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Molecular mechanisms of redoxin-mediated signalling in plant immunity

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Doctor of Philosophy

University of Edinburgh
2016
Abstract

Posttranslational modification (PTM) of proteins is essential to creating a diverse proteome with the complex functions necessary to regulate key cellular processes. Redox-based PTMs exhibit many desirable characteristics to finely modulate transcriptional regulators; they occur rapidly and can alter protein conformation, localisation and activity. The plant immune system offers an excellent model in which to study redox-based modifications due to the rapid accumulation of oxidising agents that occurs during immune invasion. This so-called “oxidative burst” causes spontaneous oxidation of cysteine residues that are present in many regulatory proteins. These modifications fine-tune the activities of proteins that harbour them, enabling them to act in a concerted effort to reprogram the transcriptome, prioritising the expression of immune-related genes over housekeeping genes. Disulphide bonds (S-S) and S-nitrosothiols (SNO, i.e. the addition of an NO group to a cysteine moiety) have been shown to play particularly important roles in plant immunity. However, what still remains unclear is how these redox-based PTMs are rendered reversible, enabling them to act as molecular signalling switches.

The work presented in this thesis explores a class of enzymes that are responsible for controlling the cellular levels of protein oxidation: the Thioredoxins. In addition to their well-established role in reducing disulphide bonds, I demonstrate in Chapter 3 that Thioredoxins are able to reverse protein S-nitrosylation during plant immune signalling. Immune-inducible Thioredoxin-h5 (TRXh5) was shown to be unable to restore immunity in gsnor1 mutants that display excessive accumulation of the NO donor S-nitrosoglutathione, but rescued impaired immunity and defence gene expression in nox1 mutants that exhibit elevated levels of free NO. This data indicates that TRXh5 discriminates between protein-SNO substrates to provide previously unrecognized specificity and reversibility to protein-SNO signalling in plant immunity. Furthermore, data is presented to show that TRXh5 reversed the effects of S-nitrosylation on many immune-related transcriptional regulators in vitro, forming the initial stages of an investigation into which proteins and pathways might be controlled by reversible S-nitrosylation in plant immunity (Chapters 3 & 4).
Although the majority of transcriptional regulators are likely modified at their site of action, the nucleus, very little is currently known about nuclear redox signalling in plants. Therefore, in Chapter 5 a subclass of the Thioredoxin superfamily was studied, the Nucleoredoxins, which have previously been shown to display disulphide reduction activity and localise in part to the nucleus. Here it is revealed that the activity and nuclear accumulation of Nucleoredoxin 1 (NRX1) is induced by the plant leaf pathogen *Pseudomonas syringae*, suggesting a key role for this protein in immune signalling. Target-capture experiments and subsequent mass spectrometry analysis identified the first *in vitro* targets of NRX1 and revealed many proteins with roles in oxidative stress, including the hydrogen peroxide scavenger Catalase 2 (CAT2). Moreover, overexpression of NRX1 was shown to be able to rescue the enhanced cell death phenotype of *cat2* knockout mutants in response to the oxidative stressor, methyl viologen. Accordingly, *nrx1* knockout mutants also exhibited an enhanced cell death phenotype in response to methyl viologen treatment. Together, these data indicate that NRX1 plays a key role in the control of oxidative stress-mediated cell death, potentially through direct regulation of Catalase proteins.

Taken together, the work in this thesis implicates members of the Thioredoxin family as key regulators of transcriptional reprogramming during plant immunity and uncovers a novel role for Thioredoxin superfamily member, NRX1, in the control of oxidative stress.
Lay summary

Plants are sessile organisms and so when they are faced with undesirable environmental conditions around them, they must be able to adapt rapidly and efficiently to enable their survival. In the field, plants are constantly under attack from pathogens, causing yield losses ranging from 10-50% for crops such as rice and maize. As a result, they have evolved a complex immune system that enables them to fight off a wide range of pathogens and insects. The functional components of the plant immune system, proteins, are often modified by oxidative molecules such as hydrogen peroxide and nitric oxide during pathogen attack, causing changes in their function, localisation and activity; this enables proteins to prioritize the correct immune response. In this thesis, the way in which these oxidative modifications, and hence the proteins that harbour them, are controlled was studied at the molecular level. The model plant Arabidopsis thaliana is used to enable rapid genetic manipulation and growth times. However, the findings in this study uncover mechanisms behind key regulatory processes that exist in many diverse organisms. The work presented here can therefore potentially be transferred into agriculturally important crops or even animal systems, thereby contributing to enhanced food security and novel therapies in biomedicine in future.
Declaration

I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or any other university. Permission has been granted by co-authors of published work for inclusion in this thesis.

Sophie Kneeshaw
Acknowledgements

First and foremost, I would like to thank my principal supervisor Dr. Steven Spoel for giving me the opportunity to undertake this PhD, for providing guidance over its course and continued support beyond it. He has been an excellent supervisor, offering timely suggestions when needed, whilst allowing me freedom to develop my own ideas and gain confidence as a research scientist. I have been presented with many exciting and special opportunities over the course of my studies that have helped enhance and progress my career.

A big thank you also to Prof. Yasuomi Tada, who was extremely supportive during my placement in his lab at Nagoya University. I thoroughly enjoyed our many conversations in which he shared valuable insights. In addition, I would like to thank Yasuomi’s entire lab for their hospitality and for providing me with assistance throughout my stay. A special thanks to Mika Nomoto for being so welcoming both inside and outside the lab. I look forward to working together again in the future.

I am grateful to my secondary supervisor Dr. Thierry Le Bihan and Lisa Imrie for their work on the mass spectrometry analysis presented in this thesis. I was always provided support on this aspect of the project whenever it was requested and I very much enjoyed working with them both.

Thank you to Dr. Rumana Keyani and Silvere Gelineau for their previous work on the NRX and TRX projects respectively. Their experiments and materials were invaluable to the progression of my project.

A big thanks to all the present and past members of Steven’s lab: Michael, James, Heather, Ellie and Lucas. Also to Dr. Gerben van Ooijen and his student Louise who share lab space and meetings with us. Everyone has been a pleasure to work with and made the lab a fun place to be.

An important thank you to the Biotechnology and Biological Sciences Research Council (BBSRC) for funding my PhD project and providing me with the means to carry out my research and attend conferences during my time as a student.

Finally, I would like to thank my family, friends and partner for their support and encouragement during the course of the PhD.
Table of Contents

Abstract ................................................................................................................. i
Lay summary ........................................................................................................... iii
Declaration .............................................................................................................. iv
Acknowledgements ................................................................................................. v
List of figures ........................................................................................................... x
List of tables ........................................................................................................... xii
List of abbreviations ............................................................................................... xiii

Chapter 1

Introduction ............................................................................................................. 1
  1.1 The plant immune system ............................................................................. 2
    1.1.1 Pathogen recognition ........................................................................... 2
    1.1.2 Hypersensitive response ....................................................................... 4
    1.1.3 Hormone signalling and systemic acquired resistance ......................... 5
  1.2 Cellular redox changes during immune activation ........................................ 6
    1.2.1 Generation of ROS ............................................................................... 7
    1.2.2 NO production ..................................................................................... 8
    1.2.3 Cellular sensing of ROS/RNS ................................................................ 9
  1.3 Redox-based posttranslational modifications ................................................ 10
    1.3.1 Disulphide linkages regulate plant immune proteins ........................... 11
    1.3.2 Protein S-nitrosylation in immune signalling ....................................... 13
  1.4 Antioxidant enzymes ................................................................................... 15
    1.4.1 Thioredoxins ..................................................................................... 16
    1.4.2 Thioredoxins in plant immunity ........................................................... 18
  1.5 Aims and objectives of this study .................................................................. 20

Chapter 2

Materials and methods ......................................................................................... 22
  2.1 Plant growth conditions ............................................................................... 23
  2.2 Plasmid constructs and plant transformation ............................................... 23
  2.3 Genomic DNA extraction ........................................................................... 24
  2.4 PCR genotyping ......................................................................................... 24
Chapter 3

Selective protein denitrosylation activity of Thioredoxin-h5 modulates plant immunity

3.1 Background ................................................................. 42
3.2 Results....................................................................... 43
  3.2.1 TRXh5 displays protein-SNO reductase activity in vitro .............................................. 43
  3.2.2 The TRX/NTR system exhibits protein-SNO reductase activity in vivo ......................... 45
  3.2.3 Trans-denitrosylation activity of TRXh5 reverses SNO modification.............................. 47
  3.2.4 TRXh5 selectively restores immune deficiencies caused by elevated protein-SNO .......... 51
  3.2.5 TRXh5 restores SA-dependent immune signalling by selective protein denitrosylation ....... 55
  3.2.6 TRXh5 restores elevated levels of polyubiquitinated proteasome subunit RPN10 in nox1 plants ......................................................................................................................... 61
3.3 Discussion .................................................................... 63
Chapter 4
Thioredoxin-h5 denitrosylates transcription (co)factors essential for plant immunity

4.1 Background

4.2 Results

4.2.1 Redox modification of NPR proteins is controlled by TRXh5

4.2.2 S-nitrosylation affects DNA binding of WRKY transcription factors

4.2.3 TRXh5 reverses the effects of NO on the DNA binding affinity of WRKY18

4.3 Discussion

Chapter 5
Nucleoredoxin 1 protects plant cells against oxidative stress

5.1 Background

5.2 Results

5.2.1 NRX proteins exhibit unique disulphide reductase activities

5.2.2 NRX1 localises to the cytoplasm and the nucleus

5.2.3 NRX1 is redox active during plant immune responses

5.2.4 Mutant nrx1 plants display autoimmune responses

5.2.5 Identification of target proteins of NRX1 oxidoreductase activity

5.2.6 In vivo validation of NRX1 interactors and substrates

5.2.7 NRX1 protects plant cells from oxidative stress

5.3 Discussion

Chapter 6
General discussion

6.1 TRXh5 as a selective protein denitrosylase

6.2 Regulation of NPR and WRKY families by S-nitrosylation

6.3 Nucleoredoxin 1 modulates responses to oxidative stress in plants

6.4 General conclusions, impact and future directions

Bibliography
Appendix I ............................................................................................................................................. 147
Supplementary figures ..................................................................................................................147
PDF of publication .......................................................................................................................148

Appendix II ....................................................................................................................................... 163
Supplementary figures ..................................................................................................................163
Supplementary tables ..................................................................................................................164
List of figures

Figure 1.1 PTI and ETI generate a “zig-zag” model for plant immunity ................................................. 4
Figure 1.2 Perturbation of the cellular redox state during immune induction is sensed by cysteine containing proteins ........................................................................................................................... 10
Figure 1.3 Redox-based cysteine modifications ........................................................................................ 11
Figure 1.4 Regulation of disulphide linkages by the NADPH-dependent TRX system ............................. 17
Figure 1.5 Redox regulation of NPR1 during plant immunity ................................................................. 20
Figure 3.1 The plant TRX/NTR system displays protein denitrosylation activity in vitro ...................... 44
Figure 3.2 The plant TRX/NTR system displays protein denitrosylation activity in vivo ...................... 46
Figure 3.3 BSA is trans-denitrosylated by TRXh5 in vitro .................................................................. 48
Figure 3.4 TRXh5 trans-denitrosylates plant protein in vitro .............................................................. 50
Figure 3.5 GSNO treatment does not affect mixed disulphide status of TRXh5 in vivo ...................... 51
Figure 3.6 Developmental abnormalities of nox1 and gsnor1 plants are not restored by TRXh5 ......... 52
Figure 3.7 TRXh5 selectively restores immune deficiencies caused by elevated protein-SNO ............ 53
Figure 3.8 TRXh5 rescues immunity in nox1 plants through its denitrosylation activity ....................... 54
Figure 3.9 TRXh5 does not exhibit differential disulphide-reduction activity in nox1 and gsnor1 backgrounds ......................................................................................................................................... 56
Figure 3.10 TRXh5 reduces SNO content in nox1 but not gsnor1 background ........................................ 57
Figure 3.11 TRXh5-mediated NPR1 monomerisation occurs in both nox1 and gsnor1 backgrounds 58
Figure 3.12 TRXh5 selectively restores immune gene expression in response to pathogen infection ........................................................................................................................................................................ 59
Figure 3.13 Denitrosylation activity of TRXh5 restores pathogen-induced immune gene expression ........................................................................................................................................................................ 60
Figure 3.14 Denitrosylation activity of TRXh5 restores SA-induced immune gene expression ........... 61
Figure 3.15 Overall polyubiquitination of protein is unaffected in SNO mutants ................................. 62
Figure 3.16 Polyubiquitination of proteasomal subunit RPN10 is increased in nox1 but restored to WT levels by TRXh5 ................................................................................................................................................ 63
Figure 3.17 Proposed model showing that the SNO reductases GSNOR1 and TRXh5 regulate different branches of protein-SNO in plant immune signalling ................................................................. 65
Figure 4.1 NPR3 and NPR4 are protein-SNO targets of TRXh5 ............................................................ 73
Figure 4.2 NPR3 and NPR4 form oligomers that are reduced by TRXh5 .............................................. 74
Figure 4.3 WRKY transcription factors are protein-SNO targets of TRXh5 ........................................ 75
Figure 4.4 S-nitrosylation of WRKY transcription factors differentially affects their DNA binding .... 77
Figure 4.5  Sequence alignments of WRKY domains from *Arabidopsis* WRKY family .......................... 80
Figure 4.6  S-nitrosylation of WRKYs affects DNA binding at distinct W-box sequences differently ... 82
Figure 4.7  S-nitrosylation of WRKY18 cysteine mutants does not increase DNA binding ................. 83
Figure 4.8  TRXh5 reverses the effects of S-nitrosylation on WRKY18 DNA binding .................. 84
Figure 5.1  Domain structures of *Arabidopsis* NRX proteins .......................................................... 94
Figure 5.2  Recombinant NRX1 and NRX2 display disulphide reduction activity *in vitro* ............. 95
Figure 5.3  Protein concentration affects the dynamics of NRX-mediated disulphide reduction ...... 96
Figure 5.4  Both active sites of NRX1 contribute towards its disulphide reductase activity .......... 98
Figure 5.5  Cytoplasmic and nuclear localisation of NRX1-GFP in protoplasts ............................... 99
Figure 5.6  NRX1 expression is induced by pathogen ....................................................................... 100
Figure 5.7  NRX1 disulphide-reduction activity increases after pathogen treatment .................. 101
Figure 5.8  NRX1 accumulates in the nucleus following pathogen treatment ............................ 102
Figure 5.9  Mutant *nrx1* plants show constitutive defence gene expression ................................. 103
Figure 5.10  Mutant *nrx1* plants exhibit enhanced disease resistance ........................................... 104
Figure 5.11  Mutant *nrx1* plants show constitutive SAR .............................................................. 105
Figure 5.12  Enhanced disease resistance of *nrx1* plants is SA-dependent ................................. 106
Figure 5.13  Active site cysteine mutant of NRX1 is used to capture targets ................................. 108
Figure 5.14  GO term analysis of NRX1(C58,378S) targets ................................................................. 110
Figure 5.15  NRX1 interacts with TRXh5 *in vivo* ........................................................................ 112
Figure 5.16  NRX1 interacts with CAT2 *in vitro* and *in vivo* ...................................................... 113
Figure 5.17  Mutant *nrx1* plants have decreased tolerance for oxidative stress ....................... 115
Figure 5.18  NRX1 restores oxidative stress responses in *cat2* mutants ..................................... 116
Figure S1  Purification of His-tagged recombinant TRXh5 and NTRA proteins ............................ 147
Figure S2  Purification of His-tagged recombinant WT and mutant NRX1 proteins .................... 163
Figure S3  Testing the *nrx1* antibody ........................................................................................... 164
List of tables

Table 2.1  Primers used in sequencing of T-DNA insertion SALK lines .......................................................... 25
Table 2.2  Primers used in sequencing of par2-1 and nox1 mutations .............................................................. 26
Table 2.3  Primers used in RT-PCR reactions ........................................................................................................ 27
Table 2.4  Primers used in qPCR reactions ............................................................................................................. 28
Table 2.5  Primers used for site-directed mutagenesis of NRX1 ............................................................................. 30
Table 2.6  Antibodies used in western blot analysis ............................................................................................... 33
Table S1  Targets of the NRX1(C58,378) protein identified by mass spectrometry ...................................... 166
Table S2  Targets of the NRX1 protein identified by mass spectrometry ............................................................ 167
List of abbreviations

AMS  4-acetamido-4’-maleimidyldistibene-2,2’-disulfonic acid
APX  ASCORBATE PEROXIDASE
Avr  avirulence
BST  biotin switch technique
CAT  CATALASE
cir1  constitutive induced resistance 1
cpr  constitutive expresser of PR genes
CTAB  cetyl trimethylammonium bromide
DAF-2T  triazolofluorescein
DNCB  1-chloro-2,4-dinitrobenzene
DTT  dithiothreitol
DVL  DISHEVELLED
EF-Tu  ELONGATION FACTOR TU
EFR  EF-Tu receptor
ET  ethylene
ETI  effector triggered immunity
ETS  effector triggered susceptibility
FLI-I  FLIGHTLESS-I
FLS2  FLAGELLIN SENSITIVE 2
FTR  FERRODOXIN-TRX REDUCTASE
GO  gene ontology
GOX  GLYCOLATE OXIDASE
GPX  GLUTATHIONE PEROXIDASE
GR  GLUTATHIONE REDUCTASE
GRX  GLUTAREDOXIN
GSH  reduced glutathione
GSNO  S-nitrosoglutathione
GSNOR1  S-NITROSOGLUTATHIONE REDUCTASE 1
GSSG  oxidised glutathione
GST  GLUTATHIONE S-TRANSFERASE
H₂O₂  hydrogen peroxide
HNO₂  nitrous acid
HR  hypersensitive response
ICS  ISOCHORISMATE SYNTTHASE
JA  jasmonic acid
KLHL12  KELCH-LIKE 12
Lsd1  lesion-stimulating disease 1
MV  methyl viologen
N₂O₄  dinitrogen tetroxide
NADH  nicotinamide adenine dinucleotide
NADPH  nicotinamide adenine dinucleotide phosphate
NB-LRR  nucleotide binding leucine rich repeat
NEM  N-ethylmaleimide
NH₄⁺  ammonium
NiR  NITRITE REDUCTASE
NO  nitric oxide
NO₂  nitric dioxide
NO₂⁻  nitrite
NO₃⁻  nitrate
NOS  NITRIC OXIDE SYNTHASE
NPR1  NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1
NPR3  NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 3
NPR4  NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 4
NR   NITRATE REDUCTASE
NRTs NITRATE TRANSPORTERS
NRX  NUCLEOREDOXIN
NTR  NADPH-DEPENDENT TRX REDUCTASE
O₂   molecular oxygen
O₂⁻  superoxide
ONOO⁻ peroxynitrite
ORA-59 octadecanoid-responsive Arabidopsis AP2/ERF-domain protein 59
PAMPs pathogen associated molecular patterns
PCD  programmed cell death
PCP  planar cell polarity
PDI  PROTEIN DISULPHIDE ISOMERASE
PFK1 PHOSPHOFRACTOKINASE 1
PR   pathogenesis-related
PRRs pathogen recognition receptors
PRX  PEROXIREDOXIN
Psm  P. syringae pv. maculicola
Pst  Pseudomonas syringae pv. tomato
PTI  pattern triggered immunity
PTMs post-translational modifications
qPCR quantitative real-time PCR
R    resistance
RBOHs respiratory burst oxidase homologues
RNS  reactive nitrogen species
ROI  reactive oxygen intermediate
ROS  reactive oxygen species
<table>
<thead>
<tr>
<th>Abbreviation</th>
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</tr>
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<tbody>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SABP3</td>
<td>SA-BINDING PROTEIN 3</td>
</tr>
<tr>
<td>SAR</td>
<td>systemic acquired resistance</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SH</td>
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<td>SNP</td>
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<tr>
<td>XOR</td>
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Chapter 1

Introduction
1.1 The plant immune system

The transcriptome of an organism is constantly changing in response to environmental cues and stresses. All organisms rely on a complex range of signalling systems in order for their cells to communicate and translate an external input to an internal action. An excellent example of this is the plant immune system, in which there is rapid reprogramming of genes in response to pathogen attack (Spoel et al., 2010). Due to the fact that plants lack a circulatory system with “immune-specific” cells as seen in the vertebrate adaptive immune system, innate signalling networks are essential for plant protection against disease (Spoel and Dong, 2012). Thus, the plant immune system consists of many overlapping networks by which to communicate information. Whilst plants possess the tools to generate a specific immune response against a wide range of pests, including insects, fungi and viruses, the work in this study will primarily focus on the defence against pathogenic bacterial invaders.

1.1.1 Pathogen recognition

There are two main routes that lead to immune activation in plants; through detection of pathogen associated molecular patterns (PAMPs) by transmembrane pattern recognition receptors (PRRs), or through intracellular nucleotide-binding leucine rich repeat (NB-LRR) containing receptors that recognise effector proteins secreted into the cell by the pathogen (Jones and Dangl, 2006; Zipfel, 2014). The former is termed pattern-triggered immunity (PTI) and consists of receptor kinases or receptor like proteins that recognise conserved structures common to a range of phytopathogens (Figure 1.1). Key examples of these PAMP-PRR couples include bacterial flagellin, which is recognised by the Flagellin Sensitive 2 (FLS2) receptor and Elongation Factor Tu (EF-Tu), which pairs with the EF-Tu Receptor (EFR) (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004, 2006). Some pathogens have evolved more complex mechanisms by which to evade recognition by PRRs; they produce effector proteins which are delivered directly into the plant cell using type III secretion systems (Cornelis and
Van Gijsegem, 2000; Mudgett, 2005). These effectors, also known as avirulence (Avr) proteins, can suppress the immune system by, for example, preventing the expression of defence genes (He et al., 2006). Therefore, the secretion of effectors into host cells promotes pathogen virulence, a process termed effector-triggered susceptibility (ETS) (Figure 1.1). To combat effector proteins, plants have developed a second type of immune response: effector triggered immunity (ETI), which is mediated by NB-LRR receptors, otherwise known as Resistance (R) proteins (Figure 1.1) (Jones and Dangl, 2006). Whereas PRRs are only able to recognise structures common amongst certain types of pathogens, the NB-LRR receptors of ETI are required to defend against a vast amount of diverse effector proteins. The original “gene-for-gene” model (Flor, 1956), in which a single NB-LRR receptor targets a single effector, is unable to explain how NB-LRR-mediated resistance is able to defend against the vast range of effectors secreted by pathogens. Instead, in order to achieve this, NB-LRR receptors monitor “altered self” molecules; proteins that have been modified by the invading pathogen. Through doing this, a single NB-LRR receptor can be effective for entire groups of pathogen effectors that operate by the same mechanisms (Spoel and Dong, 2012). Co-evolution of pathogens and plants that are in a continuous battle with one another to overcome and re-enforce defence systems, respectively, is thought to lead to a “zig-zag” model for the effectiveness of plant immune responses (Figure 1.1) (Jones and Dangl, 2006).

Recognition of pathogens by PTI and ETI trigger a variety of downstream signalling events that work to establish an immune response. These involve production of reactive oxygen/nitrogen species (ROS/RNS), phytohormone accumulation and MAPK signalling cascades, which work in a concerted effort to activate the immune transcriptome. Although many of the components of these signalling pathways are common to both PTI and ETI, the duration and effects of their activity are greater in an ETI response (Figure 1.1) (Cui et al., 2015).
Figure 1.1 PTI and ETI generate a “zig-zag” model for plant immunity

PAMPs are recognised by plant PRR receptors which signal to generate pathogen triggered immunity (PTI). Pathogens then secrete effectors into the plant cell which promote virulence and cause effector triggered susceptibility (ETS). NB-LRR receptors recognise specific effectors (Avr-R, shown in red) and then signal to initiate effector triggered immunity (ETI). When the amplitude of immune responses is strong enough, a hypersensitive response (HR) is triggered. Figure adapted from Jones and Dangl, 2006.

1.1.2 Hypersensitive response

One of the key events that occurs following recognition of pathogen effectors is the hypersensitive response (HR) (Figure 1.1). The HR is initiated by an “oxidative burst”: a rapid accumulation of ROS/RNS at the site of infection, which facilitates localised programmed cell death (PCD) (Coll et al., 2011; Grant et al., 2000). PCD acts to limit pathogen growth by cutting off resources needed from the living plant tissue and by decreasing water potentials that generate a desiccated, high osmolarity environment (Wright and Beattie, 2004); it is therefore unsurprising that the HR is an
effective mechanism against biotrophic invaders that thrive on living host cells (Morel and Dangl, 1997). Although it has been observed to be effective in many cases, some pathogens possess mechanisms by which to evade elimination by the HR. For example, necrotrophs that feed off dead tissue are more likely to thrive in this environment, allowing their continued virulence within the plant (Glazebrook, 2005). However, alongside the HR, another layer of plant defence is activated by the detection of a pathogen: the accumulation of defence phytohormones. These hormones constitute a complex signalling network that acts to defend against a wide range of stresses and are associated with “immune memory” across the plant.

1.1.3 Hormone signalling and systemic acquired resistance

Hormone signalling systems are widely studied in plants; salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the main phytohormone signals involved in the activation of plant immunity (Bari and Jones, 2009). The SA-dependent and JA/ET-dependent signalling pathways are effective against biotrophic and necrotrophic pathogens respectively (Pieterse et al., 2009). They have generally been shown to be antagonistic to one another, although they may act cooperatively in some circumstances (Derksen et al., 2013; Spoel and Dong, 2008). These hormones are responsible for activating a series of downstream molecules, many of which are transcriptional regulators that in turn control different sets of defence genes, such as the SA-induced Pathogenesis-Related genes (PR genes), the protein products of which enable plants to establish immunity against biotrophs (Pieterse et al, 2009; Spoel and Dong, 2012).

The accumulation of SA in an infected cell results in signals that spread across the rest of the plant. By this process the plant becomes “primed”, such that a secondary infection at a site independent to the initial pathogen attack is able to generate an immune response much more rapidly. This phenomenon is known as systemic acquired resistance (SAR) and is dependent on SA (Durrant and Dong, 2004; Ryals et al., 1996). In addition to providing immune memory within the
lifetime of a particular plant, SAR has also been shown to generate transgenerational resistance, meaning that the offspring of a plant that has previously launched a successful SA-dependent immune response are automatically primed against similar invaders (Luna et al., 2012). A key protein in the SA signalling pathway that is essential to the establishment of SAR, is Non expressor of Pathogenesis-Related genes 1 (NPR1) (Cao et al., 1994). NPR1 is a transcriptional coactivator and its presence has been shown to be essential to SA-dependent transcriptional reprogramming (Wang et al., 2006). In addition to its function as a coactivator in the nucleus, NPR1 has also been shown to play an important role in the cytoplasm where it is involved in the suppression of JA signalling, demonstrating its requirement in the antagonism between SA and JA signalling (Spoel et al., 2003).

Both the HR and SAR are extremely important in the generation of an effective immune response against a range of invading pests. As discussed, these processes are underpinned by both the production of ROS/RNS and phytohormones and indeed, there is active crosstalk between these two processes. Both ROS and defence hormones regulate each other during stress responses and this interplay provides a multi-layered signalling network, which is only just beginning to be dissected (Herrera-Vásquez et al., 2015; Xia et al., 2015). Whilst hormones have been known to act as signalling molecules for a great number of years, the discovery that ROS/RNS can generate redox signalling networks is much more recent. The mechanisms by which immune-related redox signalling pathways operate will be the main focus of this study.

1.2 Cellular redox changes during immune activation

A hallmark of immune activation in plants is the “oxidative burst”, which primarily involves the rapid production of ROS and RNS such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and nitric oxide (NO), all of which have the potential to be highly damaging to the cell (Apostol et al., 1989). They can contribute to immunity by causing direct damage to the pathogen, or by contributing to immune mechanisms such as cell wall strengthening or the HR (Delledonne et al., 1998; Levine et al., 1994). In
addition to triggering the HR, these molecules can be employed as signalling devices making them extremely important tools of the plant immune system. Before the effects of ROS/ RNS generation on the intracellular environment of a cell can be discussed, it is important to understand how and where they are produced.

1.2.1 Generation of ROS

The main source of ROS production in plants is thought to be through a group of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases known as the respiratory burst oxidase homologues (RBOHs) (Frederickson Matika and Loake, 2014). RBOHs generate ROS by taking electrons from intracellular NADPH and transferring them across the plasma membrane into the apoplast, where they are coupled to molecular oxygen (O₂) to generate unstable, highly reactive oxygen intermediates (Skelly and Loake, 2013). First generated is O₂⁺, which then undergoes either a superoxide dismutase (SOD) -dependent or -independent dismutation reaction to produce H₂O₂ (Noctor and Foyer, 1998). In Arabidopsis, there are 10 RBOH genes and of those, both, RBOHD and RBOHF are required in the generation of a HR during plant immunity (Frederickson Matika and Loake, 2014; Torres et al, 2002).

In addition to ROS generation through NADPH oxidases and SOD, haem-containing peroxidase enzymes have been shown to contribute to this process. The primary function of peroxidases is to metabolize H₂O₂, but these enzymes have also been shown to be capable of producing this molecule in the presence of a reductant (Bolwell and Wojtaszek, 1997). Indeed, silencing of two peroxidase genes in Arabidopsis resulted in plants that had an impaired oxidative burst and were more susceptible to pathogen infection (Bindschedler et al., 2006).

Other enzymes that have been reported to contribute to the accumulation of ROS during plant immunity include glycolate oxidases (GOXs). It has been shown that the silencing of GOX resulted in
an impaired accumulation of H$_2$O$_2$ in response to pathogen, suggesting that these enzymes also play a part in establishing the HR (Rojas et al., 2012).

### 1.2.2 NO production

In synchrony with the generation of ROS, RNS are also produced during the oxidative burst. Although RNS include nitric dioxide (NO$_2$), nitrous acid (HNO$_2$) and dinitrogen tetroxide (N$_2$O$_4$) amongst others, NO is the main RNS produced in the oxidative burst and has been shown to be a key signalling molecule during plant immunity (del Rio, 2015). In mammals, the primary source of NO is derived from nitric oxide synthase (NOS) enzymes, of which there are three main types; neuronal, endothelial and inducible (Wendehenne et al., 2001). NOS enzymes catalyse the NADPH-dependent oxidation of L-arginine, a process that also produces citrulline and NO. Despite many attempts to identify corresponding enzymes in higher plants, no homologous genes or proteins have been found for NOS (Fröhlich and Durner, 2011). However, a Ca$^{2+}$-dependent NOS-like activity has been suggested for NO generation in plants in several studies, and L-arginine-dependent NO formation has been demonstrated (Delledonne et al., 1998; Durner et al., 1998; Moreau et al., 2010). Moreover, the use of human NOS inhibitors in plants was shown to suppress L-arginine-dependent NOS activity and therefore NO production (Corpas et al., 2006; Delledonne et al., 2001). Although the enzymes in this process are yet to be identified, this represents one possible pathway to NO synthesis.

In addition to the oxidation of L-arginine, plants also utilise reductive mechanisms to produce NO from nitrite (NO$_2^-$). The main source of NO in plants comes initially from inorganic nitrate (NO$_3^-$) in the soil, which is taken up by nitrate transporters (NRTs) in the roots. NO$_3^-$ is then converted to NO$_2^-$ in the leaves by NADPH-dependent nitrate reductase (NR) before being processed by nitrite reductase (NiR) to produce ammonium (NH$_4^+$) (Frungillo et al., 2014). In addition to their function in reducing NO$_3^-$, NRs have also been shown to be able to generate NO from NO$_2^-$, although this only occurs in the presence of high NO$_2^-$ (Rockel et al., 2002). Two NR genes have been identified in Arabidopsis: NIA1 and NIA2. The nia1 nia2 double mutant was found to be impaired in NO production and hence was
unable to generate a HR. However, upon the exogenous application of NO$_2^-$, NO production was restored, demonstrating that NO synthesis from NO$_2^-$ also occurs through mechanisms independent to NIA1 and NIA2 (Modolo et al., 2006). Indeed, it was shown that NO can be generated from NO$_2^-$ through the mitochondrial electron transport system in tobacco, and subsequently, Arabidopsis (Modolo et al., 2005; Planchet et al., 2005). In anaerobic conditions, NO$_2^-$ can also be reduced to NO in the peroxisomes. This reaction is catalysed by xanthine oxidoreductase (XOR), which uses nicotinamide adenine dinucleotide (NADH) or xanthine as a reductant (Godber et al., 2000).

1.2.3 Cellular sensing of ROS/RNS

In order to protect the cell from ROS/RNS-mediated damage, antioxidant molecules and systems are in place that essentially act as “redox buffers” against ROS/RNS (Noctor and Foyer, 1998). Within a plant cell there are many different small molecule redox couples that continuously fluctuate between oxidised and reduced states. The varying reduction potentials of these small molecule couples create an electron flow from molecules with a lower reduction potential (e.g. NAD(H) or NADP(H) couples) to those with a higher reduction potential (e.g. glutathione or ascorbate couples) (Figure 1.2) (Foyer, 2005; Spoel and Loake, 2011). During the oxidative burst and accumulation of defence phytohormones, the cellular redox state environment is massively disturbed and there is an alteration of the ratio of oxidised and reduced small molecules. These changes can then cause proteins possessing reactive cysteine residues to reversibly switch between their reduced and oxidised forms (Figure 1.2). In this process, these proteins essentially act as “redox sensors”, a mechanism thought to have evolved to detect changes in the cellular redox environment and alter the transcriptional activity of a cell in response to this (Biswas et al., 2006). During plant immunity it is believe that through these redox sensors, signalling pathways are activated that facilitate reprogramming of the transcriptome to prioritize defence genes.
During pathogen attack, defence hormones such as salicylic acid (SA) accumulate synchronous with reactive oxygen and nitrogen species (ROS/RNS). This perturbs the redox state of the cell by disrupting the ratio of oxidised to reduced status of small redox couples. These changes are sensed by cysteine containing proteins, which are also subject to switching between oxidised and reduced states, potentially resulting in changes to their conformation, localisation or activity. Figure adapted from Spoel and Loake, 2011.

1.3 Redox-based posttranslational modifications

The oxidative changes that occur at cysteine residues are known as redox-based, post-translational modifications (PTMs). PTMs are chemical alterations to proteins that can subsequently alter their conformation, localisation, stability or activity, a concept originally named the allosteric theory (Monod et al., 1965). PTMs provide a way to dramatically increase the diversity of the proteome (Kwon et al., 2006). So far, over 300 types of PTMs have been identified (Jensen, 2004) and in the plant immune response, they have been implicated in not only the perception of
phytopathogens but also throughout signalling cascades ultimately leading to activation of defence genes (Stulemeijer and Joosten, 2008).

Redox modifications include S-thiolation, S-nitrosylation, and S-hydroxylation, all of which represent different levels of oxidation (Figure 1.3). The majority of redox modifications at cysteine residues are reversible, with the exception of S-sulphonation (Spadaro et al., 2010; Spoel and Loake, 2011).

![Redox-based cysteine modifications](image)

**Figure 1.3 Redox-based cysteine modifications**

Shown are cysteine thiols (SH) which can be further oxidised by S-nitrosylation (SNO), S-sulphenation (SOH), S-thiolation (SS), S-glutathionylation (SSG), S-sulphination (SO₂H) and S-sulphonation (SO₃H). All levels of oxidation, except for S-sulphonation, are reversible. Figure adapted from Spadaro et al., 2010.

Many of these redox-based cysteine modifications have been implicated in the control of immune-related proteins but two of the main ones involved in plant redox signalling are disulphide linkages (SS) and S-nitrosylation (SNO).

### 1.3.1 Disulphide linkages regulate plant immune proteins

Disulphide linkages refer to the bond formed between two cysteine thiols. As they can either form between two cysteines in the same protein or serve to unite two separate molecules through side chain cysteines, they are extremely important for protein conformation. Probably the best
studied example of how disulphide bonds can affect immune signalling in plants, is the redox post-translational control of the coactivator of SA-dependent immune signalling, NPR1. As previously discussed, NPR1 is directly involved in the activation of many defence genes, such as PR genes, and as a result is essential to the establishment of SAR (Cao et al., 1994). Moreover, in recent years, NPR1 was found to be a receptor of SA, along with its paralogs NPR3 and NPR4 (Fu et al., 2012; Kuai et al., 2015; Wu et al., 2012), reinforcing the importance of this protein in SA-mediated immunity. Redox regulation of NPR1 has been shown to be essential for both the localisation and function of this protein (Mou et al., 2003; Tada et al., 2008). It was demonstrated that disulphide bonds form between cysteine residues of different NPR1 monomers, resulting in a cytoplasm-bound oligomer. During pathogen infection, changes brought about by perturbation of the cellular redox state induce reduction of the NPR1 oligomer, allowing monomers to enter the nucleus and perform their role as transcriptional coactivators (Kinkema et al., 2000; Mou et al., 2003). In the nucleus, NPR1 binds to members of the TGA family of basic domain/leucine zipper transcription factors, whose role in activation of PR genes is dependent on this interaction (Després et al., 2000; Lebel et al., 1998; Zhang et al., 1999; Zhou et al., 2000). Indeed, disulphide bonding was also demonstrated to be important in the regulation of TGA family members. It was shown that an intramolecular disulphide bond between Cys260 and Cys266 in TGA1 precluded its interaction with NPR1. This mechanism was also shown to be the case for TGA4, but not for the other TGA family members, which lack Cys266, diminishing their ability to form this internal disulphide linkage (Després et al., 2003). Upon pathogen infection, redox changes in the cell result in reduction of the internal disulphides in TGA1, enabling this protein to interact with NPR1, which enhances its DNA binding activity (Després et al., 2003).

In addition to the formation of disulphide bonds, both NPR1 and TGA proteins can be modified by another redox-based PTM; S-nitrosylation. This adds another layer of regulation for these proteins and demonstrates not only the complexity by which redox signalling networks operate, but the importance of understanding these individual modifications and how they affect their targets.
1.3.2 Protein S-nitrosylation in immune signalling

NO is a gaseous molecule and functions as a signalling molecule in all kingdoms. It is one of the principle molecules released during an oxidative burst and as a free radical, it is a highly reactive oxidising agent that is now emerging as a key signalling molecule via the formation of S-nitrosothiols (SNO groups) (Moreau et al., 2010; Yu et al., 2014). In addition, NO also can react with tyrosine residues (tyrosine 3-nitration), transition metals (metal nitrosylation) and other reactive oxygen intermediates (ROIs) (Leitner et al., 2009). S-nitrosylation has already been identified to be extremely important during immune signalling in plants, with several immune-related proteins identified as SNO targets. Reactions of proteins with free NO are not the only way of producing SNO groups; trans-nitrosylation is the process in which an NO group is transferred from an SNO donor molecule to a free acceptor thiol on a target protein (Astier et al., 2011). A key example of this in plants is trans-nitrosylation by S-nitrosoglutathione (GSNO), the product of the reaction between glutathione (GSH) and NO.

Both NPR1 and TGA transcription factors are redox modified by both disulphide linkages and S-nitrosylation. NPR1 was shown to be S-nitrosylated by the NO donor GSNO at Cys156, and this modification was found to facilitate NPR1 oligomerisation. Interestingly, it was shown that the addition of other oxidative species, including the poor NO donor sodium nitroprusside (SNP) and H2O2, did not result in reformation of the oligomer (Tada et al., 2008). In addition to NPR1, TGA transcription factors have also been demonstrated to receive SNO modifications. Not only did treatment with GSNO improve the DNA binding capacity of TGA1 via S-nitrosylation, it also was able to improve its interaction with the coactivator NPR1 (Lindermayr et al., 2010). In addition to NPR1 and members of the TGA family, other proteins involved in the transcription of immune-related genes have been shown to be regulated by S-nitrosylation. Two members of the R2R3-MYB subfamily of transcription factors, AtMYB2 and AtMYB30, displayed decreased DNA binding after being S-nitrosylated (Serpa et al., 2007; Tavares et al., 2014). MYB transcription factors are generally involved in stress responses and indeed MYB30 has been shown to play a key role in mediating cell death during the HR (Daniel et
A conserved cysteine in the DNA binding domain, found not only in other plant MYB family members but in animal and fungi homologs, was found to be the site of redox modification, suggesting that other MYB transcription factors could be similarly affected (Serpa et al., 2007).

In addition to transcriptional regulators, several other proteins that function in plant immunity are targets of S-nitrosylation. The role of NADPH oxidases in the generation of ROS during the HR have already been discussed, but in addition to producing redox active molecules, NADPH oxidases themselves have been shown to be regulated by redox PTM. S-nitrosylation of Arabidopsis RBOHD at Cys890 inhibits its ROS-generating activity (Yun et al., 2011), creating a feedback loop by which the extent of the oxidative burst can be regulated by one of its components, NO. It has also been shown that NO can act to potentiate other RNS. Upon reaction with O$_2^-$, NO forms peroxynitrite (ONOO$^-$), which is scavenged by Peroxiredoxin II E (PrxlI E). This detoxification activity of PrxlI E was supressed by its S-nitrosylation, resulting in increased levels of ONOO' (Romero-Puertas et al., 2007). In animals, ONOO' has been associated with PCD, but this is not the case in plants (Bonfoco et al., 1995; Delledonne et al., 2001). Therefore, whilst it is apparent that NO controls the cellular levels of ONOO' through SNO modification of PrxlIE, it is unclear whether this RNS has a direct effect on cell death during plant immunity. Other redox-related enzymes have also been identified as targets of SNO regulation. S-nitrosoglutathione Reductase 1 (GSNOR1) is an enzyme that metabolises the SNO donor, GSNO, which acts as a cellular reservoir for NO bioactivity because of its ability to $\text{trans}$-nitrosylate proteins. It is therefore thought that in plants, protein-SNO levels are largely controlled indirectly by GSNOR1, as well as at the level of NO biosynthesis which occurs both non-enzymatically and through NR (Feechan et al., 2005; Modolo et al., 2005). Mutations in the GSNOR1 gene result in increased cellular SNO levels in both plants and animals, and consequently Arabidopsis gsnor1 mutant plants are highly disease susceptible, demonstrating the importance in controlling this modification during plant immunity (Feechan et al., 2005; Liu et al., 2001). Recently it has been found that GSNOR1 itself is subject to S-nitrosylation and that this modification inhibits its activity, resulting in an increased pool of GSNO. GSNO was shown to negatively inhibit the activity of NR, indicating that the S-nitrosylation
of GSNOR1 was able to indirectly regulate the production of NO. This suggests that NO is able to control its own generation and bioavailability through SNO signalling (Firungillo et al., 2014).

Other notable immune-related proteins affected by SNO modification include the SA-Binding Protein 3 (SABP3), an important protein in the SA signalling pathway. In addition to binding SA, SABP3 functions as a carbonic anhydrase and has been shown to be important in the HR and pathogen resistance (Slaymaker et al., 2002). The S-nitrosylation of this protein was found to inhibit both its ability to bind SA and act as a carbonic anhydrase, providing a regulatory mechanism for the role of this protein in plant immunity (Wang et al., 2009).

The control of immune related proteins by redox modifications such as disulphide linkages and S-nitrosylation is clearly emerging as an important signalling phenomenon. However, in order for these modifications to act as signalling switches for protein regulation, there must be a way to reverse them. Indeed, plants have evolved a wide range of antioxidant enzymes specifically for reversal of redox modifications.

1.4 Antioxidant enzymes

In addition to small molecule antioxidants such as ascorbic acid and glutathione, a plant cell contains various types of antioxidant enzymes, present in all cellular compartments, which function to protect from oxidative damage and facilitate redox signalling. Antioxidant enzymes can fall into two general categories; those which are directly responsible for ROS/RNS scavenging (i.e. Peroxidase family) and those which act to reverse the effects that ROS/RNS cause to proteins (i.e. Redoxin family). Peroxidases include enzymes such as ascorbate peroxidase (APX), glutathione peroxidases (GPXs) and thioredoxin peroxidases, better known as peroxiredoxins (PRX), all of which have been shown to metabolize \( \text{H}_2\text{O}_2 \) using the molecule suggested in their nomenclature as a reductant, i.e, APX activity uses reducing power from ascorbate (Mittler et al., 2004; Mittler, 2002). Other enzymes that perform this function include the catalase (CAT) family, but CATs differ from conventional peroxidases in that
they require no cellular reductant (Mhamdi et al., 2010). In addition to peroxidases, another key ROS scavenging enzyme is SOD, which catalyses the dismutation of $\text{O}_2^-$ into $\text{H}_2\text{O}_2$ (Mittler, 2002).

Whilst ROS/RNS scavenging enzymes are essential to controlling the levels of oxidative stress in the cell, the protein modifications caused by ROS/RNS are regulated by a different set of enzymes. These molecules are generally referred to as redoxins and despite their being many subsets, they largely belong to a protein superfamily called the Thioredoxins.

### 1.4.1 Thioredoxins

The plant Thioredoxin superfamily consists of a large group of oxidoreductase proteins including amongst others thioredoxins (TRXs), glutaredoxins (GRXs), protein disulphide isomerase (PDI) and nucleoredoxins (NRXs) (Meyer et al., 2008). Members of the TRX superfamily are classified as such due to the presence of a TRX-like fold in their structure (Jacquot et al., 2002). Each requires a cellular reductant for displaying antioxidant activity and sub-families can be formed based on the specific reductive agent that is utilized.

Classical TRXs are small proteins (10-15 kDa) and have the conserved active site sequence WCGPC (Meyer et al., 2008). This active site acts as a potent disulphide reductant for oxidised targets of TRX, the first of which identified was ribonucleotide reductase, essential for the production of deoxyribonucleotides (Laurent et al., 1964). Crystallography and NMR analysis determined the three-dimensional structure of *Escherichia coli* TRX to have a five-stranded $\beta$-sheet flanked by four $\alpha$-helices. The active site is located on a protrusion between the second $\beta$-strand and the second $\alpha$-helix (Holmgren and Bjornstedt, 1995). This position on terminal end of the $\alpha$-helix has been shown to create a dipole moment partial positive charge, which results in a low pKa value for the first cysteine in the TRX active site. As a result, this cysteine acts as a nucleophile, donating electrons to a target cysteine involved in a disulphide linkage, creating a mixed disulphide intermediate between the TRX molecule and its target protein. This action causes deprotonation of the second active site cysteine
of TRX, which then forms a second nucleophilic attack on the mixed disulphide intermediate, generating an internal disulphide bond between the two active site cysteines of the TRX molecule (Holmgren, 1995). In order to transform the oxidised TRX molecule back to its reduced state, a cellular reductant is required. In plants, two types of TRX Reductases have been identified: NADPH-dependent TRX Reductase (NTR; Figure 1.4) and Ferrodoxin-Thioredoxin Reductase (FTR). These enzymes use reducing power from NADPH (Figure 1.4) and ferrodoxin, respectively, to donate electrons to the oxidised TRX, thereby recycling its enzymatic activity (Holmgren, 1995; Meyer et al., 2008; Vieira Dos Santos and Rey, 2006).

![Diagram](image)

**Figure 1.4  Regulation of disulphide linkages by the NADPH-dependent TRX system**

Reduced TRX (TRX-(SH)₂) reduces a disulphide of a target protein. In doing so, TRX becomes oxidised (TRX-S₂). Oxidised TRX is reduced by NADPH-dependent Thioredoxin Reductase (TR-(SH)₂) and FAD, resulting in oxidation of NTR (TR-S₂), the activity of which is recycled using reducing power from NADPH. Figure adapted from Holmgren, 1995.

Plants have evolved different types of TRX to cope with different types of oxidative stress. For example, the process of photosynthesis, which is fundamental to the plants survival, puts plant cells under large amounts of photo-oxidative stress. Consequently, chloroplastic ferredoxin-dependent TRX enzymes of the m- and f-types have evolved that regulate enzymes in carbon metabolism (Ruelland and Miginiac-Maslow, 1999; Vieira Dos Santos and Rey, 2006). Indeed, almost all of the enzymes involved in the photosynthetic reactions that constitute the Calvin cycle, including Fructose 1,6-Bisphosphatase, Sedoheptulose 1,7-Bisphosphatase, Phosphoribulokinase and Rubisco activase,
require activation by one of these TRXs (Schürmann and Jacquot, 2000). The NADPH-dependent TRX system (TRX and NTRA-C) has been identified as a prominent disulphide reductase in many other cellular compartments (Reichheld et al., 2005; Serrato, 2004).

1.4.2 Thioredoxins in plant immunity

As enzymes with a clear role in the control of oxidative stress, it was anticipated that TRX superfamily members play a role in plant immunity. Indeed, GRXs, for example, have been implicated in regulating SA/JA crosstalk. GRXs are redox enzymes that use reducing power from glutathione, which in turn is maintained by Glutathione Reductase (GR). Two Arabidopsis GRX family members have been shown to be upregulated in response to SA: GRX480 and GRXS13 (Camera et al., 2011; Ndamukong et al., 2007). GRX480 was found to suppress the JA-induced gene PDF1.2, an action that was dependent on TGA transcription factors. Direct interaction between GRX480 and TGA2 was demonstrated, indicating that GRX480 may modulate the redox status of SA-induced TGA proteins and in doing so, facilitate suppression of the JA/ET signalling pathway by SA. Indeed, the activation of ET-induced gene ORA59 (Octadecanoid-Responsive Arabidopsis AP2/ERF-domain protein 59) was also found to be suppressed by GRX480 (Zander et al., 2012). Overexpression of GRXS13 was shown to increase susceptibility to the necrotrophic pathogen Botrytis cinerea, suggesting that this GRX also suppresses JA-signalling pathways (La Camera et al., 2011).

A study that investigated the direct gene targets of the immune coactivator NPR1 discovered that various TRX superfamily members were induced by this protein. Among these were several PDI proteins, and interestingly TRXh5 (Wang et al., 2005). It had previously been found that the expression of TRXh5, a member of the cytosolic \( h \)-type subfamily of TRX enzymes, was induced in response to bacterial pathogens, as well as wounding, abscission and senescence (Laloi et al., 2004). Although eight TRX-\( h \) enzymes have been identified (Meyer et al., 2002), other members of this group were not seen to be upregulated in response to these stresses (Laloi et al., 2004; Reichheld et al., 2002). A role for TRXh5 in immunity was confirmed when mutant \( \text{trhx5} \) plants were found to be unable to display
full PR-1 gene expression in response to SA and had an impaired SAR response (Tada et al., 2008). A clear role for the disulphide reduction activity of TRXh5 in plant immunity was demonstrated through its regulation of NPR1. As previously discussed, NPR1 is subject to redox-based PTMs, which are responsible for altering the conformation of this protein. Both S-nitrosylation and disulphide linkages were found to be involved in the formation of an NPR1 oligomer that is unable to enter the nucleus (Figure 1.5) (Mou et al., 2003). The nuclear activity of NPR1 is essential to the activation of PR genes which in turn are essential for SAR (Kinkema et al., 2000). Therefore, a mechanism by which the disulphide-linked NPR1 oligomer can be reduced back to its monomeric form is crucial to the activity of this protein, and hence the defence responses of the plant. The NPR1 oligomer was shown to be reduced to its monomeric state by TRXh5 (Figure 1.5) (Tada et al., 2008), demonstrating the importance of this enzyme in the activation of plant immunity through the control of immune proteins harbouring redox-based PTMs. The regulation of NPR1 by the opposing actions of redox modification and TRXh5 is a clear indication that redox signalling networks operate to control the activity of key proteins during the plant immune response (Figure 1.5).
NPR1 exists as an oligomer in the cytoplasm of plant cells, formed by NPR1 monomers held together by disulphide bonds. Changes in the redox status of the cell during pathogen attack result in increased TRX activity which then breaks these bonds, releasing NPR1 monomers. These monomers then enter the nucleus where NPR1 acts as a coactivator of defence gene expression. NPR1 monomers are also S-nitrosylated by GSNO and free NO donors, which facilitate the formation of the inactive oligomer. This continuous cycle is activated during immune attack and is essential for appropriate NPR1 protein homeostasis. Figure adapted from Spoel et al., 2010.

1.5 Aims and objectives of this study

The effects of redox-based PTMs on the activities, structures and localisations of immune-related proteins are steadily beginning to be uncovered. However, to fully understand how these modifications can be employed as signalling switches, the mechanism by which they are rendered
reversible must be studied. The TRX family has already been demonstrated to be crucial in the control of oxidative stress in plants, with some roles in plant immune signalling already identified. However, there are still many unanswered questions about the redox activities of TRX family members. The underlying aim of this study will therefore focus on the involvement of TRX and members of the TRX superfamily in the control of redox-based PTMs during the plant immune response.

The key aims of this study are as follows:

• To determine if, as they have been found to in animals (Benhar et al., 2008), plant TRX enzymes are involved in the control of protein-SNO by acting as denitrosylases.

• To assess the role of immune related TRXh5 in the removal of protein-SNO during plant immune activation.

• To study the effects of S-nitrosylation on immune-related transcription (co)factors in an attempt to understand specific branches of the complex plant immune system.

• To investigate the role of a nucleocytoplasmic TRX-family member, NRX1 in an attempt to better understand nuclear redox signalling events.

• To identify targets of NRX1 oxidoreductase activity and study how the regulation of these proteins by NRX1 affects cellular processes.

Overall, the work in this thesis aims to address gaps in our current knowledge of redox-based signalling in the plant immune response by uncovering information on the specific activities of TRX family members.
Chapter 2

Materials and methods
2.1 Plant growth conditions

*Arabidopsis thaliana* seeds were sown directly onto soil and then stratified for 2 days at 4°C before being moved to growth chambers. Growth chambers were maintained at 21°C and 100 µmol.m⁻².s⁻¹ light on a “long day” 16-h-light/8-h-dark photoperiod at 65% day humidity and 55% night humidity. After approximately 2 weeks plants were transplanted to fresh soil.

For plants grown under sterile conditions, seeds were sterilised by 5 minute incubation with 100% ethanol followed by 5 minute incubation in 10% bleach. Seeds were then washed 3 times in sterile water before being suspended in 0.1% agar solution and stratified for 2 days at 4°C. Seeds were then plated onto 1/2 strength Murashige and Skoog (MS) media plates containing 0.4% agar and 0.3% sucrose [pH 5.8]. Plates were supplemented with 1X Gamborgs’ vitamins and, if necessary, the relevant antibiotics required for selection. Plates were placed in the same lighting conditions as above at 50% humidity.

2.2 Plasmid constructs and plant transformation

According to manufacturer’s instructions, *TRXh5* (At1g45145) and *NRX1* (At1g60420) were cloned into pENTR/D-TOPO (Life Technologies). Clones were selected for on LB plates containing kanamycin (50 µg/ml) and verified by sequencing. Positive clones were then recombined with Flag-containing pEarleyGate202 using LR clonase (Life Technologies) to generate the 35S::Flag-TRXh5 and 35S::Flag-NRX1 transgenes. The 35S::Flag-TRXh5 vector was transferred into *Agrobacterium tumefaciens* strain GV3101 (pMP90) using a freeze-thaw method and subsequently transformed into 6 week old wild-type (WT) Col-0 plants by floral dipping (Clough and Bent, 1998). Transgenic plants were selected on soil by spraying glufosinate ammonium, confirmed by western blotting against FLAG and crossed into *gsnor1* (par2-1 allele) and *nox1* (cue1-6 allele) plants. The 35S::TRXh5(C42S) transgene was generated by site-directed mutagenesis (QuickChange Lightning, Agilent Technologies) and transformed directly into *nox1*. 
The 35S::Flag-NRX1 construct was transformed into Col-0, nrx1, nox1, cat2-2 and cat2-2 cat3-1 lines by floral dipping in Agrobacterium tumefaciens (Clough and Bent, 1998). Transgenic lines were selected as above. 35S::Flag-NRX1 transgenics were crossed from the WT background into gsnor1 (par2-1 allele) (Chen et al., 2009). Over-expressers of Flag-NRX1 protein and complementation lines were selected in T2 generation plants by performing western blots with an anti-NRX1 antibody generated against the full-length, recombinant NRX1 protein (ProteinTech). The NRX1 antibody was generated by Dr. Rumana Keyani and Dr. Steven Spoel.

2.3 Genomic DNA extraction

Leaf tissue was frozen in liquid nitrogen and ground to a fine powder. DNA was extracted by the addition of CTAB (cetyl trimethylammonium bromide) buffer (100 mM Tris-HCl [pH 8.0], 1.4 M NaCl, 20 mM EDTA [pH 8.0], 2% (w/v) CTAB, 1% PVP 40,000 (polyvinyl pyrrolidone 40,000)) followed by 20 minutes incubation at 65°C. Chloroform:isoamyl alcohol [24:1] was then added and samples were centrifuged at 13,000 rpm for 5 minutes. The aqueous layer was then added to an equal volume of isopropyl alcohol, mixed and incubated for 10 minutes at room temperature. Following centrifugation at 13,000 rpm for 15 minutes at 4°C, DNA pellets were washed in 70% ethanol, dried and resuspended in ddH2O.

2.4 PCR genotyping

All genotyping PCRs were performed using Crimson Taq DNA Polymerase (NEB) as per manufacturer’s instructions. All T-DNA insertion lines were analysed using two sets of primers; one set for gene amplification consisting of a gene-specific 5′ primer (LP) and a gene-specific 3′ primer (RP), and one set to confirm the presence of the T-DNA insertion, consisting of the RP and a left border primer (LBB1.3) sequence obtained from SALK (5′ ATTTTGCCGATTTCGGAAC 3′). Primers used for T-
DNA insertion lines can be found below in Table 2.1. Mutant cat2-2 and cat2-2 cat3-1 seeds were obtained from the lab of Dr. Gary Loake.

**Table 2.1 Primers used in sequencing of T-DNA insertion SALK lines**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Allele</th>
<th>T-DNA insertion</th>
<th>Gene-specific primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRX1</td>
<td>nrx1-1</td>
<td>SALK_113401</td>
<td>LP ATCCACTTTTGTGGTTGACG&lt;br&gt;RP CGATCGCAACTTCTTCTGATC</td>
</tr>
<tr>
<td></td>
<td>nrx1-2</td>
<td>SALK_100357</td>
<td>LP AACCCAACCATCTTTGGACTC&lt;br&gt;RP TCAAGACTTCAGACCAAGCC</td>
</tr>
<tr>
<td>NRX2</td>
<td>nrx2-1</td>
<td>SALK_021735</td>
<td>LP GGTTCGAGATTCTTCTTTTC&lt;br&gt;RP CCTCTCTTTACCCACAAAGG</td>
</tr>
<tr>
<td>CAT2</td>
<td>cat2-2</td>
<td>SALK_057998</td>
<td>LP AGAGGCAAGATATCCTCAGGC&lt;br&gt;RP TCTGGTGCTCCTGTATGGAAC</td>
</tr>
<tr>
<td>CAT3</td>
<td>cat3-1</td>
<td>SALK_092911</td>
<td>LP TAACCGGAGTTTGAAACCCAG&lt;br&gt;RP TCGATTGTATTAGACGTCGC</td>
</tr>
</tbody>
</table>

*All primers are written in the 5’ to 3’ direction*

The par2-1 and nox1 mutant alleles are the result of a point mutation in the PAR2 and NOX1/CUE1 genes respectively (Chen et al., 2009; Streatfield et al., 1999). Mutant par2-1 plants were genotyped by combining one of two forward primers with the same reverse primer. The WT forward primer only amplified a product from the wild-type PAR1 sequence, while the par2-1 forward primer only amplified sequences containing the point mutation (see Table 2.2). Mutant nox1 plants were genotyped by cleaved amplified polymorphism sequence (CAPS) genotyping, in which the PCR product of the NOX1 gene is amplified (see Table 2.2 for primers) and then cut using the restriction enzyme, Zra1, which digests the sequence containing the point mutation but not the WT sequence, creating differentially sized products. Mutant par2-1 seeds were obtained from the lab of Dr. Jianru Zuo and nox1 seeds were obtained from Dr. Gary Loake.
Table 2.2  Primers used in sequencing of *par2* and *nox1* mutations

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT forward (<em>par2</em>-1 sequencing)</td>
<td>GAAAACAGCTGGTGCTTCAAGGATCATTAG</td>
</tr>
<tr>
<td><em>par2</em>-1 forward</td>
<td>GAAAACAGCTGGTGCTTCAAGGATCATTTA</td>
</tr>
<tr>
<td>Reverse (<em>par2</em>-1 sequencing)</td>
<td>TTTTACATGGATGACTACCCTTGACACGACT</td>
</tr>
<tr>
<td><em>nox1</em> forward</td>
<td>TCCAGATCTCAACGATGC</td>
</tr>
<tr>
<td><em>nox1</em> reverse</td>
<td>GAAGCAAGGAGTACCT</td>
</tr>
</tbody>
</table>

*All primers are written in the 5’ to 3’ direction*

2.5  RNA extraction

For total RNA extraction, liquid nitrogen frozen plant tissue was first ground to a fine dust in a bead-mill. Subsequently, 0.5 ml of warm RNA extraction buffer (100 mM LiCl, 100 mM Tris [pH 8.0], 10 mM EDTA, 1% SDS) and 0.5 ml phenol:chloroform:isoamylalcohol [25:24:1] were added simultaneously. After thorough vortexing, the samples were centrifuged for 5 minutes at 4°C at 13,000 rpm. The aqueous phase was transferred to a new tube and mixed with 0.5 ml of chloroform:isoamylalcohol [24:1] by vortexing and then centrifuged at 13,000 rpm for 5 minutes; this step was repeated. The aqueous layer was added to 1/3 volume of 8 M lithium chloride (LiCl) and incubated overnight at 4°C. Following overnight incubation, the extract was centrifuged for 15 minutes at 4°C at 14,000 rpm to form a pellet. The pellet was washed in ice cold (-20°C) 70% ethanol and incubated in ddH₂O at room temperature for 30 minutes and then resuspended. Next, 40 µl of 5 M NaAc [pH 5.2] and 1 ml 96% ethanol (-20°C) were added and the samples incubated at -20°C for at least 1 hour. The extract was centrifuged for 5 minutes at 14,000 rpm at 4°C. The pellet was washed in 70% ethanol (-20°C) and resuspended in 25 µl of sterile H₂O.
2.6 cDNA synthesis and RT-PCR

RNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific) and appropriate dilutions were made to equalize RNA concentrations to 2 µg per sample. mRNA was reverse transcribed into cDNA using a polydT20 primer and SuperScript Reverse Transcriptase III (Invitrogen, USA) according to the manufacturer’s instructions. One microliter of cDNA was used for subsequent PCR reactions, which were carried out using Crimson Taq DNA Polymerase (NEB) as per manufacturer’s instructions, using gene-specific primers (Table 2.3).

Table 2.3 Primers used in RT-PCR reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| TRXh5  | F CATACCCCTCGAAGTTTGGAACGAGA  
|        | R TTGCCTCAACTTTGAATTCTTGAGC  |
| WRKY18 | F ACGAGAGCGCAAGTGAGTTACGAG  
|        | R AGACGGTGCAAAACGAGCATCTAAG  |
| WRKY38 | F GCGGTGCAAGCTATCGGTATGG  
|        | R CTTGTCGGCGAATAAAAATGGCATG  |
| WRKY62 | F ACTCTTGCCAACAAAGGCTATGGA  
|        | R TCGGGTTGGAAGATCTGCTATG  |
| UBQ10  | F GATCTTTGCGGAAACAAATGGAGATG  
|        | R CGACTTGTCTTAGAAAGAAGATAACGAG  |

*All primers are written in the 5’ to 3’ direction

2.7 Quantitative real-time PCR

RNA isolation and cDNA synthesis was performed as described in sections 2.5 and 2.6 respectively. Quantitative qPCR was carried out on 15-times-diluted cDNA using Power SYBR Green (Life Technologies) and gene-specific primers (Table 2.4) on a StepOne Plus Real Time PCR system (Life Technologies).
Table 2.4  Primers used in qPCR reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR-1</td>
<td>F CTAAGGGTTCACAACCAGGCC</td>
</tr>
<tr>
<td></td>
<td>R AAGGCCCAACCAGAGTGTATG</td>
</tr>
<tr>
<td>WRKY38</td>
<td>F CCGGTTCACCAAAGCACCAGAA</td>
</tr>
<tr>
<td></td>
<td>R GCTTTTCCTTCCTGCATCC</td>
</tr>
<tr>
<td>WRKY62</td>
<td>F GCCAACCAAGGACACAGAA</td>
</tr>
<tr>
<td></td>
<td>R AGAGGTGAGGAGGAAGGAGC</td>
</tr>
<tr>
<td>PR-2</td>
<td>F CAGATTCCGTACATCAACG</td>
</tr>
<tr>
<td></td>
<td>R AGTGGTGTCAGTCAGTGA</td>
</tr>
<tr>
<td>NRX1</td>
<td>F GCAAAGGTGTTTCGGGATC</td>
</tr>
<tr>
<td></td>
<td>R GTCAACGGTCACTGGAGATGAAGATC</td>
</tr>
<tr>
<td>UBQ5</td>
<td>F CCAAGCCGAAGAAGATCAAG</td>
</tr>
<tr>
<td></td>
<td>R ACTCCTTCTCAACGCTGA</td>
</tr>
</tbody>
</table>

*All primers are written in the 5’ to 3’ direction

2.8  Hormone treatments and pathogen infections

For induction of immune genes, 4-week-old soil- grown plants were sprayed directly with either 0.5 mM SA, or mock sprayed with ddH₂O.

For pathogen infections, bacterial plant pathogen strains *Pst* DC3000 or *Psm* ES4326 were grown overnight in liquid LB medium supplemented with 10 mM MgSO₄. For disease susceptibility assays, bacterial cells were collected by centrifugation, diluted to 5 x 10⁵ cells and pressure infiltrated into leaves. *In planta* bacterial growth was determined 5 days after infection by spreading serial dilutions of leaf extracts on LB plates supplemented with either rifampicin (50 mg/ml) for *Pst* DC3000 or streptomycin (100 mg/ml) for *Psm* ES4326, 10 mM MgSO₄, and 100 µM cycloheximide. Disease resistance tests were performed in the same way, but plants were infiltrated with bacterial cells diluted to 5 x 10⁶ cells and bacterial growth measured after 3 days.

To perform SAR tests, plants were first infiltrated with 2 x 10⁷ cells of avirulent *Pst* DC3000 carrying the *avrRpt2* gene. After 2 days plants were infiltrated with virulent *Psm* ES4326 at 5 x 10⁶ cells and subsequently processed as described above.
2.9 Isolation of protoplasts and SNO measurements

Protoplasts were isolated from adult leaves as described in Wu et al., 2009, resuspended in modified W5 solution (150 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose, and 2 mM MES [pH 5.7]), and treated with or without 100 mM DNBC (Sigma) for 1 hr. Subsequently, 2 mM CysNO, 2 mM GSNO, or 2 mM DEA-NO was added for the indicated times. Protoplasts were collected by centrifugation and protein extracted in PBS containing 1% Triton X-100, 2 mM HgCl₂, 100 mM DAF-2, and protease inhibitor cocktail (50 mg/ml TPCK, 50 mg/ml TLCK, and 0.6 mM PMSF). Formation of the fluorescent triazolofluorescein (DAF-2T) was monitored using excitation and emission wavelengths of 485 and 520 nm, respectively (Benhar et al, 2008).

2.10 Site-directed mutagenesis

Cys39/42 of TRXhS in pET24c were replaced with Ser using Quick-Change site-directed mutagenesis (Stratagene) and confirmed by DNA sequencing by Dr. Steven Spoel.

Site directed mutagenesis for NRX1 cysteine’s was performed using the QuickChange Lightning mutagenesis kit (Agilent technologies) according to manufacturer’s instructions. Cys55/58 and Cys375/378 of NRX1 in PET28a were replaced with Ser by Drs. Rumana Keyani and Steven Spoel. Cys58, Cys378 and Cys58/378 mutants of NRX1 were separately created by replacing the specified Cys residue with Ser using the primers listed in table 2.5.

After verification by sequencing, correctly mutated clones were transformed into BL21 (DE3) cells and the recombinant proteins produced as described in section 2.11.
Table 2.5  Primers used for site-directed mutagenesis of NRX1

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer Sequences</th>
</tr>
</thead>
</table>
| C58S     | F CTGCTTGGTGGACCGTCTCAGCGGTTT  
          | R AAACCGCTGAGAGGTCCACACCAAGCAG |
| C378S    | F GCTCACTGGTGTCCTTCTCGCTTTT  
          | R AAAAGCGCGAGAAGGAGGACACCAGTGAGC |

*All primers are written in the 5’ to 3’ direction

2.11  Recombinant protein purification

Recombinant TRXh5-His6 and His6-NTRA proteins (Marty et al., 2009; Tada et al., 2008) were produced from pET24c and pETG10a vectors, respectively, in BL21 (DE3) cells. After 2 hours of growth at 37°C bacterial cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and then grown for a further 5 hours at this temperature. Recombinant His6-NRX1 and His6-NRX2 in the pET28a vector were grown in BL21 (DE3) cells, induced with 1 mM IPTG and then moved to 16°C for growth overnight. All cells were collected at the end of the respective growth period by centrifugation at 6500 rpm for 15 minutes.

Protein was extracted in a buffer containing 1X Bugbuster (Novagen), 50 mM potassium phosphate [pH 7.4], 300 mM NaCl, 10 mM imidazole, 1 µl/ml benzonase nuclease, 10 mM β-mercaptoethanol and protease inhibitor cocktail. Protein extracts were incubated with rotation for 20 minutes at room temperature and then centrifuged at 13,000 rpm for 20 minutes at 4°C. Extracts were purified on HisPur Cobalt Resin (Pierce) by gravity flow. Columns were washed three times with Wash buffer (50 mM potassium phosphate [pH 7.4], 300 mM NaCl, 10 mM imidazole and protease inhibitor cocktail) and then eluted in a buffer containing 50 mM potassium phosphate [pH7.4], 300 mM NaCl and 500 mM imidazole. Samples were then then dialyzed against appropriate buffers using Slide-A-Lyzer dialysis cassettes (Thermo Scientific).
Proteins concentration was determined against a bovine serum albumin (BSA) standard curve, using the Bio-rad Protein Assay according to manufacturer’s instructions.

2.12 Plant protein extraction and mixed disulphide analysis

Mixed disulphides between Flag-TRXh5 and targets were assessed by extracting protein in HEN buffer (100 mM HEPES [pH7.7], 1 mM EDTA, and 0.1 mM neocuproine) containing 2.5% SDS. Following centrifugation, samples were acetone precipitated, and protein pellets were resuspended in HEN buffer containing 2% SDS and alkylated with 15 mM 4-acetamido-4’-maleimidyldistilbene-2,2’-disulfonic acid (AMS) for 1 hr.

Mixed disulphides between Myc-TRXh5 and targets were assessed in protoplasts isolated from leaves of Cl-0 and Cl-0 expressing 35S::Myc-TRXh5, as described previously (Sweat and Wolpert, 2007; Wu et al., 2009). Protoplasts suspended in W5 buffer were treated with 2 mM GSNO for 10 min, collected by centrifugation, and protein extracted in HEN buffer containing 1% SDS and 20 mM N-ethylmaleimide (NEM). Myc-TRXh5 was immunoprecipitated overnight with anti-c-Myc agarose (Bethyl labs).

Mixed disulphides between recombinant His-tagged TRXh5 mutants and targets were analysed by incubating WT leaf extracts in diluted HEN buffer (25 mM HEPES [pH7.7], 1 mM EDTA, and 0.1 mM neocuproine) containing protease inhibitor cocktail, with TRXh5-His6 mutant proteins pre-bound to HisPur Cobalt resin (Pierce). Subsequently, resin was extensively washed in diluted HEN buffer.

Mixed disulphides between TRXh5 and NPR1-GFP were assessed by incubating leaf extracts of 35S::NPR1-GFP (in npr1) plants with mutant His6-TRXh5 proteins that were immobilized on NHS-activated agarose according to the manufacturer’s instructions (Thermo Scientific Pierce). After 2 hr, agarose was washed four times with diluted HEN buffer containing 0.5% Triton X-100, and the third wash contained 1 M NaCl.
To observe mixed disulphides between NRX1 and targets, plant protein was extracted in HEN buffer containing 2.5% SDS and loaded directly onto a non-reducing SDS-PAGE gel for western blot analysis (see section 2.13).

### 2.13 SDS-PAGE, gel staining and western blotting

Samples were added to SDS-PAGE sample buffer (final concentrations: 50 mM Tris-HCl [pH 6.8], 2% SDS, 0.02% bromophenol blue and 10% glycerol) with or without 50 mM dithiothreitol (DTT) (except in the case of NPR1-GFP plant protein where 150 mM DTT was used). After heating to 70°C, samples were centrifuged for 1 minute at 13,000 rpm and then separated on polyacrylamide gels.

For silver staining, gels were incubated with Fixation buffer (50% methanol, 5% acetic acid) for 20 minutes. Gels were then rinsed once with 50% methanol for 10 minutes and 3 times with ddH₂O for 15 minutes each. Next, 0.02% sodium thiosulfate (Na₂S₂O₃) was added for 1 minute, removed and the gel washed 3 times with ddH₂O for 1 minute each. Gels were stained for 20 minutes in the dark with cold (4°C) 0.1% silver nitrate (AgNO₃). Stain was washed off twice with ddH₂O and gels developed with 2% sodium carbonate (Na₂CO₃) and 0.04% formaldehyde with intensive shaking. Developer solution was refreshed after 20-30 seconds and gel developed to the necessary degree. To end this process, gels were washed with 5% acetic acid twice for 3 minutes each.

For Coomassie Blue staining, gels were incubated in staining solution (0.25% Brilliant Blue R, 40% methanol, 7% acetic acid) for 4 hours. Gels were de-stained in de-staining solution (45% methanol, 5% acetic acid) overnight.

For western blot analysis, proteins were first transferred from gel to nitrocellulose membranes overnight at 4°C, at 20 V. Membranes were then stained with Ponceau S solution (0.1% (w/v) Ponceau S, 5% acetic acid) for 30 seconds and then rinsed with ddH₂O. They were then de-stained in PBS-T (PBS, 0.1% Tween20) and incubated with shaking for 1 hour in blocking buffer (PBS-T, 5% dried skimmed milk). Primary antibodies (see Table 2.6) were incubated for 1-4 hours at room
temperature or overnight at 4°C. Blots were washed three times with blocking buffer and then incubated with the appropriate secondary horseradish peroxidase (HRP)-linked antibodies for 1 hour at room temperature. After washing a further three times with blocking buffer and once with PBS-T, SuperSignal West Pico/Dura Chemiluminescent Substrate (Thermo Scientific) was added to the blot to detect bands by exposure on X-ray film. Table 2.6 lists the antibodies used, their manufacturer and their relevant HRP-linked secondary antibody.

Table 2.6 Antibodies used in western blot analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>HPR-linked secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-FLAG</td>
<td>Sigma</td>
<td>anti-mouse</td>
</tr>
<tr>
<td>anti-GFP</td>
<td>Roche</td>
<td>anti-mouse</td>
</tr>
<tr>
<td>anti-His</td>
<td>Cell Signalling</td>
<td>anti-mouse</td>
</tr>
<tr>
<td>anti-Myc</td>
<td>Invitrogen</td>
<td>anti-mouse</td>
</tr>
<tr>
<td>anti-BSA</td>
<td>Sigma</td>
<td>anti-mouse</td>
</tr>
<tr>
<td>anti-FK2</td>
<td>Millipore</td>
<td>anti-mouse</td>
</tr>
<tr>
<td>anti-S5α</td>
<td>Abcam</td>
<td>anti-rabbit</td>
</tr>
<tr>
<td>anti-NRX1</td>
<td>ProteinTech</td>
<td>anti-rabbit</td>
</tr>
<tr>
<td></td>
<td>(Custom-generated against full length recombinant His&lt;sub&gt;6&lt;/sub&gt;-NRX1)</td>
<td></td>
</tr>
<tr>
<td>anti-HSP90</td>
<td>Santa Cruz Biotechnology</td>
<td>anti-rabbit</td>
</tr>
<tr>
<td>anti-histone H3</td>
<td>Abcam</td>
<td>anti-mouse</td>
</tr>
<tr>
<td>anti-catalase</td>
<td>Agrisera</td>
<td>anti-rabbit</td>
</tr>
</tbody>
</table>

* All secondary antibodies were obtained from Cell Signalling
2.14 Biotin switch technique

For denitrosylation assays, BSA, in-vitro-translated Flag-tagged proteins, or plant cell extracts in diluted HEN buffer containing protease inhibitor cocktail and 40 µM MG132 were incubated with 1 mM GSNO for 30 min and desalted on Zeba spin columns (Pierce). The resulting protein-SNO were incubated for 45 min with either 5 µM TRXh5, 0.5 µM NTRA and 1 mM NADPH or with molar excess (40 µM) of TRXh5 alone. Protein S-nitrosylation was assessed using the BST as described in (Forrester et al., 2009a), except 20 mM NEM was used to alkylate free thiols. In brief, samples were mixed with 2X HEN buffer containing 5% SDS and 20 mM NEM at 50°C for 20 minutes in the dark. Following acetone precipitation, pellets were resuspended in HEN buffer containing 1% SDS and 25 mM ascorbate and 0.4 mM biotin-HPDP were added simultaneously. Samples were incubated in the dark for 1 hour at room temperature with gentle rotation before being acetone precipitated a second time and resuspended in diluted HEN buffer containing 1% SDS. Samples were then added to Neutralisation buffer (diluted HEN buffer supplemented with 100 mM NaCl and 0.5% Triton X-100) containing Streptavidin beads (Sigma) and incubated overnight at 4°C with rotation. Finally, the beads were washed with Wash buffer (Neutralisation buffer containing 600 mM NaCl) and eluted in diluted HEN buffer containing 1% β-mercaptoethanol. Samples were analysed by SDS-PAGE gel followed by silver staining or western blotting (see Section 2.13).

For non-denaturing BST, samples were incubated for 2 hrs with 100 mM NEM in diluted HEN buffer and subsequently desalted twice on Zeba spin columns. Samples were then mixed with ≥ 2 mg/ml recombinant TRXh5(C42S) or TRXh5(C39/42S) that were immobilized on NHS-activated agarose (Pierce), pre-reduced with 2 mM dithiotreitol, and washed extensively in diluted HEN buffer. After 45 minutes, supernatant was recovered by centrifugation, incubated 1 hr with biotin-HPDP (Pierce), and the conventional BST resumed, as described above. The non-denaturing BST was designed and performed by Dr. Steven Spoel.
2.15 Wheat germ protein production

*In vitro*-translated epitope-tagged proteins were produced in wheat germ extracts using a protocol developed by Mika Nomoto in the lab of Dr. Yasuomi Tada (unpublished). Full-length cDNA clones were obtained from the RIKEN BioResource Center (Tsukuba, Japan) or the *Arabidopsis* Biological Resource Center (ABRC, The Ohio State University, OH, USA). Other transcription regulators were cloned into pDONR221 using the Gateway cloning system (Gateway® BP Clonase™ Enzyme mix, Invitrogen). Protein synthesis was performed using the IN VITRO Transcription/Translation Reagents kit according to manufacturer’s instructions (BioSieg, Tokushima, Japan). For *in vitro* transcription, the coding DNA sequence of FLAG tag (DYKDDDDK) was attached to the cDNA templates of transcription factors by KOD-Plus-Neo DNA polymerase (TOYOBO, Osaka, Japan). Approximately 30 μg of RNA was prepared by T7 RNA polymerase-based transcription from the PCR product. The RNA samples were dissolved in 35 μl of RNase-free water (Invitrogen) and mixed with 10 μl of a wheat germ extract and 10 μl of amino acid mixture (BioSieg) at 16°C overnight. The synthesized proteins were confirmed by western blotting against anti-FLAG.

2.16 AlphaScreen assays

Double stranded DNA *cis*-elements were prepared by incubating 100 μM biotinylated or non-biotinylated *PR-1 LS4* W-box sequences (sense strand) and non-biotinylated complementary oligos (antisense strand) (Invitrogen) together in equal volumes at 60°C for 20 minutes, followed by overnight incubation at room temperature.

Protein-DNA interactions were analysed using the AlphaScreen system (Perkin Elmer) according to manufacturer’s instructions, with some small alterations. In brief, 2 μl *in vitro* translated FLAG-tagged WRKY transcription factors (produced as in Section 2.15) were first incubated with 1 mM DEA-NO or a DMSO control in the dark for 20 minutes. They were then dialysed against 1 X PBS twice, each for 15 minutes. Following this, FLAG tagged proteins were incubated with 2 μl biotinylated or
non-biotinylated cis-element, 2.5 µl of 10X Control buffer, 2.5 µl of 0.1% (w/v) Tween20, 2.5 µl of 1% BSA and 5.5 µl of ultrapure water at room temperature for 1 hr. The samples were then incubated with 4 µl 40-fold diluted acceptor beads for 1 hour at room temperature, followed by addition of 4 µl 40-fold diluted donor beads. Samples were then incubated at room temperature in the dark for 10 hours. After the excitation at 680 nm, the emission wavelength between 520 and 620 nm was measured using an EnSpire™ Alpha 2390 Multilabel Reader (Perkin Elmer). Samples were tested in triplicate. FLAG-WRKY18 cysteine mutants used in this experiment were created by Mika Nomoto in the lab of Dr. Yasuomi Tada.

2.17 Protein oligomerisation analysis

TRXh5-mediated reduction of NPR1-GFP oligomer was performed as described in Tada et al., 2008, with the addition of a 2 hr incubation with 500 µM oxidized glutathione prior to the start of the assay to ensure full oligomerisation of NPR1-GFP. Excess glutathione was removed by desalting on Zeba spin columns (Pierce).

Oligomerisation of FLAG-tagged in vitro-translated NPR proteins was performed by treatment of proteins with either 1 mM DEA-NO, 500 µM oxidized glutathione or a H2O control for 1 hour followed by desalting on Zeba spin columns (Pierce).

2.18 Insulin turbidity assay

Recombinant NRX1, NRX2 and TRXh5 proteins were purified from E.coli as described in Section 2.11. Insulin assays were performed as described in Holmgren, 1979, with some minor alterations. Each 200 µl reaction volume consisted of 130 µM insulin, 100mM potassium phosphate [pH 7.0], 2 mM EDTA, and indicated concentrations of purified protein ranging from 2 µM to 8 µM. To initiate the assay, either 0.33 mM DTT or 0.2 µM NTRA and 1mM NADPH were added. Measurements at 650 nm
were taken at 2 minute intervals using an Infinite® 200 NanoQuant (TECAN). Samples were tested in triplicate.

2.19 Confocal microscopy

Protoplasts from indicated genotypes were isolated and transformed as described previously (Wu et al., 2009) and viewed on a Nikon Eclipse TE2000-U confocal microscope connected to a BioRad Radiance 2100 Laser Scanning System.

2.20 Nuclear enrichment

Nuclear enrichment was performed as previously described in Zheng et al., 2013, with some minor alterations. In brief, 6 week-old Arabidopsis Col-0 plants were first infiltrated with Psm ES4326 diluted to $5 \times 10^5$ cells, or mock infiltrated with $10 \text{ mM MgSO}_4$. After 24 hours, plant tissue was harvested and frozen in liquid nitrogen. Tissue was ground in Honda’s buffer (2.5% Ficoll 400, 5% Dextran T40, 0.4 M Sucrose, 25mM Tris-HCl [pH 7.5], $10 \text{ mM MgCl}_2$, 10 mM β-mercaptoethanol and protease inhibitor cocktail) and then filtered through at 62 µm pore nylon mesh. Triton X-100 was added to a final concentration of 0.2% and samples were incubated on ice for 15 minutes. Following centrifugation at 1500g for 5 minutes, the supernatant was centrifuged at 16,000g for 10 minutes and saved as the cytoplasmic fraction. Nuclei were washed with Honda’s buffer containing 0.1% Triton X-100 and then centrifuged a further 5 minutes at 1500g. Nuclei were resuspended in 1 ml Honda’s buffer and centrifuged at 100g for 1 minute. The supernatant was transferred to a fresh tube and this step was repeated. Following centrifugation at 2000g for 5 minutes, the pellet was resuspended in 300 µl Buffer G (1.7 M Sucrose, 10 mM Tris-HCl [pH 8.0], 0.15% Triton X-100, 2 mM MgCl$_2$, 5 mM DTT and protease inhibitor cocktail) before being added to the top of 300 µl Buffer G in a new tube.
Samples were centrifuged at 16,000 g for 1 hour at 4°C and then the resulting pellet suspended in 1% SDS. Nuclear samples were quantified using the Bio-Rad DC Protein Assay and equal amounts loaded onto SDS-PAGE gels along with 10 µl of each cytoplasmic fraction and total fraction. Gels were analysed by western blot, using anti-Histone H3 and anti-HSP90 to detect nuclear and cytosolic markers, respectively.

2.21 NRX target capture and mass spectrometry

Six week-old Arabidopsis Col-0 plants were first infiltrated with Psm ES4326 diluted to 5 x 10^5 cells, or mock infiltrated with 10 mM MgSO_4. After 24 hours, plant tissue was harvested and frozen in liquid nitrogen. Tissue was ground and extracted in 50 mM potassium phosphate buffer [pH 7.0] containing protease inhibitors and then centrifuged at 14,000 rpm. The supernatant was desalted on PD-10 columns (GE Healthcare) and then added to a column containing 8 mg/ml recombinant NRX1 or NRX1(C58/C378) that were immobilized on NHS-activated agarose (Pierce). Columns were prepared according to manufacturer’s instructions and pre-reduced with 2 mM DTT, and washed extensively in 50 mM potassium phosphate buffer [pH 7.0] before addition of samples. A column containing solely NHS-activated agarose only was also included as a control and prepared exactly as protein containing columns. Following 1 hour incubation with rotation at room temperature, columns were centrifuged for 2 minutes at 1000g and then washed ten times with 50mM potassium phosphate buffer [pH 7.0] containing 0.5% Triton X-100, with the fifth wash containing 1 M NaCl. Samples were eluted by incubated 2 ml of 50 mM DTT with the agarose for 30 minutes at room temperature followed by 2 minutes centrifugation at 1000g. Samples were concentrated by Speedvac, run on SDS-PAGE until they had just entered the resolving gel and stained with Coommassie Brilliant Blue (see Section 2.13). Single bands were cut out and digested by trypsin digest and then analysed by mass spectrometry by the Kinetic Parameter Facility at the SynthSys Centre, University of Edinburgh.
2.22 Mixed disulphide co-immunoprecipitation of FLAG-tagged proteins from plants

6 week-old Arabidopsis plants from indicated genotypes were first infiltrated with Psm ES4326 diluted to $5 \times 10^5$ cells, or mock infiltrated with 10 mM MgSO$_4$. After 24 hours, plant tissue was harvested and frozen in liquid nitrogen. Tissue was first extracted in 2 ml Fixation buffer (diluted HEN buffer containing 2.5% SDS, 25 mM NEM and protease inhibitor cocktail) and then incubated at 50°C for 20 minutes with frequent vortexing. Samples were then centrifuged at 14,000g for 20 minutes at room temperature and acetone precipitated. Pellets were resuspended in diluted HEN buffer containing 0.5% SDS. Inputs were taken and the remaining samples mixed with 4 ml Neutralisation buffer (diluted HEN buffer containing 0.5% Triton X-100, 150 mM NaCl and protease inhibitor cocktail) containing 40 µl anti-FLAG M2 affinity resin (Sigma), prewashed with Elution buffer (0.1 M glycine-HCl [pH 3.5]). Samples were then incubated overnight at 4°C with rotation. Next, samples were washed three times with Neutralisation buffer and incubated for 5 minutes with Elution buffer with frequent mixing. Following centrifugation at 5,000 rpm for 1 minute, supernatants were added to a fresh tube and incubated for 5 minutes with 1/10 volume of StrataClean resin (Agilent Technologies). Samples were centrifuged at 8,000 rpm and supernatant discarded. Protein was eluted from the beads by the addition of Neutralisation buffer containing 2X SDS Sample buffer and 200 mM DTT. Samples were vortexed and then boiled for 5 minutes before being vortexed again. Following brief centrifugation, samples were loaded on SDS-PAGE gel and analysed by western blotting.

2.23 TUBE immunoprecipitation of polyubiquitinated proteins

6 week-old Arabidopsis plants from indicated genotypes were first sprayed with 0.5 mM SA, or mock sprayed with ddH$_2$O. After 6 hours, plant tissue was harvested and frozen in liquid nitrogen. Protein was extracted in 2 volumes of Extraction buffer (50 mM potassium phosphate [pH 7.4], 150 mM NaCl, 1% Triton X-100, 10 mM NEM, 40 µM MG132, 1X PPI3 and protease inhibitor cocktail)
containing 200 µg/ml His-tagged TUBE protein (Hjerpe et al., 2012). Samples were then centrifuged at 14,000 rpm for 20 minutes at 4°C and input samples taken. Remaining supernatant was added to 25 µl HisPur Cobalt resin (Pierce), pre-washed in Wash buffer (50 mM potassium phosphate [pH 7.4], 150 mM NaCl, 1% Triton X-100, 40 µM MG132, 1X phosphatase Inhibitor 3 cocktail [Sigma] and protease inhibitor cocktail). Samples were rotated for 2 hours at 4°C and the resin collected by centrifugation at 700g for 2 minutes. Samples were washed 3 times with Wash buffer containing 10 mM imidazole. Elution was performed by heating the samples to 80°C for 5 minutes with 2X SDS sample buffer containing 200 mM DTT. Samples were run on SDS-PAGE gel and analysed by western blot.

2.24 Methyl viologen treatment and electrolyte leakage assays

For analysis of cell death, 4-week-old soil-grown *Arabidopsis* plants were pressure infiltrated with 5 µM Methyl Viologen (Sigma) or a H₂O control. Plants were photographed after 24 hours under constant light.

For electrolyte leakage assays, plants were first infiltrated with Methyl Viologen as above and then left for 1 hour to ensure complete intake. Ten leaf discs (7 mm diameter) per genotype were then cut, washed extensively in H₂O and floated on 10 ml sterile H₂O in a fresh tube. Samples were then put under constant light and electrolyte leakage was measured at indicated time-points using a conductivity meter. After the final time-point, samples were boiled for 5 minutes and a total electrolyte readings taken. All samples were performed in triplicate.
Chapter 3

Selective protein denitrosylation activity of Thioredoxin-h5 modulates plant immunity

* Parts of the work in this chapter have been published in:


See also Appendix I.
3.1 Background

SNO modification of proteins is a major signalling method utilized by NO and is emerging as a key redox modification that occurs in plants during immune activation (Yun et al., 2011). Whilst the formation of disulphide bonds have been shown to be readily reversed by thioredoxin (TRX) enzymes, it is much less clear how levels of SNO modifications are controlled in plants. Identification of an enzyme that regulates the levels of S-nitrosoglutathione (GSNO), a cellular reservoir for NO bioactivity, indicated the importance of regulating GSNO as an in vivo NO donor for protein S-nitrosylation (Benhar et al., 2009; Liu et al., 2001). Arabidopsis knockout mutants of this enzyme, known as GSNO Reductase 1 (GSNOR1) accumulate high levels of protein-SNO as a result of their inability to remove cellular GSNO (Feechan et al., 2005). Consequently, they are highly susceptible to pathogen attack due to impaired SA-dependent immune signalling (Feechan et al., 2005; Tada et al., 2008; Wünsche et al, 2011), demonstrating the critical role that GSNOR1 plays in governing protein-SNO levels during plant immune responses.

Observations of stimulus-induced protein denitrosylation and the short-lived nature of many protein-SNO suggested that SNO reductases may exist that directly reduce SNO groups (Benhar et al., 2009; Forrester et al., 2009b). Indeed, a biochemical search in mammalian cells identified TRX in combination with NADPH-dependent TRX reductase (NTR) as a potent protein-SNO reductase system important for apoptosis (Benhar et al., 2008). This significantly extends the substrate repertoire of the TRX/NTR system from its well-defined disulphide-containing substrates to many potential protein-SNO targets (Benhar et al, 2010; Doulias et al., 2010; Wu et al., 2011). Analogous to enzymatic regulation of other post-translational modifications (PTMs), TRX are therefore emerging as important players in maintaining low levels of protein-SNO and limiting SNO signal duration.

Although disulphide reduction activity of TRX enzymes plays an important role in plant immunity (Tada et al., 2008), plant TRX enzymes are not yet known to exhibit protein denitrosylation activity. Moreover, it remains unclear if eukaryotic TRX enzymes in general display any specificity or preferential activity toward protein-SNO in vivo. In this chapter, immune-induced TRXh5 is shown to
act as a potent protein-SNO reductase in the plant immune response. Genetic and biochemical evidence indicates that TRXh5 discriminates between protein-SNO substrates, providing specificity and reversibility to protein-SNO signalling in SA-dependent plant immunity.

### 3.2 Results

#### 3.2.1 TRXh5 displays protein-SNO reductase activity *in vitro*

Expression of TRXh5 is highly upregulated by SA and pathogen infection, which is essential for establishment of disease resistance in plants (Laloi et al., 2004; Tada et al., 2008). Considering the importance of controlled protein-SNO levels in immune responses and the role of TRXh5 in establishing immunity, it was first tested whether TRXh5 exhibits protein denitrosylation activity.

The ability of TRXh5 to reduce S-nitrosylated BSA (BSA-SNO), used here as a model substrate, was analysed. First, BSA was S-nitrosylated with GSNO and incubated with either recombinant TRXh5 (Figure S1A), recombinant NTRA (Figure S1B), or a combination of both. BSA-SNO was then detected using the biotin switch technique (BST), in which SNO are replaced by biotin (Forrester et al., 2009a). Similar to displacement of NO by UV, only the fully reconstituted TRh5/NTRA system was able to completely denitrosylate BSA (Figure 3.1A). Next, it was investigated if the TRXh5/NTRA system was also able to denitrosylate a variety of plant proteins. Plant protein extracts were S-nitrosylated with GSNO and addition of both TRXh5 and NTRA resulted in denitrosylation comparable to omission of ascorbate during sample preparation (Figure 3.1B), which prevents switching of SNO for biotin (Forrester et al., 2009a). Taken together these data clearly indicate that plant TRXh5 displays protein denitrosylation activity *in vitro*. 
Figure 3.1 The plant TRX/NTR system displays protein denitrosylation activity *in vitro*

(A) BSA (20 µM) was S-nitrosylated with 1 mM GSNO and incubated with a TRX system consisting of TRXh5 (5 µM), NTRA (0.5 µM), and NADPH (1 mM). S-nitrosylated BSA (BSA-SNO) was detected using the biotin-switch technique (BST) and is shown relative to total BSA. UV-induced SNO photolysis served as a control.

(B) Plant protein extracts were S-nitrosylated with 1 mM GSNO and incubated with the TRXh5/NTRA system as in (A). Protein-SNO were purified with the BST in the presence or absence of ascorbate (Asc) and visualized by silver staining.
3.2.2 The TRX/NTR system exhibits protein-SNO reductase activity in vivo

It remains unknown if plant cells maintain significant TRX-mediated denitrosylation activity in vivo. To determine this, two specific inhibitors of mammalian NTR, 1-chloro-2,4-dinitrobenzene (DNCB) and auranofin were first tested for their ability to block the activity of the plant NTR/TRX system. While auranofin had little effect, DNCB effectively inhibited activity of plant NTRA in vivo (Figure 3.2A). Therefore, plant cells were treated with DNCB prior to application of the NO donor CysNO, which is cell permeable and rapidly transfers NO directly onto protein thiols. Formation of protein-SNO, as measured by NO-dependent fluorescent triazolofluorescein (DAF-2T) assays, indicated that DNCB treatment increased NO donor-induced protein-SNO accumulation (Figure 3.2B). In addition, the protein-SNO contents of trx-h3 trx-h5 double mutant cells were analysed, lacking two functional TRX-h genes that are partially redundant in immunity (Tada et al., 2008), as well as mutant ntra cells. Compared to wild-type (WT), mutant cells contained higher basal levels of protein-SNO (Figure 3.2C). Upon treatment with CysNO, trx-h3 trx-h5 cells accumulated only slightly more protein-SNO, while ntra cells accumulated over four times more protein-SNO than WT. Collectively, these data indicate that the TRX-h/NTR system strongly contributes to protein denitrosylation activity in plant cells.
Figure 3.2  The plant TRX/NTR system displays protein denitrosylation activity in vivo

(A) Oxidized insulin (130 μM) was incubated with 1 mM NADPH alone (control) or together with TRXh5 (5 μM) and NTRA (0.5 μM). Additionally, 10 μM auranofin and 100 μM DNBC were added to the reaction mixtures as indicated. Formation of reduced insulin was measured at 650 nm.

(B) WT protoplasts were pre-treated with or without 100 μM DNBC, followed by incubation with 2 mM CysNO for 20 min. Protein-SNO were detected by measuring formation of fluorescent triazolofluorescein (DAF-2T) and normalized against protein concentrations.

(C) WT, trx-h3 trx-h5 double, and ntra single mutant protoplasts were treated with 2 mM CysNO for 10 min. Protein-SNO were detected by measuring formation of fluorescent triazolofluorescein (DAF-2T) and normalized against protein concentrations. Error bars in (B) & (C) represent SD (n = 3).
3.2.3 *Trans*-denitrosylation activity of TRXh5 reverses SNO modification

The TRX active site consists of two reactive cysteines that are responsible for reducing cysteines in target substrates. TRX-mediated denitrosylation could occur in one of two ways. Analogous to disulphide reduction, the first active site cysteine of TRX could displace NO from the target cysteine via heterolytic cleavage of the S-NO bond, leading to formation of a mixed disulphide bond between TRX and its substrate. Alternatively, NO could be transferred directly from the target cysteine to one of the TRX active site cysteines by *trans*-nitrosylation via homolytic cleavage of TRX-SNO intermediates (Nikitovic and Holmgren, 1996).

To examine which of these scenarios is more likely, a BST was performed to determine if formation of a TRXh5-SNO intermediate could be detected. It was found that denitrosylation of GSNO-induced BSA-SNO was indeed associated with formation of a TRXh5-SNO intermediate (Figure 3.3A), suggesting that TRXh5 is capable of *trans*-denitrosylating protein-SNO. In addition to the active site Cys39 and Cys42, TRXh5 contains an additional Cys10 that could play a role in denitrosylation. Therefore, Cys39 and Cys42 were mutated to serines (C39S) and (C42S), to determine if *trans*-denitrosylation of BSA-SNO was dependent on the active site. Indeed, in contrast to wild-type TRXh5, mutation of TRXh5 active site cysteines eliminated denitrosylation activity and formation of the TRXh5-SNO intermediate (Figure 3.3A).

Notably, the amount of TRXh5-SNO intermediate formed in these assays was always less than the amount of BSA-SNO that was subject to denitrosylation, suggesting this intermediate is unstable. We tested if TRXh5-SNO could be stabilized by mutating single cysteine residues of the active site. Like WT, the single mutant proteins TRXh5(C39S) and TRXh5(C42S) efficiently denitrosylated BSA-SNO (Figure 3.3B). Remarkably, however, a TRXh5-SNO intermediate was undetectable for the single mutants (Figure 3.3B). This suggests that the primary *trans*-nitrosylated cysteine of the active site is either stabilized by the secondary cysteine or that it is attacked by the secondary cysteine to yield a more stable nitroxyl disulphide intermediate. Regardless of this mechanistic detail, this data demonstrates that TRXh5 exhibits *trans*-denitrosylation activity.
Figure 3.3  BSA is trans-denitrosylated by TRXh5 in vitro

(A) BSA (20 µM) was S-nitrosylated with 1 mM GSNO and incubated with WT or an active site mutant (C39/C42S) of TRXh5 (40 µM). S-nitrosylated BSA (BSA-SNO) and TRXh5 (TRXh5-SNO) were detected with the BST and are shown relative to total BSA and TRXh5. UV-induced SNO photolysis served as a control.

(B) BSA (20 µM) was S-nitrosylated with 1 mM GSNO and incubated with WT or the active site mutants TRXh5(C39S) or TRXh5(C42S) (40 µM). S-nitrosylated BSA (BSA-SNO) and TRXh5 (TRXh5-SNO) were detected with the BST and are shown relative to total BSA and TRXh5. UV- induced SNO photolysis served as a control.

It is possible that TRXh5 also utilizes a trans-denitrosylation mechanism to reduce plant protein-SNO. However, studying this is complicated by the fact that mutation of an active site cysteine prevents TRXh5 activity from being recycled by NTRA, impeding experiments similar to the one shown in Figure 3.1B. Therefore, we aimed to take advantage of the mechanistic difference in disulphide reduction and trans-denitrosylation activities of TRXh5. As opposed to trans-denitrosylation activity, disulphide reduction by TRX enzymes requires both active site cysteines (Tada et al., 2008). This
allowed the use of a non-denaturing BST, designed in the host lab, for the identification of plant protein-SNO that are specifically trans-denitrosylated by TRXh5 (Figure 3.4A). In this method, free thiol residues of native plant proteins were first alkylated with N-ethylmaleimide (NEM) and then added to a column containing immobilized mutant TRXh5(C42S) protein. Consequently, disulphide-containing protein targets were trapped on the column by formation of a mixed disulphide bond with TRXh5(C42S), while proteins with thiols that were either alkylated by NEM or trans-denitrosylated by TRXh5(C42S) passed through the column. Denitrosylated thiols were labelled with thiol-reactive biotin (Biotin-HPDP) and detected by immunoblotting with an anti-biotin antibody. This method was performed on plant protein extracts that were spiked with BSA as an internal control and treated with or without GSNO. Biotinylated BSA—i.e., BSA trans-denitrosylated by TRXh5(C42S)—was detected only after treatment with GSNO (Figure 3.4B), indicating that this method did not detect appreciable false-positives. Moreover, while some biotinylated plant proteins were recovered in absence of GSNO treatment, application of GSNO increased this amount (Figure 3.4B). These data indicate that TRXh5 subjects a large set of plant protein-SNO to trans-denitrosylation.

To determine if TRXh5 denitrosylates any plant protein-SNO via formation of mixed disulphide intermediates, recombinant His-tagged TRXh5(C42S) was used to pull down mixed disulphide intermediates directly from untreated and GSNO-treated plant extracts. In this scenario, GSNO failed to induce significant changes (Figure 3.4C). Only a complex around 80 kDa showed a small increase upon GSNO treatment, which likely represents a disulphide intermediate between TRXh5 and its main disulphide-containing target NPR1. GSNO induces the formation of intermolecular disulphide bonds between NPR1 monomers, which are major targets of TRXh5 (Tada et al., 2008). Accordingly, GSNO treatment of extracts from plants expressing 35S::NPR1-GFP stimulated interaction between TRXh5(C42S) and NPR1-GFP, which was dependent on Cys39 (Figure 3.4D). Hence, denitrosylation of plant protein-SNO via formation of mixed disulphides between TRXh5 and its substrates is not a prevalent mechanism in vitro.
Figure 3.4 TRXh5 trans-denitrosylates plant protein in vitro

(A) Schematic of non-denaturing BST, utilizing immobilized mutant TRXh5(C42S). See text for details.

(B) Protein extracts were spiked with BSA and treated with or without 1 mM GSNO and subjected to the non-denaturing BST, as shown in (A). Purified denitrosylated plant proteins and BSA (deSNO-BSA) were detected using anti-biotin and anti-BSA antibodies, respectively, and are shown relative to total BSA.

(C) Plant protein extracts were treated with 1 mM GSNO and then incubated with immobilized His-tagged TRXh5(C42S) and TRXh5(C39/42S) mutants. Mixed disulphide intermediates formed by TRXh5 (TRXh5-substrate) were separated by non-reducing (-DTT) and reducing (+DTT) SDS-PAGE and detected using an anti-His antibody.

(D) Protein extracts from NPR1-GFP plants were treated with 1mM GSNO and then incubated with immobilized TRXh5(C42S) and TRXh5(C39/42S) mutants. Mixed disulphide intermediates formed between TRXh5 and NPR1-GFP were pulled down and eluted with DTT. Subsequently, the amount of NPR1-GFP pulled down with TRXh5 was assessed relative to its input using SDS-PAGE and detection with an anti-GFP antibody.
Finally, the formation of mixed disulphides in denitrosylation reactions *in vivo* was investigated. Protoplasts expressing Myc-tagged TRXh5 were treated with GSNO and the formation of mixed disulphides between Myc-TRXh5 and its substrates assessed. As expected, GSNO treatment did not result in significant accumulation of further mixed disulphides (Figure 3.5). Taken together, this data demonstrates that TRXh5 preferentially denitrosylates protein-SNO in *trans* rather than via formation of mixed disulphide intermediates.

![Figure 3.5 GSNO treatment does not affect mixed disulphide status of TRXh5 in vivo](image)

Protoplasts from plants transformed with or without 35S::Myc-TRXh5 were treated with 2 mM GSNO for 10 minutes. Total protein was extracted under denaturing conditions and immunoprecipitated with an anti-Myc antibody. Mixed disulphide intermediates formed by TRXh5 (TRXh5-substrate) were separated by non-reducing (-DTT) and reducing (+DTT) SDS-PAGE and detected using an anti-Myc antibody.

### 3.2.4 TRXh5 selectively restores immune deficiencies caused by elevated protein-SNO

*TRXh5* is highly induced during activation of plant immunity (Laloi et al., 2004; Tada et al., 2008), but it is unknown if its SNO reductase activity is relevant to immune signalling. *TRXh5* gene expression levels were determined in mutant *gsnor1* and *cue1/nox1* plants that both contain excessive amounts of protein-SNO due to over accumulation of GSNO and free NO, respectively (Feechan et al.,
While exogenous application of SA strongly induced TRXh5 gene expression in the wild-type, expression was reduced in gsnor1 and nox1 plants (Figure 3.6A). Thus, it is plausible that accumulation of excessive protein-SNO in these mutants is exacerbated by reduced expression of TRXh5. To test this possibility, gsnor1 and nox1 plants were transformed with Flag-tagged TRXh5 driven by a constitutive 35S promoter from cauliflower mosaic virus. The resulting 35S::TRXh5 (gsnor1) and 35S::TRXh5 (nox1) plants showed strong expression of TRXh5 that was independent of immune activation by SA (Figure 3.6A). Developmental defects of gsnor1, including loss of apical dominance, elongated leaf shape, and reduced seed production (Kwon et al., 2012), were not restored by TRXh5 expression (Figure 3.6B; data not shown). Similarly, TRXh5 expression was unable to restore reduced size and interveinal paleness of nox1 plants (Figure 3.6B) (Li et al., 1995). These phenotypes suggest that TRXh5 does not act outside the plant immune system.

**Figure 3.6** Developmental abnormalities of nox1 and gsnor1 plants are not restored by TRXh5

(A) WT, gsnor1, nox1, 35S::TRXh5 (gsnor1), and 35S::TRXh5 (nox1) plants were treated with 0.5 mM SA for the indicated times. The expression of TRXh5 was assessed by RT-PCR using gene-specific primers.
(B) Morphological phenotypes of 4-week-old WT, gsnor1, nox1, 35S::TRXh5 (gsnor1), and 35S::TRXh5 (nox1) plants.
It was then assessed if TRXh5 could rescue immune deficiencies associated with elevated protein-SNO levels in these mutants. Plants were infected with the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 and bacterial growth assessed after 5 days. As reported previously (Feechan et al., 2005), *gsnor1* mutants were highly susceptible to Pst DC3000, displaying over 25-fold higher bacterial growth compared to WT (Figure 3.7A). Unexpectedly, 35S::TRXh5 (*gsnor1*) plants were as susceptible as *gsnor1* mutants (Figure 3.7A), indicating TRXh5 was unable to restore resistance in this genetic background. Infection of *nox1* mutants with Pst DC3000 demonstrated that elevated protein-SNO in this mutant also resulted in severe disease susceptibility (Figure 3.7B). Surprisingly, however, expression of TRXh5 restored resistance in the *nox1* genetic background (Figure 3.7B).

![Figure 3.7](image)

**Figure 3.7** TRXh5 selectively restores immune deficiencies caused by elevated protein-SNO

(A) WT, *gsnor1*, and 35S::TRXh5 (*gsnor1*) plants were infected with Pst DC3000 (5 x 10^5 cells) and growth of Pst DC3000 was assessed after 5 days. Cfu, colony forming units. Error bars represent 95% confidence limits (n = 8). Asterisks indicate statistically significant differences compared to the WT (Tukey-Kramer ANOVA test; α = 0.05, n = 8).

(B) WT, *nox1*, and 35S::TRXh5 (*nox1*) plants were infected and analysed as in (A).
To ensure this was due specifically to denitrosylation activity of TRXh5, 35S::TRXh5(C42S) was also expressed in nox1 plants. Whilst mutant TRXh5(C42S) protein exhibits potent denitrosylation activity (Figure 3.3), it lacks disulphide reduction activity, as clearly demonstrated by its failure to reduce disulphide-linked oligomers of its preferred natural substrate NPR1 (Figure 3.8A). Strikingly, expression of 35S::TRXh5(C42S) in nox1 mutants also restored resistance against Pst DC3000 (Figure 3.8B). Taken together, these data suggest that TRXh5 selectively restores immunity in genotypes with elevated protein-SNO derived from free NO but not GSNO.

![Figure 3.8](image)

**Figure 3.8** TRXh5 rescues immunity in nox1 plants through its denitrosylation activity

(A) Extracts from plants expressing NPR1-GFP were incubated with either recombinant TRXh5 or the active site mutant TRXh5(C42S) together with a small amount of DTT (0.33 mM) to recycle TRX activity. Reduction of NPR1-GFP oligomer (O) to monomer (M) relative to total (T) NPR1-GFP was followed for the indicated times by non-reducing (-DTT) and reducing (+DTT) SDS-PAGE and detected using an anti-GFP antibody.

(B) WT, nox1, and 35S::TRXh5(C42S)(nox1) plants were infected and analysed as in Figure 3.7.
3.2.5 TRXh5 restores SA-dependent immune signalling by selective protein

denitrosylation

Selective restoration of disease resistance by TRXh5 in gsnor1 and nox1 mutants could be due
to preferential selection of protein-SNO substrates or due to differential regulation of TRXh5
enzymatic activity. The latter was studied by assessing the disulphide reduction activity of TRXh5 in
these genetic backgrounds. 35S::TRXh5 (nox1) and 35S::TRXh5 (gsnor1) plants were treated with or
without the immune activator SA. Subsequently, proteins were extracted under denaturing conditions
in presence of the alkylation agent 4-acetamido-40-maleimidyldistilbene-2,20-di-sulfonic acid (AMS)
to prevent nonspecific interactions between TRXh5 and non-substrates during sample preparation.
Protein samples were then separated on non-reducing (-DTT) and reducing (+DTT) gels to visualize
TRXh5–substrate mixed disulphides and total amounts of TRXh5, respectively (Figure 3.9). In the nox1
mutant background, TRXh5 formed mixed disulphides that were increased by treatment with SA
(Figure 3.9, lanes 3 and 4). In the gsnor1 mutant background, TRXh5 constitutively formed mixed
disulphides independent of SA application (Figure 3.9, compare lanes 6 and 7). Thus, TRXh5 displayed
enzymatic activity regardless of genetic background, failing to explain its role in selectively restoring
disease resistance in nox1 mutants alone.
Figure 3.9  TRXh5 does not exhibit differential disulphide-reduction activity in nox1 and gsnor1 backgrounds

Mutant nox1 and gsnor1 plants with or without the 35S::Flag-TRXh5 transgene were treated with 0.5 mM SA. Total protein was extracted and incubated with or without the alkylation agent AMS, which prevents nonspecific disulphide formation. Proteins were separated by SDS-PAGE in the presence or absence of DTT and analysed by western blotting using an anti-Flag antibody. Indicated are free TRXh5 monomer, mixed disulphide intermediates between TRXh5 and substrates (TRXh5-substrate), and total levels of TRXh5.

In order to examine the ability of TRXh5 to denitrosylate protein-SNO in both nox1 and gsnor1 genetic backgrounds, SNO content was measured in these mutants along with their respective 35S::Flag-TRXh5 expressing lines. Interestingly, expression of TRXh5 reduced SNO content by over 20% in nox1 mutants, while no significant reduction in SNO was observed in gsnor1 mutants (Figure 3.10). Thus, TRXh5 probably targets a distinct repertoire of protein-SNO from those regulated by the SNO reductase GSNOR1.
Mutant nox1 and gsnor1 plants transformed with or without the 35S::Flag-TRXh5 transgene were infected with Pst DC3000 (5 x 10^6 cells). After 24 hr, SNO content was detected by measuring formation of fluorescent triazolofluorescein (DAF-2T), normalized against protein concentrations, and expressed as percentage of untransformed controls. Error bars represent SD (n = 3).

Resistance against pathogenic Pst DC3000 is conferred by the SA-induced transcriptional coactivator NPR1. Site-specific S-nitrosylation of NPR1 promotes the formation of a cytosolic disulphide-linked oligomer, thereby preventing NPR1 monomers from activating target genes in the nucleus (Tada et al., 2008). To understand how denitrosylation activity of TRXh5 can rescue immunity, it was investigated if TRXh5 was capable of denitrosylating NPR1. Figure 3.11A shows that GSNO treatment triggered S-nitrosylation of NPR1 in vitro. Subsequent incubation of S-nitrosylated NPR1 with the TRXh5/NTRA system resulted in its complete denitrosylation. To test if TRXh5-mediated denitrosylation of NPR1 affects its conformation in vivo, protoplasts were generated from nox1 and gsnor1 mutants transformed with or without Flag-tagged TRXh5 and transiently expressed NPR1-GFP (Figure 3.11B). In absence of the 35S::TRXh5 transgene, NPR1-GFP was localised to both the cytoplasm and nucleus in nox1 and gsnor1 cells. By contrast, expression of 35S::TRXh5 resulted in exclusively
nuclear-localised NPR1-GFP in both genotypes (Figure 3.11B). These data suggest that TRXh5 facilitates monomerisation and nuclear accumulation of NPR1 in both nox1 and gsnor1 genetic backgrounds.

Figure 3.11  TRXh5-mediated NPR1 monomerisation occurs in both nox1 and gsnor1 backgrounds
(A) *In vitro*-translated Flag-tagged NPR1 was S-nitrosylated with 1 mM GSNO and incubated with or without the TRX system, consisting of TRXh5 (5 µM), NTRA (0.5 µM), and NADPH (1 mM). S-nitrosylated NPR1 (NPR1-SNO) was detected with the BST in presence or absence of ascorbate (Asc) and is shown relative to total NPR1.
(B) Protoplasts from the indicated genotypes were transformed with 35S::NPR1-GFP and sub-cellular localisation analysed by confocal microscopy.
The effect of TRXh5 on NPR1 conformation fails to explain the selective nature by which this enzyme rescues immunity in nox1 but not gsnor1 mutants. Therefore, we examined if expression of TRXh5 affected SA-dependent immune gene expression upon inoculation with Pst DC3000. Expression of the marker genes PR-1, WRKY38, and WRKY62 was strongly upregulated upon infection of the WT, whereas nox1 and especially gsnor1 plants exhibited strongly reduced gene expression (Figure 3.12), explaining the enhanced susceptibility of these genotypes to this pathogen. Remarkably, expression of TRXh5 partially restored SA-responsive gene expression in nox1 mutants, while it had no significant effect in gsnor1 mutants (Figure 3.12).

Figure 3.12 TRXh5 selectively restores immune gene expression in response to pathogen infection
WT, gsnor1, nox1, 35S::TRXh5 (gsnor1), and 35S::TRXh5 (nox1) plants were infected with Pst DC3000 (5 x 10^5 cells). After 24 hr expression of SA-dependent PR-1, WRKY38, and WRKY62, genes were analysed using qPCR and normalized against constitutively expressed UBQ5. Error bars represent SD (n = 3).

To determine if this rescue of gene expression in nox1 could be attributed to denitrosylation activity of TRXh5, nox1 plants expressing the 35S::TRXh5(C42S) construct were also infected with Pst DC3000 and examined for PR-1 gene expression (Figure 3.13). Consistent with previous observations (Figure 3.8B), TRXh5(C42S) was also efficient in restoring defence gene expression indicating that denitrosylation activity of this enzyme is responsible for restoration of immune signalling.
**Figure 3.13** Denitrosylation activity of TRXh5 restores pathogen-induced immune gene expression

WT, nox1, and 35S::TRXh5(C42S) (nox1) plants were infected with Pst DC3000 (5 x 10^5 cells). After 24 hr expression of SA-dependent PR-1 gene was analysed using qPCR and normalized against constitutively expressed UBQ5. Error bars represent SD (n = 3).

To test if TRXh5 impacts immune signalling downstream of SA, we applied exogenous SA to plants and followed SA-dependent expression of PR-1, WRKY38, and WRKY62 in a time course. As expected, both nox1 and gsnor1 mutants displayed reduced expression of these genes compared to the WT (Figure 3.14). Expression of TRXh5 completely restored SA-induced gene expression in nox1 mutants to a similar level as the WT. In contrast, TRXh5 expression had little effect in the gsnor1 mutant background. In summary, these data demonstrate that TRXh5 specifically targets protein-SNO substrates to selectively modulate SA-responsive gene expression in plant immunity.
3.2.6 TRXh5 restores elevated levels of polyubiquitinated proteasome subunit RPN10 in nox1 plants

The 26S proteasome has been shown to have an important role in the regulation of SA-dependent immune responses by regulating turnover of the immune coactivator NPR1 in the nucleus (Spoel et al., 2009). It is likely that the process of ubiquitination and subsequent proteolysis by the proteasome is also required for the timely functions of other transcriptional activators and repressors and indeed this system has already been shown to function in the immune system of other plant species (Furniss and Spoel, 2015). It was therefore assessed if ubiquitination levels differed in immune-compromised nox1 and gsnor1 mutants in comparison to WT.

Plants were first treated with SA to increase overall levels of ubiquitination and then a Tandem Ubiquitin Binding Entity (TUBE) pulldown assay was performed to enrich for polyubiquitinated proteins (Figure 3.15). In comparison to WT, levels of polyubiquitinated proteins did not hugely differ in nox1 and gsnor1 mutants in the ubiquitin pulldown, although total levels of polyubiquitination were slightly elevated in nox1. Expression of TRXh5 did not appear to greatly affect the amount of polyubiquitinated proteins in either of these mutants, but moderately increased polyubiquitination levels in the WT. This suggests that overall proteasomal degradation pathways are functional in the
two SNO-overproducing mutants, although there may be subtle differences that can only be observed by looking at polyubiquitination of individual proteins.

![Poly-ubiquitinated Proteins Pulldown](image)

**Figure 3.15** Overall polyubiquitination of protein is unaffected in SNO mutants

WT, nox1, gsnor1, 35S::TRXh5 (nox1) and 35S::TRXh5 (gsnor1) plants were treated with 0.5 mM SA for 6 hours. Harvested tissue was then incubated with His-tagged TUBE protein and pulled down on HisPur Cobalt resin. Enriched polyubiquitinated proteins were detected using FK2 antibody and are shown relative to total polyubiquitinated proteins.

As it is possible that differences in polyubiquitination levels of individual proteins are too difficult to observe by looking at global cellular ubiquitination, a TUBE-assay was performed to detect differences in polyubiquitination of a protein already known to be ubiquitinated by a large variety of ubiquitin ligases; the proteasome subunit, RPN10 (equivalent of yeast S5α) (Crosas et al., 2006; Uchiki et al., 2009). Levels of polyubiquitinated RPN10 did not differ greatly between gsnor1 and WT, but there was a dramatic increase in the nox1 background. Strikingly, when TRXh5 was expressed in this background, polyubiquitinated levels of RPN10 decreased back to WT levels (Figure 3.16). This build-up of polyubiquitinated RPN10 in the nox1 background suggests that the proteasome is, at least in
part, inhibited in this mutant. As regular levels of polyubiquitination are restored following the expression of TRXh5, the data also suggests that NO-mediated S-nitrosylation may be the cause of this proteasomal inhibition.

Figure 3.16 Polyubiquitination of proteasomal subunit RPN10 is increased in nox1 but restored to WT levels by TRXh5.

WT, nox1, gsnor1, 35S::TRXh5 (nox1) and 35S::TRXh5 (gsnor1) plants were treated with 0.5 mM SA for 6 hours. Harvested tissue was then incubated with His-tagged TUBE protein and pulled down on HisPur Cobalt resin. Enriched poly-ubiquitinated proteins were detected using an S5α antibody (anti-RPN10) and shown relative to total RPN10 levels.

3.3 Discussion

Redox-based protein modifications play essential roles in cellular signalling. Protein S-nitrosylation has emerged as a particularly important post-translational signal in eukaryotes. But how this PTM is controlled and utilized as a specific signalling switch remains poorly understood. Here it is shown that in plant immunity, the oxidoreductase TRXh5 is a potent protein-SNO reductase that provides reversibility, and more importantly, specificity to protein-SNO signalling to establish SA-dependent disease resistance.
The TRXh5/NTRA system displayed potent denitrosylation activity on model and plant protein-SNO in vitro (Figure 3.1). Structurally, TRXh5 is closely related to other cytosolic TRX h-type members (h1–h9), implying that these enzymes may also function as cellular SNO reductases. Accordingly, plant cells exhibited strong denitrosylation activity that was dependent on the activity of NTR (Figure 3.2). In this respect, plant TRX-h enzymes behave similar to the mammalian TRX system, which has been described as a potent protein-SNO reductase involved in a variety of processes, including apoptosis and inflammatory signalling (Benhar et al., 2008; Kelleher et al., 2014). Mammalian TRX1 in the disulphide form, however, was shown to be S-nitrosylated at a cysteine residue away from the active site, allowing it to also function as a NO donor by trans-nitrosylating target proteins (Mitchell and Marletta, 2005; Sengupta and Holmgren, 2013). As a corresponding cysteine residue in plant TRX-h enzymes is absent, they are not expected to function as NO donors.

Despite lacking the corresponding NO donating cysteine residue of mammalian TRX1, it was observed that there is formation of S-nitrosylated TRXh5 during denitrosylation reactions. However, S-nitrosylation occurred in the active site, and reduction of protein-SNO required only a single active site cysteine (Figure 3.3), suggesting TRXh5 trans-denitrosylates its substrates; a mechanism also seen in Escherichia coli TRX (Nikitovic and Holmgren, 1996). By contrast, mammalian TRX1 was suggested to denitrosylate substrates via a mixed disulphide intermediate (Benhar et al., 2008). Here, a non-denaturing BST was performed in which mixed-disulphide intermediates were largely eliminated, while many trans-denitrosylated substrates of TRXh5 were still purified (Figures 3.4A & 3.4B). Moreover, denitrosylation by TRXh5 was not associated with a significant increase in mixed disulphide intermediates both in vitro and in vivo (Figures 3.4C & 3.5). Although these assays cannot completely rule out formation of mixed-disulphide intermediates, our results strongly imply that plant TRXh5 predominantly utilizes a trans-denitrosylation mechanism.

Remarkably, expression of TRXh5 in immune-compromised gsnor1 and nox1 mutants demonstrated that TRXh5 selectively restored immunity only in the nox1 genetic background (Figure 3.7). This was not due to differences in reduction capacity of TRXh5 in these mutant backgrounds, but
rather because of selective denitrosylation (Figures 3.8, 3.9 & 3.10). Although both *gsnor1* and *nox1* mutants accumulate high levels of protein-SNO during pathogen infection (Yun et al., 2011), these are derived from distinct NO donors (i.e., GSNO versus free NO). Stereochemistry and structure of NO donors as well as thiol microenvironment have been reported to influence site-specificity and efficiency of S-nitrosylation (Foster et al., 2009; Kovacs and Lindermayr, 2013). Whereas NO is thought to S-nitrosylate thiols directly through a radical-mediated pathway or indirectly via higher oxides of NO, GSNO is thought to trans-nitrosylate targets (Kovacs and Lindermayr, 2013). It is therefore likely that *nox1* and *gsnor1* mutants have different, albeit overlapping, sets of protein-SNO. The finding that TRX rescues immunity only in *nox1* but not in *gsnor1* mutants provides the first genetic indication that TRX enzymes may denitrosylate a distinct subset of protein-SNO from those regulated by the indirect SNO reductase GSNOR1, allowing these two enzymes to function in parallel in different protein-SNO immune signalling pathways (Figure 3.17).

![Proposed model showing the SNO reductases GSNOR1 and TRXh5 regulate different branches of protein-SNO in plant immune signalling](image)

**Figure 3.17** Proposed model showing that the SNO reductases GSNOR1 and TRXh5 regulate different branches of protein-SNO in plant immune signalling

Two classes of proteins are shown. Class I proteins are S-nitrosylated by GSNO, the level of which is regulated by the SNO reductase GSNOR1 (left panel). Class II proteins are S-nitrosylated by free NO, or other unknown intermediates and are denitrosylated by the TRXh5/NTRA system (right panel). As exemplified by the immune coactivator NPR1, class I and class II proteins partly overlap and, consequently, are regulated by both GSNOR1 and the TRXh5/NTRA system (middle panel). Both pathways contribute to SA-dependent gene expression and immunity.
As may be expected, however, some of this specificity was lost in vitro, as GSNO-derived BSA-SNO and plant protein-SNO could still be denitrosylated by TRXh5 upon reconstitution in a test tube (Figures 3.1 & 3.3). Nonetheless, it has previously been reported that the TRX-h/NTR system was unable to denitrosylate S-nitrosylated GAPDH in vitro (Zaffagnini et al., 2013), suggesting some selectively persists in vitro.

Disulphide reduction activity of TRXh5 was previously shown to facilitate SA-responsive gene expression mediated by the transcription coactivator NPR1. S-nitrosylation of Cys156 in NPR1 monomers facilitates the formation of a disulphide-linked oligomer in the cytoplasm. Upon SA signalling, these disulphides are reduced by TRXh5, releasing NPR1 monomers into the nucleus where they activate SA-responsive immune genes (Mou et al., 2003; Tada et al., 2008). The data presented here demonstrate that in addition to reducing intermolecular disulphide links in NPR1, TRXh5 is also capable of denitrosylating this co-activator (Figure 3.11A). TRXh5-mediated denitrosylation of NPR1 enhanced its nuclear translocation and was associated with increased transcriptional activity in nox1 mutants (Figures 3.11B, 3.12, 3.13 & 3.14). Thus, besides governance by GSNOR1 (Tada et al., 2008), SNO groups in NPR1 are directly targeted by TRXh5 (Figure 3.17). Denitrosylation of NPR1 is reminiscent of the mammalian immune-induced NF-kB transcription factor, whose activity is also suppressed by site-specific S-nitrosylation in respiratory epithelium. Upon cytokine stimulation, NF-kB is denitrosylated by TRX1, thereby promoting its transcriptional activity (Kelleher et al., 2014). Despite promoting nuclear translocation of NPR1, denitrosylation activity of TRXh5 was insufficient to restore immune-induced gene expression in gsnor1 mutants (Figures 3.11B, 3.12 & 3.14). This suggests that additional gene regulators normally governed by GSNOR1 remain S-nitrosylated in this genetic background and, consequently, remain transcriptionally inactive. Uncovering the identity of these S-nitrosylated gene regulators and determining how they differ from TRXh5 substrates represents a future challenge in elucidating protein-SNO signalling pathways. Furthermore, plant TRXh5 was shown to be a target of the fungal pathogen effector victorin, which binds to active site Cys39 and blocks disulphide reductase activity to induce host cell death (Lorang et al., 2012; Sweat and Wolpert, 2007).
It would be interesting to test if victorin-induced cell death also involves impairment of TRXh5 denitrosylation activity.

Interestingly, differences in the polyubiquitination of proteasomal subunit RPN10 were observed in nox1 and gsnor1 backgrounds (Figure 3.16). RPN10 plays a role in substrate recruitment to the proteasome and it has previously been shown that ubiquitination of RPN10 controls this activity by disrupting its interaction with target proteins (Isasa et al., 2010; Lipinszki et al., 2012). Defence gene expression is regulated by the proteasome, which has previously been shown to act like a cofactor in eukaryotic gene transcription (Spoel et al., 2009). Whilst it was difficult to determine small differences in total ubiquitination levels between SNO mutants and WT (Figure 3.15), elevated levels of polyubiquitinated RPN10 found in nox1 but not gsnor1 or WT backgrounds suggest that NO modifies and negatively regulates part of the machinery responsible for degrading this protein. This theory is supported by the fact that expression of TRXh5 reverses the effect on RPN10 polyubiquitination. Based on the integral role of proteasome-mediated degradation in timely transcriptional activity during plant immune responses, it would be interesting in future experiments to look further at whether or not specific subunits of the proteasome are S-nitrosylated in the nox1 and gsnor1 backgrounds and whether or not they can be denitrosylated by TRXh5 or indeed GSNOR1.

In conclusion, the findings in this chapter demonstrate that plant TRXh5 is a potent protein-SNO reductase, and this function is a key determinant of SA-dependent plant immune signalling. Selective restoration of SNO-impaired immune gene expression by TRXh5 implies that eukaryotic TRX enzymes in general may discriminate between protein-SNO substrates to provide specific, reversible protein-SNO signalling.
Chapter 4

Thioredoxin-\(h5\) denitrosylates transcription (co)factors essential for plant immunity

*The work in this chapter was performed in collaboration with the laboratory of Professor Yasuomi Tada during a rotation at the University of Nagoya, Japan*
4.1 Background

The identification of TRXh5 as a selective protein-SNO reductase in plant immunity raises a new set of questions with regard to its specific targets and how their denitrosylation controls protein function (Chapter 3; Kneeshaw et al., 2014). Indeed, although the effects of S-nitrosylation on several immune-related proteins have already been elucidated, it is unclear if these modifications are controlled by TRXh5, GSNOR1, both of these enzymes, or neither.

Many immune-related proteins in plants have already been identified to be SNO-modified and these can be sorted into different categories depending on their function. One such group are enzymes that are themselves involved in redox signalling pathways. For example, the NADPH oxidase, RBOHD, has been shown to be S-nitrosylated at Cys890, resulting in inhibition of ROS-generating activities of this protein. Consequently, a negative feedback loop is generated in which pathogen-induced NO regulates the extent of hypersensitive response programmed cell death by controlling the ROS generated by RBOHD (Yun et al., 2011). By contrast, SNO modification of Peroxiredoxin II E (PrxII E) was found to potentiate levels of ROS/RNS in the cell; peroxynitrite (ONOO⁻) detoxification activity of PrxII E was suppressed by S-nitrosylation, resulting in increased levels of this radical (Romero-Puertas et al., 2007). These feedback loops illustrate extensive interplay between NO and ROS/RNS and imply that through PTM of antioxidant machinery, NO is directly involved in controlling the level of oxidative stress in the cell. Indeed, NO has also been shown to control its own bio-availability by S-nitrosylating GSNOR1, thereby reducing the GSNO scavenging activity this enzyme (Frungillo et al., 2014). Despite these advances, it remains largely unknown how S-nitrosylation of these key immune proteins is controlled. In case of RBOHD, the extent of S-nitrosylation was proposed to be controlled indirectly by GSNOR1 (Yun et al., 2011), but it is possible that if free NO also modifies this enzyme, it could overlap as a protein-SNO target of TRXh5. Moreover, it is currently still unknown if GSNOR1 or TRXh5 control SNO modification of PrxII E and GSNOR1 itself. Whilst uncovering how reversibility of SNO modifications contributes to the regulation of these antioxidant enzymes is an interesting challenge for future studies, the focus in this chapter will be dedicated to uncovering the role of SNO regulation.
of a different category of immune proteins: those that are directly involved in SA-responsive gene transcription. In addition to targeting the aforementioned enzymes, S-nitrosylation has also been shown to control transcription regulators, such as the immune coactivator NPR1. Other proteins involved in reprogramming of the immune transcriptome are also affected by redox modification. Following pathogen attack, NPR1 physically interacts with TGA transcription factors that are involved in the activation of PR genes (Kesarwani et al., 2007). However, their interaction with NPR1 has been shown to be redox dependent; an intramolecular disulphide bond in TGA1 and TGA4 prevents association with NPR1 and must therefore be reduced before interaction can take place (Després et al., 2003). Furthermore, S-nitrosylation and S-glutathionylation of TGA1 increased DNA binding of this transcription factor by protecting against further oxidative stress (Lindermayr et al., 2010). To fully understand how these transcriptional (co)regulators are controlled, it essential to identify the mechanism by which SNO groups are reversed on these targets.

In Chapter 3, TRXh5 was demonstrated to rescue SA-dependent signalling in mutant nox1 plants over-accumulating NO-derived SNO-proteins. This suggests that many proteins involved in the activation of defence genes, or indeed the repression of housekeeping genes, may become S-nitrosylated during pathogen infection but are subsequently subjected to protective or regulatory denitrosylation by TRXh5. In this chapter, we report a preliminary investigation into which transcriptional regulators of the immune response are targets of TRXh5 denitrosylase activity and what the effect of reversible S-nitrosylation is on these proteins.

NPR1 is the master regulator of SA-mediated immunity and is essential to the establishment of SAR. As discussed in Chapter 3, previous work has demonstrated that the cysteines in the structure of NPR1 are sensitive to redox modification and that this is key to the localisation and activity of the protein (Mou et al., 2003). S-nitrosylation of Cys156 of NPR1 facilitates the formation of disulphide bonds between NPR1 monomers, resulting in an oligomer that resides in the cytoplasm. TRXh5 was identified as a reductase of disulphide bonds in NPR1 oligomer during activation of immunity. Moreover, it is able to reduce SNO modification of NPR1, which presumably promotes NPR1
monomerisation, resulting in two-tiered regulation of NPR1 conformation and activity by TRXh5 (Kneeshaw et al., 2014; Tada et al., 2008). NPR3 and NPR4 are paralogs of NPR1 and have also been shown to play an important role in plant immunity by acting as SA receptors (Fu et al., 2012). Whilst the oligomerisation status of NPR3 has not yet been studied, recombinant NPR4 was reported to spontaneously oligomerize (Fu et al., 2012). However, it is still unknown if these NPR family members are subject to redox regulation through S-nitrosylation. Therefore, the effects of SNO modification on NPR3 and NPR4 were investigated.

In addition, an in vitro study into the control of the WRKY family of transcription factors by S-nitrosylation is presented. The WRKY family is a large group of transcription factors, so named because of their conserved amino acid sequence WRKYGQK, which together with a zinc-finger-like motif, Cx_{4-5}Cx_{22-23}HxH or Cx_7Cx_{23}HxC, forms the WRKY domain. The family can be split into three main groups; group I contains two sets of the consensus WRKY sequence, whereas group II and III contain just one but differ in the number of cysteines in their zinc-finger domain. WRKY family members bind specifically to W-boxes, which have the DNA sequence (T)(T)TGAC(T/C), and this DNA binding is dependent on the WRKY domain (Eulgem et al., 2000; Maeo et al., 2001). WKRYs have been implicated in many biological processes, but most prominently they are associated with disease resistance and stress responses. (Eulgem and Somssich, 2007; Rushton et al., 2010). Indeed, W-box cis-elements have been found in, amongst others, the promotors of both NPR1 and PR genes (Maleck et al., 2000; Yu et al., 2001). WKRY proteins have been shown to act as both activators and repressors of transcription, and additionally can both positively and negatively regulate plant immunity via the regulation of SA-responsive genes (Eulgem and Somssich, 2007). This chapter presents an in vitro analysis of the control of WRKYs by S-nitrosylation and provides a framework for future investigations into the role of TRX-mediated control of this modification on immune-related transcription factors in plants.
4.2 Results

4.2.1 Redox modification of NPR proteins is controlled by TRXh5

NPR3 and NPR4 have been shown to have similar secondary structures to NPR1 and an alignment of their protein sequences indicated six conserved cysteines between all six members of the NPR family (includes NPR2, NPR6/BOP1 and NPR5/BOP2) (Hepworth et al., 2005; Shi et al., 2013). Of those conserved cysteines, Cys82 and Cys261 have previously been identified as important in the oligomerisation of NPR1 through disulphide bonding (Mou et al., 2003), suggesting that these cysteines may also be redox modified in the other NPRs. Interestingly, however, Cys156, which was shown to facilitate oligomerisation of NPR1 through S-nitrosylation, is not conserved in NPR3 and NPR4 (Shi et al., 2013; Tada et al., 2008).

To determine if like NPR1 (Figure 3.11A), the SA receptors, NPR3 and NPR4, are S-nitrosylated, a BST was performed on FLAG-tagged versions of these proteins in vitro (Figure 4.1). Despite the absence of Cys156, both proteins were found to be S-nitrosylated in response to the NO donor GSNO, indicating that one or more other cysteines are subject to this modification. Moreover, reminiscent of its effects on NPR1 (Figure 3.11A), addition of the TRXh5/NTRA system was able to completely reverse S-nitrosylation of NPR3 and NPR4 (Figure 4.1), indicating that TRXh5 is a potential denitrosylase of these proteins.
**Figure 4.1** NPR3 and NPR4 are protein-SNO targets of TRXh5

*In-vitro*-translated FLAG-tagged NPR3 (A) and NPR4 (B) were S-nitrosylated with 1 mM GSNO and incubated with or without the TRX system, consisting of TRXh5 (5 µM), NTRA (0.5 µM), and NADPH (1 mM). S-nitrosylated NPR3 (NPR3-SNO) and NPR4 (NPR4-SNO) were detected with the BST in presence or absence of ascorbate (Asc) and are shown relative to total protein.

As previously discussed, during activation of plant immunity, NPR1 forms redox-dependent oligomers that are reduced to monomers by the action of TRXh5 (Mou et al., 2003; Tada et al., 2008). Given the similarities between NPR1, NPR3 and NPR4, we studied the effect of NO on the conformation of these proteins. Thus, NPR proteins were treated with the NO donor DEA-NO or with oxidised glutathione (GSSG) to test if either of these oxidants could induce oligomer formation. Similar to NPR1, extensive oligomer formation was seen for both NPR3 and NPR4 in response to both DEA-NO and GSSG (Figure 4.2). Taken together with the observed SNO modification on these proteins, these data indicate that like NPR1, NPR3 and NPR4 can form oligomers that are facilitated by S-nitrosylation. Interestingly, the use of NPR1 as a control in this experiment highlighted the differences in the dynamics of oligomer formation between the different NPR proteins. In NPR3, the bands signifying the appropriate size of a dimer were notably more marked than for NPR1 and NPR4, where the transition to oligomer appeared occur without notable dimer formation. This suggests that disulphide-mediated interaction between two NPR3 proteins is more stable than in NPR1 or NPR4.
Finally, we tested if NO- and GSSG-induced NPR oligomerization could be counteracted by TRXh5. At low concentrations, DTT has previously been shown to effectively turnover the activity of TRXh5 while not affecting the NPR1 oligomer (Kneeshaw et al., 2014; Tada et al., 2008), and was therefore used as a recycling agent. All NPR oligomers were reduced completely to monomers by TRXh5, demonstrating that this enzyme can modulate the conformation of these proteins by reducing both SNO and disulphides (Figure 4.2).

![Figure 4.2](image)

**Figure 4.2** NPR3 and NPR4 form oligomers that are reduced by TRXh5

*In vitro* translated FLAG-tagged NPR1, NPR3 and NPR4 were incubated with either 500 µM oxidized glutathione (GSSG) or 1 mM DEA-NO. Recombinant TRXh5 (5 µM), together with a small amount of DTT (0.33 mM), which is known to recycle TRXh5 activity but not reduce NPR1 oligomers (Kneeshaw et al., 2014; Tada et al., 2008), was added where indicated. NPR oligomer (O) to dimer (D) and monomer (M) relative to total NPR protein is shown by non-reducing (-DTT) and reducing (+DTT) SDS-PAGE and detected using an anti-FLAG antibody.
4.2.2 S-nitrosylation affects DNA binding of WRKY transcription factors

Next, we investigated if in addition to NPR1, WRKY transcription factors may also be regulated by redox modifications. Despite the presence of either two or three conserved cysteines in the zinc finger, there have been no previous reports that WRKY factors are redox modified. However, a recent screen of the Arabidopsis WRKY family members has demonstrated that all of these proteins can be S-nitrosylated in vitro (M. Nomoto and Y. Tada, unpublished).

Using the BST, it was first assessed if TRXh5 was able to reverse in vitro S-nitrosylation of selected WRKY factors that play key roles in plant immunity (WRKY11, 17, 18 and 70). For all four of these proteins S-nitrosylation by DEA-NO was confirmed and in each case TRXh5 acted as a potent denitrosylase (Figure 4.3).

Figure 4.3 WRKY transcription factors are protein-SNO targets of TRXh5

In-vitro-translated FLAG-tagged WRKY18 (A), WRKY11 (B), WRKY17 (C) and WRKY70 (D) were S-nitrosylated with 1 mM DEA-NO and incubated with or without the TRX system, consisting of TRXh5 (5 µM), NTRA (0.5 µM), and NADPH (1 mM). S-nitrosylated WRKY factors (WRKY-SNO) were detected with the BST in presence or absence of ascorbate (Asc) and are shown relative to total protein.
While the above data clearly show WRKY factors can be reversibly S-nitrosylated, the molecular consequences of this modification are unknown. The primary function of WRKY proteins is to act as transcriptional activators or repressors by binding to the W-box cis-element in the promoters of target genes. Therefore, a screen was performed to determine if S-nitrosylation of WRKY proteins affects their interaction with the cis-element LS4, a known WRKY binding target in the PR-1 gene promoter (Lebel et al., 1998). To do this, the AlphaScreen system (Perkin Elmer) for protein-DNA interaction was utilised, which reports on the proximity of donor and acceptor beads coupled to DNA sequences and proteins of interest. Thus, FLAG-tagged WRKY proteins were incubated with acceptor beads conjugated to an anti-FLAG antibody, whilst biotinylated-DNA was incubated with donor beads conjugated to biotin-binding streptavidin. WRKY-DNA interactions are expected to bring the donor and acceptor beads into close proximity. Following excitation at 680 nm, a positive interaction between donor and acceptor beads emits light at 520-620 nm, which can then be measured to determine the intensity of the interaction. WRKY proteins were first treated with or without 1 mM DEA-NO, and subsequently dialysed prior to the AlphaScreen reaction. In response to DEA-NO, some of the WRKYS, but notably not all, displayed a significant change in the affinity with which they bound the PR-1 LS4 W-box (Figure 4.4). Moreover, of those that did display a difference in DNA binding pre- and post- NO treatment, some showed an increased level of binding, whereas others showed a decrease (Figure 4.4). The results from this screen suggest that despite all of the WRKY family members being subject to S-nitrosylation (Nomoto and Tada, unpublished), this modification has differential effects on individual WRKY proteins.
Figure 4.4  S-nitrosylation of WRKY transcription factors differentially affects their DNA binding

FLAG-tagged WRKY transcription factors were treated with or without 1 mM DEA-NO. Subsequently, detection of interaction with the biotinylated LS4 cis-element was performed using a protein-DNA AlphaScreen assay (Perkin Elmer). Data is presented as the fold change between control- and DEA-NO-treated samples. Significant changes (Student’s t test, P < 0.05) are shown in blue (n=3).

To investigate the reason for differential effects of S-nitrosylation on DNA binding of WRKY proteins, their protein sequences were aligned in an attempt to identify conserved cysteines around the WRKY domain of family members that displayed a change in DNA binding in response to NO (Figure 4.5). All WRKY proteins share at least two conserved cysteines that are part of the zinc-finger (Figure
4.5, dotted bracket), and these have been shown to be essential to DNA binding (Maeo et al., 2001). However, as all WRKY proteins contain these cysteines, it is unlikely that S-nitrosylation of these residues caused differential DNA binding seen in the AlphaScreen interaction assay. In addition to these cysteines, group III WRKY proteins contain a third cysteine in the zinc-finger domain, with the sequence Cx7C23HxC, as opposed to Cx4-5C22-23HxH found in group I and II proteins (Figure 4.5, black arrow). Of the eleven group III WRKY proteins in this screen, five displayed a decrease in DNA binding upon S-nitrosylation, whereas only two showed an increase. The remaining WRKYS that had improved DNA binding after treatment with NO all belonged to group II, with the exception of one group I protein. Therefore, whilst it is possible that the presence of the third zinc-finger cysteine in group III WRKY proteins is involved in disrupting WRKY-DNA interaction, it seems unlikely that its S-nitrosylation causes improved binding, and indeed, absence of this cysteine appeared more likely to enhance binding to the W-box. There did not appear to be any other cysteines around the active site that linked all, or even the majority of the different groups identified in the binding screen. However, it is possible that several different groups of cysteines are responsible for differential effects of S-nitrosylation in subsets of WRKY proteins. A cysteine conserved amongst WRKY47, WRKY6 and WRKY42 (Figure 4.5, white arrow), all of which showed increased DNA binding after S-nitrosylation, could for example, be responsible for changing DNA binding affinity of these family members, whereas S-nitrosylation of a different cysteine could be the cause of the same response in other WRKYs.
| WRKY40 | 119 | ---Q--RE--------ETVVKEKVSRYVYKTEASD----TTLVVKDGYWRKYGKQV | WRKY41 | 285 | HGHAKKRKHVR------RSIRVPAISNKVAD----IPPDYWSRKYGKPI |
| WRKY18 | 149 | ---P--VT------DSFNKAKSVTVYVPTETS----TSLTVKDGWRKYGKQT | WRKY39 | 239 | HCHSSKRKLVRK------RSIKVPAISNIAK----IPPDYWSRKYGKPI |
| WRKY60 | 125 | ---DKATV3STAYFAAEKSD----TLTVKDGWRKYGKQT | WRKY15 | 212 | HCHSSKRKIKQR------RIIRVPAISAKMD----VPPDYSWSRKYGKPI |
| WRKY36 | 173 | NHHQ--VL------EEHEQTLKKTQRVKKAS----EDPSINDGRWKRKYGKA | WRKY7 | 253 | HCHSSKRKRSRVK------RURIVPAVASSKMAD----IPDESWFRKYGKPI |
| WRKY6 | 282 | TTTT--TF------DQTAETERMKARVSRAR----EAPMTSDKRKRKYGKMA | WRKY11 | 218 | HCHSSKRKNRMK------RTTVPRPAISAKAI----IPPDYWSRKYGKPI |
| WRKY42 | 262 | SSKV--IE------QAAAEAMRKARVSRAR----EAPMSLDWRKYGKMA | WRKY17 | 215 | HCHSSKRKNRMK------RTTVPRPAISAKAI----IPPDYWSRKYGKPI |
| WRKY63 | 81 | KKRIGVKGLEIYRRDPSNPRDLGGFTWRKYGKTI | WRKY27 | 141 | PLRS--KKRNQO------KRTICHG------TQENLSDDLWARKYGKPI |
| WRKY67 | 83 | PLHK--RG------KRTSMAESDDYHRHESSTPIYDHGSDFWRKYGKPI | WRKY29 | 110 | SKSK--KKNNQO------KRVEQV------KEENLSDDAWARKYGKPI |
| WRKY38 | 86 | --QVA--HR------RKLVRAEGTVNYNDSRTMGPDNGFTWRKYGKTI | WRKY14 | 184 | NNLG--KRRLSQA------KKVVCIPAPAMNSRSGSEVPSDDLWARKYGKPI |
| WRKY3 | 105 | TSSR--KKRIHQ------KRTICHG------AEEALSDDLWARKYGKPI | WRKY22 | 205 | TSRS--KKRIHQ------KRTICHG------TQENLSDDLWARKYGKPI |
| WRKY5 | 121 | --S--KRIIHKV-------KRTICHG------AEEALSDDLWARKYGKPI | WRKY54 | 89 | S--KRIIHKV-------KRTICHG------AEEALSDDLWARKYGKPI |
| WRKY70 | 290 | --EM--KRMLRGEDEGMSIEVSAGKVEPRVQVTIS---DID----VLIDGFWRKYGKPV | WRKY44 | 323 | --EM--KRMLRGEDEGMSIEVSAGKVEPRVQVTIS---DID----VLIDGFWRKYGKPV |
| WRKY25 | 450 | --EM--KRMLRGEDEGMSIEVSAGKVEPRVQVTIS---DID----VLIDGFWRKYGKPV | WRKY34 | 335 | --EM--KRMLRGEDEGMSIEVSAGKVEPRVQVTIS---DID----VLIDGFWRKYGKPV |
| WRKY8 | 207 | --EM--KRMLRGEDEGMSIEVSAGKVEPRVQVTIS---DID----VLIDGFWRKYGKPV | WRKY33 | 324 | --EM--KRMLRGEDEGMSIEVSAGKVEPRVQVTIS---DID----VLIDGFWRKYGKPV |
| WRKY41 | 108 | --EM--KRMLRGEDEGMSIEVSAGKVEPRVQVTIS---DID----VLIDGFWRKYGKPV | WRKY6 | 124 | --EM--KRMLRGEDEGMSIEVSAGKVEPRVQVTIS---DID----VLIDGFWRKYGKPV |
| WRKY53 | 143 | --EM--KRMLRGEDEGMSIEVSAGKVEPRVQVTIS---DID----VLIDGFWRKYGKPV | WRKY55 | 78 | TKQF--RRKDEG-------EEQTVLVAALRTGN-------TDLPDDNHTWRKYGKRE |
| WRKY46 | 73 | SKNVFVKRKVSE-------KNTKEKVKVFATEQ-------ENGSIDICHWRKYGKRE |
| WRKY30 | 78 | DSQ--EPLVIKS------SKKMPVRWSKVRIPAGVDAO1DLGFSWRKYGKQRD |
| WRKY49 | 87 | VPL-----LPERS------TLSKVDRYTLKVKN--N---SNQDDGYWRKGYGKSRD |
| WRKY59 | 75 | IGKKDEIKRKR-------HKEPIHVFKTKS---SIDEKVALDDGYWRKYGKRP |
| WRKY43 | 106 | QLTQ--QKKGKE------KKKKRVEVRAMTKS---EID----HLDRGYWRKYGKRAV |
| WRKY8 | 154 | QKVV--KTKKEK------EKKKEEPRVSMKTK---EVD----HLDRGYWRKYGKRAV |
| WRKY28 | 142 | KKVGV--KTKKEV------KKKEEPRVSMKTK---EVD----HLDRGYWRKYGKRAV |
| WRKY12 | 115 | SGM--NKKVIR------RKLREPRFQTKS----VOLDGRFYWRKYGKRAV |
| WRKY13 | 262 | SSKL--MKKLST------RKLREPRFQTKS----VOLDGRFYWRKYGKRAV |
| WRKY51 | 80 | STNR--GSESQ------TKETGHRVAFTRS---KID----VMDDGFGWRYGRKSV |
| WRKY57 | 87 | ETPF--KEKKKA------TKRQPRFAMTKS----NDLEGYWRKYGKRAV |
| WRKY23 | 144 | HKQIL--KAKKNQ------KKRQREAPAMTKS----EVD----HLDRGYWRKYGKRAV |
| WRKY24 | 68 | IGK--GKELKE------SRKPRVFAPHTRS---EVD----HLDRGYWRKYGKRAV |
| WRKY56 | 84 | HNK---RKGRKKK------RTLAMQRHAFHTRS---EVD----HLDRGYWRKYGKRAV |
| WRKY45 | 35 | SSE--KPRSKK------KKKREARYAFQTRS---QVD----ILDGRYWRKYGKRAV |
| WRKY75 | 37 | EGKS--KSVESS------KKKQKRYAFQTRS---QVD----ILDGRYWRKYGKRAV |
Figure 4.5  Sequence alignments of WRKY domains from *Arabidopsis* WRKY family

Protein sequences of listed *Arabidopsis* WRKY transcription factors were aligned using Clustal Omega software [http://www.ebi.ac.uk/Tools/msa/clustalo/](http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011). The consensus WRKY sequence is highlighted in the grey box. The zinc finger motif is indicated by the dotted bracket. Cysteines are highlighted in purple. Black
and white arrows represent potential S-nitrosylated cysteines that affect DNA binding (see text). Yellow and blue highlighted titles indicate WRKYs that show enhanced or decreased DNA binding following S-nitrosylation, respectively.

Peculiarly, some of the WRKY proteins that showed a change in DNA binding after treatment with NO did not appear to possess any cysteines directly around the WRKY domain. A good example is WRKY18, where there appears to be a significant increase in DNA binding of around 4-fold post NO treatment (Figure 4.4), yet other than the two conserved zinc-finger cysteines, there are no other cysteines surrounding the WRKY domain (Figure 4.5). It could be the case that cysteines elsewhere in the WRKY18 structure, further out from the WRKY domain are S-nitrosylated, perhaps altering the 3-dimensional structure of the protein to enhance DNA binding. Another possibility is that S-nitrosylation of conserved cysteines that are present in all WRKYs does in fact have a different effect in different WRKY proteins, potentially caused by differences in the surrounding residues and structures. Indeed, it has been shown that despite the fact all WRKY proteins bind the same W-box sequence, specificity for different promoters is achieved through the DNA sequences flanking the consensus (T)(T)TGAC(T/C) sequence (Ciolkowski et al., 2008). With this level of specificity between WRKY and DNA, it is entirely possible that SNO modification of one of the conserved zinc-finger motif cysteines could alter the DNA binding to a particular cis-element differentially, depending on the neighbouring amino acids. To investigate this theory further, four WRKYs that were found to have a change in DNA binding after treatment with NO at the LS4 cis-element of the PR-1 promoter were tested in an AlphaScreen assay against a W-box in the ICS1 promotor sequence (Figure 4.6). Interestingly, in both WRKY18 and WRKY40, which had increased and decreased PR-1 LS4 binding respectively, no difference in binding to the ICS1 W-box sequence was observed upon their S-nitrosylation. Moreover, whereas WRKY70 still showed a significant decrease in binding to the ICS1 W-box in response to DEA-NO treatment, WRKY17 showed the opposite effect on the ICS1 W-box sequence to that of the PR-1 LS4 sequence, in that its DNA binding affinity was decreased rather than increased (Figure 4.6). These data suggest that DNA sequences flanking the consensus W-box not only
have an effect on which WRKYs will bind (Ciolkowski et al., 2008), but also affect the way in which post-translational S-nitrosylation alters that binding.

![Graph showing AlphaScreen Units for different WRKY samples with and without NO treatment. Asterisks indicate significant changes.](image)

**Figure 4.6** S-nitrosylation of WRKYs affects DNA binding at distinct W-box sequences differently

FLAG-tagged WRKY transcription factors were treated with or without 1 mM DEA-NO. Subsequently, detection of interaction with a biotinylated cis-element from the /ICS1 promoter was performed using the protein-DNA AlphaScreen Assay (Perkin Elmer). Asterisks indicate WRKY proteins that displayed significant changes following treatment with NO (Student’s t test P < 0.05). Error bars represent standard error (n=3).

These findings suggests that despite the fact all WRKY proteins contain two conserved cyssteines in the zinc finger motif (Figure 4.5), S-nitrosylation of one or both of these cysteines could cause different effects on the DNA binding of a particular WRKY because of its surrounding amino acids and resulting protein structure. Therefore, we used the WRKY18 mutants, WRKY18(C201S), WRKY18(C207S) and WRKY18(C201/207S), in which two zinc finger motif cysteines of WRKY18 were mutated to serines both individually or together, to test DNA binding in presence of NO. The **PR-1 LS4 cis-element** was used in this assay, as WRKY18 demonstrated a clear increase in DNA binding to this W-box sequence in response to NO (Figure 4.4). As expected, both the single and double cysteine mutants showed significantly lower DNA binding than the wild-type WRKY18 protein (Figure 4.7),
consistent with previous findings (Maeo et al., 2001). Interestingly, whilst all three mutants appeared to have a small increase in binding to DNA following treatment with NO, none of these changes were significant in a student’s $t$ test (Figure 4.7). Due to the necessity of these cysteines in DNA binding of WRKY18, it is difficult to determine whether their $S$-nitrosylation is responsible for the increases in binding affinity seen in the WT protein (Figures 4.4 & 4.7). Nonetheless, the low amount of DNA binding detected in this screen for all three of the mutants failed to be significantly improved by treatment of NO, suggesting that $S$-nitrosylation of these cysteines may be involved in increasing its affinity to bind DNA. Taken together, these data imply that $S$-nitrosylation of these two conserved cysteines adjacent to the WRKY domain and found in all WRKY proteins, results in differential effects on DNA binding in different WRKY family members depending on the surrounding amino acid sequence.

![Figure 4.7 S-nitrosylation of WRKY18 cysteine mutants does not increase DNA binding](image)

**Figure 4.7 S-nitrosylation of WRKY18 cysteine mutants does not increase DNA binding**

FLAG-tagged WRKY18, WRKY18(C201S), WRKY18(C207S) and WRKY18(C201/207S) proteins were treated with or without 1 mM DEA-NO. Subsequently, detection of interaction with biotinylated cis-element PR-1 LS4 was performed using the protein-DNA AlphaScreen Assay (Perkin Elmer). Asterisks indicate WRKY proteins that displayed significant changes following treatment with NO (Student’s $t$ test $P < 0.05$). Error bars represent standard error ($n=3$).
4.2.3 TRXh5 reverses the effects of NO on the DNA binding affinity of WRKY18

TRXh5 was shown to be a potent denitrosylase for WRKY proteins \textit{in vitro} (Figure 4.3). Therefore, using the AlphaScreen Technology we tested if TRXh5 could reverse the effects of S-nitrosylation on the DNA binding ability of WRKY18. As expected, WRKY18 showed an increase in binding to the \textit{PR-1 LS4} W-box following NO treatment, but remarkably, upon the addition of TRXh5, this effect was completely reversed (Figure 4.8). In combination with the finding that TRXh5 acts as a direct denitrosylase for WRKY18 (Figure 4.3), these results suggest that TRXh5 removes the SNO modification on WRKY18 to decrease its DNA binding affinity.

![Figure 4.8](image.png)

\textbf{Figure 4.8} TRXh5 reverses the effects of S-nitrosylation on WRKY18 DNA binding

FLAG-tagged WRKY18 was S-nitrosylated with 1 mM DEA-NO and incubated with or without the TRX system, consisting of TRXh5 (5 \textmu M), NTRA (0.5 \textmu M), and NADPH (1 mM). \textit{In vitro} detection of interaction with biotinylated cis-element \textit{PR-1 LS4} was performed using the protein-DNA AlphaScreen Assay (Perkin Elmer). Asterisks represent significant changes (Student’s \textit{t} test \( P < 0.05 \)) from the untreated WRKY18 protein. Error bars represent standard error (n=3).
4.3 Discussion

The identification of TRXh5 as a selective protein denitrosylase during the plant immune response (Chapter 3, Kneeshaw et al., 2014) raises many questions about what the targets of this enzyme are. In this chapter, two families of immune-related transcriptional regulators and their post-translational control by S-nitrosylation and subsequent denitrosylation by TRXh5 were investigated in vitro. Three members of the NPR family - the immune coactivator NPR1 and its two paralogs, the SA receptors NPR3 and NPR4 - were demonstrated to be protein-SNO targets of TRXh5, which controlled their conformation. Furthermore, the WRKY family of transcription factors that are key transcriptional regulators during plant immune responses, were found to be reversibly S-nitrosylated by NO and TRXh5, resulting in differential effects on their DNA binding ability.

S-nitrosylation of NPR1 has previously been shown to facilitate its oligomerisation through disulphide linkages that conversely, can be reduced by TRXh5 to release monomers. This has proven to be essential for the nuclear activity of the protein, as NPR1 cannot enter the nucleus as a disulphide bound oligomer (Mou et al., 2003; Tada et al., 2008). Furthermore, interaction between NPR1 and TGA transcription factors in the nucleus was reported to be enhanced by treatment with GSNO, as was NPR1-dependent DNA binding activity of TGA1 (Lindermayr et al., 2010). Here, we showed that the NPR1 paralogs, NPR3 and NPR4, were also subject to SNO modification, and could be denitrosylated by TRXh5 in vitro (Figure 4.1). Strikingly, S-nitrosylated Cys156 in NPR1 (Tada et al., 2008) is absent from NPR3 and NPR4, suggesting that these proteins are S-nitrosylated at alternative cysteine residues. Nonetheless, like NPR1, both NPR3 and NPR4 formed oligomers in response to an NO donor as well as oxidised glutathione. TRXh5 was shown to be efficient in reducing the NPR3 and NPR4 oligomers (Figure 4.2), suggesting a similar mechanism of control as that described for NPR1 (Tada et al., 2008).

Both NPR3 and NPR4 have been shown to interact with NPR1, acting as ubiquitin E3 ligase adapters to mediate the degradation of NPR1 (Fu et al., 2012). The interactions between NPR1 and NPR3/NPR4 were found to be dependent on the concentration of SA, providing a mechanism by which
to fine-tune NPR1 levels in both the absence and presence of pathogen stimuli (Fu et al., 2012; Moreau et al., 2012). However, in addition to the concentration of SA, pathogen challenge has also been shown to generate high levels of NO, which itself also potentiates SA accumulation, and vice versa (Lindermayr et al., 2010). Because S-nitrosylation of NPR3 and NPR4 can result in formation of disulphide linkages to form homo-oligomers, it is also possible that oxidative modifications of the NPR proteins could mediate (or disrupt) interactions with each other. This would provide new levels of regulation for the proposed NPR1/3/4 model (Fu et al., 2012; Tada et al., 2008). Because TRXh5 denitrosylated and monomerized all three NPR proteins (Figures 3.11A, 4.1 and 4.2), this oxidoreductase probably plays a key role in both the perception of SA and subsequent conversion of the SA signal into transcriptional output.

Furthermore, at a similar time that NPR3 and NPR4 were identified as the SA receptors in one study (Fu et al., 2012), another study identified NPR1 as the SA receptor (Wu et al., 2012). Here, it was shown that Cys521 and Cys529 were required for SA binding via the transition metal, copper. However, it was also reported that these two cysteines reside in an oxidized state irrespective of the presence of SA, making it more difficult to understand how they coordinate copper, which usually requires cysteines to be in a reduced state (Giles et al., 2003; Rochon et al., 2006). Nonetheless, it would be interesting to investigate whether the reported oxidized states of NPR1 Cys521 or Cys529 represent SNO or disulphide modification, and whether or not these are TRXh5 targets that affect interaction with SA. It is worth noting that these two cysteines are not present in NPR3 and NPR4 (Shi et al., 2013), suggesting that their interaction with SA occurs through different cysteine residues, or through a different mechanism.

In addition to NPR proteins, the WRKY family of transcription factors were shown here to be protein-SNO targets of TRXh5 (Figure 4.3). By using AlphaScreen protein-DNA interaction technology, we found that S-nitrosylation can affect the DNA binding capabilities of different WRKY proteins in distinct ways. Whilst some WRKY proteins displayed no difference in their DNA binding affinity after treatment with an NO donor, others showed either increased or decreased binding (Figure 4.4). The
WRKY domain has previously been identified as essential to DNA binding and a four stranded β-sheet structure was proposed, consisting of the zinc finger motif at one end and at the other, the WRKY consensus sequence, which is believed to make direct contact with the DNA (Maeo et al., 2001; Yamasaki et al., 2005). The zinc finger contains two cysteines conserved amongst all WRKY proteins, and a third that is specific to group III WRKY proteins. In addition, some WRKY family members possess additional non-conserved cysteines that could also be subject to S-nitrosylation (Figure 4.5). Whilst no clear pattern emerged linking WRKYs that displayed increased or decreased binding following NO treatment, it is possible that modification of these non-conserved cysteines affects DNA binding in select WRKYs. Differential WRKY-DNA binding was previously found to occur as a result of the DNA sequences neighbouring the consensus W-box sequence, providing specificity for the multiple gene promoters targeted by WRKY proteins in vivo (Ciolkowski et al., 2008). Here it is shown that S-nitrosylation of a particular WRKY protein is also perceived differently by different cis-elements (Figure 4.6). This could be because a particular cysteine is crucial to the binding of some DNA elements but is not required for others; this explanation could be relevant for both WRKY18 and WRKY40, which displayed altered changes in binding at the PR-1 promoter W-box in response to NO, but no change at the ICS1 promoter sequence. Alternatively, where S-nitrosylation of a particular WRKY improved DNA binding for one cis-element but decreased binding for another, such as was the case for WRKY17 (Figure 4.6), it could be the result of SNO-mediated structural alterations to the WRKY DNA binding domain that may favour binding of one DNA sequence over another. Future investigations into this phenomenon are essential to understanding how this modification affects the DNA binding activity of these proteins.

The involvement of the conserved zinc finger cysteines was investigated using single and double cysteine mutants of WRKY18. In the absence of both or just one of these cysteines, WRKY18 binding to the PR-1 LS4 W-box was dramatically decreased but not abolished entirely (Figure 4.7). In contrast to wild-type WRKY18, none of the cysteine mutants showed a significant increase in DNA binding upon treatment with NO. This suggests that S-nitrosylation of both of these cysteines
promotes DNA binding, although it is unclear if this is a general mechanism utilized by other WRKY proteins, particularly those that show a decrease in DNA binding after S-nitrosylation, where a different mechanism might be utilised. Nonetheless, this data indicates that whilst S-nitrosylation of non-conserved cysteines located in and around the WRKY domain may well cause alterations to DNA binding, the conserved cysteines also play a key role in this process for some WRKYs. It has been recently found that WRKY70 forms oligomers when exposed to NO donors in vitro (Nomoto and Tada, unpublished). Whilst this has not yet been investigated for other WRKY proteins, this could provide an explanation why in selected cases there is a decrease in DNA binding to both PR-1 and ICS1 promoters in response to NO (Figures 4.4 & 4.6).

Finally, we showed that TRXh5 is able to reverse the effects on DNA binding caused by S-nitrosylation of WRKY18 (Figure 4.8). This was, in part, expected, because using the BST, WRKY18 was shown to be denitrosylated by TRXh5 in vitro. Although it is still unclear if TRXh5 can restore normal DNA binding in WKRY proteins that exhibit decreased DNA binding when SNO-modified, this data indicates that the activity of at least some WRKY proteins is controlled by TRX enzymes.

The data in this chapter not only present preliminary, in vitro results on the identity of some immune-related transcriptional targets of TRXh5, but provide insight into how redox modifications and their regulation by TRX enzymes can affect protein conformation and function. The initial findings presented here open up many questions for future research. Firstly, verification of NPR and WRKY proteins as denitrosylation target of TRXh5 in vivo must be achieved. Secondly, by using the nox1 and gsnor1 mutants discussed in Chapter 3, it would be interesting to determine whether or not these protein-SNO are generated from an NO source, a GSNO source, or both (Figure 3.17). For both NPR proteins and the WRKY transcription factors, in vivo studies on the effects of their S-nitrosylation are essential to understanding how these proteins are regulated during immunity. Thirdly, by using mass spectrometry, an in depth study into the sites of S-nitrosylation could provide key information about the protein-protein and protein-DNA interactions formed by these groups, and their dependence on redox control. Recently, NPR1 and WRKY proteins have been shown to interact with each other.
(Nomoto et al., submitted), so in addition to looking at interactions amongst family members, it would be interesting to determine whether S-nitrosylation of these proteins affects their interactions with each other. Indeed, these studies could also be extended to other immune-related transcriptional regulators. The TGA family have already been discussed as targets of redox control, and members of one of the largest groups of Arabidopsis transcription factors, the MYB family, were found to have inhibited DNA binding activity following S-nitrosylation at two conserved cysteine residues, (Serpa et al., 2007; Tavares et al., 2014). It would be interesting to see if like WRKY proteins, SNO modification of these cysteines in other MYB family members would be able to increase their DNA binding dependent on the surrounding DNA sequence.

In conclusion, the findings presented here indicate that S-nitrosylation of nuclear transcriptional (co)regulators is likely a multi-layered, complex signalling tool that is utilised to orchestrate transcriptional reprogramming in effective immune response. Uncovering the role of TRX enzymes in this process will be essential to understanding how reversible regulation of transcription factors is achieved, and the framework for future in vivo studies on this topic is provided here.
Chapter 5

Nucleoredoxin 1 protects plant cells against oxidative stress

*Parts of the work in this chapter are currently in preparation for publication:


See also Appendix II.
5.1 Background

Oxidative stress in plants has been shown to cause a wide range of cysteine modifications in a variety of different cellular compartments (Mittler, 2002; Noctor and Foyer, 1998). As a result, plants possess a large number of different oxidoreductase enzymes to ensure correct spatial and temporal control of these protein modifications. The *Arabidopsis* TRX family consists of enzymes whose reductive capabilities apply to many different oxidative cysteine modifications and they have been shown to be present in almost all compartments of the plant cell; in chloroplasts, TRX enzymes act in conjugation with ferredoxin-thioredoxin reductase (FTR), using the reducing power of ferredoxin to combat oxidative stress caused during photosynthetic reactions, while in other compartments, including the cytosol and mitochondria, TRX-h enzymes act as disulphide reductants and denitrosylases that are recycled by NADPH-dependent TRX Reductase (NTR), using reducing power from NADPH (Kneeshaw et al., 2014; Meyer et al., 2009; Vieira Dos Santos and Rey, 2006). In addition to canonical TRX enzymes, the TRX superfamily consists of, amongst others, Protein disulphide isomerases (PDI), Glutaredoxins (GRX) and Nucleoredoxins (NRX); and each of these sub-groups is thought to target defined cysteine modifications in specific cellular locations (Jacquot et al., 2002; Meyer et al., 2008). Whilst TRX, PDI, and GRX enzymes have long been the focus of attention and have been studied in detail, the role of NRX enzymes has only just started to be uncovered.

NRXs have been identified in both plants and animals, albeit with slightly different structures. Importantly, as their nomenclature suggests, these enzymes have been shown to localise, although not exclusively, to the nucleus (Kurooka et al., 1997; Laughner et al., 1998; Marchal et al., 2014). Whilst TRX enzymes have been demonstrated to accumulate in the nucleus during stress responses (Serrato and Cejudo, 2003), there is still very little known about nuclear redox signalling and whether or not there are dedicated nuclear oxidoreductases. Redox control of nuclear localized proteins such as transcription factors and co-factors is likely essential to their timely behaviour in the activation or repression of genes. Indeed, in Chapter 3 and 4 we showed that S-nitrosylation directly regulates the activities of immune transcriptional regulators NPR1 and WRKY proteins. Understanding the role of
NRX proteins could therefore be crucial to determining how nuclear processes incorporate and utilise redox-based modifications as signalling tools.

NRX proteins were first identified in mice, with homologs subsequently discovered in other organisms, including maize (Funato and Miki, 2007; Kurooka et al., 1997; Laughner et al., 1998). However, little subsequent work has been performed on these proteins, though they were reported to have a role in controlling the levels of Dishevelled (Dvl) protein during Wnt–β-catenin signalling, involved in animal cell growth and development. Mis-regulated levels of Dvl can cause tumours, so tight regulation of this protein is necessary (Funato and Miki, 2007; Funato et al., 2010). NRX was shown to interact directly with Dvl, which was dependent on its TRX-like active site and this association was disrupted by treatment with H$_2$O$_2$. Through this interaction, NRX was demonstrated to inhibit Dvl and supress Wnt–β-catenin signalling (Funato et al., 2006). A later study from the same group discovered that whilst NRX directly inhibited Dvl by binding to it, it was also responsible for maintaining an inactive pool of Dvl. NRX achieved this by competing for Dvl interaction with Kelch-like 12 (KLHL12), a protein responsible for ubiquitinating Dvl and in doing so, targeting it for degradation. NRX-mediated disruption of the KLHL12-Dvl interaction therefore prevents degradation of Dvl by the proteasome, so that there are sufficient levels of this protein available to activate Wnt–β-catenin signalling following Wnt stimulation (Funato et al., 2010). NRX has also been shown to play a role in the planar cell polarity (PCP) branch of the Wnt signalling pathway in Xenopus laevis and the negative regulation of Toll-like receptor 4 signalling via interaction with Flightless-I (Fli-I) in mice (Funato et al., 2008; Hayashi et al., 2010). Whilst Fli-I and Dvl are both present in the nucleus, they have also been found to localise to the cytoplasm and in fact, this is the predominant localisation for Dvl (Funato et al., 2006; Hayashi et al., 2010). The interaction of these proteins with mammalian NRX, which has also been shown to have both nuclear and cytosolic localisation, suggests that despite the nomenclature of this enzyme suggesting otherwise, this enzyme can also have targets in, and therefore function in the cytoplasm. Indeed, phosphofructokinase I (PFK1), a cytosolic glycolytic enzyme was also identified as a target for NRX in mice, suggesting a role for NRX in glycolysis (Funato et al., 2013).
Two Arabidopsis NRX enzymes, NRX1 and NRX2, have recently been characterised. Although NRX2 was found to have only very low disulphide reductase activity in vitro, NRX1 was demonstrated to be a potent disulphide reductase. Genetic analysis of the progeny of selfed heterozygous nrx1 and nrx2 mutants indicated that whilst nrx2 progeny displayed a normal segregation ratio, those from the nrx1 mutant background showed higher ratios of WT plants. After further analysis it was determined that NRX1 plays a role in pollen fertility, although what this role entails is still unclear (Marchal et al., 2014). NRX1 has also previously been implicated in the role of pollen tube growth in the pistil (Qin et al., 2009). Beyond this, little is known about the function of NRX proteins in plants and how these enzymes might be involved in oxidative stress responses, or indeed, redox signalling events.

In this chapter, NRX1 is shown to play a role in SA-dependent plant immunity, with its redox activity and nuclear accumulation being induced by strains of the plant leaf pathogen, Pseudomonas syringae. Moreover, genetic and proteomic data show that it is involved in protection against oxidative stress, a process intimately associated with pathogen infection, and that this is probably mediated, at least in part, through its direct interaction with the ROS scavenger, Catalase 2 (CAT2). These data define a new mechanism by which NRX enzymes control signalling during plant immune responses and associated cellular oxidative stress.

5.2 Results

5.2.1 NRX proteins exhibit unique disulphide reductase activities

In contrast to typical TRX proteins, NRX enzymes possess multiple TRX or TRX-like domains (two or three), thereby drawing structural similarities to PDI, particularly in the case of animal NRX, which possesses a C-terminal domain similar to that also found in PDI. Plant NRX proteins lack this PDI-like C-terminal domain, instead possessing divergent C1 domains at the carboxyl terminus; cysteine rich regions that are involved in ligand recognition and binding (Colón-González and Kazanietz, 2006; Funato and Miki, 2007; Laughner et al., 1998). Whilst animal NRX proteins all appear
to have only one TRX-like active site with the typical WC(G/P)PC sequence (the N-terminal TRX domain is atypical with sequence CxxSAPC), plant NRX proteins can be classified into three different types; type I contain two classical TRX active site motifs and a third TRX-like domain that does not include a conventional redox active site, whereas types II and III each possess two atypical TRX-like active sites (Marchal et al., 2014). Arabidopsis NRX1 and NRX2 belong to type I and III subgroups, respectively (Figure 5.1).

When plant NRX proteins were first identified in maize, these enzymes were shown to have some disulphide reducing activity albeit much lower than a classical TRX from Spirulina (Laughner et al., 1998). More recently, this activity was also shown in vitro for the Arabidopsis NRX enzymes, and the TRX reductase, NTRA, was also shown to be a reductase for NRX1 (Marchal et al., 2014). Here we independently verified these results by performing insulin turbidity assays using recombinant His$_6$-NRX1 (Figure S2) and His$_6$-NRX2 proteins. As expected, in presence of the electron donor DTT, NRX1 was able to reduce insulin, while NRX2 performed poorly in comparison, displaying only very low levels of disulphide reduction activity (Figure 5.2A). We then assessed if this disulphide reduction activity of

Figure 5.1  Domain structures of Arabidopsis NRX proteins

C-terminal C1 domain and redox active TRX-like domain structures are shown for AtNRX1 and AtNRX2. Redox active TRX-like domains are represented as green boxes with the active site sequence amplified in yellow ovals. C-terminal C1-like domains are shown in red boxes. NRX1 also contains a third TRX-like domain that does not contain a conventional redox active site, which is not shown here.
NRX1 could be recycled by NTRA in presence of NAPDH reducing equivalents. As a positive control the prototypical TRX enzyme, TRXh5, was included and as expected showed strong disulphide reducing activity (Figure 5.2B). Interestingly, contrary to previous findings, recombinant NTRA in combination with NADPH and the cofactor FAD was found to be unable to stimulate NRX1 activity, resulting in failure to reduce insulin (Figure 5.2B).

![Graph A](image1)

**Figure 5.2** Recombinant NRX1 and NRX2 display disulphide reduction activity in vitro

(A) Oxidized insulin (130 µM) was incubated with 0.3 mM DTT either alone (control), or together with NRX1 (6 µM) or NRX2 (6 µM). Formation of reduced insulin was measured at 650 nm. Error bars indicate SD (n=3).

(B) Oxidized insulin (130 µM) was incubated with 1 mM NADPH alone (control) or together with NRX1 (6 µM), NTRA (0.5 µM) and FAD (10 µM). As a positive control, TRXh5 (2 µM) together with NTRA (0.2 µM) and 1 mM NADPH was included. Formation of reduced insulin was measured at 650 nm. Error bars indicate SD (n=3).

Although NRX proteins showed disulphide reduction activity in DTT-mediated insulin turbidity assays, the dynamics of NRX-mediated disulphide reduction still remain unclear. Because NRX enzymes contain two active sites, they are likely to perform disulphide reduction via a different mechanism than conventional TRX enzymes. It was therefore first tested how the concentration of NRX enzyme affected the rate of disulphide reductase activity (Figure 5.3). Although higher concentrations of NRX1 appeared to initiate activity slightly faster and at a greater rate than lower
concentrations, their disulphide reduction activity became saturated much sooner. This resulted in greater levels of activity by lower concentrations of NRX1 after longer periods of time. These data could possibly indicate that if highly concentrated, NRX1 proteins inhibit one another, causing a decrease in the availability of active sites for target reduction.

Figure 5.3 Protein concentration affects the dynamics of NRX-mediated disulphide reduction

Oxidized insulin (130 µM) was incubated with 0.3 mM DTT either alone (control), or together with TRXh5 (2 µM) or NRX1 at the indicated concentration. Formation of reduced insulin was measured at 650 nm. Error bars indicate SD (n=3).

It has previously been reported that removing the C-terminus of Arabidopsis NRX1 does not affect its ability to reduce disulphides in an insulin turbidity assay (Marchal et al., 2014). Moreover, truncation of maize NRX such that the first TRX-like active site was removed, did not abolish activity of the protein (Laughner et al., 1998). However, the exact contributions of each of the two active sites in NRX1 on the overall enzymatic activity of this protein are yet to be uncovered. It is possible that disulphide reduction is carried out by one active site alone, or by a coordinated effort from both sites. Moreover, should one active site inhibit the other in some way, its removal could trigger an increase in the proteins overall enzymatic activity. To test which of these scenarios is the case, active site
mutants of NRX1 were produced in which both Cys55 and Cys58 of the first active site, or both Cys375 and Cys378 of the second active site were mutated to serines. A mutant with all four cysteines replaced by serines was also produced (Figure S2). In comparison to wild-type NRX1, both NRX1(C55,58S) and NRX1(C375,378S) mutants displayed reduced disulphide reduction ability. However, NRX1(C375,378S) was much more efficient than NRX1(C55,58S), indicating that the majority of NRX1 disulphide reduction activity occurs at the first active site (Figure 5.4). Both active sites were necessary for maximal disulphide reduction activity, as removal of all four active site cysteines in the NRX1(C55,58,375,378S) mutant protein completely abolished its ability to reduce insulin (Figure 5.4).

To determine if the full enzymatic capacity of the native NRX1 protein could be reconstituted from two different mutant proteins, equal amounts of the two active site mutants were combined (Figure 5.4). Interestingly, this produced lower activity than the NRX1(C375,378S) mutant alone, suggesting there is either an inhibitory interaction between the two active sites, or that a collaboration between the active sites is required. In latter case, NRX1(C55,58S) could, for example, have a much higher affinity for substrate binding than NRX1(C375,378S), but a lower reducing potential, such that the second active site binds the substrate and then transfers it to the first site for further reduction.
Figure 5.4  Both active sites of NRX1 contribute towards its disulphide reductase activity
Oxidized insulin (130 μM) was incubated with 0.3 mM DTT either alone (control), or together with 6 μM NRX1 or indicated NRX1 active site mutants. Formation of reduced insulin was measured at 650 nm. Error bars indicate SD (n=3).

5.2.2 NRX1 localises to the cytoplasm and the nucleus

In animals, NRX proteins were so named as a result of being largely localised to the nucleus but have been shown in various studies to reside in the cytoplasm as well (Funato et al., 2006; Kurooka et al., 1997). In plants, nuclear localisation of NRX was reported in maize kernels (Laughner et al., 1998), and *Arabidopsis* NRX1 was found in both the nucleus and cytoplasm of flowers and root tips (Marchal et al., 2014). To determine cellular localisation of NRX1 in plant mesophyll cells, protoplasts were generated from wild-type Col-0 plants and GFP-tagged NRX1 was transiently expressed (Figure 5.5). Consistent with previous reports, NRX1-GFP signal was seen in both nuclear and cytoplasmic compartments.
5.2.3 NRX1 is redox active during plant immune responses

In plants, the rapid accumulation of ROS/RNS that are associated with pathogen attack require an upregulation of many different redoxins to prevent cellular damage and control redox signalling cascades. As is the case for TRXh3 and TRXh5, the expression of many of the genes encoding these reductase enzymes are therefore induced in response to pathogen recognition (Laloi et al., 2004). To determine if this is the case for NRX proteins, leaves of WT plants as well as knockout mutant nrx1 plants were inoculated with *P. syringae* pv. *maculicola* (Psm) ES4326 and the expression of *NRX1* examined by qPCR (Figure 5.6). Indeed, *NRX1* was induced by pathogen attack, suggesting that it may play a role during plant immune responses.
WT and *nrx1* plants were infected with *Psm* ES4326 (5 x 10^5 cells) for the indicated times. Expression of the *NRX1* gene was analysed using qPCR and normalized against constitutively expressed *UBQ5*. Error bars represent SD (n=3).

As *NRX1* is the more potent disulphide reductant of the *Arabidopsis* NRX enzymes (Figure 5.2A), an antibody against this enzyme was produced (Keyani and Spoel, unpublished results), and its specificity verified with *nrx1* knockout mutant plants (Figure S3). We subsequently used this antibody to assess if disulphide reduction by NRX enzymes is associated with activation of plant immune responses by investigating the formation of mixed disulphides between NRX1 and unknown substrates. Wild-type plants were either mock-treated, sprayed with the immune inducer SA or inoculated with *Psm* ES4326, and protein was extracted under denaturing conditions and then analysed on reducing (+ DTT) or non-reducing (- DTT) SDS-PAGE gels, the latter of which enabled the visualisation of mixed disulphides formed between NRX1 and its unknown substrates (Figure 5.7). There was a clear increase in the number of mixed disulphides formed by NRX1 after pathogen treatment, indicating that NRX1 activity is induced by pathogen attack. Interestingly, disulphide reductase activity did not increase upon treatment with SA, suggesting that signalling pathways upstream of NRX1 activity are SA-independent. Nonetheless, this data demonstrates that NRX1
enzyme activity is pathogen-inducible, and therefore strongly suggests a role for this protein in immunity.

![Figure 5.7](image)

**Figure 5.7** NRX1 disulphide-reduction activity increases after pathogen treatment

WT plants were treated with 0.5 mM SA, *Psm* ES4326 (5 x 10⁵ cells) or 10 mM MgSO₄ (control, lane 1). Total protein was extracted and separated by SDS-PAGE in the presence or absence of DTT. Analysis was by western blot against anti-NRX1. Indicated are free NRX1 monomer, mixed disulphide intermediates between NRX1 and substrates (NRX1-substrate) and total levels of NRX1.

The increase in redox activity of NRX1 after pathogen treatment coupled with its previously reported cytoplasmic and nuclear localisation could indicate that NRX1 is involved in immune-related redox signalling events in multiple cellular compartments. Therefore we determined if NRX1 localisation is affected by pathogen treatment by performing nuclear enrichment on WT plants treated with or without *Psm* ES4326 (5 x 10⁵ cells) (Figure 5.8). Using the NRX1 specific antibody, a clear increase was seen in the amount of nuclear NRX1 after pathogen treatment. There was no clear difference in cytoplasmic NRX1 or the total amount of this protein before or after infection. Taken
together with the pathogen-induced increase in NRX1 activity, these data suggest that nuclear disulphide reductase activity of NRX1 becomes more prominent following pathogen treatment. Thus, NRX1 likely plays an important role in nuclear redox signalling during plant immune responses, though its constitutive high abundance in the cytoplasm suggests it also functions outside the nuclear compartment.

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**Figure 5.8** NRX1 accumulates in the nucleus following pathogen treatment

WT plants were treated with *Psm* ES4326 (5 x 10^5 cells) or control-treated with 10 mM MgSO_4. Total protein was extracted and a nuclear enrichment performed. Equal amounts of nuclear fractions were loaded on gel. NRX1 protein was detected using an NRX1-specific antibody. Histone H3 antibody and HSP90 antibody were used to detect nuclear and cytosolic markers respectively.

**5.2.4 Mutant nrx1 plants display autoimmune responses**

Pathogen-induced changes in NRX1 activity and localisation suggest that this protein is important for plant immunity. It was therefore assessed if *nrx1* knockout plants displayed altered immune phenotypes. First, immune gene expression was studied by assessing the levels of SA-responsive *PR-1* and *PR-2* gene expression in uninduced *nrx1* plants. To ensure any changes seen could be attributed solely to the mutation of *nrx1*, two lines of *nrx1* were used (denoted *nrx1*-1 and *nrx1*-2), as well as a complementation line in which a 35S::Flag-NRX1 construct was overexpressed in the *nrx1*-1 mutant background (Figure S3). Interestingly, a higher basal expression level of both *PR-1* and *PR-2*
was observed in the *nrxi* mutant lines, which returned to wild-type levels when 35S:*Flag-NRX1* was expressed in *nrxi-1* (Figure 5.9).

![Figure 5.9](image)

**Figure 5.9** Mutant *nrxi* plants show constitutive defence gene expression

Expression of SA-dependent *PR-1* (A) and *PR-2* (B) genes was analysed in WT, *nrxi-1*, *nrxi-2* and 35S:*Flag-NRX1* (*nrxi-1*) plants using qPCR and normalized against constitutively expressed *UBQ5*. Error bars represent SD (n = 3).

The elevated levels of basal *PR* genes in *nrxi* mutants is reminiscent of the *cpr1* (constitutive expresser of PR genes) mutant, which was identified in a screen for mutants with dysregulated *PR* gene expression (Bowling et al., 1994). In *cpr1* plants, and indeed other identified *cpr* mutants such as *cpr5* and *cpr6*, constitutive SA-responsive *PR* gene expression was found to be associated with enhanced disease resistance to *P. syringae* (Bowling et al., 1994; Bowling et al., 1997; Clarke et al., 1998). To examine if this is also true for *nrxi* plants, a disease resistance test was performed; plants were infected with *Psm* ES4326 and bacterial growth assessed after 3 days. Compared to the wild type, mutant *nrxi* plants displayed increased resistance to this pathogen with reduced bacterial growth levels similar to wild-type plants after SA treatment (Figure 5.10).
Figure 5.10 Mutant *nrx1* plants exhibit enhanced disease resistance

WT, *nrx1-1*, and *nrx1-2* plants were mock-treated or sprayed with 0.5 mM SA. Plants were then infected with *Psm ES4326* (5 x 10⁶ cells) and growth of *Psm ES4326* was assessed after 3 days. Cfu, colony forming units. Error bars represent 95% confidence limits (n = 8). Asterisks indicate statistically significant differences compared to the WT (Tukey-Kramer ANOVA test; α = 0.05, n = 8).

SA-dependent SAR develops upon localized infection by a virulent or avirulent pathogen and provides long-lasting protection against future pathogen challenge in uninfected tissues (Fu and Dong, 2013). Because mutant *nrx1* plants exhibited enhanced *PR*-gene expression and disease resistance, we assessed if further resistance could be induced by establishment of SAR. To test this, the lower leaves of wild-type and *nrx1-1* plants were first either infected with avirulent *Pst DC3000* carrying the *avrRpt2* effector or mock infiltrated. After 2 days upper leaves were challenge infiltrated with virulent *Psm ES4326* and pathogen growth assessed after 3 days (Figure 5.11). Consistent with previous findings, bacterial growth in wild-type plants that were pre-treated with avirulent pathogen was reduced from those that had been mock-treated, demonstrating an effective SAR response. As seen in previous experiments, *nrx1* plants displayed a decreased level of bacterial growth to WT plants when infiltrated with virulent pathogen following mock treatment, indicating increased resistance.
However, when inoculated with avirulent *Pst* DC3000 first to induce SAR, no further increase in resistance against virulent *Psm* ES4326 was observed in the *nrx1* mutant, suggesting that the constitutive expression of *PR* genes in this mutant results in a SAR “primed” state (Figure 5.11).

![Graph showing CFU per leaf disc](image)

**Figure 5.11** Mutant *nrx1* plants show constitutive SAR
Lower leaves of wild-type (WT) and mutant *nrx1-1* plants were infiltrated with *Pst* DC3000/*avrRst2* (5 x 10^7) or 10mM MgSO_4_. After 2 days upper leaves were infected with *Psm* ES4326 (5 x 10^6 cells) and pathogen growth assessed after 3 days. CFU, colony forming units. Error bars represent 95% confidence limits (n = 8). Asterisks indicate statistically significant differences compared to the WT (Tukey-Kramer ANOVA test; \( \alpha = 0.05, n = 8 \)).

SAR has been shown to be dependent on accumulation of SA in systemic tissues and indeed can be mimicked by spraying SA prior to pathogen treatment (Fu and Dong, 2013; White, 1979). Therefore we tested if the disease resistance of *nrx1* mutant plants was dependent on SA. *ICS1* is one of two genes encoding isochorismate synthase (ICS), which has been shown to be essential in the synthesis of SA. Mutant *ics1* plants have normal basal levels of SA but are unable to accumulate SA in response to pathogen attack, resulting in failure to upregulate SA-responsive genes and launch an effective SAR response (Wildermuth et al., 2001). To determine the effects of the *nrx1* mutation in the absence of pathogen-induced SA accumulation, *ics1 nrx1* double mutant plants that had previously...
been generated (Keyani and Spoel, unpublished results) were used. Wild-type, single and double mutant plants were subjected to infection with *Psm* ES4326 as described above. As expected, the *ics1* mutant displayed a much higher level of bacterial growth than wild type, demonstrating the susceptibility of this mutant, while *nrx1* mutants were more resistant (Figure 5.12). Interestingly, the *ics1 nrx1* double mutant was also highly disease susceptible, indicating that the resistance observed in *nrx1* single mutants is indeed dependent on SA.

![Figure 5.12](image)

**Figure 5.12** Enhanced disease resistance of *nrx1* plants is SA-dependent

Wild-type (WT), *nrx1*-*1*, *ics1* and *nrx1*-*ics1* plants were infected with *Psm* ES4326 (5 x 10⁶ cells) and pathogen growth assessed after 5 days. Cfu, colony forming units. Error bars represent 95% confidence limits (n = 8). Asterisks indicate statistically significant differences compared to the WT (Tukey-Kramer ANOVA test; α = 0.05, n = 8).

5.2.5 Identification of target proteins of NRX1 oxidoreductase activity

Whilst we showed that the localisation and activity of NRX1 are pathogen inducible, and that genetic data confirmed a role in plant immune signalling for this protein, it is still unclear what the substrate targets of this protein are. A strategy for the capture and identification of targets that exploits the mechanism by which disulphides are reduced by TRX family members has previously been
designed (Motohashi et al., 2001). Mutation of the second active site cysteine to serine in column bound mutant TRX proteins was used to prevent resolution of the substrate reduction reaction, resulting in trapping of targets via a mixed disulphide bond. These targets were then eluted from the column and analysed by mass spectrometry (Motohashi et al., 2001). Performing this type of experiment with NRX1 is complicated by the presence of two active sites, so we decided to mutate the second cysteine to serine in both TRX-like active sites (C58 & C378). The resulting NRX1(C58,378S) mutant protein was purified (Figure S2) and tested in an insulin turbidity assay. As expected, the active site mutant could not complete disulphide reduction reactions and consequently was unable to reduce insulin (Figure 5.13A). Therefore a target capture experiment was designed based on a protocol previously described for chloroplastic TRX enzymes (Balmer et al., 2003). NRX1(C58,378S) protein was immobilised on NHS-activated resin and pre-reduced with DTT to ensure all available active site cysteines were in the reduced state. Based on the fact NRX1 redox activity is pathogen induced (Figure 5.7), it is likely that some targets may only present themselves after infection and so plants were first infiltrated with Psm ES4326. Subsequently, protein was extracted and added to the immobilised NRX1(C58,378S) column where targets were trapped via the formation of mixed disulphide bonds. Targets were then eluted from the column using DTT and analysed by mass spectrometry in order to identify them (Figure 5.13B). An immobilized wild-type NRX1 protein column was included so that distinctions could be drawn between targets that interact at the active site as substrate or as non-substrate. Background binding was monitored by the inclusion of a control column containing only quenched resin without NRX1 protein. A comprehensive list of targets identified in this screen that were enriched in either the wild-type or active site mutant NRX columns compared to the control are listed in Tables S1 and S2. All targets with a p-value of 0.10 or less are shown, with those statistically significantly different from the control by a p-value of 0.05 or less highlighted in red. Of these significant targets, 24 were found to be enriched by the NRX1(C58,378S) column and 17 by the wild-type NRX1 column, with only 6 overlapping (Figure 5.13C, Tables S1 & S2). This data suggests that the 18 targets that are found to exclusively bind to NRX1(C58,378S) interact at its active site and
are subject to its reductase activity. In contrast, those proteins found in the wild-type NRX1 column are possibly binding at other cysteine residues and/or may interact with NRX1 as non-substrates.

**Figure 5.13** Active site cysteine mutant of NRX1 is used to capture targets

(A) Oxidized insulin (130 µM) was incubated with 0.3 mM DTT either alone (control), or together with NRX1 (6 µM) or NRX1(C58,378S) (6 µM). Formation of reduced insulin was measured at 650 nm. Error bars indicate SD (n=3).

(B) Schematic of NRX1 target capture experiment, utilizing immobilized mutant NRX1(C58,378S). See text for details.

(C) Venn diagram of targets found to be enriched by immobilised NRX1(C58,378S) (blue) and NRX1 (red) containing columns from mass spectrometry analysis. Overlapping proteins are shown in the middle.
Next, gene ontology (GO) analysis was performed for the 24 proteins identified as active site interactors to determine enriched GO biological processes and molecular functions (Figure 5.14). The most prominent biological process identified from this list was “electron transport or energy pathways” (Figure 5.14A), which may be explained by the fact that the components of these processes likely undergo significant levels of oxidation and reduction, the latter of which may be partially controlled by NRX1. In addition, both “response to stress” and “response to abiotic or biotic stimulus” were found to be significant GO term processes for the targets of NRX1 activity. This is consistent with the previous findings of this chapter and could provide an explanation for why the nrx1 knockout mutants have an altered immune phenotype.

The largest associated molecular function of targets was “other enzyme activity”, which indicates NRX1 could be involved in determining the activity of a variety of cellular enzymes, many of which often contain active site cysteine residues (Figure 5.14B). Interestingly, enzymatic function can also be associated with some of the targets identified from the wild-type NRX1 column (Table S2), suggesting that some enzymes are not substrates of the NRX1 active site, but rather interact as non-substrates and/or treat cysteines in NRX1 itself as a target of their own activity. Collectively, these data indicate that interaction with enzymes is potentially of high importance to the functional role of NRX1.
Figure 5.14 GO term analysis of NRX1(C58,378S) targets

GO term analysis for biological processes (A) and molecular function (B) were performed using Classification Superviewer on [http://bar.utoronto.ca/](http://bar.utoronto.ca/). Normalised frequencies, standard deviation and p-values were determined from absolute values as described in (Provart and Zhu, 2003). Error bars represent SD. P-values ≤ 0.5 are printed bold.
5.2.6 *In vivo* validation of NRX1 interactors and substrates

As previously discussed, the method by which NRX1 activity is recycled remains elusive. Despite previous reports that NTRA, together with FAD and NADPH, is able to reduce the oxidised active site of NRX1 (Marchal et al., 2014), insulin assays performed during this study suggested that the turnover of NRX1 activity may not be this simple (Figure 5.2B). As is the case for other oxidoreductase enzymes, such as PRXs which are reduced by the TRX/NTR system, it is possible that the reductase for NRX1 is itself another redoxin. The list of proteins identified to interact with NRX1 at its active site (Table S1) contains two members of the TRX superfamily; one uncharacterised and PRX Q. Thus it is possible that members of the TRX family could be capable of interacting with the NRX1 active site. However, this is very difficult to capture with our immobilized NRX1(C58,378S) column because absence of the second active site cysteines prevents internal disulphide bond formation, thereby limiting the chances of capturing a redoxin that targets an oxidised active site. In addition, the reactive nature of redoxin active sites mean that interaction with its target it likely transient and therefore difficult to capture. In order to determine whether other TRX family members that have not been identified in the mass spectrometry screen do indeed interact with NRX1, a denaturing co-immunoprecipitation was performed between TRXh5 and NRX1. TRXh5 is an immune-induced TRX and its upregulation therefore coincides with the pathogen-induced increase in NRX1 activity (Chapter 3, Laloï et al., 2004; Tada et al., 2008). A FLAG pulldown was performed on pathogen-treated Col-0 plants transformed with or without 35S::Flag-TRXh5 followed by a western blot against NRX1. Remarkably, it was seen that TRXh5 was able to pulldown NRX1 (Figure 5.15), confirming the interaction of NRX1 and thioredoxin family members, and demonstrating that there may be some redox enzymes that we were not able to identify in the *in vitro* target capture experiment.
Figure 5.15 NRX1 interacts with TRXh5 in vivo

Wild-type Col-0 plants transformed with or without 35S::Flag-TRXh5 were infected with Psm ES4326 (5 x 10^5 cells) for 24 hours. Total protein was extracted under denaturing conditions and incubated with the alkylating agent NEM, which prevents nonspecific disulphide formation. Extracts were then diluted and incubated with anti-FLAG M2 affinity resin (Sigma). Immunoprecipitated proteins and input samples were separated by SDS-PAGE and analysed by western blotting using an anti-NRX1 antibody.

As potential NRX1 substrates identified by mass spectrometry are involved in stress responses, this prompted us to verify some of these interactions in vivo. Notably, Catalase 2 (CAT2) was identified to interact with NRX1(C58,378) but not wild-type NRX1 (Table S1 & S2, Figure 5.16A), and is an enzyme important in the control of oxidative stress via its H_2O_2 scavenging activity. H_2O_2 is one of the key ROS that accumulate in the oxidative burst during pathogen recognition and catalase genes have been shown to be upregulated in response to H_2O_2 (Du et al., 2008; Xing et al., 2007). There are three Arabidopsis catalases, CAT1, CAT2 and CAT3, which perform the same function but vary in the tissues in which they are expressed. CAT2 represents the major isoform in leaves, accounting for approximately 90% of leaf catalase activity (Mhamdi et al., 2010; Queval et al., 2007). CAT3 also contributes to overall catalase activity in leaves, whilst CAT1 was shown to have limited activity here. Interestingly, CAT3 was also identified as a potential target of NRX1(C58,378) but was less significant than CAT2 with a p-value of 0.071 (Table S1). To determine if NRX1 and CAT2 interact in vivo, co-immunoprecipitation with anti-Flag and anti-CAT antibodies was performed on pathogen-treated
wild-type plants transformed with or without 35S::Flag-NRX1. As the anti-CAT antibody recognizes all three isoforms of catalase, we also included cat2 cat3 double mutant plants expressing 35S::Flag-NRX1 as a control. Immunoprecipitation with an anti-Flag antibody pulled down CAT protein specifically in 35S::Flag-NRX1 (WT) plants but not in the cat2 cat3 mutant background or untransformed wild-type (Figure 5.16B). Taken together with the main functions and processes associated with other targets identified by mass spectrometry, this data suggests that NRX1 is involved in the control of oxidative stress, and that this is accomplished at least in part through the regulation of redox-based modification of other oxidative stress enzymes, such as CAT2.

![Image](image.png)

**Figure 5.16** NRX1 interacts with CAT2 *in vitro* and *in vivo*

(A) Peptide abundance from mass spectrometry analysis of CAT2 interaction with NRX1, NRX1(C58,378S) and control columns. Error bars represent standard error of the mean (n = 3). Asterisks indicate statistically significant differences compared to the control (p-value ≤0.05).

(B) WT, 35S::Flag-NRX1 (Col-0) and 35S::Flag-NRX1 (cat2/cat3) plants were infected with *Psm* ES4326 (5 x 10^5 cells). Total protein was extracted under denaturing conditions, incubated with the alkylation agent NEM, which prevents nonspecific disulphide formation. Extracts were then diluted and incubated with anti-FLAG M2 affinity resin (Sigma). Immunoprecipitated proteins and input samples were separated by SDS-PAGE and analysed by western blotting using an anti-CAT antibody. Anti-FLAG and anti-HSP90 were used as input controls.
5.2.7 NRX1 protects plant cells from oxidative stress

The identification of CAT2 as a target of NRX1 prompted an investigation into the role of NRX1 in oxidative stress. Strikingly, the previously observed immune phenotype of nrx1 mutant plants (see Section 5.2.4) holds many similarities to that of cat2 mutant plants. Indeed, cat2 mutants have also been reported to exhibit enhanced basal PR-1 gene expression and enhanced disease resistance, which was shown to be SA-dependent (Chaouch et al., 2010). However, like lsd and cpr mutants, cat2 mutants also form spontaneous lesions, which are not observed macroscopically on nrx1 plants (data not shown). Due to the role of catalases in H$_2$O$_2$ scavenging, it is unsurprising that another distinctive feature of cat2 plants is their increased sensitivity to oxidative stress. To investigate the role of NRX1 in oxidative stress, WT and nrx1 mutant plants were treated with methyl viologen and then monitored for ion leakage, a well-established marker of cell death. Methyl viologen is an acceptor of electrons which are then transferred to molecular oxygen to produce ROS, hence causing oxidative stress in the cell. Compared to wild type, mutant nrx1 plants displayed a clear phenotypic difference when treated with methyl viologen, exhibiting cell death that spreads across the entire leaf as opposed to being contained in lesions (Figure 5.17A). When cell death was measured by ion leakage over a time-course of 36 hours, methyl viologen-treated nrx1 mutants were found to have higher electrolyte leakage than WT plants, indicating an increased amount of cell death and therefore a decrease in tolerance to oxidative stress (Figure 5.17B).
Figure 5.17  Mutant *nrxi* plants have decreased tolerance for oxidative stress

(A) Phenotypes of 4-week-old WT and *nrxi*-1 leaves following infiltration with 5 µM methyl viologen. Photographs taken 6 hours after infiltration.

(B) Leaves of WT and *nrxi*-1 mutant plants were infiltrated with 5 µM methyl viologen (MV) or ddH₂O (control). Electrolyte leakage measurements taken at indicated time-points with values presented as a percentage of total electrolyte leakage measured after boiling of the leaves. Error bars represent standard deviation of the mean (n = 3).
Because these data indicate that NRX1 plays a key role in oxidative stress responses, we assessed if its overexpression had an effect on the enhanced cell death phenotype previously observed in cat2 mutants. Electrolyte leakage analysis was performed on wild-type, cat2 and cat2 plants expressing 35S::Flag-NRX1. As expected, methyl viologen-treated cat2 plants displayed an increased rate of electrolyte leakage compared to WT plants. Strikingly, when NRX1 was overexpressed in cat2 mutants, levels of ion leakage were restored back to wild-type (Figure 5.18). In summary, these data demonstrate that NRX1 is required for protection of the plant cell against oxidative stress.

![Graph showing electrolyte leakage over time](image)

**Figure 5.18**  NRX1 restores oxidative stress responses in cat2 mutants
Leaves of wild-type, cat2 and 35S::Flag-NRX1 (cat2) plants were infiltrated with 5 µM methyl viologen (MV) or ddH2O (control). Electrolyte leakage measurements taken at indicated time-points with each value presented as a percentage of total electrolyte leakage measured after boiling of the leaves. Error bars represent standard deviation of the mean (n = 3).
5.3 Discussion

Redox signalling is key to many cellular processes and is controlled by a plethora of oxidoreductase enzymes; each specific to different stimuli, subcellular compartments and targets. Although TRXs are generally a very well-studied group of reductase enzymes, with their actions having been linked to several redox-based modifications in various different cellular locations, the functions and roles of some members of the wider TRX family remain elusive. Moreover, despite many reports of nuclear proteins such as transcription factors being post-translationally redox modified, little is known about the nuclear localized redoxin enzymes potentially responsible for controlling these modifications. In this chapter, partly nuclear localized NRX enzymes of the TRX superfamily are shown to have a key role in plant immune signalling through the protection of the cell against oxidative stress.

Unlike mammalian NRX, plant NRX proteins have been shown to possess two TRX-like active sites (Figure 5.1). Despite the structural differences to classical TRX proteins, *Arabidopsis* NRX1 has previously been reported, and was found here, to be a potent disulphide reductase *in vitro* (Figure 5.2A) (Marchal et al., 2014). NRX2 displayed low levels of disulphide reduction which may be attributed to the fact that it is a type III NRX, possessing only one atypical redox active site with two cysteine residues (Figure 5.2A). In contrast to previous reports, NTRA was not found to be able to recycle NRX1 activity (Figure 5.2B) (Marchal et al., 2014). One potential reason for these contrasting results is that whilst we measured insulin precipitation, previous data was obtained by measuring NADPH reduction, which usually indicates its consumption by NTRA. However, these different data sets suggest that NADPH may have been consumed for other purposes in this previous assay (Marchal et al., 2014). Nonetheless, our data suggest that the reductase for this enzyme may still be unidentified (Figure 5.2B). Indeed, there are no other reports of an identified reductase for NRX in other organisms, despite a recent screen for *in vivo* targets of mammalian NRX1 (Nakao et al., 2015). NRX1 was seen to be less potent in disulphide reduction than TRXh5, which, as a larger protein of ~65kDa, could be due to its decreased ability to access disulphide bonds in insulin compared to the far less bulky ~15kDa TRXh5. Increasing the concentration of NRX1 in an insulin assay resulted in a faster rate of reduction, but
saturation was reached at an earlier point (Figure 5.3). Therefore, it is possibility that high concentration of NRX protein may result in self-inhibition, either through direct interaction between two active sites of different molecules, blocking interaction with the substrate insulin, or through the redox-independent dimerization of NRX1 (Marchal et al., 2014). Indeed, the dimerization of TRX superfamily member PDI at high concentrations has been shown to decrease its redox activity by blocking substrate binding (Bastos-Aristizabal et al., 2014; Gruber et al., 2009). The contributions of each respective active site in NRX1 have not previously been reported, but here we showed that both sites possess redox activity, albeit not in equal measure (Figure 5.4). Combining the two single active site knockouts did not reconstitute wild-type levels of disulphide reduction, suggesting that a coordinated effort of both active sites may be required for full redox activity of this enzyme. Future analysis of the NRX1 structure could provide further information on the dynamics of the two active sites.

Consistent with previous findings, NRX1 was demonstrated to localise to both the nucleus and cytoplasm in protoplasts (Figure 5.5). However, the induction of NRX genes following treatment with pathogenic \textit{Psm} ES4326 (Figure 5.6) prompted us to investigate the influence of this stimulus on the localisation and activity of NRX1 protein. The data presented here show that the redox activity of NRX1 is pathogen-inducible but that this process is SA independent (Figure 5.7). It is possible that either NRX1 is subject to a PTM which increases its affinity for disulphide reduction, or that the majority of NRX1 targets only present themselves after exposure to this stress, or indeed both of these scenarios could be true. The nuclear localisation of NRX1 was also found to be pathogen-inducible (Figure 5.8), which favours the argument that NRX1 may be subject to PTM. Whilst it is tempting to postulate that NRX1 dimerization in the cytoplasm could prevent its nuclear translocation in the same way as the oligomerisation of the immune coactivator NPR1, dimerization of NRX1 has been shown to be redox-independent, so a different mechanism for this would have to be in place (Marchal et al., 2014).

Mutant \textit{nrx1} plants were shown to constitutively express immune genes, which expectedly provided them with enhanced disease resistance to \textit{Psm} ES4326 and a constitutive SAR phenotype.
Several gain-of-function mutants with enhanced disease resistance have been previously reported in *Arabidopsis* and tend to fall into two categories; those which exhibit spontaneous HR-like cell death phenotypes, such as *cpr5* or *lesion-stimulating disease 1* (*lsd1*) mutants, and those which do not, such as *cim, cpr6* or the more recently identified *constitutive induced resistance 1* (*cir1*) mutants (Carstens et al., 2014). *nrx1* mutants appear to belong to the latter category of mutants, although it is possible that potential cell death lesions in these plants are only visible microscopically. Disease resistance of *nrx1* mutants was abolished in the *ics1 nrx1* double mutant indicating that resistance is SA dependent (Figure 5.12). Constitutive SAR and PR gene expression in *cpr6* were shown to require SA but not NPR1 (Clarke et al., 1998), so it would be interesting in the future to see whether these phenotypes of *nrx1* are dependent on NPR1. Unfortunately the NRX1 and NPR1 loci appear to be genetically linked, so it has not been possible to create a double mutant (Keyani and Spoel, unpublished results).

Several targets of mammalian NRX have been identified *in vivo*, including the Wnt–β-catenin signalling pathway component, Dvl, and glycolysis component, PFK1 (Funato et al., 2013; Funato et al., 2006), although these targets were unable to be confirmed in a more recent *in vivo* target screen (Nakao et al., 2015). However, targets of plant NRX1 have not yet been studied. In using an active site cysteine mutant of NRX1 to trap interacting proteins, this study identified the first potential targets of *Arabidopsis* NRX1. The use of both wild-type NRX1 and a NRX1(C58,378) mutant, which cannot complete disulphide reduction reactions, enabled identification of proteins that interact with the active site as substrates or non-substrates (Figure 5.13). We found that of those proteins interacting at the active site of NRX1, a large proportion were involved in electron transport and stress responses; both of these processes are associated with high levels of oxidative stress and it is therefore likely that modification by reductases such as NRX1 is essential to the redox activity of these enzymes. Interestingly, many of the identified targets were also classified as enzymes (Figure 5.14). A previous report on intrinsic cysteine reactivity showed that functional cysteines are actually a rare feature and in addition to sites of oxidative modification, hyper-reactive cysteines were predicted to be localised
to enzymatic active sites (Weerapana et al., 2010). It is therefore highly likely that the active sites of some of these identified enzymes are redox modified and require reduction by NRX1 to attain functionality. Also, notable interactors with NRX1(C58,378) were both members of the TRX superfamily and NADPH-dependent Thioredoxin Reductase C (NTRC). As previously mentioned, a reductase for NRX1 has remained largely elusive, with reports on the ability of NTRA to recycle NRX1 activity being inconsistent (Figure 5.1B) (Marchal et al., 2014). However, NTRC was found here to interact at the NRX1 active site, although the p-value did not quite fall within the 0 - 0.5 range. NTRC is a chloroplastic NTR protein homologous to NTRA and NTRB, localised to the cytosol/nucleus and mitochondria, respectively (Reichheld et al., 2005; Serrato et al., 2004). In combination with other targets identified, such as high cyclic electron flow 1, and the association of target functions with electron transport and energy pathways, it also suggests that NRX1 may be associated with processes in the chloroplasts, although these potential targets should be interpreted with caution as NRX1 was not found to localize to the chloroplasts. Another set of candidates for the NRX1 reductase are other redoxin enzymes. Members of the TRX superfamily were identified in this screen, including a PRX and an uncharacterized TRX superfamily protein (At3g11630). Indeed, PRXs have been shown to be reduced by TRX (Verdoucq et al., 1999), demonstrating that NRX redox activity could also be dependent on TRX family members. Whilst the in vitro method of target trapping used here is able to effectively identify substrates of NRX1 redox activity, it may be inefficient at capturing proteins for which NRX1 itself is a target. Indeed, through a denaturing co-immunoprecipitation procedure, designed to catch mixed disulphide targets of TRX molecules, TRXh5 was shown to directly interact with NRX1 (Figure 5.15), suggesting that other members of the TRX family could do the same, particularly within the TRX-h subset in which members have closely related structures.

Of proteins identified in the screen for NRX1 targets, several enzymes that metabolise H$_2$O$_2$ were identified, including two catalases (Table S1 & Figure 5.16A). Confirmation of catalase interaction was provided by a denaturing co-immunoprecipitation between FLAG-tagged NRX1 and catalase in vivo (Figure 5.16B). The role of catalases in oxidative stress protection make it quite likely that whilst
functioning as detoxifying enzymes, they may also receive redox PTMs. As CAT2 was identified as a target that interacts with the NRX1 active site, it is plausible that NRX1 is responsible for controlling CAT2 activity through the regulation of a redox modification on this enzyme. It was previously shown that treatment with NO donors can inhibit the activity of catalase in tobacco (Clark et al., 2000), although denitrosylation activity of NRX1 is yet to be studied. Experiments performed previously in the green alga *Chlamydomonas reinhardtii* have also indicated that catalase activity may be inhibited as a result of PTM. The data in this study demonstrated that the reduction of a single cysteine in catalase was responsible for the inhibition of its activity. The conserved residue, Cys230, found in the single *C. reinhardtii* catalase and all three *Arabidopsis* isoforms, was suggested as a candidate for this modification (Michelet et al., 2013). An earlier study in *C. reinhardtii* that identified catalase as a target of TRX indicated that catalase is activated by redox modification and that TRX- or DTT-mediated removal of this modification could abolish CAT activity. However, this study was performed in uninduced protein extracts and was proposed to be specific to *C. reinhardtii* CAT as *Arabidopsis* catalases did not behave in the same way (Lemaire et al., 2004). Collectively and together with this work, these studies demonstrate the possibility of a redox-based modification on catalase proteins that could be controlled by NRX1. Interestingly, Ascorbate Peroxidase 1 (APX1) was also identified in the screen for NRX1 active site interactors. Like catalase, APX1 is a $\text{H}_2\text{O}_2$ scavenging enzyme, but whereas CAT enzymes are primarily localised to the peroxisomes, APX1 is cytosolic. Removal of APX1 has been associated with high levels of protein oxidation as a result of uncontrolled ROS accumulation (Davletova et al., 2005). Like CAT, APX activity in tobacco was inhibited following the addition of NO donors (Clark et al., 2000). In addition, APX has been shown to be regulated by various other redox modifications and was shown to be a target of the TRX/NTR system (Correa-Aragunde et al., 2015a; Marchand et al., 2004; Yamazaki et al., 2004).

According to its observed interaction with the oxidative stress response enzymes CAT2 and APX1, *nrx1* mutant plants displayed an increased level of cell death in response to ROS-generating methyl viologen (Figure 5.17). Should redox modifications of CAT2 or APX inhibit the $\text{H}_2\text{O}_2$
metabolising activity of either of these enzymes, then NRX1 may be responsible for the reversal of that modification, providing a clear explanation for the observed intolerance to oxidative stress in \textit{nrx1} mutants. Another explanation for increased sensitivity to oxidative stress in the \textit{nrx1} mutant is that based on its constitutive \textit{PR} gene expression and SAR phenotype, it is reasonable to assume that \textit{nrx1} mutants may have elevated levels of SA. SA has been shown to potentiate ROS production, in part by binding to and inhibiting the activities of H$_2$O$_2$ scavengers CAT and APX (Chen et al., 1993; Vlot et al., 2009). In the case of elevated SA levels in an \textit{nrx1} mutant, it is therefore possible that H$_2$O$_2$ levels are unable to be controlled and lead to increased levels of cell death. This is however a less likely explanation, because constitutive elevated levels of SA are usually associated with penalties in growth and development, a phenotype that is not observed in \textit{nrx1} mutants.

Remarkably, over-expression of NRX1 in the \textit{cat2} background was able to rescue the cell death in this mutant in response to methyl viologen (Figure 5.18). There is a certain amount of redundancy is ROS-scavenging enzymes so in absence of CAT2, it is likely that other peroxidases try to prevent over-accumulation of H$_2$O$_2$ (Mhamdi et al., 2010). As NRX1 was also found to interact with APX1, PRX Q, glutathione S-transferase (GST), and CAT3 (Table S1), we postulate that its overexpression could serve to facilitate the activity of these enzymes, allowing them to compensate for the absence of CAT2.

In conclusion, the data presented in this chapter demonstrate a novel role for \textit{Arabidopsis} NRX1 in plant immunity through the protection against oxidative stress. Future studies into what modifications occur to catalase enzymes and how they are controlled by NRX1 are essential to understanding how this protein functions in oxidative stress pathways. Furthermore, elucidation of the NRX1 structure would facilitate advances in knowledge about the redox properties of this enzymes and the mechanism by which it reduces its targets.
Chapter 6

General discussion
Plants are continuously under stress from a range of invading pathogens and have developed a complex, layered immune system by which to defend against them. Redox-based protein modifications have emerged as a crucial signalling mechanism for controlling protein behaviour during the plant immune response. Antioxidant enzymes of the TRX family have been demonstrated to be invaluable in the reversal of these redox PTMs, enabling a protein to switch between oxidative and reductive states, which often corresponds to switching between active and inactive states, or between two different functions, conformations or localisations. In order to fully understand redox signalling during the plant immune response, it is therefore essential to uncover the activities of TRX family members and their regulation of specific cysteine modifications.

This study focused on two main areas: (i) understanding the role of protein-SNO formation and its reversal by TRX enzymes in immune gene expression, and (ii) revealing the function of NRX enzymes that are novel nucleocytoplasmic TRX superfamily members, in the control of oxidative stress during immunity.

6.1 TRXh5 as a selective protein denitrosylase

There is relatively little information on the exact mechanisms behind denitrosylation of SNO groups, but studies have shown this redox modification to be a stable way of storing or utilizing bioactive NO (Benhar et al., 2009). A proteomics study identified that whilst some S-nitrosothiols are reversed readily by small reducing compounds such as glutathione, others are protected by the local conformational protein landscape, such that enzymes may be required to reverse them (Paige et al., 2008). Indeed, human TRX1 was initially found to denitrosylate Caspase 3 in lymphocytes in order to keep S-nitrosylation of this protein at a low, steady state level (Benhar et al., 2008; Mitchell and Marletta, 2005). Subsequently, TRX1 was shown to have many potential protein-SNO targets in human cells (Benhar et al., 2010).
In Chapter 3, TRXh5 was identified as the first potent denitrosylase in plants and trans-denitrosylation was put forward as the predominant mechanism utilised by this enzyme. A previously unrecognised feature of TRX-mediated denitrosylation is the ability to discriminate between SNO targets based on the NO donor that was responsible for their formation. Here, plant TRXh5 is demonstrated to act as a selective denitrosylase providing novel insight into the specificity of redox modifications. It is possible that cysteines buried within the structure of a protein may not be accessible to certain types of NO donor, such as GNSO, but are to free NO. A study using protein microarrays identified that whilst certain protein structures or motifs can generate a particular chemical micro-environment for cysteines that seemingly makes them more likely to be S-nitrosylated, knowledge of protein structure alone is not sufficient to predict sites of S-nitrosylation. Instead, the stereochemistry of the NO donor and how it interacts with a particular target was proposed to be an important determinant in SNO generation and reactivity (Foster et al., 2009). Other studies have suggested a specific “GSNO binding motif”, in which GSNO docks onto a site such that the Cys residue and the NO are ideally positioned to react (Hess et al., 2005). Based on these collective findings, it is quite plausible that GSNO and NO target different, albeit partly overlapping sets of reactive cysteines. A previous study identified that reversal of “stable” SNO groups required enzymatic reduction due to their inaccessible nature, which could explain why TRX mediates denitrosylation at sites only accessible by free NO (Paige et al., 2008). The newly identified selectivity described in this thesis is therefore an important advancement in the field of (S)NO signalling and further studies into whether this is also a feature of other eukaryotic TRX that mediate denitrosylation, could be vital to biomedical research.

Using two genetic mutants that over accumulate protein-SNO, nox1 and gnsor1, it was shown that denitrosylation activity of TRXh5 on protein-SNO groups derived from free NO plays a key role in plant immune signalling. Whilst the TRXh5-mediated rescue of immunity in the nox1 background but not the gnsor1 background was not caused by selective denitrosylation of NPR1, NPR1 did appear to be a protein-SNO target of TRXh5. In addition to both disulphide linkages and S-nitrosylation, NPR1
has been shown to be the target of other non-redox PTMs which are essential to controlling its activity and turnover (Saleh et al., 2015; Spoel et al., 2009), reinforcing the idea that the multiple functions of this protein require many levels of regulation. Indeed, redox regulation surrounding this protein alone has already been implicated in the control of its localization, modification of DNA-binding activity of its interacting partner proteins, the TGA transcription factors, and more recently, its role in mediating the interplay between redox rhythms and the circadian clock (Lindermayr et al., 2010; Mou et al., 2003; Zhou et al., 2015). With such a wide variety of roles for this protein, it is possible that NO-mediated and GSNO-mediated S-nitrosylation of NPR1 have some differential effects on this protein, which may explain why it is an overlapping protein-SNO target of TRXh5 and GNSOR1. Plant TRX denitrosylation activity is not expected to be exclusive to only TRXh5 or to immunity and indeed, a recent study has already identified a role for the TRX-NTR system as a denitrosylase in auxin-mediated root development (Correa-Aragunde et al., 2015b).

It was also shown in Chapter 3 that the proteasome subunit RPN10 has increased levels of ubiquitination in nox1 plants but not gsnor1, and that these levels are reduced following overexpression of TRXh5. Whilst this branch of the study is still in its preliminary stages, these results suggest that proteasomal machinery is affected by SNO modifications that are targets of TRXh5. In yeast, it was shown that the switch between open and closed states of the 20S proteasomal particle is controlled by redox modification, demonstrating that redox control is important for appropriate functioning of the proteasome system (Silva et al., 2012). In a subsequent study, the intracellular redox status of yeast was studied in different growth media and it was shown that in cells displaying lower reductive power, the α-5 subunit of the 20S proteasome particle was S-glutathionylated. This opened the proteasome gate, enabling it to degrade oxidized proteins that accumulate in the absence of reductive power (Demasi et al., 2014). Our data, however, indicates an accumulation of ubiquitinated RPN10 following S-nitrosylation. RPN10 functions as one of the main ubiquitin receptors of the proteasome but additionally has been shown to exist as a free monomeric protein that is proposed to have extraproteasomal function (Lipinszki et al., 2012). Ubiquitination of RPN10 was seen to block its
ubiquitin-interacting motif which disrupted its interaction with substrates and extraproteasomal polyubiquitin receptors (Isasa et al., 2010; Lipinszki et al., 2012). The accumulation of polyubiquitinated RPN10 in nox1 plants suggests therefore, that proteasome activity could be affected either directly or indirectly by SNO modification. In plants, the proteasome plays a key role not only in the removal of damaged proteins but also in the turnover of transcriptional (co)activators and (co)repressors. The immune coactivator NPR1 paradoxically requires proteasome-mediated degradation to facilitate its transcriptional activity (Spoel et al., 2009). A reduction in proteasome activity caused by S-nitrosylation of its subunits could therefore prevent NPR1 from efficiently activating defence genes; a phenotype that we have observed in nox1 plants. Regulation of proteasomal subunits by S-nitrosylation could therefore be crucial to many molecular processes, including plant immunity.

In summary, the work presented in Chapter 3 provides not only novel information about the activities of plant TRXs in the control of S-nitrosylation, but begins to dissect the specificity of SNO modifications as selective, reversible signalling molecules.

6.2 Regulation of NPR and WRKY families by S-nitrosylation

The identification of TRX as a denitrosylase generates many questions about the SNO targets of this enzyme. In Chapter 3 it was demonstrated that the impairment of SA signalling pathways caused by high protein-SNO levels was rescued by TRXh5 suggesting that the SNO targets of this enzyme may include transcriptional activators or repressors of defence genes. To this end, two families of immune-related proteins, the NPRs and the WRKYs, were studied and identified as potential protein-SNO targets of TRXh5 in Chapter 4.

NPR1 paralogs, NPR3 and NPR4, were shown to be protein-SNO targets of TRXh5 in vitro. Moreover, treatment with an NO donor resulted in oligomerisation of these proteins similarly to that seen for NPR1. In addition, TRXh5 was able to reduce these oligomers to monomers, suggesting that
the localisations of NPR3 and NPR4 may also be controlled by TRX enzymes. The transcriptional co-activator function of NPR1 in the nucleus is reliant on its degradation by the 26S proteasome. It is thought that target gene expression requires turnover of NPR1, whereby “spent” NPR1 monomers that have already initiated transcription are constantly degraded and replaced with “fresh” NPR1 at the promoter (Spoel et al., 2009). NPR3 and NPR4 have been suggested as adaptors for CUL3 (Fu et al., 2012), the E3 ligase responsible for NPR1 degradation. So there is a requirement for these adaptors to localise to the nucleus in order to be able to regulate NPR1 turnover and associated transcriptional activity. The redox regulation of NPR3 and NPR4 by S-nitrosylation, disulphide linkage and TRXh5-mediated reduction could provide a mechanism by which NPR3 and NPR4 availability in the nucleus is regulated similar to NPR1 (Tada et al., 2008). As NPR3 and NPR4 do not possess Cys-156, the cysteine identified as the site of S-nitrosylation in NPR1, this modification must occur at one or more alternative sites. It would be interesting in the future to determine which cysteines are S-nitrosylated either by point mutations or mass spectrometry analyses. In addition, whether or not this modification affects their ability to act as CUL3 adapters either by altering their interaction with CUL3 itself, or with the substrate NPR1, remains to be investigated. Indeed it is also possible that SNO modification has the potential to affect the SA binding affinities of NPR3 and NPR4, which is a crucial factor in the proposed model for NPR1 regulation by NPR3/4 mediated turnover in both the absence and presence of pathogen challenge.

In addition to NPR1, 3 and 4, the WRKY family of transcription factors were shown here to be regulated by SNO modification. Of those WRKY factors tested, all were found to be denitrosylated by TRXh5, suggesting a role for this enzyme in reversing the effects of S-nitrosylation of these transcription factors. Strikingly, S-nitrosylation caused increased DNA binding in some WRKY family members, decreased DNA binding in others, or no effect on the remainder. This could indicate differences in the site of S-nitrosylation. Whilst an alignment of WRKY family protein sequences was unable to identify candidate cysteines common to all of the WRKYS that responded to NO in a particular way, it is possible that there are subsets among these groups of WRKYs that share regulatory
cysteines. In mammalian studies, both increased and decreased DNA binding following S-nitrosylation of transcription factors have been observed, and can be a result of a modified cysteine within the DNA binding region or neighbouring regulatory region. Typically cysteine modifications within the DNA binding region are associated with inhibitory effects on DNA binding (Hess et al., 2005). In the case of WRKY proteins, all members contain the same two cysteines within the DNA binding region, although group III proteins contain a third. The majority of group III WRKY factors displayed either decreased or unaltered DNA binding activity after NO treatment, suggesting that modification of this additional active site cysteine in group III WRKYS is indeed more likely to be associated with disrupted DNA binding. It has also previously been shown that small changes in the DNA sequence flanking the consensus W-box binding motif can result in different binding affinities for different promotor elements (Ciolkowski et al., 2008). This level of specificity might mean that S-nitrosylation of a conserved zinc finger motif cysteine in the WRKY DNA-binding domain could alter its overall structure, resulting in enhanced or suppressed affinity for the W-box depending on flanking sequences. Indeed, changing the cis-element sequence demonstrated that this is the case for at least some WRKY factors (Chapter 4). Future studies on this topic would first have to confirm the data produced in the AlphaScreen assay using protein-DNA pulldowns or electrophoretic mobility shift assays (EMSA) before proceeding to examine theories on the causes of the differential effects that S-nitrosylation has on the DNA binding of WRKY proteins. In addition, studies on whether or not TRXhS is able to reverse these effects on all family members, as was found in case of WRKY18, is crucial to understanding the control of these transcription factors.

It should be noted that Chapter 4 of this study presents a set of in vitro experiments that provide the framework for future investigations into the control of transcriptional regulators by S-nitrosylation and TRX. The preliminary findings presented here are encouraging in suggesting that SNO modification of both NPR and WKRY family proteins is crucial to their activity, and demonstrates once more the specificity with which this modification acts.
6.3 Nucleoredoxin 1 modulates responses to oxidative stress in plants

Classical TRX enzymes have been very well studied across many different organisms and their activities implicated in many cellular processes. However, there are many TRX-like proteins that belong to the TRX superfamily of enzymes, whose redox properties and roles are much less understood. Despite being identified nearly two decades ago, one such group are NRX enzymes, of which little is known, especially in plants. A recent study characterised the Arabidopsis NRX1 and NRX2 enzymes, and demonstrated their involvement in aspects of plant development (Marchal et al., 2014). Similar to this previous report, we found that NRX1 localized to both the cytoplasm and the nucleus (Marchal et al., 2014). Because currently there is little known about nuclear redox signalling, and how redox-based modifications of transcription factors are regulated, we set out to investigate the role of NRX enzymes in plant immune responses that are associated with dramatic cellular redox changes.

In Chapter 5, NRX1 was identified as a key regulator of oxidative stress during the immune response. Both the disulphide reduction activity of NRX1 and its nuclear localisation were shown to increase following pathogen challenge, suggesting a role in nuclear signalling for this enzyme during plant immunity. Previously, it has been suggested that TRX enzymes in wheat seeds relocate to the nucleus in response to stress, and indeed NTRA has also been found to partially reside in this compartment (Marchal et al., 2014; Serrato and Cejudo, 2003). Whilst it is possible that there is redundancy in the TRX and NRX systems in the nucleus, it could also be the case that these two enzymes target different nuclear proteins. Indeed, compared to single active site TRX enzymes, the two redox active sites of NRX1 displayed unusual cooperative disulphide reduction activity. Additionally, TRX and NRX may target different redox modifications. Previous studies and the work in Chapter 4 of this thesis have demonstrated the need for denitrosylases in the regulation of transcription factors (Serpa et al., 2007), so it would be interesting to test if NRX enzymes are also able to perform denitrosylation reactions. Unlike overexpression of TRXh5 (Chapter 3), overexpression of NRX1 did not rescue the immune-deficient phenotype of nox1 mutants (Kneeshaw and Spoel, unpublished results), suggesting that TRXh5 and NRX1 do not have overlapping protein-SNO targets.
Nonetheless, a role for NRX1 in immunity was revealed in this study by using the \textit{nrx1} knockout mutant, which displayed constitutive defence gene expression and SAR. Constitutive resistance was found to be dependent on SA synthesis. To determine what role this enzyme plays in plant immunity, a search for its targets was performed using pathogen-infected plant tissue. Targets were identified using an \textit{in vitro} target capture approach in combination with mass spectrometry and subsequently selected NRX1 enzyme-substrate interactions confirmed by \textit{in vivo} co-immunoprecipitations. The most notable set of proteins that were found to interact with the active sites of NRX1 were enzymes involved in the control of oxidative stress. The role of oxidative stress enzymes is typically described in the literature as protective against cellular damage from an over accumulation of ROS/RNS. However, the redox enzymes that are identified here as targets of NRX1 suggest that whilst performing these detoxifying reactions, oxidative stress enzymes themselves are modified and perhaps even “damaged” by ROS/RNS; that is, they receive redox-based PTMs that then require other oxidoreductase enzymes to reverse them. Indeed, should oxidative stress enzymes be inhibited by redox-based PTMs, their ROS/RNS detoxifying activity may be dependent on reductase enzymes, such as NRX1. Catalase enzymes, most prominently CAT2, were identified as targets of NRX1. Their H$_2$O$_2$ scavenging activity makes them essential to the control of oxidative stress (Mhamdi et al., 2010). Consistent with this and similarly to cat2 mutants, \textit{nrx1} mutant plants displayed increased susceptibility to oxidative stress generated by application of the oxidative stressor, methyl viologen. Constitutive disease resistance as seen in \textit{nrx1} plants, has also been found in cat2 mutants (Chaouch et al., 2010), suggesting that this phenotype may result from a deficiency in CAT2 activity or regulation. However, one aspect of cat2 mutants not yet found in \textit{nrx1} mutants is the formation of spontaneous cell death lesions. Previous studies have found that over-accumulation of H$_2$O$_2$ cannot necessarily be coupled to cell death and that this phenomenon may occur through an active process (Dat et al., 2003). It would therefore be interesting to test the levels of H$_2$O$_2$ in untreated \textit{nrx1} mutants in order to determine if the lack of lesions is a result of lower basal levels of oxidative stress, or the mechanism of cell death being inactive in these mutants. It is also possible that these lesions do exist in \textit{nrx1} plants,
but are only visible microscopically. This must therefore be confirmed before further studies into this phenomenon can be initiated. Interestingly, over-expression of NRX1 in the cat2 mutant background resulted in rescue of the increased level of cell death induced by methyl viologen treatment. This result indicates that NRX1 may also be responsible for the regulation of other H$_2$O$_2$ scavenging enzymes, enabling upregulation of their activity to compensate for the absence of CAT2. Indeed, both APX and CAT3 were identified as in vitro targets of NRX1 and therefore may be subject to redox modifications that are controlled by this enzyme. The focus of future studies would be to firstly identify PTMs of oxidative stress enzymes that could act as a regulatory switch controlled by NRX1. S-nitrosylation of catalase and APX has previously been reported to inhibit the activity of these enzymes and so it must also be established if NRX1 is able to act as a protein-SNO reductase (Clark et al., 2000). Whilst no work has currently been done to identify if NRX1 is able to denitrosylate model substrates in vitro, nox1 and gnsor1 mutant plants have been transformed with Flag-tagged NRX1 under the control of a constitutive 35S promoter from cauliflower mosaic virus. As stated above, in a disease susceptibility test on the 35S::Flag-NRX1 expressing nox1 plants, it was seen that NRX1 was unable to rescue the immune compromised phenotype of nox1 (data not shown). It will be interesting to test if this is also the case for the gnsor1 mutant, which, as discussed in Chapter 3, form protein-SNO through a different NO donor. The inability of NRX1 to rescue immunity in nox1 plants also highlights the specificity of TRXh5, which in Chapter 3 was found to rescue immune signalling in this mutant. Whilst the TRX superfamily is a large family of enzymes that all share certain features and perform similar enzymatic functions, this data emphasises the importance of uncovering and understanding the specific roles that individual members play. These reductase enzymes differ not only in the biological processes that they regulate, but in the reactions which they are able to participate in. It is through these differing features that such a wide range of oxidative modifications be not only protected against, but utilised as signalling devices in the correct special and temporal fashion by the TRX superfamily.

Finally, it is also interesting to note that mammalian NRX has previously been found to interact with phosphofructokinase 1 (PFK1), inhibiting its catalytic activity (Funato et al., 2013). Here, a
member of the phosphofructokinase family was identified as a target of NRX1 enzymatic activity in vitro. This suggests that there may be a conserved role for NRX proteins in glucose metabolism although this remains to be investigated further.

In conclusion, Chapter 5 demonstrates a novel role for Arabidopsis NRX1, showing it is involved in the protection against oxidative stress that occurs during pathogen challenge. As a result it has an altered immune phenotype and is sensitive to treatment with ROS generating chemicals. It is proposed that the activities of antioxidant enzymes such as CAT and APX are regulated by redox-PTMs and that these modifications are rendered reversible by NRX1. This information suggests novel, unexpected mechanisms by which the complex antioxidant system functions on many different levels to regulate, protect against and utilise cellular oxidation.

6.4 General conclusions, impact and future directions

The work in this study uncovers novel mechanisms and roles of redoxin molecules in the regulation of redox signalling during plant immunity. The data presented has impact and relevance in a wide variety of fields, including agriculture, where the findings related to immune signalling in Arabidopsis can be used as a basis for research in crops and ultimately disease prevention strategies. In addition, as previously discussed, TRX and TRX-like enzymes are not specific to plants; they have homologues in mammals and humans, and have already been shown to play crucial roles in many processes, including human disease. Findings about the specific mechanisms that these enzymes exhibit in plants could therefore be exploited for new therapies should their human counterparts be found to act in similar ways. For example, mammalian TRX has already been shown to act as a denitrosylase – but as revealed here for plants, does it target only specific protein-SNO? Similarly, several targets for mammalian NRX have been identified – but it is currently unknown if they too are responsible for the regulation and protection of other antioxidant enzymes. Advances on these questions could have huge impact in the medical field.
Going forward, there are still many gaps in our knowledge of redox signalling in both the mammalian and plant fields. The specificity that TRXh5 exhibits during denitrosylation reactions suggests that it is highly likely that other TRX family enzymes will exhibit specificity, whether it be in the reversal of SNO groups, or other oxidative modifications. This specificity is likely the key to redox-based PTMs being utilised as signalling tools and therefore a better understanding of this is crucial to moving forward in this field. Another area in which there are still many questions to address is compartmentalised redox signalling; that is, which enzymes have dedicated function to select subcellular localisations. Whilst NRX1 has been found to be localised to both the nucleus and the cytoplasm, a nuclear role for these enzymes in plants is yet to be uncovered. In addition, there are likely other TRX superfamily members that act as dedicated nuclear reductases that have not yet been characterized in detail. Whilst some work has already been performed by looking at TRX family members in other locations such as the chloroplasts, understanding compartmentalised redox signalling remains a big challenge faced by both the mammalian and plant fields in the future.
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145


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Appendix I

Supplementary figures

**Figure S1  Purification of His-tagged recombinant TRXh5 and NTRA proteins**

(A) *Escherichia coli* expressing His₆-TRXh5 was treated with or without IPTG and total protein extracted. Extracts from IPTG-treated cells were incubated with His-binding cobalt resin and the flow through (FT), first (W1) and second (W2) washes, as well as first (E1) and second (E2) elutions with imidazole separated by SDS-PAGE and stained by Coomassie blue.

(B) *Escherichia coli* expressing His₆-NTRA was treated with or without IPTG and total protein extracted. Extracts from IPTG-treated cells were incubated with His-binding cobalt resin and the first elution (E1) separated by SDS-PAGE and stained by Coomassie blue.
Selective Protein Denitrosylation Activity of Thioredoxin-\(h5\) Modulates Plant Immunity

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http://dx.doi.org/10.1016/j.molcel.2014.08.003

SUMMARY

In eukaryotes, bursts of reactive oxygen and nitrogen species mediate cellular responses to the environment by modifying cysteines of signaling proteins. Cysteine reactivity toward nitric oxide (NO) leads to formation of S-nitrosothiols (SNOs) that play important roles in pathogenesis and immunity. However, it remains poorly understood how SNOs are employed as specific, reversible signaling cues. Here we show that in plant immunity the oxidoreductase Thioredoxin-\(h5\) (TRX\(h5\)) reverses SNO modifications by acting as a selective protein-SNO reductase. While TRX\(h5\) failed to restore immunity in gsnor1 mutants that display excessive accumulation of the NO donor S-nitrosoglutathione, it rescued immunity in nox1 mutants that exhibit elevated levels of free NO. Rescue by TRX\(h5\) was conferred through selective denitrosylation of excessive protein-SNO, which reinstated signaling by the immune hormone salicylic acid. Our data indicate that TRX\(h5\) discriminates between protein-SNO substrates to provide previously unrecognized specificity and reversibility to protein-SNO signaling in plant immunity.

INTRODUCTION

Cellular redox changes are a common feature of evolutionarily conserved innate immune responses in plants and animals. Abundantly produced reactive oxygen and nitrogen species are thought to attack invading pathogens and orchestrate immune signaling in the host. In plants, pathogen attack has been shown to induce the accumulation of nitric oxide (NO) (Delledonne, 2005). NO is required for effective immune responses against biotrophic pathogens and modulates the expression of a large suite of genes (Delledonne et al., 1998; Durner et al., 1998; Parani et al., 2004; Polverari et al., 2003; Vitor et al., 2013).

In recent years, it has become clear that NO relays information by covalently modifying reactive cysteine residues of signaling proteins to generate S-nitrosothiols (SNOs). This process, termed S-nitrosylation, has been shown to alter the function, localization, conformation, and activities of proteins in a large variety of eukaryotic signaling pathways (Hess et al., 2005). Pivotal regulators of plant immune responses mediated by the signaling hormone salicylic acid (SA) are subject to S-nitrosylation. SA induces transient S-nitrosylation of nonexpressor of pathogenesis-related genes 1 (NPR1), a key transcription coactivator of broad-spectrum plant immunity, thereby regulating its conformation and stability (Spoel et al., 2009; Tada et al., 2008). Moreover, S-nitrosylation of the transcription factor TGA1, an interaction partner of NPR1, was shown to enhance its DNA binding ability (Lindermayr et al., 2010). In addition to transcriptional regulators, other components of SA signal transduction are regulated by S-nitrosylation. S-nitrosylation of SA binding protein 3 (SABP3) inhibited both the SA binding and carbonic anhydrase activities of this protein, the latter of which was required for disease resistance (Wang et al., 2009). Moreover, production of superoxide by the NADPH oxidase, RBOHD, required for the onset of programmed cell death in plant immunity, is regulated by S-nitrosylation. Site-specific S-nitrosylation is thought to sterically eject the cofactor flavin adenine dinucleotide, impairing the ability of RBOHD to produce superoxide (Yun et al., 2011).

Despite our increasing knowledge of the S-nitrosylated plant proteome, little is known about the mechanisms by which this modification is regulated. Identification of an enzyme that regulates the levels of S-nitrosogluthathione (GSNO), a cellular reservoir for NO bioactivity, indicated the importance of regulating GSNO as an in vivo NO donor for protein S-nitrosylation (Benhar et al., 2009; Liu et al., 2001). Arabidopsis knockout mutants of this enzyme, known as GSNO Reductase 1 (GSNOR1), fail to remove cellular GSNO and consequently accumulate high levels of protein-SNO (Feechan et al., 2005). Importantly, these mutants are impaired in SA-dependent immune signaling and are highly disease susceptible (Feechan et al., 2005; Tada et al., 2008; Wünsche et al., 2011), indicating that GSNOR1 plays a critical role in governing protein-SNO levels during plant immune responses.

Observations of stimulus-induced protein denitrosylation and the short-lived nature of many protein-SNO suggested SNO reductases may exist that directly reduce SNO groups (Benhar et al., 2009; Forrester et al., 2009b). Indeed, a biochemical search in mammalian cells identified thioredoxin (TRX) in combination with NADPH-dependent TRX reductase (NTR) as a potent protein-SNO reductase system important for apoptosis (Benhar et al., 2008). This significantly extends the substrate repertoire of...
Selective Denitrosylation by Immune-Induced TRXh5

RESULTS

TRXh5 Displays Protein-SNO Reductase Activity In Vitro

Expression of TRXh5 is highly upregulated by SA and pathogen infection, which is essential for establishment of disease resistance in plants (Laloi et al., 2004; Tada et al., 2008). Considering the importance of controlled SNO levels in immune responses and the role of TRXh5 in establishing immunity, we examined if TRXh5 exhibits protein denitrosylation activity. We tested if TRXh5 could reduce S-nitrosylated BSA (BSA-SNO), used here as a model substrate. BSA was S-nitrosylated with GSNO and incubated with either recombinant TRXh5 (Figure S1A available online), recombinant NTRA isoform A (NTRA; Figure S1B), or a combination of both. BSA-SNO was then detected by the biotin switch technique (BST) in which SNO are replaced by biotin (Forrester et al., 2009a). Similar to displacement of NO by UV, only the fully reconstituted TRXh5/NTRA system was able to completely denitrosylate BSA (Figure 1A). Together, these data clearly indicate that plant TRXh5 displays protein denitrosylation activity in vitro.

The TRX/NTR System Exhibits Protein-SNO Reductase Activity In Vivo

It remains unknown if plant cells maintain significant TRX-mediated denitrosylation activity in vivo. Therefore, we protoplasted plant cells and examined the time-dependent formation and decay of protein-SNO after treatment with S-nitrosylated cysteine (CysNO). CysNO rapidly induced abundant protein S-nitrosylation within 5 min (Figure 1C). However, 10 min post CysNO treatment protein-SNO levels decreased and were nearly undetectable after 20 min. To determine if this cellular denitrosylation activity can be attributed to the TRX/NTR system, we sought to inhibit its activity with two specific inhibitors of mammalian NTR, 1-chloro-2,4-dinitrobenzene (DNCB) and...
Selective Denitrosylation by Immune-Induced TRXh5

Protoplasts from plants transformed with or without anti-His antibody were immunoprecipitated with an anti-Myc antibody. Mixed disulphide intermediates formed by TRX-disulphide intermediates formed by TRX.

Plant protein extracts were treated with 1 mM GSNO and then incubated with immobilized His-tagged TRX-nitrosylated plant proteins and BSA (deSNO-BSA) were detected using anti-biotin and anti-BSA antibodies, respectively, and are shown relative to total BSA and TRXh5.

Protein extracts were spiked with BSA and treated with or without 1 mM GSNO and subjected to the nondenaturing BST, as shown in (F). Purified denitrosylated plant proteins and BSA (deSNO-BSA) were detected using an anti-Myc antibody and are shown relative to total BSA.

Plant protein extracts were treated with 1 mM GSNO and then incubated with immobilized His-tagged TRX5(C42S) and TRXh5 (TRXh5-SNO) were detected with the BST and are shown relative to total BSA and TRXh5.

GSNO to trigger conversion of oxymyoglobin (Fe2+) to metmyoglobin (Fe3+). An analogous to disulphide reduction, the first active site cysteine of TRX could displace NO from the target cysteine via heterolytic cleavage of TRX-SNO intermediates (Figures 1D–1F). In addition, we analyzed protein-SNO contents of trx-h3 trx-h5 double mutant cells, lacking two functional TRX-h genes that are partially redundant in immunity (Tada et al., 2008), as well as mutant ntra cells. Compared to wild-type (WT), mutant cells contained higher basal levels of protein-SNO (Figure 1G). Upon treatment with CysNO, trx-h3 trx-h5 cells accumulated only slightly more protein-SNO, while ntra cells accumulated over four times more protein-SNO than WT. Collectively, these data indicate that the TRX-h/NTR system strongly contributes to protein denitrosylation activity in plant cells.

Figure 2. Trans-Denitrosylation Activity of TRXh5 Reverses SNO Modifications

(A) Incubation of oxymyoglobin (peaks at 542 and 580 nm) with GSNO and TRXh5 released NO-, converting oxyhemoglobin into metmyoglobin (peak at 632 nm). (B) Incubation of oxymyoglobin with GSNO and either TRXh5 or mutant TRXh5(C42S) released NO-, resulting in conversion of oxymyoglobin into metmyoglobin.

(B) BSA (20 μM) was S-nitrosylated with 1 mM GSNO and incubated with TRXh5 (40 μM). S-nitrosylated BSA (BSA-SNO) and TRXh5 (TRXh5-SNO) were detected with the BST and are shown relative to total BSA and TRXh5.

(D) BSA (20 μM) was S-nitrosylated with 1 mM GSNO and incubated with WT or an active site mutant (C39/42S) of TRXh5 (40 μM). S-nitrosylated BSA (BSA-SNO) and TRXh5 (TRXh5-SNO) were detected with the BST and are shown relative to total BSA and TRXh5.

(E) BSA (20 μM) was S-nitrosylated with 1 mM GSNO and incubated with WT or the active site mutants TRXh5(C39S) or TRXh5(C42S) (40 μM). S-nitrosylated BSA (BSA-SNO) and TRXh5 (TRXh5-SNO) were detected with the BST and are shown relative to total BSA and TRXh5. UV-induced SNO photolysis served as a control.

(F) Schematic of nondenaturing BST, utilizing immobilized mutant TRXh5(C42S). See text for details.

Trans-Denitrosylation Activity of TRXh5 Reverses SNO Modifications

The TRX active site consists of two reactive cysteines that are responsible for reducing cysteines in target substrates. TRX-mediated denitrosylation could occur in one of two ways.

Analogous to disulphide reduction, the first active site cysteine of TRX could displace NO from the target cysteine via heterolytic cleavage of the S-N bond, leading to formation of a mixed disulphide bond between TRX and its substrate, as well as liberation of HNO. Alternatively, NO could be transferred directly from TRX- mediated denitrosylation could occur in one of two ways. NOHM (Nikitovic and Holmgren, 1996). To examine which of these scenarios is more likely, we first tested if TRXh5 could liberate NO from GSNO to trigger conversion of oxyhemoglobin (Fe2+) to metmyoglobin (Fe3+). Incubation of oxyhemoglobin (Fe2+) with GSNO alone had little effect (Figure S2A). However, when GSNO and TRXh5 were added together, oxyhemoglobin (Fe2+) was rapidly converted into metmyoglobin (Fe3+) (Figure 2A), indicating NO-release. Moreover, mutant TRXh5(C42S) protein, carrying a...
cysteine-to-serine mutation in the second cysteine of the active site, was as efficient as the WT protein in liberating NO from GSNO to trigger time-dependent conversion of oxymyoglobin (Fe²⁺) to metmyoglobin (Fe³⁺) (Figure 2B). These data strongly suggest that TRXh5 trans-denitrosylates its targets using a single active site cysteine, which is subsequently subject to homolytic cleavage to yield NO⁺.

Next, we examined if formation of a TRXh5-SNO intermediate could be detected. We found that denitrosylation of GSNO-induced BSA-SNO was indeed associated with formation of a TRXh5-SNO intermediate (Figure 2C), suggesting that TRXh5 also trans-denitrosylates protein-SNO. In addition to the active site Cys39 and Cys42, TRXh5 contains an additional Cys10 that could play a role in denitrosylation. Therefore, we determined if trans-denitrosylation of BSA-SNO was dependent on the active site. Indeed, in contrast to WT TRXh5, mutation of Cys39 and Cys42 to serines (C39S and C42S) eliminated denitrosylation activity and formation of the TRXh5-SNO intermediate (Figure 2D).

Notably, the amount of TRXh5-SNO intermediate formed in these assays was always less than the amount of BSA-SNO that was subject to denitrosylation, suggesting this intermediate is unstable. We tested if TRXh5-SNO could be stabilized by mutating single cysteine residues of the active site. Like WT, the single mutant proteins TRXh5(C39S) and TRXh5(C42S) efficiently denitrosylated BSA-SNO (Figure 2E). Remarkably, however, a TRXh5-SNO intermediate was undetectable for the single mutants (Figure 2E). This suggests that the primary trans-nitrosylated cysteine of the active site is either stabilized by the secondary cysteine or that it is attacked by the secondary cysteine to yield a more stable nitrosoyl disulfide intermediate. Regardless of this mechanistic detail, our data demonstrate that TRXh5 exhibits trans-denitrosylation activity.

We then sought to explore if TRXh5 also utilizes a trans-denitrosylation mechanism to reduce plant protein-SNO. However, this is complicated by the fact that mutation of an active site cysteine prevents TRXh5 activity from being recycled by NTRA, impeding experiments similar to the one shown in Figure 1B. Therefore, we aimed to take advantage of the mechanistic difference in disulfide reduction and trans-denitrosylation activities of TRXh5. As opposed to trans-denitrosylation activity, disulfide reduction by TRX enzymes requires both active site cysteines. Indeed, while TRXh5 rapidly reduced disulfide bonds in the model substrate insulin, mutant TRXh5(C42S) protein was ineffective in this respect (Figure S2B). This allowed us to design a nonnaturating BST for the identification of plant protein-SNO that are specifically trans-denitrosylated by TRXh5 (Figure 2F). In this method, free thiol residues of native plant proteins were first alkylated with N-ethylmaleimide (NEM) and then added to a column containing immobilized mutant TRXh5(C42S) protein. Consequently, disulfide-containing protein targets were trapped on the column by formation of a mixed disulfide bond with TRXh5(C42S), while proteins with thiols that were either alkylated by NEM or trans-denitrosylated by TRXh5(C42S) passed through the column. Denitrosylated thiols were labeled with thiol-reactive biotin (Biotin-HPDP) and detected by immunoblotting with an anti-biotin antibody. We performed this new method on plant protein extracts that were spiked with BSA as an internal control and treated with or without GSNO. Biotinylated BSA—i.e., BSA trans-denitrosylated by TRXh5(C42S)—was detected only after treatment with GSNO (Figure 2G), indicating that our method did not detect appreciable false-positives. Moreover, while some biotinylated plant proteins were recovered in absence of GSNO treatment, application of GSNO increased this amount and was dependent on the presence of an active site cysteine of TRXh5 (Figures 2G and S2C). These data indicate that TRXh5 subjects a large set of plant protein-SNO to trans-denitrosylation.

To determine if TRXh5 denitrosylates any plant protein-SNO via formation of mixed disulfide intermediates, we also eluted the TRXh5(C42S) column with the reducing agent dithiotreitol (DTT) after the nonnaturating BST. The eluate thus contains proteins that formed mixed disulfides with TRXh5(C42S). Figure S2C shows that GSNO treatment did not stimulate the formation of additional mixed disulfides. Similarly, when recombining His-tagged TRXh5(C42S) was used to pull down mixed disulfide intermediates directly from untreated and GSNO-treated plant extracts, GSNO failed to induce significant changes (Figure 2H). Only a complex around 80 kDa showed a small increase upon GSNO treatment, which likely represents a disulfide intermediate between TRXh5 and its main disulfide-containing target NPR1. GSNO induces the formation of intermolecular disulfide bonds between NPR1 monomers, which are major targets of TRXh5 (Tada et al., 2008). Accordingly, GSNO treatment of extracts from plants expressing 35S::NPR1-GFP stimulated interaction between TRXh5(C42S) and NPR1-GFP, which was dependent on Cys39 (Figure S2D). Hence, denitrosylation of plant protein-SNO via formation of mixed disulfides between TRXh5 and its substrates is not a prevalent mechanism in vitro.

Finally, we sought to investigate the formation of mixed disulfides in denitrosylation reactions in vivo. We treated protoplasts expressing Myc-tagged TRXh5 with GSNO and assessed the formation of mixed disulfides between Myc-TRXh5 and its substrates. As expected, GSNO treatment did not result in significant accumulation of further mixed disulfides (Figure 2I). Taken together, our data demonstrate that TRXh5 preferentially denitrosylates protein-SNO in trans rather than via formation of mixed disulfide intermediates.

**TRXh5 Selectively Restores Immune Deficiencies Caused by Elevated Protein-SNO**

TRXh5 is highly induced during activation of plant immunity (Laloi et al., 2004; Tada et al., 2008), but it is unknown if its SNO reduction activity is relevant to immune signaling. We determined TRXh5 gene expression levels in mutant gsnor1 and cue1/nox1 plants that both contain excessive amounts of protein-SNO due to over accumulation of GSNO and free NO, respectively (Feechan et al., 2005; He et al., 2004; Yun et al., 2011). While exogenous application of SA strongly induced TRXh5 gene expression in the WT, expression was reduced in gsnor1 and nox1 plants (Figure 3A). Thus, it is plausible that accumulation of excessive protein-SNO in these mutants is exacerbated by reduced expression of TRXh5. To test this possibility, we transformed gsnor1 and nox1 plants with Flag-tagged TRXh5 driven by a constitutive 35S promoter from cauliflower mosaic virus.
The resulting 35S::TRXh5 (gsnor1) and 35S::TRXh5 (nox1) plants showed strong expression of TRXh5 that was independent of immune activation by SA (Figure 3A). Developmental defects of gsnor1, including loss of apical dominance, elongated leaf shape, and reduced seed production (Kwon et al., 2012), were not restored by TRXh5 expression (Figure 3B; data not shown). Similarly, TRXh5 expression was unable to reduce restored size and interveinal paleness of nox1 plants (Figure 3B) (Li et al., 1995). These phenotypes suggest that TRXh5 does not act outside the plant immune system.

We then assessed if TRXh5 could rescue immune deficiencies associated with elevated protein-SNO levels. Plants were infected with the bacterial leaf pathogen Pseudomonas syringae pv. tomato (Pst) DC3000 and bacterial growth assessed after 5 days. As reported previously (Feechan et al., 2005), gsnor1 mutants were highly susceptible to Pst DC3000, displaying over 25-fold higher bacterial growth compared to WT (Figure 3C). Unexpectedly, 35S::TRXh5 (gsnor1) plants were as susceptible as gsnor1 mutants (Figure 3C), indicating TRXh5 was unable to restore resistance in this genetic background. Infection of nox1 mutants with Pst DC3000 demonstrated that elevated protein-SNO in this mutant also resulted in severe disease susceptibility (Figure 3D). Surprisingly, however, expression of TRXh5 restored resistance in the nox1 genetic background (Figure 3D). To ensure this was due specifically to denitrosylation activity of TRXh5, we also expressed 35S::TRXh5(C42S) in nox1 plants. While we reported above that mutant TRXh5(C42S) protein exhibits potent denitrosylation activity (Figure 2), it lacks disulphide reduction activity, as clearly demonstrated by its failure to reduce disulphide-linked oligomers of its preferred natural substrate NPR1 (Figure 3E). Strikingly, expression of 35S::TRXh5(C42S) in nox1 mutants also restored resistance against Pst DC3000 (Figure 3F). Taken together, these data suggest that TRXh5 selectively restores immunity in genotypes with elevated protein-SNO derived from free NO but not GSNO.

**TRXh5 Restores SA-Dependent Immune Signaling by Selective Protein Denitrosylation**

Selective restoration of disease resistance by TRXh5 in gsnor1 and nox1 mutants could be due to preferential selection of protein-SNO substrates or due to differential regulation of TRXh5 enzymatic activity. We studied the latter by assessing the disulphide reduction activity of TRXh5 in these genetic backgrounds. 35S::TRXh5 (nox1) and 35S::TRXh5 (gsnor1) plants were treated with or without the immune activator SA. Subsequently, proteins were extracted under denaturing conditions in presence of the alkylating agent 4-acetamido-4′-maleimidyldistilbene-2,2′-disulfonic acid (AMS) to prevent nonspecific interactions between TRXh5 and nonsubstrates during sample preparation. Protein samples were then separated on nonreducing (−DTT) and reducing (+DTT) SDS-PAGE and detected using an anti-GFP antibody.!!!small amount of DTT (0.33 mM) to recycle TRX activity. Reduction of NPR1-GFP oligomer (O) to monomer (M) relative to total (T) NPR1-GFP was followed for the indicated times by non-reducing (−DTT) and reducing (+DTT) SDS-PAGE and detected using an anti-GFP antibody. (F) WT, nox1, and 35S::TRXh5(C42S) (nox1) plants were infected and analyzed as in (C).
both enzymes being localized to similar subcellular locations (Figure 4C).

Resistance against pathogenic Pst DC3000 is conferred by the SA-induced transcriptional coactivator NPR1. Site-specific S-nitrosylation of NPR1 promotes the formation of a cytosolic disulphide-linked oligomer, thereby preventing NPR1 monomers from activating target genes in the nucleus (Tada et al., 2008). To understand how denitrosylation activity of TRXh5 can rescue immunity, we investigated if TRXh5 was capable of denitrosylating NPR1. Figure 5A shows that GSNO treatment triggered S-nitrosylation of NPR1. Subsequent incubation of S-nitrosylated NPR1 with the TRXh5/NTRA system resulted in its complete denitrosylation. To test if TRXh5-mediated denitrosylation of NPR1 affects its conformation in vivo, we generated protoplasts from nox1 and gsnor1 mutants transformed with or without Flag-tagged TRXh5 and transiently expressed NPR1-GFP (Figure 5B).

In absence of the 3SS::TRXh5 transgene, NPR1-GFP was localized to both the cytoplasm and nucleus in nox1 and gsnor1 cells. By contrast, expression of 3SS::TRXh5 resulted in exclusively nuclear-localized NPR1-GFP in both genotypes (Figure 5B). These data suggest that TRXh5 facilitates monomerization and nuclear accumulation of NPR1 in both nox1 and gsnor1 genetic backgrounds.

The effect of TRXh5 on NPR1 conformation fails to explain the selective nature by which this enzyme rescues immunity in nox1 but not gsnor1 mutants. Therefore, we examined if expression of TRXh5 affected SA-dependent immune gene expression upon inoculation with Pst DC3000. Expression of the marker genes PR-1, WRKY38, and WRKY62 was strongly upregulated upon infection of the WT, whereas nox1 and especially gsnor1 plants exhibited strongly reduced gene expression (Figure 5C), explaining the enhanced susceptibility of these genotypes to this pathogen. Remarkably, expression of TRXh5 partially restored SA-responsive gene expression in nox1 mutants, while it had no significant effect in gsnor1 mutants (Figure 5C). To test if TRXh5 impacts immune signaling downstream of SA, we applied exogenous SA to plants and followed SA-dependent expression of PR-1, WRKY38, and WRKY62 in a time course. As expected, both nox1 and gsnor1 mutants displayed reduced expression of these genes compared to the WT (Figure 5D). Expression of TRXh5 completely restored SA-induced gene expression in nox1 mutants to a similar level as the WT. In contrast, TRXh5 expression had little effect in the gsnor1 mutant background.

In summary, these data demonstrate that TRXh5 specifically targets protein-SNO substrates to selectively modulate SA-responsive gene expression in plant immunity.

**DISCUSSION**

Redox-based protein modifications play essential roles in cellular signaling. Protein S-nitrosylation has emerged as a particularly important posttranslational signal in eukaryotes. But how this posttranslational modification is controlled and utilized as a specific signaling switch remains poorly understood. In this study, we discovered that in plant immunity the oxidoreductase TRXh5 is a potent protein-SNO reductase that provides reversibility, and more importantly, specificity to protein-SNO signaling to establish SA-dependent disease resistance.

The TRXh5/NTRA system displayed potent denitrosylation activity on model and plant protein-SNO in vitro (Figures 1A and 1B). Structurally, TRXh5 is closely related to other cytosolic proteins...
Selective Denitrosylation by Immune-Induced TRXh5

TRX h-type members (h1–h9), implying that these enzymes may also function as cellular SNO reductases. Accordingly, plant cells exhibited strong denitrosylation activity that was dependent on the activity of NTR (Figures 1C–1G). In this respect, plant TRX-h enzymes behave similar to the mammalian TRX system, which has been described as a potent protein-SNO reductase involved in a variety of processes, including apoptosis and inflammatory signaling (Benhar et al., 2008; Kelleher et al., 2014). Mammalian TRX1 in the disulphide form, however, was shown to be S-nitrosylated at a cysteine residue away from the active site, allowing it to also function as a NO donor by trans-nitrosylating target proteins (Mitchell and Marietta, 2005; Sengupta and Holmgren, 2013). As a corresponding cysteine residue in plant TRX-h enzymes is absent, they are not expected to function as NO donors.

Despite lacking the corresponding NO donating cysteine residue of mammalian TRX1, we observed formation of S-nitrosylated TRXh5 during denitrosylation reactions. However, S-nitrosylation occurred in the active site, and reduction of protein-SNO required only a single active site cysteine (Figure 2), suggesting TRXh5 trans-denitrosylates its substrates. Trans-denitrosylation activity has been proposed for Escherichia coli TRX, which, like plant TRXh5, cleaves GSNO with release of NO•, a strong indicator that homolytic cleavage of an S-NO bond within the TRX active site underpins denitrosylation reactions (Nikitovic and Holmgren, 1996). By contrast, mammalian TRX1 was suggested to denitrosylate substrates via a mixed-disulphide intermediate (Benhar et al., 2008). Here, we designed a non-denaturing BST in which mixed-disulphide intermediates were largely eliminated, while many trans-denitrosylated substrates of TRXh5 were still purified (Figures 2F, 2G, and S2C). Moreover, denitrosylation by TRXh5 was not associated with a significant increase in mixed disulphide intermediates both in vitro and in vivo (Figures 2H, 2I, and S2C). Although these assays cannot completely rule out formation of mixed-disulphide intermediates, our results strongly imply that plant TRXh5 predominantly utilizes a trans-denitrosylation mechanism.

Remarkably, expression of TRXh5 in immune-compromised gsnor1 and nox1 mutants demonstrated that TRXh5 selectively restored immunity only in the nox1 genetic background (Figure 3). This was not due to differences in reduction capacity of TRXh5 in these mutant backgrounds, but rather because of selective denitrosylation (Figures 3F, 4A, and 4B). Although both gsnor1 and nox1 mutants accumulate high levels of protein-SNO during pathogen infection (Yun et al., 2011), these are derived from distinct NO donors (i.e., GSNO versus free NO). Stereochemistry and structure of NO donors as well as thiol microenvironment have been reported to influence site-specificity and efficiency of S-nitrosylation (Foster et al., 2009; Kovacs and Lindermayr, 2013). Whereas NO• is thought to S-nitrosylate thiols directly through a radical-mediated pathway or indirectly via higher oxides of NO, GSNO is thought to trans-nitrosylate targets (Kovacs and Lindermayr, 2013). It is therefore likely that nox1 and gsnor1 mutants have different, albeit overlapping, sets of protein-SNO. Our finding that TRX rescues immunity only in nox1 but not in gsnor1 mutants provides the first genetic indication that TRX enzymes may denitrosylate a distinct subset of protein-SNO from those regulated by the indirect SNO reductase GSNOR1. As TRXh5 and GSNOR1 exhibit similar subcellular...
Selective Denitrosylation by Immune-Induced TRXh5

Figure 6. Proposed Model Showing that the SNO Reductases GSNOR1 and TRXh5 Regulate Different Branches of Protein-SNO in Plant Immune Signaling

Two classes of proteins are shown. Class I proteins are S-nitrosylated by GSNO, the level of which is regulated by the SNO reductase GSNOR1 (left panel). Class II proteins are S-nitrosylated by free NO$^\bullet$ or other unknown intermediates and are denitrosylated by the TRXh5/NTRA system (right panel). As exemplified by the immune coactivator NPR1, class I and class II proteins partly overlap and, consequently, are regulated by both GSNOR1 and the TRXh5/NTRA system (middle panel). Both pathways contribute to SA-dependent gene expression and immunity.

localization (Figure 4C), it is unlikely either enzyme has privileged access to a subset of protein-SNO substrates. More likely, these SNO reductases have at least partially distinct repertoires of substrates, allowing them to function in parallel in different protein-SNO immune signaling pathways (Figure 6). As may be expected, however, some of this specificity was lost in vitro, as GSNO-derived BSA-SNO and plant protein-SNO could still be denitrosylated by TRXh5 upon reconstitution in a test tube (Figures 1 and 2). Nonetheless, it was recently reported that the TRX-h/NTRA system was unable to denitrosylate S-nitrosylated GAPDH in vitro (Zaffagnini et al., 2013), suggesting some selectively persists in vitro.

Disulphide reduction activity of TRXh5 was previously shown to facilitate SA-responsive gene expression mediated by the transcription coactivator NPR1. S-nitrosylation of Cys156 in NPR1 monomers facilitates the formation of a disulphide-linked oligomer in the cytoplasm. Upon SA signaling, these disulphides are reduced by TRXh5, releasing NPR1 monomers into the nucleus where they activate SA-responsive immune genes (Mou et al., 2003; Tada et al., 2008). Our data demonstrate that in addition to reducing intermolecular disulphide links in NPR1, TRXh5 is also capable of denitrosylating this coactivator (Figure 5A). TRXh5-mediated denitrosylation of NPR1 enhanced its nuclear translocation and was associated with increased transcriptional activity in nox1 mutants (Figures 5B–5D). Thus, besides governance by GSNOR1 (Tada et al., 2008), SNO groups in NPR1 are directly targeted by TRXh5 (Figure 6). Denitrosylation of NPR1 is reminiscent of the mammalian immune-induced NF-κB transcription factor, whose activity is also suppressed by site-specific S-nitrosylation in respiratory epithelium. Upon cytokine stimulation, NF-κB is denitrosylated by TRX1, thereby promoting its transcriptional activity (Kelleher et al., 2014). Despite promoting nuclear translocation of NPR1, denitrosylation activity of TRXh5 was insufficient to restore immune-induced gene expression in gsnor1 mutants (Figures 5B–5D).

This suggests that additional gene regulators normally governed by GSNOR1 remain S-nitrosylated in this genetic background and, consequently, remain transcriptionally inactive. Uncovering the identity of these S-nitrosylated gene regulators and determining how they differ from TRXh5 substrates represents a future challenge in elucidating protein-SNO signaling pathways. Furthermore, plant TRXh5 was shown to be a target of the fungal pathogen effector victorin, which binds to active site Cys39 and blocks disulphide reductase activity to induce host cell death (Lorang et al., 2012). It would be interesting to test if victorin-induced cell death also involves impairment of TRXh5 denitrosylation activity.

In conclusion, our findings demonstrate that plant TRXh5 is a potent protein-SNO reductase, and this function is a key determinant of SA-dependent plant immune signaling. Selective restoration of SNO-impaired immune gene expression by TRXh5 implies that eukaryotic TRX enzymes in general may discriminate between protein-SNO substrates to provide specific, reversible protein-SNO signaling.

EXPERIMENTAL PROCEDURES

Plant Transformation, Chemical Induction, and Pathogen Infection

According to the manufacturer’s instructions, TRXh5 (At1g455145) and GSNOR1 (At5g43940) were cloned into pENTR(3D)-TOPO (Life Technologies) and recombined with Flag- or YFP-containing pEarleyGate 202 and 103 (Earley et al., 2006) using LR clonase (Life Technologies) to generate the 35S::Flag-TRXh5, 35S::YFP-TRXh5, and 35S::YFP-GSNOR1 transgenes. The 35S::Flag-TRXh5 vector was transferred into Agrobacterium tumefaciens strain GV3101 (pMP90) using a freeze-thaw method and subsequently transformed into WT Col-0 plants by floral dipping (Bent, 2000). Transgenic plants were selected on soil by spraying glucosinate ammonium and crossed into gsnor1 (par2-1 allele) and nox1 (cue1-6 allele) plants. The 35S::TRXh5(C42S) transgene was generated by site-directed mutagenesis and transformed directly into nox1 plants.

Pat DC3000 was grown overnight in liquid LB medium supplemented with 10 mM MgSO$_4$. Bacterial cells were collected by centrifugation, diluted to
Selective Denitrosylation by Immune-Induced TRXh5

5 x 10^6 cells, and pressure infiltrated into leaves. In planta bacterial growth was determined 5 days after infection by spreading serial dilutions of leaf extracts on LB plates supplemented with rifampicin (50 μg/ml), 10 mM MgSO₄, and 50 μM cycloheximide. For induction of immune genes, 4-week-old soil-grown plants were pressure infiltrated as described above or sprayed with a solution containing 0.5 mM SA.

SNO Measurements

Protoplasts were isolated from adult leaves as described in Wu et al. (2009), resuspended in modified W5 solution (150 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose, and 2 mM MES [pH 5.7]), and treated with or without 100 μM DNB (Sigma) for 1 hr. Subsequently, 2 mM CysNO, 2 mM GSNO, or 2 mM DEA-NO was added for the indicated times. Protoplasts were collected by centrifugation and protein extracted in PBS containing 1% Triton X-100, 2 mM Hepes, 100 μM DAF-2, and protease inhibitor cocktail (50 μg/ml TPCK, 50 μg/ml TDC, and 0.6 mM PMSF). Formation of the fluorescent triazolofluorescein (DAF-2T) was monitored using excitation and emission wavelengths of 478 and 520 nm, respectively (Benhar et al., 2008).

Enzymatic Assays

TRXh5-mediated reduction of GSNO was measured by incubating oxymyoglobin (30 μM; Sigma) with TRXh5 (35 μM) and GSNO (50 μM) in a buffer containing 100 mM potassium phosphate (pH 7.4) and 1 mM EDTA. Release of NO was measured by monitoring the conversion of oxymyoglobin to metmyoglobin spectrophotometrically. Disulphide reduction activity of TRXh5 was measured using an insulin turbidity assay as described in Tada et al. (2008). Auranofin (10 μM) and DNB (100 μM) were added to the mixture prior to the start of assays.

Proteins prebound to HisPur cobalt resin (Pierce). Subsequently, resin was extensively washed in diluted HEN buffer. Mixed disulphides between His-tagged TRXh5 mutants and targets were analyzed by incubating WT leaf extracts with TRXh5-His₅ mutant proteins prebound to HisPur cobalt resin (Pierce). Subsequently, resin was extensively washed in diluted HEN buffer containing 0.5% Triton, and the third wash contained 1 M NaCl.

For western blot analysis SDS sample buffer with or without 50 mM DTT (except 150 mM DTT for NPR1-GFP protein) was added to antibody agarose or total protein extracts. Samples were heated to 70°C for 10 min, separated on SDS-PAGE gels, and transferred to nitrocellulose membranes. Western blots were probed with anti-Flag (Sigma), anti-Myc (Invitrogen), anti-His (Cell Signaling), anti-GFP (Roche), anti-BSA (Sigma), and anti-biotin (New England Biolabs) antibodies.

Gene Expression Analysis

RNA extractions and cDNA synthesis were performed as described previously (Sporel et al., 2009). Undiluted cDNA was analyzed by qualitative RT-PCR, while quantitative qPCR was carried out on 20-times-diluted cDNA using Power SYBR Green (Life Technologies) and gene-specific primers on a StepOne Plus Real Time PCR system (Life Technologies).

Confocal Microscopy

Protoplasts from indicated genotypes were isolated and transformed as described previously (Wu et al., 2009) and viewed on a Nikon Eclipse TE2000-U confocal microscope connected to a BioRad Radiance 2100 Laser Scanning System.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.08.003.

ACKNOWLEDGMENTS

We thank Dr. Andreas Meyer for the NTRA expression clone, Dr. Jianru Zuo for par2-1 seeds, Dr. Thomas Wolpert for 35S::Myc-TRXh5 seeds, Michael Skelly for the GSNOR1 donor clone, and Dr. Nisha Philip for technical assistance. S.H.S. was supported by a Royal Society University Research Fellowship (UF090321), Royal Society Research Grant (Rg110495), European Molecular Biology Organization Long-Term Fellowship, and Netherlands Science Foundation Rubicon Fellowship. S.K. was supported by a studentship from the Biotechnology and Biological Sciences Research Council (BBSRC) and G.J.L. by a BBSRC grant (BB/D011809/1).

Received: January 30, 2014 Revised: June 5, 2014 Accepted: July 30, 2014 Published: September 4, 2014

REFERENCES


Molecular Cell 56, 153–162, October 2, 2014 ©2014 Elsevier Inc. 161


Appendix II

Supplementary figures

Figure S2  Purification of His-tagged recombinant WT and mutant NRX1 proteins

*Escherichia coli* expressing His$_6$-NRX1 proteins was treated with or without IPTG and total protein extracted. Extracts from IPTG-treated cells were incubated with His-binding cobalt resin and the proteins eluted with imidazole. Following separation with SDS page and transfer to nitrocellulose membrane, Ponceau staining enabled visualisation of purified proteins.
Figure S3  Testing the *nrx1* antibody

Protein was extracted from WT, 35S::Flag-NRX1 (Col-0), 35S::Flag-NRX1 (nrx1-1), nrx1-1 and nrx1-2 plants and separated by SDS-PAGE. A previously generated anti-NRX1 antibody (Keyani and Spoel, unpublished results) was used to detect accumulation of endogenous NRX1 protein.

Supplementary tables

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### Table S1  Targets of the NRX1(C58,378) protein identified by mass spectrometry

NRX1(C58,378) protein was immobilised on NHS-activated resin and incubated with extracts from pathogen-infected plants. Targets were eluted with DTT, and then identified by mass spectrometry. P-values signify differences to a control column which contained quenched NHS-activated beads in the absence of NRX1 protein. P-values ≤ 0.05 are highlighted in red. Asterisks represent targets identified to also interact with wild-type NRX1; double asterisks indicate that significant P-values (≤ 0.05) were seen for a particular target in both NRX1(C58/378) and NRX1 columns (see Table S2)

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Table S2  Targets of the NRX1 protein identified by mass spectrometry

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