The role of S-nitrosothiols in the establishment of disease resistance in *Arabidopsis*

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The role of S-nitrosothiols in the establishment of disease resistance in *Arabidopsis*

Following pathogen recognition NO and ROI's are rapidly produced in plants. NO may react with O_2, O_2^- and thiol compounds to generate S-nitrosoglutathione (GSNO). S-nitrosothiols (SNO's) can also be derived from S-nitrosylated proteins. S-nitrosylation is a redox-related modification of a single critical cysteine residue usually within an acid-base or hydrophobic motif. In animals SNO's are central to signal transduction and host defences and therefore it is necessary to regulate SNO levels. The enzyme GS-FDH exhibits strong GSNO reductase activity, which is highly conserved in bacteria, animals and plants.

Using a biotin switch method we show that proteins are S-nitrosylated in *Arabidopsis* following challenge with both virulent *Pseudomonas syringae* pv. *tomato* strain *Pst* DC3000 and avirulent *Pst* carrying (*avrB*). Purification and MALDI analysis indicates that one of these S-nitrosylated proteins is a carbonic anhydrase.

A single copy *Arabidopsis GS-FDH* (glutathione-dependent formaldehyde dehydrogease) was identified which shows 75% homology in amino acid sequence to mouse *GS-FDH*. The induction of *GS-FDH* by GSNO in *Arabidopsis* was confirmed by northern analysis. *GS-FDH* expression in *Arabidopsis* is constitutive at basal levels. During the HR, which was induced by *Pst* DC3000 (*avrB*), GS-FDH expression and activity were found to be rapidly suppressed. Whereas, no GS-FDH suppression takes place following infection with virulent *Pst* DC3000. Therefore GS-FDH expression and activity are tightly regulated during the HR.

Three *GS-FDH* T-DNA insertion lines were identified from the SAIL and SIGnAL databases. Two of these lines *fdh1-1* and *fdh1-2* are allelic and both contain a T-DNA insert in the promoter of GS-FDH. Northern analysis and enzyme activity assays found that GS-FDH is over-expressed in these lines. Characterisation of *fdh1-1* plants revealed the constitutive expression of high levels of the defence gene *PDF1.2* and slightly elevated levels of *PR1* expression. *fdh1-1* plants exhibit resistance against the virulent biotroph *Pst* DC3000. Moreover, *fdh1-1* mutants show a delayed HR response following *Pst* DC3000 (*avrB*) infiltration.
fdh1-3 mutants contain a T-DNA insert in the coding sequence of *GS-FDH*. Northern analysis and enzyme activity assays demonstrate that the transcription and translation of GS-FDH is knocked-out in *fdh1-3* mutants. Interestingly, *fdh1-3* plants are susceptible to virulent *Pst* DC3000 and display an accelerated HR phenotype in response to *Pst* DC3000 (*avrB*). Therefore, GS-FDH is important for both R gene-mediated and basal disease resistance against bacterial challenge in *Arabidopsis*.

In addition, an activation tagged population was screened to isolate mutants resistant to nitrosative stress, from which ten NO resistant mutant candidates were identified.

Abbreviations

µg	Microgram
µl	Microlitre
AOX	Alternative Oxidase
At	*Arabidopsis thaliana*
Avr	Avirulent gene
BLAST	Basic Local Alignment Search Tool
Bp	base pair
BZIP	Basic Leucine Zipper
CaM	Calmodulin
CaMV	Cauliflower Mosaic Virus
CC	Coiled-Coil
Cfu	Colony forming units
CGMP	Guanosine 3,5-cyclic monophosphate
Col-0	*Arabidopsis* ecotype
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
ET	Ethylene
G	Gram
GS-FDH	Gluthatione-dependent formaldehyde dehydrogenase
GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione Redutase
GST	Glutathione S-Transferase
HR	Hypersensitive Response
iNOS	Immune Nitric Oxide Synthase
JA	Jasmonic Acid
KB	King's broth media
Kda	Kilodalton
LRR	Leucine-Rich Repeat
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation Time-of-Flight
MMTS	Methyl methanethiosulfonaste
MAPK	Mitogen Activated Protein Kinase

Me-JA	Methyl-jasmonate
MS	Murashige and Skoog media
MW	Molecular weight standard
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NahG	Salicylate hydroxylase gene
NBS	Nucleotide-Binding Site
NO	Nitric oxide
NOS	Nitric Oxide Synthase
NR	Nitrate Reductase
$ONOO^-$	Peroxynitrite
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDF	plant defensin
PR	Pathogen Related protein
Pst DC 3000 (*avrB*)	*Pseudomonas syringae* pv *tomato* DC3000 carrying *avrB*
Pst DC 3000	*Pseudomonas syringae* pv *tomato* DC3000
R	Resistance gene
RNA	Ribonucleic acid
RNI	Reactive Nitrogen Intermediate
ROI	Reactive Oxygen Intermediate
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SNAP	S-nitroso-N-acetyl-penicillamine
SNO	S-nitrosothiol
SNP	Sodium Nitroprusside
TAIR	The *Arabidopsis* Information Resource
T-DNA	Transfer DNA
TIR	Toll/Interleukin-1 Receptor
TMV	Tobbaco Mosaic Virus

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Chapter 1

1. Introduction

1.1 General Introduction

Despite the substantial advances in controlling plant disease brought about by the use of chemical pesticides, crop yields are still dramatically reduced by pathogens and pests. The impact of such is particularly acute in developing nations. It is estimated that 12% of potential global crop production is lost annually to pre-harvest plant disease (Agrios 1997, Shah 1997). The United Nations median population assessments predict that by the year 2020 the population will have increased from 6 billion to around 8 billion (Trewavas, 2001). In order to be able to feed this increasing population, an annual increase of 1.3% in food production would be necessary and a 3-fold increase in cropland. However, it is not feasible to increase crop land since most of the best agricultural land is in use and the use of marginal lands would not give good crop yields. From 1961 to 1993 the population almost doubled, increasing from 3 billion to 5.5 billion whereas there was a relatively small increase in cropland from 1340Mha to 1450 Mha (Trewavas, 2001). These improvements in agricultural efficiency and crop yield were brought about by technological advances; including better plant breeding, irrigation and the use of chemical fertiliser and pesticides (McDowell, 2003). Although pesticides provide effective protection against plant pathogens, they are often expensive and their application may have adverse environmental effects.

Therefore new technology is required to provide solutions to sustain the increasing population though more efficient food production. Recent advances in biotechnology including the genetic engineering of crops have shown promising results in some countries. Despite this there remains widespread scepticism about such biotechnology especially in the UK (Coghlan, 2003). In China, many farmers are already growing GM crops including pest and disease-resistant rice, cotton, tomatoes and sweet peppers (Huang et al., 2002). *Bt* (*Bacillus thuringiensis*) cotton, which carries a gene encoding an insect toxin, is the biggest success (Pearce, 2002). Around 2 million Chinese cotton farmers now grow *Bt* cotton. Production costs have dropped by 28 per cent and the use of toxic pesticides such as organophosphates has plummeted by 80 per cent (Pearce, 2002).

Thus, by gaining a greater understanding of the genetic and molecular signalling processes involved in plant disease resistance it may be possible to apply this knowledge to develop transgenic crops with increased disease resistance or through novel pesticides which activate plant defence responses.

1.2 Disease resistance in plants

Plants are sessile organisms and therefore must be able to cope with a wide range of biotic and abiotic environmental challenges. Biotic stresses come from plant pathogens and parasites, including fungi, bacteria, nematodes, viruses and insects. To prevent colonisation by these pathogens and parasites, plants have evolved elegant defence mechanisms. Their first means of defence are physical and chemical barriers provided by the outer waxy cuticle of the plant and preformed anti-microbial compounds (Osbourn, 1996). Once these defences are breached by pathogens there is a rapid production of reactive oxygen intermediates (ROI) and programmed cell death (PCD) at the site of attempted pathogen infection. These events are part of the hypersensitive response (HR) (Lamb and Dixon, 1997). The recognition of pathogens is dependent upon the expression of a corresponding pair of genes in the plant and pathogen, known as resistance (*R*) and avirulence (*avr*) genes (Flor, 1971). Following the HR, levels of salicylic acid (SA) increase locally and systemically. The accumulation of SA is known to activate the expression of pathogenesis related (*PR*) genes, which leads to the establishment of systemic acquired resistance (SAR). This provides immunity to subsequent attack by a broad range of pathogens (Durner et al., 1998).

The oxidative burst is one of the most rapid plant defence responses, leading to the production of reactive oxygen intermediates (ROI), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Apostol et al., 1989). Recent evidence suggests nitric oxide (NO) (Delledonne et al., 1998; Durner et al., 1998) and reactive nitrogen intermediates (RNIs) such as S-nitrosoglutathione are also important as early signalling molecules (Delledonne et al., 1998). Although these molecules are emerging as key signals involved in plant defence responses, little is known about their generation, regulation and the downstream signal transduction pathways.

1.3 Gene-for-gene resistance

1.3.1 R-genes

Plants remain vigilant to pathogens which circumvent the initial physical plant defences, by the expression of an array of plant resistance (*R*) genes. Recognition of pathogen attack by plants requires the presence of *R*-gene products and the corresponding pathogen (*Avr*) avirulence gene product. This was first described by Flor as gene-for-gene resistance (Flor, 1971). Plant *R*-genes fall into five different classes.

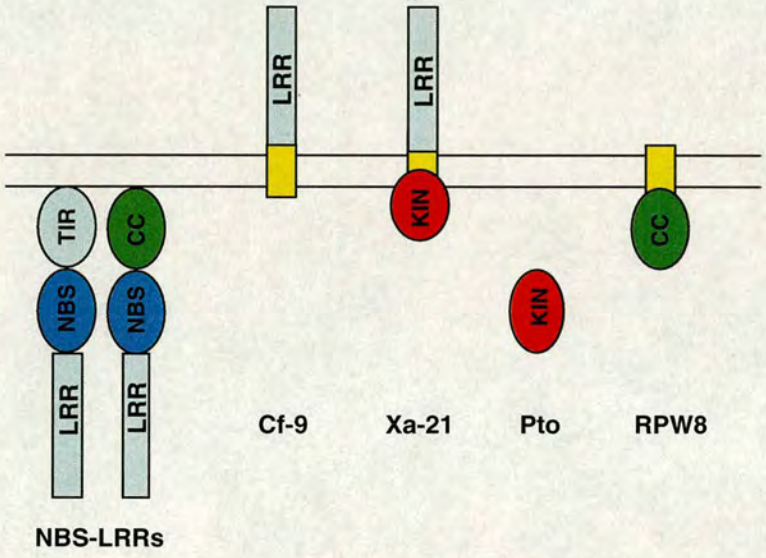

Figure 1.1 Structure and function of the five main classes of *R*-gene products.

The largest class of R-proteins the NBS-LRR (light blue) class are cytoplasmic with distinct N-terminal domains. Xa21 and Cf-X proteins carry transmembrane domains (yellow) and extracellular LRRs. Pto encodes a cytoplasmic serine/threonine kinase. RPW8 carries a putative signal anchor at the N-terminus. Adapted from Dangl and Jones, 2001.

The majority of *R*-genes encode proteins with a nucleotide binding site (NBS) and an intracellular leucine rich repeat (LRR) region. LRR domains are found in diverse proteins and function in protein-protein interactions and protein-carbohydrate interactions. These can be subdivided into two subclasses based on N-terminal features; one with a 'Toll and interleukin-1 receptor' homology region and the other with a predicted coiled-coil (CC) motif (Dangl and Jones, 2001). The remaining four classes of *R*-genes encode structurally diverse proteins (Figure 1.1). The rice *Xa21* gene for resistance against *Xanthomonas* encodes a transmembrane receptor carrying a large extracellular LRR domain and an intracellular protein kinase domain (Song et al., 1995). Whereas the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* encodes a

transmembrane protein with extracellular LRRs but no intracellular region (Jones et al., 1994). *Pto* from tomato encodes a Ser/Thr kinase that confers resistance to *Pseudomonas syringae* strains expressing *avrPto*. *Pto* has no transmembrane or extracellular domain and requires the function of the NBS-LRR protein *Prf* (Salmeron et al., 1996). Finally, a new *R*-gene has been discovered in *Arabidopsis* called *RPW8* which encodes a small, probable membrane protein, with a possible intracellular CC domain, but no other homology to known proteins (Dangl and Jones 2001) (Figure 1.1).

Most NBS-LRR *R*-genes signal through either EDS1 (enhanced disease susceptibility) or NDR1 (non-race specific disease resistance) signal transduction pathways (Aarts et al., 1998). EDS1 has homology to lipases although no catalytic function has been yet been demonstrated (Falk et al., 1999) and NDR1 encodes a putative glycosylphosphatidylinositol (GPI) anchored protein (Holt et al., 2003). *R*-genes which are dependent on EDS1 are TIR-NB-LRRs class *R*-genes, whereas NDR1 dependent *R*-genes are of the CC-NB-LRRs class. Mutations in EDS1 or NDR1 block signalling through the cognate *R*-gene signalling pathway and result in the loss of disease resistance (Aarts et al, 1998). However, some *R*-genes, for example *RPP13*, require neither EDS1 nor NDR1 and therefore may act via a novel signalling pathway (Bittner-Eddy and Beynon, 2001). Alternatively, EDS1 and NDR1 may function redundantly in *RPP13* signalling (Bittner-Eddy and Beynon, 2001).

1.3.2 Avr Genes

Avr genes can be chromosomal or plasmid borne and encode diverse hydrophilic, signalling molecules (Bonas and Van den Ackerveken, 1999). *Pseudomonas syringae avr* genes are located in regions flanking the pathogenicity gene *hrp* (hypersensitive response and pathogenicity) cluster. Bacterial Avr protein function requires the *hrp* genes, which encode type III protein secretion machinery (Ham et al., 1998). Among the proteins secreted by the type III secretion system of *Pseudomonas* species, is the subunit of a pilus-like bacterial surface structure. This appendage allows the bacteria to deliver virulence/avirulence factors into the plant cell cytoplasm (Ham et al., 1998). Avirulence proteins are thought to contribute to pathogen virulence in the absence of a corresponding *R*–gene product. Thus Avr proteins are effectors interacting with particular target proteins in the plant to manipulate the host's metabolism in favour of

the pathogen. For example, *Arabidopsis* lines without the *R*-gene *RPS2* were found to be more susceptible to the virulent *Pseudomnas syringae* pv. *tomato* strain *Pst* DC3000 carrying *avrRpt2*, than to *Pst* DC3000 lacking *avrRpt2*. Transgenic *Arabidopsis* lines expressing *avrRpt2* were generated and this was also found to promote the virulence of *Pst* DC3000. Thus, AvrRpt2 appears to promote pathogen virulence from within the plant cell (Chen et al., 2000).

1.4 The guard hypothesis

Initially it was proposed that there may be a direct interaction between *R*-proteins and *Avr* gene products in the cytoplasm. However, the *in vitro* interaction of the *Pi-ta* R-protein from Rice and the corresponding avirulence protein, AvrPita from the pathogen *Magnoporthe grisea* is one of the few examples of a direct interaction between an R-protein and the corresponding Avr protein (Jia et al., 2000). Pto from tomato is a serine/threonine kinase that can phosphorylate protein targets with defence functions. Pto also interacts directly with its corresponding avirulence protein, avrPto (Loh and Martin, 1995). However, Pto requires an NBS-LRR type protein called Prf to trigger defence responses against *Pseudomonas syringae* strains expressing avrPto (Salmeron et al, 1996). The guard hypothesis has been suggested to explain why Pto requires Prf to activate defence upon recognition of AvrPto (Dangl and Jones, 2001). This theory proposes that R-proteins 'guard' plant proteins (guardee), which are targeted for modification by the pathogen in order to create a favourable environment for themselves (Figure 1.2). Once this guardee protein has been modified it is detected by the R-protein and resistance is activated (Dangl and Jones, 2001). For example, Avr Pto functions as a virulence factor targeting the Pto Kinase in an attempt to disrupt disease resistance. Prf guards Pto and detects Pto-AvrPto interactions activating defence responses. Therefore the Avr-guardee interaction in the absence of an R-protein is a positive virulence mechanism (Holt et al, 2003). Furthermore, *Arabidopsis* plants expressing *RPM1* are resistant to *Pseudomoas syringae pv maculicola* expressing *avrRPm1*. No physical interaction between RPM1 and avrRPm1 has been detected. The recently discovered RIN4 protein has been found to interact with both RPM1 and avrPM1 in yeast two-hybrid assays and *in vivo*. The interaction of RIN4 with RPM1 and avrRpm1 is necessary for the establishment of the HR (Mackey et al, 2002). Thus RPM1 may guard RIN4 from avrRPM1.

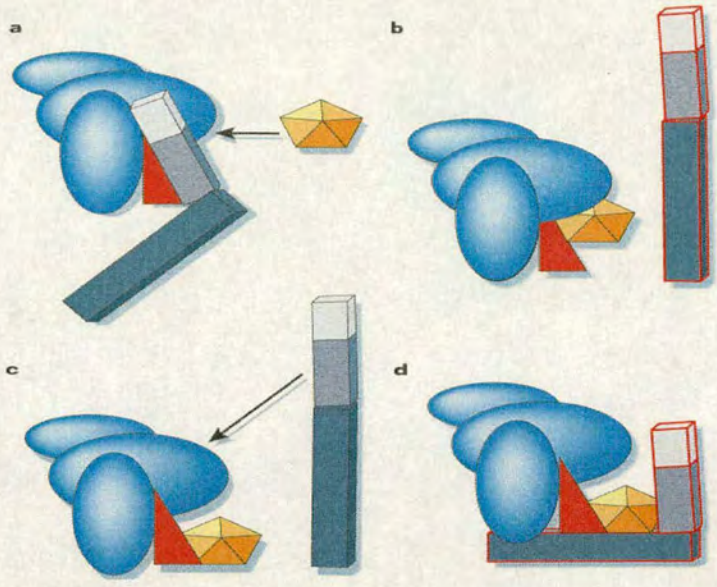

Figure 1.2 The guard model for R protein function

The cellular complex of proteins is in blue, whereas the guardee/virulence target is in red, the R-protein in grey, and the avirulence factor of disease in orange (Dangl and Jones, 2001).

1.5 The Nitric Oxide (NO) and oxidative burst

1.5.1 Synthesis of NO

Recent evidence has shown that following pathogen recognition there is a rapid burst of NO production in plants (Delledonne et al., 1998). The important role of NO as a signalling molecule in animal cells has been established for over a decade. It has a range of physiological effects including the relaxation of smooth muscle, neural communication and immune regulation (Wendehenne et al., 2001). Recent studies suggest that NO is also a key signalling molecule in plant physiology, where it has been implicated in development and defence responses (Delledonne et al., 1998; Beligni and Lamattina, 2000).

Measurements of gaseous emissions from plants have shown that NO can be synthesised in plants both by non-enzymatic and enzymatic reactions (Wendehenne et al., 2001). It can be produced non-enzymatically from NO_2^- by a reductant such as

ascorbate under acidic conditions or by carotenoids in the presence of light (Wojtaszek, 2000). For example NO_2^- was shown to be reduced to NO in the apoplast of barley aleurone layers (Bethke et al., 2004). Alternatively, NO can be produced enzymatically by the enzyme nitrate reductase (NR), which is found in the cytosol. NR is a key enzyme involved in nitrogen assimilation in plants, catalysing the transfer of electrons from NADPH to nitrate to produce nitrite. However, NR can also catalyse the NADPH dependent reduction of nitrite to NO and such activity has been measured in soybean (Dean and Harper, 1988), maize (Yamasaki et al., 1999) and corn (Yamasaki et al, 2000). NO synthesis in *Arabidopsis* guard cells which is required for stomatal closure was found to be mediated by NR activity (Desikan et al, 2002). It has been proposed that NR activity constitutively produces NO in non-elicited leaves (Rockel at al., 2002). In mammals NO biosynthesis is catalysed by NO synthase(s) (NOS), a group of evolutionarily conserved cytosolic or membrane bound isoenzymes which catalyse NO and L-citrulline formation from O_2 and L-arginine (Figure 1.3) (Mayer and Hemmens, 1997). There are three main NOS isoforms. The neural and endothelial isoforms (nNOS and eNOS respectively), which are constitutively expressed in mammals, whereas the macrophage NOS (iNOS) is induced by immunological stimuli (Bogdan et al., 2000). Moreover, a new NOS isoform was found in mitochondria isolated from rat liver, named mtNOS (Wendehenne et al., 2001). The activity of nNOS and eNOS is controlled by the concentration of intracellular free Ca^{2+} unlike that of iNOS activity, which is independent of intracellular free Ca^{2+} (Nathan and Xie, 1994).

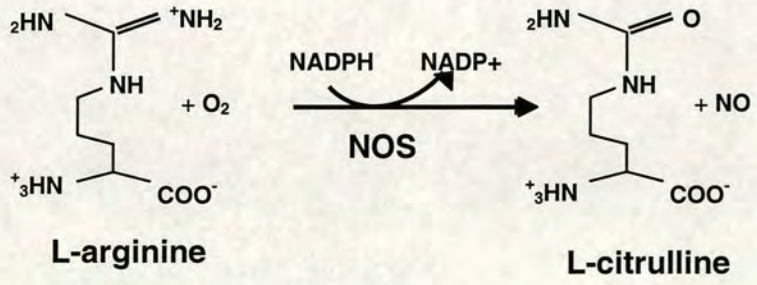

Figure 1.3. The reaction catalysed by mammalian NOS.

The conversion of L-arginine to L-citrulline and NO requires oxygen and NADPH. Adapted from Wendehenne et al., 2001.

The source of NO production in plants has been the focus of much debate. Until recently no plant NOS genes had been identified; although several studies suggested the presence of a NOS type enzyme in plants. For example, the conversion of L-arginine to L-citrulline has been measured in maize (Ribero et al., 1999), pea (Lesham and Haramaty, 1996; Barroso et al., 1999) and tobacco (Durner et al., 1998) and this activity was found to be sensitive to NOS inhibitors in the latter two species. In several studies this NOS activity was found to be Ca^{2+} dependent suggesting similarities with the constitutive nNOS and eNOS in mammals (Wendehenne et al., 2001). Low constitutive levels of NOS activity have been measured in tobacco and this activity was found to increase upon infection with tobacco mosaic virus (TMV) (Durner et al., 1998). NO was also found to increase in soybean (Delledonne et al., 1998) and *Arabidopsis* (Clarke et al., 2000) cell suspensions in response to inoculation with *Pseudomonas syringae* pv *glycinea* and *Pseudomonas syringae* pv *maculicola* respectively. NO production is biphasic showing a similar pattern to that of the oxidative burst, an initial peak of NO is seen about 1-2 hours after infection followed by a second greater peak 3-6 hours after infection. However, the second peak is only observed after inoculation with an avirulent *Pseudomonas* species (Clarke et al., 2000; Delledonne et al., 1998)

Recently two NOS genes were discovered in plants, which have no sequence similarity to known mammalian type NOS. The first NOS to be discovered was from tobacco and encodes a variant of the P protein of glycine decarboxylase (GDC). This NOS is induced in response to tobacco mosaic virus (TMV), in TMV-resistant (Xanthi nc [NN]) plants, but not in TMV-susceptible plants (Chandok et al, 2003). The same study found that NOS activity is also induced in resistant *Arabidopsis* plants after inoculation with turnip crinkle virus (TCV) (Chandok et al, 2003). It was shown recently that virus-induced gene-silencing of a putative tomato ortholog of the tobacco iNOS, led to the complete suppression of *Pst* DC3000-induced iNOS activation and increased bacterial growth in both resistant and susceptible tomato plants (Chandok et al., 2004). The induction of this plant NOS is similar to that of the animal iNOS, which is only induced in response to pathogen infection. Although, the activity of the two are controlled differently, since the plant iNOS requires both Ca^{2+} and calmodulin (CaM) for activity, whereas the animal iNOS activity is independent of intracellular free Ca^{2+}.

The second plant NOS to be discovered (*AtNOS1*) (Guo, et al 2003) has sequence similarity to a gene implicated in NO synthesis in the central nervous system of the snail

Helix pomatia (Huang et al, 1997). *Arabidopsis* T-DNA knockout mutants of *AtNOS1* were shown to have lower levels of NO production using the NO fluorescent indicator dye, diaminofluorescein diacetate (DAF-2 DA). These mutants have shorter roots as well as reduced reproductive growth and fertility compared to wild type plants. *AtNOS1* mutants also have impaired abscisic acid–induced stomatal movement. *AtNOS1* unlike the plant *iNOS* is constitutively expressed but similar in that Ca^{2+} and CaM also control its activity. Therefore, *AtNOS1* resembles the constitutive mammalian eNOS and nNOS that are stimulated by Ca^{2+}.

There is little information available about the subcellular localisation of NOS activity in plants. Western blot analysis of soluble root fractions from maize using anti-mouse and anti-rabbit iNOS revealed the presence of an immunoreactive protein localised in the cytoplasm (Ribeiro et al., 1999). This protein was found to be translocated to the nucleus depending on the phase of cell growth. NOS activity has also been reported in the peroxisomes of pea. Further analysis in pea with anti-NOS antibodies revealed an immunoreactive protein localised to peroxisomes and chloroplasts (Barroso et al., 1999). Using DAF-2DA to visualise NO, tobacco cells treated with the fungal elicitor cryptogein were found to respond by producing a burst of NO. The earliest increases in NO production were found in chloroplasts, where NO accumulation occurred after 3 minutes. The cytosol and the plasma membrane showed NO production after 6 minutes (Foissner et al., 2000). Similarly, DAF-2DA studies in *Arabidopsis* cells after wounding stress showed the production of NO localised to chloroplasts and the cytosol (Garces et al., 2001).

Thus, plants like animals employ different enzymes for NO synthesis, which are present in different cellular locations. These enzymes which are either constitutive or inducible perform diverse roles from hormonal signalling and growth to defence responses.

1.5.2 Metabolism of NO

Both pathogens as well as their respective hosts try to reduce nitrosative stress by eliminating and/or scavenging NO derivatives. In bacteria and yeast, flavoheamoglobins are thought to function as dioxygenases, breaking down NO to protect the cell from nitrosative stress. *Escherichia Coli* (*E-Coli*) possesses a flavohaemoglobin (*Hmp*) which catalyses NO consumption, *hmp* mutants are more sensitive to the NO donors Sodium

Nitroprusside (SNP) and S-nitrosoglutathione (GSNO) (Membrillo-Hernandez et al., 1999). Studies with the human fungal pathogen *Cryptococcus neoformans* that replicates in macrophages, demonstrated that a mutation in flavoheamoglobin denitrosylase (*FHB1*) leads to reduced fungal virulence (de Jesus-Berrios et al, 2003). Furthermore, by combining mutations in *FHB1* and in glutathione-dependent formaldehyde dehydrogease (*GS-FDH*), which encodes a GSNO reductase, pathogen virulence was further attenuated. The virulence of these *fhb1* mutants could be restored in NOS-/- animals that are unable to produce an NO burst in response to pathogens (de Jesus-Berrios et al, 2003). There is evidence that plant pathogens also use flavoheamoglobins to avoid nitrosative stress. For example, *Erwinia chrysanthemi* deploys a flavoheamoglobin to scavenge NO in response to the tobacco NO burst (Favey et al., 1995). NO/heam complexes have been detected in maize cell cultures and significant amounts of NO have been shown to be formed in hypoxic maize cells. Transformed maize lines with reduced stress-induced haemoglobin expression produced greater amounts of NO than wild-type. This suggests that stress-induced haemoglobins may function as dioxygenases, detoxifying NO produced during hypoxia (Dordas et al., 2003). Furthermore, large increases in NO production were found in alfalfa root cultures under hypoxic stress. Transgenic alfalfa expressing antisense barley haemoglobin transcripts were found to have 2.5 fold higher levels of NO than transgenic lines expressing sense transcripts, suggesting that haemoglobin modulates NO in the hypoxic cell (Dordas et al., 2003). An alfalfa haemoglobin (Mhb1) was identified which is induced by hypoxia (Sergelyes et al., 2000). Immunolabelling using a polyclonal antibody against Mhb1 revealed that it is localised in the nucleus and to a lesser extent the cytosol. Transgenic tobacco plants over expressing *Mhb1* from alfalfa were found to be more resistant to SNP. Leaves treated with SNP and tobacco necrosis virus showed reduced necrosis in transgenic tobacco compared to wild type. These plants also showed elevated levels of ROI and SA (Seregelyes and Dudits, 2003).

Bacterial peroxiredoxins have peroxynitrite reductase activity and are widespread among bacterial genera. The peroxiredoxin alkylhyroperoxide reductase subunit (*AhpC*) from *Salmonella typhimurium* was found to catalytically detoxify peroxynitrite and similar results were found with peroxiredoxins from *Myxobacterium tuberculosis* and *Helicobacter pylori* (Bryk et al, 2000). There are 10 peroxiredoxin genes in *Arabidopsis* which can be grouped as 1-Cys Prx, 2-Cys Prx, type II Prx, and Prx Q (Dietz et al., 2002). Plant 1-cysteine peroxiredoxin (1-CP PER1) was identified as a 'dormancy-

related protein', which is expressed in the aleurone cell layer of barley. Immunocytochemistry revealed that PER1 1-CP is preferentially localised in the nucleus (Stacy *et al.*, 1996). Two peroxiredoxin genes, 2-cysteine peroxiredoxin A and B (*2CP-prx A* and *2CP-prx B*) show high sequence homology to bacterial peroxiredoxins, (Dietz et al., 2002). Both these genes contain transit peptides for post-translational import into the chloroplast. Antisense *2CP-prxA Arabidopsis* plants show disturbed early shoot development and impaired photosynthesis. They also have increased expression of the antioxidant genes *SOD* and peroxidase, indicating that these lines suffer from oxidative stress (Dietz, et al 2002). The third class of plant peroxiredoxin-like proteins (type II peroxiredoxins) were identified in a screen for proteins binding to a modified thioredoxin (Choi et al., 1999). This class of peroxiredoxins is suggested to be targeted to the peroxisomes of eukaryotic cells and to be involved in detoxifying H_2O_2 or alkyl hydroperoxide, produced by the reaction of lipids with reactive oxygen species in the peroxisomal membrane. Recently, the fourth type of peroxiredoxin, a homolog of the bacteriferritin co-migratory protein of *E. coli*, was identified in *Sedum lineare* (Kong *et al.*, 2000). As yet there is no report of plant peroxiredoxins metabolising peroxynitrite.

A mechanism to cope with nitrosative stress, which is unique to plants, operates in the mitochondria. NO inhibits cellular respiration by affecting the terminal enzyme of the respiratory chain, cytochrome *c* oxidase. Plant mitochondria possess a second terminal oxidase, called the alternative oxidase (AOX), which catalyses the oxidation of ubiquinol and the reduction of oxygen to water, bypassing the cytochrome c step (Huang et al., 2002). Treatment of *Arabidopsis* plants with NO gas was found to induce *AOX1a* and this induction was suppressed using the NO scavenger cPTIO (carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl) (Huang et al., 2002). The induction of *AOX* by NO was not found to be dependent on SA, since *NahG* plants (which express the SA-degrading enzyme salicylate hydroxylase) were able to express *AOX* in response to NO. Plants may also be able to reduce ROI generation during periods of high respiration via the induction of *AOX*. Since ROI and NO synergistically induce cell death (Delledonne et al., 2001) the action of AOX may be important to protect from cell death. In fact the AOX inhibitor salicylhydroxamic acid (SHAM) together with NO treatment was found to increase cell death compared to controls

treated with SHAM or NO only (Huang et al., 2002). Thus AOX may have an important role in cell death induction and NO's redox-regulating properties.

1.5.3 S-nitrosoglutathione (GSNO)

Evidence from animals indicates that NO biology involves S-nitrosothiols such as S-nitrosoglutathione (GSNO), which are central to signal transduction and host defences (Liu et al., 2004; Mayer et al., 1998). In mammals S-nitrosothiols circulates in the blood as S-nitrosoheamoglobin, acting as both an intra and intercellular NO carrier (Rassaf et al., 2002; 2004; Gow and Stamler, 1998). GSNO is an adduct of NO with glutathione (GSH), formed primarily by nitrosation of GSH with NO in the presence of oxygen, once formed GSNO is more stable than NO (Fernandez et al., 2003). It is thought that GSNO acts as an NO reservoir, which can release NO after reaction with Cu^{2+} (Gorren et al., 1996). It has been found to be biologically active as a vasodilator in animals, preventing platelet adhesion and aggregation (Radomski et al., 1992; Ignarro et al., 1981). GSNO can also post-translationally modify proteins by the S-nitrosylation of critical cysteine residues (Stamler et al., 1992; Jaffery et al., 2001). GSNO may be produced as a result of NOS activity, since O_2^- as well as NO is produced by NOS, therefore in the presence of GSH it is expected that GSNO would be formed. There is HPLC evidence using NOS incubates that this is in fact the case (Tsikas et al., 2000).

GSNO has strong antioxidant properties it can protect from oxidative brain injury in low nanomolar doses *in vivo* (Chiueh, 1999). It has been found to be 100 fold more potent than GSH at suppressing the iron-induced generation of •OH, protecting the brain from lipid peroxidation (Rauhala et al., 1998). It can also protect against oxidative stress in endothelium tissue (Chiueh, 1999). Thus GSNO may be part of an antioxidative cellular defence system.

GSNO in animals can also have a pro-apoptotic function depending on the concentration and the cell type involved. GSNO induced apoptosis in RAW 264.7 mouse macrophages causing DNA fragmentation after four hours (Messmer et al., 1995). It has also been found to induce apoptosis and DNA fragmentation in human neutrophils, where the apoptosis-to-total cell death ratio of the neutrophils increased as GSNO levels increased. Interestingly, these effects could be attenuated, by the co-incubation of superoxide dismutase (Fortenberry et al., 1999).

GSNO has also been implicated in the plant defence response. The administration of GSNO to tobacco plants or tobacco suspension cells triggered expression of the plant defence-related genes encoding PR1 and phenylalanine ammonia lyase (PAL) (Durner et al., 1998). As mentioned above GSNO may be produced as a result of NOS activity (Tsikas et al., 2000). The discovery of NOS in plants (Guo et al., 2003) suggests that GSNO could also be produced by NOS in plants. There is evidence, which shows NOS activity is present in peroxisomes (Barroso et al., 1999). Studies with anti-NOS antibodies have shown immunoreactive proteins localised in the nucleus, cytosol, peroxisome and chloroplasts (Ribeiro et al.,1999; Barroso et al., 1999). Furthermore, NO production has also been visualised in the cytosol and chloroplast using DAF-2DA staining (Foissner et al., 2000; Garces et al., 2001). Therefore in plants, GSNO has the potential to be formed in the cytosol, peroxisome or chloroplast from the reaction of NO and GSH in the presence of ROIs.

1.5.4 GSNO reductase

Since GSNO is a biologically active signalling molecule it is therefore necessary to regulate levels of GSNO. Glutathione-dependent formaldehyde dehydrogenase (GS-FDH) or *S*-Nitrosoglutathione reductase (GSNOR) is an enzyme which has high affinity for GSNO (Liu et al., 2001). GS-FDH is conserved in animals, bacteria and plants (Liu et al., 2001). In yeast this enzyme is localised in the cytosol and nuclear compartments (Fernandez et al., 2003). This localisation of GS-FDH is in agreement with previous studies in rat hepatocytes (Iborra et al., 1992). It is member of the class III alcohol dehydrogenase (ADH) family of genes. Although GS-FDH has overall similarities to the domains of other ADHs it is unable to breakdown ethanol (Dolferus et al., 1997). Instead GS-FDH has GSNO reductase activity determined by the GSNO specific oxidation of NADH that allows the reduction of GSNO to S-amino-L-glutathione via an S-hydroxylamine intermediate. In *E.Coli* the final products of GSNO reduction were found to be glutathione disulphide (GSSG) and ammonia (NH_3), as shown in Figure 1.4 (Liu et al., 2001).

$$GSNO + NADH + H^+ \rightarrow GSNHOH + NAD^+$$

$$GSNHOH + NADH + H^+ \rightarrow GSNH_2 + NAD^+ + H_2O$$

$$SNH_2 + GSH \rightarrow GSSG + NH_3$$

Figure 1.4 The reduction of GSNO by GSNOR.

The main products of the *E.Coli* GSNO reductase are GSSG and NH_3 (Liu et al., 2001)

GS-FDH knockout mutants in mice and yeast demonstrated that these mutants were hypersensitive to nitrosative challenge (Liu et al, 2001). Moreover *GS-FDH* knock-out mice were found to show increased whole-cell S-nitrosylation, tissue damage and mortality following endotoxic or bacterial challenge (Liu et al., 2004).

GS-FDH has been identified in *Arabidopsis* (Sakamoto et al., 2002), Pea (Shafqat et al., 1996), Rice (Dolferus et al., 1997) and maize (Fliegmann et al., 1997). However, the subcellular localisation of GS-FDH in plants has not yet been demonstrated. The expression of *GS-FDH* cDNA from *Arabidopsis* in *E.Coli* confirmed its ability to reduce GSNO. This cDNA was also found to functionally complement yeast mutants hypersensitive to GSNO, identifying it as a GSNOR (Sakamoto et al., 2002). Recently, the *Arabidopsis GS-FDH* was found to be down regulated by wounding and up regulated by salicylic acid (Diaz et al., 2003).

In addition to its ability to metabolise GSNO, GS-FDH is also able to catalyse the NAD+ dependent formation of S-formylglutathione from S-hydroxymethylglutathione (HM-GSH) that occurs through the spontaneous interaction between formaldehyde and glutathione (Sakamoto et al., 2002). Thus it was predicted that GS-FDH is also involved in the metabolism and detoxification of formaldehyde. This housekeeping function of GS-FDH was discovered before its GSNOR activity. It is worth noting that GSNO and HM-GSH have similar structures and that this may explain the catalytic efficiency of GS-FDH to metabolise both substrates (see Figure 1.5). However, GSNOR/GS-FDH has far higher affinity for GSNO (Liu et al., 2001).

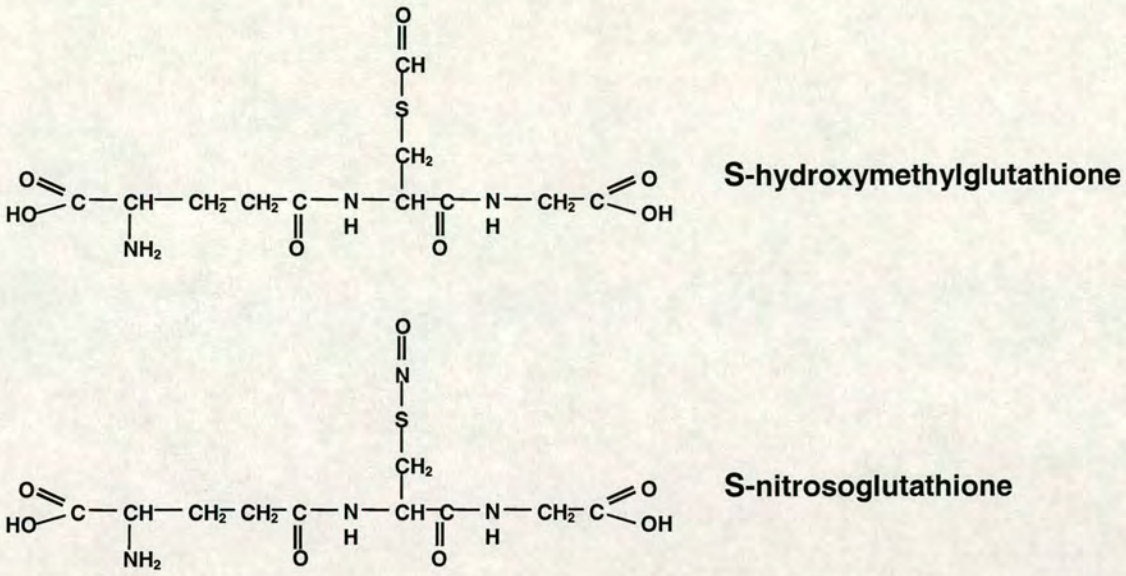

Figure 1.5 Structure of S-nitrosoglutathione and S-hydroxymethylglutathione

Both GSNO and HM-GSH are substrates for GSNOR/GS-FDH.

The housekeeping function of GSNOR/GS-FDH is now being challenged. In *situ* hybridisation studies in *Drosophila* and zebra fish have shown that *GS-FDH* expression is not ubiquitous; it is expressed at particular developmental stages and gradients in expression occur along the anterior-posterior axis in these organisms (Canestro et al., 2003). Moreover, in mammalian tissue up to a 30-fold difference in *GS-FDH* expression has been described (Uotila and Koivusalo, 1997). It has been proposed that GS-FDH is involved in development and growth in mammals by stimulating the production of retinoic acid (RA). Vitamin A (retinol) is oxidised sequentially to retinal and then RA, which is perceived by RA receptors that are essential in the development of specific tissues. *GS-FDH* null mutant mice exhibit reduced survival compared to wild type, when fed with a vitamin A deficient diet (Molotkov et al., 2002). It was shown that *GS-FDH* has retinol oxidation activity *in vitro* and as a consequence *GS-FDH* null mice were found to have a 3.8 fold reduction in RA production compared to wild-type (Molotkov et al., 2002).

1.5.5 Synthesis of Reactive Oxygen Intermediates (ROIs)

The oxidative burst in plants is a biphasic response to pathogens. An initial peak of ROI production is seen about 1-2 hours after infection followed by a second greater peak 3-6 hours after infection. This second peak is only seen following infection with an avirulent pathogen (Grant and Loake, 2000). There has been some debate about the origin of the oxidative burst and several sources are known to exist for the generation of ROI. These include a plasma-membrane NADPH oxidase, cell wall peroxidases, oxalate oxidases and protoplastic sources from mitochondria, chloroplasts and peroxisomes. Of these possible systems the neutrophil-like NADPH oxidase system, analogous to the mammalian neutrophil NADPH oxidase, has received the most attention. This complex consists of five components, $gp91^{phox}$, $p22^{phox,}$ $p47^{phox}$, $p40^{phox}$ and $p67^{phox}$ (Lal et al., 1999). The first two are membrane bound, whereas $p47^{phox}$, $p40^{phox}$ and $p67^{phox}$ are cytosolic, associating with the plasma membrane after several phosphorylation events (Lal et al., 1999). The small GTP-binding protein $p21^{rac2}$ is also involved in the assembly of the five components to form an active NADPH complex (Diekmann et al., 1994). Superoxide is produced by the active NADPH complex (O_2+ NADPH$\rightarrow$ O_2^- + $NADP^+ + H^+$), and is then converted to H_2O_2 by dismutation of O_2^- to H_2O_2. Analogues to the mammalian $gp91^{phox}$ have been cloned from rice and there are at least six homologs (*Atrboh* A-F) that have been identified in *Arabidopsis*, suggesting that a similar system exists in plants. Atrboh D and F are required for the accumulation of ROIs in the plant defence response (Torres et al., 2002). However, most of the plant equivalents of the mammalian components have only been demonstrated by cross reactivity with antibodies (Bowell, 1999). These data are controversial since no homologs of the mammalian burst oxidase components ($p47^{phox}$, $p40^{phox}$ and $p67^{phox}$) other than $p21^{rac2}$ have been identified in the *Arabidopsis* genome (The *Arabidopsis* genome initiative, 2000). The discovery that G-proteins may be involved in the control of the mammalian NADPH oxidase complex led to the discovery of a rice gene homolog *OsRac1*, which may be involved in the regulation of a NADPH oxidase in plants (Kawasaki et al., 1999). The expression of a constitutively active *OsRac1* in transgenic rice plants resulted in ROI production, whereas expression of a dominant negative form of *OsRac1* blunted ROI generation (Kawasaki et., 1999). An alternative way in which ROI production may be regulated is through calcium signalling. The interaction of either *avr* and *R* gene products or the exogenous application of fungal

elicitors are known to elevate cytosolic [$Ca2^+$] rapidly by activating plasma membrane calcium channels (Zimmermann et al., 1997). The $gp91^{phox}$ plant homologs ATRBOH A-F contain two EF hand (calcium binding) motifs and thus it is possible that elevated cytosolic [$Ca2^+$] promotes the oxidative burst through the activation of the NADPH oxidase complex (Keller et al., 1998).

Phospholipase D is particularly significant in animal cells for the activation of NADPH oxidase; thus equivalent roles for this enzyme have been searched for in plants. Phospholipase D (PLD) expression has been demonstrated in response to infection with *Xanthomonas orzyae* in rice. Using an antibody PLD was shown to be recruited to the plasma membrane at the point at which the *X. orzyae* attacked the cell (Young et al., 1996). PLD was also shown to be activated in tomato cells within minutes of treatment with the elicitor xylanase (Laxalt et al., 2001). Phospholipase A (PLA) is also thought to be involved in ROI production, as inhibitors of PLA blunt the oxidative burst in tobacco cells following recognition of avirulent *Cladosporium fulvum* (Xing et al., 1997). Genes have been identified in *Arabidopsis* that encode phospholipase-like enzymes. For example *PAD4* (phytoalexin deficient) and *EDS1* (enhanced disease susceptibility), which are required for disease resistance (Falk et al., 1999; Jirage et al., 1999). Thus it is possible that *PAD4* and *EDS1* may be involved in the regulation of the oxidative burst via modulating the activity of a NADPH oxidase. Moreover, ROI levels were shown to be lower in *eds1* and *pad4* mutants than in wild-type plants after attempted infection with the non-host pathogen *Blumeria graminis f.* sp. *tritici* (*Bgt*) (Yun et al., 2003).

Cell wall peroxidases have also been shown to generate H_2O_2 during the oxidative burst, through a superoxide–binding intermediate that requires alkaline conditions (Bowell, 1996). Apoplastic alkalisation is a common feature following attempted pathogen infection; thus rapid alkalinisation of the apoplast via plasma membrane ion channels could activate cell wall peroxidases. It has also been demonstrated that a peroxidase is secreted to bacterial infection sites (Bowell, 1999). Potential reductants for a cell wall peroxidase include ascorbate and NADPH. Although, a recent study to identify reductants in the apoplastic fluid has established that the best producers of H_2O_2 are saturated fatty acids, so far products of such oxidation reactions have failed to be identified (Bowell, 1999).

In barley, a germin-like oxalate oxidase, which generates H_2O_2, has been shown to accumulate during resistance to *Erisyphe graminis f.sp. Hordei*, suggesting an alternative mechanism of ROI generation may exist in monocots (Zhou et al., 1998)

1.5.6 Metabolism of ROIs

The main enzymes involved in the scavenging of ROI in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Figure 1.6) (Mittler, 2002). SOD and APX are found in most cellular compartments (Bowler et al., 1992). APX requires an ascorbate and GSH regeneration system called the ascorbate glutathione cycle (Figure 1.6c). Glutathione peroxidase (GPX) can also detoxify H_2O_2 to H_2O in the cytosol but uses GSH directly as a reducing agent (Figure 1.6d) (Mittler, 2002). CAT is only found in peroxisomes (Willekwens et al., 1997). CAT and APX have different affinities for H_2O_2 (APX micromolar and CAT millimolar). The different compartmentalisation and H_2O_2 affinities of APX and CAT suggest different roles in H_2O_2-scavenging: APX may be responsible for the fine modulation of ROIs for signalling, whereas CAT might be responsible for the removal of excess ROIs generated in peroxisomes during stress (Mittler, 2002). Antisense *APX* and *CAT* transgenic plants have reduced capability to detoxify ROIs. Therefore, programmed cell death (PCD) was activated in these plants in response to low amounts of pathogens that were not sufficient to trigger PCD in wild-type plants (Mittler et al., 1999).

In addition, ROIs also induce the expression of stress related genes that detoxify lipid peroxidation products, such as glutathione-S-transferases (*GSTs*). There is a large family of GST enzymes in *Arabidopsis*, many classes of *GST* are induced by oxidative stress since they are involved in the detoxification of a wide variety of xenobiotic compounds (Marrs, 1996). *GST*'s are often used as molecular markers of oxidative stress and cell death due to their rapid induction (Grant et al., 2000).

a) Superoxide Dismutase

$$2\,O_2 + 2\,H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

b) Catalase

$$2\,H_2O_2 \xrightarrow{CAT} 2\,H_2O + O_2$$

c) Ascorbate-Glutathione Cycle

1. H_2O_2 + ascorbate $\xrightarrow{APX}$ 2 H_2O + monodeydroascorbate (MDA)
2. MDA + NAD(P)H $\xrightarrow{MDAR}$ ascorbate + $NAD(P)^+$
3. Dehydroascorbate + GSH $\xrightarrow{DHAR}$ ascorbate + GSSG
4. GSSG + NAD(P)H $\xrightarrow{GR}$ GSH + $NAD(P)^+$

d) Glutathione Peroxidase Cycle

1. H_2O_2 + GSH $\xrightarrow{GPX}$ H_2O + GSSG
2. GSSG + NAD(P)H $\xrightarrow{GR}$ GSH + $NAD(P)^+$

Figure 1.6 Enzymatic ROI scavenging mechanisms

The principal modes of enzymatic ROI scavenging by superoxide dismutase (SOD), catalase (CAT), the ascorbate-glutathione cycle and the glutathione peroxidase cycle. **(a)** SOD converts superoxide to hydrogen peroxide. **(b)** CAT converts hydrogen peroxide to water. **(c)** The first reaction of the ascorbate-glutathione cycle is catalysed by ascorbate peroxidase (APX) which converts ascorbate to monodehydroascorbate (MDA). MDA reductase (MDAR) converts MDA to ascorbate. Dehydroascorbate (DHA) is produced spontaneously from MDA and can be reduced to ascorbate by DHA reducatse (DHAR) with the help of GSH which is oxidised to GSSG. The cycle closes with glutathione reductase (GR) converting GSSG back to GSH with the reducing agent NAD(P)H. **(d)** The glutathione peroxidase (GPX) cycle converts hydrogen peroxide to water using the reducing equivalents from GSH. Oxidised GSH is again converted to GSH by GR.

1.6 The Hypersensitive Response (HR)

1.6.1 The role of ROIs in the HR

The generation of ROIs is one of the earliest events in the HR and is thought to play an important role in the induction of PCD. The discovery of *Arabidopsis* lesion mimic mutants such as *lsd* (lesion stimulating disease)*1* suggests that the HR is under genetic control (Jabs et al., 1996). Superoxide, pathogen infection and SA induce runaway cell death in this mutant. It was subsequently found that *LSD1* encodes a zinc finger transcription factor, which is thought to act by inducing CuZn superoxide dismutase in response to SA accumulation (Kliebenstein et al., 1999). Hence, the lack of CuZn superoxide dismutase in the *lsd1* mutant may result in PCD due to high levels of O_2^- accumulation. Moreover, knockout mutants of the *Arabidopsis* NADPH oxidase *AtrbohD* and *AtrbohF* genes which are required for full ROI production during incompatible interactions, show reduced cell death after *Pst* DC3000 (*avrRpm1*) inoculation compared to wild-type plants (Torres et al., 2002).

H_2O_2 has also been implicated in the induction of PCD and the activation of *PR1* gene expression, which leads to the establishment of SAR (Chamnongpol et al., 1998). Transgenic tobacco plants were generated which express an antisense CAT gene and so are compromised in their ability to remove H_2O_2 from their cells. When these transgenic plants were exposed to high light intensities (thus generating high levels of H_2O_2) they subsequently developed HR-like lesions. These plants were shown to accumulate SA and PR proteins in leaves that had been completely covered from the light, indicating that H_2O_2 functions as a trigger for the HR preceding both local and systemic PR responses (Chamnongpol et al., 1998). In a separate study transgenic tobacco plants with reduced CAT activity were also found to accumulate high levels of H_2O_2 under photorespiratory conditions. This perturbation of H_2O_2 homeostasis resulted in the induction of cell death in palisade parenchyma cells, primarily along the veins. This transgenic tobacco line also showed enhanced transcript levels of mitochondrial defence genes such as AOX. (Dat et al., 2003)

Further evidence for the involvement of ROIs in the HR came from the discovery that inoculation of *Arabidopsis* plants with avirulent bacteria (*Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst*)) expressing *avrRpt2* induced low-frequency systemic

microbursts (Alvarez, 1998). These systemic microbursts could also be induced by the infiltration of a H_2O_2 generating system (glucose/glucose oxidase) and were found to be necessary for the establishment of SAR. Thus, ROI are important for the establishment of the HR and function as a reiterative signal underlying local and systemic acquired resistance (Alvarez et al., 1998).

ROIs have been shown to engage a stress-activated class of mitogen-activated protein kinases (MAPKs) in animals. The C-Jun N-terminal kinase cascade is activated in response to H_2O_2 and induces apoptosis in animal cells (Kovtun et al, 1999; Wang et al., 2002). Twenty MAPKs have been identified in *Arabidopsis*, a higher number than in any other eukaryote (The Arabidopsis genome, 2000). Studies in *Arabidopsis* suggest that ROIs also engage MAPK cascades in plants. For example, H_2O_2 initiates a phosphorylation cascade that activates at least two MAPKs including AtMPK3 and AtMPK6 (Kovtun et al., 2000). Furthermore, a 48kDa MAPK may be required for the successful transmission of ROI signalling in the establishment of plant disease resistance (Grant et al., 2000). A study using transgenic *Arabidopsis* plants that express the active mutants of *AtMEK4* and *AtMEK5* (two closely related *Arabidopsis* MAPKKs) under the control of a steroid-inducible promoter has shown that expression of these transgenes leads to HR-like cell death, which is preceded by the activation of endogenous MAPKs and the generation of H_2O_2 (Ren et al., 2002). Thus similarities may exist between ROI signal transduction pathways for the induction of PCD in plants and animals.

1.6.2 The role of NO in the HR

In animals NO can either induce apoptosis or protect from cell death. This apparent paradox is thought to depend on the cell type and NO concentrations. For example, high concentrations of exogenous NO (100-200μM) were found to be proapoptotic whereas lower concentrations of NO protect the cell from apoptosis (Bogdan et al., 2000).

Anti-apoptotic

In animals anti-apoptotic effects of NO are associated with low levels (1nM -1μM) (Curtin et al., 2002), which have been reported to have protective effects in lymphocytes (Mannick et al.,1994;1999), neural cells (Estevez et al., 1998) and endothelial cells

(Dimmeler et al., 1999). NO at physiological concentrations may act as antioxidant, scavenging ROIs. In animal cells NO has been found to protect from oxidative damage resulting from O_2^-, H_2O_2 and alkyl peroxides. In the human brain NO concentrations range from between about 25-125nM (Sharpe et al., 2003). Some areas of the brain contain high levels of iron; therefore the brain is likely to be at risk of Fenton/Haber-Weiss chemistry. This leads to the generation of •OH, which is biologically very damaging.

Haber –Weiss reaction:

1. $H_2O_2 + O_2^- \rightarrow O_2 + OH^- + \bullet OH$
2. $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$
3. $Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$

NO has been found to block the production of •OH at physiological conditions protecting the brain from Fenton chemistry (Sharpe et al, 2003). It also has been found to act against oxidative stress in plants. Potato leaves that were initially treated with the NO donor SNP subsequently showed less lipid peroxidation, protein loss, and RNA degradation following the application of diquat which mediates ROI generation (Beligini and Lammatina, 2002). Treatment with the NO donors 3-Morpholinosydnonimine N-ethyl-carbamide (SIN-1) and SNP reduced paraquat toxicity in rice leaves through a decrease in lipid peroxidation and protein loss (Hung et al, 2002). Furthermore, NO was also found to delay PCD in giberellin treated (GA) barley aleurone layers, without inhibiting metabolism. This reduction in PCD could be mimicked by the antioxidant butylated hydroxy toluene, supporting the idea that PCD was slowed by NO acting as an antioxidant (Beligini et al., 2002).

One of the best-characterised ways in which NO protects against PCD in animal cells is via the inhibition of caspases. Caspases are a family of cysteine proteases that play a crucial role in activating apoptosis (Kim et al., 1997). Mitochondria respond to stress-related cellular messengers by releasing cell death activators such as cytochrome c into the cytosol. Cell death activators trigger caspase cascades in mammals (Yang et al., 1997). One of the caspases important for executing apoptosis (caspase-3) was found to

be S-nitrosylated on its catalytic cysteine residue (Rossig et al., 1999). It is thought that this S-nitrosylation helps to maintain the enzyme in an inactive form and that the activation of caspase by de-nitrosylation is part of the apoptotic pathway (Rossig et al., 1999). The S-nitrosylation of caspase-1(Kim et al., 1998) and caspase-8 (Kim et al., 2000) by NO have also been reported (Li et al., 1998). In plants cysteine proteases are involved in the regulation of protein turnover and in resistance to insects and pathogens (Pernas et al., 1998). Although no caspase homologs have been identified in plants, there is some evidence for caspase-like protease activity. In tobacco tissue undergoing the HR, specific peptide inhibitors of caspases abolished the HR in tobacco leaves induced by *Pseudomonas syringae* pv *phaseolicola* (del Poxo and Lam, 1998). In *Arabidopsis* NO induced cell death was shown to be inhibited by an irreversible inhibitor of caspase-1 (Clarke et al., 2000). The *AtCYS1* gene in *Arabidopsis* belongs to the cystatin family of cysteine protease inhibitors. *AtCYS1* is strongly induced by pathogen challenge and NO. The over expression of *AtCYS1* blocks cell death in *Arabidopsis* suspension cultures induced by avirulent *Pseudomonas syringae* pv. *maculicola* carrying *avrRpm1* and by SNP plus the H_2O_2 generating system glucose/glucose oxidase. Similar results were found with transformed tobacco where the HR was inhibited following treatment with *Pseudomonas syringae* pv. *phaseolicola* (Belenghi et al., 2003).

Pro-apoptotic

High concentrations of NO can cause cell injury, for instance NO is overproduced in macrophages to kill bacteria and tumour cells (Nathan, 1992). NO can induce apoptosis in several cell types in animals including pancreatic islets (McDaniel et al., 1997), macrophages (Messmer et al., 1996) and certain neurons (Heneka et al., 1998). In animals apoptotic cell death is linked to the release of mitochondrial cytochrome c into the cytosol and by the subsequent activation of caspases (Yang et al., 1997). NO can directly induce cytochrome c release through the potential loss of the mitochondrial membrane (Brookes et al., 2000). NO has also been shown to reversibly bind to mitochondrial iron-sulfur cluster-containing enzymes, such as aconitase and complexes I and II of the mitochondrial respiratory chain thus inhibiting ATP generation (Drapier et al., 1988; Drapier and Hibbs, 1986). Similar processes may take place in plants, since the treatment of carrot cells with SNP led to a reduction in respiration (Zottini et al.,

2002). SNP treatment also caused a decrease in mitochondrial membrane potential and the release of cytochrome c in carrot cells (Zottini et al., 2002).

Cyclic GMP (cGMP) is an important downstream messenger in many NO mediated signalling pathways in animals (McDonald and Murad, 1995; Hobbs, 1997). NO initiates' cGMP- dependent pathways through the activation of adenylate cyclase by binding to its heme iron moiety. This activation results in a transient increase in cGMP levels, which results in physiological changes, for example smooth muscle relaxation (Stamler, 1994). Such physiological changes are brought about by the targets of cGMP, including protein kinases, cyclic nucleotide gated channels and phosphodiesterases (Beck et al., 1999). The targets for cGMP in plants are as yet unclear. However, a cyclic nucleotide-gated channel (AtCNGC2) has been identified recently in *Arabidopsis* that mediates Ca^{2+} and K^{+} influx in response to cGMP (Leng et al., 1999). The *Arabidopsis* mutant *dnd1* is defective in the gene encoding AtCNGC2 and fails to produce a HR in response to the avirulent pathogen *Pseudomonas syringae* pv *tomato* (Clough et al., 2000). It is not yet known whether *AtCNGC2* is regulated by an NO-cGMP dependent pathway. In animals cGMP pathways have been shown to be pro-apoptotic, although they can also be anti-apoptotic. The incubation of pancreatic B-cell line (HIT-TI5) and cardiomyocytes with the NO donor S-nitroso-N-acetyl-penicillamine (SNAP) induces apoptosis (Taimor et al., 2000; Shimojo et al., 1999; Loweth et al., 1997). This induction of apoptosis by SNAP could be blocked by the inhibition of soluble guanylyl cyclase or the cGMP-dependent protein kinase G (PKG) (Taimor et al., 2000; Loweth et al., 1997). Evidence suggests that NO can induce apoptosis in plants using cGMP as a secondary messenger (Clarke et al., 2000) (Figure 1.7). For example, the use of a specific guanylate cyclase inhibitor blocked NO-induced cell death in *Arabidopsis* cells and this inhibition was reversed by a cGMP analogue (Clarke et al., 2000). Administration of NO donors or recombinant mammalian NOS to tobacco plants or tobacco suspension cells triggered expression of the defence-related genes encoding *PR1* and *PAL*. This induction could also be blocked using a specific guanylate cyclase inhibitor (Durner et al, 1998). Consistent with cGMP acting as a second messenger in tobacco, NO treatment induced dramatic and transient increases in endogenous cGMP levels (Durner et al, 1998).

Although many NO-mediated effects in animals are cGMP dependent, alternatives include the S-nitrosylation of thiol-containing proteins or the direct interaction of NO

with metal containing proteins, altering protein activity (Stamler, 1994). Those S-nitrosylated proteins identified in animals include the small GTP–binding protein $p21^{ras}$, protein kinase C, phosphatases, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphate channels (Jaffrey et al., 2001; Lander et al., 1997). $p21^{ras}$, a key signalling target for NO in mammals, is activated by S-nitrosylation, which in turn modulates downstream MAPK cascades (Lander et al., 1997, Lander et al., 1996). Depending on the stimuli and the interaction of the components of the MAPK signalling cascade, apoptosis may be triggered in certain cell types (Beck et al., 1999). In-gel protein kinase assays showed that NO activates a potential MAPK in *Arabidopsis* suspension cells (Figure 1.7). The pre-treatment of cells with the NO scavenger PTIO reduced the activation of this kinase. However, the specific mammalian MAPK inhibitor, PD98059 (2'-amino-3'-methoxyflavone) was not found to block NO induced cell death (Clarke et al., 2000).

Another important NO sensor in animals is aconitase, which is sensitive to inactivation by NO. Tobacco aconitase has also been found to be inhibited by NO (Navarre et al., 2000) (Figure 1.7). In mammals NO can also convert cytosolic aconitase to an mRNA binding protein called an iron regulatory protein (IRP) (Kaptain et al., 1991, Klausner et al., 1993). IRPs promote elevated intracellular iron levels by reducing the translation of proteins that utilise or sequester iron (for example ferritin) and by increasing the stability of the mRNA for the transferrin receptor, which imports iron into the cell (Hentze and Kuhn, 1996). Plant aconitases show high homology to mammalian–IRP. It is therefore possible that aconitases function as IRPs in plants to elevate free iron levels in response to pathogen attack. High levels of free iron would promote Fenton/Haber-Weiss chemistry, generating the extremely reactive hydroxyl radical (•OH). This would create a highly toxic environment, which may promote the HR and directly kill pathogens (Navarre et al., 2000). Furthermore, NO has been found to inhibit the H_2O_2 scavenging CAT and APX in tobacco; thus it is possible that by modulating H_2O_2 levels, NO regulates the HR (Clark et al., 2000).

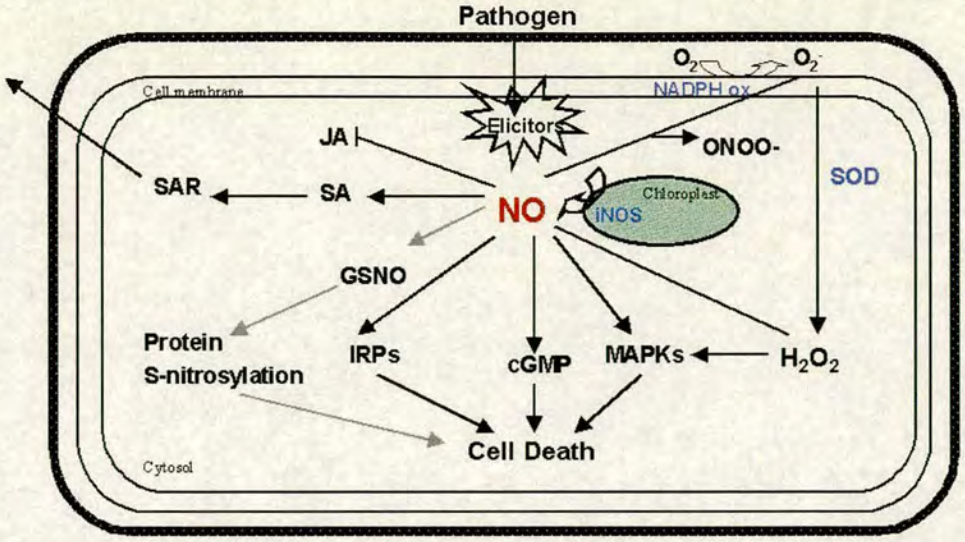

Figure 1.7 NO signalling during the HR.

Grey arrows represent potential HR functions. Black arrows represent experimental supported results. Jasmonic acid (JA), Salicylic acid (SA), systemic acquired resistance (SAR), S-nitrosoglutathione (GSNO), iron regulatory protein (IRP), cyclic GMP (cGMP), mitogen activated protein kinase (MAPK), immune nitric oxide synthase (iNOS), peroxynitrite ($ONOO^-$), superoxide dismutase (SOD).

1.6.3 The interplay of NO and ROI during the HR

It is likely that the interplay between ROIs and NO is highly complex and that the two can act both in synergy as well as antagonistically. In animals NO and O_2^- react to form the highly cytotoxic molecule peroxynitrite ($ONOO^-$), which has a key role in the animal immune system (Koppenol et al., 1992; Groves, 1999). O_2^- can be generated through the dismutation of H_2O_2 to O_2^- or by mammalian NOS which are also capable of producing O_2^- ions (Pou et al, 1992). Once formed in animals $ONOO^-$ can induce apoptosis in tumour cells, cause oxidative tissue damage and it is used by macrophages to kill bacteria. The exposure of animal cells to $ONOO^-$ in the range of 1-1000 μM induces a concentration dependent cell death (Cookson et al., 1998; Lin et al., 1995). The treatment of *Arabidopsis* leaves with the peroxynitrite scavenger urate was found to reduce cell death post inoculation with avirulent *Pseudomonas syringae* pv. *phaseolicola* (Alamillo and Garcia-Olmedo, 2001). This suggests that $ONOO^-$ is also required for cell death in *Arabidopsis*. However, the exposure of soybean cell suspensions of up to 1mM SIN-1 (3-Morpholinosydnonimine N-ethyl-carbamide) a commercial $ONOO^-$ donor did not induce cell death (Delledonne et al., 2001). It was found to induce protein nitration in soybean and *Arabidopsis*, leading to changes in the

redox state (Delledonne et al., 2001). It may be that whereas in mammalian systems peroxynitrite is a key mediator of cell death this is not the case for plants. Although ONOO- does not appear to be an essential intermediate of NO-induced cell death in plants it may have important physiological functions.

Alternatively, it has been proposed that cell death in plants is mediated by a synergistic interaction between NO and H_2O_2 (Delledonne et al., 2001). In animals the reaction between NO and H_2O_2 does not appear to be involved in the direct killing of cells. There is some evidence that NO co-operates with H_2O_2 to induce DNA fragmentation and cell lysis in murine lymphoma cells and endothelial cells (Filep et al., 1997; Farias-eisener et al., 1996). This may be due to NO and H_2O_2 reacting to produce hydroxyl radicals, or alternatively singlet oxygen, which are both highly reactive and cytotoxic (Delledonne et al., 2003). The addition of the NO donor sodium nitroprusside (SNP) at concentrations between 0.1 and 1mM to rapidly agitated soybean cells, which produce a steady-state H_2O_2 concentration of ~1 μM causes up to 90% cell death. However if the SNP concentration is increased to 5mM the percentage of soybean cell death is reduced to 10% (Delledonne et al., 2001). Therefore high levels of NO do not affect cell viability unless compensated by high levels of H_2O_2. A balance model has been proposed to account for this phenomenon (Figure 1.8). If the NO/O_2^- balance is in favour of O_2^- then NO is scavenged before reaction with H_2O_2 (Figure 1.8c). While if the balance is in favour of NO, O_2^- is scavenged before dismutation to H_2O_2 (Figure 1.8d). Either way the product is $ONOO^-$, which was not found to induce cell death in plants (Delledonne et al, 2001). Thus it is possible that cell death is induced only in the balanced presence of NO and H_2O_2 (Figure 1.8a). Moreover, the treatment of *Arabidopsis* suspension cells with the O_2^- donor methyl viologen and the NO donor roussins black salt (RBS) did not significantly increase cell death compared with cells treated only with RBS, but 20mM H_2O_2 was found to have an additive effect on cell death with RBS (Clarke et al., 2000). These experiments suggest that NO and H_2O_2 are required to trigger cell death. However, studies which followed the kinetics of NO production using the NO stain 4-amino-5-methylamino-2', 7'-difluoroescein (DAF-FM) post inoculation with *pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 carrying *avrB* challenge this hypothesis (Zhang et al., 2003). Cell death and NO production were first visualised in this study 3 hours post inoculation with *Pst* DC3000 (*avrB*). Since these events happen in close proximity it may be that NO cannot be a signal to trigger

programmed cell death. Subsequent experiments with the NOS inhibitor L-NMMA found a delay in the progression of the HR post *Pst* DC3000 (*avrB*) inoculation. Therefore it was suggested that NO may not be required for cell death but contributes to the HR as a cell-to-cell signal (Zhang et al., 2003).

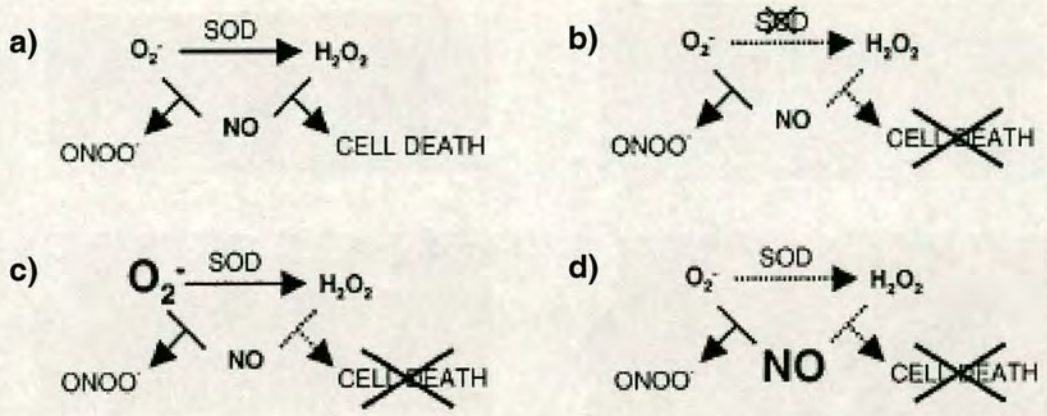

Figure 1.8 Balance model for NO and ROI interactions.

(a) Although NO/ H_2O_2 co-operation triggers PCD, the reaction of NO/O_2^- leads to the formation of ONOO-, which is not an essential intermediate of NO-mediated cell death. **(b)** SOD activity is required for H_2O_2 accumulation during the HR. **(c)** When the NO/ O_2^- balance is in favour of O_2^- then there is no NO left for interaction with H_2O_2. **(d)** When the NO/ O_2^- balance is in favour of NO there is no O_2^- left for SOD mediated dismutation to H_2O_2. Delledonne et al., 2001.

1.7 Defence Signalling Pathways

1.7.1 Salicylic (SA) and NO

Salicylic acid (SA) plays a central role in plant defence against pathogens. Following pathogen infection SA levels increase both locally and systemically. Exogenous application of SA results in enhanced resistance to a broad range of pathogens. SA is required for the expression of several defence genes, such *PR* genes and the establishment of systemic acquired resistance (SAR) (Ryals et al., 1996). *SID2* encodes an isochorismate synthase necessary for the synthesis of SA (Nawrath et al, 1999). The *Arabidopsis* mutant SA *induction deficient2* (*sid2*) and the transgenic line *NahG*, cannot accumulate SA. Both exhibit enhanced diseased susceptibility to a broad range of pathogens including; the fungal pathogen *Peronospora parasitica* and the bacterial

pathogen *Pseudomonas syringae* pv *tomato* DC3000 (Nawrath et al., 1999). *NPR1* (*non-expression of PR1*) encodes a protein with a BTB/BOZ domain and an ankyrin-repeat domain; both these domains are involved in protein-protein interactions. NPR1 has been shown to be translocated to the nucleus where it interacts with proteins of the basic leucine zipper (bZIP) family of transcription factors. These transcription factors are involved in the SA-dependent activation of *PR*-genes (Spoel et al., 2003). Mutants of *NPR1* also exhibit increased susceptibility to *Peronospora parasitica* and *Pseudomonas syringae* pv *tomato* DC3000 (Cao et al., 1998). It has been proposed that SA is a systemic signal that initiates SAR. However, experiments using grafts between wild-type tobacco plants and plants expressing *NahG* demonstrated that a signal for SAR could be communicated from inoculated *NahG* leaves to wild-type plants but not from wild-type leaves to *NahG* plants (Vernooij et al., 1994). This implies that SA is a local cell-to-cell signal but is unlikely to function as systemic signal initiating the development of SAR.

NO action in plants is tightly linked to SA, and NO appears to act through an SA-dependent signalling pathway. The administration of NO donors or recombinant mammalian NOS to tobacco plants or tobacco suspension cells induced a significant accumulation of SA, which led to induction of the defence genes *PR-1* and *PAL*. SA is required for the NO mediated activation of *PR-1* expression, since *PR-1* was not induced in tobacco plants expressing the bacterial gene *NahG* (Durner et al., 1998). Furthermore, the treatment of soybean with SA was found to lead to the enhanced production of NO (Klepper, 1991). NO can induce SAR. Tobacco plants pre-treated with NO-releasing compounds displayed enhanced disease resistance against subsequent TMV infection, both in local and systemic leaves. This was found to be SA dependent as NO failed to induce resistance to TMV in *NahG* transgenic tobacco (Song and Goodman, 2001). NO is able to alter the activity of many SA-regulated enzymes, including aconitase, APX and CAT, which are inhibited by NO as well as SA (Clark et al., 2000; Navarre et al., 2000). The inhibition of CAT and APX may promote H_2O_2 levels in the plant and the inhibition of aconitase may promote the Fenton reaction producing $^{\bullet}OH$. NO donors were found to cause the transient activation of the SA-inducible protein kinase SIPK, a MAPK. The activation of SIPK by NO was also mediated via a SA-dependent pathway, as NO failed to activate SIPK in *NahG* transgenics (Klessig et al, 2000).

Thus it is likely that NO and SA may work synergistically to transduce the defence signal by regulating the same target proteins and genes. An initial NO and ROI burst induces SA synthesis, while elevated SA in turn leads to further production of NO and ROI, acting in an self amplifying feed back loop (Klessig et al., 2000).

1.7.2 Jasmonic acid (JA) and NO

Jasmonic acid (JA) is a fatty acid that is involved in plant reproduction, where it is required for the development of seed and pollen (Creelman and Mullet, 1997; Reymond and Farmer 1998 and Li et al., 2001). It also has an important role in wounding, insect predation and plant defence. Wounding strongly induces enzymes involved in JA biosynthesis such as allene oxide synthase (AOS) and lipoxygenase (LOX). These enzymes are part of the octadecanoid pathway through which JA is synthesised from α-linolenic acid, abundant in membrane lipids (Schaller, 2001). Following JA accumulation after wounding, JA dependent genes are induced including *plant defensin 1.2* (*PDF1.2*), *thionin* (THI 2.1) and *chitinase B* (CH1B) (Reymond and Farmer, 1998). *Arabidopsis* mutants that are unable to perceive JA (for example *coi1 coronatine insensitive1 and jar1 jasmonic acid response*) or produce JA (*fatty acid desaturase fad3-2,7-2,8)* show enhanced susceptibility to fungal and some bacterial pathogens (Vijayan et al., 1998; Staswick et al., 1998; Norman-Setterblad et al., 2000). These pathogens are all necrotrophs or hemi-biotrophs that kill plant cells. *COI1* encodes an F-box protein, such proteins form part of the SCF complex that selectively recruits regulatory proteins for ubiquitination and degradation (Devoto et al., 2002). It is anticipated that this complex regulates the expression of JA-responsive genes by targeting proteins that are negative regulators of JA responses, for degradation (Devoto et al., 2002). The *JAR1* gene encodes an adenylating enzyme with specific activity for jasmonate, implying that *JAR1* encodes an enzyme that biochemically modifies JA to control its activity (Staswick et al., 2002). Fatty acid desaturases play an important role in regulating the levels of desaturated fatty acids in the cell and these are important in the JA biosynthetic pathway (Kachroo et al., 2001).

NO does not appear to be a key component of the JA signalling pathway. NO levels were found to remain unchanged in wounded tomato leaves (Orozco-Cardenas and Ryan, 2002). However, the exogenous application of NO by the donor SNP to wounded

tomato leaves was found to inhibit wound inducible defence genes including proteinase Inh I and InhII, a cathepsin D inhibitor and a metallocarboxylase inhibitor, respectively. SNP was also found to block the production of H_2O_2 induced by JA in tomato (Orzoco-Cardens and Ryan, 2002). The inhibition of these genes and H_2O_2 production by NO was reversible using a NO scavenger. NO was still able to inhibit these genes in *NahG* plants, therefore the inhibition of these genes in tomato is independent of SA. Experiments with DAF-2 DA in tobacco also showed no increase in NO levels following wounding (Gould et al., 2003). However, in *Arabidopsis* NO production was visualised in response to wounding using DAF-2 DA and this production was localised to chloroplasts, and in the cytosol of epidermal and sub-epidermal cells. This NO production was inhibited by L-NMMA suggesting that NOS activity is required (Garces et al, 2001). Consistent with these findings Huang et. al. also found that *Arabidopsis* shows an NO burst in response to wounding (Huang et al., 2004). In these experiments NO was found to induce AOS and LOX enzymes but the induction of these genes was not blocked using the NO scavenger cPTIO. Furthermore, JA-responsive genes such as, *PDF1.2* were not induced by NO in wild type plants but they were induced in *NahG* plants, suggesting that *PDF1.2* is only induced by NO in the absence of SA. Subsequently it was shown that the treatment of *NahG* plants with NO induces accumulation of high levels of JA within 1 hour whereas, wild-type plant show an increase in SA but not JA levels (Huang et al., 2004).

Therefore NO appears to be induced in *Arabidopsis* following wounding, but the evidence suggests that although NO may activate JA biosynthesis genes (*AOS* and *LOX*), it is unlikely to be a key signal for the induction of the wound response. This is similar in tomato where NO appears to antagonise wound response genes during the plant defence response against pathogens. The differences observed between NO production following wounding in tomato and *Arabidopsis* may be due to differences in signalling between the *Solanaceae* and *Cruciferaceae* families (Haung et al 2004).

1.7.3 Ethylene (ET) and NO

Ethylene (ET) is involved in germination, flowering, fruit ripening, senescence, wounding and pathogen attack (Johnson and Ecker, 1998; Wang et al., 2002). ET is produced following a pathogen attack by activating expression of the genes encoding

enzymes responsible for ET biosynthesis, 1-aminocyclopropane-1-carboxylate (ACC) synthase genes. The role of ET in plant defence is controversial as it contributes to resistance in some interactions (Thomma et al., 1999; Norman-Setterblad et al., 2000) but promotes disease in others (Bent et al., 1992; Lund et al., 1998). In *Arabidopsis,* ET is perceived by a family of receptors including: ethylene receptor1 (*ETR1*), *ETR2*, ethylene insensitive (*EIN4*) and ethylene response sensor 1(*ESR1*) and *ESR2* (Hua and Meyerowitz, 1998). These receptors are believed to activate a Raf-like serine/ threonine kinase called *constitutive triple response1* (*CTR1*) (Kieber et al., 1993). *EIN2* is thought to be a positive regulator of the ET response and encodes a metal–ion transporter-related integral-membrane protein (Alonso et al., 1999). The *ein2* mutant is susceptible to *Botrytis cinerea* (Thomma et al., 1999) and *Erwinia carotovora* (Norman-Setterblad et al., 2000), but shows decreased disease symptoms following infection with *Pseudomonas syringae* pv *tomato* and *Xanthomonas campestris pv campestris* (Bent et al., 1992). This pattern of pathogen responses is similar to that of *coi1* (Norman-Setterblad et al., 2000; Penninckx et al., 1998). Also, *EIN2* is required for the production of PDF 1.2 and THI 2.1 (Norman-Setterblad et al., 2000; Penninckx et al., 1998). Moreover, JA and ET have been shown to function synergistically in the activation of *PDF 1.2* (Penninckx et al., 1998).

NO is considered to be an endogenous maturation and senescence-regulating factor in plants (Siegel-Itzkovich, 1999). In unripe fruits NO levels are high, whereas ET levels are low; in ripe fruit the converse is true. The exogenous treatment of plants with NO results in the inhibition of ET production, which is a natural inducer of plant senescence (Leshem and Pinchasov, 2000). Contrary to this there are reports of ET levels increasing in *Arabidopsis* after exposure to NO gas (J. R. Magalhaes et al., 2000) and in tobacco after treatment with SNP (Mur et al., 2003). Drought stress has been reported to reduce NO emission and ET production in *Arabidopsis*. By contrast, waterlogging enhanced ET and reduced NO emission. Two mutants of *Arabidopsis*, *etr-1* (*insensitive to ethylene*) and *eto* (an *ethylene over-producer*), generated much less NO compared to the wild type plants (J. R. Magalhaes et al, 2000).

1.8 Cross-talk between defence signalling pathways

1.8.1 JA and ET cross talk

Several studies provide evidence for positive interactions between the JA and ET signalling pathways. The *Arabidopsis* mutants *ein2* and *coi* are defective in ET and JA signal transduction pathways respectively, and both fail to induce *PDF1.2*, *PR3* and *PR4* (Penninckx et al., 1998). Both JA and ET are required for the expression of *PDF 1.2* in response to *Alternaria brassicicola* and *E. caraotovora* (Staswick et al., 1998). Thus, the induction of *PDF1.2* requires both functional JA and ET signalling pathways. Further evidence that JA and ET function co-ordinately to regulate defence genes was obtained through an *Arabidopsis* microarray experiment, which monitored gene expression in response to various defence-related stimuli (Schenk et al., 2000). Interestingly, nearly half of the genes that were induced by ET were also induced by JA. However, the microarray data also showed that JA and ET independently regulate separate sets of genes (Schenk et al., 2000). This evidence suggests that JA and ET pathways interact with each other co-regulating some genes.

1.8.2 SA and ET cross-talk

Data suggest both positive and negative interactions exist between the SA and ET signalling pathways. In tomato, the development of disease symptoms in response to infection with *Xathomonas campestris* pv. *vesicatoria* requires SA and ET (O Donnell et al., 2001). Microarray experiments suggest that SA and ET may function together co-ordinately to induce several defence genes (Schenk et al., 2000).

Furthermore, when the *Arabidopsis* mutants *cpr5* and *cpr6* (which constitutively express *PR* genes) are crossed with *npr1, PR* gene expression is partially blocked. Only when this double mutant is crossed to *ein2* is *PR* gene expression completely blocked (Clarke et al., 2000). The expression of *PR1* genes in *Arabidopsis* does not require ET but ET does potentiate *PR1* induction (Lawton et al., 1994). Thus in *Arabidopsis* ET may potentiate SA mediated responses. A mutation in the *EDR1* gene enhances resistance to *Pst* DC3000 and *Erisyphe cichoracearm* and causes the accelerated induction of *PR1*. The expression of *PR1*, which is dependent on SA, is highly induced by ET in *edr1* but not in wild type plants. In this case ET potentiates SA-mediated *PR1*

gene expression, whereas EDR1 negatively regulates this process. *EDR1* encodes a MAPKKK similar to CTR1 (Frye et al., 2001). However, contrary to this, in *ein2* mutants the basal levels of *PR1* appears to be elevated suggesting that ET negatively regulates SA responses (Lawton et al., 1994).

1.8.3 SA and JA cross talk

The interactions between SA and JA are complex; there is evidence for both positive and negative interactions between these pathways. Overall the pattern appears to be that of mutual antagonism. The *cpr6* mutant that constitutively expresses *PR* genes also accumulates SA, when *cpr6* is crossed to *eds5,* thus reducing levels of SA, an increase in *PDF1.2* expression is observed (Clarke et al., 2000). Treatment of tobacco plants with *Erwinia carotovora* activates JA signalling (Norman-Setterblad et al., 2000) but inhibits the expression of SA dependent genes (Vidal et al., 1997). *coi1* mutants that are unable to perceive JA, infected with *Pst* DC3000 accumulate higher levels of SA than wild-type plants and as a result also show increased levels of *PR1* induction. This activation of *PR1* is inhibited by crossing *coi1* with *NahG* and *npr1*, thus *PR1* expression in *coi1* is SA dependent (Kloek et al., 2001).

An *Arabidopsis* mutant of the MAPK 4 gene, *mpk4* is unable to induce *PDF1.2* in response to JA. It also shows elevated levels of SA and constitutive expression of *PR1*, *PR2* and *PR5*. The induction of these *PR* genes is blocked when *mpk4* is crossed with *NahG*, indicating SA is required. The *mpk4NahG* double mutant is still unable to induce *PDF1.2* expression. Consequently, *MPK4* is required to repress SA signalling as well as being required for JA mediated gene expression. Therefore, MPK4 may be required to integrate SA and JA signalling pathways (Petersen et al., 2000). Microarray data again shows that more than 50 defence genes are co-induced by SA and JA, suggesting that the two signals co-ordinately regulate these genes (Schenk et al., 2000).

As previously mentioned recent evidence suggests that NO functions synergistically with SA but antagonises JA signalling, thus NO may have an important role in the crosstalk between these two pathways.

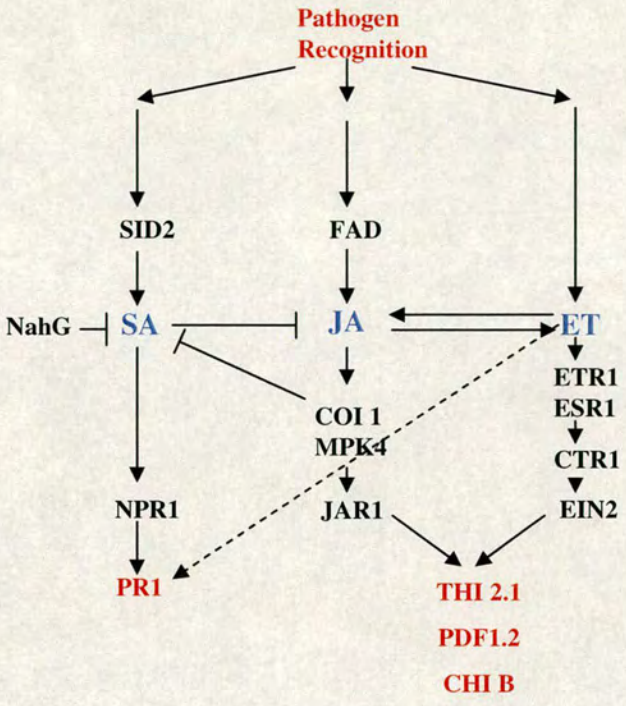

Figure 1.9. SA, JA and ET Cross-Talk

A model of the position and interactions of plant proteins (black) and hormones (blue) in the pathogen defence pathways are shown, which lead to the expression of defence proteins (red), that confer pathogen resistance. Adapted from Kunkel and Brooks et al., 2002.

Chapter 2

2. Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma (Sigma-Aldrich, UK)

2.1 *Arabidopsis thaliana* seed and growth conditions

Arabidopsis thaliana (Arabidopsis) seeds of ecotype Columbia (Col-0) and Landsberg erecta (L*er*) were used. All *Arabidopsis* transgenic lines and mutant strains used were in a Col-0 background and are outlined in Table 2.1. Seeds were placed on potting medium consisting of peat moss, vermiculite and sand (4:1:1), and allowed to vernalise for 48 hours at 4°C after which they were transferred to 20°C. Plants were placed 6 to a pot and grown in long days (16 hours light, 8 hours dark) at 20°C.

Table 2.1 *Arabidopsis* transgenic lines and mutant strains

Wild type and Mutant Strains			
Strains	**Phenotype**	**Reference**	**Source**
Col-0	wild-type		NASC
L*er*	wild-type		NASC
PR-1a:luc	*PR-1a:luc* transgenic	Thomson & Loake	Thomson & Loake
sid1	Salicylic Acid Induction deficient	Nawrath & Metraux	Nawrath, Freiburg
sid2		1990	University
NahG	Salicylate hydroxylase transgenic	Lawton et al., 1995	Novartis, USA
npr1-1	SAR insensitive	Cao et al., 1995	Dong, Duke University
ein2-1	Ethylene insensitive	Guzman & Ecker 1990	NASC
ein3	Ethylene insensitive	Chao et al., 1997	Milner, Glasgow university
etr1-1	Ethylene insensitive	Bleeker et al., 1988	NASC
coi1-1	Jasmonate insensitive	Feys et al., 1994	Turner, University of East Anglia

2.2 Plate based chemical treatments

2.2.1 Screening for NO Resistant Mutants

Around 250 seed from each pool of PR1a:luc tagged lines (Tani et al., 2004) were surface sterilised in sterile water containing 10% (v/v) bleach with a drop of Triton® X-100 for 20 minutes. The seeds were then washed 5 times with sterile water before resuspension in 0.1% (w/v) agarose. Seeds were screened on 1/2MS (1% sucrose and 0.8% agar) containing 2mM SNP (Sodium Nitroprusside). As described in Chapter 3, 2mM SNP was used to screen for NO resistant mutants as wild-type seeds fail to germinate at this concentration. Control plates contained light inactivated 2mM SNP or 0mM SNP. Seedlings showing resistance to 2mM SNP were transferred to soil and allowed to set seed. These candidates were screened again in the F_2.

2.2.2 Reactive Nitrogen Intermediates (RNI) and ROI sensitivity plates

T-DNA insert mutant lines (*fdh1-1* and *fdh1-3*) and Col-0 seeds were surface sterilised as before with bleach. Plates containing the following concentrations of ROI and NO donors were used;

Hydrogen peroxide 1mM, 2mM, 3mM and 4mM,

Paraquat 0.25mM, 0.5mM, 0.75mM and 1mM.

Rose Bengal 15μM, 20μM, 25μM and 30μM.

SNP 0.75mM, 1mM, 1.25mM and 1.5mM

GSNO (ALEXIS biochemicals) 0.500 mM, 0.625 mM, 0.750 mM and 1mM

These concentrations of ROIs and RNIs were selected after preliminary experiments using Col-0 seed to establish which concentration ranges are toxic to wild-type plants. Twenty five seed from each line were placed on a plate for each concentration and the percentage germination recorded. This was replicated three times.

2.3 Pathogen growth and disease resistance assays

2.3.1 Growth of *Pseudomonas Syringae* pv *tomato* DC3000 (*avrB*) and inoculation

Pseudomonas Syringae pv *tomato* DC3000 (*avrB*) was grown in Kings broth (KB) liquid media (King et al.,1954) supplemented with 50mg.l^{-1} rifampicin and 50mg.l^{-1} kanomycin at 30°C. Cells were harvested at OD_{600} equal to 0.2 (the equivalent of 10^6 cfu.cm^{-2}) and pelleted by centrifugation before re-suspension in 10mM $MgCl_2$. Four week old plants were infiltrated with a *Pst* DC3000 (*avrB*) suspension ($OD_{600=}$ 0.2 or 0.002 for leaf staining) on the abaxial side of the leaf using a 1ml syringe (Cao et al., 1994; Grant et al., 2000).

2.3.3 Pseudomonas syringae pv *tomato* DC3000 resistance assay

Pst DC3000 (Whalen et al., 1991) was grown in KB medium supplemented with 50mg.l-*1* rifampicin. Four week old plants were infected with a *Pst* DC3000 suspension (OD_{600}= 0.002) in 10mM $MgCl_2$ on the abaxial side of the leaf using a 1ml syringe (Cao et al., 1994). Three leaves per plant and three plants per line were infected. After four days, plants were examined for disease symptoms. Leaves were also harvested at this time point for analysis of bacterial growth. Three leaf discs (0.5cm^2) were collected from each plant using a cork borer and then ground in 990µl 10mM $MgCl_2$ with a pestle and mortar. Serial dilutions were made of the resulting bacterial suspension and 100µl of each dilution plated onto KB plates containing 50mg.l^{-1} rifampicin. The plates were incubated for 3 days at 30°C and the number of bacterial colonies for each sample counted and recorded.

2.3.4 Fungal disease resistance assays

2.3.4.1 Penospera parasitica Noco 2

P. paraisitica Noco2 (gift from Jane Parker) was maintained on Col-0 and *eds1* (Parker et al., 1996) under humid conditions (Bowling et al., 1994). For the disease resistance assay conidiospores were harvested by vortexing infected seedlings in water. A haemocytometer was used to determine spore concentration, which was adjusted to

$1X10^5$ spores per ml. Four week old plants were sprayed with the conidiospore solution and maintained under humid conditions. After 14 days infected plants were scored for the extent of downy mildew growth by eye. Scoring was as follows: 0=no infection, 1=less than 25% of one leaf with conidiophore growth, 2=25 to 50% of one or two leaves covered with conidiophores, 3=25 to 50% of three or four leaves covered with condiophore growth, 4=25 to 50% of all leaves covered with conidiophore growth and 5=all leaves covered with more than 50% conidiophore growth.

2.3.4.2 Botrytis Cinerea

Disease resistance assays against *Botrytis cinerea* were carried out according to Grant et al., (2003). *Botrytis* was maintained by growth on oat meal plates for one life cycle followed by growth on Col-0 to ensure virulence. Spores were collected by covering sporulating fungus growing on an oatmeal plate with 10mls of water. The suspension was vortexed then filtered and spores resuspended in potato dextrose broth. Spore density was determined using a haematocytometer and adjusted to $1X10^9$ spores per ml. Spore suspensions were sprayed onto 4 week old plants. Infected plants were watered then placed in a covered chamber so that the humidity was close to 100%. After 8 days the plants were examined by eye for disease symptoms. Scoring was as follows: 0=no infection, 1= faint chlorosis of one or more leaves, 2= strong chlorosis of 75% of leaves, 3= 50% necrosis on 75% of leaves, 4= 50-100% necrosis on 75% of leaves, 5=total necrosis of all leaves.

2.3.4.3 Erysiphe cichoracearum

Powdery mildew, *Erysiphe cichoracearum* UED1 (Grant et al., 2003) which is endemic to the university greenhouses was maintained on Col-0 plants. Plants were inoculated via rubbing infected leaves against the leaves of plants to be infected, ensuring the whole leaf surface was covered. Ten days post-inoculation plants were scored for the presence of conidiophores and chlorotic symptoms. Scoring was as follows 0=no infection, 1=1-2 leaves infected less than 25%, 2=3-4 leaves infected less than 50%, 3=3-4 leaves infected more than 50%, 4= most leaves infected 75% and 5= all leaves in infected at least 75%.

For all fungal disease resistance assays the plants in different replicates were assigned a disease index as follows D.I.= Σ I X j/n, where i=infection class, j =number of plants scored for that infection class and n=the total number of plants

2.4 Histochemical staining

2.4.1 Trypan Blue (TB) Staining

Dead plant cells were visualised by TB staining (Yun et al., 2003). Leaves were boiled for 2 minutes in a lactic acid-phenol-trypan blue solution (2.5mg/ml trypan blue, 25% (w/v) lactic acid, 23% water saturated phenol, 25% glycerol and water). After cooling the leaves were rinsed in water before destaining with chloral hydrate solution (250g/l). After 24 hours the chloral hydrate solution was removed and the leaf samples mounted onto microscope slides with glycerol. The slides were examined for cell death using a Lecia Wild M3C microscope.

2.4.1 3'3'-Diaminobenzidine (DAB) staining

H_2O_2 accumulation *in planta* was visualised by DAB staining (Yun et al., 2003). DAB is rapidly absorbed by the plant and is polymerised locally in the presence of H_2O_2 and peroxidase giving a visible brown stain. Leaves were placed in 50ml tubes, covered with 0.1% (w/v) 3,3-diaminobenzidine solution and incubated overnight. The stain was removed and the chlorophyll cleared by boiling in 96% (v/v) ethanol for 10 minutes.

2.4.3 Nitroblue tetrazolium (NBT) stain

O_2^- accumulation *in planta* was visualised by NBT staining (Jabs et al., 1996). Leaves were immersed in 10mM KH_2PO_4 buffer (pH 7.8) containing 600µM NBT for 2 hours in the dark. The stain was removed and chlorophyll cleared by boiling in 96% (v/v) ethanol for 10 minutes.

2.4.4 4,5-Diaminoflurescein diacetate (DAF-2DA) stain

NO production in leaves was visualised by DAF-2DA staining (Huang et al., 2004). Leaves were placed in a 50ml tube containing 5ml of loading buffer (10mM Tris/KCl, pH 7.2) and DAF-2 DA at a final concentration of 10μM (from a 5mM stock in DMSO). The leaf samples were then incubated at 25°C in the dark for 1 hour. After incubation, the samples were washed in water and mounted in water. Confocal images were obtained using a BioRad Radiance 2100 system mounted on a Nikon Eclipse TE300 inverted microscope. Samples were imaged using UV x 40 objectives, with 488 nm excitation from an argon ion laser. Emissions were collected at 515 (DAF-2 DA) and 590 nm (chlorophyll autofluorescence).

2.5 RNA Extraction

Total RNA was extracted from leaves of 4 week old plants using the guanidine thiocyanate (GTC) phenol chloroform extraction method (Sambrook et al., 1989). Leaf tissue (approximately 200mg) was ground in liquid nitrogen using a pestle and mortar. Leaf tissue was transferred to a 1.5ml eppendorf containing 450µl GTC solution (4M guanidine thiocyanate, 25mM sodium citrate, 0.5% (w/v) sarcosyl, 0.1M ß mercaptomenthanol). Samples were vortexed for 30 seconds and 0.05ml of 2M sodium acetate pH 4.0, 0.45ml phenol and 0.1ml chloroform: iso-amylalcohol (49:1) added. After further mixing samples were incubated on ice for 15 minutes before centrifuging at 10,000 rpm for 20 minutes. The supernatant was transferred to a new tube and an equal volume of isopropanol added to each sample. The samples were incubated at -20°C for between 2-12 hours in order to precipitate the RNA. The RNA was then recovered by centrifugation (10,000rpm for 20 minutes). The RNA pellet was then re-dissolved in 0.15ml GTC solution and re-precipitated by the addition of an equal volume of isopropanol and further incubation at -20°C for 1 hour. Following centrifugation (10,000rpm for 20 minutes), the RNA pellet was washed twice in 70% ethanol, dried and dissolved in 50µl DEPC-treated water. The absorbance of each sample was measured at 260nm and used to calculate the RNA concentration of each sample.

2.6 Northern Blot

RNA samples (10μg) were separated on a formaldehyde-agarose (Sambrook et al.,1989) gel and then transferred to a Hybond^{TM}-N hybridisation membrane according the suppliers instructions (Amersham Life Sciences). To check for equal RNA loading of lanes, membranes were stained with methylene blue (0.3M Sodium acetate pH 5.5, 0.03%w/v Methylene Blue). Methylene blue was removed by washing in 1XSSC, 1%SDS w/v for 15 minutes. Probes (described below, table 2.2) for hybridisation were labelled with α-^{32}P-dCTP by random priming prepared using a Prime-a-Gene® labelling kit (Promega). The pre-hybridisation/hybridisation buffer solution included dextran sulphate (10%w/v) to improve efficiency of probe binding (Sambrook et al.,1989). After hybridisation overnight at 65°C, membranes were washed at 65°C, twice for 30 minutes in 4X SSC, 1% (w/v) SDS and twice in 4X SSC, 0.5% (w/v) SDS for 15 minutes. Blots were exposed to X-Omat-AR ^{TM} imaging film (Kodak). The strippng of blots was done by incubating membranes in boiling 0.1% SDS, before washing in 0.5X SSC for 30 minutes at room temperature.

Probes were prepared by amplification of appropriate sequences by PCR and purified using a PCR purification kit (QIAGEN). The size of the PCR product was verified by gel electrophoresis and sequencing.

Table 2.2 Primers used to generate probes

Gene	Template	Forward primer	Reverse primer	Probe (kb)
PR1	TA-*PR1* [a]	TGCAGACTCATACACTCTGG	TATGTACGTGTGTATGCATGATC	0.3
GST1	genomic DNA[c]	GGTTCTTTAAGTGAATCTCAAAC	CAAGACTCATTATCGAAGATTAC	1.0
PDF1.2	genomic DNA[c]	TCATGGCTAAGTTTGCTTCC	AATACACACGATTTAGCACC	0.3
GS-FDH	CDNA	GAGGTTCGGATCAAGATCCT	CTTGGAACGGACGAGTTGAT	0.8

[a] Uknes *et al.*. 1992.

[c] Penninckx *et al.*, 1996

2.7 Protein extraction

Leaf tissue from 4 week old plants (200mg) was ground in liquid N_2, then added to 2 volumes of protein extraction buffer (50mM Hepes, pH7.4, 5mM EDTA, pH7.4, 5mM EDTA, 5mM DTT, 10mMNaF, 10mM Na_3VO_4 50mM β-glycerophosphate, 1mM PMSF, 2μg/μl antipain, 2μg/μl aprotinin, 2μg/μl leupeptin) (Grant et al., 2000). Samples were centrifuged for 30 minutes at 13,000rpm in the cold room (4 °C) and the supernatant collected. The protein content was quantified by Bradford analysis (Bradford et al., 1976).

2.8 Western Blot Analysis

Western blots were performed as described by Sambrook et al., (1989). Proteins were separated on a 12% SDS-PAGE gel at 150V for 45 minutes. A PVDF membrane (Amersham) was soaked in 100% Me-OH and 3mM Whatman's paper was soaked in transfer buffer (25mM Tris , 200mM glycine and 20% Me-OH). Blotting was performed by electrotransfer for 1 hour. Ponceau solution was used to stain the membrane to ensure the equal loading of protein samples. Blocking was performed in [1xTBS (10xTBS=12g of Tris and 40g of $NaCl_2$ per litre, adjusted to pH 7.6), 0.1% Tween and 0,5% BSA] for 1 hour and the membrane washed 3 times with TBST (1xTBS and 0.1% Tween). The blot was then incubated with a primary antibody at the appropriate dilution in 15mls of antibody dilution buffer (1xTBS, 0.1% Tween and 5% BSA) overnight at 4 °C with agitation, before washing 3 times in TBST. The secondary HRP-conjugated secondary antibody (New England Biolabs) was then incubated with the blot in 15 ml of antibody dilution buffer for 1 hour at room temperature. Finally the blot was washed 3 times with TBST. Protein detection was performed using a chemi-luminescent New England Biolabs Photo-HRP kit. The blot was incubated with 500μl Lum GLO and 500μl of 20x Peroxide in 9mls of sterile water for 1 minute. The blot was exposed to x-ray film (Kodak).

2.9 PCR based methods

2.9.1 Identification of T-DNA insertion lines

T-DNA insertion lines of gene At5g43940 (*GS-FDH*) were obtained from the following organisations; 1288D09 SAIL (Syngenta Arabidopsis Insertion Lines), N594956 SIGnAL (Salk Institute Genomic and Analysis Laboratory) and 315D11 GABI-KAT (Genomanalyse im Biologischen System Planze). PCR was used to verify the T-DNA insertion/position in these lines and to identify homozygous lines for T-DNA inserts by using two gene specific primers and a T-DNA left border (Sessions et al., 2000). Primers used to identify lines with a T-DNA insert in the *GS-FDH* region are shown in table 2.3.

Table 2.3 Primers used to identify T-DNA insertion lines

Line	Left border	Forward	Reverse	Wild-type product	T-DNA insert product
1288D09	TTCATAACCAATCTGG ATACAC	TATATAATGGTTCGACG CATATTT	GTCGGGTCGGGTCG TTAATA	500bp	300bp
N594956	GCGTGGACCGCTTGCT GGAAC	TATATAATGGTTCGACG CATATTT	GTCGGGTCGGGTCG TTAATA	550bp	270bp
315D11	ATATTGAACATCATAC TCATTGC	TATATAATGGTTCGACG CATATTT	CCACCAACACTCTCA ACAATC	1300bp	550bp

2.9.2 RT-PCR

RNA was extracted and the concentration determined as before. RNA was DNAase (Promega) treated by adding 1µl of DNAase per µg of RNA in 1X promega DNase buffer and incubating at 37 °C for 30 minutes. The reaction was stopped by adding 1X promega stop buffer and incubating at 65 °C for 10 minutes. RT-PCR was carried out using QIAGEN One Step RT-PCR kits according to the manufacturer's instructions. The PCR program was as follows; Reverse Transcription for 30 minutes at 50 °C, PCR activation 15 minutes at 95 °C, 3-step cycling x35 1 minute at 94°C, 1 minute at 64 °C, 1 minute at 72°C and final extension 10 minutes at 72 °C. Primers used were as shown in table 2.4.

Table 2.4 Primers used for RT-PCR

Gene	Forward primer	Reverse primer	Product (kb)
At5g43935 (FLS)	TGGCCTAACAATCCTTCAGA	ATACTGTGCTTTATCGCCAC	0.12
At5g43935 (FLS)	TTGTCCTCATTGGGGATCAG	CATCAATCACGAAGAACCTT	0.25
At5g43940 (GS-FDH)	GTGATGAGAGCTGCATTGGA	GTTGGAACGGACGAGTTGAT	0.10
At5g43940 (GS-FDH)	GAGGTTCGGATCAAGATCCT	CACCAACACTCTCAACAATC	0.13
At3g26650 (GAPDH)	CTTGCTCCCTTTGTCAAAGTTCTT	CGCGTCTAGCAACCTCTGGT	0.11

2.10 Biochemical analyses

2.10.1 S-nitrosylation assay

The S-nitrosylation assay was carried out according to Jaffrey et al., (2001). To 100µl of protein extract (proteins were extracted using the same extraction buffer as described above) 4 volumes of blocking buffer (9 volumes of extraction buffer, 1 volume of 25% SDS and 20mM MMTS) was added and incubated at 50 °C for 20 minutes with frequent vortexing. The blocking buffer was removed using a MicroBioSpin–6 column (Bio-Rad), which had been pre-equlibrated in extraction buffer, 3 times. Then 12.5µl of 4mM Biotin-HPDP (N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio) propionamide) (Pierce) in DMSO and 1µl of sodium ascorbate (Sigma) to a final concentration of 1mM was added. Samples were incubated for 1 hour at room temperature in the dark. SDS-PAGE buffer without the reducing agent β-mercaptoethanol was added to the samples without boiling before electrophoresis.

2.10.2 Immunoprecipitation using streptavidin agarose

The immunoprecipitaion of biotinylated proteins using streptavidin agarose was carried out according to Jaffrey et al., (2001). Four volumes of blocking buffer were added (9 volumes of extraction buffer, 1 volume of 25% SDS and 20mM MMTS) to at least 1ml of protein extract and incubated at 50 °C for 20 minutes with frequent vortexing. The protein samples were then incubated at -20 °C with two volumes of chilled acetone for

30 minutes. Samples were centrifuged at 2000g for 10 minutes at 4 °C and the pellet resuspended in 100µl of extraction buffer (50mM Hepes, pH7.4, 5mM EDTA, pH7.4, 5mM EDTA, 5mM DTT, 10mMNaF, 10mM Na_3VO_4 50mM β-glycerophosphate, 1mM PMSF, 2µg/µl antipain, 2µg/µl aprotinin, 2µg/µl leupeptin) for every mg of starting protein. Biotin-HPDP to a final concentration of 200µM was added and the samples incubated for 1 hour at room temperature. The biotin-HPDP was removed by using 2 volumes of acetone and incubating the samples at -20 °C for 30 minutes, then centrifuging the samples (2000g for 10 minutes 4 °C) and washing the protein pellet with chilled acetone. The pellet was then resuspended in 100µl of neutralisation buffer (20mM Hepes pH 7.7, 100mM $NaCl_2$, 1mM EDTA and 0.5% triton) for every mg of starting protein and then a further 2 volumes of neutralisation buffer was added. To purify the S-nitrosylated protein samples were incubated at 4°C with streptavidin agarose (15µl per mg of starting protein). The protein samples plus streptavidin agarose were centrifuged for 10 seconds at 200g and the supernatant removed. The protein-agarose pellet was washed 5 times in neutralisation buffer containing 600mM $NaCl_2$ using the centrifge. Finally samples were incubated in elution buffer (20mM Hepes pH 7.7, 100mM $NaCl_2$, 1mM EDTA and 100mM 2-β Mercaptoethanol) for 15 minutes. The eluted protein samples were resolved by SDS-PAGE electrophoresis.

2.10.3 Coomassie staining of SDS-PAGE Gels for MALDI analysis

After electrophoresis gels were fixed in a 50% methanol and 10% acetic acid solution for 15 minutes, then washed in distilled water for 5 minutes. Proteins were stained with colloidal Coomassie Blue (Pierce CodeBlue) according to the manufacturers instructions by adding 5ml of stain and microwaving for 40 seconds. The gel was stained for between 1-12 hour, then destained with distilled water. Protein bands were then exicised for MALDI (matrix assisted laser desorption/ionization) analysis at the proteomics facility at the John Innes Centre in Norwich.

2.10.4 Silver staining of SDS-PAGE Gels

Silver staining of protein gels was carried out using plusone™ kits from Phamacia Biotech according to the manufacturers instructions.

2.10.5 2-D SDS-PAGE electrophoresis

2-D SDS-PAGE electrophoresis was carried out according to protocols developed by Syngenta using Amersham products. Protein extracts of 100µg were made up to 125µl with solubilisation buffer (8M Urea) that was dissolved in 25ml of distilled water and after the addition of 0.5g mixbed resin was filtered through Whatman No1 filter paper, 0.2% DTT, 0.5% CHAPS and 0.2% Phamalyte), then vortexed. The samples were centrifuged at 15000rpm for 5 minutes at room temperature before dispensing equally along IEF (Isoelectric Focusing) coffins. The 7 cm IEF gel strips (Amersham) were placed over the protein samples ensuring the end of the gel strip is over the cathodic electrode and avoiding air bubbles. Mineral oil was added to prevent evapouration of the samples. The IEF was run for 10 hours at 20˚C on an Ettan™ IPGphor™ II IEF System from Amersham. This seperates the proteins along the IEF gel strips according to their isoelectric points (first dimension). The IEF strips are then removed and placed in 3ml of equilibration buffer (6M urea, 30% glycerol, 2% SDS, 50mM Tris-HCl pH 8.8 and 0.002% of bromophenol blue) for 15 minutes with agitation at RT. Equilibration was repeated in buffer 2 (6M urea, 30% glycerol, 2% SDS, 50mM Tris-HCl pH 8.8, 0.002% bromophenol blue and 0.02% Iodoacetamide). The strips were then lowered into the loading well of Novez NuPAGE 4-12% Bis-Tris ZOOM gels for electrophoresis, separating the proteins by size (second dimension). Proteins were visualised by silver staining.

2.10.6 GS-FDH in gel enzyme activity assay

GS-FDH enzyme activity was determined as described previously by Dolferus et al., (1997). Proteins were extracted in 2 volumes of 50mM KH_2PO_4, 1mM phenylmethanesulfonyl fluoride (PMSF). Proteins were then separated on a non-denaturing 7.5% (w/v) polyacrylamide gel. To stain for GS-FDH activity, gels were equilibrated for 1 minute in 0.1mM Na-phosphate buffer (pH 7). Native gels were then incubated at 25°C in 0.1mM Na-phosphate buffer (pH 7), 0.1mM NAD+, 0.1mM NBT, 0.1mM PMS, 1mM formaldehyde and 1mM glutathione for between 1 hour and 12 hours until bands of enzyme activity could be clearly visualised.

2.10.7 GS-FDH enzyme activity spectrophotometric assay

Enzyme activity was measured spectrophotometrically at 25°C by monitoring the decomposition of NADH at 340nm as described by Liu et al., (2001). GS-FDH activity was determined by incubating 100μg of protein in 300μl of assay mix that contained 20mM Tris-HCl (pH 8.0), 0.2mM NADH and 0.5mM EDTA. The reaction was started by adding GSNO (ALEXIS biochemicals) to the mix at a final concentration of 400 μM.

2.11 Ethylene evolution

Ethylene evolution was performed using 4 week old plants according to Iannetta and co-workers (1999). Ten plants per line were enclosed individually in 50ml syringes and ethylene allowed to accumulate over a 4 hour period. One ml of space was expelled from the 50ml syringe and ethylene determined by gas chromatography. The fresh weight of each plant was determined after incubation.

2.12 Quantification of Anthocyanin and Flavonols

Anthocyanins and flavonols were quantified using methods modified from Kubasek et al., (1992) and Gislefoss et al., (1992) respectively. Leaf samples (100mg) from ten individual plants per line were collected. The tissue samples were ground in 300µl of acidified methanol (1%HCl v/v) and incubated for 2 hours at 30°C. Following centrifugation (13,000rpm for 10 minutes) the supernatant was transferred to a new tube. Two volumes of chloroform were then added and the samples vortexed before centrifugation (13,000rpm for 5 minutes). The supernatant (methanol phase) was carefully collected and transferred to a new tube. The chloroform step was repeated and the supernatant collected and added to 600µl of water and vortexed. This fraction was read spectrophotometrically at 550nm for anthocyanin and at 350nm for flavonols.

Chapter 3

3. Screen for RNI resistance genes

3.1 Introduction

NO is produced in plants both by non-enzymatic and enzymatic reactions (Wendehenne et al., 2001). NO_2^- can be reduced under acidic conditions by ascorbate or by carotenoids in the presence of light to HNO_2, which subsequently dismutates to NO, NO_2 and higher oxides of nitrogen (Ehrt et al., 1997;Wojtaszek, 2000). Alternatively, NO can be produced by the cytosolic enzyme Nitrate Reductase (NR) which has been found to catalyse the NADPH dependent reduction of NO_2^- to NO (Dean and Harper, 1988, Yamasaki et al., 2000). It is thought that NO is constitutively formed in this way from NR (Rockel et al., 2002; Yamasaki et al., 2000). Finally, NO can be produced by two recently identified plant NO synthases (NOS). The first to be identified is a variant of the P protein of glycine decarboxylase (GDC) (Chandok et al., 2003). This NOS was found to be induced following TMV treatment, in resistant tobacco plants (Xanthi nc [NN]) and in response to TCV in resistant *Arabidopsis* plants. Therefore, this NOS is similar to the animal iNOS, which is only activated in response to pathogen infection (Nathan, 1997; Xie et al., 1992). The second plant NOS to be discovered is AtNOS, which is constitutively expressed (Guo et al., 2003). Both the plant iNOS and AtNOS are controlled by Ca^{2+} and calmodulin (CaM) (Chandok et al., 2003; Guo et al., 2003). The subcellular localisation of these plant NOS's has not yet been identified but evidence from studies using anti-NOS antibodies show immunoreactive proteins localised in the peroxisomes, chloroplasts and cytosol (Barroso et al., 1999; Riberio et al., 1999).

Thus, given the diverse and abundant sources of NO in plants, mechanisms to regulate NO levels and reduce nitrosative stress are required. GS-FDH is an enzyme which is specific for the metabolism of GSNO, which exists in plants, animals and bacteria (Liu et al., 2001, Sakamoto et al., 2002). Other genes important for resistance to nitrosative stress have also been identified in bacteria. For example, flavoheamoglobins (*Hmp*) catalyse NO consumption and *hmp* mutants in bacteria and yeast are hypersensitive to NO donors (Membrillo-Hernandez et al, 1999). Evidence suggests that plant

flavoheamoglobins also probably function as NO dioxygenases (Dordas et al., 2003). Transgenic alfalfa expressing antisense barley haemoglobin transcripts were found to have 2.5 higher levels of NO than transgenic lines expressing sense transcripts. In addition, transgenic tobacco plants over expressing an alfalfa haemoglobin were found to be more resistant to Sodium Nitroprusside (SNP) than wild type plants (Seregelyes and Dudits, 2003). Peroxiredoxins, which have $ONOO^-$ reductase activity, are widespread among bacterial genera. The peroxiredoxin alkylperoxide reductase subunit (*AhpC*) from *Salmonella typhimurium* was found to detoxify $ONOO^-$ (Bryk et al., 2000). There are 10 peroxiredoxin genes in *Arabidopsis*, which have been shown to be localised to different subcellular compartments including peroxisomes, chloroplasts and the nucleus (Dietz et al., 2002). However as yet none have been reported to metabolise $ONOO^-$.

Therefore, in order to uncover genes which are important for resistance to NO and RNI, a screen was developed using the NO donor SNP. Around 7000 activation tagged lines were screened and 10 mutant candidates were subsequently identified which show resistance to SNP

3. 2 Activation Tagged Lines

In plants mutant screens have been carried out using EMS mutagenesis or T-DNA tagging. These methods can be used to generate large numbers of mutations that effectively saturate the genome. However, these methods largely result in the identification of loss-of-function mutations.

Activation tagging was developed as a means of isolating gain-of-function mutations and corresponding genes (Weigel et al., 2000; Tani et al., 2004). The generation of mutant phenotypes by activation tagging is based on gene activation by transcriptional enhancers. In this system, four CaMV *35S* enhancer repeats within the T-DNA insert, can promote the over-expression of immediately adjacent genes, resulting in dominant phenotypes (Figure 3.1b). This method can potentially identify genes with either redundant or essential functions. In addition, the insertion of the T-DNA into a gene coding sequence may result in a knock-out mutant, which can be uncovered in the T_2 generation. The T-DNA tag can subsequently be used to clone the mutant gene of

interest by thermal asymmetric interlaced (TAIL)-PCR (Sessions et al., 2002), although chromosome rearrangements that inhibit cloning are possible.

Previously activation tagged lines were generated in the lab using Col-0 plants (Tani et al., 2004). Plants were transformed *via Agrobacterium* with the activation-tagging binary vector pSKI015 (Walden *et al.*, 1994; Weigel *et al.*, 2000) (Figure 3.1a). The bar gene is integrated into the transformation vector, which confers resistance to the herbicide BASTA® (glufosinate) and is useful for high-throughput selection of transgenic plants on soil. Primary transformants (T_1) were thus selected on soil for BASTA® resistance and allowed to self, T_2 seeds were then harvested from each T_1 plant.

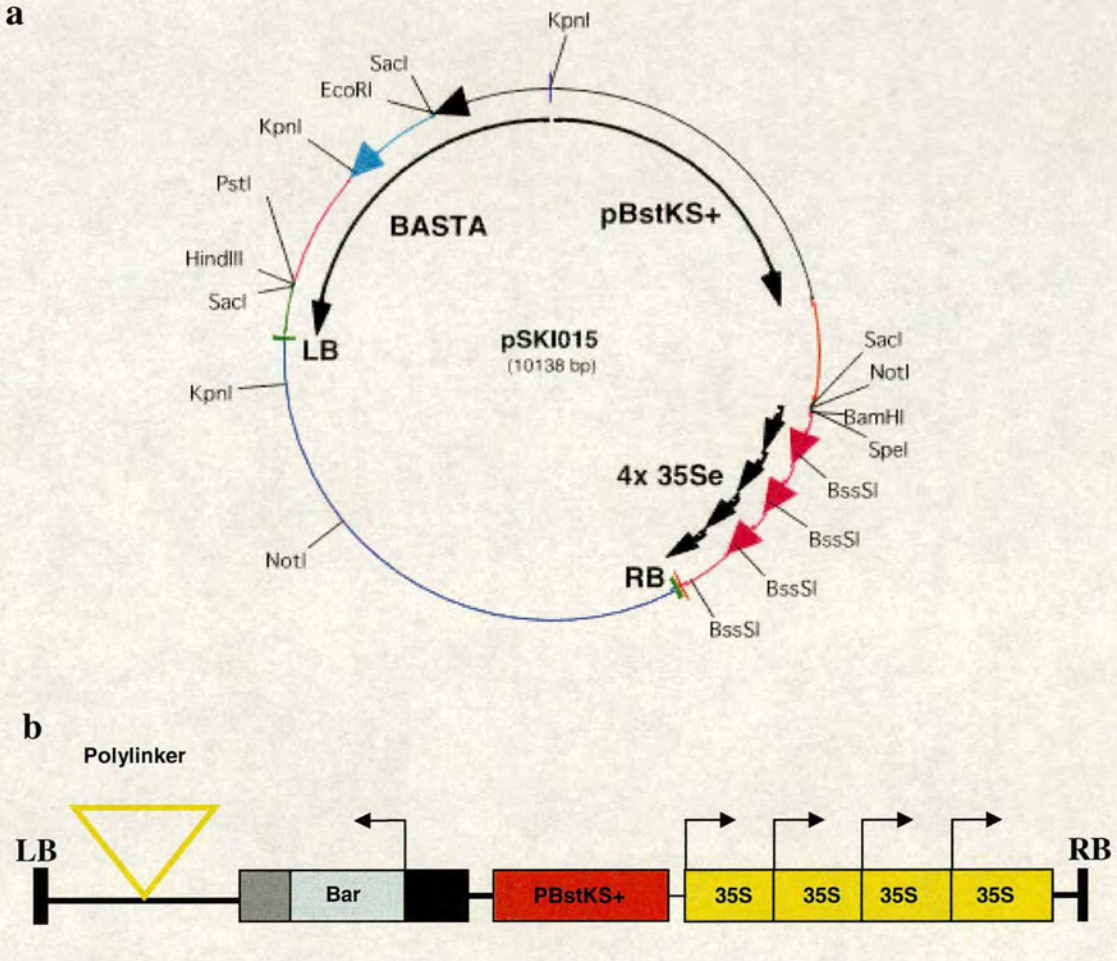

Figure 3.1 The activation tagging vector pSKI015

(a) Activation tagging vector pSKI015. **(b)** T-DNA insertion cassette enclosed by LB left border and RB-right border; containing BASTA® resistance gene, origin of replication (BluescriptSK+), and tetramer of CaMV 35S enhancer (Walden et al., 1994; Weigel et al., 2000).

3.3 Nitric Oxide Donors

To carry out a screen for mutants resistant to NO and RNI it was necessary to select an appropriate NO source. It was decided to use a plate-based screen to select for mutants resistant to an NO donor present in MS media. This would allow the NO to be easily administered as well as the rapid selection of mutant candidates. However, many commercially available NO donors are expensive. Acidified $NaNO_2$ and SNP were selected as economically viable NO donors to use, since it would be necessary to use large amounts of NO donor for routine screening.

3.3.1 $NaNO_2$

In acidic conditions $NaNO_2^-$ is protonated to HNO_2, which is then dismutated to NO, and other RNI (Ehrt et al., 1997; Ruan et al., 1999). Thus, acidified $NaNO_2$ can be used as an RNI/NO donor. To investigate the effect of $NaNO_2$ on the germination and development of *Arabidopsis*, Col-0 seeds were germinated on plates containing between 0-15mM $NaNO_2$. This concentration range was chosen, since a previous study with *E.coli* found that 10mM $NaNO_2$ completely suppressed growth (Ehrt et al., 1997). The inhibition of Col-0 germination was found to be complete on 12.5mM $NaNO_2$ (Figure 3.2a). Although, Col-0 was able to germinate on lower concentrations of $NaNO_2$, secondary leaf development was impaired and bleaching was observed. For example, on 7mM $NaNO_2$, 94% of Col-0 seeds germinated, but 91% had bleached leaves and only 24% of seedlings developed secondary leaves (Figure 3.3a). Therefore, although $NaNO_2$ does not completely inhibit germination until 12.5mM, it impairs development and causes severe bleaching, at lower concentrations.

The release of NO_2^- and its subsequent protonation / dismutation to NO and other RNI requires an acidic pH (Ehrt et al., 1997; Ruan et al., 1999). Therefore, the germination of Col-0 on 10mM $NaNO_2$ at pH5, pH6 and pH7 was investigated. The germination of Col-0 seeds on 10mM KNO_2, which is also reported to generate NO in acidic conditions, was examined for comparison (Agani et al., 2002; O'Donnell et al., 1999; Nakano, 2001). MS plates and 10mM $NaCl_2$ plates were also used as controls.

Surprisingly, the percentage germination of Col-0 was not found to be lower on 10mM $NaNO_2$ at pH5 (Figure 3.2b). In fact the percentage of germination at pH5 was similar. to that found at pH7, (32% and 37% respectively). The lowest germination percentage

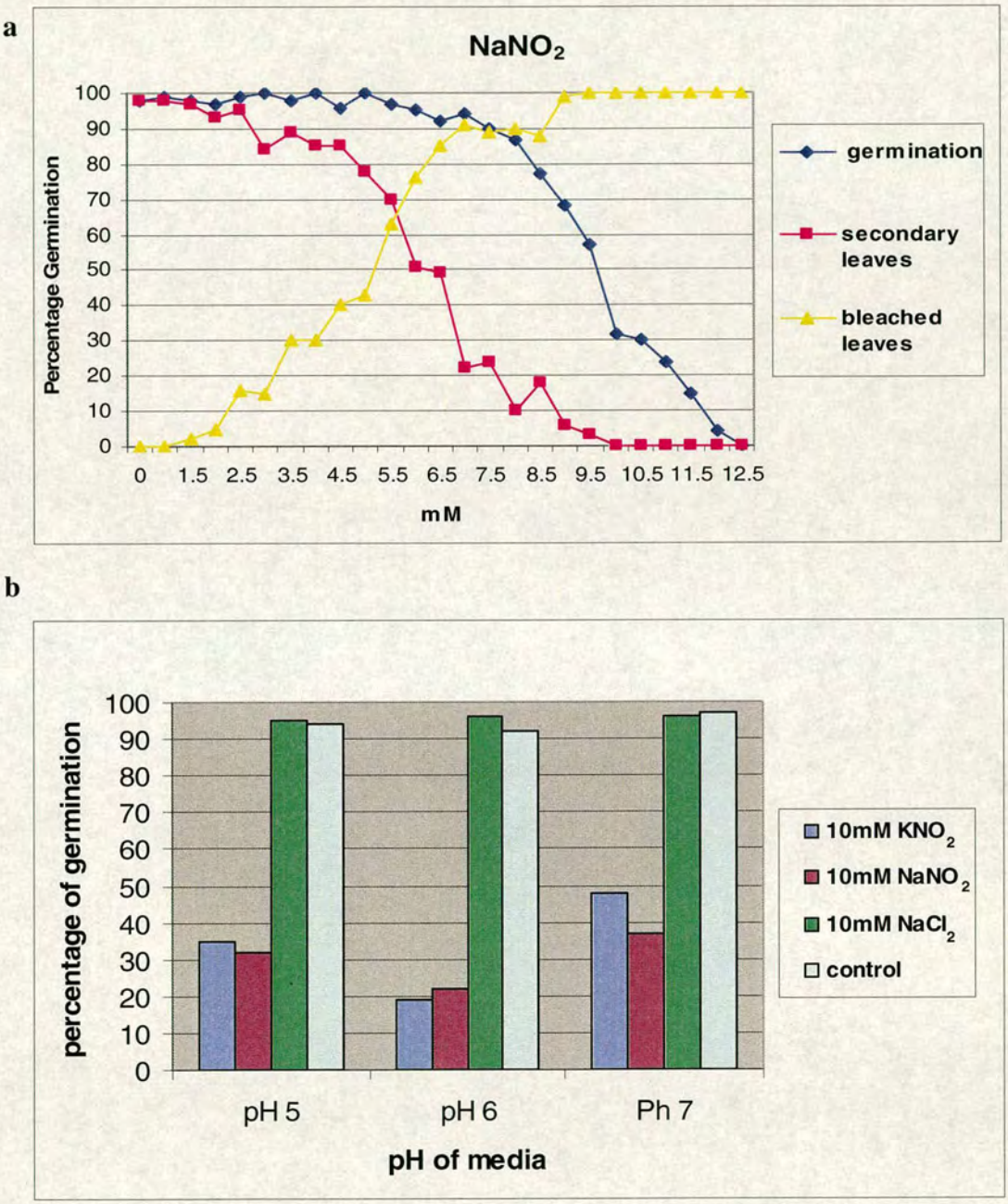

Figure 3.2 The effect of $NaNO_2$ on germination

(a) Percentage germination, secondary leaf formation and bleaching of Col-0 on MS plates containing increasing concentrations of $NaNO_2$ **(b)** Germination of seedlings at pH5, pH6 and pH7 on MS plates containing 10mM KNO_2, $NaNO_2$ and $NaCl_2$. ~250 seeds per plate

of 22% was found at pH6 (Figure 3.2b). Similar results were found on 10mM KNO_2^- at pH5, 6 and 7 (Figure 3.2b). Moreover, the germination of Col-0 on 10mM $NaCl_2$ was similar to that found on MS plates for all three pH values (Figure 3.2b). These results suggest that the lower percentage of Col-0 germination found on 10mM $NaNO_2$ and 10mM KNO_2 compared to that found on 10mM $NaCl_2$ is due to the generation of NO. However, these results are not consistent with the idea that an acidic pH accelerates the conversion of NO_2^- to NO and RNIs (Figure 3.2b)

3.3.2 SNP

The NO donor Sodium nitroprusside (SNP) requires light to release NO in solution and this release depends on the intensity of light (Harrison and Bates, 1993). The nitroprusside dianion is a complex of ferrous ion with five cyanide anions (CN^-) and a nitrosonium ion (NO^+) (Yamamoto and Bing, 2000). Interaction of SNP with a reducing agent such as a thiol leads to the formation of NO (Bates et al., 1991).

The germination of Col-0 was investigated on increasing concentrations of SNP between 0-3mM. It was found that at 2mM, SNP completely inhibits the germination of Col-0 seedlings (Figure 3.3a). The development of secondary leaves was also inhibited by SNP; only 2% of seedlings formed secondary leaves on 0.6mM SNP (Figure 3.3a). Therefore, SNP and $NaNO_2$ both inhibit the development of secondary leaves, although no bleaching of leaves was found with SNP.

To ensure this inhibition of germination and development was due to the effect of NO, the germination of Col-0 on light inactivated (LI) SNP was investigated. A stock solution of SNP was exposed to light for one week in order to allow the release of NO. Over this time a colour change from brown to blue was observed. Potassium ferricyanide (KFC) is structurally similar to SNP except for the absence of a nitroso group (Li et al., 1994; Li et al., 2001). It was found that the germination percentage of Col-0 on 1mM (88%) and 2mM (54%) KFC was similar to that on 1mM (76%) and 2mM (47%) LI SNP, respectively (Figure 3.3b). The percentage germination on 1mM SNP (20%) was significantly lower compared to that on MS (96%), 1mM LI SNP (76%) and 1mM KFC (88%). Therefore, the difference in germination between these controls and 1mM SNP is probably due to the generation of NO. Furthermore, the

germination of Col-0 on 2mM SNP (0%) was completely inhibited but not on 2mM LI SNP (47%) and 2mM KFC (54%) (Figure 3.3b). Again these results suggest that the germination difference is due to NO. The lower percentage of germination found

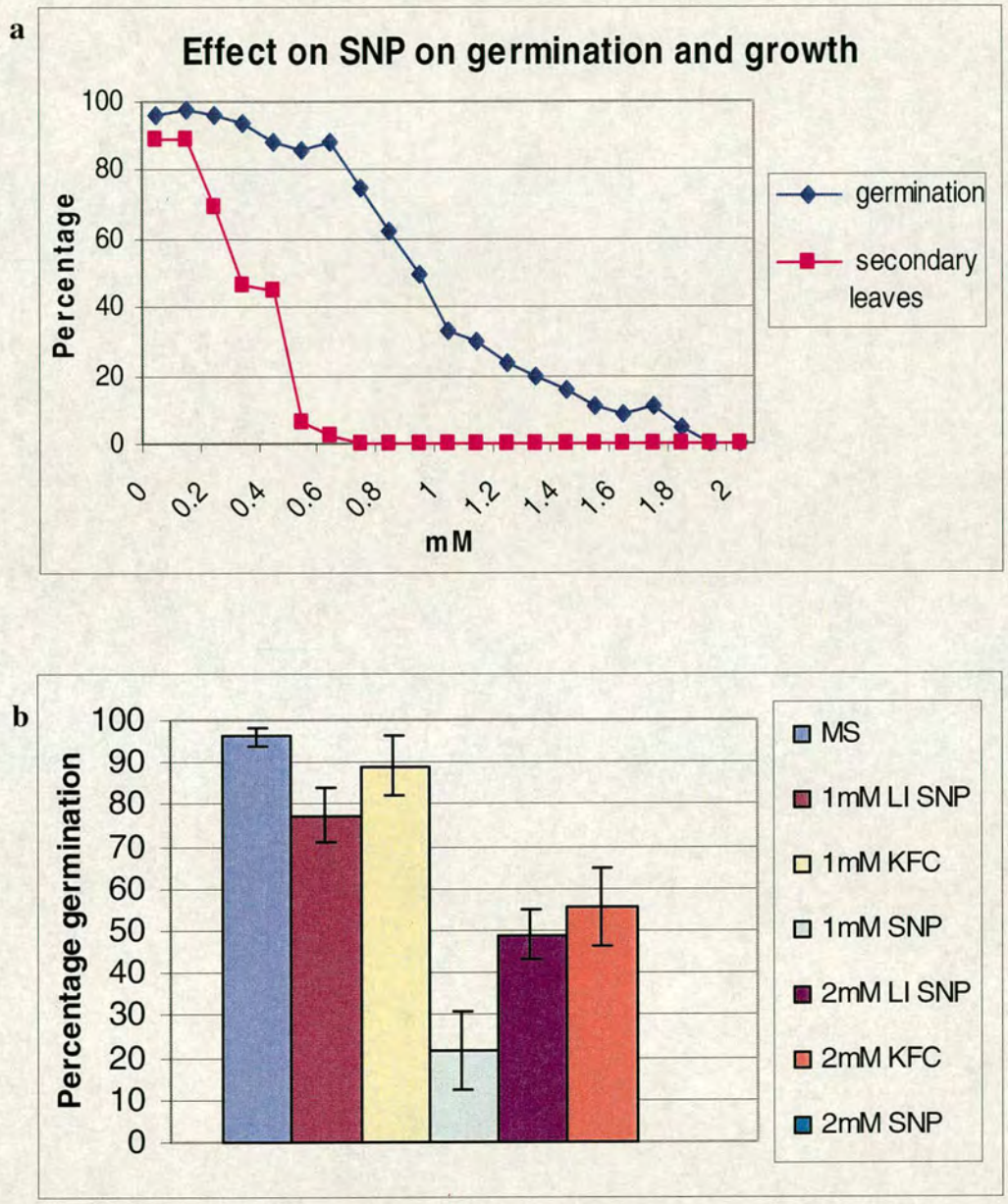

Figure 3.3 The effect of SNP on germination

(a) Germination and secondary leaf development of seedlings on MS plates containing increasing concentrations of the light activated NO donor SNP. ~250 seeds per plate **(b)** Germination of seedlings on MS plates containing 1mM or 2mM SNP, Light Inactivated (LI) SNP and Potassium Ferricyanide (KFC). Values shown are the average of 3 experiments (250 seed) and error bars represent the standard error between values at the 95% confidence level.

on 2mM LI SNP (47%) and 2mM KFC (54%) compared to MS plates (96%) also suggests that at 2mM these compounds are slightly toxic (Figure 3.3b). However, it was decided to use 2mM SNP for screening in order to avoid any 'leakiness', which may be allowed by lower concentrations.

3.4 Identification of SNP resistant mutants

The NO donor SNP was used to screen T_2 activation tagged lines for mutants resistant to NO and RNIs. Activation tagged lines were screened on MS plates containing 2mM SNP, which was found to completely inhibit the germination of Col-0 (Figure 3.4c). Around 200 seeds from each pool of activation tagged lines (50 lines per pool) were sown on 2mM SNP plates. After 3 weeks T_2 seedlings, which had successfully grown on the NO donor were selected as mutant candidates (Figure 3.4d). In this primary screen, 7000 lines were screened and 68 mutant candidates were selected. These were first transferred to MS plates before soil and then allowed to self. However, some of these mutant candidates were lost as they became bleached when transferred from 2mM SNP to MS plates and died as a result (Figure 3.5). The mutant candidates were screened again on 2mM SNP plates in the T_3 generation (Figure 3.4f). Following this secondary screen 10 putative mutants were isolated which displayed resistance to the NO donor 2mM SNP.

These NO resistant candidates have been backcrossed to Col-0 in order to test the segregation ratio of the F_2 population on 2mM SNP plates, this will establish if the mutation is dominant or recessive. Progeny, which segregate for resistance to SNP, will then be sprayed with BASTA® in order to determine if NO resistance segregates with a T-DNA tag. BASTA® analysis of the F_2 population will also reveal how many T-DNA tags are present in these mutant candidates.

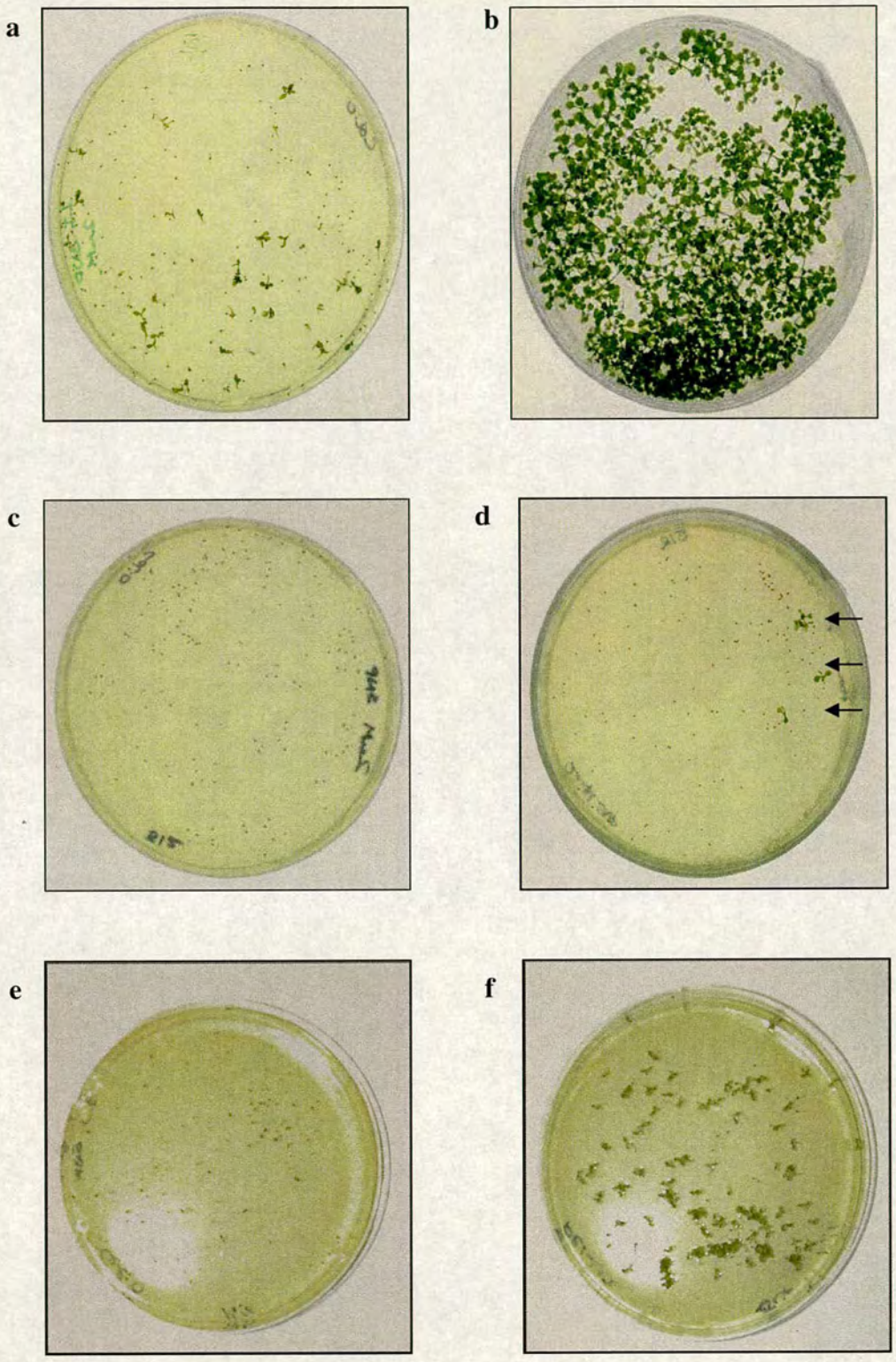

Figure 3.4 Screen for SNP resistant mutants

Pools of activation tagged lines were screened on plates of 2mM SNP which is a light sensitive nitric oxide donor. Plates (**a**) Col-0 seeds on Light Inactivated 2mM SNP (**b**) Col-0 on MS (**c**) and (**e**) Col-0 seed on 2mM SNP (**d**) arrows indicateT_2 mutant candidates (**f**) T_3 mutant candidate

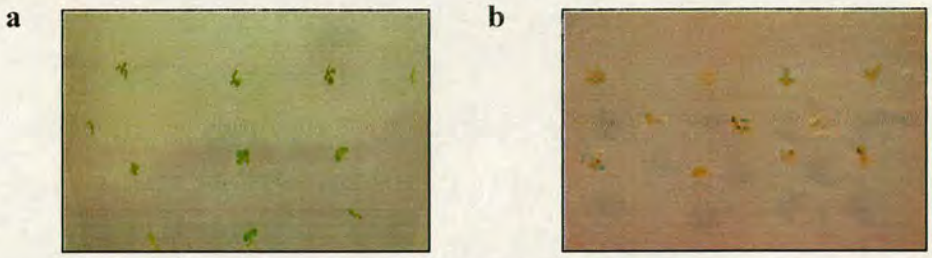

Figure 3.5 Bleached SNP resistant candidates on MS media

F_2 progeny were transferred to MS plates before transfer to soil. Mutant candidates on MS media **(a)** normal development **(b)** bleached

3.5 Discussion

Since there are diverse and abundant sources of NO in plants, mechanisms to regulate NO levels and reduce nitrosative stress are required. In order to try and elucidate genes involved in resistance to nitrosative stress a screen was developed using activation tagged lines. $NaNO_2$ and SNP were investigated as economically viable NO donors to use for screening mutants. It was decided to use SNP as an NO donor rather than $NaNO_2$ since the control experiments for SNP were consistent with the hypothesis that SNP is a light sensitive NO donor (Figure 3.3b). Control experiments for $NaNO_2$ were not consistent with the idea that NO is generated under acidic conditions (Figure 3.2b). In order to avoid any 'leakiness' of the mutant screen 2mM SNP was used which had previously been found to completely inhibit the germination of Col-0 (Figure 3.3 a and b). In addition, previous studies with *Arabidopsis* demonstrated that SNP promotes cell death in synergy with ROIs and is able to induce the expression of the defence genes *PAL, CHS* and *GST1* (Delledonne et al., 1998).

Using this screen 10 putative mutants were found that show increased resistance to SNP in comparison to Col-0. It will be necessary to confirm that these mutants are resistant to other NO donors such as *S*-nitroso-*N*-acetylpenicillamine (SNAP) or diethylenetriamine/NO (DETA/NO). Segregation analysis of the F_2 population from a Col-0 backcross will establish if the mutations for SNP resistance are dominant. This is likely since activation tagging was designed as a method to isolate gain-of function mutations. If SNP resistance is also found to segregate with BASTA® resistance then the

corresponding gene will be T-DNA tagged. BASTA® selection will also reveal how many T-DNA tags are present in the mutant. A TAIL-PCR approach can then be used to clone the RNI resistance gene (Session et al., 2002). However, if the gene is not tagged it is possible to use mapping to identify the gene of interest.

Once confirmed these mutants will provide a useful tool to further investigate the role of NO in plant disease resistance. For example, they may display enhanced susceptibility or resistance to virulent pathogens. It will be interesting to investigate if these NO resistant mutants also show resistance to ROIs. Screens to identify RNI resistance genes from the bacterium *M. tuberculosis* identified two genes *noxR1* and *noxR3* both of which provide resistance to H_2O_2 in addition to RNIs (Raun et al., 1999; Ehrt et al., 1997). Therefore, NO and ROI levels may differ in these mutants compared to wild-type plants and this in turn may affect the progression of the HR and defence responses. It is possible that the mutant candidates identified in this screen may over-express RNI catabolic enzymes. Therefore, it will be particularly interesting to compare the results of these studies with those found for the *GS-FDH* over-expressing mutant *fdh1-1*. Catabolic enzymes that confer resistance to RNIs have been identified in micro-organisms These include flavoheamoglobins which are NO dioxygenases (*hmp*) and peroxiredoxins (*AhpC*) that detoxify $ONOO^-$ (Bryk et al., 2000). Evidence suggests that plant flavoheamoglobins also probably function as NO dioxygenases. Transgenic tobacco plants expressing an alfalfa haemoglobin (*Mhb1*) were found to be more resistant to SNP than wild type plants (Seregelyes and Dudits, 2003). Immunolabelling using a polyclonal antibody against Mhb1 revealed that it is localised in the nucleus and to a lesser extent the cytosol (Seregelyes et al., 2000). There are also 10 peroxiredoxin genes in *Arabidopsis*, however as yet none have been reported to metabolise $ONOO^-$. Other mechanisms have been implicated in RNI resistance in bacteria. For example, screening of *M. tuberculosis* transposon mutants led to the identification of 12 mutants with increased susceptibility to acidified $NaNO_2$, 5 of which were found to be in proteasome associated genes (Darwin et al., 2003). In eukaryotic cells the proteasome has been shown to selectively degrade proteins in the damaged by oxidative stress (Tsirigotis et al., 2001).

In conclusion, these mutant candidates may help to uncover mechanisms, which confer resistance to RNIs in plants. In addition the investigation of defence responses in these mutants will further our understanding of the role of NO in plant disease resistance.

Chapter 4

4. Identification and characterisation of a *GS-FDH* over-expressor mutant

4.1 Introduction

Following pathogen recognition there is a rapid burst of NO production in plants (Delledonne et al., 1998; Durner et al., 1998). Recent studies have shown that NO is a key signalling molecule in plant physiology where it has been implicated in plant development and defence responses (Delledonne et al., 1998; Beligini and Lamattina, 2000). In animals NO initiates cGMP dependent signalling pathways through the activation of adenylate cyclase by binding to its heme iron moiety. This activation results in a transient increase in cGMP levels, which triggers physiological changes such as smooth muscle relaxation (Stamler, 1994). These physiological changes are brought about by the targets of cGMP, including protein kinases and cyclic nucleotide gated channels (Beck et al., 1999). cGMP pathways can be both pro-apoptotic (Taimor et al., 2000; Shimojo et al., 1999) or anti-apoptotic (Ciani et al., 2002; Kolb, 2000; Fiscus et al., 2002). Evidence suggests that NO can also induce apoptosis in plants using cGMP as a secondary messenger (Clarke et al., 2000).

In animals an alternative pathway for NO signalling is through the S-nitrosylation of thiol containing proteins. S-nitrosylation has emerged as a redox-based post-translational modfication which regulates protein activity (Foster et al., 2003). For example, the S-nitrosylation of caspase-1 maintains this enzyme in an inactive form, wheras the de-nitrosylation of caspase-1 is part of an apoptotic pathway (Rossig et al., 1999; Kim et al., 1998).

NO may interact with oxygen, superoxide anion, and thiol compounds, generating *S*-nitrosothiols (SNO's) including *S*-nitrosoglutathione (GSNO) (Rauhala et al., 1998). Evidence from animals indicates that GSNO is central to signal transduction and host defences, providing a reservoir of NO bioactivity (Liu et al., 2004; Mayer et al., 1998). GSNO has also been shown to be a potent antioxidant protecting from oxidative stress in endothelium tissue and lipid peroxidation by $\cdot OH$ in the brain (Chiueh, 1999; Rauhala et al., 1998). Since GSNO is a biologically active signalling molecule and an

important antioxidant, it is necessary to regulate GSNO levels. The enzyme GS-FDH exhibits strong GSNO reductase activity, which is highly conserved in animals, bacteria and plants (Lui et al., 2001). In yeast and rat hepatocytes GS-FDH is localised in the nucleus and cytosol (Fernandez et al., 2003; Iborra et al., 1992). GS-FDH knock-out mutants in mice and yeast are hypersensitive to nitrosative challenge (Lui et al., 2001). Recently, it was found that GS-FDH knock-out mice have increased protein S-nitrosylation, tissue damage and mortality following endotoxic or bacterial challenge (Lui et al., 2004). These mice were also found to have increased basal levels of SNOs in red blood cells and were hypotensive under anesthesia (Lui et al., 2004). Thus, GS-FDH in animals regulates GSNO and SNO levels, which are central to innate immune and vascular function.

The discovery that GS-FDH is highly specific for the metabolism of GSNO (Lui et al., 2001) prompted us to investigate the role of this enzyme in plant disease resistance. A single copy *Arabidopsis* GS-FDH (At5g43940) was identified by searching the TAIR database, which shows 75% homology in amino acid sequence to mouse GS-FDH (P28474). The induction of *GS-FDH* in *Arabidopsis* by GSNO was confirmed by northern analysis. Syngenta's SAIL (Syngenta's Arabidopsis Insertion Lines) database was then searched for an insertion line in *GS-FDH* and a line identified, which was subsequently found to be an over-expressor (*fdh1-1*). Since GSNO and SNO's exists in equilibrium with S-nitrosylated proteins, presumably the metabolism of GSNO by the over-expression of GS-FDH will drive the equilibrium away from protein S-nitrosylation in *fdh1-1* plants.

fdh1-1 plants exhibit perturbed developmental phenotypes affecting roots, flowering and anthocyanin accumulation. *fdh1-1* was used to investigate the role of GS-FDH in resistance to oxidative and nitrosative stress, using different ROI and NO donors. The response of *fdh1-1* to different bio-trophic and nectrotrophic pathogens was also investigated to elucidate a role for GS-FDH in plant disease resistance.

During this work Sakamoto and co-workers confirmed the ability of *Arabidopsis* GS-FDH to metabolise GSNO by expressing At5g43940 in *E.coli*. GS-FDH was also shown to functionally complement yeast mutants hypersensitive to GSNO (Sakamoto et al.,2002). As yet the subcellular location of GS-FDH in plants has not been demonstrated.

4.2 *GS-FDH* expression is induced by GSNO and H_2O_2 in wild-type plants

An *Arabidopsis* homologue of GS-FDH (At5g43940) was identified which has 75% amino acid homology to mouse GS-FDH (P28474) and it was found to be a single copy gene. To investigate if *GS-FDH* in *Arabidopsis* is responsive to GSNO, wild-type, Col-0 plants were infiltrated with 500μm GSNO. Wild-type plants were also infiltrated with 20mM H_2O_2 to investigate whether ROIs can also regulate *GS-FDH*. Samples were collected at the time points indicated and RNA extracted for northern analysis. Infiltration with 500μm GSNO strongly induced *GS-FDH* expression in *Arabidopsis* after 1 hour and *GS-FDH* expression had increased further, 6 hours after treatment. Surprisingly, infiltration of H_2O_2 also induces *GS-FDH* expression, although not as strongly as GSNO after 1 or 6 hours. However, 24 hours after H_2O_2 treatment the levels of *GS-FDH* transcript are comparable with those induced by GSNO after 6 hours. These results are shown in Figure 4.1.

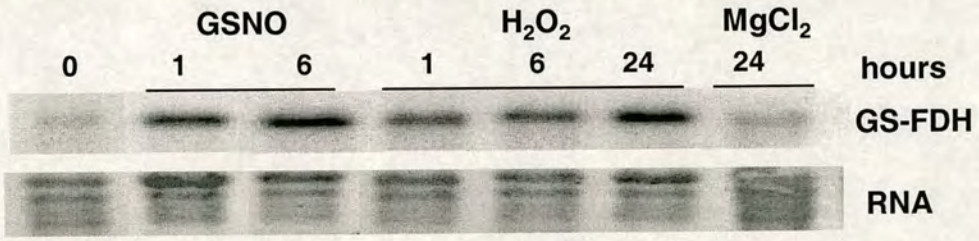

Figure 4.1 *GS-FDH* expression in wild-type plants

Wild-type Col-0 plants were infiltrated with 500 μm GSNO or 20mM H_2O_2 and harvested at 1, 6 and 24 hours post infiltration. Infiltration of $MgCl_2$ was used as a control and samples collected at 24 hours post infiltration. Northern blots were probed with a *GS-FDH* cDNA probe.

4.3 Identification of the *fdh1-1* mutant line

Syngenta's SAIL collection was searched for lines containing a T-DNA insert in *GS-FDH*. TAIL-PCR analysis (performed by Syngenta) identified two lines (1288-D09 and 678-D05) with a possible T-DNA insert in *GS-FDH*. Blasting the sequenced TAIL-PCR

product indicated that the T-DNA insert in both these lines was situated in the 5' UTR promoter region of *GS-FDH*. PCR was then undertaken to confirm the presence of T-DNA, using two gene specific primers and one left border T-DNA primer (Figure 4.3). PCR using Col-0 genomic DNA produces a 600bp product, whereas PCR with DNA from plants homozygous for the T-DNA insert will produce a 350bp product and PCR with DNA from heterozygotes will produce both bands. Figure 4.2a shows plants from a segregating F_2 population (1288-D09). A homozygote (subsequently called *fdh1-1*) for the T-DNA insert was identified from this population and allowed to set seed (Figure 4.2a). The progeny were tested again by PCR, which confirmed *fdh1-1* was a homozygous line for the T-DNA insertion (see Figure 4.2b). This PCR analysis established that the T-DNA insert of *fdh1-1* is situated in the 5' UTR promoter region of *GS-FDH*, 350bp upstream from the start codon (Figure 4.3). It was also found that there is an flavonol synthase (*FLS*) gene situated 450bp upstream of the *fdh1-1* T-DNA insert (Figure 4.3).

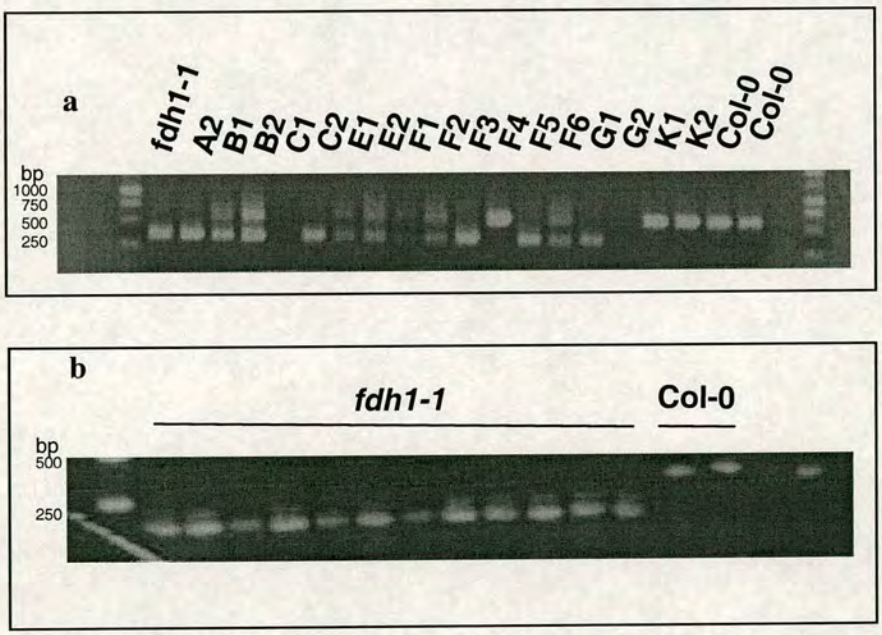

Figure 4.2. Identification of T-DNA insertion line using PCR

PCR with two gene specific and one T-DNA left border primer were used to confirm the *GS-FDH* T-DNA insert in a **(a)** segregating population, *fdh1-1* was selected as a homozygote and allowed to set seed **(b)** *fdh1-1* progeny were confirmed as homozygous for the T-DNA insertion

4.4 Phenotypic characterisation of *fdh1-1* plants

fdh1-1 plants show interesting developmental phenotypes including, anthocyanin accumulation, short inflorescences, short roots and increased root hair formation. In order to confirm that these phenotypes are due to the presence of T-DNA in the 5'UTR of *GS-FDH*, an additional allele *fdh1-2* was identified from the SIGnAL database. *fdh1-2* plants have a T-DNA insert 100bp downstream of the insert of *fdh1-1* plants and 200bp upstream of the *GS-FDH* start codon (Figure 4.3). The position of this insert was confirmed as before using PCR. Importantly, *fdh1-2* mutants display a similar phenotype to *fdh1-1* mutants (Figure 4.4b). *fdh1-1* plants were also back-crossed to Col-0 and the F_1 progeny allowed to self. PCR analysis of 30 of the F_2 plants identified six individuals, which were homozygous for the *GS-FDH* insert. The *fdh1-1* phenotype was found to segregate with the T-DNA insert in *GS-FDH*.

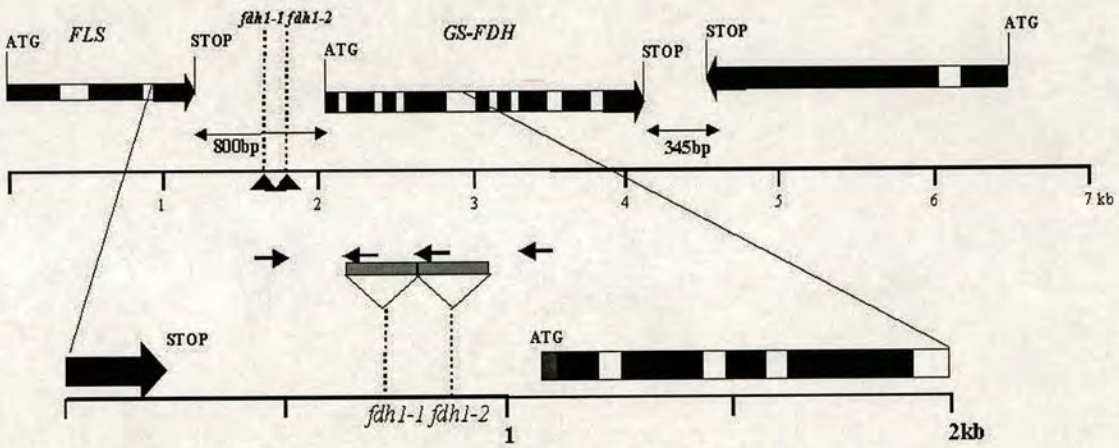

Figure 4.3 Position of the T-DNA insert in *fdh1-1* and *fdh1-2* mutants

fdh1-1 and *fdh1-2* T-DNA inserts are 350 and 200bp upstream of the *GS-FDH* start codon. Arrows show position of gene specific and T-DNA left border primers. T-DNA inserts are shown in grey (~4.7kb) but are not shown to scale.

4.4.1 Flavonoid accumulation in *fdh1-1* plants

The most obvious phenotype of *fdh1-1* is the precocious accumulation of anthocyanin around 4 weeks old, which is also seen in *fdh1-2* (Figure 4.4b). At 3 weeks old anthocyanin levels in *fdh1-1* are not significantly higher than those seen in wild-type Col-0 (Figure 4.4c). However, flavonol levels in 3 week old *fdh1-1* plants are more than double those found in Col-0 (Figure 4.4a). Between 3-5 weeks, *fdh1-1* accumulates high levels of anthocyanin and at 5 weeks old *fdh1-1* has approximately ten times the anthocyanin content of Col-0 (Figure 4.4c). Flavonol levels are also still significantly

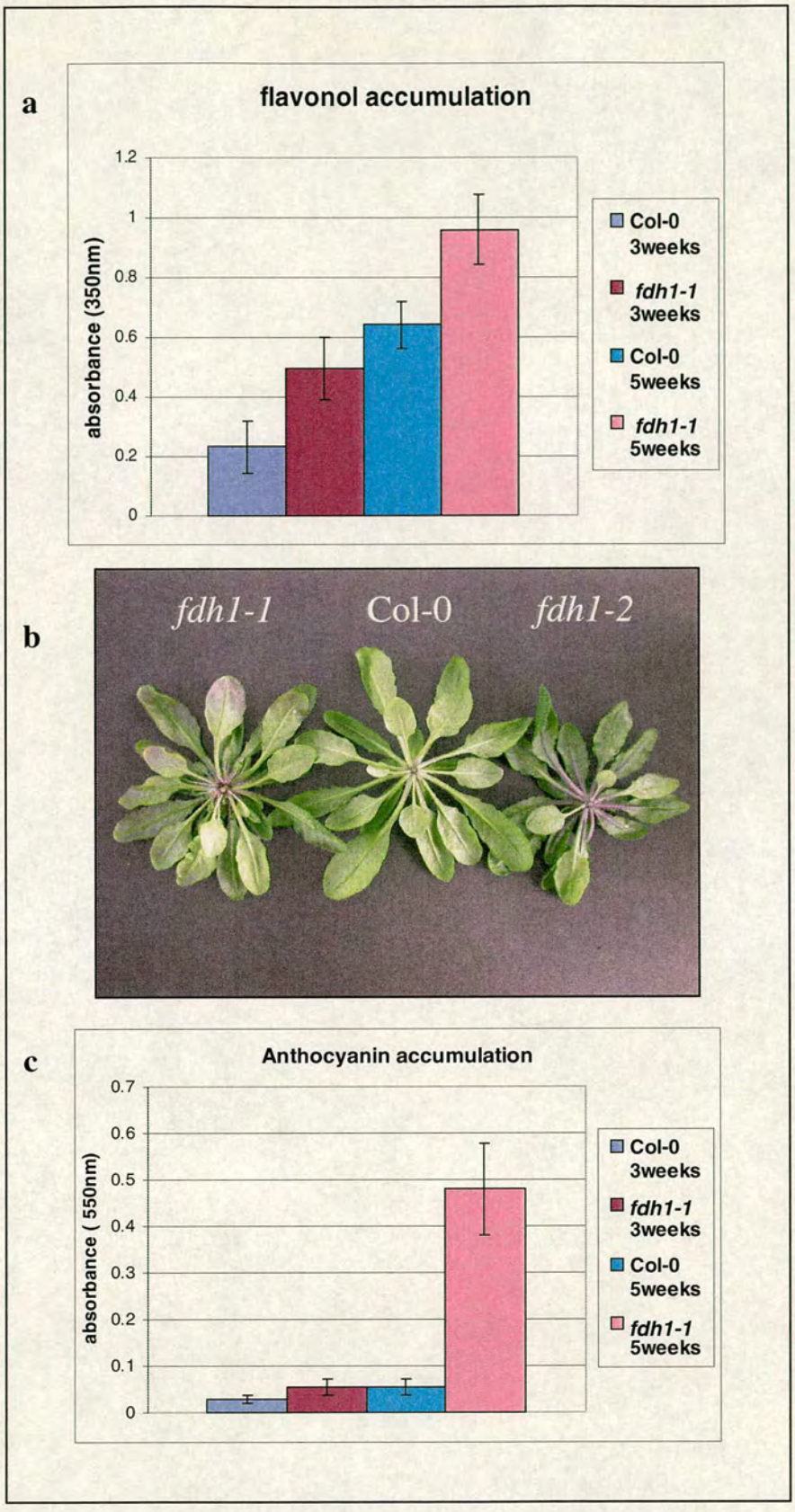

Figure 4.4. Anthocyanin and flavonol accumulation in *fdh1-1* mutants

Spectrophometric assay for **(a)** flavonol content (350nm) **(c)** anthocyanin (550nm) content in 3 week and 5 week old Col-0 and *fdh1-1* plants. Values shown are averages of 10 plants and error bars represent the standard error between values at the 95% confidence level. **(b)** Col-0, *fdh1-1* and *fdh1-2* plants at 4 weeks old, abaxial side of leaves.

higher in *fdh1-1* than Col-0 at this time (Figure 4.4a). Although, the T-DNA insert in *fdh1-1* and *fdh1-2* plants was found to be situated in the promoter region of *GS-FDH* it is also 450 bp downstream of an *FLS* gene. Therefore, it is possible that the high levels of anthocyanin accumulation in *fdh1-1* and *fdh1-2* plants may be caused by the perturbed expression of *FLS*.

4.4.2 Root development in *fdh1-1* plants

Recently ROIs have been proposed to control root tip growth (Foreman et al., 2003). Moreover, T-DNA knockouts of *AtNOS1* have shorter roots than wild-type plants (Guo et al., 2003), suggesting that NO as well as ROIs have a role in root development. To investigate the root development of *fdh1-1,* forty seedlings of *fdh1-1* and Col-0 were grown vertically on MS plates. The root length of these *fdh1-1* and Col-0 seedlings were measured after 14 days. *fdh1-1* was found to have shorter roots than wild-type Col-0. The average root length for Col-0 is 2.3cm whereas *fdh1-1* has an average root length of 1.1cm (see Figure 4.5a and b). This observation led to a more detailed examination of *fdh1-1* roots microscopically (Figure 4.5c). It was found that *fdh1-1* has prolific root hair formation and that these root hairs are longer than those of Col-0 (Figure 4.3 c and d).

Mutants which have a constitutive JA and ET signalling pathways, such as *cev1*, also have short roots and prolific, long root hairs (Ellis and Turner, 2001). In addition these mutants have reduced sensitivity to MeJA (Ellis and Turner, 2001). Thus to investigate if *fdh1-1* is sensitive to MeJA, Col-0, *fdh1-1* and *coi1* seedlings were germinated on 150μm MeJA plates and examined after 14 days. Col-0 seedlings showed anthocyanin accumulation and inhibition of root elongation on 150 μm MeJA. The average Col-0 root length on MS plates is 2.3cm compared to around 1.1cm on 150 μm MeJA (Figure 4.5 b and f). *coi1* seedlings which are insensitive to MeJA were light green and had long roots of around 2.5cm (Figure 4.5 f). Whereas *fdh1-1* showed strong anthocyanin accumulation and had the shortest roots (0.5mm) (Figure 4.5 f). Thus *fdh1-1* does not have reduced sensitivity to MeJA since *fdh1-1's* root length is around half that of Col-0 both on MS and MeJA plates. The root hairs of *fdh1-1* were also prolific and long on 150μm MeJA plates (Figure 4.5e). Col-0 showed an increased number of root hairs on MeJA plates compared to MS plates (Figure 4.5 h and d) and *coi1* was found to have shorter root hairs than both Col-0 and *fdh1-1* (Figure 4.5g).

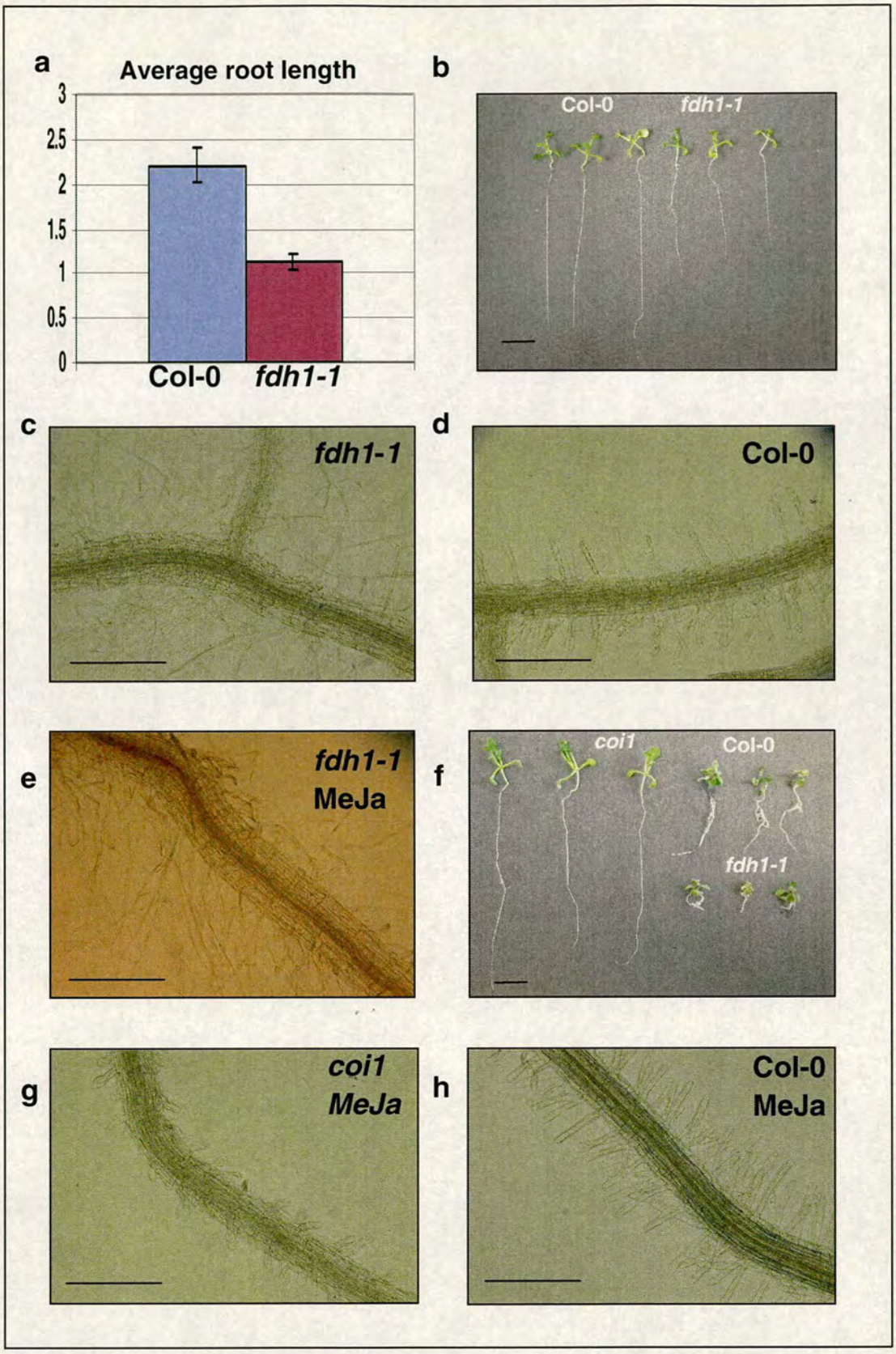

Figure 4.5 Root development in *fdh1-1* mutants

a) average root length of Col-0 and *fdh1-1* 14 day old seedlings. Values shown are the average of 40 seedlings and error bars represent the standard error between values at the 95% confidence level. 14 day old seedlings of **b)** Col-0 and *fdh1-1* grown on MS plates **f)** Col-0, *fdh1-1* and *coi1* grown on 150 µm MeJa plates. Scale bars 0.5mm. Root hair growth of **c)** Col-0 **d)** *fdh1-1* on MS plates and of **e)** *fdh1-1* **g)** *coi1* **h)** Col-0 on 150 µm MeJa plates. Scale bars 50µm.

4.4.3 Flower development in *fdh1-1* plants

T-DNA knockouts of *AtNOS1* have shorter inflorescence's than wild-type plants (Guo et al., 2003) implicating NO in flowering. *fdh1-1* shows flowering abnormalities such as short inflorescences, small siliques compared to those of Col-0 and some flower buds fail to develop (Figure 4.6). These abnormalities are most pronounced when *fdh1-1* is grown in short days. *fdh1-1's* perturbed flowering phenotypes suggests GSNO and SNO's are important during flower development

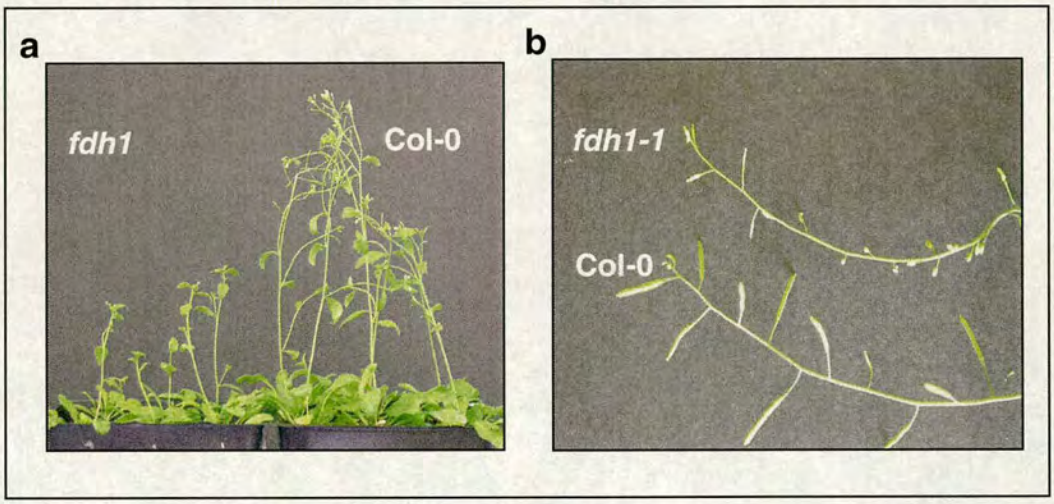

Figure 4.6. Flowering abnormalities in *fdh1-1* mutants

(a) 5 week old flowering *fdh1-1* and Col-0 grown in short days **(b)** florescence's from 6 week old *fdh1-1* and Col-0 grown in short days.

4.5 *fdh1-1* mutants over-express *GS-FDH*

The T-DNA insert of *fdh1-1* is situated in the promoter region of *GS-FDH* (Figure 4.3) and this results in perturbed development. To establish the effect of the T-DNA on *GS-FDH* expression, RT-PCR and northern analysis were used to detect *GS-FDH* transcript. RNA was extracted from untreated Col-0 and *fdh1-1* plants. Primers for *GS-FDH*, the upstream flavonol synthase gene (*FLS*) and the housekeeping gene *GAPDH* (as a control) were used for RT-PCR. Figure 4.7b shows that *GS-FDH* transcript and *FLS* transcript are present in both Col-0 and *fdh1-1*. These initial RT-PCR results indicate that both *FLS* and *GS-FDH* are over-expressed in *fdh1-1* plants. However the control gene *GADPDH*, also appears to be over-expressed in *fdh1-1* plants. Thus there is probably not equal loading of RNA in the Col-0 and *fdh1-1* samples.

Northern analysis also confirms the presence of *GS-FDH* transcript in both Col-0 and *fdh1-1*. The northern results also clearly show that there is more *GS-FDH* transcript present in *fdh1-1* than in Col-0 plants (figure 4.7a). Probes for the defence genes *PR-1* and *PDF1.2* were also used. It was found that *fdh1-1* strongly constitutively expresses the defence gene *PDF1.2* in addition to low levels of *PR1*. The higher levels of *GS-FDH* transcript present in *fdh1-1* prompted us to investigate GS-FDH protein levels.

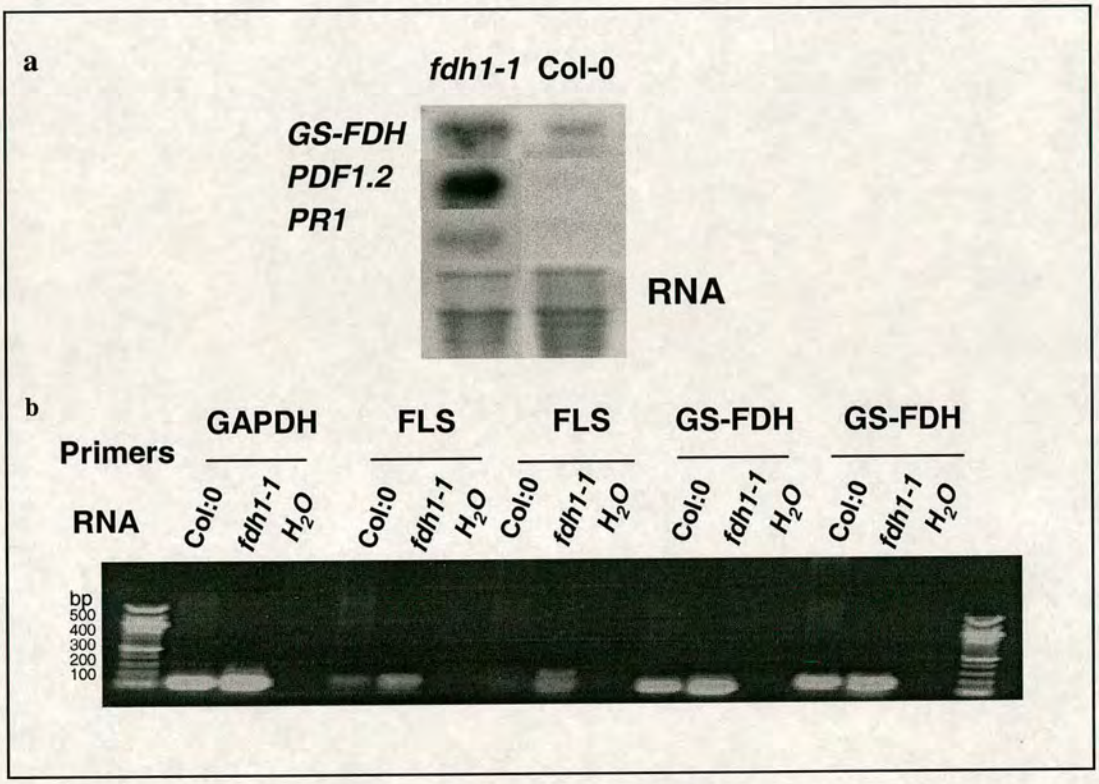

Figure 4.7 *GS-FDH* transcript levels in *fdh1-1* mutants

RNA samples were extracted from naïve *fdh1-1* and Col-0 plants and used for **(a)** Northern blot analysis with *GS-FDH* cDNA, *PDF1.2* and *PR1* probes **(b)** RT-PCR with primers for flavonol synthase (*FLS*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *GS-FDH*.

The metabolism of GSNO by GS-FDH is dependent on NADH. Using a spectrophometric assay which measures the consumption of NADH, protein extracts from *fdh1-1*, *NahG* and Col-0 were assayed for GSNOR activity. *fdh1-1* was found to have a higher GSNOR activity than Col-0 (figure 4.8a). Interestingly extracts from *NahG* plants (which are depleted in SA) were found to have a lower GSNOR activity than Col-0 (figure 4.8a). GS-FDH is also able to use S-hydroxymethylglutathione (HM-GSH) as a substrate although, it has higher affinity for GSNO (Lui et al., 2001).

Evidence suggests that GSNO and HM-GSH bind in a similar if not an identical manner in the active site of GS-FDH. Substituting Arg115 for either Lys or Ser in GS-FDH impairs the reduction of both GSNO and HM-GSH (Hedberg et al., 2003). An in-gel enzyme activity assay was therefore performed (figure 4.8b), based on the ability of GS-FDH to metabolise HM-GSH. Protein extracts from *fdh1-1, fdh1-2,* Col-0 and *NahG* were separated on NATIVE-PAGE gels, before incubation with the substrate HM-GSH. *fdh1-1* and *fdh1-2* extracts exhibited the highest GS-FDH enzyme activity and *NahG* the lowest, compared to wild type Col-0 plants. The low GS-FDH activity of *NahG* plants is in agreement with a recent report, which shows GS-FDH activity is activated by SA (Diaz et al., 2003). These GS-FDH activity assays, in addition to the northern data show that the T-DNA insert in the 5'UTR of *GS-FDH* has caused its over-expression in *fdh1-2* and *fdh1-2* plants.

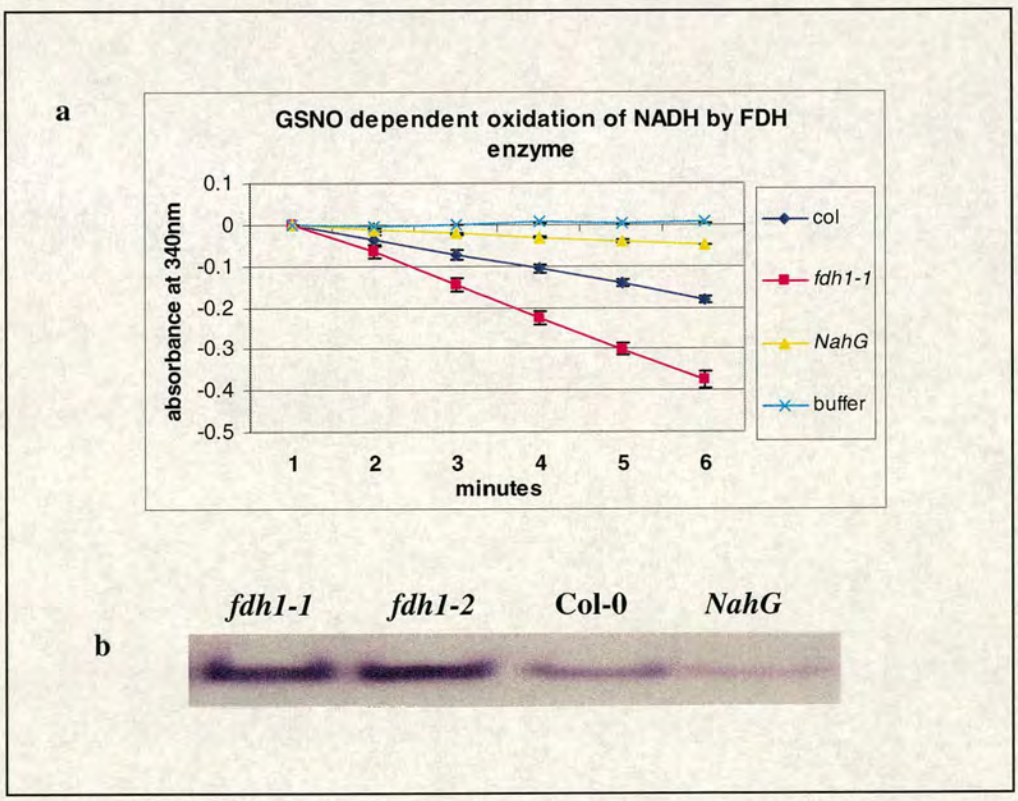

Figure 4.8 GS-FDH enzyme activity

Protein extracts from naïve *fdh1-1*, *fdh1-2*, Col-0 and *NahG* plants were collected and used for **(a)** spectrophometric assay monitoring NADH dependent GSNO consumption. Values shown are the average of 5 plants and error bars represent the standard error between values at the 95% confidence level. This experiment was repeated twice with similar results. **(b)** In gel enzyme activity assay using HM-GSH as a substrate.

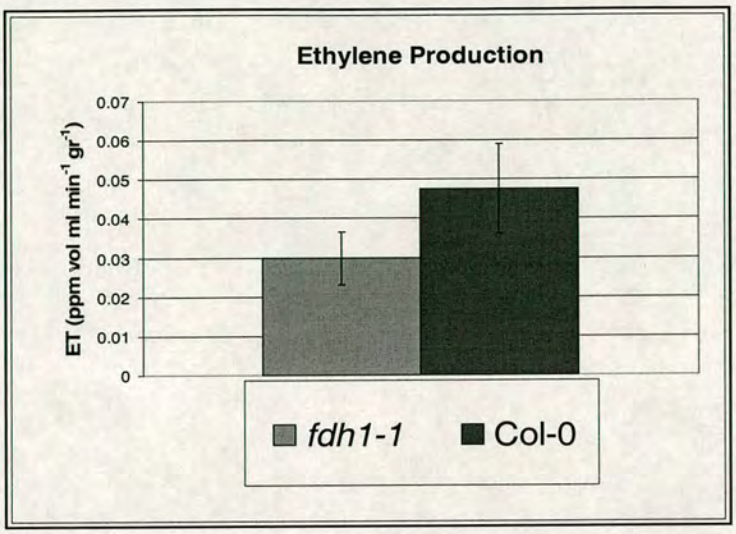

Figure 4.9 Ethylene levels in *fdh1-1* plants

3 weeks old plants grown on soil were used for measurements and the ethylene concentration was determined by GC (Gas Chromatography) analysis. Values shown are the average of ten plants per line and error bars represent the standard error between values at the 95% confidence level

4.6 Ethylene evolution in *fdh1-1* plants

The *Arabidopsis* mutants *ein2* and *coi* are defective in ET and JA signal transduction pathways respectively, and both fail to induce *PDF1.2* (Penninckx et al., 1998). Thus, the induction of *PDF1.2* is thought to require both functional JA and ET signalling pathways. Since *PDF1.2* expression is constitutive in naive *fdh1-1* plants, ET levels were investigated. Surprisingly, there was no significant difference found between ET levels in Col-0 and *fdh1-1* plants (Figure 4.9) despite the constitutive expression of *PDF1.2* (Figure 4.7).

NO is considered to be an endogenous maturation and senescence-regulating factor in plants (Siegel-Itzkovich, 1999). The exogenous treatment of plants with NO results in the inhibition of ET production, which is a natural inducer of plant senescence (Leshem and Pinchasov, 2000). However, increased GS-FDH activity in *fdh1-1* plants does not significantly alter ET levels compared to those found in Col-0 plants. Thus it may be NO only which, affects ET levels in plants rather than SNO's.

4.7 *fdh1-1* mutants are resistant to GSNO but hypersensitive to $O_2^{\cdot}$ and O_2^{-}

A previous study by Lui and co-workers found that *GS-FDH* knockout yeast were hypersensitive to GSNO but not H_2O_2 treatment (Lui et al., 2001). To investigate the role of GS-FDH in resistance to nitrosative and oxidative stress in *Arabidopsis, fdh1-1* and Col-0 seed were germinated on different NO (SNP and GSNO) and ROI (Rose Bengal, paraquat and H_2O_2) donors at increasing concentrations.

4.7.1 fdh1-1 plants and nitrosative stress

fdh1-1 plants which over-express GS-FDH exhibit striking resistance to GSNO, the percentage germination of *fdh1-1* was significantly greater than Col-0 on 0.625mM, 0.75mM and 1mM GSNO (Figure 4.10). For example, the germination frequency of wild type plants on MS media containing 1mM GSNO was 4% compared to 47% for *fdh1-1* (Figure 4.10). These results demonstrate that the over-expression of GS-FDH provides protection from high concentrations of GSNO.

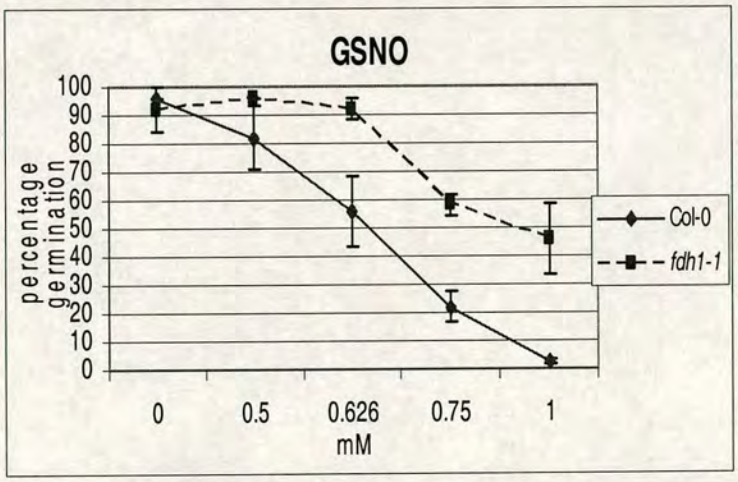

Figure 4.10 *fdh1-1* mutants show increased resistance to GSNO

25 seed of *fdh1-1* and Col-0 were germinated on MS plates containing **a)** 0mM, 0.5mM, 0.625mM, 0.7mM and 1mM GSNO **b)** Values shown are the average of 3 experiments (75 seed) and error bars represent the standard error between values at the 95% confidence level.

The germination frequency of Col-0 and *fdh1-1* on SNP are very similar. On 1.5mM SNP, *fdh1-1* germination frequency is 11% compared to 13% for Col-0 (Figure 4.11). Thus increased GS-FDH activity does not protect from NO produced by SNP.

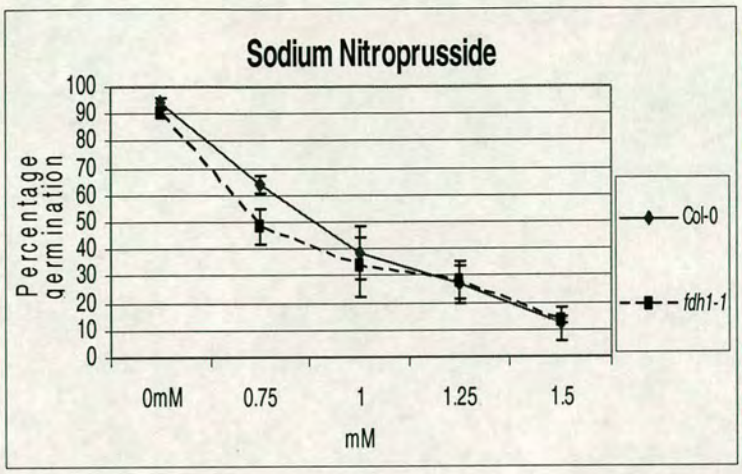

Figure 4.11 *fdh1-1* mutants do not show increased resistance to SNP

25 seeds of Col-0 and *fdh1-1* were germinated on 0mM, 0.75mM, 1mM, 1.25mM and 1.5mM SNP. Values shown are the average of 3 experiments (75 seed) and error bars represent the standard error between values at the 95% confidence level.

4.7.2 fdh1-1 plants and oxidative Stress

The germination frequency of *fdh1-1* and Col-0 on H_2O_2 are also similar. On 4mM H_2O_2 the germination frequency was 5% and 8% for *fdh1-1* and Col-0 respectively (Figure 4.12). Thus GS-FDH does not provide protection form H_2O_2. This is consistent with findings by Lui et al, 2001.

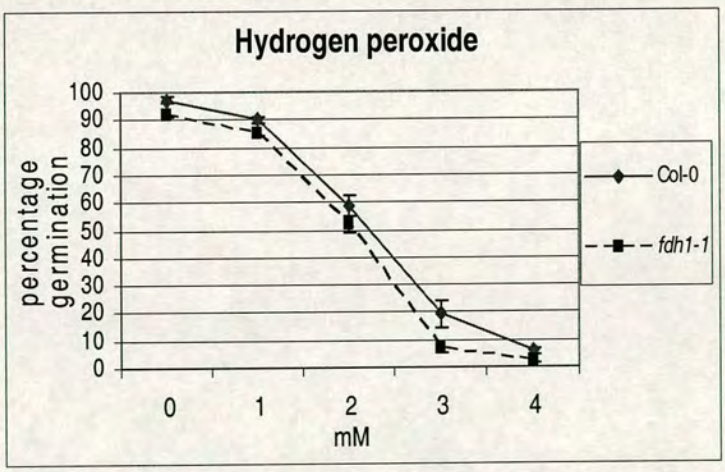

Figure 4.12. *fdh1-1* mutants do not show increased resistance to H_2O_2

25 seeds of Col-0 and *fdh1-1* were germinated on 0mM, 1mM, 2mM, 3mM and 4mM H_2O_2. Values shown are the average of 3 experiments (75 seed) and error bars represent the standard error between values at the 95% confidence level.

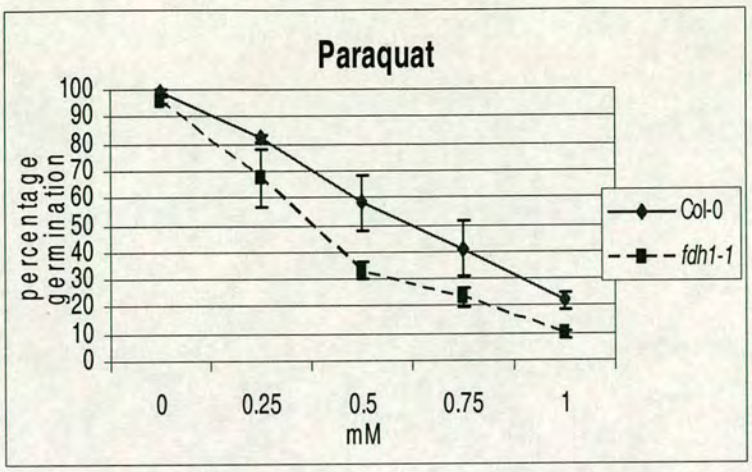

Figure 4.13. *fdh1-1* mutants are hypersensitive to paraquat

25 seed of *fdh1-1* and Col-0 were germinated on 0mM, 0.25mM, 0.5mM, 0.75mM and 1mM paraquat. Values shown are the average of 3 experiments (75 seed) and error bars represent the standard error between values at the 95% confidence level.

Interestingly, *fdh1-1* is hypersensitive to O_2^- released by paraquat. For example, on 0.5mM paraquat the germination frequency of *fdh1-1* (33%) is almost half that of Col-0 (59%) (Figure 4.13). Thus the over-expression of GS-FDH in plants leads to increased O_2^- sensitivity. *fdh1-1* was also found have increased sensitivity to $O_2^{\cdot}$ released by rose bengal. The germination frequency of *fdh1-1* on 0.05mM rose bengal was 26% compared to 48% for Col-0 (Figure 4.14). Therefore, the over-expression of GS-FDH causes increased sensitivity to the oxygen species $O_2^{\cdot}$ as well as O_2^-.

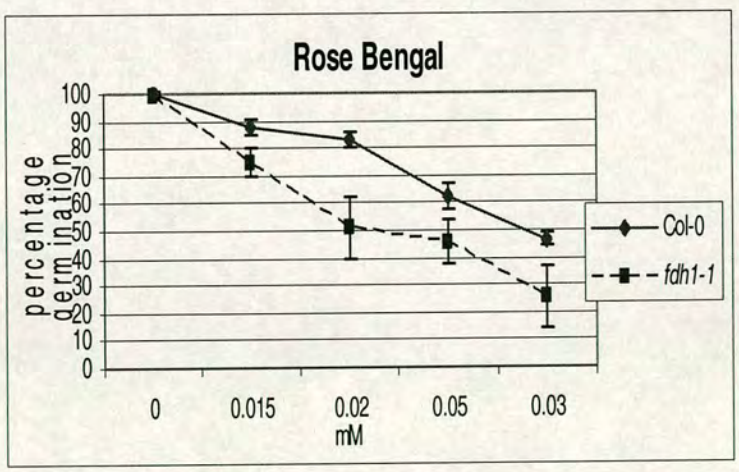

Figure 4.14. *fdh1-1* mutants are hypersensitive to Rose Bengal

25 seed of *fdh1-1* and Col-0 were germinated on 0.015mM, 0.02mM, 0.03mM and 0.05mM Rose Bengal. Values shown are the average of 3 experiments (75 seed) and error bars represent the standard error between values at the 95% confidence level.

4.8 Disease resistance assays

The first events following pathogen challenge include the generation of NO (Delledonne et al., 1998; Clarke et al., 2000) and ROIs (Grant and Loake, 2000). GSNO has been shown to be involved in defence responses in animals (Lui et al., 2004, Gaston et al., 1993; Mayer et al., 1998). To investigate the role of GS-FDH in plant disease resistance, *fdh1-1* was used in disease resistance assays using different pathogens including the biotrophic pathogens *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000), *Peronospora parasitica, Erysiphe cichoracerum* and the necrotrophic pathogen *Botrytis cinerea.*

4.8.1 fdh1-1 mutants are resistant to *Pseudomonas syringae* pv *tomato* DC3000

Four week old Col-0, *NahG* and *fdh1-1* plants were inoculated with *Pst* DC3000. *NahG* transgenic plants express the SA-degrading enzyme salicylate hydroxylase and as a consequence are unable to accumulate SA. Therefore, *NahG* plants show enhanced disease susceptibility to *Pst* DC3000 compared to wild-type Col-0 plants. Development of disease symptoms (chlorosis, wilting of leaves) was monitored daily and the development of symptoms in wild-type Col-0 was found to be optimal at four days post inoculation (Figure 4.15a). At this time point *NahG* plants had undergone extreme chlorosis and wilting compared to Col-0 plants which showed chlorosis but no wilting. Whereas in *fdh1-1* plants, disease symptoms such as chlorosis and leaf wilting were absent (figure 4.15a).

Three plants per line were inoculated with *Pst* DC3000 and the leaves harvested after 3 days. This time point was chosen as *Pst* DC3000 growth in *Arabidopsis* peaks after 3 days (Whalen et al., 1991). Plant extracts were made by grinding in 10mM $MgCl_2$ and dilution's plated out on KB media. The number of bacterial colonies were recorded after 3 days (shown in figure 4.15b). Col-0 samples show a bacterial titre of around 10^7 cfu/leaf disk which corresponds to the bacterial titres in Col-0 at this time point in previous studies (Bowling et al., 1997). *NahG* was the most susceptible to *Pst* DC3000, with a bacterial titre two hundred and fifty times higher than found in Col-0. The growth of *Pst* DC3000 in *fdh1-1* was ten times lower than found in wild-type Col-0 plants. The reduced bacterial titre and lack of disease symptoms in *fdh1-1* plants after infection with virulent *Pst* DC3000 indicate a role for GSNO/GS-FDH in disease resistance.

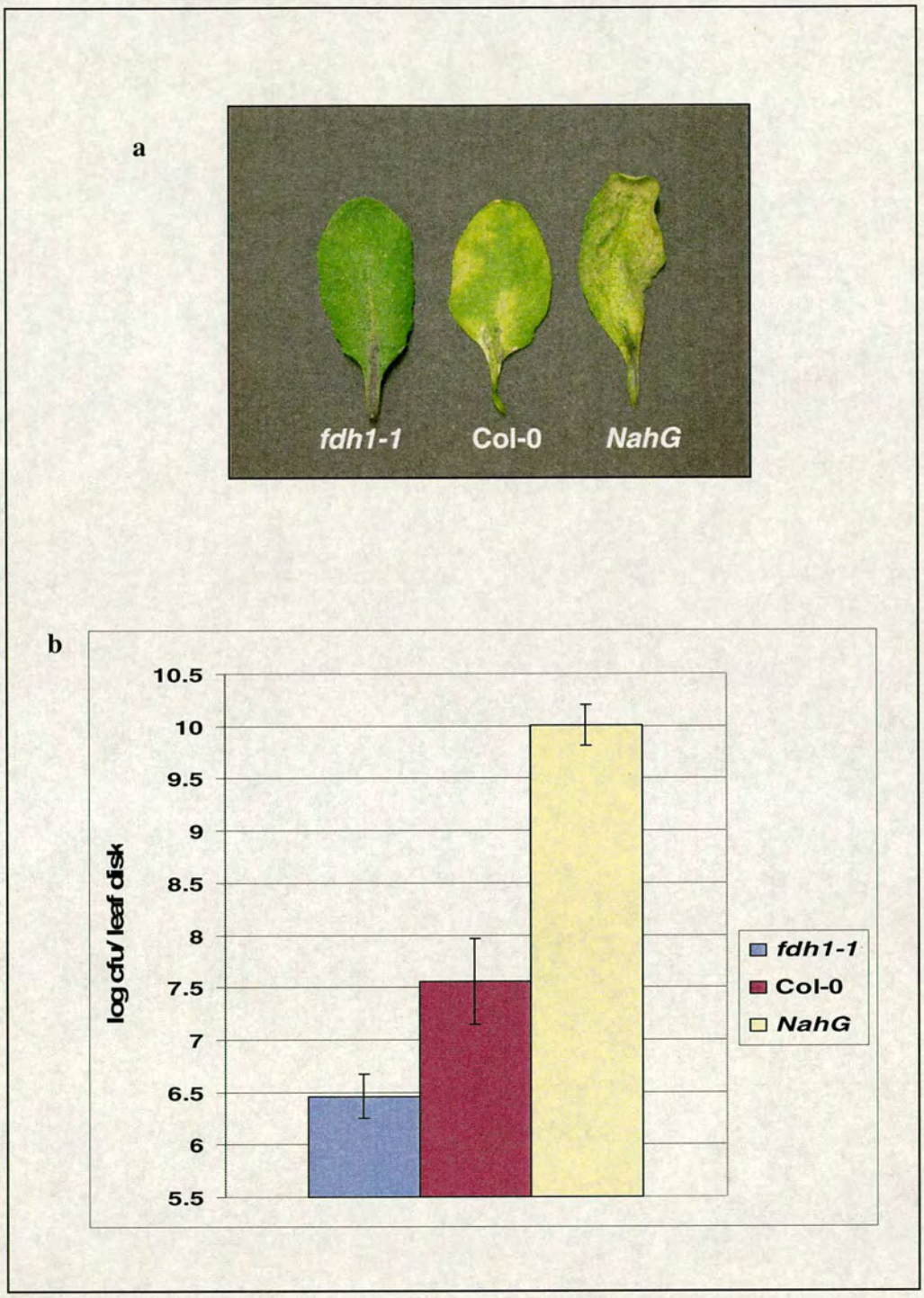

Figure 4.15. *fdh1-1* plants are resistant to *Pst* DC3000.

a) Development of disease symptoms in *fdh1-1*, Col-0 and *NahG* plants 4 days after inoculation with *Pst* DC3000. **b)** Bacterial titre 4 days after *Pst* DC3000 inoculation in *fdh1-1*, Col-0 and *NahG*. Values shown are the average of three plants per line and error bars represent the standard error between values at the 95% confidence level. This experiment was repeated twice with similar results.

4.8.2 fdh1-1 mutants do not show resistance to *Erysiphe cichoracerum*

Four week old Col-0, *NahG* and *fdh1-1* plants were infected with powdery mildew (*Erysiphe cichoracearum)* by rubbing infected Col-0 leaves over the whole area of leaves to be infected. Ten days post-inoculation, plants were scored for the presence of conidiophores and chlorotic symptoms. *NahG* was found to be the most susceptible to *Arabidopsis* powdery mildew. However the disease index of Col-0 and *fdh1-1* were similar (2.3 and 1.9 respectively) (Figure 4.16). Therefore *fdh1-1* was not found to be resistant to powdery mildew.

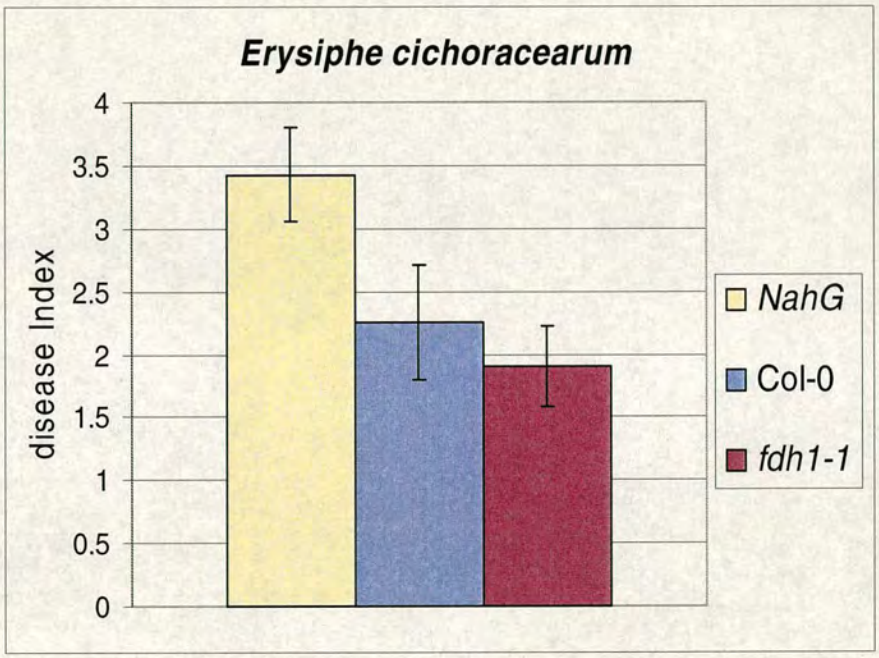

Figure 4.16. *fdh1-1* plants are not resistant to *E.cichoreacerum*

Disease rating of *E.cichoreacerum* infection 10 days after inoculation of Col-0, *fdh1-1* and *NahG.* Values shown are the average of fifteen plants per line and error bars represent the standard error between values at the 95% confidence level. This experiment was repeated twice with similar results.

4.8.3 fdh1-1 mutants show resistance to *Botrytis cinerea*

B.cinerea spore suspensions (adjusted to $1X10^9$ spores per ml) were sprayed on 4 weeks old *fdh1-1*, Col-0 and *coi1* plants. Infected plants were then kept in a humid chamber for 8 days. The plants were scored by eye for disease symptoms such as chlorosis and necrosis. *coi1* was found to be the most susceptible to *B.cinerea* with a disease index almost double (3.9) that of Col-0 (2). *fdh1-1* showed a small but significant increase in resistance to *B. cinerea*, with a disease index of 1.4 (Figure 4.17).

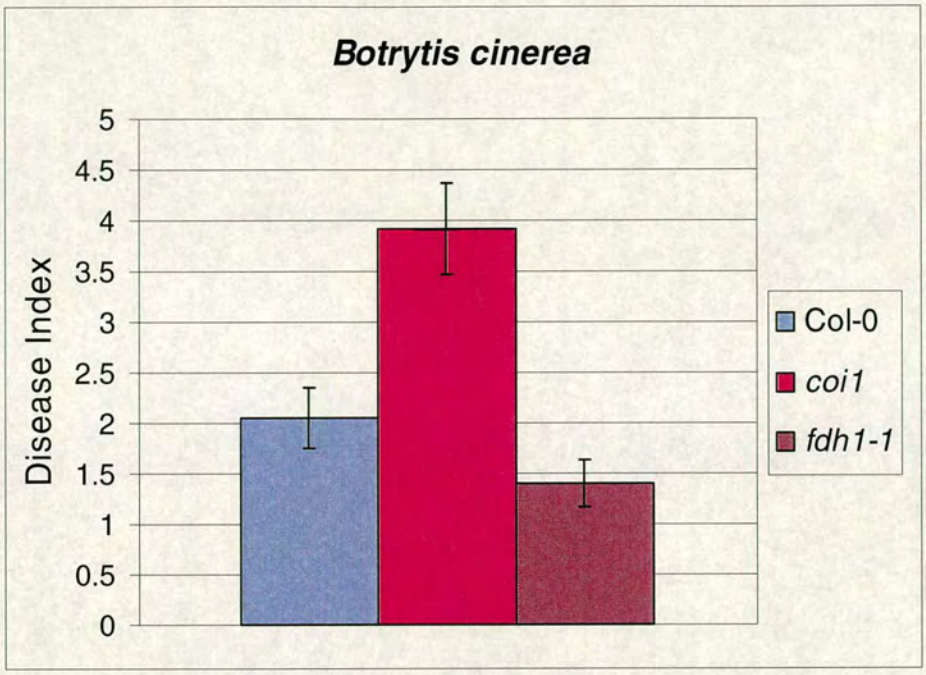

Figure 4.17. *fdh1-1* plants are resistant to *B. cinerea*

Disease rating of *B. cinerea* infection 8 days after inoculation of Col-0, *fdh1-1* and *coi1*. Values shown are the average of fifteen plants per line and error bars represent the standard error between values at the 95% confidence level. This experiment was repeated twice with similar results.

4.8.4 fdh1-1 mutants are not resistant to *Peronospora parasitica* Noco 2

P. parasitica conidospores were harvested by vortexing infected seedlings in water. Four week old Col-0, *fdh1-1, eds1* and *NahG* plants were sprayed with the conidiospore solution (adjusted to $1X10^5$ spores per ml) and maintained under humid conditions. After 14 days infected plants were scored by eye for the extent of downy mildew growth by eye. *eds1* and *NahG* had similar disease indexs of 4.2 and 4.3 respectively. This is more susceptible than wildtype Col-0, which had a disease index of 1.8. *fdh1-1* shows similar susceptibility to *P. parasitica* as Col-0 with a disease index of 2 (Figure 4.18).

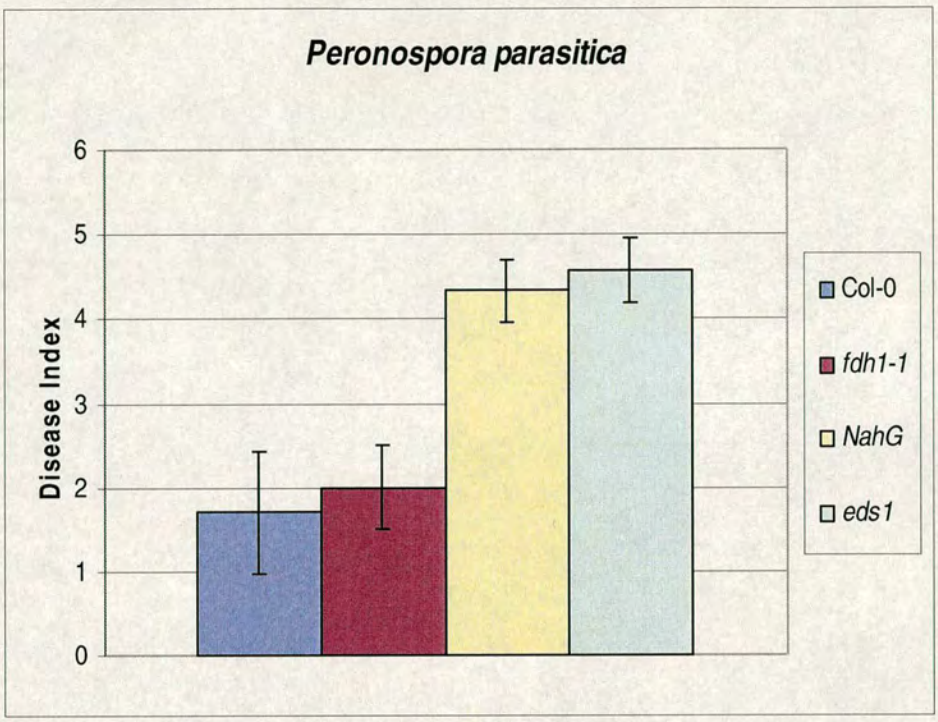

Figure 4.18. *fdh1-1* mutants are not resistant to *P. Parasitica* Noco 2

Disease rating of *P. parasitica* Noco 2 infection 14 days after inoculation of Col-0, *fdh1-1*, *eds1* and *NahG*. Values shown are the average of fifteen plants per line and error bars represent the standard error between values at the 95% confidence level. This experiment was repeated twice with similar results.

4. 9 Discussion

The T-DNA insert in *fdh1-1* is situated downstream of *FLS* (Figure 4.3) in the 5' UTR promoter region of *GS-FDH* and this was found to cause the over-expression of *GS-FDH* (Figure 4.7 and 4.8). The T-DNA insertional vector, pDAP101 (Sessions et al., 2002) used to generate Syngenta's SAIL collection contains no plant promoter besides that driving expression of the *BAR* marker gene. Therefore the over-expression of *fdh1-1* presumably reflects ablation of a negative regulatory element in *GS-FDH* or the introduction of an adventitious promoter or enhancer element as a result of the insertion event. Similarly, a recent study found that a SAIL T-DNA line (used to investigate ethanolamine biosynthesis) with an insertion ~100bp upstream of the start codon was also an over-expressor (Rontein et al., 2003).

Evidence from animals indicates that GSNO is central to signal transduction and host defences (Liu et al., 2004; Mayer et al., 1998). GS-FDH has GSNOR activity and has recently been shown to deplete cellular GSNO and SNO levels (Lui et al., 2001). Recently, it was found that GS-FDH knock-out mice have increased protein S-nitrosylation, tissue damage and mortality following bacterial challenge (Lui et al., 2004).

To elucidate a role for GSNO in plant disease resistance we identified a *GS-FDH* T-DNA insert line (*fdh1-1*), which was subsequently found to be an over-expressor of *GS-FDH*. *fdh1-1* shows increased resistance to GSNO (Figure 4.10), which is consistent with the over-expression of *GS-FDH*. However, *fdh1-1* does not show increased resistance to the NO donor SNP (Figure 4.11). This may indicate that other enzymes are important for controlling NO levels in plants. In addition, SNP has been reported to have different physiological effects on animals from GSNO. For example, most NO donors including GSNO were found to suppress $^{\cdot}OH$ generation and lipid peroxidation in the brain, whereas SNP was found to be neurotoxic (Rauhala et al., 1997). In fact, SNP is thought to generate mainly NO^+ whereas GSNO is a physiological NO donor (Stamler 1992; Hu et al., 1999). Interestingly, *fdh1-1* was also found to have increased sensitivity to O_2^- (Figure 4.13) and $O_2^{\cdot}$ (Figure 4.14) but not H_2O_2 (Figure 4.12). GS-

FDH knock-out yeast were also shown to have no increased sensitivity to H_2O_2 (Lui et al., 2001). Paraquat acts as a terminal oxidant of photosystem I in the chloroplast, light reduces O_2 to O_2^- promoting the accumulation of O_2^- (op den Camp et al., 2003). Rose Bengal is a photosensitiser that gives rise to $O_2^{\cdot}$ upon illumination. $O_2^{\cdot}$ then accumulates within the internal membranes of chloroplasts (op den Camp et al., 2003). $O_2^{\cdot}$ and O_2^- are highly reactive and have short half-lives of ~0.2μs and ~2μs respectively. Therefore, paraquat and rose bengal cause the production and accumulation of high levels of reactive oxygen species within the chloroplast. These are likely to react with molecules localised in or close to the chloroplast membrane. In contrast H_2O_2 is a more stable molecule with a half-life of ~1ms which can diffuse freely through membranes (Mittler, 2002).

GSNO has strong antioxidant properties, for example it has been shown to protect endothelium tissue from oxidative stress (Chiueh, 1999). Therefore in *fdh1-1* seedlings germinated on paraquat or rose bengal, the over-expression of GS-FDH will remove GSNO allowing high levels of $O_2^{\cdot}$ and O_2^- to accumulate in the chloroplast. This may overwhelm the ROI scavenging mechanisms in the chloroplast causing lipid peroxidation and the damage of photosynthetic apparatus. However, it is not know if GS-FDH is present in the chloroplast. In yeast and mammalian cells GS-FDH is localised in the nucleus and cytosol (Iborra et al., 1992; Fernandez et al., 2003). Anti-NOS immunoreactive proteins have been shown to be localised in the chloroplast and DAF-2DA staining also shows NO production in the chloroplast (Ribeiro et al., 1999; Foissner et al., 2000). Thus GSNO may be formed in the chloroplast from the nitrosation of GSH (which is present at concentrations of 1-5mM) by NO (Mittler, 2002; Fernandez et al., 2003). Similar to H_2O_2, GSNO can diffuse among cell compartments (del Rio et al., 2002). Increased levels of GS-FDH in the cytosol of *fdh1-1* plants may cause oxidative stress as a result of decreased cellular GSNO. This may be more pronounced in the chloroplast of *fdh1-1* plants since this is the site of paraquat and rose bengal action.

Moreover, maintaining a high level of GSH is essential for ROI scavenging in cells (Mittler et al., 2002). Therefore, it is also possible that a reduction in GSNO levels owing to the over-expression of GS-FDH in *fdh1-1* plants may lead to a depletion of GSH levels also impairing ROI scavenging in the cell.

fdh1-1 mutants show developmental abnormalities during flowering, root formation and also accumulate high levels of flavonoids (Figure 4.4-4.6). These phenotypes implicate a role for SNO's in regulating plant development. However it is important to remember that there is an *FLS* gene upstream of *GS-FDH* (Figure 4.3). The T-DNA insertion in the promoter region of *GS-FDH* may have also caused the over-expression of *FLS* as well as *GS-FDH*. This would explain the increased accumulation of anthocyanin and flavonols in *fdh1-1* plants.

NO has already been shown to be involved in plant development promoting germination, seedling de-etiolation and the inhibition of hypocotyl elongation (Beligni and Lamattina, 2000). In tomato NO was found to promote the formation and elongation of lateral roots, while inhibiting elongation of the primary root (Correa-Aragunde et al., 2004). *Arabidopsis* knock-out mutants of *AtNOS1* (which is a constitutive NOS) were found to have shorter roots and infloresences than wild-type plants (Guo et al., 2003). These phenotypes of *AtNOS* mutants are similar to those found in *fdh1-1*.

As well as increased anthocyanin accumulation, stunted roots and elongated root hairs (Figure 4.4-4.6). *fdh1-1* also expresses *PDF1.2* constitutively (Figure 4.7a). These phenotypes are similar to those of *cev1* mutants, which have a constitutively active JA and ET pathway (Ellis and Turner, 2001). However, *fdh1-1* was not found to have significantly different ET levels compared to Col-0 plants (Figure 4.9) despite the constitutive expression of *PDF1.2*. In addition, *cev1* mutants were also found to have increased resistance to powdery mildew (*E. cichoracearum*). This is not the case for *fdh1-1*, which shows similar disease symptoms to that of wild-type plants, following powdery mildew infection (Figure 4.16). There is some evidence from tomato that NO antagonises the induction of wound induced JA signalling (Orozco-Cárdenas and Ryan, 2002). It is possible that the JA pathway and *PDF1.2* expression is constitutive in *fdh1-1,* due to the action of GS-FDH removing GSNO. Although studies from *Arabidopsis* indicate that NO does activate JA signalling and the expression of *PDF1.2,* this has only been shown in *NahG* plants which cannot accumulate SA (Haung et al., 2004).

fdh1-1 shows strong resistance to a virulent strain of the biotroph *Pst* DC3000 (Figure 4.13) and a small but significant increase in resistance to the necrotroph *B. cinerea* (Figure 4.17). *fdh1-1* shows increased levels of flavonoids (Figure 4.4). This may be due to the over-expression of *GS-FDH* or possibly the over-expression of the upstream

FLS gene (Figure 4.3 and 4.7). Flavonoids are involved in stress responses to cold, drought, UV-B radiation (Shirley, 1996) and also have anti-microbial properties (Dixon et al., 1983; Kemp and Burden, 1986).

Anthocyanins are antioxidants, which scavenge H_2O_2, O_2^- and $O_2^{\cdot}$ (Yamasaki et al., 1997; Nagata et al., 2003; Tournaire et al., 1993). Therefore, reduced levels of the antioxidant GSNO in *fdh1-1* plants may lead to increased anthocyanin accumulation in order to compensate for impaired ROI scavenging. Elevated levels of ROIs may themselves be directly antimicrobial. For example, ROIs damage DNA and protein chemical moieties, including sulfhydryls and Fe-S clusters (Nathan and Shiloh, 2000). Evidence in plants suggest that ROIs are directly toxic to the virulent plant pathogens *Erwinia chrysanthemi* (Hassouni et al.,1999) and *Phytophthora infestans* (Wu et al., 1995). If ROI levels are elevated in *fdh1-1* this would also explain the constitutive expression of *PDF1.2* which is induced by ROIs (Penninckx et al., 1998; Alonso et al., 1999).

PDF1.2 expression is required for resistance to the necrotrophic pathgens such as *B. cinerea* and *A. brassicicola* but is not thought to required for resistance to biotrophic pathogens such as *P. syringae* and *P. parasitica* (Thomma et al., 1998). Furthermore *Pst* has been shown to produce the phytotoxin coronatine, which mimics JA (Zhao et al., 2003). *Pst* may employ coronatine to repress SA mediated defence genes, since the JA signalling pathway is antagonistic towards the SA pathway (Kloek et al., 2001). Therefore, it is unlikely that the constitutive expression of *PDF1.2* in *fdh1-1* confers resistance to virulent *Pst* DC3000, although it may confer resistance to the necrotroph *B. cinerea*. *npr1* mutants which can no longer accumulate *PR1* transcripts and fail to establish SAR in response to SA, show increased susceptibility to *Pst* DC3000 (Cao et al., 1994; Cao et al, 1998). *fdh1-1* plants constitutively express slightly elevated levels of the defence gene *PR1*, which may confer resistance to *Pst* DC3000.

Conclusion

The GS-FDH over-expressor, *fdh1-1* has perturbed developmental phenotypes affecting flowering, root growth and flavonoid accumulation. *fdh1-1* is resistant to higher levels of GSNO, but shows increased sensitivity to the oxygen species O_2^- and $O_2^{\cdot}$ compared to wild-type plants. Furthermore, *fdh1-1* shows resistance to the virulent biotroph *Pst*

DC3000 and necrotroph *B. cinerea.* Thus GS-FDH may regulate developmental and defence responses in plants through modulating GSNO and SNO levels. In order to further characterise the role of GS-FDH in plant defence responses; *GS-FDH* expression was examined during the HR and the kinetics of the HR were investigated in *fdh1-1* plants.

Chapter 5

5. The role of GS-FDH in the HR

5.1 Introduction

Following pathogen recognition the oxidative burst is one of the most rapid plant defence responses, producing ROI's including O_2^- and hydrogen peroxide (Apostol et al., 1998; Grant et al., 2000). Recent evidence suggests that NO is also rapidly produced in response to pathogen challenge (Durner et al., 1998; Delledonne et al., 1998). ROIs and NO are key signalling molecules for the induction of PCD, which is characteristic of the HR (Delledonne et al., 1998; Lamb and Dixon 1997).

In animals, NO and O_2^- react to form ONOO-, a highly cytotoxic molecule that induces cell death (Cookson et al., 1998; Lin et al., 1995). However, this does not appear to be the case in plants, since treatment of soybean cell suspensions with ONOO- was not found to induce PCD (Delledonne et al., 2001). As an alternative it has been proposed that a synergistic reaction between NO and H_2O_2 mediates PCD in plants. A balance model has been proposed, where if the NO/O_2^- balance is in favour of O_2^- then NO is scavenged before reaction with H_2O_2. While if the balance is in favour of NO, O_2^- is scavenged before dismutation to H_2O_2. Either way the product is $ONOO^-$, which was not found to induce cell death in plants (Delledonne et al, 2001). Furthermore, NO has been found to inhibit the H_2O_2 scavenging enzymes catalase (CAT) and ascorbate peroxidase (APX) in tobacco; thus it is possible that NO regulates the onset of the HR by modulating H_2O_2 levels (Clark et al., 2000).

The induction of PCD by NO in plants is thought to be dependent on cGMP. A specific guanylate cyclase inhibitor was found to block NO-induced cell death in *Arabidopsis* and this inhibition could be reversed by a cGMP analogue (Clarke et al., 2000).

In animals S-nitrosylation is an alternative signalling pathway which also modulates apoptosis. $P21^{ras}$ a key signalling target for NO in mammals is activated by S-nitrosylation, which in turn modulates downstream MAPK cascades (Lander et al., 1997). Depending on the stimuli and the interaction of the components in the MAPK

signalling cascade, apoptosis may be triggered (Beck et al., 1999). Caspases which are important executors of PCD are also S-nitrosylated, helping to maintain them in an inactive form (Li et al., 1998). Moreover, cytochrome c has been found to be S-nitrosylated in animal cells prior to release from the mitochondria, which in turn increases caspase activation (Stamler et al., 2001). Thus both de-nitrosylation and S-nitrosylation events are involved in the apoptotic pathway.

In addition to inducing apoptosis NO can also protect from PCD in animals (Sharpe et al., 2003). This apparent paradox depends on the cell type and the concentrations of NO involved. For example, high concentrations of NO (100-200μm) are proapoptotic and low concentrations (1nM-1μM) can protect the cell from apoptosis (Bogdan et al., 2000; Curtin et al., 2002). At physiological concentrations, NO can act as an antioxidant scavenging ROIs (Sharpe et al., 2003). This may also be true in plants, where NO donors were found to protect potato (Beligini and Lammatina, 2002) and rice (Hung et al., 2002) from oxidative stress induced by paraquat.

NO's role in the regulation of PCD in plants has only recently emerged, however, nothing is yet known of the involvement of GSNO and SNO's. In order to elucidate a role for GS-FDH and GSNO in the HR, gene expression and enzyme activity of GS-FDH was monitored in wild-type plants during the HR. In addition, ROI accumulation and PCD were investigated in *fdh1-1* plants (which over-express *GS-FDH*) for comparison with wild-type plants.

5.2 *GS-FDH* expression is suppressed during the HR

In order to investigate the expression of *GS-FDH* during the HR, wild-type Col-0 plants were infiltrated with avirulent *Pst* DC3000 (*avrB*). Samples were then collected at the time points indicated and RNA extracted for northern analysis. The expression of *GS-FDH* is constitutive and present at basal levels in untreated tissue (Figure 5.1). Following *Pst* DC3000 (*avrB*) infiltration, transcript levels of *GS-FDH* decreased dramatically after 1 hour and *GS-FDH* expression was off 3 hours post infiltration. *GS-FDH* transcript levels then started to increase again 24 hours after treatment (Figure 5.1). In comparison, infiltration of virulent *Pst* DC3000 only induced a small decrease in *GS-FDH* expression, which otherwise remains relatively constant between 1-24 hours (Figure 5.1).

The expression of other known defence genes involved in the HR were investigated using this blot. Strong *GST1* expression was quickly induced between 1-5 hours post infiltration with *Pst* DC3000 (*avrB*) but not with virulent *Pst* DC3000. The defence gene *PR1* was strongly induced in response to *Pst* DC3000 (*avrB*) between 9-24 hours, but again not following treatment with virulent *Pst* DC3000. *PDF 1.2* was also found to be induced by *Pst* DC3000 (*avrB*) (Figure 5.1). In addition, *PDF1.2* expression was induced by the infiltration of $MgSO_4$ buffer, this is likely to be as a result of wounding.

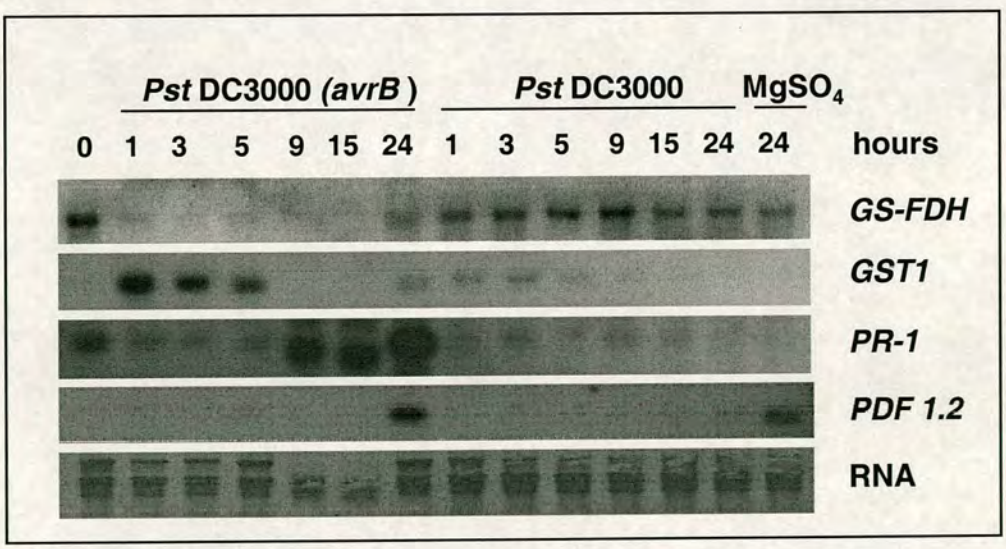

Figure 5.1 *Pst* DC3000 (*avrB*) treatment suppresses *GS-FDH* during the HR

Wild-type Col-0 plants were infiltrated with *Pst* DC3000 (*avrB*) and virulent *Pst* DC3000. Northern blots were probed with *GST1, PR1, PDF1.2* probes and a *GS-FDH* cDNA probe

5.3 Is *GS-FDH* suppression EDS 1 or NDR1 dependent ?

Most NBS-LRR *R*-genes signal though either EDS1 or NDR1 signalling pathways (Aarts et al., 1998). For example, a mutation in *NDR1* abolished resistance conferred by *RPM1* (which is a CC-NB-LRRs class *R*-gene) to the bacterial gene *avrB*. Whereas, a mutation in *EDS1* blocked resistance conferred by a subset of *R* loci *RPP2*, *RPP4*, *RPP5*, *RPP21*, and *RPS4* (which are TIR-NB-LRRs class *R*-genes) (Aarts et al., 1998). In order to investigate if the suppression of *GS-FDH* is triggered via both EDS1 and NDR1 dependent signalling pathways, Col-0 plants were infiltrated with *Pst* DC3000 (*avrRps4*), which is recognised by RPS4 (Gassmann et al., 1999). Subsequently, the suppression of *GS-FDH* expression with *Pst* DC3000 (*avrRps4)*, was found to be weak compared to that found with *Pst* DC3000 *(avrB)* (Figure 5.1 and 5.2). *GS-FDH* expression remained constant with a decrease at 5 hours post *Pst* DC3000 (*avrRps4*) infiltration (Figure 5.2). Similar to the previous experiment, virulent *Pst* DC3000 induced a small decrease in *GS-FDH* transcript levels, compared to basal levels and transcript levels remain fairly constant between 1-24 hours (Figure 5.2).

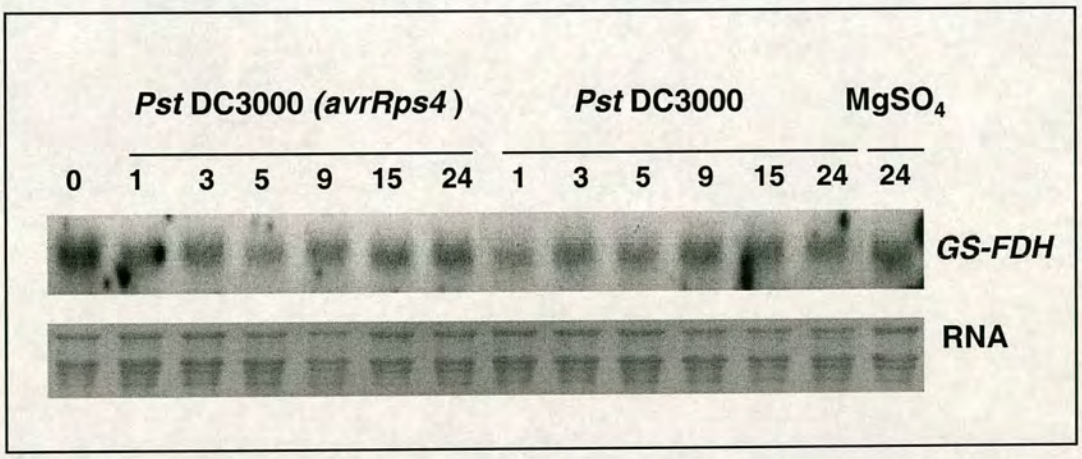

Figure 5.2 *GS-FDH* suppression is weak following *Pst* DC3000 (*avrRps4*) treatment

Wild-type Col-0 plants were infiltrated with *Pst* DC3000 (*avrRps4*) and virulent *Pst* (DC3000). Northern blots were probed with a *GS-FDH* cDNA probe.

5.4 GS-FDH activity is suppressed during the HR

GS-FDH transcript levels were found to be strongly suppressed by treatment with *Pst* DC3000 (*avrB*) but not *Pst* DC3000 (*avrRps4*). In order to investigate if this is also true at the post-transcriptional level, GS-FDH activity was examined following inoculation with these two different strains. Wild-type Col-0 plants were infiltrated with *Pst* DC3000 (*avrB*) or *Pst* DC3000 (avr*Rps4*) and samples collected at the time points indicated for protein extraction. Proteins were then separated on NATIVE-PAGE gels before incubation with the substrate HM-GSH.

The infiltration of *Pst* DC3000 (*avrB*) induced a decrease in GS-FDH activity after 5 hours and low GS-FDH activity was visible between 9-24 hours (Figure 5.3a). Treatment with *Pst* DC3000 (*avrRps4*) also caused a decrease in GS-FDH activity between 9-15 hours (Figure 5.3b), although this suppression was not as pronounced as that induced by *Pst* DC3000 (*avrB*). The activity of GS-FDH altered very little in response to virulent *Pst* DC3000 treatment in both experiments (Figure 5.3a and b)

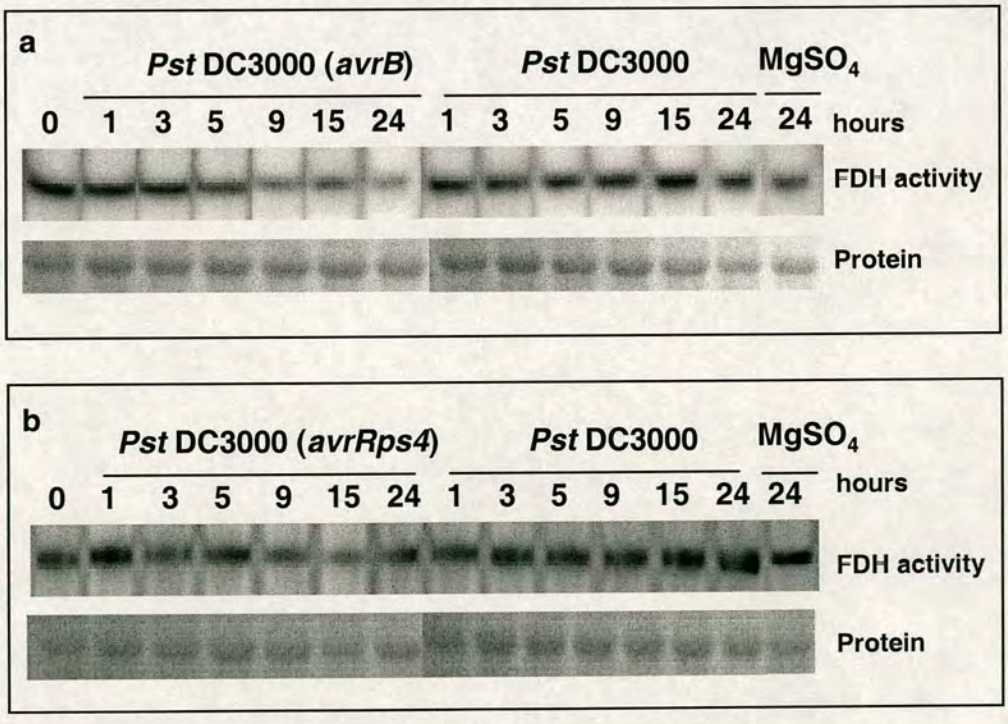

Figure 5.3 GS-FDH activity during the HR

In gel enzyme activity assays using HM-GSH as a substrate. Protein extracts were made from Col-0 plants infiltrated with **(a)** *Pst* DC3000 (*avrB*) and virulent *Pst* DC3000 or **(b)** *Pst* DC3000 (*avrRps4*) and virulent *Pst* DC3000. $MgSO_4$ infiltration was used as a control.

5.5 *GS-FDH* expression is suppressed in defence signalling mutants

To elucidate which signalling molecules are required for the suppression of *GS-FDH*, different mutants compromised in disease resistance signalling were infiltrated with *Pst* DC3000 (*avrB*) and samples collected for northern analysis. *GS-FDH* expression was found to be suppressed in Col-0 plants 5 hours post infiltration with *Pst* DC3000 (*avrB*), before recovering to a similar level found in untreated leaves at 24 hours (Figure 5.4). Comparable, results were found with the mutants *etr1, ein2.1, eds1, npr1, sid1, sid2* and the transgenic *NahG,* again *GS-FDH* expression decreased 5 hours post infiltration before returning to basal levels at 24 hours (Figure 5.4). These results suggest that neither ETR1, EIN2, SID1, SID2, NPR1 or EDS1 are required for the suppression of *GS-FDH* expression. Thus SA and ET are not required as signals to switch off *GS-FDH.* In *coi1* plants, *GS-FDH* expression was suppressed after 5 hours, but did not return to basal levels at 24 hours (Figure 5.4). Thus, although COI1 is not required for the suppression of *GS-FDH*, it may be required for the recovery of *GS-FDH* expression to basal levels. In contrast to the other mutants, *GS-FDH* expression increases in *fdh1-1* mutants 5 hours after *Pst* DC3000 (*avrB*) treatment and expression is still higher than found in untreated leaves at 24 hours (Figure 5.4).

This blot was also probed for the expression of the defence genes *PR1* and *PDF1.2.* Col-0 plants showed induction of *PR1* and *PDF1.2,* 24 hours after *Pst* DC3000 (*avrB*) treatment (Figure 5.4). Mutants perturbed in ET and MeJA signalling such as *coi1*, *ein2.1* and *etr1* did not show *PDF1.2* expression following *Pst* DC3000 (*avrB)* infiltration but did show *PR1* expression (Figure 5.4). Whereas, mutants upstream of, or required for SA signalling (*npr1, eds1, sid1* and *sid2*) showed *PDF1.2* expression but not *PR1* expression in response to *Pst* DC3000 *(avrB*) (Figure 5.4). The *fdh1-1* over-expressor mutant constitutively expresses *PDF1.2* and shows potentiated *PR1* expression compared to wild-type plants in response to *Pst* DC3000 (*avrB*) (Figure 5.4).

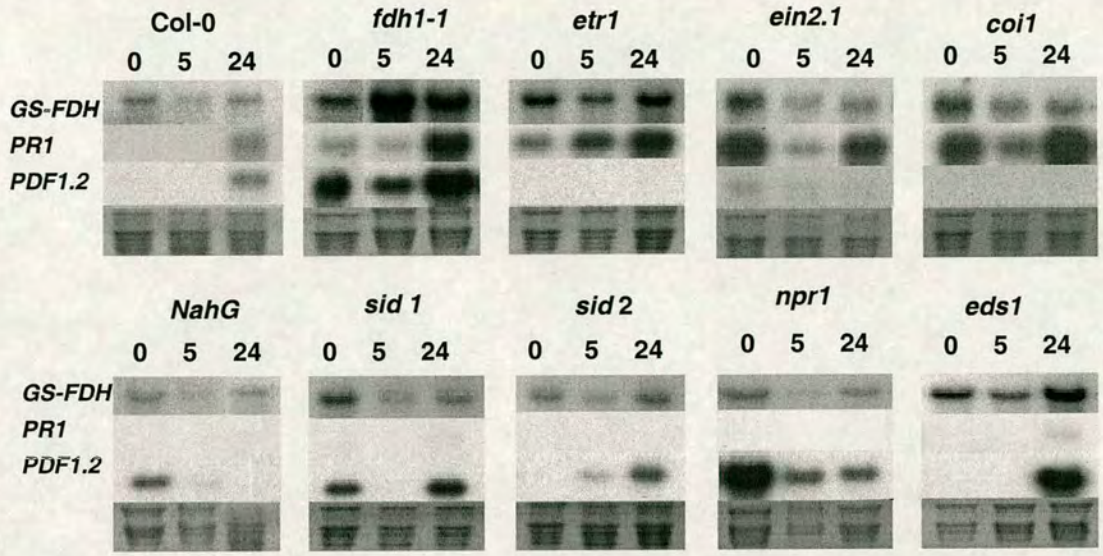

Figure 5.4 *GS-FDH* expression in defence signalling mutants

Col-0, *fdh1-1*, *etr1*, *ein2.1*, *coi1*, *NahG*, *sid1*, *sid2*, *npr1* and *eds1* mutants were infiltrated with *Pst* DC3000 (*avrB*) and samples collected 0, 5 and 24 hours post infiltration. Northern blots were probed with *PR1*, *PDF1.2* and *GS-FDH* cDNA probes.

5.6 The HR is delayed in *fdh1-1* plants

To examine the effect of *GS-FDH* over-expression on the HR, *fdh1-1* and wild-type Col-0 plants were infiltrated with *Pst* DC3000 (*avrB*) or *Pst* DC3000 (*avrRps4*) and the HR monitored. Leaf collapse was first visible in Col-0 plants 6 hours following *Pst* DC3000 (*avrB*) treatment. Whereas, no wilting of *fdh1-1* leaves was apparent until 15 hours post infiltration (Figure 5.5a). At 24 hours leaf collapse was severe in Col-0 and PCD visible, compared to *fdh1-1* which showed less severe HR progression (Figure 5.5a). The treatment of Col-0 with *Pst* DC3000 (*avrRps4*) induced the HR slower than found with *Pst* DC3000 (*avrB*) (Figure 5.5b). Following, *Pst* DC3000 (*avrRps4*) infiltration, leaf collapse could not be seen in Col-0 plants until 15 hours and not in *fdh1-1* over-expressor plants until 24 hours (Figure 5.5b).

These results suggest that the over-expression of *GS-FDH* leads to a delay in the progression of the HR.

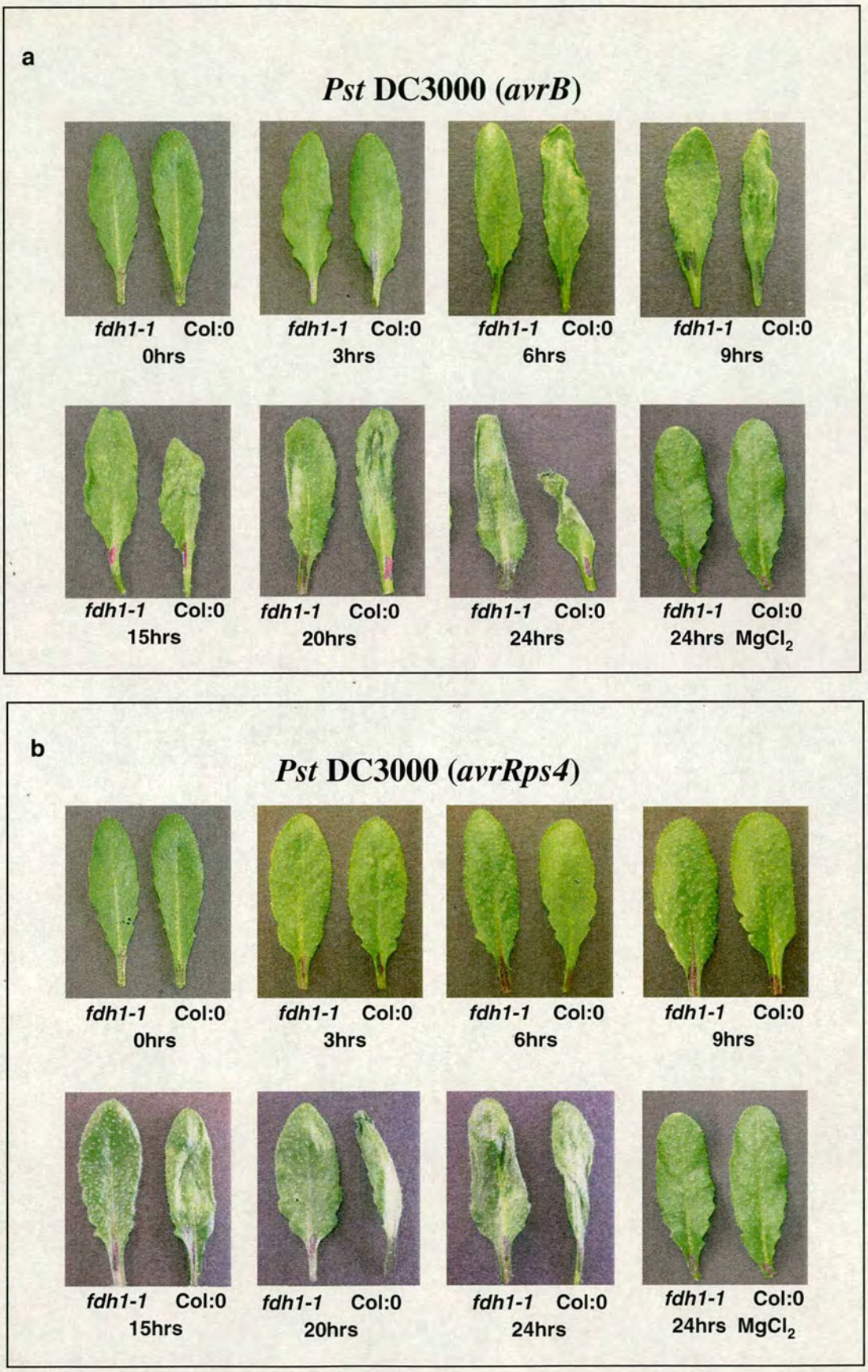

Figure 5.5 The HR is delayed in *fdh1-1* plants.

Col-0 and *fdh1-1* plants were infiltrated with **(a)** *Pst* DC3000 (*avrB*) or **(b)** *Pst* DC3000 (*avrRps4*) and leaves monitored for HR progression.

5.7 O_2^- accumulation in *fdh1-1* plants

A burst of O_2^- which, dismutates spontaneously or via superoxide dismutase to H_2O_2 is one of the first events in the HR (Bestwick et al., 1997). GSNO has been reported to have antioxidant properties (Chiueh, 1999). Therefore, the over-expression of *GS-FDH* in *fdh1-1* mutants may lead to perturbed ROI levels. To investigate O_2^- levels in *fdh1-1* plants compared to Col-0, both lines were infiltrated with *Pst* DC3000 (*avrB*). Leaves were removed at the indicated time points and NBT staining used to detect O_2^- *in situ*. In wild-type Col-0 plants NBT staining and therefore O_2^- production was strongest 3 hours post infiltration (Figure 5.6a). Lower levels of O_2^- production were visible in Col-0 at 6 and 15 hours following *Pst* DC3000 (*avrB*) infiltration and no NBT staining could be detected after 15 hours (Figure 5.6a). However, in *fdh1-1* plants O_2^- production was strong at 3 and 6 hours post infiltration with *Pst* DC3000 (*avrB*) and low levels of O_2^- could still be detected up to 48 hours (Figure 5.6b).

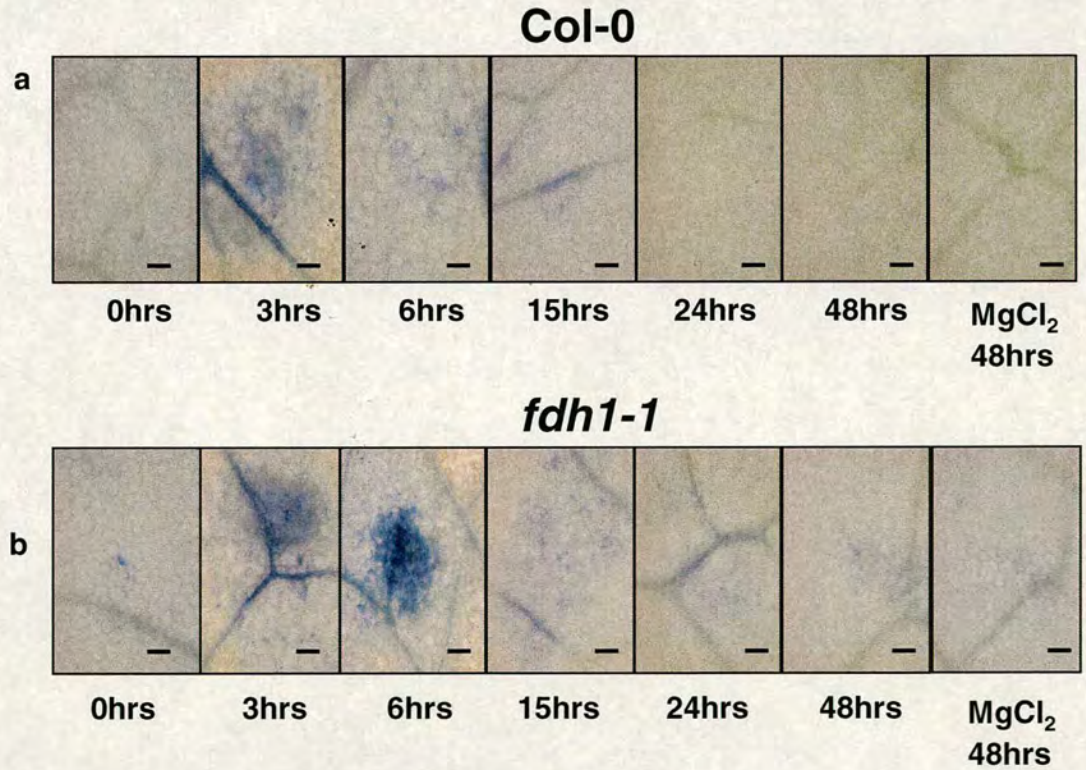

Figure 5.6 The superoxide burst is prolonged in *fdh1-1* plants

NBT (nitroblue tetrazolium) staining for superoxide accumulation in **(a)** Col-0 and **(b)** *fdh1-1* plants following *Pst* DC3000(*avrB*) treatment. x10 magnification, scale bars 50µm.

5.8 H_2O_2 accumulation in *fdh1-1* plants

To further investigate ROI levels in *fdh1-1*, H_2O_2 accumulation was visualised using DAB staining. *fdh1-1* and Col-0 plants were infiltrated with *Pst* DC3000 (*avrB*), before removing leaves for DAB staining at the same time points as the previous experiment. In Col-0 plants between 3-15 hours, the brown precipitate formed from the reaction between DAB and H_2O_2 is most abundant (Figure 5.7a). Lower levels of H_2O_2 accumulation were then apparent at 24 and 48 hours in Col-0 (Figure 5.7a). *fdh1-1* plants showed a similar pattern of H_2O_2 accumulation following *Pst* DC3000 (*avrB)* treatment (Figure 5.7b). However, the background DAB staining in *fdh1-1* leaves was more pronounced than in Col-0 leaves. This is most obvious in the untreated and buffer control samples (Figure 5.7a and b). Using a higher magnification to examine the untreated leaves, shows that this background DAB staining/H_2O_2 accumulation is localised to the chloroplasts (Figure 5.8). This suggests that there are higher levels of H_2O_2 localised in the chloroplasts of *fdh1-1*.

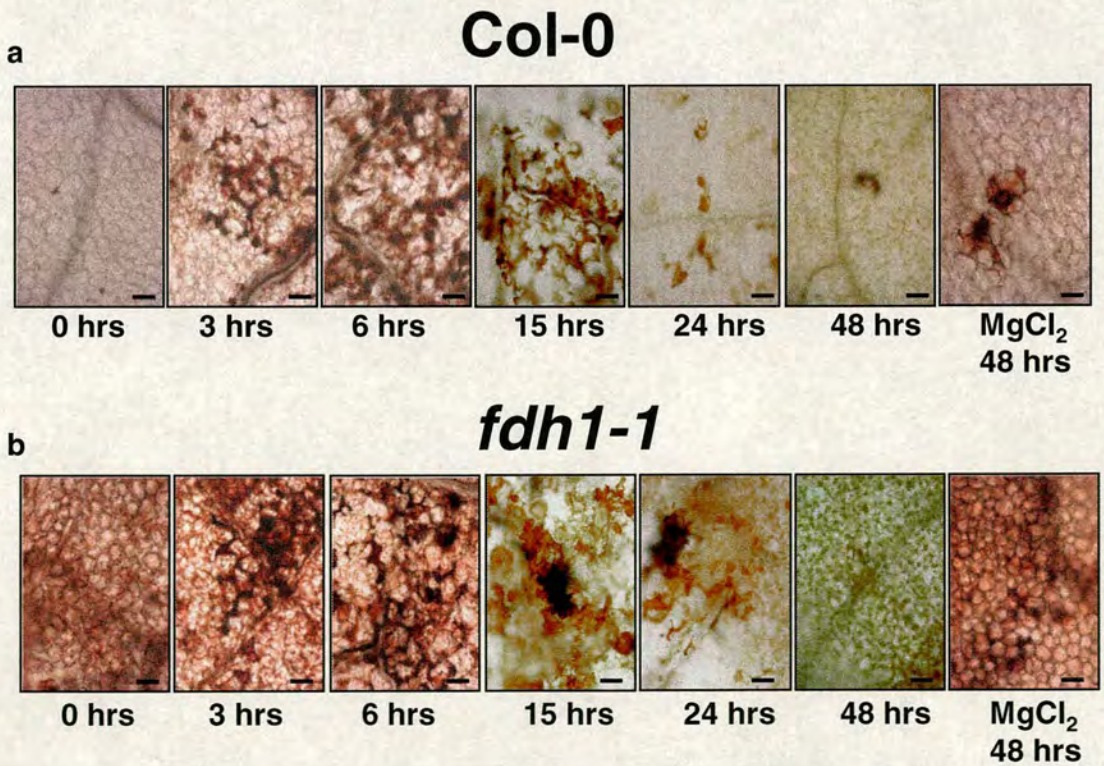

Figure. 5.7 Hydrogen Peroxide accumulation in *fdh1-1* plants

DAB (Diaminobenzidine) stain for hydrogen peroxide accumulation 0-48hours after infiltration of *Pst* DC3000(*avrB*)in **(a)** Col-0 and **(b)** *fdh1-1*. x10 magnification, scale bars 50µm

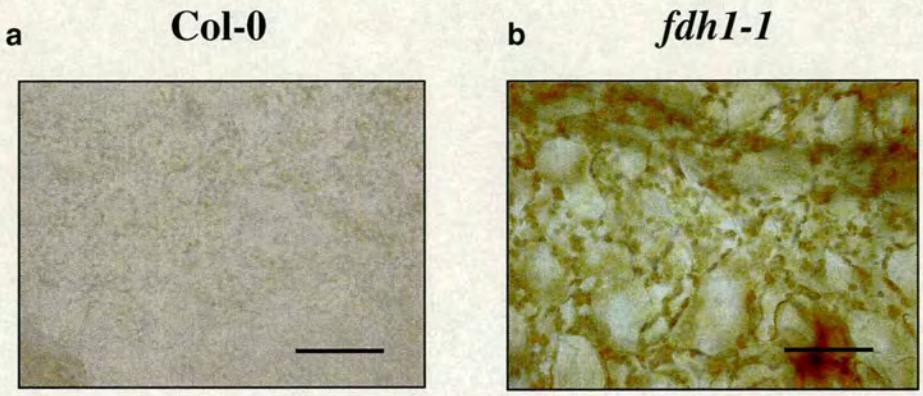

Figure. 5.8 Hydrogen Peroxide accumulation in the chloroplasts of *fdh1-1* plants

DAB staining of naïve **(a)** Col-0 and **(b)** *fdh1-1* plants. x40 magnification, scale bars 50μm.

5.9 Programmed cell death in *fdh1-1* plants

The signalling molecules H_2O_2 and NO are thought to act synergistically to induce PCD during the HR (Delledonne et al., 2001). In order to investigate if the perturbed levels of ROIs found in *fdh1-1* mutants affect PCD, Col-0 and *fdh1-1* were infiltrated with *Pst* DC3000 (*avrB*). The progression of PCD was monitored by staining with trypan blue (TB), which is taken up by dead cells. Following *Pst* DC3000 (*avrB*) infiltration the onset of PCD in Col-0 plants could be seen at 6 hours (Figure 5.9a). In comparison TB staining was not as strong in *fdh1-1* plants 6 hours after treatment (Figure 5.9b). By 15 hours dead cells in Col-0 plants had collapsed and TB staining was pronounced (Figure 5.9a). Although TB staining in *fdh1-1* plants was also obvious at 15 hours, the stained dead cells had not collapsed (Figure 5.9b). Similarly, at 24 hours in Col-0 plants dead cells are condensed and collapsed whereas in *fdh1-1* dead cells are still round and full (Figure 5.9b). The lack of collapsed cells in *fdh1-1* reflects the delayed HR phenotype seen in the leaves of *fdh1-1* plants compared to Col-0 (Figure 5.5a) .

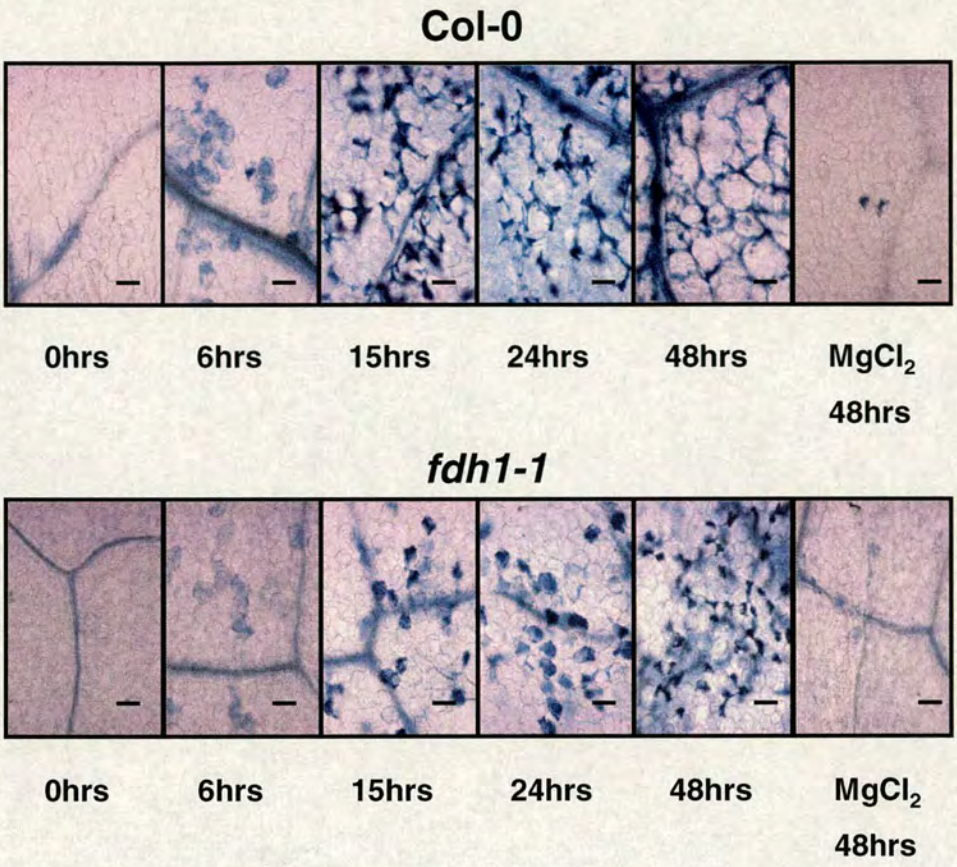

Figure 5.9 Programmed cell death in *fdh1-1* plants

TB (Trypan Blue) staining for cell death in in Col-0 and *fdh1-1* plants following *Pst* DC3000(*avrB*) treatment. x10 magnification, scale bars 50μm

5.10 NO accumulation in *fdh1-1* plants

Confocal laser-scanning microscopy in conjunction with the NO-selective fluorescence indicator DAF-2 DA revealed that there is more NO present in excised Col-0 leaves than *fdh1-1* leaves (Figure 5.10 a and b). The trichome and the surrounding area in Col-0 leaves showed strong DAF-2 DA staining (Figure 5.10a). In comparison, only the trichome in *fdh1-1* leaves showed the presence of NO (Figure 5.10b). The stomata in Col-0 leaves stained as areas of intense fluorescence (Figure 5.10 a). Using a higher magnification, it was found that the chloroplasts in the stomata were the source of this fluorescence (Figure 5.10c). Therefore, in Col-0 leaves NO was localised in the chloroplasts of the stomata (Figure 5.10c). However, NO could not be detected in the stomata of *fdh1-1* leaves (Figure 5.10d).

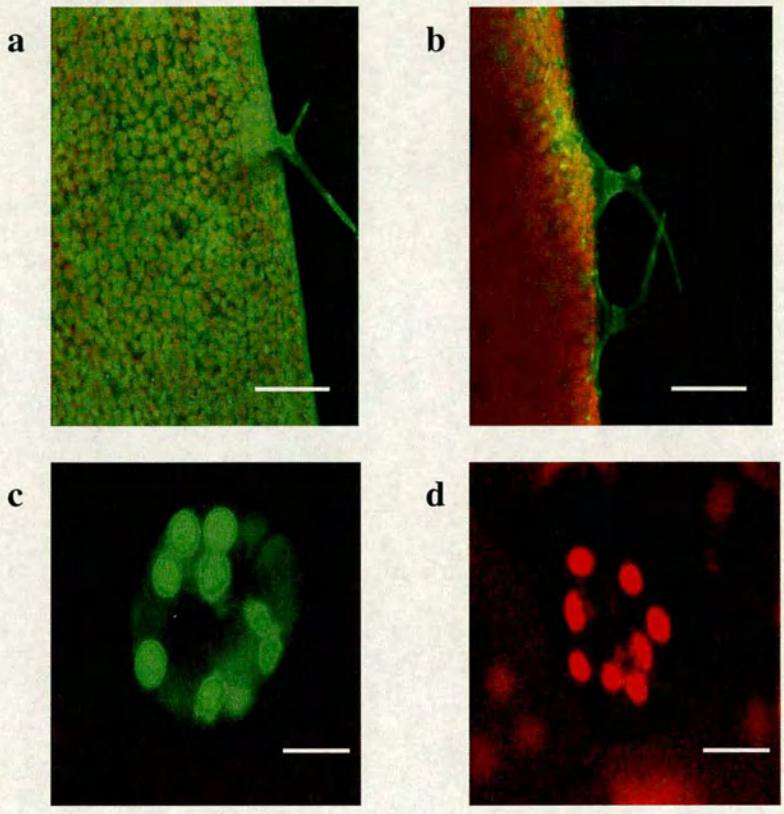

Figure 5.10. Nitric oxide accumulation in *fdh1-1* plants.

DAF-2 DA (4,5-diaminoflurecein diacetate) staining of NO accumulation and red chlorophyll autoflouresence in excised **(a)** Col-0 and **(b)** *fdh1-1* leaves in an area with a trichome. NO accumulation and chlorophyll autoflourescence in the stomata of **(c)** Col-0 and **(d)** *fdh1-1* leaves. Scale bars **(a)** and **(b)** 150 μm **(c)** and **(d)** 10 μm

5.11 Discussion

NO can S-nitrosylate proteins (SNO's) and glutathione to form GSNO (Foster et al., 2003; Fernanadez et al., 2003). In animals SNO's derived from proteins and GSNO supply the cell with reserviors of NO bioactivity (Foster et al., 2003). Furthermore, S-nitrosylation has been shown to regulate the onset of apoptosis by activating and deactivating proteins such as caspases and cytochrome c (Stamler et al., 2001; Li et al., 1998). However, it is not known if similar processes occur during the HR in plants.

GS-FDH is an enzyme specific for GSNO metabolism and has recently been implicated in animal immune responses (Lui et al., 2001; Lui et al., 2004). To investigate a possible role for GSNO during the plant HR, *GS-FDH* expression and enzyme activity were investigated following pathogen challenge. In addition, *fdh1-1* plants were used to investigate if PCD and ROI accumulation are perturbed as a result of *GS-FDH* over-expression.

It was found that the treatment of Col-0 plants with avirulent *Pst* DC3000 (*avrB*) but not virulent *Pst* DC3000 strongly suppressed the expression of *GS-FDH* after just 1 hour (Figure 5.1). The defence genes *GST1, PR1* and *PDF1.2* were induced by *Pst* DC3000 (*avrB*) at 1, 9 and 24 hours respectively (Figure 5.1). ROIs and NO are produced as signals that act synergistically to induce PCD (Delledonne at al., 2001). Enzymes, such as APX and CAT that scavenge H_2O_2, have also been shown to be down regulated during the HR. This enables the rapid progression of PCD (Dorey et al., 1998; Mittler et al., 1998). It is likely that GSNO is also an important signalling molecule, possibly S-nitrosylating downstream proteins which induce PCD. Therefore, the down-regulation of *GS-FDH* would also allow the progression of the HR.

Most NBS-LRR *R*-genes signal through either EDS1 or NDR1 signal transduction pathways (Aarts et al., 1998). *R*-genes, which are dependent on EDS1 are TIR-NB-LRRs class *R*-genes, whereas NDR1 dependent *R*-genes are of the CC-NB-LRRs class. (Aarts et al, 1998). The *R*-gene, *RPM1,* that confers resistance to bacteria expressing *avrB,* is dependent on *NDR1*, whereas *RPS4*, which confers resistance to the avirulence gene *avrRps4*, is EDS1 dependent (Aarts et al., 1998). It was found that the infiltration of *Pst* DC3000 (*avrB)* strongly suppresses the expression of *GS-FDH,* in contrast *Pst* DC3000 a*vrRps4* treatment does not (Figure 5.1 and Figure 5.2). This suggests that the suppression of *GS-FDH* may be dependent on NDR1. However, GS-FDH protein

activity was found to be down-regulated by both *Pst* DC3000 (*avrB)* and *Pst* DC3000 (*avrRps4)* treatment (Figure 5.3). GS-FDH activity was strongly down-regulated 5-24 hours post *Pst* DC3000 *avrB* infiltration (Figure 5.3a). In comparison *Pst* DC3000 (*avrRps4)* treatment induced the down-regulation of GS-FDH activity between 9-15 hours, but this supression was not as pronounced as that with seen with *Pst* DC3000 (*avrB*) (Figure 5.3b). Therefore the suppression of GS-FDH is not strictly NDR1 dependent. *RPS4* is a 'weaker' *R*-gene than *RPM1*, inducing the HR with slower kinetics. For instance, leaf collapse in Col-0 plants treated with *Pst* DC3000 (*avrB)* can be seen after 6 hours (Figure 5.5.a). Whereas, leaf collapse after treatment with *Pst* DC3000 (*avrRps4*) is not visible until 15 hours (Figure 5.5b). Thus *RPS4* may be a slower acting *R*-gene because it suppresses GS-FDH activity less effectively.

Furthermore, the suppression of *GS-FDH* activity but not expression by *Pst* DC3000 (*avrRps4*) suggests that GS-FDH levels are controlled both at the transcriptional and post-transcriptional level. This is also true for cytosolic APX, which detoxifies H_2O_2 during the HR, thus regulating PCD. In tobacco during a TMV induced HR, it was found that APX transcripts increased whereas, APX protein and activity levels were down-regulated (Mittler et al., 1998).

In order to elucidate which signalling molecules are required for the suppression of *GS-FDH* expression different mutants compromised in disease resistance signalling were infiltrated with *Pst* DC3000 (*avrB*). *GS-FDH* expression was suppressed in wild-type Col-0 plants after 5 hours, before returning to basal levels again at 24 hours (Figure 5.4). A similar *GS-FDH* expression pattern was found in the mutants *etr1, ein2.1, eds1, nrp1, sid1, sid2* and in transgenic *NahG* plants (Figure 5.4). These mutants are still responsive to signals, which repress *GS-FDH*. Thus neither ETR1, EIN2, SID1, SID2, NPR1 nor EDS1 are required for *GS-FDH* suppression. *coi1* mutants also showed *GS-FDH* repression at 5 hours post infiltration, however, transcript levels failed to increase again at 24 hours (Figure 5.4). Therefore, COI1 may be required for the recovery of *GS-FDH* expression to basal levels. Recently, *GS-FDH* was demonstrated to be down regulated by wounding and up regulated by SA in wild-type Col-0 plants (Diaz et al., 2003). In *coi1* plants wounding was found to up-regulate *GS-FDH*. However, the application of JA to Col-0 plants did not down-regulate *GS-FDH* (Diaz et al., 2003). Our results show that *GS-FDH* is suppressed in *coi1* plants following pathogen challenge (Figure 5.4). Thus COI1 is required for suppression of *GS-FDH* during

wounding but not pathogen challenge. Therefore the signalling network required for *GS-FDH* suppression following pathogen challenge differs from that induced by wounding. Further work on the response of *GS-FDH* to wounding in different mutant backgrounds would help elucidate these differences in signalling.

Interestingly, *GS-FDH* expression increases in *fdh1-1* mutants 5 hours after *Pst* DC3000 (*avrB)* treatment and transcript levels are higher than in untreated leaves after 24 hours (Figure 5.4). This demonstrates that *fdh1-1* plants are no longer responsive to signals, which normally repress *GS-FDH* expression in wildtype plants. Thus the T-DNA insertion of *fdh1-1* mutants may be perturbing a negative regulatory element of the *GS-FDH* promoter. Alternatively, GSNO may be required either directly or indirectly for *GS-FDH* repression in a positive feedback mechanism. Therefore, the over-expression of *GS-FDH* would reduce GSNO levels preventing *GS-FDH* down regulation. However, it should be noted that a high concentration of exogenously applied GSNO was found to induce *GS-FDH* expression (Figure 4.1)

The presence of O_2^- in *fdh1-1* plants following pathogen challenge was found to be prolonged compared to Col-0 plants (Figure 5.6). *fdh1-1* was also found to have increased levels of H_2O_2 localised in the chloroplast (Figure 5.8). Furthermore, *fdh1-1* plants show increased anthocyanin levels. This may be due to the over-expression of *GS-FDH* or alternatively to the perturbation of the downstream *FLS* gene by the T-DNA insertion (Figure 4.3). Anthocyanins are antioxidants, which scavenge H_2O_2, O_2^- and $O_2^{\cdot}$ (Yamasaki et al., 1997; Nagata et al., 2003; Tournaire et al., 1993). It is possible that the high levels of anthocyanin in *fdh1-1* plants are produced to mop up excess H_2O_2 and O_2^- in these mutants. In addition to oxidative stress flavonoids have anti-microbial properties (Dixon et al., 1983; Kemp and Burden, 1986). Therefore, the delayed HR phenotype in *fdh1-1* plants (Figure 5.5 and 5.9) may be a result of lower bacterial numbers caused by the antimicrobial properties of flavonoids.

GSNO is a source of NO bioactivity, but it also has antioxidant properties protecting from oxidative stress in endothelium tissue and lipid peroxidation in the brain (Chiueh, 1999; Rauhala et al., 1998). NO has been shown to act as an anti-oxidant in rice (Hung et al., 2002) and potato (Beligini and Lammatina, 2002), protecting from oxidative stress. Thus it is possible that the metabolism of GSNO by *GS-FDH* over-expression may perturb ROI levels in *fdh1-1*. It has been proposed that when H_2O_2 and NO are balanced they act synergistically to induce PCD (Delledonne et al., 2001). However, if

either O_2^- or NO production is in favour, then ONOO- is formed which was not found to induce PCD (Delledonne et al. 2001). For example, the addition of 0.5mM SNP to rapidly agitated soybean cells, which produce a steady-state H_2O_2 concentration of ~1μM, caused cell death in 90% of cells. However, if the SNP concentration is increased to 5mM the percentage of soybean cell death decreases to 10% (Delledonne et al., 2001). The presence of O_2^- in *fdh1-1* plants following pathogen challenge was found to be prolonged compared to Col-0 plants (Figure 5.6). *fdh1-1* was also found to have increased levels of H_2O_2 localised in the chloroplast (Figure 5.8). Confocal laser scanning microscopy using the fluorescent NO indicator DAF-2 DA revealed lower levels of NO in *fdh1-1* leaves than in Col-0 leaves particularly in the chloroplasts (Figure 5.10). As previously discussed in Chapter 4 it is possible that GSNO is produced in chloroplasts from the nitrosation of GSH by NO (Fernandez et al., 2003). Anti-NOS immunoreactive proteins have been shown to be localised in chloroplasts and DAF-2DA staining has also shown NO production in chloroplasts (Ribeiro et al.,1999; Foissner et al., 2000). However, it is not known if GS-FDH is present in chloroplasts. In yeast and mammalian cells GS-FDH is localised in the nucleus and cytosol (Iborra et al., 1992; Fernandez et al., 2003). Lower levels of GSNO in the chloroplasts of *fdh1-1* plants due to high GS-FDH activity would reduce the ROI scavenging potential of the cell, since GSNO is an antioxidant. It may also deplete levels of GSH which is required for ROI scavenging (Mittler, 2002). An impaired ability to scavenge ROIs may explain why *fdh1-1* plants accumulate H_2O_2 particularly in chloroplasts and show a prolonged O_2^- burst following pathogen attack.

Since ROIs and NO are required to be in balance to induce PCD (Figure 1.6), the perturbed ROI and NO levels in *fdh1-1* plants, as a result of GSNO metabolism are likely to account for the delayed HR (Figure 5.5) and lack of collapsed cells (Figure 5.9) observed in *fdh1-1* plants. In addition, low levels of GSNO due to GS-FDH activity may prevent necessary signalling events from taking place such as protein S-nitrosylation, which could delay the onset of the HR.

Conclusion

In conclusion, these results show that *GS-FDH* expression and activity are tightly regulated during the HR. This regulation may be both at the transcriptional and post-transcriptional level.

Furthermore, the over-expression of *GS-FDH* in *fdh1-1* mutants perturbed ROI and NO levels as well as delaying the onset of the HR. Thus GS-FDH controls the progression of the HR by modulating GSNO levels.

Chapter 6

6. Identification and characterisation of a *GS-FDH* Knock-out mutant

6.1 Introduction

The enzyme GS-FDH exhibits strong GSNO reductase activity (Lui et al., 2001). In animals, GS-FDH regulates SNO levels, which are central to regulating innate immunity and vascular function (Lui et al., 2004).

Previously we identified *fdh1-1*, a T-DNA mutant that over-expresses *GS-FDH*. This mutation conferred resistance to high levels of GSNO. Furthermore, *fdh1-1* mutants showed altered developmental phenotypes including precocious anthocyanin accumulation, short roots with long prolific root hairs and short inflorecences, when grown in short days. To further assess the effect of GS-FDH and therefore GSNO levels on plant disease resistance, the response of *fdh1-1* to different necrotrophic and biotrophic pathogens was investigated. It was found that *fdh1-1* plants are more resistant to the virulent biotrophic pathogen *Pst* DC3000. During the HR induced by *Pst* DC3000 (*avrB*) treatment, *fdh1-1* plants exhibited a prolonged oxidative burst and less collapsed cells than found in Col-0 plants. Overall, the progression of the HR in *fdh1-1* was delayed compared to Col-0.

These findings prompted us to find a GS-FDH knockout mutant to further investigate GS-FDH function and to compare with the *GS-FDH* over-expressor, *fdh1-1*. The SIGnAL database was searched for an insertion within the *GS-FDH* coding sequence and a knockout line, *fdh1-3* was subsequently identified. The initial characterisation of this line shows that it has more pronounced developmental abrmormalities than *fdh1-1*. Interestingly, *fdh1-3* is susceptible to virulent *Pst* DC3000 and shows an accelerated HR phenotype in response to *Pst* DC3000 (*avrB*).

6.2 Identification of the *fdh1-3* mutant line

The SIGnAL (Salk Institute Genomic Analysis Laboratory) database was searched for lines containing a T-DNA insert in the *GS-FDH* coding sequence. One line (315D11) was identified from GABI-KAT's (German plant genomics research program) T-DNA mutagenised population with a possible insert in *GS-FDH* (Rosso et al., 2003). T-DNA flanking sequences from PCR analysis (performed by GABI-KAT) indicated that the T-DNA insert was situated in the coding sequence of *GS-FDH*.

In order to confirm the presence and position of the T-DNA insert in this line, PCR was performed using two gene specific primers and one left border T-DNA primer. PCR using Col-0 genomic DNA produces a 1.2Kb product, whereas PCR with DNA from plants homozygous for the T-DNA insert will produce a 650 bp product and PCR with DNA from heterozygotes will produce both bands (Figure 6.1). Using this method the presence of a T-DNA insert in *GS-FDH* was confirmed, however only heterozygotes for the T-DNA insert were identified in the T_2 generation (Figure 6.2a). Therefore heterozygotes were allowed to self and a homozygous line was subsequently identified in the T_3 generation (Figure 6.2b). Progeny from the homozygous line were then tested by PCR to confirm that this line (subsequently called *fdh1-3*) was homozygous for the T-DNA insertion.

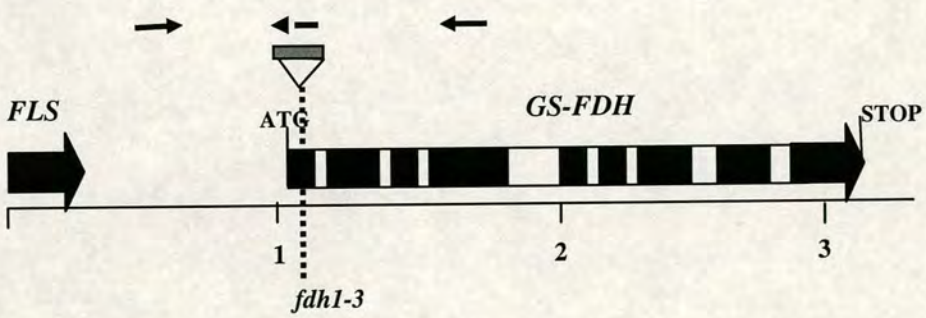

Figure 6.1 Position of the T-DNA insert in *fdh1-3* mutants

The T-DNA insert in *fdh1-3* is 30bp downstream from the ATG of *GS-FDH*, in the first exon. The arrows show the position of gene specific and T-DNA left border primers. The T-DNA insert (~4.5kb) is shown in grey but is not to scale

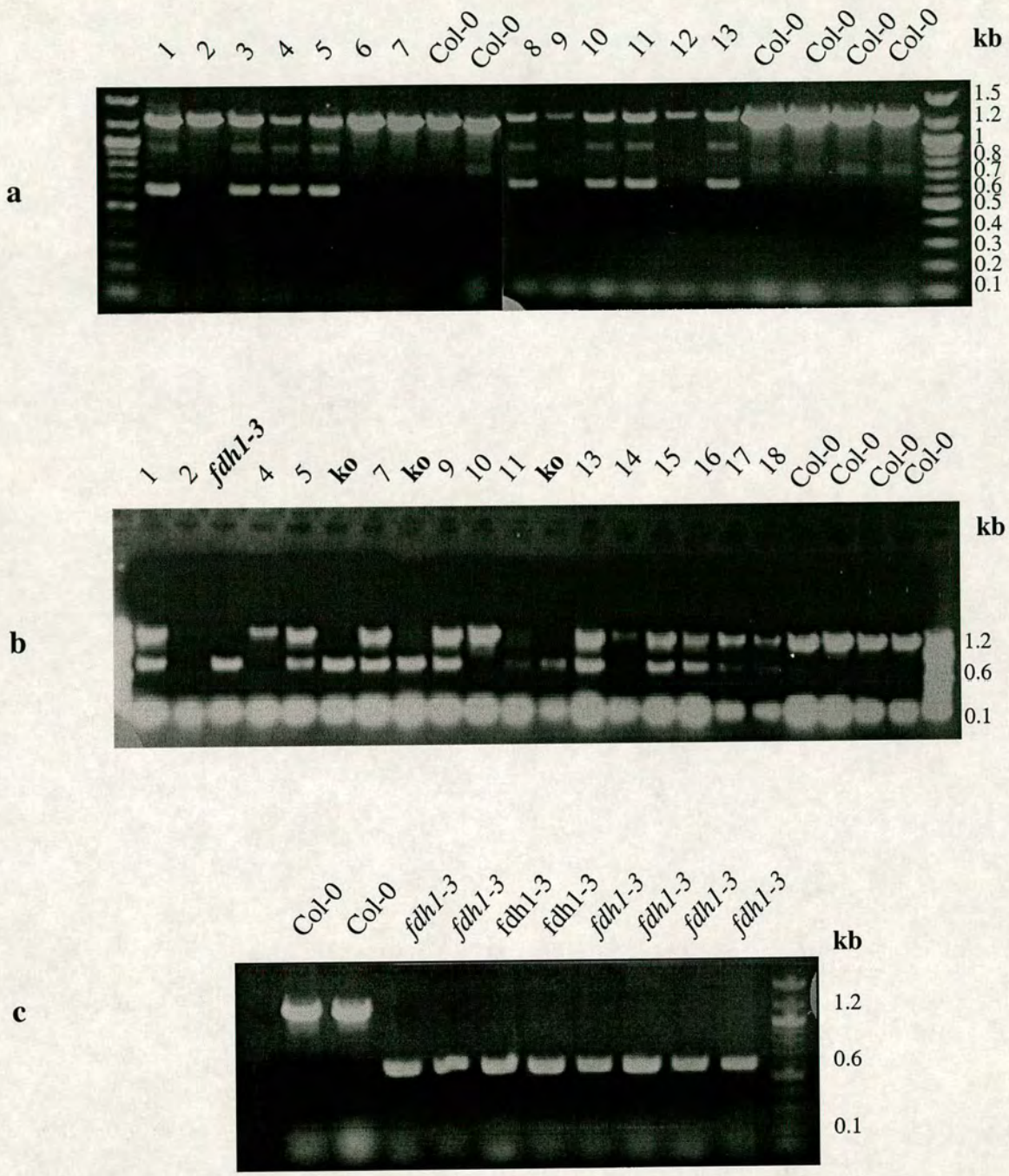

Figure 6.2 Identification of a *GS-FDH* Knock-out mutant by PCR.

PCR with two gene specific and one T-DNA left border primer were used to confirm the T-DNA insert in *GS-FDH*. In **(a)** F_2 segregating population **(b)** homozygous knock-out (KO) mutants were identified in the T_3 segregating population and allowed to set seed **(c)** Progeny of the KO line *fdh1-3* were confirmed as homozygous for the T-DNA insertion in *GS-FDH*.

6.3 Phenotypic characterisation of *fdh1-3* plants

fdh1-3 knock-out mutants show altered developmental phenotypes affecting growth, root development and flowering.

fdh1-3 mutants have broad pale green leaves and are smaller than wild-type plants (Figure 6.3a). During flowering *fdh1-3* forms many secondary inflorescences which are stunted and develop small siliques (Figure 6.3 b and c). As a result fertility is reduced in *fdh1-3*; the average seed yield per plant is ~70 for *fdh1-3* whereas the average seed yield from Col-0 is ~1500. *fdh1-3*'s perturbed flowering phenotypes suggests GSNO and/or SNO's are important during reproductive development.

Figure 6.3 The Phenotype of *fdh1-3* mutants

(a) *fdh1-3* left and Col-0 right **(b)** Flowering *fdh1-3* **(c)** Flowering, Col-0 left and *fdh1-3* right.

6.4 Root development of *fdh1-3* plants

To investigate the root development of *fdh1-3* mutants, Col-0 and *fdh1-3* seedlings were grown vertically on MS plates and root length was measured after 10 days. The average root length for Col-0 was found to be 2.3cm whereas, *fdh1-3* has an average root length of 0.7cm (see Figure 6.4.e). Roots from Col-0 and *fdh1-3* were also examined microscopically at 10 days old. *fdh1-3* was found to have decreased root hair formation compared to Col-0 (Figure 6.4a and b). In addition, the root tip of *fdh1-3* was found to be aberrant, lacking organised cell layers found in wild-type root tips (Figure 6.4c and d). After 21 days some *fdh1-3* mutants were able to grow longer roots but these had few or no lateral roots (Figure 6.4f). These results suggest that GS-FDH is important for different stages of root development

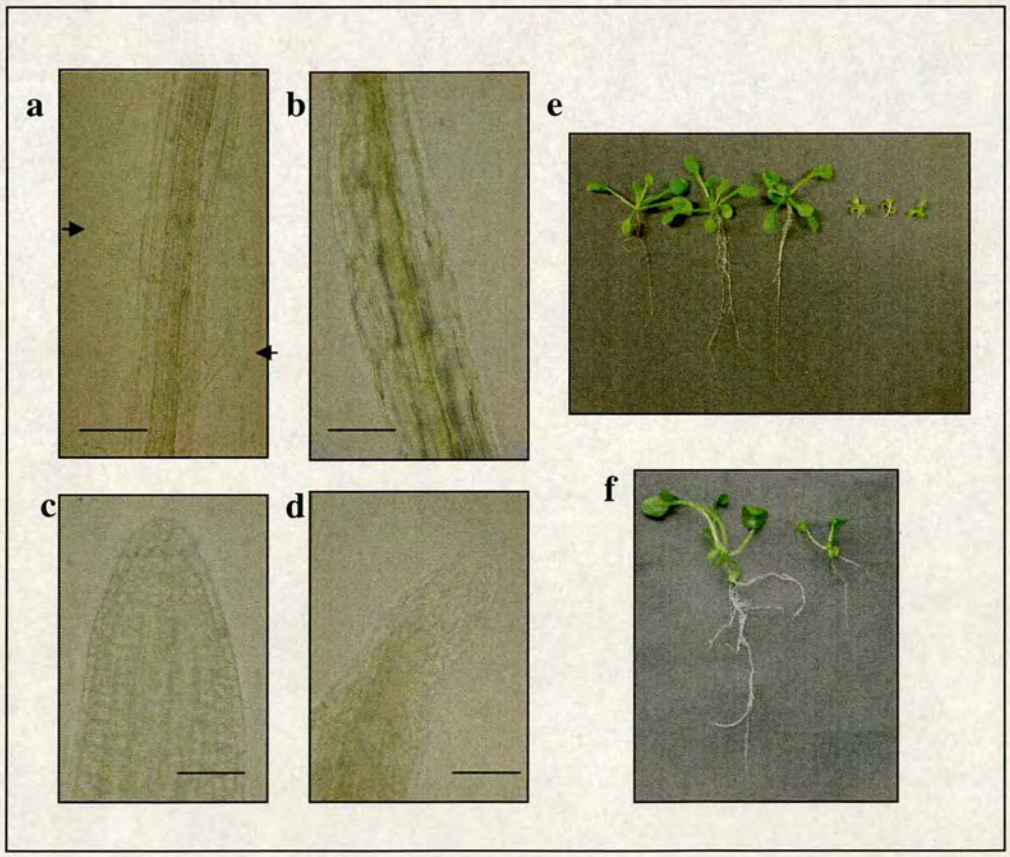

Figure 6.4 Root Development in *fdh1-3* mutants

At 10 days **(a)** Col-0 root, arrows indicate root hairs **(b)** *fdh1-3* root **c)** Col-0 root tip **(d)** *fdh1-3* root tip **(e)** root length of Col-0 and *fdh1-3*, Scale bars 50μm . **(f)** root length of Col-0 and *fdh1-3* at 21 days, Scale bars 0.5mm.

6.5 *fdh1-3* is an Knock-out mutation

The T-DNA insert of *fdh1-3* is situated in the coding sequence of *GS-FDH* and this was found to cause perturbed developmental phenotypes. To establish the effect of the T-DNA on *GS-FDH* expression, northern analysis was used to detect *GS-FDH* transcript in naïve Col-0 and *fdh1-3* plants. Basal expression of *GS-FDH* was present in Col-0 but not in *fdh1-3* plants (Figure 6.5a). GS-FDH is able to use S-hydroxymethylglutathione (HM-GSH) as a substrate although, it has higher affinity for GSNO (Lui et al., 2001). Evidence suggests that GSNO and HM-GSH bind in a similar if not an identical manner in the active site of GS-FDH (Hedberg et al., 2003). To investigate GS-FDH activity in *fdh1-3* and Col-0 plants, an in-gel enzyme activity assay was performed based on the ability of GS-FDH to metabolise HM-GSH. Protein extracts from naïve *fdh1-3* and Col-0 plants were separated on NATIVE-PAGE gels, before incubation with the substrate HM-GSH. *fdh1-3* extracts exhibited no GS-FDH enzyme activity, whereas wild type Col-0 plants did show activity (Figure 6.5b).

The lack of *GS-FDH* transcript and enzyme activity in *fdh1-3* mutants confirms that the T-DNA insert in the coding sequence of *GS-FDH* has knocked-out its transcription and translation.

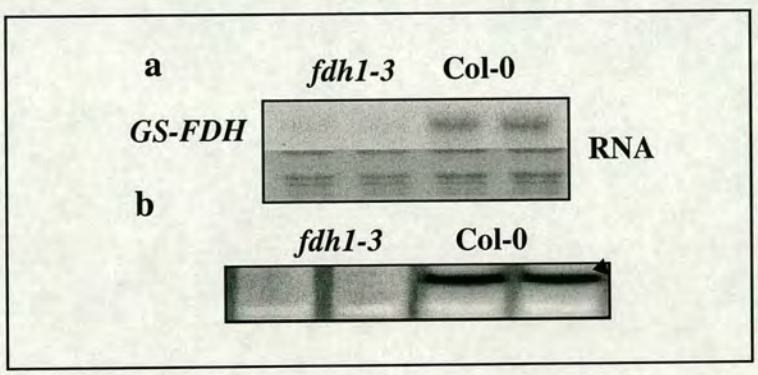

Figure 6.5 *fdh1-3* **is a knock-out mutant**

(a) RNA samples were extracted from naïve *fdh1-3* and Col-0 plants and used for northern blot analysis with a *GS-FDH* cDNA probe **(b)** Protein extracts from naïve *fdh1-3* and Col-0 plants were collected and used for an in gel enzyme activity assay using HM-GSH as a substrate. Arrow indicates GS-FDH activity band.

6.6 GSNO sensitivity in *fdh1-3* plants

GS-FDH knock-out yeast were found to be hypersensitive to GSNO treatment (Lui et al., 2001). To investigate if this was also the case for *Arabidopsis*, *fdh1-3* and Col-0 seed were germinated on MS media containing increasing concentrations of GSNO. The germination of *fdh1-3* plants (74%) was found to be lower than that of wild-type seedlings (100%) on MS media containing no GSNO. Therefore, GS-FDH may have a role in germination and seedling establishment. The percentage germination of *fdh1-3* was significantly lower than Col-0 on MS containing between 0.375mM and 1mM GSNO (Figure 6.6). For example, the germination frequency of wild type seedlings on 1mM GSNO was 48% compared to 4% for *fdh1-3* (Figure 6.6). These results demonstrate that knocking out *GS-FDH* in *Arabidopsis* leads to increased GSNO sensitivity.

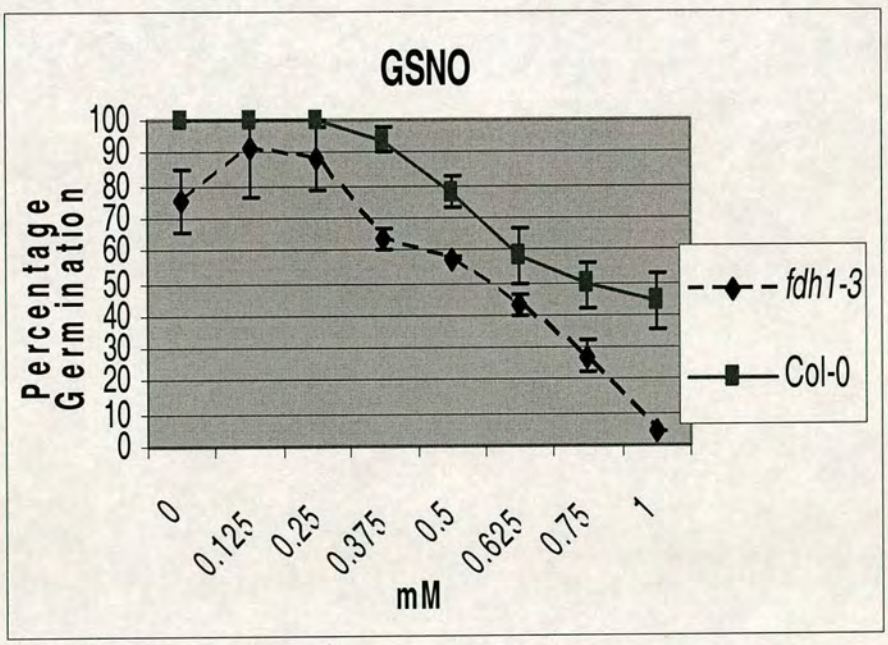

Figure 6.6 *fdh1-3* mutants are hypersensitive to GSNO

25 seed of Col-0 were germinated on MS plates containing 0mM, 0.125mM, 0.250mM, 0.375mM, 0.5mM, 0.625mM, 0.75mM and 1mM GSNO. Values are the average of two experiments and error bars represent the standard error between values at the 95% confidence level.

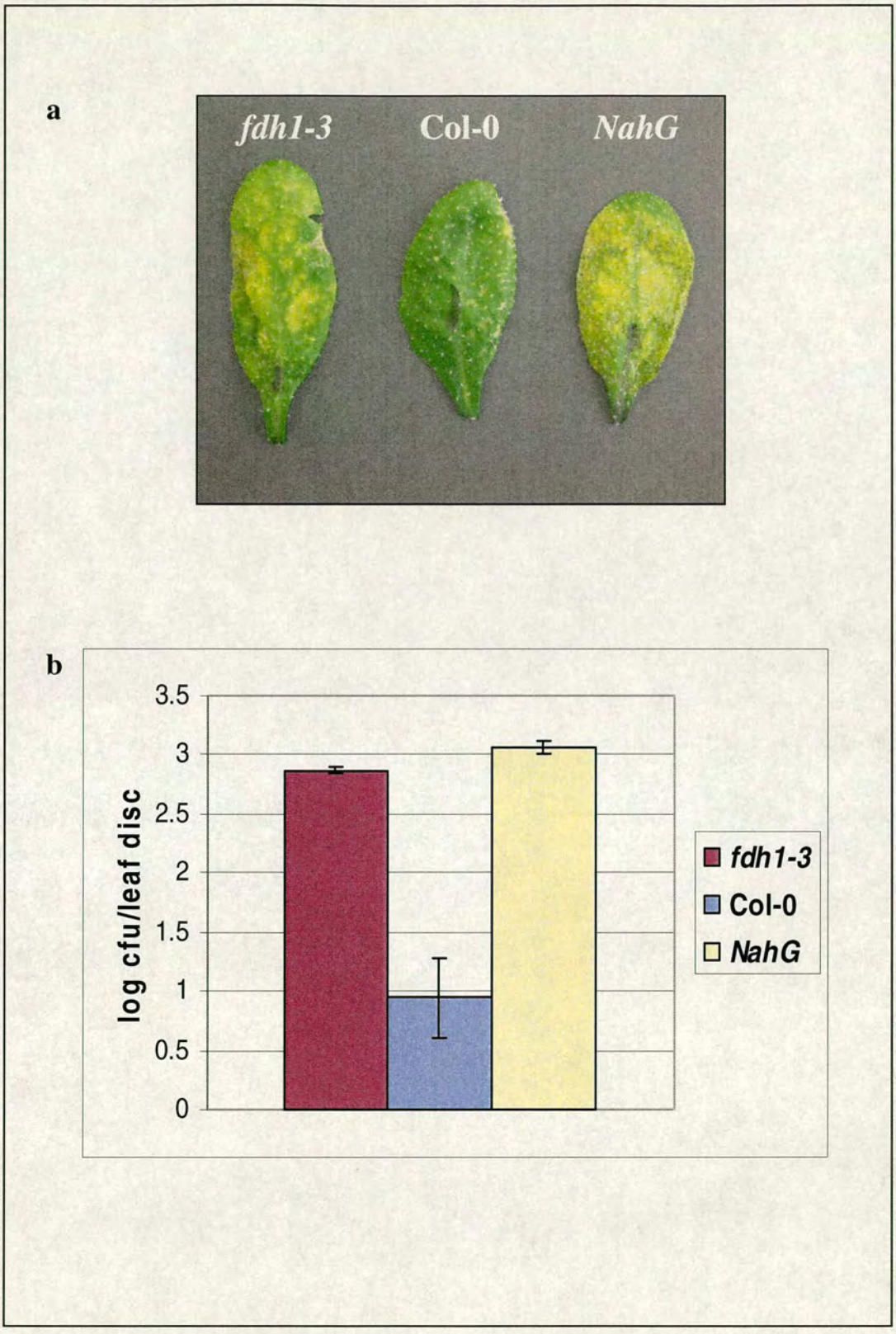

Figure 6.7 *fdh1-3* mutants are susceptible to *Pst* DC3000

a) Development of disease symptoms in *fdh1-3*, Col-0 and *NahG* plants 4 days post inoculation with *Pst* DC3000. **b)** Bacterial titre 4 days after *Pst* DC3000 inoculation in Col-0, *NahG* and *fdh1-3*. Values shown are the average of three plants per line and error bars represent the standard error at the 95% confidence level.

6.7 *fdh1-3* plants are compromised in basal disease resistance

At four weeks old three plants of Col-0, *NahG* and *fdh1-3* plants were inoculated with a low concentration of *Pst* DC3000 (OD 0.0002). After 4 days leaves from each line were examined for the development of disease symptoms. At this time *NahG* and *fdh1-3* plants had undergone extreme chlorosis, whereas Col-0 plants showed few disease symptoms (Figure 6.7a). Leaf extracts were made by grinding in 10mM $MgCl_2$ and dilution's were plated out on KB media. The number of bacterial colonies was recorded after 3 days (shown in Figure 6.7b). *NahG* plants were most susceptible to virulent *Pst* DC3000 with a bacterial titre of around 10^3 cfu/leaf disk. Interestingly, *fdh1-3* had a similar bacterial titre to *NahG*, whereas Col-0 plants had a bacterial titre around one hundred times less than *NahG* and *fdh1-3* (Figure 6.7b). Therefore, GS-FDH is important for resistance to the virulent biotroph *Pst* DC3000.

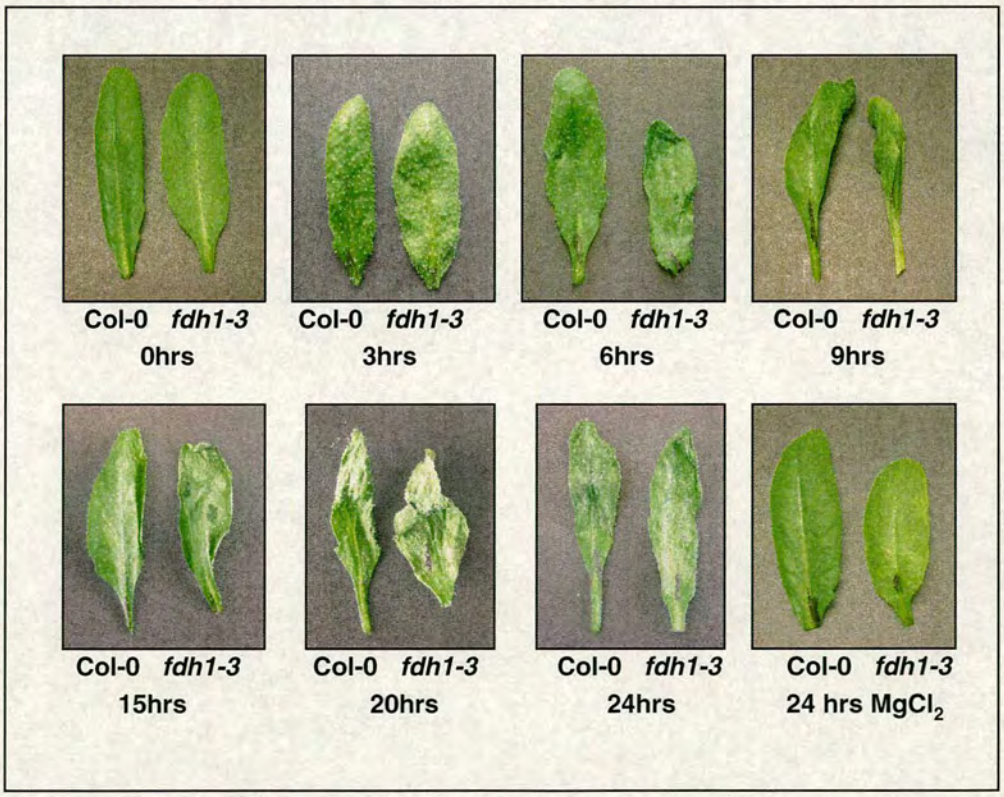

Figure 6.8 *fdh1-3* mutants show an accelerated HR phenotype.

Col-0 and *fdh1-3* plants were infiltrated with *Pst* DC3000(*avrB*) and leaves were monitored for the progression of the HR.

6.8 The HR is accelerated in *fdh1-3* plants

Previously we demonstrated that the GS-FDH over-expressor *fdh1-1* has a delayed HR phenotype. Therefore the HR was investigated in the GS-FDH knock-out line, *fdh1-3*. Following the infiltration of *Pst* DC3000 (*avrB)* the development of the HR was monitored in Col-0 and *fdh1-3* plants. Leaf collapse was found to be visible in both Col-0 and *fdh1-3* after 6 hours, however, leaf wilting and PCD was more pronounced in *fdh1-3* (Figure 6.8). HR symptoms in *fdh1-3* were also more obvious than in Col-0 at 9 and 15 hours after *Pst* DC3000 (*avrB*) treatment (Figure 6.8). From 20 hours onwards the HR symptoms in Col-0 and *fdh1-3* were similar (Figure 6.8). Thus the loss of GS-FDH function results in an more rapid HR response.

6.9 Discussion

fdh1-3 mutants have a T-DNA insert situated in the coding sequence of GS-FDH. No GS-FDH transcript or enzyme activity was detected in *fdh1-3* plants. Therefore, the T-DNA insert has disrupted the transcription and consequently the translation of GS-FDH (Figure 7.4a and b). Consistent with the knockout of GS-FDH function, *fdh1-3* mutants show increased susceptibility to GSNO compared to wild-type plants. This is in agreement with previous studies by Lui and co-workers who found that GS-FDH knockout yeast are hypersensitive to GSNO (Lui et al., 2001).

fdh1-3 mutants have perturbed growth, flowering and root development. For example, *fdh1-3* plants are smaller than wild-type with pale green, broad leaves. During flowering *fdh1-3* displays reduced apical dominance with stunted inflorescences and small siliques. The roots of *fdh1-3* are also very stunted at 10 days old and lack root hairs. Although some recovery in root growth occurs after 20 days, they are still shorter than wild-type and lack lateral roots. These phenotypes suggest that GS-FDH activity is required to regulate SNO levels during development.

Evidence suggests a role for NO in root tip development. In wild-type *Arabidopsis* plants DAF-2 DA staining of root tips showed strong NO production, whereas DAF-2 DA staining was lacking in *AtNOS* mutants, which, have shorter roots than wild-type plants (Guo et al., 2003). Further studies have shown that NO induces adventitious and lateral root formation in cucumber and tomato respectively. In cucumber NO was shown to be required for an auxin induced MAPK kinase signalling cascade during adventitious root development (Pagnussat et al., 2004). In tomato NO is able to promote lateral root initiation in roots treated with an auxin transport inhibitor. Furthermore, the promotion of lateral root development by a synthetic auxin was prevented by scavenging NO. Thus it is possible that NO and auxin are involved in a linear signalling pathway in the process of lateral root formation in tomato (Correa-Aragunde et al., 2004). Auxin drives cell wall loosening processes that lead to leaf initiation and possibly affect the shape of developing leaves (Kessler and Sinha, 2004). Therefore it is possible that NO also affects leaf shape through an auxin-signalling pathway. The auxin transport mutants *asa1*, *umb1* and *tir3-1* share common development abnormalities with *fdh1-3* including; more secondary inflorescences, short siliques and few lateral roots

(Kanyuka et al., 2003). Thus it is possible that SNO's may also be involved in auxin signalling.

fdh1-3 is no longer able to metabolise GSNO via GS-FDH. SNO's have been demonstrated to be directly cytotoxic to pathogens such as the malarial parasites *Plasmodium falciparum* (Venturini et al., 2000a). However, *fdh1-3* was found to be susceptible to virulent *Pst* DC3000. Extreme chlorosis was observed 4 days after infection with virulent *Pst* DC3000 and these symptoms were comparable to those found in infected *NahG* plants (which have depleted SA levels). Bacterial titres from the infected leaves of *NahG* and *fdh1-3* were also found to be similar, around 20 times higher than found in Col-0 plants. These results demonstrate that GS-FDH is important for the establishment of basal disease resistance in plants. Recently, GS-FDH knockout mice were found to have increased levels of protein S-nitrosylation and mortality following bacterial challenge (Lui et al., 2004). The lymphatic tissue of GS-FDH knockout mice showed substantial cell death 48 hours after lipopolysaccharide challenge (Lui et al., 2004). Therefore, in animals, GS-FDH is required to protect the immune system from nitrosative stress, which disrupts critical protein function and causes endotoxic injury. (Lui et al., 2004; Stamler et al., 2001). It is conceivable that a similar situation takes place in plants, where GS-FDH protects protein activity by regulating SNO levels during pathogen challenge in order to avoid the inappropriate S-nitrosylation of proteins. Therefore, proteins involved in signalling pathways required for the establishment of disease resistance would still be able to function. For instance, signals which are required to induce the expression of defence genes such as *PR1* and *PDF1.2* may be impaired by the loss of GS-FDH activity. Analysis of defence gene expression by northern and microarray analysis will elucidate the impaired defence responses of *fdh1-3* mutants. It will also be interesting to compare defence gene expression in *fdh1-3* knock-out mutants with the over-expressor *fdh1-1*, which constitutively expresses *PDF1.2*.

fdh1-3 shows accelerated HR symptoms compared to wild-type plants following the infiltration of *Pst* DC3000 (*avrB*). The balance model proposes that a synergistic reaction between H_2O_2 and NO is required for the induction of PCD in plants. If either O_2^- or NO production is favoured then $ONOO^-$ is formed which has not been found to induce PCD (Delledonne et al., 2001). GSNO has antioxidant properties protecting animal tissues from oxidative stress (Chieueh, 1999; Rahala et al., 1998). Therefore, it

is conceivable that ROI levels may be lower in *fdh1-3* plants due to the knock-out of GS-FDH promoting the accumulation of the antioxidant GSNO. To confirm this hypothesis, it will be necessary to further characterise *fdh1-3* by monitoring the kinetics of ROI accumulation during the HR. If this is found to be the case, then the balance model proposes that without a balance between H_2O_2 and NO, PCD will be delayed in *fdh1-3* mutants. However, *fdh1-3* shows accelerated HR symptoms compared to Col-0 in response to *Pst* DC3000 (*avrB*). Thus it may be that higher GSNO levels in *fdh1-3* mutants cause the more rapid S-nitrosylation of signalling molecules required for the onset or progression of the HR. In animals cytochrome c is S-nitrosylated prior to release from the mitochondria, which in turn increases caspase activation, inducing apoptosis. In addition, it is also likely that high levels of SNO's accumulate in *fdh1-3* mutants following pathogen challenge. This would cause nitrosative stress damaging proteins and accelerating cell death.

Conclusion

In conclusion, these results show that GS-FDH is important for mediating both *R* gene-mediated and basal disease resistance in *Arabidopsis*. GS-FDH is also involved in regulating plant growth and development. It is likely that GS-FDH is able to have such pleiotropic affects by regulating SNO levels, which can modulate or disrupt protein function depending on the concentration.

Chapter 7

7. Identification of S-nitrosylated proteins in *Arabidopsis*

7.1 Introduction

NO biosynthesis by the mammalian iNOS has been found to coincide with an increase in S-nitrosylated proteins (Eu et al., 2000; Marshall and Stamler, 2002). S-nitrosylation is the redox-related modification of a single critical cysteine residue within an acid-base or hydrophobic structural motif (Stamler et al., 2001). The reversible alteration of protein function by S-nitrosylation has led to the proposal that S-nitrosylation functions as a post translational modification analogous to that of phosphorylation (Stamler et al., 1997).

S-nitrosothiols (SNO's) derived from S-nitrosylated proteins supply the cellular compartments and extracellular fluids with reservoirs of NO. SNO's operate both as second messengers and as a precisely regulated mechanism for the control of protein function (Foster et al., 2003). For examples, caspase-3 (Kim et al., 1997) and caspase-8 (Kim et al., 2000) which execute apoptosis in animals are S-nitrosylated to maintain them in an inactive form. Whereas, p21 Ras a key signalling molecule in animals is activated by S-nitrosylation, which in turn modulates downstream MAPK cascades involved in controlling apoptosis (Lander et al., 1997). The S-nitrosylation of proteins can be used to fight invasion by microbes and cancer cells, disrupting the function of critical proteins (Stamler et al., 2001). For example, malarial parasites can grow in NO saturated solutions but are highly sensitive to SNO's (Rockett et al., 1991). It has been suggested that red blood cells might exploit SNO-Hb (haemoglobin) to defend against malarial infection (Taylor–Robinson and Looker, 1998). In fact SNO-Hb is a potent antimalarial compound (Foster et al., 2003). S-nitrosylation of a catalytic cysteine residue has also been shown to inhibit proteases of the parasitic pathogens *Leishmania infantum* (Salvati et al., 2001), *Plasmodium falciparum* (Venturini et al., 2000a), and *Trypanosoma cruzi* (Venturini et al., 2000b), as well as Human Immunodeficiency Virus-1 (Persichini et al., 1998), suggesting that the inactivation of cysteine proteases is a general mechanism of NO-related antimicrobial activity (Stamler et al., 2001). The

emerging picture of NO action in microbes is in many ways similar to apoptotic models of nitrosative stress which, are believed to simulate various human diseases. Nitrosative stress in microbes and mammals may share overlapping mechanisms and sets of cellular targets (Stamler et al., 2001). In support of this notion, it has been found that both macrophage cells undergoing NO-mediated apoptosis and NO-inhibited yeast cells accumulate S-nitrosylated proteins (Eu et al., 2000a; Liu et al., 2001). Despite the emerging importance of NO signalling in plants, S-nitrosylation has not been demonstrated and its protein targets remain unknown.

7.2 S-nitrosylation assay

Recently, Jaffery and co-workers developed a three step method to convert S-nitrosylated cysteines into biotinylated cysteines, which is outlined in Figure 7.1 (Jaffery et al., 2001). Using this S-nitrosylation assay they sucessfully identified S-nitrosylated proteins such as creatin kinase, α -, β - tubulin, β -, γ -actin, inducible heat shock protein 72 and Retinoblastoma gene product (Rb) in rat brain lysates (Jaffery et al., 2001). The first step of this method is to block free thiols by incubation with the thiol specific methylating agent methyl methanesthiosulfonate (MMTS). After blocking free thiols, nitrosothiol bonds are decomposed using ascorbate, which reduces nitrosothiols to thiols. Finally, these newly formed thiols are reacted with N-[6-(biotinamido)hexyl]-3'-2'-pyridyldithio) propionamide (biotin-HPDP), a sulphydryl-specific biotinylating reagent. The samples are then resolved using SDS-PAGE and the biotinylated proteins detected by immunoblotting with a biotin specific antibody. S-nitrosylated protein are purified using streptavidin agarose and resolved using SDS-PAGE. Proteins of interest can be excised and digested by trypsin for MALDI-TOF (Matrix Assisted Laser Desorption/Ionization Time-of-Flight) MS (Mass Spectrometry) analysis. The peptide mass fingerprint obtained is then used to search for matching proteins (Jaffery et al., 2001).

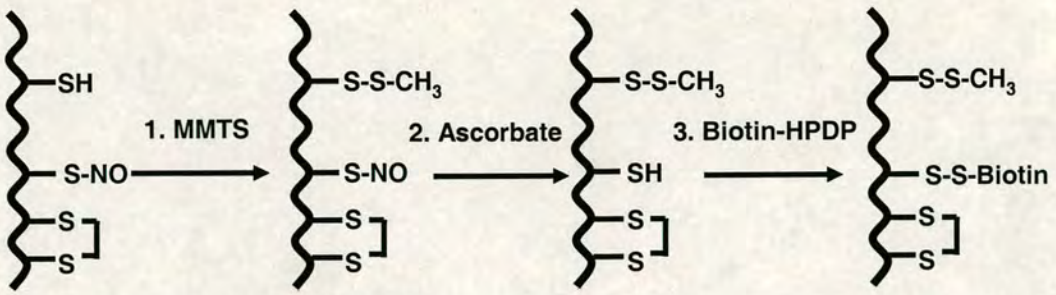

Figure 7.1. S-nitrosylated protein assay.

Schematic diagram of the S-nitrosylation assay, modified from Jaffery et al., 2001. Protein showing cysteines in the free thiol, nitrosothiol and disulphide conformation. In step 1, free thiols are rendered unreactive by methylthiolation with MMTS. In step 2, MMTS is removed by passing the protein through a spin column and ascorbate is added which reduces nitrosothiols to thiols. In step 3, biotin-HPDP reacts with the newly formed thiols.

7.3 Basal levels of S-nitrosylation are present in *Arabidopsis*

The identification of S-nitrosylated proteins in animal tissue using this assay prompted us to establish whether this method could be used with plant extracts. In the first step, free thiols are blocked using MMTS, this ensures that only nitrosothiols which are reduced by ascorbate are subsequently biotinylated by Biotin-HPDP (see Figure 7.1). To confirm that MMTS efficiently blocks free thiols in plant extracts, the S-nitrosylation assay was carried out on extracts from untreated Col-0 plants, with and without the MMTS blocking step (Figure 7.2a). By omitting this blocking step all proteins containing free thiols should be detected since all available thiols are converted to Biotin. Figure 7.2a shows the detection of a large number of proteins containing free thiols, ranging from 14 - 165 kDa. In order to identify any endogenously biotinylated proteins (since these would be false positives) this assay was also carried out with and without the final biotinylation step on extracts from untreated Col-0 . Five proteins were subsequently identified that are endogenously biotinylated (Figure 7.2b).

The use of this assay confirms that plants use protein S-nitrosylation as a post-translational modification.

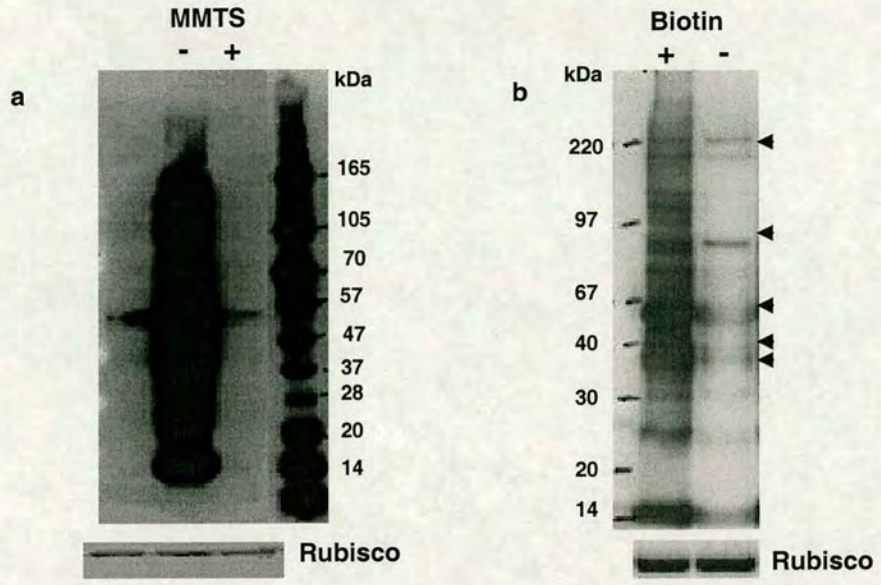

Figure 7.2 Detection free thiols and biotinylated proteins.

The S-nitrosylation assay carried out on untreated *Arabidopsis* extracts **(a)** with (+) and without (-) blocking free thiols with MMTS. **(b)** with (+) and without (-) the addition of the biotinylating agent, Biotin-HPDP. Endogenously biotinylated proteins are indicated by black arrows. Loading control is shown below by ponceau staining of Rubisco.

7.4 Protein S-nitrosylation is induced in response to pathogen challenge

In animals S-nitrosylated proteins have signalling roles in apoptosis and also anti-microbial functions. This prompted us to investigate whether pathogen challenge induces the S-nitrosylation of proteins in *Arabidopsis*. Wild-type (Col-0) plants were inoculated with virulent *Pseudomonas syringae* pv. *tomato* strain *Pst* DC3000 or avirulent *Pst* carrying *avrB*. Leaves were harvested at the time points indicated post inoculation (see Figure 7.3) and protein extracts prepared. These protein extracts were then subjected to the S-nitrosylation assay. Two low molecular weight proteins of approximately 20- and 25 kDa were found to be consistently S-nitrosylated, 60 minutes post inoculation with *Pst* DC3000 *(avrB)* and to a lesser extent with virulent *Pst* DC3000 (see Figure 7.3b). Two high molecular weight proteins of approximately 150- and 180 kDa were found to be S-nitrosylated from 30 minutes to 360 minutes post infection with virulent *Pst* DC3000 and with *Pst* DC3000 *(avrB)* 360 minutes post inoculation (see Figure 7.3a). One protein of ~100 kDa was S-nitrosylated at basal

levels in untreated plants and the S-nitrosylation of this protein was found to increase in response to virulent *Pst* DC3000 after 30, 60 and 360 minutes. However, the same protein was de-nitrosylated in response to avirulent *Pst* DC3000 (*avrB*) from 60 to 180 minutes post inoculation.

Therefore pathogen challenge was found to induce protein S-nitrosylation in *Arabidopsis*. Moreover, the proteins S-nitrosylated in response to virulent *Pst* DC3000 and avirulent *Pst* DC3000 (*avrB)* appear to be similar, but the time at which this post-translational modification occurs following pathogen challenge differs between the two.

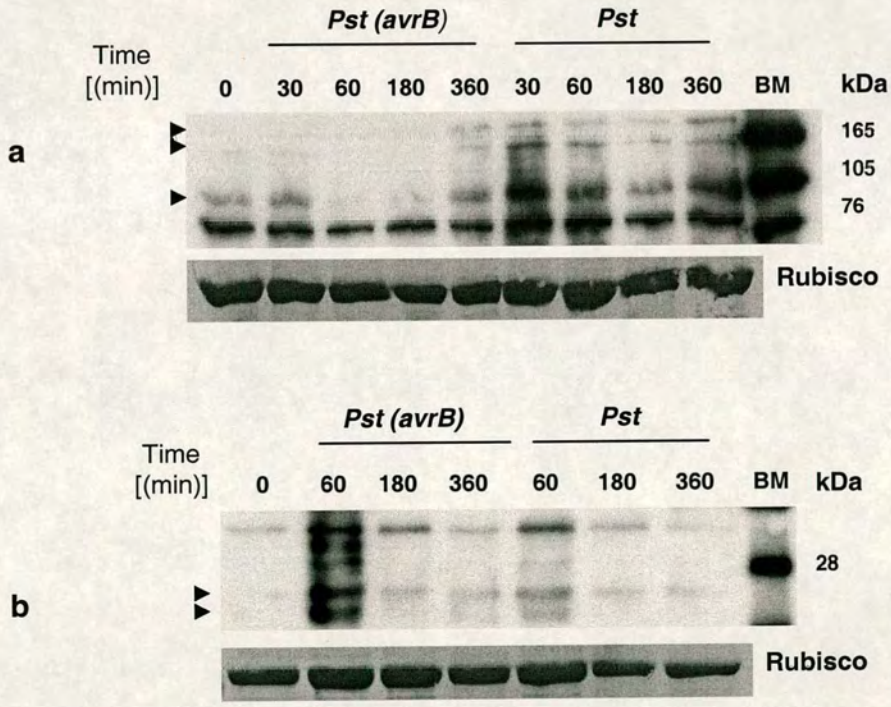

Figure 7.3 Protein S-nitrosylation is induced by inoculation with *Pst*.

(a) and **(b)** Col-0 were inoculated with *Pst (avrB)* or *Pst* and extracts were subjected to the S-nitrosylation assay. **(a)** high molecular weight proteins **(b)** low molecular weight proteins. Arrow heads show S-nitrosylated proteins in response to inoculation with *Pst* DC3000 *(avrB)* or *Pst* DC3000. BM; Biotinylated protein markers. Loading is shown below by ponceau staining of Rubisco. This experiment was repeated 3 times with similar results.

7.5 2-D analysis of S-nitrosylated proteins

Using one-dimensional (1D) electrophoresis we were able to identify protein bands that were S-nitrosylated in response to either virulent *Pst* DC3000 or avirulent *Pst* DC3000 *(avrB)* however, many bands were likely to contain more than one protein, especially in the middle range of proteins from 30-70 kDa (see Figure 7.2b). This could make the identification of these proteins difficult. In order to try and resolve proteins more efficiently two–dimensional electrophoresis was used. Wild-type plants were treated with virulent *Pst* DC3000 and samples collected after 180 and 360 minutes. These extracts and controls were run on IEF (Isoelectric Focusing) gel strips that separate proteins according to isoelectric point (first dimension). The protein strips were then run on SDS-PAGE gels to separate proteins by size (second dimension) before immunoblotting.

The S-nitrosylation of an alkaline protein (pH 10), ~50 kDa was induced 180 minutes following inoculation with *Pst* DC3000 (Figure 7.4b), which was not present in the control extract (Figure 7.4a). The S-nitrosylation of neutral proteins of ~105 and ~50 kDa was induced 360 minutes after inoculation with *Pst* DC3000, as well as a group (~20-165 kDa) of slightly acidic (pH 6) proteins (Figure 7.4d) that are not present in the control extract (Figure 7.4c).

The proteins separated on these 2-D blots are smeared and individual protein spots are difficult to identify. This may be due to the high levels of SDS required for the S-nitrosylation assay, preventing the proteins from iso-electric focusing. Therefore proteins separated on 2-D gels could not be individually picked for identification by MALDI-TOF MS analysis and it was decided to use 1D gels for further analysis.

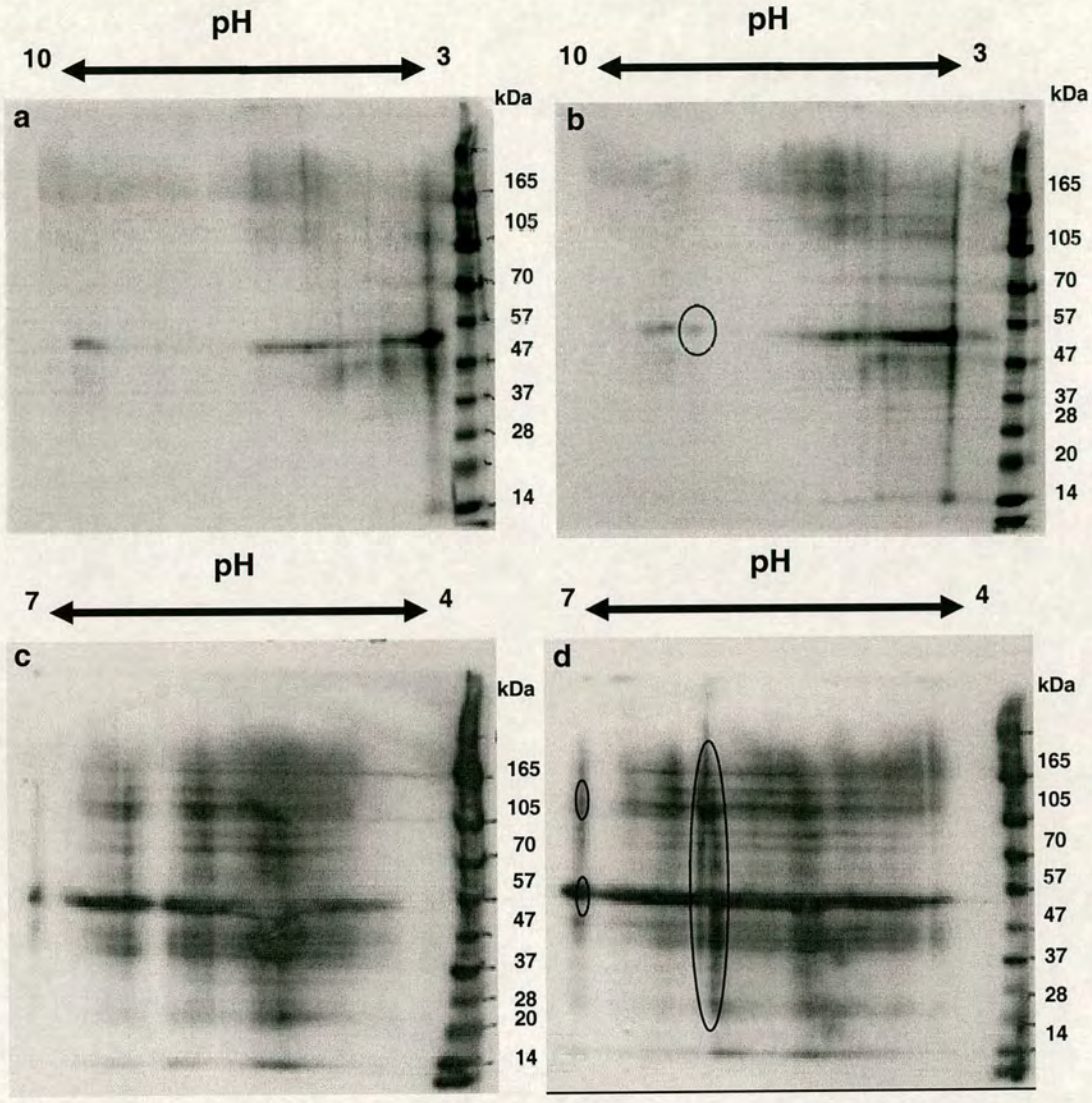

Figure 7.4. 2-D analysis of S-nitrsoylated proteins

Col-0 untreated extracts **(a)** and **(c)** extracts collected from Col-0 plants treated with *Pst* DC3000 **(b)** 180 minutes post inoculation **(d)** 360 minutes post inoculation

7.6 Identification of protein S-nitrosylated in response to *Pst* DC3000 (*avrB*)

In order to identify proteins S-nitrosylated in response to pathogen challenge Col-0 plants were inoculated with *Pst* DC3000 (*avrB*) and protein samples collected after 60 minutes (this time point was chosen based on the results shown in Figure 7.3b, where proteins ~25 kDa were S-nitrosylated in response to *Pst* DC3000 (*avrB*). These extracts were subjected to the S-nitrosylation assay converting S-nitrosylated cysteines to Biotin. Samples were then purified using streptavidin agarose beads, which bind

biotinylated proteins. After resolving the proteins by SDS-PAGE, gels were stained with coomassie blue (Figure 7.5b and c) and silver stain (Figure 7.5a). Silver staining is 10 times more sensitive than coomassie, therefore it was necessary to use double the amount of protein extract for coomassie staining to compensate for its reduced sensitivity. Silver staining is not compatible with MALDI-TOF MS due to the presence of formaldehyde in the staining procedure.

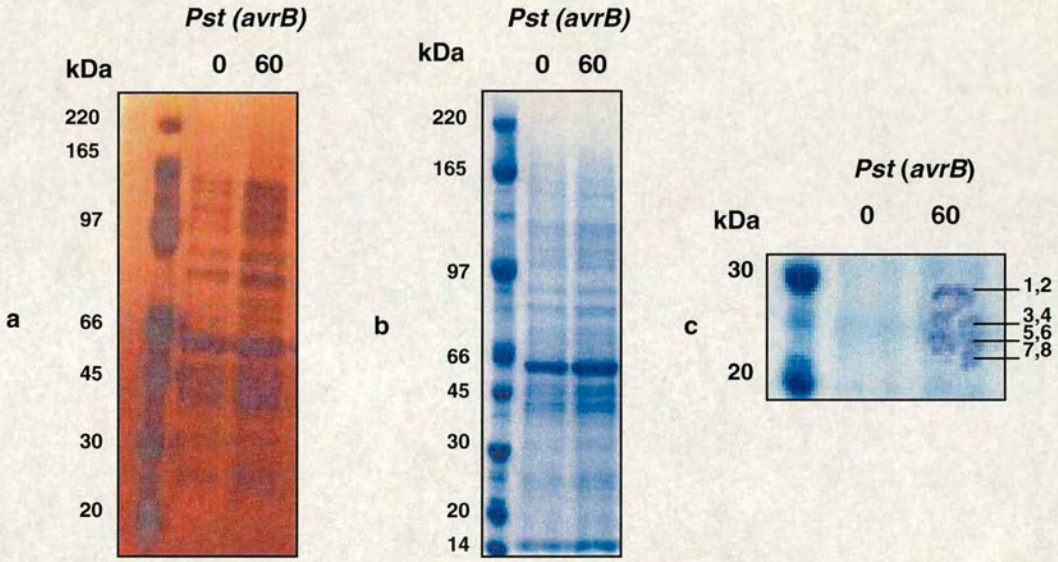

Figure 7.5. Silver and coomassie stained S-nitrosylated proteins

Following treatment with *Pst* DC3000 (*avrB*) protein samples were purified using streptavidin agarose. SDS-PAGE gels were stained with **(a)** Silver stain and **(b)** coomassie stain. **(c)** Proteins were excised from coomassie gels for matrix-assisted laser desorption/ionization (MALDI) analysis to identify the proteins. A duplicate coomassie gel was run and proteins excised from the same area on the gel.

Proteins between ~22-29 kDa from the *Pst* DC3000 (*avrB*) treated sample were excised from the coomassie stained gels. Two spots from four bands were excised for MALDI-TOF analysis, shown in Figure 7.5c. A duplicate coomassie gel was run and proteins excised from the same area of the gel as shown in Figure 7.5c. Rubisco was also

excised as a control. The resulting MALDI-TOF peptide mass fingerprint data was analysed using the search engine MASCOT. Twelve candidate S-nitrosylated proteins were matched from the MASCOT search which are shown in table 7.1, along with the location from which they were excised (see Figure 7.5c). To assess whether the protein matches are successful, the predicted size of the protein should match the size where the analyte was excised from the gel. Furthermore, protein matches are given a probability based score to predict whether they are likely to be significant or not. Scores above 57 indicate that the probability of the protein match being a random event, is less than 5%. Unfortunately, the only excised protein with a significant score (97) from this study was the control protein, Rubisco (Table 7.1).

However, the precursor of the chloroplast carbonic anhydrase (At3g01500) was matched from both of the excised spots 5 and 6 from the same band (see Figure 7.5c) and these have a score of 42 and 55 respectively (see table 7.1). The excised protein analytes from spots 5 and 6 have a size of around ~27 kDa. This is close to the predicted size of 29 kDa for At3g01500 from the TAIR (The Arabidopsis Information Resource) database. Recently, chloroplast carbonic anhydrase was implicated in the defence response as an SA binding protein and it may also be involved in regulating the HR (Slaymaker et al., 2002). An auxin response protein (At3g20220) was also matched from both of the excised spots 5 and 6 from the same band (see Figure 7.5c), with respective scores of 49 and 31 (Table 7.1). However, the predicted size of this protein from the TAIR database is 14 kDa, whereas from the analytes position on the gel, this protein has a size of ~27 kDa. Therefore this protein match is unlikely to be correct. A legume lectin family protein with similarities to protein kinases was matched to the analyte from spot 8. Although, this match has a score of 29 (Table 7.1), the predicted TAIR protein size of 27 kDa corresponds well with the size (~29 kDa) of the excised spot from the gel (Figure 7.5c). Lectin-like protein kinases have been shown to be induced in response to fungal elicitors (Ndimba et al., 2003).

MALDI MS Candidate S-nitrosylated proteins					
Spot	**score**	**Gene**	**name**	**Gel (kDa)**	**TAIR (kDa)**
1	36	At3g54550	Putative protein	22	32
1	32	At2g42820	abscisic acid-responsive HVA22 family protein	22	73
2	32	At5g14740	Carbonic anhydrase 2	22	37
3	24	At3g46640	MYB family transcription factor	24	35
3	38	At3g07310	Unknown protein	24	41
4	26	At5g63070	40S ribosomal protein S15-like protein	24	19
4	38	At1g12720	Hypothetical protein	24	/
5	49	At3g20220	Auxin response protein	27	14
5	42	At3g01500	Carbonic anhydrase, chloroplast precursor	27	29
6	55	At3g01500	Carbonic anhydrase, chloroplast precursor	27	29
6	31	At3g20220	Auxin response protein	27	14
7	21	At3g42900	Hypothetical protein	29	/
7	22	At3g12800	short-chain dehydrogenase/reductase (SDR) family protein	29	31
8	29	At1g53060	Legume lectin family protein / similar to protein kinases	29	27
	97	ATCG00490	Rubisco	50	53

Table 7.1 Putative S-nitrosylated proteins

7.7 Discussion

Using this assay S-nitrosylated proteins were successfully detected in plant extracts, both in untreated and pathogen challenged samples. The detection of S-nitrosylated proteins in untreated plant extracts is in agreement with findings in animals where S-nitrosylated proteins have been found at basal levels in tissues and extra-cellular fluids (Gow et al., 2002; Gaston et al., 2003; Jaffrey et al., 2001). Recent evidence suggests that NO is produced in plants constitutively by NR (Rockel et al., 2000; Yamasaki and Sakihama et al., 2000) and AtNOS (Guo et al., 2003), thus the S-nitrosylation of proteins in unchallenged plant samples may be expected. The presence of S-nitrosylated proteins at basal levels is consistent with the importance of S-nitrosylation as a signal to transduce NO bioactivity (Lui et al., 2004).

In animals S-nitrosylated proteins can acts as signals (Lander et al., 1997; Kim et al., 1998) or they can be directly anti-microbial (Foster et al., 2003; Taylor-Robinson et al., 1998). In order to elucidate if protein S-nitrosylation is induced in plants in response to pathogens, this assay was carried out on extracts from wild-type *Arabidopsis* plants treated with avirulent *Pst* DC3000 (*avrB*) or virulent *Pst* DC3000. Two proteins (~20- and 25 kDa) were found to be consistently S-nitrosylated, 60 minutes post inoculation with *Pst* DC3000 (*avrB*) and to a lesser extent with virulent *Pst* DC3000 (see Figure 7.3b). Two proteins (150- and 180 kDa) were found to be S-nitrosylated from 30 minutes to 360 minutes post infection with virulent *Pst* DC3000 but only after 360 minutes were the same proteins S-nitrosylated in response to *Pst* DC3000 (*avrB*). One protein of ~100 kDa was S-nitrosylated at basal levels in untreated plants and the S-nitrosylation of this protein was found to increase in response to virulent *Pst* DC3000 after 30, 60 and 360 minutes. However, this protein was de-nitrosylated in response to avirulent *Pst* DC3000 (*avrB)* from 60 to 180 minutes post inoculation. Thus proteins are quickly S-nitrosylated in plants following pathogen challenge (30-60 minutes). This coincides with an increase in NO production found in Soybean and *Arabidop*sis cell suspensions, following attempted infection by avirulent pathogens (Clarke et al., 2000; Delledonne et al., 1998). Interestingly, the proteins S-nitrosylated in response to virulent and avirulent pathogens are similar, however the timing and the intensity of these S-nitrosylation events differs between the two.

MALDI MS analysis of *Pst* DC3000 (*avrB*) treated samples gave twelve S-nitrosylated protein candidates. The probability scores of the twelve protein candidates are all below 57 which, indicates that there is more than a 5% chance that many of the protein matches are the result of random events. The low scores are likely to be due to a low protein content in the samples. Since low amounts of protein give few peptides to match when searching the protein databases. Therefore to improve protein identification it would be necessary to purify more protein extract.

Although, the probability scores were below 57, some of the protein candidate sizes matched well with where the analyte spot had been excised form the gel. For example, chloroplast carbonic anhydrase (At3g01500) has a predicted size of ~29 kDa and the excised analyte was ~27 kDa. Carbonic anhydrases (CA) catalyse the reaction $CO_2+H_2O \leftrightarrow HCO_3^- + H^+$ which, is important for photosynthesis. An antioxidant role has also been proposed for CAs, yeast mutants with a deletion in a CA gene were found to be more sensitive to oxidative stress (Gotz et al., 2002). The chloroplast CA (CA1) cDNA from tobacco was found to complement this yeast mutant (Slaymaker et al., 2002). Recently, CA1 has been implicated in the plant defence response as a SA binding protein in tobacco. SA is known to bind other antioxidant enzymes such as CAT and APX (Slaymaker et al., 2002). Furthermore, silencing CA1 in leaves of tobacco was found to suppress the Pto:avrPto mediated HR (Slaymaker et al., 2002). *Pto* confers resistance in tomato to *Pseudomonas syringae* pv *tomato* strains expressing *avrPto,* which trigger a HR. However, tobacco also exhibits a HR after challenge with *P. syringae* pv *tabaci* expressing *avrPto,* as the essential components of a Pto-mediated signal transduction pathway are conserved in tobacco (Thilmony et al., 1995). There is evidence from animals that CAs are S-nitrosylated, CA III from rat liver extracts were shown to be S-nitrosylated by GSNO (Ji et al., 1999). Using DAF-2DA to visualise NO, tobacco cells were found to produce an NO burst following treatment with the fungal elicitor cryptogein (Foissner et al., 2000). The earliest increases in NO production were found in the chloroplasts. This is consistent with the S-nitrosylation of CA1 in response to pathogen treatment.

Another protein candidate size (~27 kDa) which corresponds well to the size of the excised analyte (~29 kDa) is a legume lectin family protein (At1g53060) with similarities to protein kinases. Legume lectins are carbohydrate binding proteins that

are involved in plant defence (Chrispeels et al., 1991). Lectin kinase receptors have been found to be induced in response to wounding and fungal elicitors (Nishiguchi et al., 2002; Ndimba et al., 2003).

Conclusion

In conclusion this has study demonstrated S-nitrosylation in plants and identified some candidate S-nitrosylated proteins. However, to improve the identification of S-nitrosylated proteins by MALDI MS analysis the use of more protein extract is required. This assay has the potential to identify further S-nitrosylated proteins in response to different pathogens and shed light on the role of S-nitrosylation in the plant defence response.

Chapter 8

8. General Discussion

Despite the importance of SNO's in immune regulation in animals (Liu et al., 2004) nothing is known of the role of SNO's in plant disease resistance. During this study different approaches have been used to elucidate the role of SNO's in disease resistance in *Arabidopsis*. These include using a biotin switch method to identify S-nitrosylated proteins in response to pathogen attack and identifying and characterising T-DNA insertion mutants in GS-FDH, which encodes a GSNO reductase. In addition, an activation tagged population was screened to isolate mutants resistant to nitrosative stress, from which ten NO resistant candidates were identified. The results from the first two approaches will be discussed since the NO resistant candidates identified require further characterisation, which was previously described.

8.1 Pathogen challenge induces protein S-nitrosylation in *Arabidopsis*

Using the biotin switch method it was demonstrated that S-nitrosylated proteins exist at basal levels in *Arabidopsis*. This is in agreement with studies in animals, which have also detected basal levels of S-nitrosylated proteins in tissues and extra-cellular fluids (Gow et al., 2002; Gaston et al., 2003; Jaffery et al., 2001). It is likely that the constitutive production of NO by the enzymes NR (Rockel et al, 2000) and AtNOS (Guo et al., 2003) may be responsible for these basal levels of protein S-nitrosylation. NO can also be produced by the reduction of nitrite under acidic conditions by ascorbate or phenolics (Berthke et al., 2004; Yamasaki et al., 2000). Recently the production of NO was demonstrated via the non-enzymatic reduction of apoplastic nitrite in seeds (Berthke et al., 2004).

Pathogen inoculation was found to induce further protein S-nitrosylation in *Arabidopsis*. Similar S-nitrosylated proteins were detected following the inoculation of both *Pst* DC3000 *(avrB)* and virulent *Pst* DC3000. However, the intensity and timing of these S-nitrosylation events was found to differ between the two. Virulent bacteria cause only a modest increase in NO levels compared to avirulent bacteria (Clarke et al., 2000).

However, some proteins which were S-nitrosylated quickly in response to virulent *Pst* DC3000 were found to be weakly S-nitrosylated in response to *Pst* DC3000 (*avrB*). Therefore, it cannot only be the level of NO production, which promotes the S-nitrosylation of proteins. S-nitrosylation is a specific post-translational modification targeting the metal centre of a protein or a single critical cysteine residue within an acid-base or hydrophobic structural motif. Levels of other molecules such as O_2 and calcium-calmodulin (CaM) can promote S-nitrosylation by changing the conformation of target proteins (Stamler et al., 2001). The production of different signalling components such as ROIs or ion fluxes, at different times and intensities in response to *Pst* DC3000 (*avrB)* and virulent *Pst* DC3000 may cause specific conformational changes in proteins targeting them for protein S-nitrosylation. Moreover, in animals the spatial distribution as well as the activity of NOS isoforms allows the S-nitrosylation of nearby target proteins (Stamler et al., 2001). For example, the NMDA receptor (NMDAR) in animals is S-nitrosylated by nNOS which is brought into close proximity with NMDAR by the scaffolding protein PSD94 (Choi et al., 2000). In addition, these signals are also temporally controlled. This is demonstrated by the transient S-nitrosylation of proteins found in response to pathogen challenge. It is also important to remember that both S-nitrosylation and de-nitrosylation can activate/de-activate proteins depending on the target protein.

The purification and subsequent MALDI analysis of proteins rapidly S-nitrosylated following *Pst* DC3000 *(avrB*) treatment provided 12 S-nitrosylated protein candidates. The strongest of which was CA1 (chloroplast carbonic anhydrase). CA1 has three splice variants of differing sizes 29, 36 and 37 kDa, the first of which matches the predicted size of the excised S-nitrosylated protein induced by *Pst* DC3000 (*avrB*) treatment. This 29 kDa CA1 amino acid sequence contains six cysteines; one of which is within the acid-base motif typical of S-nitrosylation, [**G**STCYNQ]-[K**R**HDE]-**C**-[D**E**] (Stamler et al., 1997). Moreover, CA1 is a metalloenzyme and therefore its metal centre is also a potential target for S-nitrosylation. Genes involved in signalling and stress responses are often prone to alternative splicing in plants and animals (Kazan, 2003). The rapid S-nitrosylation of CA1 following *Pst* DC3000 (*avrB*) treatment may indicate a possible role in triggering the HR. In agreement with this hypothesis, no protein of 29 kDa was found to be S-nitrosylated following virulent *Pst* DC3000 treatment. Silencing CA1 in the leaves of tobacco was found to suppress the Pto:avrPto mediated HR (Slaymaker et

al., 2002) Furthermore, CA1 from tobacco has been found to bind SA (Slaymaker et al., 2002). Thus, it is conceivable that CA1 is S-nitrosylated in order to trigger an HR response. Further studies are currently underway with CA1 T-DNA insertion mutants and purified CA1 protein. These may help to elucidate the role and mechanism of CA1's involvement in the HR.

8.2 GS-FDH regulates S-nitrosothiol levels during the HR

In animals GS-FDH controls SNO levels in the cell and is therefore central to immune function (Liu et al., 2004). The expression of *GS-FDH* in wild-type plants was found to be suppressed quickly following *Pst* DC3000 *avrB* challenge before recovering to basal level after 24 hours. *GS-FDH* suppression was also found in *ein2, etr1, sid1*, *sid2*, *npr1, coi1* and *eds1* mutants following *Pst* DC3000 (*avrB*) challenge. Therefore neither EIN2, ETR1, SID1, SID2, NPR1 nor EDS1 is required for *GS-FDH* suppression. This suggests a novel signalling pathway is required for *GS-FDH* suppression following pathogen challenge. However, COI1 may be required for the recovery of *GS-FDH* to basal levels since *coi1* mutants failed to recover basal *GS-FDH* levels after 24 hours.

In wild-type plants *GS-FDH* expression and activity were found to be strongly suppressed in response to *Pst* DC3000 (*avrB*), but not virulent *Pst* DC3000. Furthermore, the expression of *GS-FDH* was not repressed by *Pst* DC3000 (*avrRps4),* but GS-FDH activity was. These results indicate that GS-FDH levels may be regulated both at the transcriptional and post-transcriptional level. This has been observed for cytosolic APX, which detoxifies H_2O_2 during the HR, thus regulating PCD (Mittler et al., 1998). GS-FDH is also a metalloenzyme, which binds zinc and contains 15 cysteine residues. It is possible that GS-FDH activity may be regulated post-translationally by S-nitrosylation on one of its cysteine residues or metal centre.

fdh1-1 T-DNA mutants over express *GS-FDH* and show increased resistance to GSNO compared to wild type plants. The insertion in *fdh1-1* mutants is situated in the promoter of *GS-FDH.* Presumably this has caused the ablation of a negative regulatory element causing the constitutive over-expression of *GS-FDH.* Moreover, *fdh1-1* plants accumulate extremely high levels of *GS-FDH* after *Pst* DC3000 (*avrB*) treatment. Therefore, these mutants are no longer responsive to signals, which normally repress *GS-FDH* expression in wild-type plants following pathogen challenge. Thus, *fdh1-1*

mutants may have lost a cis-element due to the T-DNA insert situated in the *GS-FDH* promoter. Alternatively, GSNO may be required either directly or indirectly for *GS-FDH* repression in a positive feedback mechanism. Therefore, the over-expression of *GS-FDH* in *fdh1-1* mutants would reduce GSNO levels preventing *GS-FDH* down regulation. Although it should be noted that a high concentration of exogenously applied GSNO was found to induce *GS-FDH* expression .

fdh1-3 mutants contain a T-DNA insert in the coding sequence of *GS-FDH* which has caused the knockout of *GS-FDH* transcription and translation. As a consequence *fdh1-3* shows increased susceptibility to GSNO in comparison to wild-type plants.

The GS-FDH over-expressor, *fdh1-1* displays delayed HR symptoms following *Pst* DC3000 (*avrB)* treatment. Microscopic examination of *fdh1-1* plants during the HR revealed delayed cell collapse, a prolonged O_2^- burst and higher levels of H_2O_2 localised in the chloroplast. Whereas, the knockout line *fdh1-3* shows an accelerated HR phenotype in relation to wild-type plants. These observations suggest that the suppression of GS-FDH activity is required for the progression of the HR.

Pathogen challenge leads to the rapid production of NO through an increase in iNOS activity, probably localised in the chloroplast (Chandok et al., 2003; Chandok et al., 2004). Glutathione (GSH) is present at concentrations of 2-3mM in various plant tissue (Ball et al., 2004; Noctor et al., 2002; Meyer and Fricker, 2002). Due to these high levels of GSH, it is likely that any NO produced by iNOS will quickly react with GSH to form GSNO (Liu et al., 2004). An oxidative burst is also rapidly triggered following pathogen challenge, one of the sources of which is a plasma membrane NADPH oxidase (Torres et al., 2002). In addition, a transient influx of Ca^{2+} across the plasma membrane is induced (Zimmermann et al., 1997; Xu and Heath, 1998). NO itself has been found to promote increased levels of cytosolic Ca^{2+} in tobacco cells that are responding to cryptogein (Lamotte et al., 2004). Therefore, it is possible that elevated levels of ROIs or Ca^{2+} triggered by pathogen challenge may induce conformational changes in proteins revealing critical thiols, thus allowing S-nitrosylation by GSNO (see Figure 8.1). For example, in animals O_2 induces a change in the conformation of haemoglobin allowing the S-nitrosylation of a reactive thiol (Pawloski et al., 2001). The subsequent increase in S-nitrosylated proteins may lead to downstream signalling and trigger the onset of the HR and PCD. This is true in animals where de-nitrosylation and S-nitrosylation regulate caspases and cytochrome c, respectively which are involved in

apoptosis (Li et al., 1998; Stamler et al., 2001). However, some studies suggest that NO may not be a prerequisite for initiating PCD in plants but that NO facilitates the cell-to-cell spread of the HR (Zhang et al., 2003). The repression of GS-FDH activity during the first stages of the HR in wild-type plants probably allows the S-nitrosylation of proteins important for the progression of the HR and the activation of downstream defence responses.

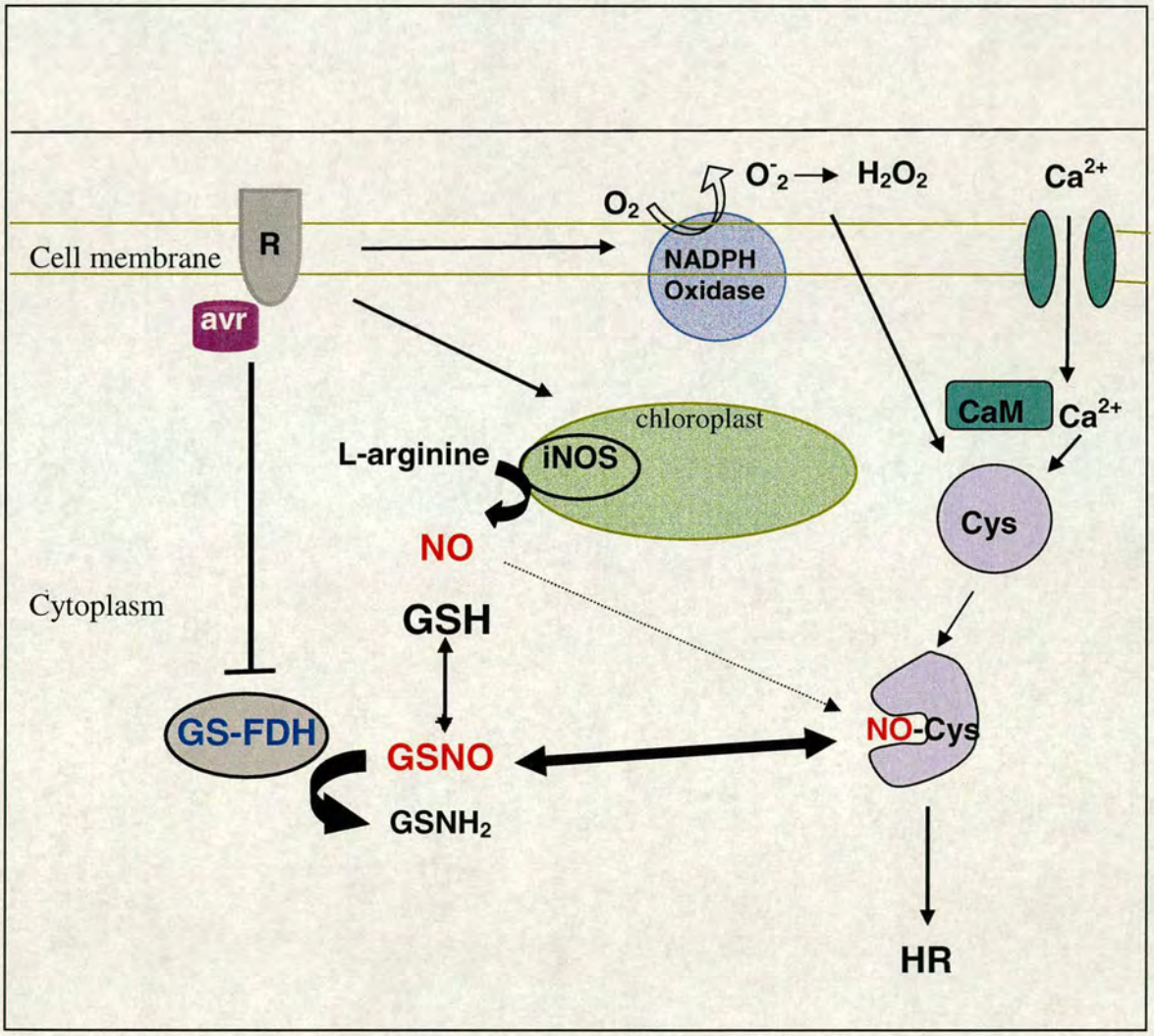

Figure 8.1. S-nitrosylation regulates the HR.

Schematic model for the regulation of the HR by protein S-nitrosylation. Pathogen recognition triggers the production of ROIs by NAPDH oxidase and NO by iNOS. Ca^{2+} influx and ROIs may induce conformational changes in proteins containing thiol groups within an acid base motif. High GSH levels in the cell promote the reaction of NO and GSH to form GSNO. The suppression of GS-FDH activity following pathogen recognition leads to an increase in GSNO which S-nitrosylates proteins that are revealing active thiol groups. These proteins may regulate the HR and downstream defence signalling.

Therefore, the over-expression of GS-FDH in *fdh1-1* mutants may delay the S-nitrosylation of signalling molecules due to low SNO levels, which in turn delays cell collapse and the HR. The opposite presumably take place in *fdh1-3* knock-out mutants, which may be compromised in GSNO metabolism. The accumulation of SNO's in *fdh1-3* mutants may lead to accelerated cell death and nitrosative stress. Although, the repression of GS-FDH activity during the HR may be required for the progression of the HR and downstream signalling, GS-FDH activity in wild-type plants is never completely repressed following *Pst* DC3000 (*avrB*) challenge. This indicates that GS-FDH activity is also necessary to alleviate excessive nitrosative stress.

8.3 GS-FDH is required for basal disease resistance

fdh1-1 mutants show strong resistance to the virulent biotroph *Pst* DC3000 compared to wild-type plants. In contrast, the knockout mutant *fdh1-3* is susceptible to *Pst* DC3000. Thus, GS-FDH is required for basal disease resistance in *Arabidopsis*. *fdh1-1* plants show increased levels of flavonoid accumulation compared to Col-0 plants. This may be due to *FLS* rather than *GS-FDH* over-expression. Flavonoids have anti-microbial properties (Dixon et al., 1983; Kemp and Burden, 1986) and therefore may be providing resistance to virulent *Pst* DC3000 in *fdh1-1* plants. However, *fdh1-3* plants are susceptible to virulent *Pst* DC3000. These opposite phenotypes suggest that *fdh1-1* plants are resistant to virulent *Pst* DC3000 due to the over-expression of *GS-FDH* not *FLS*.

Interestingly, *fdh1-3* plants show similar disease symptoms and levels of *Pst* DC3000 growth as those found in infected *NahG* plants. GSNO has been shown to induce *PR1* expression (Durner at al., 1998). The administration of NO donors or recombinant mammalian NOS to tobacco plants induced the accumulation of SA and *PR1* expression (Durner et al., 1998). Moreover, tobacco treated with NO donors displayed SAR against TMV infection. This was dependent on SA, since NO failed to induce SAR in *NahG* transgenics (Song and Goodman, 2001). The absence of GS-FDH activity in *fdh1-3* presumably results in elevated GSNO levels, although this remains to be substantiated. It is therefore perhaps surprising that *fdh1-3* is susceptible to *Pst* DC3000 since GSNO was found to induce *PR1* expression (Durner et al., 1998). However, previous studies show that it is possible to uncouple *PR1* expression from disease resistance, for example

acd5 (*accelerated cell death*) plants show SA accumulation and constitutive *PR1* expression but are susceptible to *Pseudomonas syringae* (Greenberg et al., 2000). Alternatively, increasingly high levels of GSNO in *fdh1-3* plants following pathogen challenge may cause nitrosative stress and the inappropriate S-nitrosylation of proteins, disrupting critical protein function. For example, the lymphatic tissue of GS-FDH knockout mice 48 hours after lipolysaccharide challenge showed substantial apoptosis (Lui et al., 2004). Nitrosative stress may therefore inhibit defence responses by disrupting the function of proteins required for the establishment of disease resistance.

fdh1-1 plants constitutively express high levels of *PDF1.2* and the levels of *PR1* transcripts are also slightly elevated. *npr1* mutants can no longer accumulate *PR1* transcripts and fail to establish SAR in response to SA (Cao et al., 1994). Furthermore, *npr1* mutants pre-treated with *pseudomonas syringae* pv *phaseolicola* strain NPS3121 carrying the *avrRpt2* gene fail to induce SAR and therefore show susceptibility to subsequent challenge with virulent *Pseudomonas syringae* pv *maculicola* ES4326 (Cao et al., 1994). In contrast *coi1* mutants which cannot accumulate *PDF1.2* but show strong *PR1* potentiation are resistant to virulent *Pst* DC3000 (Penninckx et al., 1998; Kloek et al., 2001). Thus low constitutive levels of *PR1* expression in *fdh1-1* plants may provide resistance to *Pst* DC3000. The induction of *PR1* expression in *fdh1-1* is also higher than in wild-type plants following *Pst* DC3000 (*avrB)* treatment. Therefore this potentiation of *PR1* expression in *fdh1-1* may confer disease resistance against *Pst* DC3000. However, it would be necessary to confirm *PR1* potentiation in *fdh1-1* mutants in response to virulent *Pst* DC3000. High ROI levels have been found to induce *PDF1.2* (Penninckx et al., 1998; Alonso et al., 1999). It is also possible that in *fdh1-1* plants elevated levels of H_2O_2 in the chloroplast and the prolonged O_2^- burst following pathogen challenge are directly toxic to *Pst* DC3000 in *fdh1-1* plants. ROIs have been reported to be directly toxic to the virulent pathogens *Erwinia chrysanthemi* (Hassouni et al., 1999) and *Phytophthora infestans* (Wu et al., 1995).

The constitutive *PDF1.2* expression in *fdh1-1* plants suggests that GSNO negatively regulates the JA pathway in wild-type plants. In tomato NO inhibits the wound inducible defence genes including proteinases *Inh I* and *InhII*, which are a cathepsin D inhibitor and a metallocarboxylase inhibitor respectively (Orzoco-Cardens and Ryan, 2002). The inhibition of these genes is independent of SA since NO was still able to inhibit them in *NahG* plants (Orzoco-Cardens and Ryan, 2002). In *Arabidopsis* NO was

only found to induce the JA responsive gene *PDF1.2* in *NahG* plants, but not wild-type plants. Thus, NO can only induce *PDF1.2* expression in the absence of SA (Huang et al, 2004). It was also subsequently found that NO induces a rapid increase in JA levels in *NahG* plants, conversely wild-type plants show a rapid increase in SA levels (Huang et al., 2004). Therefore, basal levels of GSNO in wild-type plants may negatively regulate the JA pathway and *PDF1.2* expression by inducing low levels of SA accumulation. The over-expression of *GS-FDH* in *fdh1-1* mutants may therefore lead to low SA levels by the depletion of GSNO, thus no longer antagonising the JA pathway. However, it is unlikely that there are lower levels of SA in *fdh1-1* plants since they constitutively express low levels of *PR1* and show strong potentiated *PR1* expression after *Pst* DC3000 (*avrB*) treatment. Therefore, the inhibition of the JA pathway by GSNO may be independent of SA, similar to the results found for wound inducible genes in tomato (Orzoco-Cardens and Ryan, 2002).

It is also possible that GSNO strongly inhibits the JA pathway and to a lesser extent the SA pathway, potentially through protein S-nitrosylation. Low levels of SNOs in *fdh1-1* plants would allow the activation of both pathways. This may explain why *fdh1-3* plants are susceptible to virulent *Pst* DC3000 since they would constitutively negatively regulate SA and JA disease resistance pathways due to high SNO levels. Alternatively GSNO may act downstream of SA and JA to negatively regulate transcription factors through S-nitrosylation. Thus low SNO levels in *fdh1-1* mutants would allow the transcription of the defence genes *PDF1.2* and *PR1*. Conversely, the high SNO levels in *fdh1-3* mutants would inhibit the transcription of these defence genes. ET levels were not found to be significantly different in *fdh1-1* plants compared to wildtype plants. Therefore, ET may not be regulated by GSNO/SNOs.

Northern and microarray analysis of *fdh1-1* and *fdh1-3* plants following *Pst* DC3000 infection will help us to shed light on which genes are up-or-down regulated in these mutants following pathogen infection. This will allow us to understand how GS-FDH activity regulates disease resistance pathways and why it is required for basal disease resistance.

8.4 GS-FDH and development

fdh1-1 and fdh1-3 mutants have perturbed growth, flowering and root development. *fdh1-1* mutants show precocious flavonoid accumulation, short roots with long prolific root hairs and short inflorescences when grown in short days. However it is possible that increased levels of anthocyanin in *fdh1-1* plants is due to the over-expression of *FLS* rather than *GS-FDH* (Figure 4.3). *fdh1-3* plants are smaller than wild-type plants with pale green, broad leaves and stunted roots lacking root hairs and lateral roots. *fdh1-3* plants also display reduced apical dominance, stunted inflorescences and small siliques. NO has recently been found to be involved in auxin signalling pathways during adventitious and lateral root formation in cucumber and tomato respectively (Pagnussat et al., 2004; Correa-Aragunde et al., 2004). *fdh1-3* shares some common developmental abnormalities with the auxin transport mutants *asa1*, *umb1* and *tir3-1* including; more secondary inflorescences, short siliques and few lateral roots (Kanyuka et al., 2003). Thus it is conceivable that SNO's may also be involved in regulating auxin signalling.

8.5 Conclusion

The importance of S-nitrosylation in plants is now just emerging. This redox based post-translational modification is a reversible and highly specific way of controlling protein function. The evidence presented here suggests that protein S-nitrosylation is central to disease resistance as well as development in *Arabidopsis*. The GS-FDH over-expressor, *fdh1-1* and knockout mutant, *fdh1-3* identified in this study will continue to be invaluable tools to dissect the role of SNO signalling in disease resistance.

Furthermore, the continued identification of S-nitrosylated proteins in response to different pathophysiological and physiological conditions will help us to understand how S-nitrosylation regulates different signal transduction pathways.

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