active sites work in collaboration or at least first active site may have

comparatively higher affinity towards the substrate (Fig. 5.9). The oxidoreductase activity of NRX1 is comparable to study by Laughner et al., 1998 at intermolecular level that Maize NRX exhibited the oxidoreductase activity but truncated NRX (without third module) exhibited a significantly higher reduction activity compare to full length NRX. However the data in Fig. 5.9 also showed that both active sites contribute to the oxidoreductase activity indicating NRX1 behaves different from Laughner's study at the intramolecular level.

6.1. Conclusions and future plans

This project's findings are summarised in the following bullet points:

- *ICS1* is down regulated in *gsnor1-3* plants probably because of lower *CBP60g* and *SARD1* transcript abundance. In addition, since both proteins contain cysteine residues, they may be prone to *S*-nitrosylation and probably get *S*-nitrosylated in *gsnor1* mutant. Hence it is suggested that GSNO might inhibit SA synthesis via *S*-nitrosylating CBP60g and SARD1 preventing their binding to *ICS1* promoter. This can be further tested through biotin switch assays to assess *S*-nitrosylation of CBP60g and SARD1 (synthesized through *in vitro* protein synthesis).

- T-DNA insertion in *NRX1* results in enhanced disease resistance. Complementation of both *nrx1* and *nrx2* mutations is being performed in the lab of Dr Steven Spoel to confirm the mutant phenotypes are due to deletion of *NRX1*.

- *NRX1* negatively regulates SA-responsive immune genes in an SA-dependent manner

- Like TRX5, NRX1 and NRX2 reduce disulphide bond formation in target proteins and both proteins have different physical properties i.e. NRX1 is highly soluble while NRX2 is insoluble

- Unlike other TRXs, NRX1 activity is not recycled by NTRA indicating NRX1 is a novel member of TRX

- NRX1 is not recycled by GSH either demonstrating it does not behave like PDI

- Both active sites in NRX1 contribute to the oxidoreductase activity.

  Further, the targets of NRX1 can be detected through pull-down/immunoprecipitation assays by using a recently developed anti-NRX1 antibody in these assays

Chapter 7

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Appendix 1

Primers used for genetic analysis with their name and sequence

Gene ID	Forward sequence	Reverse sequence	Primer length
ICS1/SID2 At1g74710	GGTGCACCAGCTTTTATCGGAAAC	TGAGAACCCCTTATCCCCCATACAA	24/25
EDS5/SID1 At4g39030	CTGCGTCAGAATTGATCGGGAA	CAGCCCAAGGACCGAATAATCTTG	22/24
PAD4 At3g52430	ATGACATCGCCGGGATTACATACG	GATGTTCCTCGGTGTTTTGAGTTGC	24/25
PBS3 At5g13320	CGACTTGCCTCTCGTTTCAACAAC	GCAAGGCTTTCCCTGACTTAATGC	24/24
NRX1 At1g60420	ATGGCCGAAACCTCGAAGCAAG	GGCGAAATCACAAAGTCTCG	22/20
NRX2 At4g31240	CACGAGTCCCGAAATTACGTTGTTG	AAGAAAACGCCCAAAACCTGCC	25/22
PR1 At2g14610	ACCTCACTTTGGCACATCCG	GGTGACTTGTCTGGCGTCTC	20/20
CBP60g At5g26920	ATCGCAGCACATCGACTTTCAAGG	TGGCCGGGATCCATTTATCTAACC	24/24
SARD1 At1g73805	CCTCGCCAATTTCCAGTGTGTTTG	ACCCGAAGGAAAATCTCCGTGAAG	24/24

Appendix 2

Primers used for genotyping with their name and sequence

Gene ID	Forward primer	Reverse primer	Primer length
Ics1	CAATCTTGATGCTCTGCAGCTTC	GAAGATAGTTGAACCAAGG	23/19
NahG	GCCTTAGCACTGGAACTCTG	TCGGTGAACAGCACTTGCAC	20/20
*pad4-1*_CAPS	GCGATGCATCAGAAGAG	TTAGCCCAAAAGCAAGTATC	17/20
npr1-1	CTCGAATGTACATAAGGC	CGGTTCTACCTTCCAAAG	18/18

Appendix 3

Primers used in cloning of genes

Gene ID	Primer sequence	Primer length
pET28a/NRX1-NdeI/EcoRI	GGGAATTCCA^TATGGCCGAAACCTCGAAG/CAACGG^AATTCTCAGGCCTTGGTGCATAC	29/29
pET28a/NRX2-NdeI/EcoRI	GGGAATTCCA^TATGGCAGTATCAGCTG/CGG^AATTCAAATCACGACGACCGTCTC	27/27
pET28a/CBP60-Nde1/ EcoR1	TACATATGATGAAGATTCGGAACAGCCCTAG/AATTGAATTCTTACAAGCCTTCCCTCGGATTTC	31/33
pET28a/SARD1-Nde1/ EcoR1	TACATATGATGGCAGGGAAGAGGTTATTTCAAG/AATTGAATTCTTAGAAAGGGTTTATATGATTTTGAGACG	33/39
pGEX 6p-1/CBP60g-BamH1/EcoR1	CGCGGATCCATGAAGATTCGGAACAGCCC/CGCGGATCCATGAAGATTCGGAACAGCCC	29/29
pGEX 6p-1/SARD1-BamH1/EcoR1	CGCGGATCCATGGCAGGGAAGAGGTTATTTC/CAACGGAATTCTTAGAAAGGGTTTATATGATTTTGAGACG	31/40