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# Manipulation of host S-nitrosylation by *Pseudomonas syringae*

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## Declaration

I hereby declare that the work presented here is my own and has not been submitted in any form for any degree to any other university.

Noor Baity Saidi

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To Abah, Ma, Abg and Asyraf,

with lots of love...

## Manipulation of host S-nitrosylation by *Pseudomonas syringae*

Nitric oxide (NO) and S-nitrosothiols (SNOs) are widespread signalling molecules that regulate immunity in animals and plants (Wendehenne et al., 2001). Previously, we have reported that *Arabidopsis thaliana S-nitrosoglutathione reductase*, (*AtGSNOR1*) modulates the extent of total cellular SNO formation, which subsequently regulates multiple modes of plant disease resistance (Feechan et al., 2005). Loss-of-function mutations in *AtGSNOR1*, leading to increased SNO levels, have recently been shown to result in S-nitrosylation of the key defence regulators *NPR1* and *AtSABP3*, blunting their activity and subsequently leading to increased pathogen susceptibility (Tada et al., 2008; Wang et al., 2009). Thus, inhibiting *AtGSNOR1* function leading to increased SNOs, would potentially provide a good strategy for bacterial effector proteins, delivered by the type III secretion system (TTSS), to promote infection.

*AtGSNOR1* is constitutively expressed in all organs in *Arabidopsis* and its expression is induced by wounding stress avirulent and non-host pathogen. Using gas phase chemiluminescence, we show that infection with *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst*DC3000) resulted in increase SNO levels which is TTSS. At the same time, RT-PCR and GUS analysis indicated that *AtGSNOR1* expression was transiently suppressed by *Pst*DC3000 which is also TTSS-dependent. Therefore, *Pst*DC3000 infection suppresses denitrosylase function of *AtGSNOR1* to increase SNO levels and this virulence effect is delivered by at least one of the effector protein secreted through TTSS.

Several putative cis-acting elements were identified in *AtGSNOR1* promoter through deletion analysis including GT-box, W-box and MYB/MYC binding motif. These elements comprise of positive and negative regulators which are critical for the induction and suppression of *AtGSNOR1* in response to pathogen infection.

A few transgenic plants expressing effector proteins were selected and tested for their suppressive effect on *AtGSNOR1* expression during *Pst*DC3000 infection. HopAM1 effector proteins showed the ability to suppress *AtGSNOR1* when expressed *in planta.*

## Abbreviations

| | |
|---|---|
| µg | Microgram |
| µl | Microlitre |
| ABA | Abscisic acid |
| At | *Arabidopsis thaliana* |
| *Avr* | Avirulent gene |
| *BAK1* | *BRI1-associated kinase 1* |
| *Bgh* | *Blumeria graminis* f. sp. *Hordei* |
| *Bgt* | *Blumeria graminis f. sp. tritici* |
| *BRI1* | *Brassinosteroid insensitive 1* |
| *CaMV* | *Cauliflower Mosaic Virus* |
| Cfu | Colony forming units |
| Col-0 | Arabidopsis ecotype Columbia 0 |
| cor | Coronatine |
| CSH | Cysteine |
| CSNO | S-nitrosocysteine |
| CTAB | Cetyltrimethyl Ammonium Bromide |
| CuCl | Cuprous Chloride |
| DAMP | Danger-Associated Molecular Pattern |
| DEPC | Diethylpyrocarbonate |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic Acid |
| EF-Tu | Elongation Factor Tu |
| *EFR* | *EF-Tu Receptor* |
| eNOS | Epithelial NOS |
| ET | Ethylene |
| ETI | Effector-Triggered Immunity |
| FALDH | Formaldehyde Dehydrogense |
| flg22 | Flagellin |
| *FLS2* | *Flagellin-sensing 2* |

| | |
|---|---|
| GS-FDH | Glutathione-Dependent Formalydehyde Dehydrogenase |
| GSNO | S-nitrosoglutathione |
| GSNOR | S-Nitrosoglutathione Reductase |
| GUS | β-glucoronidase |
| Hg | Mercuric |
| $HgCl_2$ | Mercuric Chloride |
| HR | Hypersensitive Response |
| *hrc* | HR and conserved |
| *hv* | Chemiluminescence |
| iNOS | Inducible NOS |
| JA | Jasmonic Acid |
| Kan | Kanamycin |
| KB | King's Broth media |
| LPS | Lipopolysaccharides |
| LRR | Leucine Rich Repeat |
| MAMP | Microbe-Associated Molecular Pattern |
| MAPK | Mitogen Activated Protein Kinase |
| MS | Murashige and Skook media |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| *NahG* | Salicylate hydroxylase gene |
| NASC | Nottingham Arabidopsis Stock Center |
| NBS-LRR | Nucleotide Binding Site And Leucine Rich Repeat |
| NED | N-(1-napthyl)ethylenediamine) |
| *NHO1* | *Non-Host Resistance 1* |
| NHR | Non-Host Resistance |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| nNOS | Neuronal NOS |
| *NPR1* | *Non-expressor of PR-1* |
| $ONOO^-$ | Peroxynitrate |

| | |
|---|---|
| PAMP | Pathogen-Associated Molecular Pattern |
| PCD | Programmed Cell Death |
| PCR | Polymerase Chain Reaction |
| PMSF | Phenylmethanesulfonylfluoride |
| PR | Pathogensis Related protein |
| PRR | Pattern-Recognition Receptor |
| *Psp* | *Pseudomonas syringae phaselicola* |
| *Pst*DC3000 (*avrB*) | *Pseudomonas syringae* pv. *tomato* DC3000 carrying *avrB* |
| *Pst*DC3000 | *Pseudomonas syringae* pv. *tomato* DC3000 |
| PTI | PAMP-triggered immunity |
| PVDF | Polyvinylidene Fluoride |
| *R* | Resistance Gene |
| *RNA* | Ribonucleic Acid |
| ROI | Reactive Oxygen Intermediate |
| RSNO | Protein SNO |
| RT | Reverse Transcriptase |
| SA | Salicylic Acid |
| SABP | Salicylic Acid Binding Protein |
| SAR | Systemic Acquired Resistance |
| SERKs | Somatic Embryogenesis Receptor Kinase |
| SNO | S-nitrosothiol |
| TAIR | The Arabidopsis Information Resource |
| TBE | Tris-Boric-EDTA |
| T-DNA | Transfer DNA |
| TIR | Toll and Human Interleukin-1 Receptor |
| TLR | Toll-Like Receptors |
| TMV | Tobacco Mosaic Virus |
| TTE | Type III Effector Proteins |
| TTSS | Type III Secretion System |

| | |
|---|---|
| UV | Ultraviolet |
| WRKY | conserved amino acid sequence WRKYGQK |
| Ws-0 | *Arabidopsis* ecotype Wassilewskija 0 |

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## 1. Introduction

### 1.1 Biotechnology and agriculture

In agriculture today, the persistent threat of loss of yield and quality from diseases and insect attack is one of the most disruptive factors. Insects alone destroy about 25 percent of food crops worldwide each year (http://www.gmo-compass.org). Although better farm machinery and development of fertilizers, insecticides, and herbicides have been extremely useful, biotechnology through genetic modification to increase crop diversification may offer more promising solutions and greater impacts (Gurr et al., 2005; Vain, 2006; Toenniessen et al., 2009). In fact, worldwide use of pest-resistant genetically modified (GM) crops substantially reduced pesticide spraying by nearly 300 million kilogram (Lomborg, 2009). Transgenic science and technology are fundamental to the molecular genetics and crop improvement for sustainable agriculture (Vain, 2006). Development of transgenic crop plants that can tolerate adverse weather and soil condition, adapt to different climates and resistant to pathogens will potentially improve food security and enhance human nutrition (Abdalla et al., 2003; Toenniessen et al., 2009).

GM crops such as the well known *Bacillus thuringiensis* (Bt) corn has been commercially available since 1996 (Sivasupramaniam et al., 2007) and is already being grown on hundreds millions of acres worldwide with no ill effects. In 2008, after thirteen years of commercialization, millions of small and resource-poor farmers around the world continue to plant more hectares of transgenic crops as a result of the consistent and substantial economic, environmental and welfare benefits offered by these transgenic crops. The expanding list of transgenic crops now includes soybean, maize, cotton, canola, squash, papaya, alfalfa, sugarbeet, sweet pepper and petunia (Clive, 2008).

Biotechnology advancement is no panacea to the food insecurity and poverty problems in developing countries, but it must be looked at as one of the most important tools that will contribute to boost food production and thus, poverty reduction. The best way to control crop disease is to use naturally occurring genetic mechanisms that have evolved in plants to counter pathogen attack. The way pathogens cause disease in plants, and how plants defend themselves against pathogens are crucial research issues for global food security. Fruitful lines of research are currently in progress towards a better understanding the of molecular basis of plant disease resistance in order to precisely engineer plants with increased resistance which will benefit farming and lead to a more secure food supply in the future.

### 1.2 Plant-pathogen interactions: An arms race

A plant is a living organism that makes its own food from organic substances and thus is a rich source of nutrients and water for microbes. Therefore, they can be infected by a plethora of pathogenic microorganisms and pests such as bacteria, viruses, fungi, protozoa, nematodes and insect herbivores. Plants also become infected by microbes with a different lifestyle. Biotrophic pathogens such as powdery mildews, rusts and the downy mildews (Agrios, 2005) are specialized to feed on living cells of their hosts, rather than killing the host cells as part of infection process (Heath, 2002; de Wit, 2007). Some of them have developed an intimate relationship with their host plants, co-evolving into obligate biotrophs that are completely dependent on their hosts and cannot be cultured on synthetic media. Biotrophs have a narrow host range, and strains of these pathogens have often develop complex adaptations enabling them to engage in the interaction with a specific line of a given plant species (de Wit, 2007). Necrotrophic pathogens, for example *Alternaria brassicicola* and *Botrytis cinerea* fungi are less specialized and have much less intimate relationship with their host plants. They grow on host tissue that are wounded, weakened or senescent and frequently excreting toxins or exoenzymes to kill their host cells prior to colonization (de Wit, 2007). Many plant pathogens display both lifestyles, depending on the stage of their life cycle, and are called hemibiotrophs. Hemibiotrophs are initially biotrophic in their interaction with the hosts, but become more necrotrophic as the pathogenesis process progresses (Hammerschmidt, 2006).

Recent works has revealed striking similarities between immune systems across kingdom borders (Nurnberger et al., 2004). However, significant differences remain. For example, vertebrates are equipped with adaptive immune system, which is characterized by the creation of antigen-specific receptors through somatic recombination in maturing lymphocytes. Other key players of the animal immune system are specialized cell types that exist as parts of a circulatory blood system, which are not found in plants (Nunberger et al., 2004). Compared with animals, plants are at an apparent disadvantages in these battles, because they are rooted in place and do not have circulating antibodies or dedicated immune cell lineages. Nevertheless, the world is still green because plants have developed a variety of sophisticated defence systems to cope with an environment in which many microbes live. Thus, disease is an exception rather than the rule in natural plant communities and on the farm.

Interactions between plants and bacteria are a complex process and very specific, based on pathogen recognition and cell-to-cell communication. It present us with some of the most intricate and fascinating examples of ecological and evolutionary interplay between organisms. Antagonistic coevolution between a plant and its enemy can be simplified as a three-step process. First, the enemy attacks and use a wide variety of virulence factors to confound host surveillance and gain access to the resources available in their hosts while hosts respond by selecting a novel defence that spreads through the plant population aimed at restricting and eliminating infecting pathogens; second, effectively defended plants decrease pathogen fitness, thus selecting for a genotype that can overcome the defence barrier by acquiring effector molecules to suppress the basal immune responses; and third, plants in return acquire surveillance proteins to either directly or indirectly monitor presence of the effectors. This long-standing dance of adaptation and counter-adaptation is often called an arms race (Figure 1.1). Ultimately, the final outcome of the battle depends on the balance between the ability of the pathogen to suppress the plant's immune system and the capacity of the plant to recognize the pathogen and to activate effective defences.

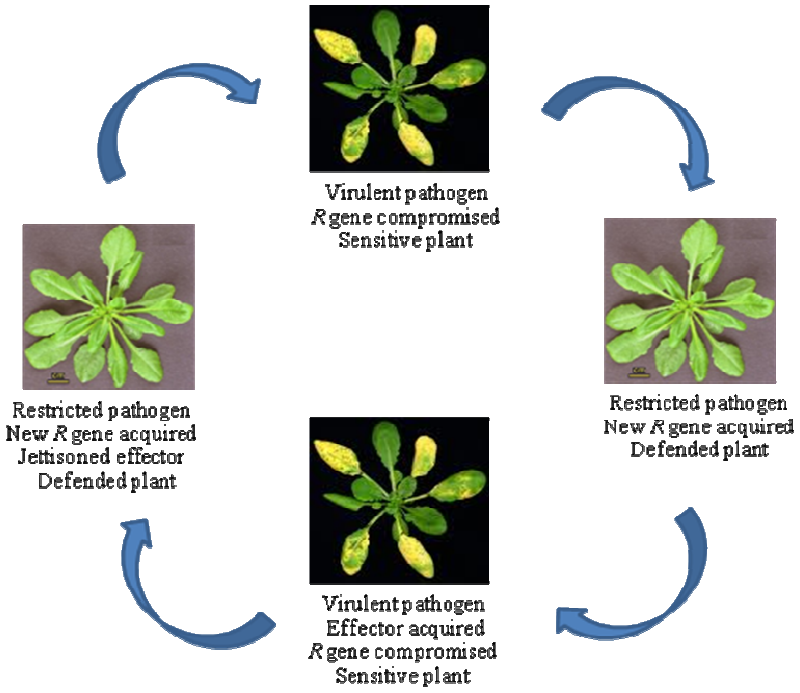



**Figure 1 .1 Co-evolution of host *R* genes and the pathogen effector complement**

### 1.3 Non-host and basal disease resistance

As discussed previously, the evolutionary arms race between plants and their attackers provide plants with a highly sophisticated defence system that efficiently detects and wards off potentially dangerous microbes. The ability of a given plant species to resist infection by all isolates of a given pathogen species is termed non-host disease resistance (NHR) or species-specific resistance (Heath, 2000; Thordal-Christensen, 2003; Mysore et al., 2004). Being the most common form of disease resistance, NHR confers strong and durable crop protection against the majority of potentially pathogenic microorganisms. Conceptually, the stability of NHR is proposed to be the consequence of several successive layers of protective mechanisms that comprise both pre-formed constitutive barriers as well as inducible defence reactions.

Pre-formed physical and chemical barriers constitutively present on a plant surface such as wax layers, rigid cell wall and toxic phytoanticipins are frequently cited as controlling invasion success of some non-adapted pathogen (Thordal-Christensen, 2003; Mysore et al., 2005; Reina-Pinto and Yephremov, 2009). For example, a disruption in plant actin microfilaments leads to the loss of non-host resistance against several nonhost fungi (Kobayashi et al., 1997). Furthermore, a combination of loss of actin cytoskeletal function and a disease resistant gene activity severely compromises non-host resistance in *Arabidopsis thaliana* (*Arabidopsis*) against wheat powdery mildew, *Blumeria graminis f. sp. tritici* (Yun et al., 2003). These data provide evidence that the plant cytoskeleton plays a significant role during non-host disease resistance. Despite the diversity of these constitutive defences that physically impede the growth and spread of the potential pathogen, many microbes succeed in breaking through this pre-invasive layer of defence. However, once the plant exterior has been breached, microbes are subjected to recognition at the plasma membrane by extracellular surface receptors that recognize a large variety of microbe-associated products leading to signal transduction and induced innate immune responses (Zipfel and Robatzek, 2010).

Inducible defence responses in non-host plant comprise the synthesis and accumulation of antimicrobial reactive oxygen species (ROS; also known as *reactive* oxygen intermediates, ROI), phytoalexins, and translation products from pathogenesis-related (PR) genes as well as the localized reinforcement of the plant cell wall and hypersensitive response (HR) (Mysore and Ryu, 2004; Thordal-Christensen, 2003). A well-known example of an inducible structural barrier during NHR is papilla. Papilla is cell wall apposition composed of callose,

phenolics, hydroxyproline-rich glycoproteins and other materials (Hauck et al., 2003). This local cell wall fortification is formed on the inner side of plant cell walls at the site of fungal infection representing a physical and chemical blockage deployed to arrest infection. It has been studied mostly in the interaction between barley (Hordeum vulgare L.) and *Blumeria graminis* f. sp. *hordei* (*Bgh*) (Thordal-Christensen et al., 2000; Zeyen et al., 2002). In wild-type *Arabidopsis*, attack from the non-host pathogen *Bgh* are as well stopped at the pre-invasive stage of penetration in association with papillae (Thordal-Christensen, 2003).

Induction of the innate immune response also occurs in susceptible host plants to inhibit pathogen spreads after successful infection and onset of disease, defined as basal resistance (Dangl and Jones, 2001, reviewed in Jones and Dangl, 2006; Pieterse et al., 2009). However, this type of defence is not well defined and still poorly understood. In a recent review, Niks and Marcel (2009) integrated the concepts of NHR and basal resistance to account for the specificity of defence suppression and argue that NHR and basal resistance to adapted pathogens may rest on similar principles. As both type of resistance utilise the same defence mechanisms, NHR may represent the success of basal defence system to control the pathogen growth while host susceptibility is the consequence of ineffective induction or suppression of the system by pathogen (Ingle et al., 2006).

The ability of a potential host to discriminate between self and non-self is the key to the activation of innate defence mechanism. Plants recognize general elicitors that are released during attack by both host and non-host pathogens in a non-specific manner to activate defence responses. Flagellin, lipopolysaccharides (LPS), peptidoglycans, microbial cell wall fragments, phospholipids, proteins, double stranded RNA and methylated DNA serve as such elicitors (Heath, 2000). These general elicitors are often indispensable for the microbial lifestyle. Pathogen recognition in non-host plants can be brought about by pathogen-associated molecular patterns (PAMPs), synonymously called general or exogenous elicitors (Gomez-Gomez and Boller, 2002; Nurnberger et al., 2004; Zipfel, 2009). Perception of PAMPs is mediated by pattern-recognition receptors (PRRs), located on the cell surface. Recognition of such signals is very likely to activate defence responses in natural plant microbe encounters. However, in basal disease resistance, PAMP-induced defence is insufficient to stop infection.

Several plant signalling components are involved during the induction of plant defence. The analysis of mutants impaired in hormone homeostasis revealed that jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) are indispensable for maintenance of non-host

resistance (Mysore and Ryu, 2004). An ET-insensitive tobacco has been shown to lack non-host resistance to several soil-borne fungi (Knoester et al., 1998). Moreover, non-host resistance of *Arabidopsis* to *Alternaria brassicicola* is dependent on JA as *coi1* (coronatine-insensitive 1) mutants are susceptible to fungal infection (Thomma et al., 1998). SA is one of the key signalling molecules that activate plant defence responses against invading pathogens. The *Arabidopsis* mutant *sid2* (defective in an enzyme required for SA synthesis) and *Arabidopsis NahG* line (expresses salicylate hydroxylase that can degrade SA) support growth of non-host cowpea rust fungus, *Uromyces vignae* (Mellersh and Heath, 2003).

An *Arabidopsis* NHR gene, *NHO1*, encodes a glycerol kinase and is required for resistance against *Botrytis cinerea* and *Pseudomonas syringae* isolates from bean and tobacco for which *Arabidopsis* is a non-host (Kang et al., 2003). NHO1 is required for resistance to only certain pathogens since the *nho1* mutation does not compromise resistance to several other non-host pathogens. Interestingly, a virulent pathogen of *Arabidopsis*, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) appears to actively suppresses *NHO1* transcription (Kang et al., 2003). These results suggest that *NHO1* plays a key role in NHR and general resistance against some pathogens in *Arabidopsis* and is targeted for bacterial virulence.

Programmed cell death or HR is one of the components in defence signalling cascades induced by a pathogen on a non-host plant and commonly used as visual marker for incompatible plant-pathogen interactions. In many cases, inoculation of a pathogen into non-host plant elicits HR, but interestingly in some cases, NHR is not associated with induction of the HR. Based on these observations, Mysore and Ryu (2004) proposed that NHR could be classified into two types: type I and type II, dependent on both the plant and the pathogen species. The type I NHR, does not produce any visible symptoms and the type II NHR is always associated with HR.

Even though many components of NHR appear to be well known from host resistance, there is obviously a long way to go before we fully understood NHR. One of the reasons for this could be that NHR operates at the plant-species level making our understanding of the roles played by this type of resistance relatively poor and often beyond the reach of breeding programs that are based on allele combinations within a given crop species (Thordal-Christensen, 2003; Schweizer, 2007). Nevertheless, recent progress in functional genomic technologies has made available tools that can be used to dissect the complex phenomenon of NHR.

### 1.4 PAMP recognition and PAMP-triggered immunity

Recognition of non-self and subsequent activation of defence against the attacking pathogen in all multicellular organisms, is collectively referred as innate immunity (Chisholm et al., 2006). PAMP recognition represents the major trait of innate immunity common to plants and animals, and it has been revealed that its molecular basis shows remarkable evolutionary conservation across kingdom borders (Nurnberger and Brunner, 2002; Nurnberger et al., 2004; Iriti and Faoro, 2007). PAMPs are also known as microbe-associated molecular patterns (MAMPs) (Zipfel, 2008; Zipfel, 2009). Such patterns are invariant surface structures that are characteristic of whole class of microbes and therefore are difficult to mutate or delete. They do not exist in the host, allowing the host to recognize them as non-self to fend off invading pathogens. Besides pattern of microbial origin, endogenous host molecules can also trigger defence reactions in animals and plants. These so-called danger-associated molecular patterns (DAMPs) are signals that are encoded by the host and released upon plant damage (Postel and Kemmerling, 2009).

In animals as well in plants, the recognition of the invading pathogens is based on perception of the slowly evolving PAMPs by PRRs which are predominatly located on the plasma membrane, but can also localize to endosomal compartments or cytoplasm (Nurnberger et al., 2004). In animals, Toll (from *Drosophila melanogaster*) and Toll-like receptors (TLRs) (from mammals) recognize PAMPs through an extracellular LRR domain and transduce the signal through a cytoplasmic TIR domain (Toll and human interleukin-1 receptor) (Lemaitre et al, 1996). Interestingly, this structure is similar to the first identified PRR from plants, the flagellin receptor *FLS2* (*flagellin-sensing 2*) that also encodes putative transmembrane receptor-kinase with an extracellular LRR domain (Nurnberger and Brunner, 2002; Postel and Kemmerling, 2009). Although the extracellular LRR domains of *FLS2* and *TLR5* (both responsible for flagellin sensing in plants and animal, respectively) do not share much sequence similarity, it obvious that during evolution, the same LRR were selected for PAMP recognition in the animal and plant lineages (Nurnberger et al., 2004).

It is now clear that there are, in essence, two distinct branches but interconnected surveillance system for defence against pathogens (Nurnberger et al., 2004; Chisholm et al., 2006; Jones and Dangl, 2006). Primary innate immunity, conferred by PTI (Figure 1.2a), is achieved through a set of defined PRR at the plant's cell surface that recognize conserved MAMPs or PAMPs. Immune responses in plants triggered by PAMPs comprise the change in cytoplasmic $Ca^{2+}$ levels, the production of ROS as well as the activation of MAPK

cascades that lead to the activation of defence-related genes (Nurnberger et al., 2004; Zipfel, 2008; Postel and Kemmerling, 2009). Intriguingly, most of these components have also been described to be of central importance to PAMP-induced activation of innate immune responses in animal cells (Nurnberger et al., 2004). These elements add to the growing list of parallels in the molecular organization of innate immunity in both plant and animal kingdoms.

In many cases, successful pathogens secrete effectors in the apoplast or directly into the cytoplasm of host cells to inhibit PTI, dubbed as effector-triggered susceptibility (ETS) (Figure 1.2b) (Jones and Dangl, 2006; Postel and Kemmerling, 2009; Zipfel, 2009). Plants, in turn, can perceive such effectors through additional receptors, typically polymorphic nucleotide binding site and leucine rich repeat (NBS-LRR) protein products encoded by resistance (*R*) gene to mount a second layer of defence called effector-triggered immunity (ETI) (Figure 1.2c). ETI is an accelerated and amplified PTI response, involves the direct or indirect recognition of effectors by the NBS-LRR proteins (Jones and Dangl, 2006; Chisholm et al., 2006; Boller and He, 2009). Consecutively, pathogens have evolved effectors capable of suppressing ETI, and so the arms-race between host and pathogens unfolds.

The best-characterized PAMP known to activate innate immunity in plants is flagellin, the protein subunit of eubacteria flagella (Felix et al., 1999). Flagellum-based motility is important for bacterial pathogenicity in plants (Zipfel and Felix, 2005). A conserved N-terminal 22-mer fragment of eubacterial flagellin, flg22, is a potent elicitor of defence responses in *Arabidopsis* and with slightly different epitope specificity in tomato and *Nicotiana benthamiana* (Felix et al., 1999; Gomez-Gomez et al., 1999; Robatzek et al., 2007). In *Arabidopsis*, flg22 is perceived by *FLS2*. *FLS2* consists of an N-terminal signal peptide, 28 LRRs, a transmembrane domain, and a cytoplasmic kinase domain (Gomez-Gomez and Boller, 2000). Although the exact flg22-binding site is unknown, *FSL2* directly binds to flg22 and contributes to recognition specificity (Chinchilla et al., 2006). Flg22-induced immune responses restrict the growth of the virulent *Pst*DC3000, whereas mutant deficient in *FLS2* are more susceptible to bacterial infection (Zipfel, 2004). These studies provide genetic evidences that PTI acts as the first layer of plant innate immunity and contributes to disease resistance.

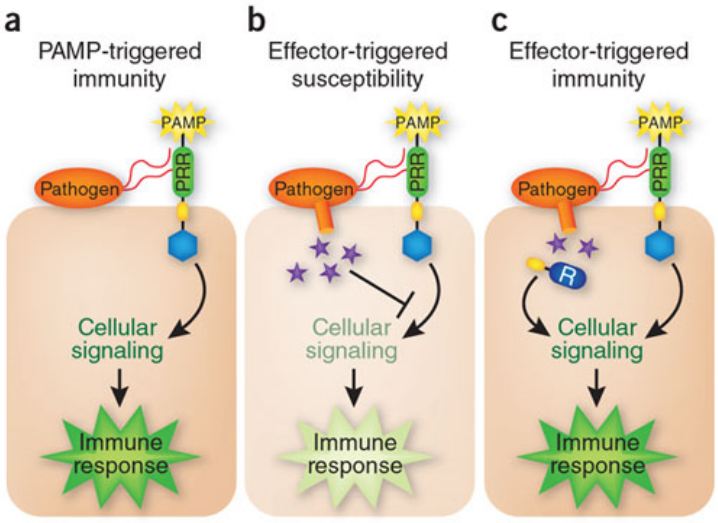



**Figure 1.2 Simplified schematic representation of the plant immune system**

(**a**) Upon pathogen attack, pathogen-associated molecular patterns (PAMPs) activate pattern-recognition receptors (PRRs) in the host, resulting in a downstream signaling cascade that leads to PAMP-triggered immunity (PTI). (**b**) Virulent pathogens have acquired effectors (purple stars) that suppress PTI, resulting in effector-triggered susceptibility (ETS). (**c**) In turn, plants have acquired resistance (R) proteins that recognize these attacker-specific effectors, resulting in a secondary immune response called effector-triggered immunity (ETI). (Pieterse et al., 2009)

Elongation factor Tu (EF-Tu) is the most abundant bacterial protein and is recognized as another PAMP that triggers innate immune responses in *Brassicaceae* including *Arabidopsis* (Kunze et al., 2004). A highly conserved N-acetylated 18 amino acid peptide, elf18, is sufficient to trigger those responses induced by full-length EF-Tu. EF-Tu is recognized by *EF-Tu Receptor* (*EFR*), a close homolog of *FLS2* and belongs to the same subfamily XII of leucine-rich repeat receptor-like kinase (LRR-RLK). The structural and functional similarity of *FLS2* and *EFR* suggests that more members of the LRR-RLK family may be receptor for yet unidentified PAMPs (Nurnberger and Kemmerling, 2006; Postel and Kemmerling, 2009). Unlike *FLS2*, *EFR* does not contribute significantly to plant defence since bacterial growth is not altered in *efr* mutants but the mutants were more susceptible to *Agrobacterium tumefaciens*-mediated transformation than wild-type plants (Zipfel et al., 2006). This supports a role of *EFR* in defence against *Agrobacterium* infection and harbours the potential to enhance biotechnological tools for plant transformation.

Receptor oligomerization is a common principle of animal innate immune receptors. Homo- and heterooligomerization of receptors also take place in plants. *Brassinosteroid insensitive 1* (*BRI1*) is the receptor of brassnosteroid hormones that controls plant growth and development. It forms a protein complex with another LRR-RLK named *BRI1-associated kinase 1* (*BAK1*), a member of a small subfamily of somatic embryogenesis receptor kinase (SERKs), during perception of brassinosteroids (BR) (Li et al., 2002). Unexpectedly, *BAK1* was recently identified as a positive regulator in *FLS2* and *EFR* signalling and *FLS2* was found to heterodimerize with *BAK1* in the perception of flagellin (Chinchilla et al., 2007). Mutants deficient in *BAK1* are impaired in brassinosteroid, flagellin, pathogen-induced cell death control and other PAMP responses (Kemmerling et al., 2007; Chinchilla et al., 2007; He et al., 2007) and show defects that cannot be explained by the interaction with the known interacting *BRI1* and *FLS2*. Given the central role of *BAK1* in transmitting signals from *FSL2* and possibly from other PAMPs such as EF-Tu, it would seem plausible that it becomes the potential target for pathogen effectors rather than the PRR themselves (Shan et al., 2008; Boller, 2008).

In general, even though PTI only induces a relatively weak resistance response compared to ETI, it is undoubtedly crucial for non-host disease resistance and contributes to the basal resistance of host plants. Moreover, the interference of effector proteins with the PRRs accentuates the relevance of these receptor proteins for basal defence and the importance of PTI suppression for triggering defence.

### 1.5 Effector protein and their delivery

#### 1.5.1 Type III secretion system (TTSS)

PTI is known to be effective in preventing invasion by the vast majority of microorganisms with which plants come into contact. Somehow, in the co-evolution of host-microbe interactions, pathogens acquired the ability to deliver effector proteins to the plant cells primarily through type III secretion system (TTSS) to suppress PTI and promote parasitism or referred to as ETS. Thus, it is now clear that PTI emerges as a dominant target of plant bacterial effectors, and it is very likely that the selective pressures imposed by pathogens in general are responsible for shaping and driving the evolution of plant immune systems.

TTSS is a key pathogenicity factor of many Gram negative plant and animal pathogens such as *Yersinia, Pseudomonas syringae, Shigella, Xanthomonas*, including pathogenic *Escherichia coli* and also present in some symbiotic bacteria (Büttner and Bonas, 2006; Yip

and Strynadka, 2006; McCann and Guttman, 2007). This system is a highly conserved, complex molecular injection apparatus that translocates type III effectors (TTEs) into the host cell cytosol (Figure 1.3) where they promote diseases by targeting various plant cellular systems, including plant innate immunity, transcription, cell death, proteasome and ubiqutination systems, RNA metabolism, hormone pathways and chloroplast function (Lewis et al., 2009; Cunnac et al., 2009).

In *Pseudomonas syringae*, TTSS pathway is encoded by *hrp* (HR and pathogenicity) and *hrc* (HR and conserved) genes (Bogdanove et al., 1996) and associated with an extracellular filamentous appendage, called the Hrp pilus (Jin et al., 2003; He et al., 2004). *hrp/hrc* genes also encode *avirulence* (*avr*) and *hrp*-dependent outer proteins (*hop*) genes. The *hrp* region is conserved in pathogenic microbes and affects the ability of a bacterium to induce resistance or susceptibility depending on the nature of the host plants (He, 1998). The Hrc proteins direct secretion of TTSS substrates through host cell barriers, whereas a subset of the Hrp proteins are themselves secreted by the TTSS as a molecular chaperones or helper proteins (Bogdanove et al., 1996; van Dijk, 2002; Yip and Strynadka, 2006). *hrp* or *hrc* mutants which are defective in TTSS, do not usually multiply or cause disease in otherwise susceptible host plants. The TTSS shares many functional, structural and sequence similarities with the bacterial flagellum (a rotating motility structure widely distributed among the bacteria), except for the ability to translocate proteins into eukaryotic cells (Blocker et al., 2003; McCann and Guttman, 2007).

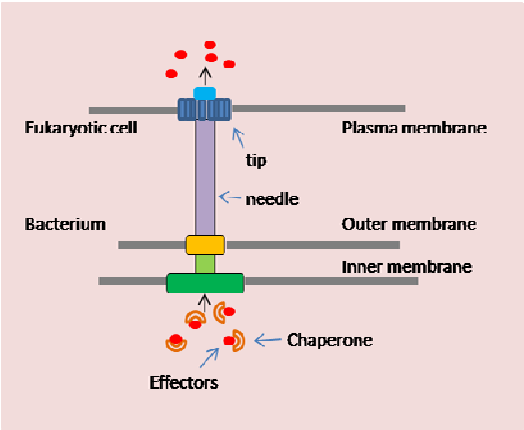



**Figure 1.3 The type III secretion system (TTSS)**

The TTSS provides a conduit for the passage of TTEs that travel through the needle-like structure across three membranes into the cytosol of targeted cells. (Adapted from http://carbon.bio.ku.edu/research.htm)

For a successful infection, plant pathogens must penetrate the polysaccharide-rich host cell wall, form a conduit between the bacteria and the host membrane, and generate a pore to facilitate translocation of TTEs into the host cell. Specialized chaperone proteins are often required to guide the TTEs into the TTSS (Grant et al., 2006). Delivery of TTEs and the associated helper proteins into the host cell is essential for the successful life histories of many Gram-negative plant-associated bacteria.

Resistance to bacterial infection by plants is often determined by the presence of *R* genes in plants and *avr* genes in bacteria (Martin, 1999). *avr* genes were originally determined as incompatibility factors of a pathogen which elicit host plant defence in a gene-to-gene manner but the current view is that, the *avr* gene product is actually bifunctional and also has a role in pathogenicity. In incompatible interactions, Avr proteins are recognized by the corresponding R proteins and results in activation of a suite of defence responses in plants. Conversely, Avr proteins also contribute to virulence of a pathogen on plants lacking the corresponding R protein through direct or indirect interaction with host proteins, inhibiting them from establishing a specific defence. Hence, avirulence became a conceptually restrictive term since the same protein with an avirulence activity in incompatible interactions may display a virulence activity in compatible interactions (Hogenhout et al., 2009).

Recently, the term effector and its associated concepts have been routinely used by plant scientists to describe secreted proteins that exert effect on plant cells. In a broader definition, effectors include small molecules that alter host cell structure and function such as PAMPs, toxins, and degradative enzymes (Hogenhout et al., 2009). Several best-studied TTEs are designated as Avr proteins because they were detected through gain-of-function avirulence phenotypes (Keen, 1990). More effectors were subsequently identified by their ability to travel the TTSS pathway, for example Hop as designated for *Pseudomonas syringae* (Alfano and Collmer, 1997).

Over the past few years, virulence promoting function of pathogen effectors and their host targets has become a centre of attention. Despite vast documentation on effector proteins in animals, very little is known about the molecular mechanism by which bacterial protein trigger diseases in plants. *Pst*DC3000, which causes bacterial speck in tomato, is an excellent model for investigating the possible operation of TTEs mainly because the DC3000 genome has been fully sequenced (Buell et al., 2003) and it has the ability to infect the experimentally tractable plants *Arabidopsis* and *Nicotiana benthamiana*. Moreover, its TTSS

is being extensively studied and molecular function of several of the TTEs has been determined. Recently, the complete repertoire of *Pseudomonas syringae* TTEs have been identified (>50) and 47 are present in *Pst*DC3000 (Cunnac et al., 2009). Apparently, only 28 of them are considered to be fully active, 12 are effector pseudogenes and the rest appear only weakly expressed. Although several effectors indeed have the ability to promote virulence when expressed individually or produced as a transgene in plant cells, all mutations in single effector genes tested so far just cause weak reductions in virulence or growth *in planta* (Cunnac et al., 2009).

### 1.5.2 Type III effectors in action

Consistent with previous findings that PTI is actively inhibited by *Pseudomonas syringae* effectors, several reports demonstrated that regulatory proteins in the PTI pathways are directly targeted by these effectors. For example, earlier studies indicated that HopAI1 directly inactivates *Arabidopsis* MAP kinases MPK3 and MPK6 through phosphothreonine lyase activity to block downstream events associated with PTI (Zhang et al., 2007). HopAI1 is an OspF-related TTEs that is highly conserved in animal pathogens and *Pseudomonas syringae*. Interestingly, another member of this effector family dephosphorylates kinases involved in mammalian innate immunity (Li et al., 2007), showing that pathogens can apply the same virulence mechanism to both plant and mammals.

Pathogens may also acquire or evolve additional effectors that target both PTI and ETI. Most spectacularly, AvrPto and AvrPtoB (unrelated effectors from *Pst*DC3000), suppress very early immune responses mediated by MAPK cascades, suggesting that suppression can occur immediately after signal perception or before MAPKKK signalling (He et al., 2006). Recent publications have elegantly demonstrated that AvrPto and AvrPtoB target the kinase domains of BAK1, FLS2, EFR and chitin receptor CERK1 (Gohre et al., 2008; Shan et al., 2008; Xiang et al., 2008; Gimenez-Ibanez, 2009). Both AvrPto and AvrPtoB are believed to block PTI through direct binding to BAK1 and interfere with the interaction between BAK1 and FLS2 (Gohre et al., 2008; Shan et al., 2008).

In addition to their PTI-suppression abilities, AvrPto and AvrPtoB also inhibit ETI through interaction with their host targets, tomato kinases Pto and/or Fen (Xing et al, 2007; Rosebrock et al, 2007). AvrPtoB via its C-terminal E3 ubiquitin ligase domain specifically targets Fen kinase, which is part of a unique and presumably ancient ETI pathways, for degradation by proteosome (Rosebrock et al., 2007) while AvrPto appear to exert its

virulence function by inhibiting the kinase activity of Pto (Xiang et al., 2008). This is one of the clearest examples of how effector proteins hijack plant ubiquitination/proteosome systems. Three other effectors from *Pseudomonas syringae*, AvrB, AvrRpm1 and AvrRpt2 were found to interact and differentially manipulate plant-specific *Arabidopsis* protein RIN4, which allows it to be recognized by the R protein RPM1 and RPS2, thus activating defence components in plant (Kim et al., 2005; Ong and Innes, 2006). Conversely, a recent publication from Cui et al. (2010) shows that *Pseudomonass syringae* effector protein AvrB activates components of MAPK pathway and RIN4 for the benefit of bacterium by perturbing hormone signalling and enhance susceptibility. RIN4 also has been shown to be a direct target for *Pseudomonas syringae* effector HopF2$_{Pto}$ and this is the first evidence showing that RIN4 is manipulated to promote pathogen virulence (Wilton et al., 2009).

Pathogen effectors target more than PRRs or the MAPK cascade to suppress PTI. These effectors also attack processes directly downstream of PRR signalling and other consequent events. The miRNA pathway is a component of RNA metabolism that is important for plant immunity (Navarro et al., 2008; Li et al., 2010). HopT1, AvrPto and AvrPtoB suppress miRNA activity but their direct targets related to the miRNA pathway remain to be determined (Navarro et al., 2008). A number of TTEs manipulate plant hormone signalling pathways to alter host defence response. There is growing evidence that *Pseudomonas syringae* injects several TTEs to promote virulence by reducing levels of SA and increasing levels of ET, JA, absisic acid (ABA) and auxin (Valls et al., 2006; He et al., 2004; de Torres-Zabala et al., 2007; Chen et al., 2007). *Pst*DC3000 also produces polyketide toxin coronatine, which is a jasmonate mimic that suppress SA levels through activation of JA signalling (Uppalapati et al., 2005). In addition, TTEs can modify protein levels by altering host transcription, which can lead to increased susceptibility. AvrBs3/PthA family of TTEs is widely distributed in *Xanthomonas* species and the family members have features of transcription factors (Grant et al., 2006). AvrBs3 alters transcription by binding to the promoter region of the pepper gene *upa20* to activate the transcription of this gene and enhance hypertrophy, which may prime the host physiology for optimum bacterial colonization or dispersal (Kay et al., 2007).

Figure 1.4 shows how TTEs directly alters a component of PTI and/or ETI signalling, or usurps another plant system to indirectly affect plant immunity as reviewed in Lewis et al. (2009). The many examples of physical associations between pathogen effectors and regulators of host immune responses imply that pathogen effectors can be used as a molecular probe to identify unknown components of the plant innate immune system.

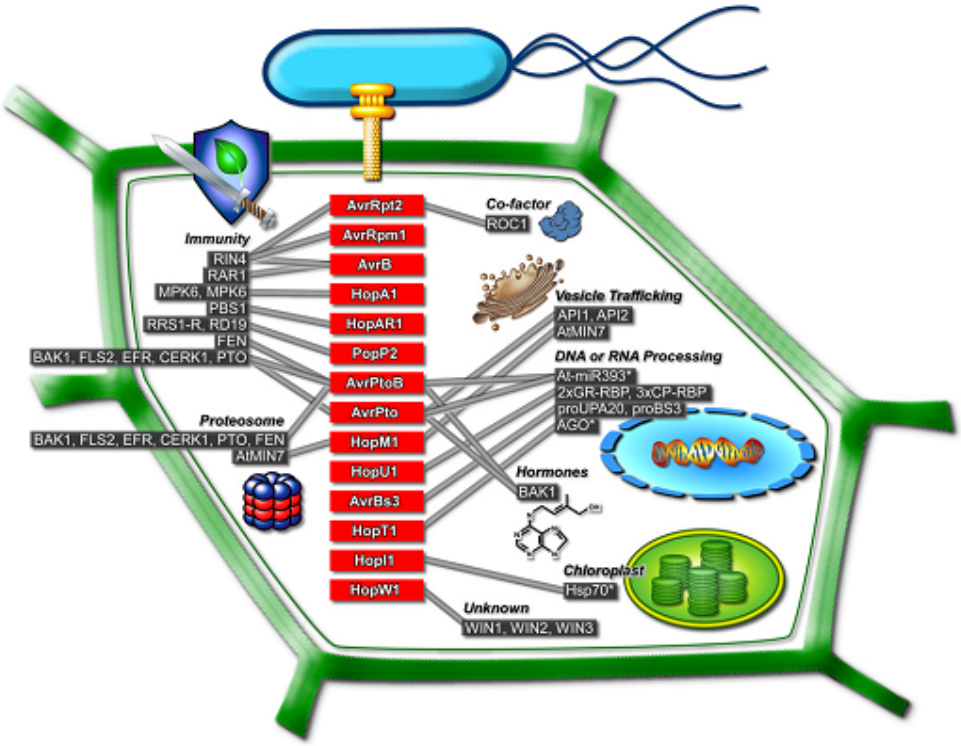



**Figure 1.4 Plant systems targeted by phytopathogenic TTEs**

Plant systems targeted by phytopathogenic type III effector proteins. The direct targets of phytopathogenic T3SEs are grouped according to the plant systems to which they belong as outlined in the review. Effectors are shown with a red background, while host interactors are shown with a grey background. Asterisks indicate proteins or genes predicted to be targets of the corresponding effector but for which a direct interaction has not yet been demonstrated (Lewis et al., 2009).

There are established and emerging themes regarding TTEs targets as reviewed by Lewis et al. (2009) to pave ways for future studies to identify host targets of this vast repertoire of pathogen effectors. First, single TTEs may target multiple host factors. Second, TTEs target critical steps in key host processes, the immune system. Third, distinct TTEs can congregate on specific host targets, perhaps providing redundancy and sturdiness. Finally, important host targets of TTEs can directly interact with R proteins containing NBS-LRR.

### 1.6 *R* gene-mediated resistance (Gene-for-gene model)

#### 1.6.1 Resistance (*R*) genes and proteins

ETI or classically known as gene-for-gene resistance denotes matching specificity between dominant *R* genes in plants whose products recognize those products of pathogen complementary *avr* alleles (Dangl and Jones, 2001; Grennan, 2006). *R* genes specify a polymorphic component of a particular recognition event. Very often, interaction involving the products of *R* and *Avr* genes culminates in HR (Jones and Dangl, 2006). In contrast to basal defence, *R* genes control heightened state of disease resistance, commonly specific to particular pathogen strains. Genetic overlap between specific and basal resistance responses suggests that *R* mediated signalling is more rapid and trigger stronger disease resistance by boosting basal defence reactions (Dangl and Jones, 2001). We have achieved remarkable progress in understanding *R* gene-mediated resistance since the development of a model based on classical genetics using flax, *Linum ultissimom*, and the fungal pathogen *Melampsora lini* by Flor in 1940s (Campbell et al., 2002).

Years ago, the major hub in plant molecular pathology was cloning and characterization of *R* genes. In fact, many R genes have now been cloned, conferring resistance to numerous classes of pathogens such as bacteria, viruses, fungi, and even nematode and insect pathogens. Despite *R* genes confer resistance to different pathogens, the encoded proteins shared a limited number of conserved elements (van Ooijen et al., 2007). To date, 5 classes of effector-specific R proteins are known based primarily upon their combination of a limited number of structural motifs and their sequence suggests roles in both effector recognition and signal transduction (Martin et al., 2003). The functions of various R proteins require posttranslational modification such as phosphorylation, protein degradation, or specific localization within host cell (Martin et al., 2003).

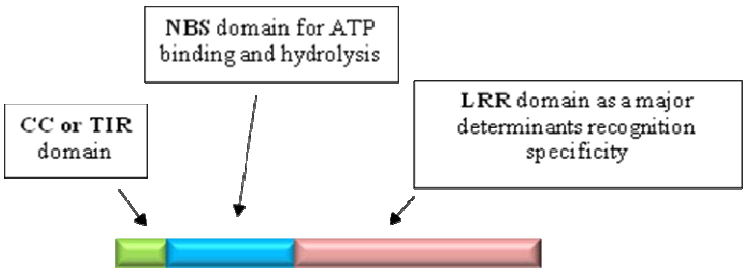



**Figure 1.5 Domain structure of NBS–LRR proteins**

The great majority of them are intracellular NBS-LRR proteins (Figure 1.5), forming the largest group of plant R proteins with about 150 genes found in *Arabidopsis* and about 600 in rice (Rafiqi et al., 2009). Other types of R proteins that have been identified contain an extracellular LRR domain. They are either members of the RLK or the receptor-like protein (RLP) family. However, while RLKs contain a cytoplasmic serine/threonine kinase domain, no recognizable intracellular signalling domains can be discerned in RLP (Tameling and Taken, 2007; Tameling and Joosten, 2007).

Plant NBS-LRR contains a C-terminal LRR domain, a varying N-terminal effector domain, and a central NBS-domain (Tamling and Joosten, 2007). The most striking structural feature is a variable number of LRRs. LRR domains are characterized by a 25-30 amino acid repeat motif that forms barrel-like structures with a parallel β-sheet lining the inner concave surface and α-helical structures comprising much of the rest of the domain (Kobe and Kajava, 2001). The LRR domain is found in diverse proteins appears to be the major determinants of recognition specificity. The NBS domain (also called NB or NB-ARC) contains blocks of sequence that are conserved in both plant and animal proteins. It is part of a larger domain that includes additional homology between R proteins, human apoptotic protease activating factor-1 (Apaf-1) and *Caenorhabitis elegans* death-4 (Ced-4) (van der Biezen and Jones, 1998). This domain is proposed to act as a nucleotide-dependent molecular switch regulating the conformation and signalling activity of R proteins (Takken et al., 2006). By analogy with Apaf-1 and Ced-4 functions, the presence of a specific signal or elicitor, for example Avr proteins, would induce conformational changes in R proteins, allowing nucleotide exchange for further signalling events (Rafiqi et al., 2009). The N-terminal domain found in plant NBS-LRRs can either be a TIR-NBS-LRR or a non-TIR, containing putative coiled-coil domain (CC-NBS-LRR) (Martin et al., 2003; Takken et al., 2006; Tameling and Takken., 2007). TIR-NBS-LRR class represents 60% of overall NBS-LRR proteins (Dangl and Jones, 2001). The CC-NBS-LRR class probably comprises multiple subfamilies, varying in size and in the location of the coiled-coil domain (Dangl and Jones, 2001). The function of CC and TIR domain in pathogen perception and signalling is ambiguous.

The complete *Arabidopsis* sequence permits a comprehensive analysis of the diversity of NBS-LRR R-gene sequence in one plant. There are 149 genes encodes for NBS-LRR and 58 related genes that do not encode LRRs in *Arabidopsis* Col-0 and these number represent approximately 0.8% of all open reading frames (ORFs) so far annotated in Col-0 (Tan et al., 2007). This seems surprisingly a small of genes to mediate recognition of all possible pathogen-encoded-ligands. Perhaps R proteins actually perceive the presence of more than

one Avr protein. In fact, dual recognition has been observed, for example RPM1 disease resistance loci of *Arabidopsis* confer resistance to *Pseudomonas syringae* strains that carry two non-homologous avr genes, AvrB and AvrRpm1 (Bisgrove et al., 1994). In addition, the tomato *Mi* gene confers not only nematode resistance but also aphid resistance (Rossi et al, 1998). Alternatively, it is also possible that some R protein recognize conserved pathogen molecules of ancient origin and may not evolve rapidly in response to pathogen pressure (Riely and Martin, 2001).

### 1.6.2 Guard and decoy model

Following the characterization of many *R-avr* gene pairs, the exact nature of the *R-avr* interaction leading to recognition of pathogen is becoming more comprehensible. Initially it was widely thought that R proteins behave like 'receptors' that directly interact with 'specific ligands', encoded by *avr* genes. This receptor-ligand model was supported by the fact that some *avr* gene products co-localize with *R* gene products, most of which encode LRR domains. In fact, a few direct physical interactions between LRR-containing R proteins and corresponding *avr* effectors were found, consistent with a receptor-ligand mode of action (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). However, for a number of *R-avr* combinations, physical interactions have not been observed, and perception is thought to be indirect. In the light of such observations, the original receptor-ligand model was amended to add a new dimension to the *R-avr* interaction that provide intriguing conceptual framework for the action of effectors and R protein complex.

In this so-called guard model (Dangl and Jones, 2001; Belkhadir et al, 2004), R proteins, postulated to act as a 'guard' are likely to be part of a multiprotein complex that should includes protein that are targeted by pathogen virulence factors. Avr proteins, presumably acting as virulence factors, interact with cellular targets or 'guardees' inside the host cell. These targets are probable partners of R proteins. The perturbation of these guardee proteins may or may not be required for virulence but still leads to R protein activation. R proteins either constitutively bind to their partner(s) and then dissociate after modification of the complex by the TTEs or form a new interaction with a cellular target that leads to activation.

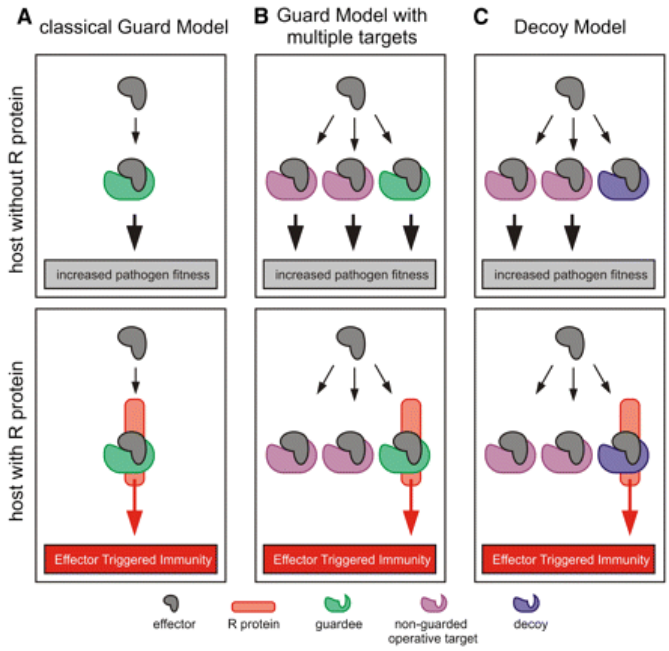



**Figure 1.6 Comparisons of the Guard and Decoy Models**

The classical Guard Model (**A**) is contrasted with a modified Guard Model in which the effector targets multiple plant proteins (**B**) and the Decoy Model (**C**). Effectors are depicted in grey, operative targets in purple, guardee in green, decoy in blue and the R protein in orange. (van der Hoorn and Kamoun, 2008).

This model was initially proposed to elucidate the role of NBS-LRR protein Prf in AvrPto–Pto signalling (Van der Biezen and Jones, 1998). In this model, Prf protein was found to associate with the Pto protein (a serine/threonine kinase) and render Prf in inactive state. Prf is activated upon AvrPto-Pto interaction which disrupt the inhibitory action of Pto and allows Prf to induce plant defence responses. However, new findings on effector activities (Zhou and Chai, 2008; Zipfel and Rathjen, 2008) are inconsistent with the original description of guard model and a new concept has been proposed, arguing that some host targets act as decoy to detect pathogen effectors via R proteins (van der Hoorn and Kamoun, 2008). In this variation, known as the decoy model, the guardee protein is a decoy that mimics the operative host target but only functions in perception of pathogen effectors without benefiting pathogen fitness in the absence of its cognate R protein. For this reason, the decoy model is distinct from the classical receptor-ligand and refined guard models (Figure 1.6). Both indirect and direct recognition model present a fascinating example of the diversity and the complexity of mechanism employed by host plants to detect the broad range of effectors.

### 1.7 Hypersensitive response

A ubiquitous feature of plant-pathogen interactions is HR that is manifested as rapid and localized necrosis of cells at the inoculation site and often associated with resistance responses (Dangl et al., 1996; Heath, 2000). Resistance responses conditioned by initial recognition events between host plant and pathogen is often mediated by plant *R* gene and a cognate microbial *avr* gene, which then triggers the HR. Despite many observations that most resistance responses are accompanied by an HR (Zhou et al., 1995; Mackey et al., 2002), in some cases, they can occur with very little or without cell death (Bendahmane et al., 1999). It has been suggested that the HR is a form of programmed cell death (PCD) in plants based on some similarities between HR and well-characterized animal PCD known as apoptosis (Jacobson et al., 1997; Heath, 2000; Greenberg and Yao, 2004).

It appears that the signal transduction requirements as well as structural and cytological changes that lead to hypersensitive cell death may vary depending on the host-pathogen combination (Morel and Dangl, 1997; Heath, 2000). HR-linked cell death in plants requires active plant metabolism and depends on the activity of the host transcriptional machinery. It has also long been recognised that the HR can generate signals that cause local and systemic changes in the plant. One of the most rapid plant responses following pathogen recognition is the alterations of ion fluxes characterized in particular by an uptake of $Ca^{2+}$. An oxidative burst producing ROS, including superoxide anion ($O_2^-$) that can be dismutated rapidly to hydrogen peroxide ($H_2O_2$) through the action of superoxide dismutase (SOD), constitute another likely signal in the cascade leading to HR (Lamb and Dixon, 1997; Grant and Loake, 2000). ROS derived from this oxidative burst are generated by plasma membrane NADPH oxidases, anchored by $gp91^{phox}$ proteins related to those responsible for the respiratory oxidative burst activated in mammalian neutrophils during infection (Torres et al., 2002). Loss-of-function mutations in two *Arabidopsis respiratory burst oxidase homologue* (*rboh*), *rbohD* and *rbohF* indicated that *AtrbohD* is the major source of extracellular ROS after pathogen infection, whereas *AtrbohF* mainly functions in HR control, potentially in a pathogen-dependent manner (Torres et al., 2002).

Induction of HR is often associated with elevated levels of SA (Lamb and Dixon, 1997). It has been suggested that SA functions as both pro-death and pro-survival signals (Lorrain et al., 2003; Lam, 2004) and that the ROS generated by Atrboh proteins can antagonize the SA-dependent pro-death signals (Torres et al., 2005). However, neither ROS nor SA is sufficient to activate HR on their own. The generation of NO has been shown to enhance $H_2O_2$-

mediated cell death and defence mechanisms (Delledone et al., 1998). Moreover, it has been observed that NO production occurs within the same time frame with $H_2O_2$ and a critical balance between this two molecules governs whether cell death is initiated or suppressed (Delledone et al., 2001). Thus, multiple secondary signals, such as ROS, SA and NO, appear to be essential second messengers for the activation and execution of HR.

In animals, there are three established morphological type of PCD: apoptosis, autophagy and non-lysosomal PCD. Whereas apoptosis and non-lysosomal PCD are uncommon in plants, autophagy is more recognized since it generally coincides with PCD during plant development (reviewed in van Doorn and Woltering, 2005). Autophagy is a major degradation and recycling system in eukarotic cells involving the turnover of cellular components by delivering portions of the cytoplasm into lysosome and vacuoles where they are digested (Klionsky and Emr, 2000). Recent developments in the study of HR-PCD suggest that evolutionarily conserved autophagy genes and the autopaghy pathway play an important role in the regulation of HR (Liu et al., 2005; Van Doorn and Woltering, 2005; Patel et al. 2006; Love et al., 2009). Autophagic pathways can both promote survival and death in plants (Kwon and Park, 2008; Love et al., 2008), both of which are a function of the degree of its activation which is subjected to tight regulations (Mizushima, 2007; Kwon and Park, 2008; Love et al., 2008).

Considerable efforts have been made to identify genes essential for the hypersensitive cell death, for example, by identifying mutant lines in which cell death is misregulated. Lesion mimic mutants, which exhibited spontaneous cell death in the absensce of pathogenic infection resembling pathogen-inducible HR have been identified in a few plant species including *Arabidopsis* (Lorrain et al., 2003). *Lesion simulating disease 1* (*LSD1*) gene is known as a negative regulator of both HR-like cell death and basal defence responses (Dietrich et al., 1994). *Arabidopsis* plants carrying the recessive null *lsd1* allele are unable to restrict cell death development after infection by various incompatible pathogens and exhibit a 'runaway cell death' (RCD) phenotype that is dependent on SA production (Jabs et al., 1996). *lsd1* induced-RCD requires *enhanced disease susceptibility 1* (*EDS1*) and *phytoalexin deficient 4* (*PAD4*), which are mediators for disease resistance conveyed by *R* genes encoding TIR-NBS-LRR (Rusterucci et al., 2001). Depending on ROS status, *non race-specific disease resistance 1* (*NDR1*), which mediate disease resistance conferred by CC-NBS-LRR class of *R* gene is also required for *lsdl*-induced RCD (Rusterucci et al., 2001).

In essence, there are two major signalling pathways following *R-avr* interactions that lead to HR, depending on the structure of the particular R protein that is activated by pathogen. The first one is defined by the NDR1 protein, and the second is defined by the EDS1 and PAD4 proteins (Loake, 2001; Mur et al., 2007). Overall, it is rather difficult to quantify the contribution of HR to pathogen resistance since its establishment is concomitant with the activation of other defence mechanisms, such as accumulation of PR proteins. On one hand, rapid elimination of infected cells may be responsible for protecting the neighbouring cells from further invasion and in the other hand, the HR itself can be uncoupled from resistance and thus may not be the primary barrier during plant-pathogen interactions.

### 1.8 Systemic Acquired Resistance (SAR)

Apart from the localized HR at the infection sites, defence responses can also be activated, or primed for rapid activation, in distal, uninoculated organs of the infected plants. This enhanced state of broad-spectrum disease resistance that develops in the whole plant in response to a local infection of leaves with microbial pathogens was termed systemic acquired resistance (SAR) (Durrant and Dong, 2004; Conrath, 2006; Grant and Loake, 2008). In this state of systemic acquired resistance, plants are primed to more quickly and more efficiently activate defence responses the second time they encounter pathogen attack. Protection is also observed after an attack by herbivorous arthropods, mechanical injuries or following contact with certain chemicals. SAR is distinguished from race-specific resistance in that it does not decay with time and is generally effective against a broad and distinctive spectrum of pathogens including viruses, bacteria, oomycetes, and fungi (Agrios, 2005; Conrath, 2006). SAR is associated with activation of a large number of *pathogenesis-related* (*PR*) genes in local and systemic tissues and these genes serve as powerful molecular markers for the onset of SAR (Maleck et al., 2000; Ryals et al., 1996).

Induction of SAR is not restricted to HR-inducing pathogens but also takes place upon treatment with high inoculum of non-pathogenic microbes onto the leaf surface or after treatment with PAMPs (Mishina and Zeier, 2007). SAR has been shown to involve various different signals with different mode of actions such as SA, JA, isonicotinic acid, lipid-derived signal, peptidic mobile signal, nitric oxide, hydrogen peroxide and less well characterized MAP kinases (Conrath, 2006; Vlot et al., 2008; Shah, 2009). Whereas SAR signal generation appears to be a general feature of SA-dependent defence signalling, the mobile signal itself has been elusive for decades. SA was initially proposed to serve as the mobile signal that is transmitted from the inoculated leaves via the phloem to the uninfected

portions of the plant (Durrant and Dong, 2004). SA was found to accumulate both at inoculation sites and distant leaves concomitant with the onset of SAR, and *NahG* plants that are unable to accumulate SA are SAR deficient (Malamy et al., 1990; Gaffney et al., 1993). In addition, SA was also detected in the phloem of pathogen-infected plants and radio-tracer study suggested that a significant amount of SA in the systemic leaves of pathogen-infected tobacco and cucumber was transported from the infected leaf (Molders et al., 1996; Shulaev et al., 1995). However, grafting experiments involving tobacco plants expressing the *NahG* gene have dismissed SA as the systemic signal in SAR (Vernooji et al., 1994).

Recently, a study showed that SA-derivative methyl-salicylate (MeSA) acts as a critical long-distance mobile signal for SAR because it is not degraded by salicylate hydroxylase *in vitro* and accumulates in *nahG* transgenic tobacco (Park et al., 2007). A very recent study in *Arabidopsis* further supports the occurrence of MeSA as a mobile SAR signal (Liu et al., 2010). However, Attaran et al. (2009) argued that MeSA is dispensable for SAR in *Arabidopsis*, and that SA accumulation in distant leaves appears to occur by de novo synthesis via isocharismate synthase. The discrepancies in these studies are probably due to the complexity in SAR signalling and differences in experimental conditions. There has been a considerable argument for multiple mobile signals for SAR as these signals could function redundantly, synergistically or perhaps antagonistically depending on various factors (Park et al., 2007; Vlot et al., 2008; Shah et al., 2009; Liu et al., 2010). Interestingly, for MeSA, in addition to serving as an engoenous SAR signal, it can serve as an airborne signal that is emitted from infected plants and induces defence gene expression in neighbouring wild type plants (Shulaev et al., 1997).

Several mutant screens have been performed to identify *Arabidopsis* mutants defective in the SA-dependent SAR signal transduction pathway (Conrath, 2006). *Non-expressor of PR-1* (*npr1*) mutant is probably the most prominent of these mutants. *npr1* mutants accumulate high level of SA after infection, but fail to activate *PR* genes and are highly susceptible to a wide range of pathogens (Cao et al., 1994). Pathogen-induced SA accumulation or treatment with SAR inducers results in an early transient increase in cellular reduction potential followed by a rapid decrease in reduction potential. The SA-induced redox changes lead to the reduction of NPR1 from cytosolic, disulphide-bound oligomers to active monomers that accumulate in the nucleus where they interact with the TGA class of basic leucine zipper transcription factors (Mou et al., 2003; Kinkema et al., 2000; Fan and Dong, 2002). This interaction induces the expression of defence response genes via a largely unknown mechanism to activate SAR (Fan and Dong, 2002; Mukhtar et al., 2009). The NPR1

promoter region contains several W-boxes that function as binding sites for plant-specific WRKY transcription factors suggesting that basal and SA-induced expression of NPR1 appears to be controlled by yet unidentified WRKY transcription factors (Yu et al., 2001; Eulgem and Somssich, 2007). Constitutive induction of disease resistance in plants might incur fitness costs and it explains why plants have evolved inducible defence mechanism. Mutants that constitutively express *PR* genes and accumulate SA often have reduced plant size, loss of apical dominance, curly leaves, and decrease fertility (Heil and Baldwin, 2002), while constitutive expression of SAR in uninfected plants is detrimental (Durrant and Dong, 2004).

## 1.9 Nitric Oxide (NO) and defence signal transduction

### 1.9.1 Nitric oxide: general properties

Nitric oxide (NO) acts as a signalling molecule within species from every biological kingdom. Because of its unique chemistry, which permits both stability and reactivity, NO and its exchangeable redox-activated forms are ideally suited to its cellular signalling function. At room temperature and at atmospheric pressure, NO is a free radical colourless diatomic gas with lipophilic property. It's small Stoke's radius and neutral charge allows rapid membrane diffusion (Kiger et al., 1993) and can play a part in cell-to-cell signalling in a brief period of time. Due to the presence of unpaired electron and free radical nature of NO, it readily reacts with oxygen ($O_2$), superoxide ($O_2^-$), transition metals and thiols, which largely shape its cellular function within the cell (Mur et al., 2006; Neill et al., 2007; Hong et al, 2007). The reaction of NO with $O_2$ results in the generation of $NO_x$ compounds (including $NO_2$, $N_2O_3$, and $N_2O_4$), which can either react with cellular amines and thiols, or simply hydrolyze to form end metabolites nitrite ($NO_2^-$) and nitrate ($NO_3$) (Wendehenne et al., 2001).

### 1.9.2 Nitric oxide production in animals and plants

NO is a multifunctional effector involved in numerous mammalian physiological processes, including neurotransmission, immunological and inflammatory responses, and relaxation of vascular smooth muscle (Schmidt and Walter, 1994). However, the use of NO is not confined to the animal kingdom. NO is also involved in diverse physiological processes in plants, such as defence response, metabolism, cellular detoxification, transport, iron

homeostasis, signalling, flowering, and lignin biosynthesis (He et al., 2004; Bason-Bard et al., 2008). Despite the importance to elucidate the biosynthesis of NO in plants, there is still much uncertainty after years of research. In animals, NO is synthesized primarily by the enzyme nitric oxide synthase (NOS), which catalyzes the NADPH-dependent oxidation of L-arginine to L-citrulline and NO (Stuehr et al., 2004). Three NOS isoforms have been identified (Nathan and Xie, 1994); neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS in macrophages (iNOS). nNOS and eNOS are considered as constitutive and both show fast and transient activation. iNOS is induced in macrophages and many other cell types in response to inflammatory agents and cytokines (Mayer and Hemmens, 1997; Beck et al., 1999). Compared to constitutive NOSs, iNOS activity is sustained longer, more stable and generates more NO, thus exerting cytotoxic and antimicrobial effects on the immune systems (Beck et al., 1999).

NO synthesis in plants includes both arginine and nitrite-dependent pathways. It is well documented that potential enzymatic sources of NO in plant cells include nitrate reductase (NR) and NOS-like activity (Neill et al., 2003; Romero-Puertas et al., 2004; Wang et al., 2006). NR catalyzes the *in vitro* production of NO through a one-electron reduction of nitrite via the use of NAD(P)H as an electron donor (Yamasaki and Sakihama, 2000). It has been viewed as a candidate for NO production during plant-pathogen interaction (Neill et al., 2003), but there are a few contradictory evidences that collectively suggest that NR is not likely to be the major generator of NO synthesized during the pathogen-triggered nitrosative burst (Hong et al., 2008). In addition, significant NO production from NR is dependent upon high levels of nitrite and anoxia or the absence of photosynthetic activity (Yamasaki, 2000), which are not the common scenario for the plants under natural condition. Although there is no obvious homolog of animal NOS in the *Arabidopsis* genome, several NOS-like activities have been reported (Cueto et al., 1996; Barroso et al., 1999; Corpas et al., 2006). In addition, mammalian NOS inhibitors have been shown to effectively abrogate the pathogen-triggered NO production in plants (Delledone et al., 1998; Neill et al., 2003). Corpas et al. (2009) had elaborately compared animal and plant NOS and concluded that plant also possesses L-arginine-dependent NOS activity which is differ from canonical animal NOS.

A search for the enzyme(s) that catalyze(s) the pathogen-triggered NO production in *Arabidopsis* led to the cloning of the *Arabidopsis* NOS 1 (*AtNOS1*) gene, which exhibited significant sequence similarity to a snail gene that encoded a NOS-like activity, but no homology to mammalian NOS (Guo et al., 2003). It now appears that *AtNOS1* may not actually be NOS at all because it has been difficult to demonstrate reproducibility of typical

NOS activity through recombinant *AtNOS1* (Crawford et al., 2006; Zemojtel et al., 2006). Instead, *AtNOS1* was found to serve as a chloroplast-targeted GTPase essential for proper ribosome assembly (Flores-Perez et al., 2008). AtNOS1 has therefore been renamed *Arabidopsis* nitric oxide associated 1 (*AtNOA1*). Several other mutants with altered NO levels has been reviewed to show increased NO accumulation correlates with concentrations of putative substrates for NO biosynthesis but none of them is exclusively affected in NO production (Leitner et al., 2009). Finally, researchers have reported that NO can also be formed non-enzymatically in a reaction between nitrogen dioxide and plant metabolites, nitrous oxide decomposition or as a result of chemical reduction of $NO_2^-$ at acidic pH (Wendehenne et al., 2001).

### 1.9.3 S-nitrosylation as a redox-based signalling in plants

NO-related signalling can be attributed to various NO derivatives, collectively referred to as reactive nitrogen species (RNS), which comprise not only the NO radical ($NO^{\cdot}$) and its nitroxyl ($NO^-$) and nitrosonium ($NO^+$) ions, but also peroxynitrite ($ONOO^-$), S-nitrosothiols (SNO), higher oxides of nitrogen and dinitrosyl-iron complexes (Leitner et al., 2009). NO and RNS exert their biological actions through the chemical modification of targets by reacting with different amino acids or prosthetic groups. They mostly act through the binding to transition metals of metalloproteins (metal nitrosylation) and the covalent modifications of cysteine (S-nitrosylation) and tyrosine (tyrosine 3-nitration). These processes are emerging as specific posttranslational protein modifications and the best characterised of these is S-nirosylation.

S-nitrosylation, the covalent attachment of an NO moiety to the thiol side chain of cysteine to form SNO, is emerging as a prototypic redox-based post-translational modification by which NO orchestrates cellular functions in animals (Stamler et al., 2001). Recently, S-nitrosylation has been shown to regulate small numbers of plant proteins *in vitro* (Lindermayr et al., 2005; Lindermayr et al., 2006). In this framework, the formation of SNO may serve to stabilize and diversify NO-related signals. Being a new paradigm in signal transduction, S-nitrosylation presents unique features, the main one being the fact that its formation and degradation depend solely on chemical reactions (Martinez-Ruiz and Lamas, 2007). In the latest review by Lindermayr and Durner (2009), direct reaction between NO and thiol groups was said to be too slow to operate physiologically. Instead, the radical combination reaction of the thiyl radical ($RS^{\cdot}$) with $NO^{\cdot}$ seems to be more efficient and extremely fast. The fact that $NO^+$, $N_2O_3$, and $ONOO^-$ serve as effective nitrosylating agents

further supports this notion. Other important S-nitrosylating agents include metal-NO complexes, which are the product of NO and transition metals that are able to transfer their NO moiety to the thiol group of cysteine residues and operate efficiently in hydrophilic environments (Lane et al., 2001; Lindermayr and Durner, 2009).

Specificity of S-nitrosylation within and between proteins is conferred by acid-base and hydrophobic motifs aiming at critical cysteine residues and by protein-protein interactions that impound the signals (Hess et al., 2001). For example, in animals, acid-base catalysis has been shown to promote nitrosylation and denitrosylation of haemoglobin (β-Cys93) in a conformation-dependent fashion (Stamler et al., 1997). In *Arabidopsis*, S-nitrosylation of S-adenosylmethionine synthetase 1, metacaspase 9 and recently salicylic acid binding protein 3 (AtSABP3) also have been shown to specifically occur at cysteine residues that are directly surrounded by basic and acidic amino acids (Lindermayr et al., 2006; Belenghi et al., 2007; Wang et al., 2009). As a regulatory mechanism in plants and animals, S-nitrosylation is a reversible process. Indeed, S-nitrosylated proteins can be easily de-nitrosylated as the S-NO bond is labile in a cytoplasm's reducing environment, allowing cells to flexibly and precisely accommodate protein function in response to environmental signals. S-nitrosylated proteins are in dynamic equilibrium with de-nitrosylated proteins largely due to the action of glutathione (GSH) with the subsequent formation of S-nitrosoglutathione (GSNO), reconstituting the protein thiol as a consequence. GSNO has the ability to release NO or function as a transnitrosylationg agent, thus it is considered as a natural reservoir of NO (Besson-Bard et al., 2007; Leitner et al., 2009).

Two of the enzymes that are known to metabolize GSNO are S-nitrosoglutathione reductase (GSNOR) and thioredoxin. The presence of GSNOR is conserved between bacteria, animals and plants (Liu et al., 2001) and due to its ubiquitous nature, this enzyme was suggested to confer protection against nitrosative stress rather than as a cell signalling factor. In contrast, thioredoxin or thioredoxin reductase denitrosylation reactions seem to be a part of a signal transduction mechanisms (Lindermayr and Durner, 2009). GSNOR controls intracellular levels of GSNO and limits NO toxicity through NADH-dependent reduction of GSNO to glutathione disulfide (GSSG) and ammonia ($NH_3$) (Lamotte et al., 2005). Though highly specific for GSNO, GSNOR seems to modulate the extent of total cellular SNO formation (Liu et al., 2001; Feechan et al., 2005).

### 1.9.4 NO and SNO functions in plant disease resistance

The function of NO in signalling defence responses during plant-pathogen interactions have been well documented in many experiments conducted years ago. A widespread feature of plant disease resistance is the HR. NO is suggested to play a key signalling role during HR, next to the accumulation of ROS and SA (Delledonne et al., 2002). In animals, many biological effects of NO including apoptosis are mediated by the highly toxic molecule, $ONOO^-$, which is relatively non-toxic in plants (Bonfoco et al., 1995; Delledone et al., 2001). On the contrary, HR-associated cell death in plants is proposed to be mediated by the relative level of NO and $H_2O_2$ that is formed by dismutation of $O_2^-$ by SOD. In plants, $ONOO^-$ is continuously produced in healthy cells, exposing them to an environment rich in $ONOO^-$. Therefore, plants have developed some detoxification mechanism, for example, through the action of peroxiredoxin II E (PrxIIE), a member of the peroxiredoxin family of antioxidant enzymes responsible for lipidoxidation and tyrosine nitration (Romero-Puertas et al., 2007). Interestingly, PrxIIE has been found to be S-nitrosylated during the HR resulting in inhibition of its hydroperoxide-reducing peroxidise activity together with its ability to detoxify $ONOO^-$ and also increasing the amount of tyrosine nitration (Romero-Puertas et al., 2007). In conclusion, NO regulates the effect of its own reactive species through S-nitrosylation of crucial components of the antioxidant defence system. NO also controls cell death in plants through S-nitrosylation of *Arabidopsis* metacaspase 9 and cytosolic glyceraldehyde 3-phosphate dehydrogenase, both of which can act as a potential executioner of PCD (Belenghi et al., 2007; Holtgrefe et al., 2008).

NO is not only thought to function during the development of hypersensitive cell death but also in the establishment of plant disease resistance complementary to and independent of ROS. Administration of NO donors induced the expression of defence-related genes encoding phenylalanine ammonia lyase (PAL), the first enzyme of phenylpropanoid biosynthesis pathway and pathogenesis-related protein 1 (PR-1) (Durner et al., 1998). NO action in plants, at least partially, is mediated through the SA-dependent signalling pathway. NO treatment induces endogenous SA accumulation required for *PR-1* gene induction in tobacco (Durner et al., 1998). *NPR1*, a master regulator of SA-mediated defence genes and a crucial component of disease resistance and signal cross-talk is known to be redox-regulated (Tada et al., 2008), adding an important clue to understanding NO's signalling functions. S-nitrosylation of *NPR1* controls its subcellular localization through oligomer-monomer exchange and thus its transcription co-factor activity. Mutations at critical cysteine residues in NPR1 increased monomer accumulation, constitutive nuclear localization and *NPR1*-

mediated gene expression in the absence of pathogen (Mou et al., 2003; Tada et al., 2008). Another very interesting example for the regulatory function of NO is S-nitrosylation of *AtSABP3*, which may interfere with the signal cross-talk as both carbonic anhydrase and SA-binding activity of the protein are inhibited (Wang et al., 2009).

In addition to data presented above, the importance of NO and SNO in plant disease resistance were presented through the analysis of a GSNOR knock-out mutant. Loss-of-function mutation in *Arabidopsis GSNO reductase 1* (*AtGSNOR1*) resulted in an increased cellular levels of SNOs and compromised all modes of disease resistance (Feechan et al, 2005). Conversely, enhanced *AtGSNOR1* activity results in increased protection against ordinarily virulent microbial pathogens. *AtGSNOR1* also positively regulates the signalling network controlled by the plant immune system activator, SA (Feechan et al., 2005). Subsequently, similar results were obtained by Tada et al. (2008) through *NPR1* studies. Surprisingly, using antisense strategy, basal resistance has been reported to increase in *atgsnor1* antisense plants, correlating with higher levels of intracellular SNOs and constitutive activation of *PR-1* (Rusterrucci et al., 2007), which is the opposite result to that obtained by Feechan et al. (2005). Probably the contradictory reactions of the GSNOR modified plants might be a result of different cellular levels of SNO that change dramatically in *atgsnor1* mutants (Feechan et al., 2005) compared to a minor changes in the antisense plants (Rusterucci et al., 2007) (Hong et al., 2008). Nevertheless, both works underlines the physiological importance of SNO formation and turnover in regulating multiple modes of plant disease resistance.

### 1.10 Defence signalling – crosstalk between the signalling molecules

Phytohormones play important roles in regulating developmental processes and signalling networks involved in plant responses to a wide range of biotic and abiotic stresses. Induced plant defences against microbial pathogens and insects are differentially regulated by cross-communicating signalling pathways involving various types of phytohormones in plants resulting in fine-tuning of the expression of defence-related genes (Grant et al., 2009) (Fig 1.7). Three key signal molecules, namely SA, JA and ET are produced in a specific blend upon pathogens attack and mediate expression of both specific (*R* gene-mediated) as well as basal defence responses (Glazebrook et al., 2003; Koornneef and Pieterse, 2008; Pieterse et al., 2009). In response to infection by biotroph and hemibiotroph pathogens, endogenously accumulating SA antagonizes JA/ET-dependent defences, thereby prioritizing SA-dependent resistance to this type of pathogens over JA/ET-dependent defence against necrotrophic

pathogens and herbivorous insects (Glazebrook, 2005). Although SA and JA/ET defence pathways are mutually antagonistic, evidences of synergistic interactions also have been reported (Beckers and Spoel, 2006; Mur et al., 2006; Loake and Grant, 2007), suggesting that the defence signalling network activated and utilized by the plant is dependent on the nature of the pathogen and its mode of pathogenicity.

Through mutational or ectopic expression analyses, several genes have been identified as a molecular player in SA and JA signalling such as MAPK4, EDS1, PAD4, NPR1, glutaredoxin GRX480, PDF1.2 (plant-defensin 1.2) and WRKY transcription factor such as WRKY70 (Broderson et al., 2006; Spoel et al., 2003 ; Ndamukong et al., 2007; Li et al., 2004). *NPR1* acts downstream of *EDS1* and *PAD4* in the SA signalling pathway (Brodersen et al., 2006) and downstream of *NPR1*; several WRKY transcription factors play important roles in the regulation of SA-dependent defence response in plants (Eulgem and Somssich, 2007). WRKY trancription factors are newly identified transcription factors involved in many plant specific processes including plant responses to biotic and abiotic stresses (Zhang and Wang, 2005). *Arabidopsis* WRKY70 has been found to positively regulate SA-dependent defences and negatively regulate JA-dependent defences and plays a pivotal role in determining the balance between these two pathways (Li et al., 2004; Li et al., 2006).

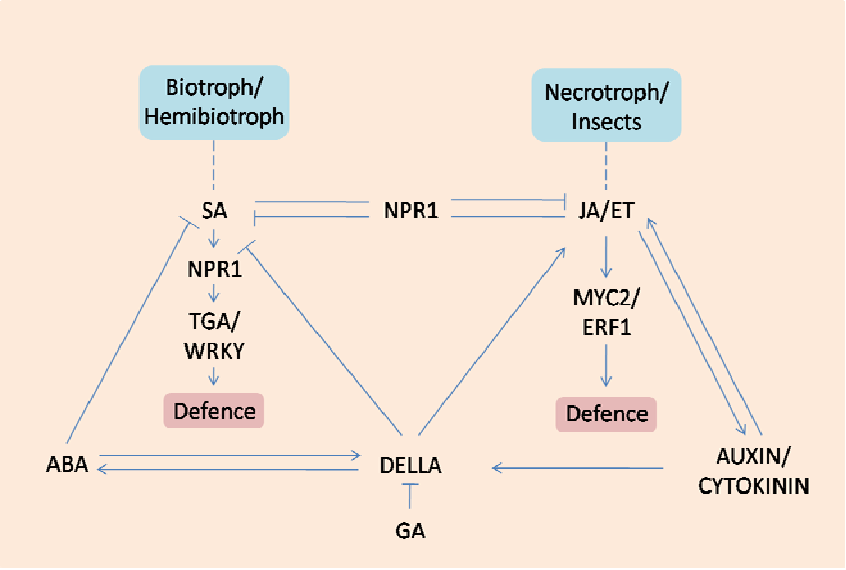



**Figure 1.7 Possible interaction between hormone signalling pathways**

Recently, it was demonstrated that ET bypasses the need of *NPR1* in SA-JA crosstalk, while it enhances *NPR1*-dependent, SA-responsive *PR-1* expression (Leon-Reyes et al., 2009). This study highlights the dual role of NPR1 in regulating SA-mediated activation of SA-dependent defences and SA-mediated suppression of JA-dependent defences. There is a tight connection between the SA, JA and ET responses pathway, and several studies indicate that JA- and ET-signalling often operate synergistically (Penninckx et al., 1998; Thomma et al., 2001). The basic helix-loop-helix leucine zipper transcription factor MYC2 and ethylene response factor 1 (ERF1) have been shown to play an important role in regulating the interaction between the two hormones (Lorenzo and Solano, 2005; Lorenzo et al., 2003).

Although the SA, JA and ET response pathways serve as the backbone of the induced defence signalling network, other hormone response pathways feed into it. ABA is commonly involved in the regulation of many aspects of plant development and abiotic stress, but it is becoming increasingly evident that ABA also influences many plant pathogen interactions depending on pathogen lifestyle. In general, ABA is shown to be involved in the negative regulation of plant defence against various biotrophic and necrotrophic pathogens (Bari and Jones, 2009; Pieterse et al., 2009). Another enzyme that originally plays a role in plant development and is connected to the SA-JA-ET signalling network is auxin. Auxin promotes disease susceptibility and repression of auxin signalling could potentially result in enhanced resistance in plants (Thilmony et al., 2006; Wang et al., 2007). In *Pst*DC3000 challenged plants, elevated auxin and ABA responsiveness are TTSS- and coronatine-dependent (Thilmony et al., 2006). Isolation of virulence factors that target auxin and ABA signalling (AvrBs3, AvrRpt2, coronatine and AvrPtoB) further indicates that pathogens actively target these pathways to promote virulence (Robert-Seilaniantz et al., 2007). The role of cytokinin in plant defence is elusive, but it displays some similarities to that of auxin (Robert-Seilaniantz et al, 2007; Piertese et al., 2009).

Recently, gibberelin (GA) has been shown to affiliate with the SA-ET-JA network as well with the opposite effect on plant defence. GA promotes plant growth by inducing the degradation of growth-repressing DELLA proteins, a family of putative transcriptional repressors that inhibit cell proliferation and expansion, which drives the growth of plant organs (Hartberd, 2003). Navarro et al. (2008) demonstrated that DELLA proteins promote susceptibility to biotrophic pathogens and resistance to necrotrophic pathogens by modulating the relative strength of the SA and JA signalling pathways. In a recent review by Grant and Jones (2009), DELLA proteins are proposed to play a central role in fine-tuning the defences mounted through the SA, JA, or ET pathways.

Emerging evidences indicates that BR treatments can also affect induction of plant defence (Nakashita et al., 2003). However, a connection between BR signalling and the SA-JA-ET network remains to be established. In conclusion, plant hormones regulate complex signalling networks involving developmental processes and plant responses to environmental stresses. Thus, the involvement of multiple hormones in plant-pathogen interaction cannot be overlooked to fully understand the tight interaction between a host and its pathogen.

### 1.11 Aims and Objectives

1. To determine the cellular level of S-nitrosothiol in response to virulent *Pst*DC3000 and *Pst*DC3000 *hrcC* mutant

2. To explore if *AtGSNOR1* might constitute a target of pathogen effector proteins

3. To identify the specific effector protein(s) that target *AtGSNOR1* and the cognate mechanism that underlies the manipulation of *AtGSNOR1* gene function.

## 2. Materials and Methods

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

### 2.1 Plant materials and growth conditions

*Arabidopsis* seeds of ecotype Columbia (Col-0) were used. Unless otherwise stated, all *Arabidopsis* transgenic and mutant lines used are in a Col-0 background and were summarised in Table 2.1.

Seeds were placed on potting medium consisting of peat moss, vermiculite and sand (4:1:1) and vernalized at 4°C in dark for 2-3 days. Then they were transferred to a long-day growth chamber at 20°C with 16 hours light and 8 hours dark. For aseptic growth, seeds were placed onto MS medium (1/2 Murashige and Skoog salt, 1% sucrose, 0.4% phytoagar, pH 5.8) after being sterilized with commercial bleach (25%) for 5 minutes and washed 5-10 times in distilled water. Petri dishes were transferred to a growth chamber with a long-day condition (16 hours light and 8 hours dark, 22°C, 60% humidity).

**(a)**

| Mutant lines | Protein | Accession No. | Reference | Source |
|---|---|---|---|---|
| At3g01080 | WRKY58 | SALK_150041C | Wang et al., 2006 | NASC |
| At4g31800 | WRKY18 | SALK_093916C | Xu et al., 2006 | NASC |
| At3g56400 | WRKY70 | SALK_025198C | Li et al., 2006 | NASC |
| At2g30250 | WRKY25 | SALK_136966C | Zheng et al., 2007 | NASC |
| At5g49520 | WRKY48 | SALK_066438C | Xing et al., 2008 | NASC |

NASC (Nottingham Arabidopsis Stock Center)

**(b)**

| Transgenic line | Background accession | Reference | Source |
|---|---|---|---|
| *hopAO1(Dex)* | *Col gl1* | Underwood et al., 2007 | He, MSU |
| *hopAI1(Est)* | *Col-0* | Li et al., 2005 | Zhou, National Institute of Biological Sciences, Beijing |
| *hopAM1(Dex)* | *Ws-0* | Goel et al., 2007 | Grant, Univ. of North Carolina at Chapel Hill |
| *avrPto(Dex)* | *Col-0* | Hauck et al., 2003 | He, MSU |

MSU (Michigan State University)

**Table 2.1 *Arabidopsis* accessions, mutant and transgenic lines.**

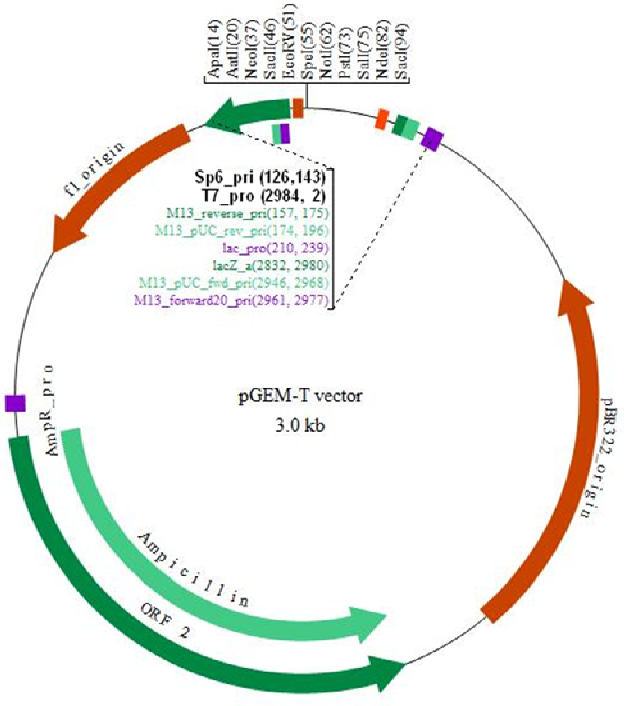
(a) SALK T-DNA insertional mutants were identified from SALK T-DNA database (http://signal.salk.edu/cgi-bin/tdnaexpress) and seeds for the lines were ordered from NASC. (b) Transgenic lines expressing either dexamethasone- (Dex) or estradiol (Est) – inducible effector protein from *Pseudomonas syringae*.

## 2.2 Generation of *AtGSNOR1::GUS*

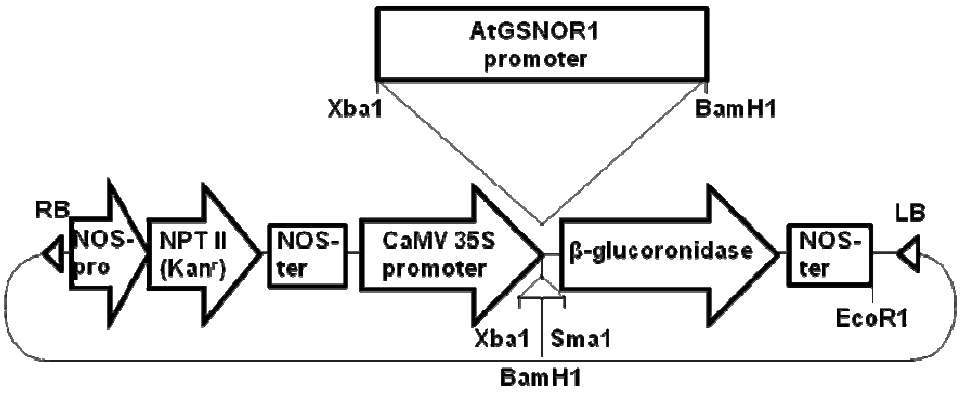
Transgenic *Arabidopsis* lines harbouring *AtGSNOR1::GUS* deletion constructs was generated in collaboration with Dr. Jeum Kyu Hong. The promoters sequences of *AtGSNOR1* were amplified from *Arabidopsis* genomic DNA using primers designed to amplify DNA fragments from -747 to +342 bp covering the 5' flanking region upstream of *AtGSNOR1*, first and second exon as well as first intron. Promoter sequences amplified by PCR were cloned into pGEM®-T Easy (Promega) and verified by nucleotide sequencing. Deletion promoter fragments were generated using site-specific primer pairs demonstrated in Table (2.2). The promoter was then subcloned into the XbaI and BamHI sites of the pBI121 expression vector (Clonetech Laboratories, USA). The insertion site was between the *CaMV* 35S promoter and *GUS* gene and the promoter was placed in the same orientation as the 35S-*GUS* gene. Subsequently, the complete cassette was released by digestion with XbaI and EcoRI and cloned into the pGreen-0229 binary vector (Hellen et al., 2000). The vector was then transferred into *Agrobacterium tumefaciens* strain GV3101 and used to transform *Arabidopsis* plants using the floral dip method (Clough and Bent, 1998). Transformation of female gametes was accomplished by dipping developing *Arabidopsis* influorescenses for a few seconds into a 5% sucrose solution containing 0.01-0.05% (v/v) Silwet L-77 and resuspended *Agrobacterium* cells carrying the genes to be transferred. Independent transgenic lines were screen by application of Basta.

| DNA fragments | Forward primer | Reverse primer |
|---|---|---|
| (-747 ~ +342) | TCTAGATGCTAAACCTCAGCAAAATCATGTGTT | GGATCCGTAAGCGTCGGTGTGACAAAG |
| (-531 ~ +342) | TCTAGAATAATTGTGAAATAAACCTAATTGCTATG | GGATCCGTAAGCGTCGGTGTGACAAAG |
| (-361 ~ +342) | TCTAGATAGCTTTATGGTAACGAGAAAGAAA | GGATCCGTAAGCGTCGGTGTGACAAAG |
| (-221 ~ +342) | TCTAGATGTTAACAATGAGCCGGCGTGA | GGATCCGTAAGCGTCGGTGTGACAAAG |
| (+1 ~ +342) | TCTAGAAAGACCACACTACTCTCTCTATCTCTCTT | GGATCCGTAAGCGTCGGTGTGACAAAG |
| (+72 ~+342) | TCTAGAATGGCGACTCAAGGTCAGGTTATCA | GGATCCGTAAGCGTCGGTGTGACAAAG |

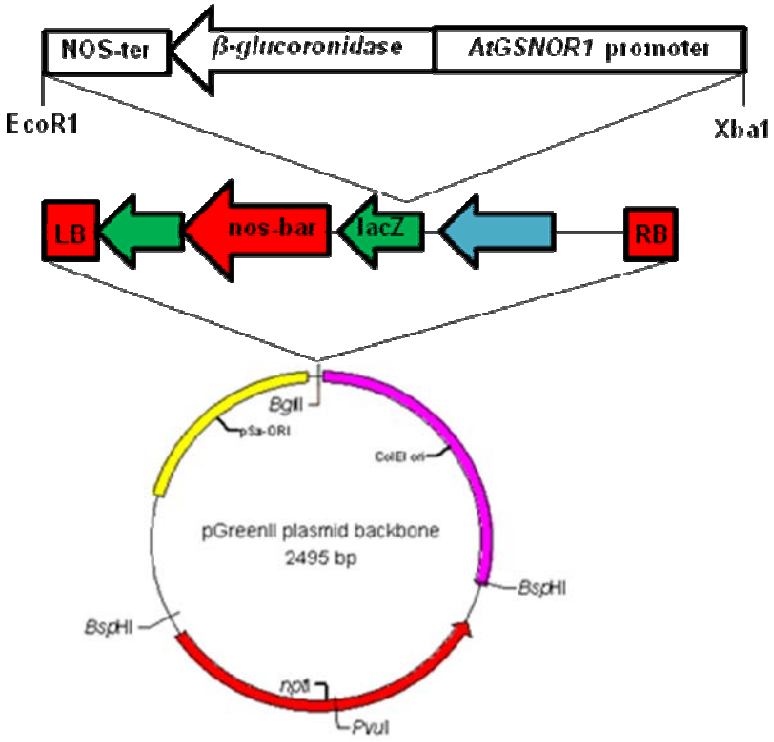
**Table 2.2 Primer pairs for generation of *AtGSNOR1::GUS* promoter deletion constructs.**




**(a) pGEM-T Easy vector**




**(b) pBI121 expression vactor**




**(c) pGreen-0229**

**Figure 2.1 Schematic diagram of vectors used for construction of *AtGSNOR1::GUS* transgenic lines**

(a) *AtGSNOR1* promoter fragment was cloned into pGEM-T Easy and putative positive clones sequenced for confirmation using Sp6 and T7 primers. (b) *AtGSNOR1* was cloned into Xba1 and BamH1 sites of pbBI121 expresion vector. (c) The complete expression cassette containing *AtGSNOR1* promoter fused to *GUS* reporter gene was cloned into the lacZ region of pGreen 0229 binary vector at EcoR1 and Xba1 sites.

### 2.3 Chemical treatments

The transgenic lines containing the BASTA resistance gene as a selection marker were selected by spraying with a 150 mg/l BASTA herbicide solution (Agrevo, Germany). Seedlings were sprayed 10 days after germination and again 4 days later.

Selection for antibiotic resistance and chemical treatments to induce transgene in the transgenic lines were carried out according to the references (Table 2.1)

### 2.4 Pathogens growth and inoculation

Bacterial pathogens carrying antibiotic resistance marker were grown in King's broth (KB) liquid media (King et al., 1954) supplemented with specific antibiotics for selection (Table 2.3). *Psp* did not carry any antibiotic resistance and was grown in KB liquid media without antibiotic. The culture was incubated at 30°C overnight under agitation. The bacterial cells were harvested by centrifugation at 4000 g for 5 minutes and resuspended in 10mM $MgCl_2$ to an $OD_{600}$ of 0.02 or 0.0001 (the equivalent of $10^7$ or $10^5$ colony forming units/ml) for gene expression analysis and SNO measurement. The leaves of 4 to 5 weeks old plants were infiltrated with 1 ml needleless syringe on the abaxial side and harvested at a certain time points for analysis.

*Bgt* was obtained from Syngenta (Jealott's Hill) and maintained on wheat cultivar Hereward. The wheat plants maintained in a growth cabinet and heavily infested with *Bgt* were shaken one day before the harvest of spores to displace older spores and ensure that freshly formed conidia were available. Leaf segments bearing conidia were harvested, and one leaf segment per pot was used to inoculate the leaves of Arabidopsis plants (4 to 5 weeks old) by dusting the conidia onto the leaves to be inoculated, then inoculated plants were incubated in greenhouse (Yun et al., 2003).

| Pathogens | Antibiotic selection | Reference |
|---|---|---|
| *Pst*DC3000 | 50µg/ml Rifampicin (Generics, UK) | Whalen et al., 1991 |
| *Pst*DC3000 *hrcC*$^-$ | 50µg/ml Chloroamphenicol | Yuan and He, 1996 |
| *Pst*DC3000 (avrB) | 50µg/ml Kanamycin | Whalen et al., 1991 |
| *Pst*DC3000 coronatine-deficient (cor$^-$) DB4G3 | 50µg/ml Kanamycin | Brooks et al., 2003 |
| *Pseudomonas syringae phaselicola (Psp)* 1448A | - | Taylor et al., 1996 |
| *Blumeria graminis f.sp. tritici (Bgt)* | - | Yun et al., 2003 |

**Table 2.3 Bacterial pathogens and selection marker**

PstDC3000, PstDC3000 hrcC$^-$, PstDC3000 (avrB) and PstDC3000 cor$^-$ were grown in KB liquid media supplemented with specific antibiotic at 50µg/ml for selection. Psp 1448A were grown in KB medium in the absence of antibiotic and Bgt growth was maintained on wheat.

### 2.5 Genomic DNA extraction

Genomic DNA was extracted from leaves of about 4 weeks old plants using cetyltrimethyl ammonium bromide (CTAB) extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, pH 8, 20mM EDTA and 0.2% 2-mercaptoethanol)(Ausubel et al., 1994). Excised leaf was ground with micro-pestle in 1.5 ml microtube in the presence of 500 µl pre-warmed CTAB buffer at 65°C and incubated for 30 minutes with grinding at 15 minutes interval. An equal volume of chloroform was added into the tube and vortexed for 10 seconds before being centrifuged at 12,000 g for 10 minutes at room temperature. This condition applies to all centrifugation steps. The upper layer was taken into fresh tube and an equal volume of chloroform was added. The tube was vortexed again and centrifuged. The upper clear part was removed into a new tube and added with 1 ml of 100% cold ethanol. The tube was kept at -20°C for one hour. After the incubation, the tube was centrifuged and the supernatant discarded. Pellet was washed with 1 ml of 75% (v/v) cold ethanol and vortexed to mix. The tube was centrifuged again and residual ethanol was taken out using a pipette. The pellet was dissolved in 50 µl deionized distilled water.

### 2.6 β-glucoronidase (GUS) assay

#### 2.6.1 Fluorometric assay

GUS enzyme activity was measured in fluorometric assay as described by Jefferson et al. (1987). Leaf samples were collected from 4-5 weeks old plant (~ 0.1 gram per plant) at 0, 2, 4, 8, 12, and 24 hour after pathogen inoculation or chemical treatments and immediately frozen in liquid nitrogen. The plant materials were ground in liquid nitrogen using mortars and pestles and were immediately transferred to 1.5 ml pre-chilled eppendorf tube. For each tube, 1ml of GUS extraction buffer (50 mM $NaH_2PO_4$, pH 7, 10 mM EDTA, 0.1 % Triton X-100, 0.1% Sodium lauryl sarcosine and 10 mM β-mercaptoethanol) was added and vortexed vigorously. The tubes were kept on ice before being centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant (~600 µl) was taken out into a new tube and protein concentration was measured by Bradford assay (Bradford, 1976).

A 100 µl aliquot of the supernatant (protein extract) was added into 1.5 ml reaction tubes containing 900 µl pre-warmed GUS assay buffer (GUS extraction buffer and 4-methylumbelliferyl – β-D-glucoronide trihydrate) to the final concentration of 1 mM 4-MUG and incubated at 37°C for 1 hour. A volume of 200 µl of the reaction mixture was immediately added into 2 ml GUS stop buffer (0.2 M $Na_2CO_3$) and vortexed for a 0 time control. The step was repeated for the same sample after 1 hour incubation and the amount of fluorescence was measured using spectrofluorometer (SPEX FluoroMax3, Horiba Scientific) with excitation filter at 365 nm (UV) and emission filter at 455nm (blue) in disposable plastic cuvettes. Freshly prepared 4-methylumbelliferone at different concentration in stop buffer was used to make a standard curve. GUS activity was expressed as picomoles MU (4-methylumbelliferone) per minute per milligram fresh tissue.

#### 2.6.2 Histochemical assay

Histochemical assay was carried out based on method described by Jefferson et al., 1987). Seedlings or excised plant organs (leaf, flower, root, stem or silique) were washed with 100 mM potassium phosphate buffer (pH 7) for 3 times. They were immersed in the staining solution (1mg/ml X-gluc, 100 mM potassium phosphate buffer, pH 7, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 10mM EDTA) in 1.5 ml microtubes and incubated at 37°C in the dark for overnight. After incubation, the staining solution was discarded and a sufficient volume of fixation solution (formalin:acetic acid:ethanol in a ratio 10:5:20 (v/v/v)) was added to the

same tube just to cover the sample and incubated for 10 minutes. The sample was cleared in 50% ethanol for 30 minutes followed by overnight incubation in 100% ethanol at room temperature. Samples were placed on a clean microscope slide and photographed using Nikon digital camera (Nikon, Japan).

**2.7 RNA blot hybridisation**

Total RNA was extracted from leaves of 4 weeks old plants using the TRIzol method according to the manufacturers instruction. The leaf tissues (approximately 100mg per sample) were ground to fine powder in liquid nitrogen using mortars and pestles and 1 ml of TRI™ Reagent or TRIzol (Invitrogen) was added immediately to dissolve it. The slurry was transferred into 1.5 ml microtube and vortexed to mix. The sample was centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was taken out into fresh tube and 200µl of choloroform was added. The was vortexed vigorously for 15 seconds and allowed to sit on ice for 3 minutes followed by centrifugation at 12,000 g for 15 minutes at 4°C. About 600µl of the aqueous phase was transferred to a new tube followed by addition of 300µl of each isopropanol and NaCl/Na-Citrate salt solution (1.2 M and 0.8 M respectively) with gentle mixing by inversion. The tubes were allowed to sit at room temperature for 10 minutes. Then they were centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was discarded carefully and pellet was washed with 1 ml of 75% (v/v) ethanol diluted in diethylpyrocarbonate (DEPC) water by vortexing briefly. After centrifugation at 7,500 g for 5 minutes at room temperature, the supernatant was carefully discarded. The residual supernatant was pipette out after a short spin. The pellet was resuspended in 40µl DEPC-treated water and incubated for 30 minutes at 60°C to dissolve it. The absorbance of each sample was measured at 260 nm and used to calculate the RNA concentration.

RNA samples (10µg) were separated on a formaldehyde-agarose (Sambrook et al., 1989) gel and then transferred to a Hybond$^{TM}$-N hybridisation membrane (GE Healthcare) according to the suppliers instruction. 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 DEPC-treated water for 5 minutes followed by 1X SSC, 1% SDS (w/v) for 15 minutes. Probes were prepared by amplification of appropriate sequences by PCR (Table 2.4) and purified using a PCR purification kit (QIAGEN). The size of the PCR product was verified by gel electrophoresis and sequencing. For hybridisation,

probes 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 minute. Blots were exposed to X-Omat-AR™ imaging film (Kodak). The stripping of blots was done by incubating membranes in boiling 0.1% SDS, before washing in 0.5X SSC for 30 minutes at room temperature.

| Probe name | Forward primer | Reverse primer | Size (kb) |
|---|---|---|---|
| AtGSNOR1 | GAGGTTCGGATGAAGATCCT | CTTGGAACGGAGTTGAT | 0.8 |
| GUS | CCGACGAAAACGGCAAGAAAA AGCTGT | CCAGAAGTTCTTTTTCCAGT ACCT | 1.0 |

**Table 2.4 Primers used for probe synthesis**

Forward and reverse primers used to amplify specific DNA fragments to generate probe for northern blot.

### 2.8 Reverse transcriptase – polymerase chain reaction (RT-PCR)

RNA was extracted and the concentration determined as before. One µg of RNA was used for RT-PCR using QIAGEN Omniscript RT (Qiagen) kit according to manufacturer's instructions. The PCR was carried out using 1µl cDNA as a template with specific primer pairs (listed in Table 2.5). The PCR program was roughly as follows: 94°C, 5 minutes; 94°C, 30 seconds; 57°C, 30 seconds; 72°C, 1 minutes; 27 cycles (*AtGSNOR1*) or 28 cycles (*Actin-2*) followed by additional extension at 72°C; 7 minutes. The optimal conditions for amplification (cycle number and annealing temperature) were experimentally determined by preliminary experiments. Optimal number of PCR cycles appropriate for optimal semiquantitative PCR analysis was done by identifying the number of PCR cycles that permitted detection of signals from a specific gene mRNA, by demonstrating that the amount of its products increased proportionally with the cycle number. In this manner, amplification was stopped before the signals for the cDNA reached saturation. Amplification was gel verified by electrophoresis.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *AtGSNOR1* | GAGGTTCGGATGAAGATCCT | CTTGGAACGGAGTTGAT |
| *Actin* | AATTGACGCAGATTATGTTTG | GCTCGTAGTGAGGGAGTACC |
| *HopAM1* | GCGCTCGAGCATATGGCACGCA | CAGAACCCAGCCACGCTGGCGTTATGAAG |
| *HopAI1* | AACTCGAGCGATAGAAAGCAGGAAAACAAC | TTTTGCAAGCGAGTCCAGGGCGGTGGCATC |
| *HopAO1* | TACTCGAGCGAGATAGTTCATACAGCTATG | CAACTAGTGCGAGAAACACTAAAGGGC |
| *AvrPto* | CCGCTCGAGACCATGGGAAATATATGTGTC | GACTAGTTCATTGCCAGTTACGGTACG |

**Table 2.5 Primers used for RT-PCR**

### 2.9 Polyclonal anti-AtGSNOR1 antibody production and optimization

To generate the antibody, 3mg histidine-tagged AtGSNOR1 protein (generated earlier by Dr. Yiqin Wang, Institute of Genetics and Developmental Biology Chinese Academy of Sciences) was purified. Half amount of the purified protein, 1.5mg was used to immunize the rabbit for the first time and another 1.5mg was used to repeat the immunization after 15 days. After 30 days, small amount of blood were collected and tested for the presence of antibody. Another round of immunization with more purified protein is required if there is no antibody detected in the test blood. Following successful detection of antibody, blood were collected from the immunized rabbit and freeze dried into powder.

In order to determine the right antibody concentration to be used, serial dilutions were performed with an initial 1:1000 dilution in 1X TBST [diluted from 10X TBS (12g Tris, 40g NaCl per litre, adjusted to pH 7.6) with 0.1% Tween] and tested with plant protein sample in western blot. Optimum antibody concentration was determined at 1:20,000 dilutions in 1X TBST in the presence of 5% milk powder to reduce non-specific background signal. Higher or lower dilutions will generate signals that are either too strong (dark blot) or too weak (almost no band detected).

### 2.10 Western blot analysis

Leaf tissues from 4 weeks old plants (200mg) were ground in liquid nitrogen, then added to 2 volumes of proteins extraction buffer (50mM Hepes pH 7.4, 5mM EDTA pH 8.0, 5mM DTT, 10mM NAF, 10mM $Na_3VO_4$, 50mM β-glycerophosphate, 1mM PMSF, 2ug/ul antipin, 2ug/ul aprotinin, 2ug/ul leupeptin). Samples were centrifuged for 15 minutes at 13,000 rpm at 4°C. The supernatant was collected and spun again at 13,000 rpm for 20 minutes at 4°C. The protein content was quantified by Bradford analysis (Bradford et al., 1976). Proteins were separated on a 12% SDS-PAGE gel at 80V for the first 30 minutes and at 100V for 2 hours. A PVDF membrane (GE Healthcare) was soaked in 100% methanol and 3mM Whatman paper was soaked in transfer buffer (25mM Tris, 200mM glycine and 20% methanol). Blotting was performed by electrotransfer at 25V for overnight in the cold room (4°C). Coomassie blue solution was used to stain the membrane to ensure the equal loading of protein samples. Blocking was performed in 1X TBS [diluted from 10X TBS (12g Tris, 40g NaCl per litre, adjusted to pH 7.6), 0.1% Tween and 5% milk powder for 1 hour at room temperature and the membrane washed 3 times with TBST (1X and 0.1% Tween). The blot was then incubated with a primary antibody at 1:20000 dilutions in 20ml of TBST at 4°C on a shaker for overnight. The HRP-conjugated secondary antibody (New England Biolabs) was then incubated with the blot in 20ml TBST for 1 hour on a shaker at room temperature. Finally the blot was washed 3 times with TBST. Protein detection was performed using Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare). The blot was incubated with solution A and solution B for 1 minute. The blot was exposed to x-ray film (Kodak).

### 2.11 S-nitrosoglutathione reductase in-gel activity assay

Proteins were extracted in 2 volumes of 50mM sodium phosphate, 1mM phenylmethylsulphonyl (PMSF) and protease inhibitor. Proteins were then separated on a non-denaturing 7.5% (w/v) polyacrylamide gel in Tris-boric-EDTA (TBE) buffer, pH 8. Gel**s** were soaked in 0.1 M sodium phosphate, pH 7.4, containing 2 mM NADH, for 15 minutes, in an ice-bath. Excess buffer was drained and gel**s** were covered with filter paper strips soaked in freshly prepared 3 mM GSNO. After 30 minutes, the filter paper was removed and gels were exposed to ultraviolet light and analysed for the disappearance of the NADH fluorescence, indicating GSNOR activity.

### 2.12 Densitometry quantification

PCR products, in gel assay products or autoradiograms of the immunoblots were scanned and loaded for analysis using Adobe Photoshop CS4 (Adobe System, Inc., San Jose, CA). The images were set to greyscale mode to discard any colour information and inverted so that the band was set to dark and the background was light. The mean and pixel value for each band were analyzed using lasso tool and recorded in a spreadsheet. The mean value was multiply by the pixel value for each band to give an integrated measure of the intensity and size of the band, or absolute intensity. To obtain a relative intensity, the absolute intensity of each band was divided by the absolute intensity of a standard which was set to 1. Standard error was calculated as the mean of at least three separate experiments repeated with similar results.

### 2.13 Determination of SNO levels

#### 2.13.1 Saville assay

Proteins were extracted in 100 mm Tris HCl, pH 6.8. The extracts were incubated for 5 min with an equivalent volume of solution A (1% sulfanilamide dissolved in 0.5 m HCl) in the presence or absence of solution B (solution A plus 0.2% HgCl2), allowing the development of the diazonium salt. The formation of the azo dye product was obtained by reacting the two samples for an additional 5 min with an equal volume of solution C [0.02% of *N*-(1-naphthyl) ethylenediamine dihydrochloride dissolved in 0.5 m HCl], and the absorbance was subsequently read at 550 nm with a multimode plate reader (SpectraMax M5, Molecular Devices). SNO content was quantified as the difference of absorbance between solution B and A (B − A), comparing the values with a standard curve made from a solution of GSNO.

#### 2.13.2 Gas-phase chemiluninescence

Proteins were extracted in 0.5 mM phosphate buffer and protease inhibitor and protein samples were analyzed as described (Pinder et al., 2008). SNO measurement was achieved by using Nitric Oxide Analyzer (NOATM 280i, SIEVERS, USA) according to the supplier's manual with CuCl in 1mM cysteine solution as a reductive agent.

## 3. Regulation of host S-nitrosylation and manipulation of *AtGSNOR1* during pathogenesis

### 3.1 Introduction

Plants have evolved remarkable defence strategies to counter microbial infections, and so do pathogens which have evolved virulence systems to overcome host defence and acquire nutrients. A variety of pathogenic microorganisms including viruses, fungus, bacteria and nematodes possess their own unique pathogenicity factors to effectively overcome several layers of defence and successfully colonize their hosts. Bacterial pathogen for example the well-characterized *Pseudomonas syringae* equipped with a *hrp*-gene-encoded TTSS which is essential for bacteria to cause disease in susceptible plants by secreting a plethora of effector proteins (Hueck, 1998; Galan and Collmer, 1999). In addition, *P. syringae* are also known to produce various phytotoxins which are required for its full virulence (Bender et al., 1999). Suppression of basal defence, gene-for-gene resistance and non-host resistance are established as the major virulence function of effector proteins and toxins.

One of the most important component of plant defence response is NO, a small free radical bioactive molecule that is well known as an important messenger in key biological processes in animals and plants (Lamattina et al., 2003). Predominantly, NO mediates signalling related to plant defence by regulating multiple nodes of plant immunity through NO-dependent protein modifications such as tyrosine nitration (Saito et al., 2006; Chaki et al., 2009; reviewed in Corpas et al., 2009) and S-nitrosylation (Stamler et al., 2001; Feechan et al., 2005; Tada et al., 2008).

S-nitrosylation is a form of reversible post-translational modification involving transfers of an NO moiety to a critical cysteine residue on a target protein to form SNO. S-nitrosylation of GSH forms GSNO, a low molecular weight SNO that may function as a natural NO reservoir. Additionally, GSNO has been implicated in systemic resistance against tobacco mosaic virus (TMV) in tobacco (Song and Goodman, 2001), as a strong inducer of plant defence gene (Durner et al., 1998) and generally in redox regulation (Wang et al., 2006; Lindermayr et al., 2006; Tada et al., 2008). Although there are still uncertainties about specific enzymatic mechanism involved in protein S-nitrosylation, degradation of GSNO promotes a denitrosylated state and so GSNO turnover, significantly influences the S-nitrosylation status of whole cells.

GSNO is mainly degraded by an NADPH-dependent GSNOR, also known as glutathione-dependent formaldehyde dehydrogenase (FALDH), an enzyme that is conserved in most major life forms (Liu et al., 2001). GSNOR plays a role in both glutathione-dependent formaldehyde oxidation and GSNO reduction but with high specificity towards the latter (Jensen et al., 1998). This is further supported by the fact that yeast, mouse and *Arabidopsis* GSNOR knockouts devoid of GSNOR activity show a substantial increase in SNO (Liu et al., 2004; Liu et al., 2001; Feechan et al., 2005). Diaz et al. (2003) first demonstrated that *Arabidopsis* GSNOR is transcriptionally regulated by signals related to plant defence but no experiments addressed any direct interactions between plant and pathogens. This work is followed by major findings from Feechan et al. (2005) where an *AtGSNOR1* loss-of-function mutation (*atgsnor1-3)* increased SNO levels which negatively affected multiple modes of plant disease resistance. More detailed study on S-nitrosylation status in *Arabidopsis* showed that a master regulator of salicylic acid-induced defence gene, *NPR1* is S-nitrosylated in *atgsnor1-3* thus hampering the expression of *NPR1* dependent defence gene *PR-1* (Tada et al., 2008). In conclusion, both studies successfully showed that *AtGSNOR1* is one of the most important components of the resistance signaling network through explicit control of SNO homeostasis.

NO and SNO is thought to follow distinct functional roles during establishment of disease resistance (Feechan et al., 2005). Thus, while NO accumulation is required for resistance against pathogen (Delledonne et al., 1998; Mur et al., 2005), an increase in SNO levels will inhibit the normal function of plant disease resistance components and thus promote pathogen susceptibility (Feechan et al., 2005; Wang et al., 2006; Tada et al., 2008). Therefore, to see whether an increase in host SNO levels are indeed required to aid pathogenesis, SNO levels were measured in wild type *Arabidopsis* in response to virulent *Pst*DC3000 and *Pst*DC3000 *hrcC* mutants which is defective in TTSS. For this reason, *AtGSNOR1* could be an attractive candidate for pathogens to promote pathogenicity through the suppression of its denitrosylase activity to induce cellular SNO levels. In order to investigate the possibility of *AtGSNOR1* being a virulence target for bacterial TTEs during pathogenesis, gene expression and enzyme activity of *AtGSNOR1* was monitored in wild-type plants during pathogen infection.

### 3.2 SNO measurement

Considering the sometimes extremely labile nature of the SNO bond, it is often demanding to identify and measure. In addition, SNOs are present in very low concentrations in vivo and are exquisitely sensitive to changes in protein structure and location. There are various available methods to measure SNO level in biological sample and they include chemuliminescense-based assays, colorimetric assay, assays using antibody against S-nitrosocysteine (CSNO), biotin-switch method and mass-spectrophotometric identification of the SNO containing species.

In the following experiments, a colorimetric assay (Saville-Griess) and a chemiluminescense-based assay were used to determine SNO level. Saville-Griess is considered as most commonly used method (Zhang and Loscalzo, 2000; Miranda et al., 2001; Basu et al., 2006) because it uses simple chemical reagents, does not require expensive instrumentation and suitable for routine analysis of large samples (Miranda et al., 2001). The Griess assay was formulated by Griess (1879) where in this reaction, nitrite reacts with sulfanilic acid under acidic condition to form the diazonium ion which couples to α-napthylamine to form a readily water-soluble, red colored azo dye. In the modified Griess method, sulfanilamide and N-(1-napthyl)ethylenediamine (NED) were used as nitrosable and coupling components respectively. This so-called Saville-Griess assay (Saville, 1958) works via mercury ($HgCl_2$)-mediated decomposition of SNO by releasing $NO^+$ followed by subsequent Griess reaction to form azo dye (Fig 3.1). Given that this method will as well measure contaminating nitrate and nitrite formed by SNO cleavage, the SNO concentration is taken as the difference between $NO_2^-$ concentration with and without addition of $HgCl_2$ in absorbance of 540nm. However, the only drawback of this method is the limit of detection for this assay (approximately 100nM to 500nM) which is ordinarily close to biological concentrations (Gow et al., 2008).

Chemulinescence-based techniques have been reported to detect SNO levels at least 1000 times smaller than the Saville assay, hence this assay is recognized as the most accurate and sensitive technique available to measure SNO (Stamler and Feelisch, 1996). The principle of chemiluminescent NO detection is based on the rapid reaction of NO in the gas phase with ozone ($O_3$), which forms excited $NO_2^*$ (Equation 1 and 2). As the excited electron decays back to its ground state, a photon is emitted that can be quantified as chemiluminescence ($h\nu$) and can be quantified by a photomultiplier to generate electrical signal (MacArthur et al., 2007).

$$NO + O_3 \rightarrow NO_2^* + O_2 \quad \text{(Eqn 1)}$$
$$NO_2^* \rightarrow NO_2 + h\nu \quad \text{(Eqn 2)}$$

This step is critically dependent upon the equal rate of mixing of NO and ozone (Sexton et al., 1994). A number of chemical reagents are available to selectively reduce NO-modified species for example S-nitrosothiols, iron nitrosyls or N-nitrosamines. In this analysis, cuprous chloride (CuCl)/I-Cysteine (CSH) was used to release NO from RSNOs in the presence of $Cu^+$. In addition to its sensitivity and accuracy, the neutrality of the CuCl/CSH reagent ensures that other metabolites such as nitrite or nitrate is not detected, rendering high specificity for RSNO detection (Fang et al., 1998). One of the difficulties encountered with the CuCl/CSH assay is foaming following injection of biological samples. However, this can be dealt with the addition of anti-foaming agent in limited quantities (0.1volume %) (MacArthur et al., 2007).

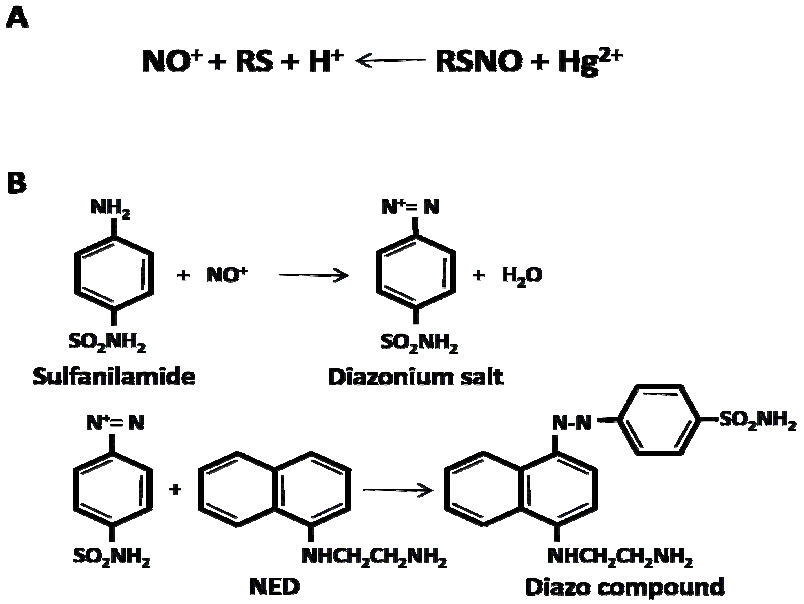



**Figure 3.1 Saville-Griess reaction.**

(A) Hg displaced NO from thiols in the form of nitrite. (B) Under acidic conditions nitrite reacts with the amino group of sulphanilamide to form the diazonium salt, which couples to N-(1-napthyl)ethylenediamine in *para*-position to form the diazo compound (Modified from Tsikas, 2006).

### 3.3 TTSS-dependent increased of S-nitrosothiol during pathogenesis

SNO levels were measured at early time points (within 24 hours following pathogen inoculation) during the course of pathogen infection. Samples were collected at the indicated time points and subjected to Saville-Griess analysis. The increase in SNO levels was evident from 2 hpi with 11.5% induction from initial level at time 0. At six hours after infection with *Pst*DC3000 ($1x10^7$ cfu/ml), SNO levels continue to increase to 15% followed by a minor drop at 12 hpi back to initial level. However, at 24 hpi SNO levels rise back up to 39% from initial level. Conversely, SNO levels barely increase in wild-type plant inoculated with *Pst*DC3000 *hrcC* ($1x10^7$cfu/ml). The levels only climbed as high as 2% from the initial level at 2 hpi preceded by 8% decrease at 6 and 12 hpi before it went back to initial level at 24 hpi (Fig 3.2a, statistical significance confirmed by ANOVA single factor, $p<0.05$).

To waive the possibility that changes in SNO levels following pathogen inoculation might be influence by high bacterial titre, SNO levels were measured in plants infected with lower bacterial titre, at $1x10^5$ cfu/ml, which probably better reveals a weak resistance effect and thus more sensitive. This time, the chemiluminescence-based method was utilized to measure SNO level using the NO analyzer. The initial level of SNO detected with the analyzer was similar to the level detected with Saville-Griess assay indicating the detectable level of SNO in wild-type Col-0 is around 50-52 pmole/mg protein (Fig 3.2b). After infection with *Pst*DC3000, SNO level increase gradually from the initial level to 44% at 1 dpi and 56% at 2 dpi. The next time point was 7 dpi and SNO levels had gone up to 300% compared to the initial level.

On the other hand, the increment of SNO level observed during *Pst*DC3000 *hrcC* infection was only half that of *Pst*DC3000. In fact, the level actually went down for as much as 39% at 1 dpi before it rise up to a level similar to the uninfected sample at 2 dpi. At 7 dpi, SNO level increased to 63% but the level was far less compared to *Pst*DC3000 infected sample measured at the same time. Both *Pst*DC3000 and *Pst*DC3000 *hrcC* cause a significant increase in SNO levels ($p<0.05$, ANOVA single factor) at 1 and 2 dpi. However, the increase in SNO levels at 7dpi were not significant ($p=1$) and this can be due to the fact that bacterial growth has reached stationary phase or the infected tissues were dying.

From this analysis, SNO levels were found to increase significantly during *Pst*DC3000 infection and only marginally during *Pst*DC3000 *hrcC* infection. *Pst*DC3000 *hrcC* can only grow poorly in wild type plant and lost the ability to cause virulence due to its inability to deliver TTEs into host plants. Therefore, the increase in SNO levels in host plant during *Pst*DC3000 infection is largely caused by the TTEs which are delivered through the TTSS.

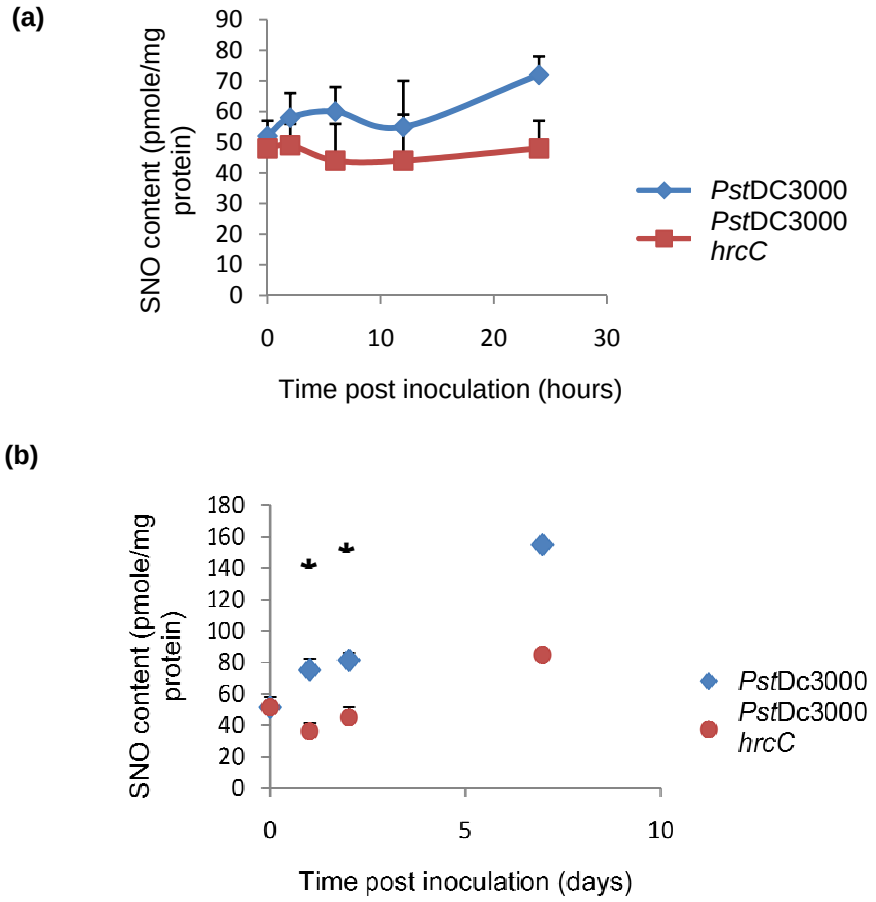
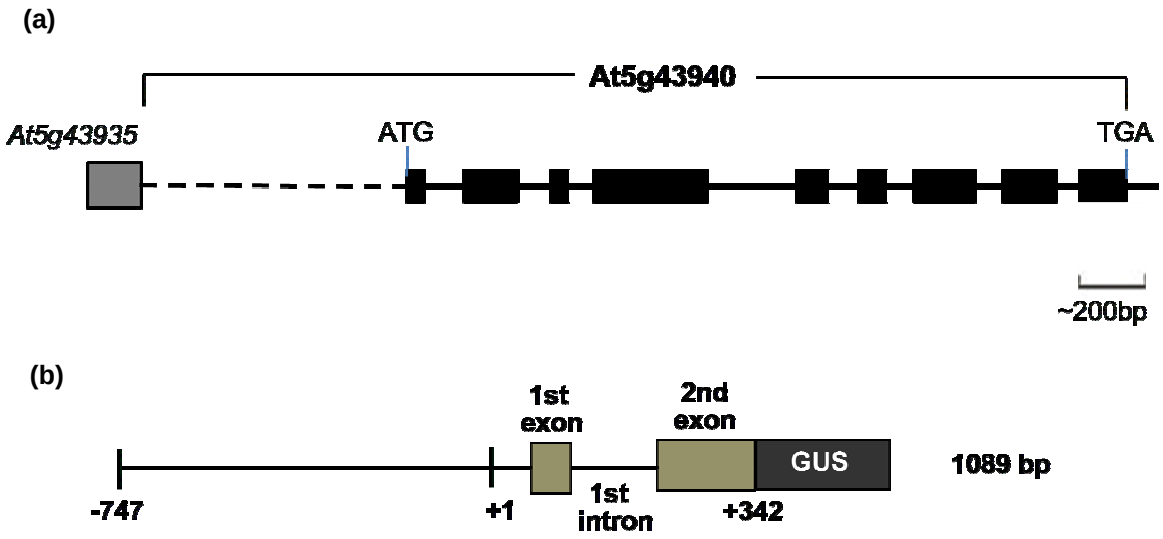



**Figure 3.2 Effect of pathogen infection of intracellular SNO levels**

*Arabidopsis* plants were infected with *Pst*DC3000 (blue line) or *Pst*DC3000 *hrcC* (red line) and leaf samples were collected for SNO measurement using (a) Saville-Griess assay or (b) chemiluminescence-based assay. Each data point consisted of two or three biological replicates. Asterisks indicate significant ($p<0.05$) increase in intracellular SNO levels. Error bars indicate standard deviation (SD).

### 3.4 Expression of *AtGSNOR1*

As a first step in the functional analysis of *AtGSNOR1* gene expression, cellular expression patterns of this gene during normal growth and developmental conditions were characterized. Previous attempts to generate AtGSNOR1 promoter β-glucoronidase (GUS) fusion construct by a previous members in our lab were not successful as GUS activity was failed to be detected in the resulting transgenic lines. Presumably, the promoter segment was incapable of conferring basal gene expression by itself. To overcome this problem, a fragment consisting of 747 bp of the 5' flanking promoter region and 342 bp of the protein coding was fused to GUS reporter gene and introduced into *Arabidopsis* Col-0 by *Agrobacterium*-mediated transformation. More than ten independent lines were generated and subjected to GUS histochemical analysis.



(c)

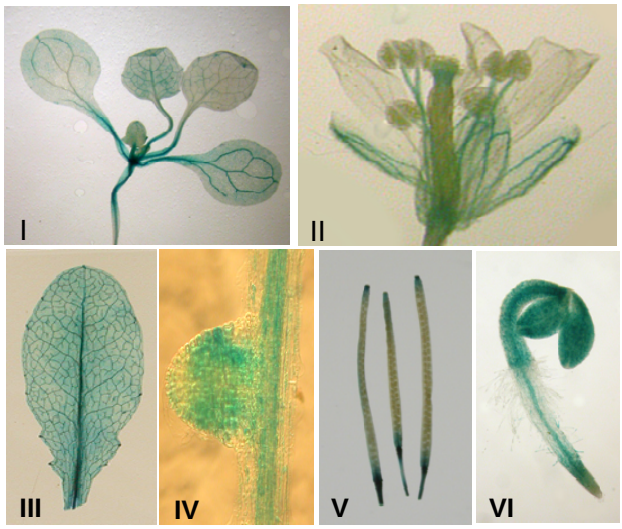


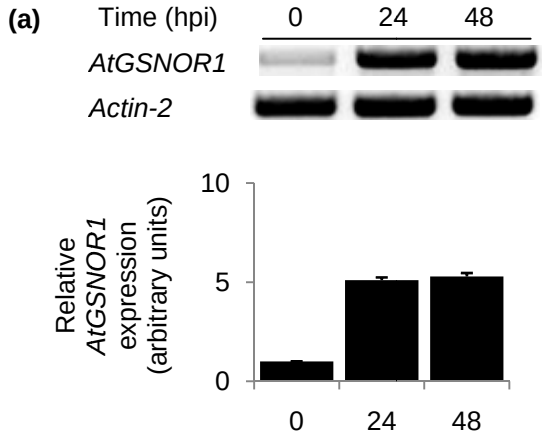

**Fig 3.3 Structure and histochemical localization of *AtGSNOR1::GUS***

(a) Schematic diagram of full-length *AtGSNOR1*. Putative promoter region is denoted by dash line. Exons and introns are represented by filled box and solid lines, respectively. (a) Schematic diagram of AtGSNOR1::GUS (b) X-Gluc staining for GUS activity in 14-days old seedling (I), flowers (II), rosette leave (III), roots (IV), siliques (V) and 3-days old seedlings (VI).

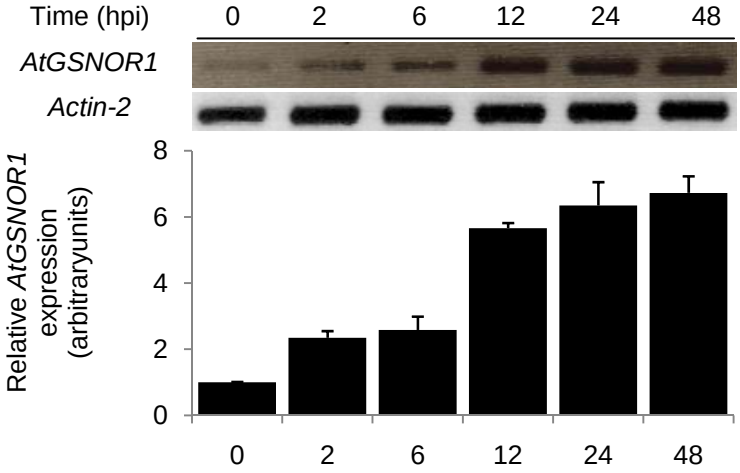
The *AtGSNOR1::GUS* translational fusion appeared to be active in the vascular region in most of the organs examined, with emphasize on leaf tissue. GUS staining was first detected in 3 days old seedling (Fig 3.3, IV) throughout the whole plant except the elongation zone of root tissue. 14 days after germination, GUS staining was confined to vascular region of cotyledons and upper part of hypocotyls (Fig 3.3, I) and as the plant matured, GUS activity was visible throughout the leaf blade (Fig 3.3, III). In the flowers, GUS activity was found in vascular region of sepal and stamen, and stigma (Fig 3.3, II). Some patchy staining was observed in root and lateral root primordium (Fig 3.3, IV) while in siliques, GUS staining was localized to the tip and base (Fig 3.3, V).

### 3.5 Induction of *AtGSNOR1* transcripts by non-host pathogens and wounding

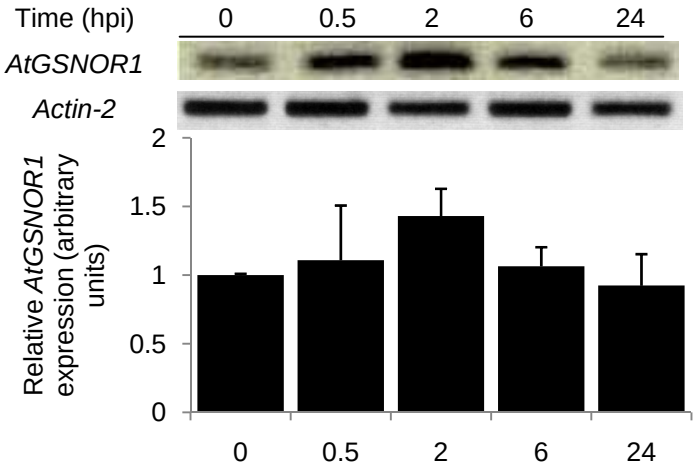
In an earlier study, *AtGSNOR1* has been proposed to define a general resistance mechanism (Feechan et al., 2005). In the study, an *AtGSNOR1* loss-of-function mutation supported a marked increase in haustoria formation and bacterial growth when tested with *Blumeria graminis* f. sp. *triciti* (*Bgt*) and *Pseudomonas syingae* pv. *phaseolicola* (*Psph*) (NPS3121), respectively (Feechan et al., 2005). To gain further insight into the non host interaction, *AtGSNOR1* expression was examined in response to *Bgt* and *Psph* (1448A) (Fig 3.4a). Induction of *AtGSNOR1* expression with *Psph* was evident from 2 hpi and increasing over time. Following infection with *Bgt*, *AtGSNOR1* expression was strongly induced at 24 hpi. Unfortunately, there was no data available for *AtGSNOR1* expression at earlier time points (2, 6 and 12 hpi) upon infection with Bgt. Based on *AtGSNOR1* induction profile in response to Psph, it is expected that *AtGSNOR1* would be similarly induced in response to Bgt infection at earlier time points, probably differ in kinetics and timing. Both pathogens induced *AtGSNOR1* transcript to higher levels at 48 hour post inoculation (hpi). Wounding treatment which was done using sharp laboratory forceps as well as mock infiltration with $MgCl_2$ solution marginally induced *AtGSNOR1* expression (Fig 3.4c, d). The transcript levels only increased to half of the wild type with very rapid and transient induction observed with wounding treatment whereas mock infiltration induced *AtGSNOR1* transcript to a slightly higher levels compare to wounding treatment and was clearly evident after 24 hours. Collectively, these results establish *AtGSNOR1* a pathogen-inducible gene in *Arabidopsis* whose expression promotes disease resistance by reducing SNO levels (Feechan et al., 2005).

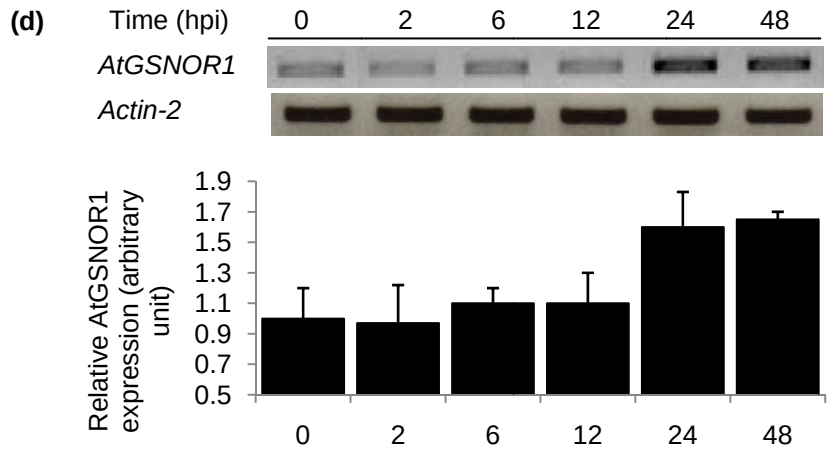


**(b)**


Time (hpi)
0
2
6
12
24
48
AtGSNOR1
Actin-2
Relative AtGSNOR1 expression (arbitraryunits)
8
6
4
2
0
0
2
6
12
24
48

**(c)**


Time (hpi)
0
0.5
2
6
24
AtGSNOR1
Actin-2
Relative AtGSNOR1 expression (arbitrary units)
2
1.5
1
0.5
0
0
0.5
2
6
24



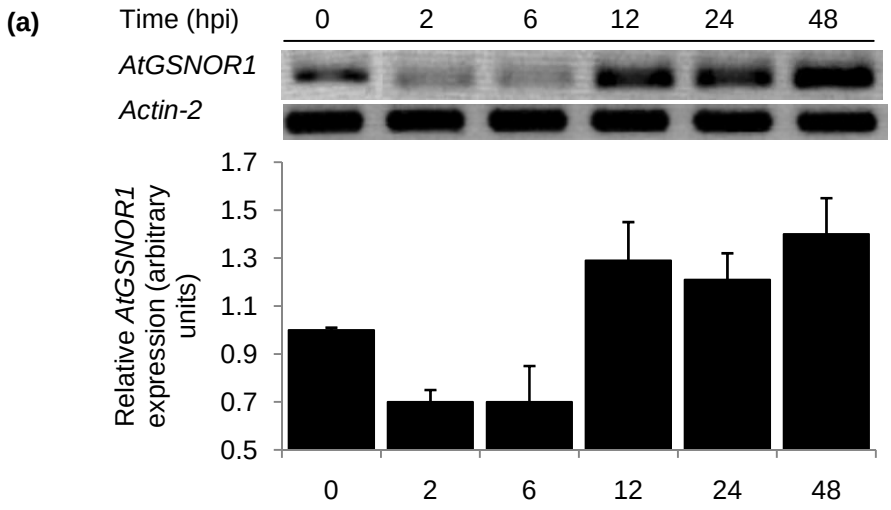

**3.4 *AtGSNOR1* expression is induced by non host pathogen and wounding.**

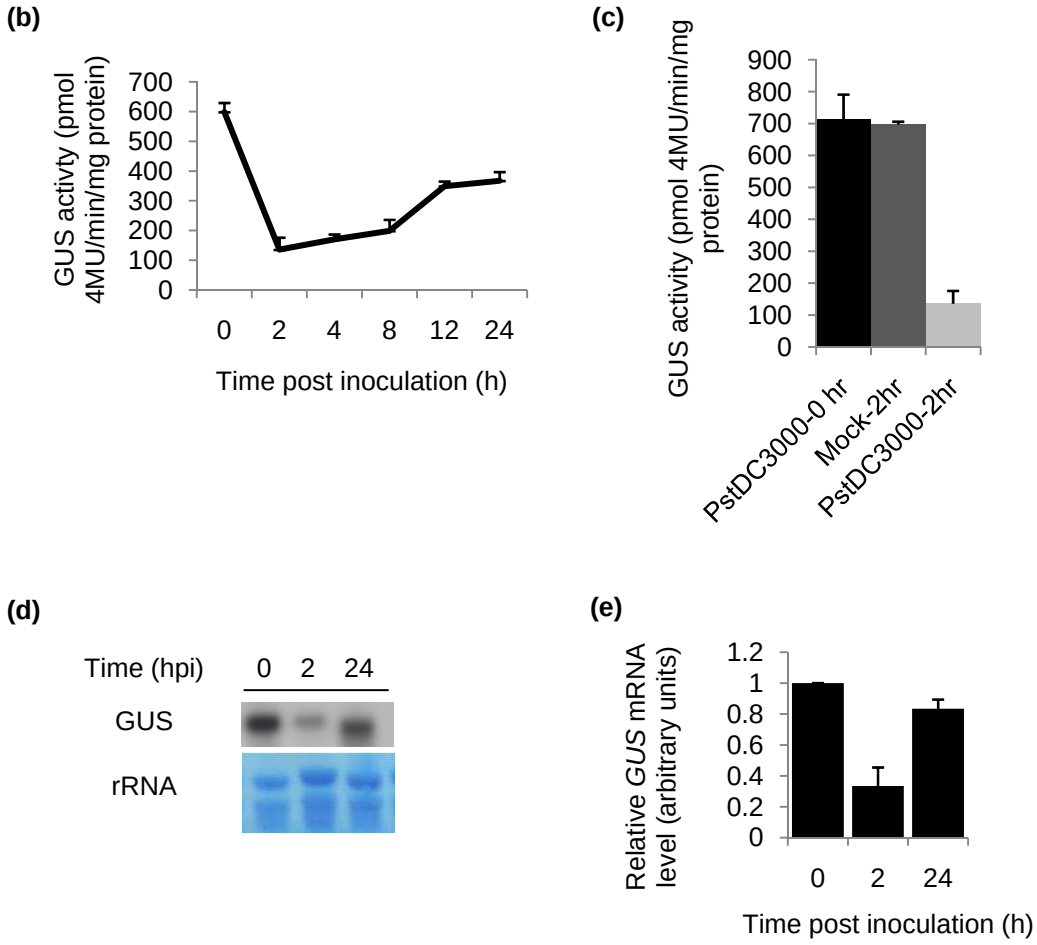
Col-0 was inoculated with (a) *Blumeria graminis* f. sp. *triciti* (*Bgt*) (b) *Pseudomonas syingae* pv. *phaseolicola* (*Psph*) (NPS3121), (c) wound treated and (d) mock infiltrated. RNA was isolated at indicated time for RT-PCR and A*ctin-2* was analyzed as a control. RT-PCR products were quantified by densitometry and all intensity readings are relative to the respective A*ctin-2* transcript. Results represent the mean and standard deviation of at least three biological replicates.

**3.6 TTSS-dependent suppression of *AtGSNOR1* expression during early stage of infection**

An important aspect of any host-pathogen interaction is the mechanism by which a pathogen infects its host. In order to investigate the expression of *AtGSNOR1* during compatible interaction, wild-type Col-0 plants were infiltrated with virulent *Pst*DC3000. Samples were then collected at the time points indicated and RNA extracted for RT-PCR. Following *Pst*DC3000 infiltration, transcript levels of *AtGSNOR1* decreased dramatically after 2 hours and remained suppressed for up to 12 hours (Fig 3.5a). *AtGSNOR1* transcript levels then started to increase again 24 hours after treatment and remained stable for the next 24 hours. Moreover, as discussed previously, mock infiltration with $MgCl_2$ did not suppress *AtGSNOR1* expression (Fig 3.5d); suggesting that *Pst*DC3000 actively suppressed *AtGSNOR1* expression at least at the level of transcription.

To see the suppression effect at the translational level, the transgenic plants harbouring full length *AtGSNOR1::GUS* construct (Fig 3.3b) were infected with *Pst*DC3000, *Pst*DC3000 *hrcC* or mock and examined for GUS activity using fluorometric analysis. The activity was measured from the infected leaves of at least four independent lines at indicated times. Upon infection with *Pst*DC3000, GUS expression driven by *AtGSNOR1* translational fusion reproduced the data from RT-PCR analysis (Fig 3.5b). There were transient reductions of GUS activities in the infected leaves, indicating a translational repression of the gene. The repression was apparent after 2 hours of inoculation, where there was a 5-fold reduction in GUS activity. After 24 hours of inoculation, gradually GUS activities were recovered to half that of the control. There was no reduction in GUS activity observed with mock inoculation at 2 hpi (Fig 3.5c). The reduction in the level of GUS activity following *Pst*DC3000 infection was correlated to the reduced level of GUS mRNA accumulation that was determined by northern blot using GUS specific primers (Fig 3.5d, e). Therefore, this data further confirmed that *AtGSNOR1* was down-regulated as a consequence of pathogen infection and this response requires both transcriptional and translational regulations.

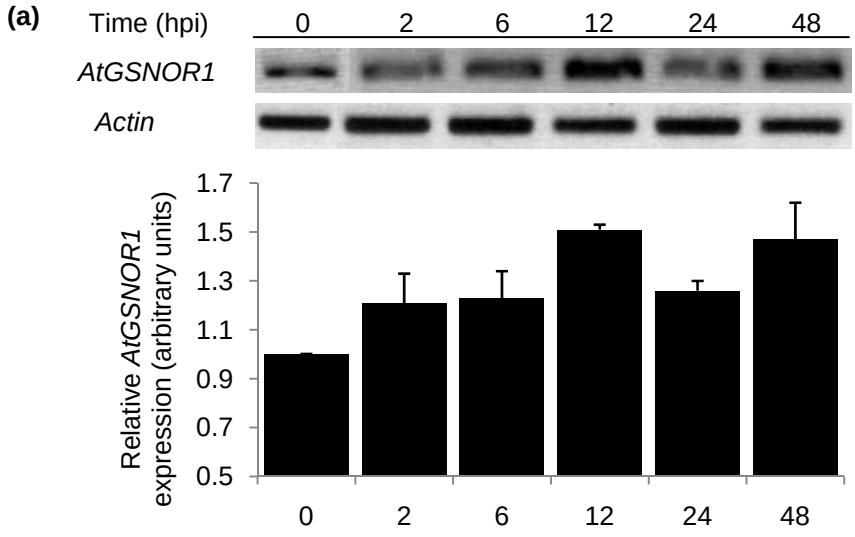


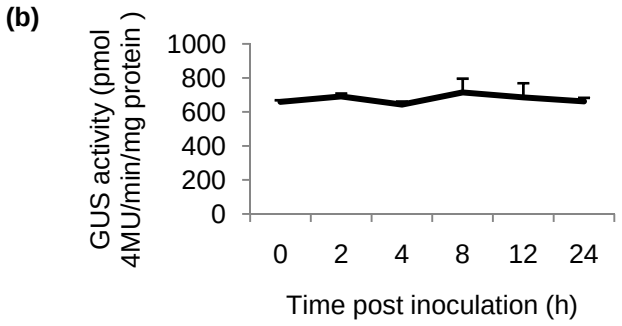



### 3.5 Transcriptional suppression of *AtGSNOR1* by *Pst*DC3000

Col-0 (a) and *AtGSNOR1::GUS* (b) were inoculated with *Pst*DC3000. Infected leaves were collected at indicated times for RT-PCR (a), GUS flurometric analysis (b, c) and northern blot with GUS specific primers (d). Mock inoculation and pathogen infection at 2hpi (c). *Actin-2* was analyzed as an internal control for RT-PCR. RT-PCR and northern blot products were quantified by densitometry. Results represent the mean and standard deviation of at least three biological replicates.

For a successful infection, *Pst*DC3000 requires a functional type three secretion system (TTSS) in order to deliver the type three effectors (TTEs) into the host cells and cause disease in susceptible plants (Nomura et al., 2005). To justify that the observed outcome is due to the virulence effect of TTEs, wild-type Col-0 was infiltrated with *Pst*DC3000 *hrcC* which is defective in TTSS. The hypersensitive response (HR) and conserved (*hrcC)* gene encodes an outer membrane protein that is essential for the TTEs secretion and has a primary role in protein translocation across the outer membrane (Charkowsky et al., 1997). Contrary to the suppression effect observed with virulent *Pst*DC3000, there was no reduction in *AtGSNOR1* transcript levels in all time points tested (Figure 3.6a) indicating that the TTSS is largely responsible for the suppression of this gene. Similarly, inoculation of *AtGSNOR1::GUS* with *Pst*DC3000-*hrcC* did not decrease GUS activity in the cognate transgenic plants (3.6b). Together, these observations indicated that *AtGSNOR1* transcript accumulation is transcriptionally suppressed in the early stage of infection by *Pst*DC3000 and that the suppression is TTSS-dependent.



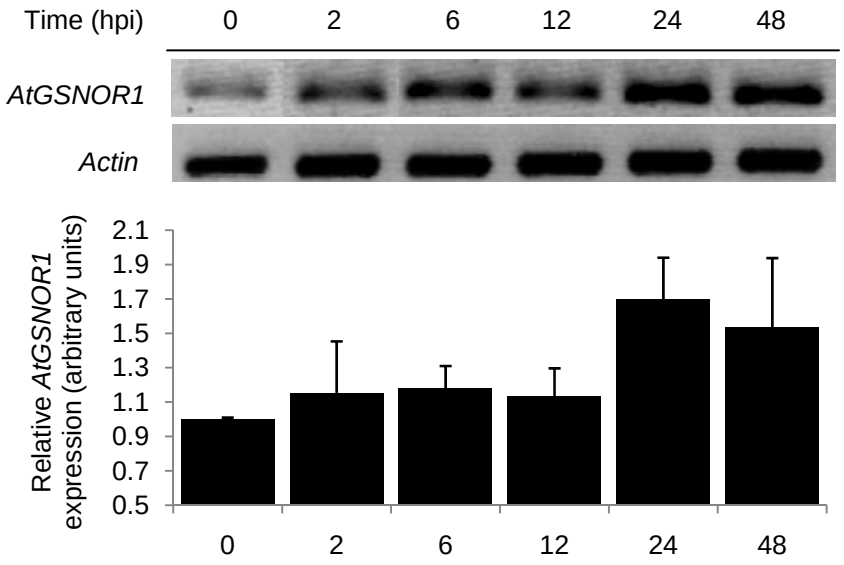



### 3.6 TTSS-dependent suppression of *AtGSNOR1*

Col-0 (a) and *AtGSNOR1::GUS* (b) were inoculated with *Pst*DC3000 *hrcC*. Infected leaves were collected at indicated times for RT-PCR (a) and GUS flurometric analysis (b). *Actin-2* was analyzed as an internal control for RT-PCR. RT-PCR products were quantified by densitometry. Results represent the mean and standard deviation of at least three biological replicates.

### 3.7 Gene-for-gene resistance activates *AtGSNOR1* expression

In response to virulence gene products which can cause disease symptoms in host cells, plant have evolved a repertoire of resistance (R) proteins that can recognize either directly or indirectly delivered effector proteins triggering the expression of defence mechanisms. Such recognized effectors are also termed avirulence proteins. *AtGSNOR1* is required for *R* gene-mediated resistance against *Pst*DC3000 containing an avirulence gene (Feechan et al., 2005). Wild-type plants expressed *R* gene-mediated resistance against *Pst*DC3000 (*avrB*) due to the presence of the *R* gene *RPM1* (Grant et al., 1995). Inoculation with *Pst*DC3000 bacteria containing *avrB* was unable to suppress *AtGSNOR1* expression (Fig 3.7). Instead, there was a small increase in the transcript level from 2 hpi which peaked at 24 hpi before it went slightly down at 48 hpi.



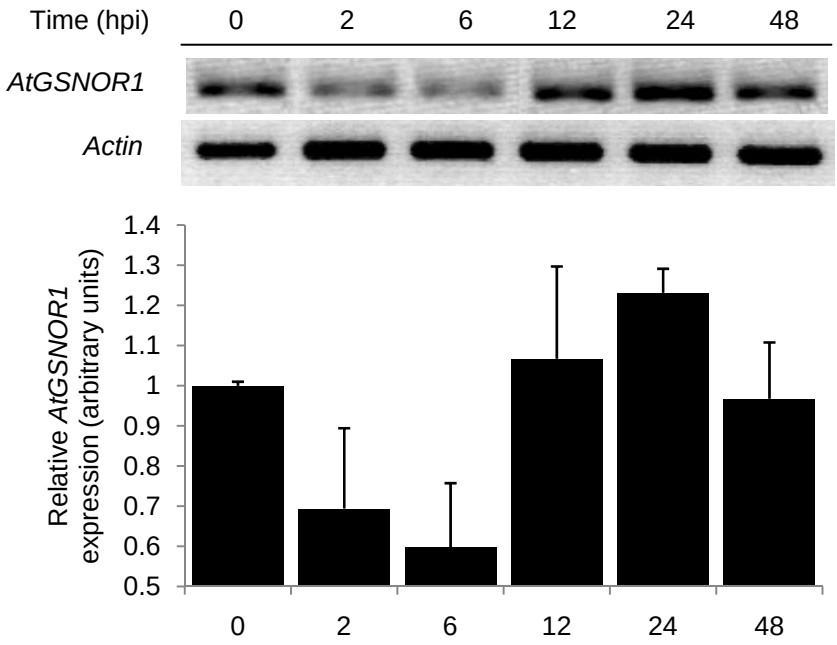

**3.7 Induction of *AtGSNOR1* expression in response to *Pst*DC3000 (*avrB*)**

Col-0 was inoculated with *Pst*DC3000 (*avrB*) and RNA was isolated at indicated times for RT-PCR. *Actin-2* was analyzed as an internal control. RT-PCR products were quantified by densitometry. Results represent the mean and standard deviation of at least three biological replicates.

**3.8 Coronatine is not required for *AtGSNOR1* suppression**

Production of the phytotoxin coronatine appears to be an important component in plant pathogenesis for most *P. syringae* pathovars including *Pst*DC3000 (Bender et al., 1987; Gross, 1991). Moreover, previous works proposed that coronatine and TTEs modulate the expression of a similar set of plant genes (Alfano et al., 2000; He et al., 2004). Coronatine exerts its virulence function by masquerading as one or more jasmonate analogues, stimulating jasmonates signaling during *Pst*DC3000 infection in order to suppress SA-dependent defences (Brooks et al., 2003). This suggestion led to an experiment to test whether coronatine also contributes to the suppression of *AtGSNOR1* expression. In this experiment, wild-type Col-0 was infiltrated with

*Pst*DC3000 *cor*-, a mutant of *Pst*DC3000 that is blocked in the synthesis of coronatine. There was a slight decrease in the transcript level observed at 2 and 6 hours post inoculation (Fig 3.8). Suppression of *AtGSNOR1* transcript by *Pst*DC3000 *cor*- was almost similar to the suppression observed with virulent *Pst*DC3000 (Fig 3.5a). There was a transient decrease in the transcript levels at 2 and 6 hpi and the levels went up again at 12 hpi . This result suggests that coronatine may not be required for *AtGSNOR1* suppression by *Pst*DC3000.




**3.8 Suppression of *AtGSNOR1* expression in response to *Pst*DC3000 *cor*-**

Col-0 was inoculated with *Pst*DC3000 *cor*- and RNA was isolated at indicated times for RT-PCR. *Actin-2* was analyzed as an internal control. RT-PCR products were quantified by densitometry. Results represent the mean and standard deviation of at least three biological replicates.

### 3.9 TTSS-dependent suppression of *AtGSNOR1* enzyme activity and protein levels

In principal, changes in transcript levels don't necessarily reflect the response at the protein level or at the level of enzyme activity (Steinmetz and Deutschbauer, 2002; Glanemann et al., 2002; Greenbaum et al., 2003). The impact of changes in transcript level on subsequent cellular events depend on rates of turnover of the encoded proteins, their contribution to the control of particular pathways in which they are involved in and the rates of turnover of the final products. Therefore it is important to analyze changes in protein levels and activity to understand the molecular function of specific protein and hence the gene which encode them.

To establish the effect of the *AtGSNOR1* transcript suppression on the enzyme activity, an in gel activity assay was performed based on the ability of *AtGSNOR1* to specifically metabolize GSNO. This assay was developed based on the original method by Seymour and Lazarus (1988) to detect pyridine nucleotide–linked dehydrogenases. Generally, crude protein extracts from Col-0 inoculated with *Pst*DC3000 or *Pst*DC3000 *hrcC* were first separated on native-PAGE. Following incubation with substrate and cofactor, in this case GSNO and NADH, respectively bands were visualized under UV light where the oxidized cofactor appears black (Seymour and Lazarus, 1988; Barroso et al., 2006).

The staining for GSNOR activity in *Arabidopsis* Col-0 showed a single band, as shown by a black arrow in Fig 3.9a. There was a delay in the suppression of enzyme activity as compared to the suppression of the transcript levels shown in Fig 3.5a. In the compatible interaction with *Pst*DC3000, the activity gradually reduced starting from 2 hpi and maximum suppression in enzyme activity is observed at 12 hpi (Fig 3.9a). The activity started to increase at 24 hpi and at 48 hpi, the activity was slightly higher compared to the infected sample at time 0. However, the enzyme activity in *Pst*DC3000 *hrcC* inoculated plants remained stable at most time points with exception of 12 hpi where there was a slight increase in activity (Fig 3.9b).

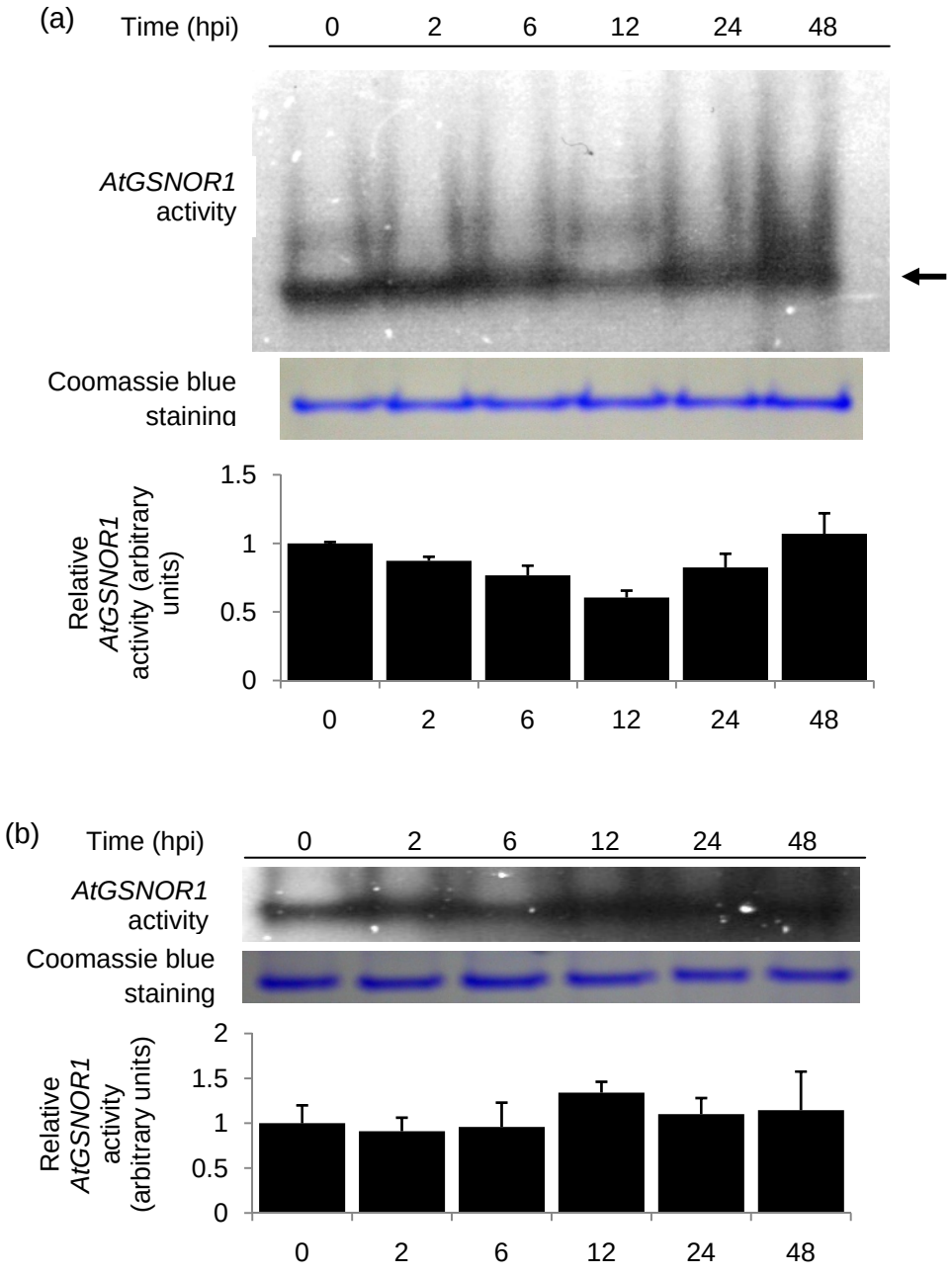
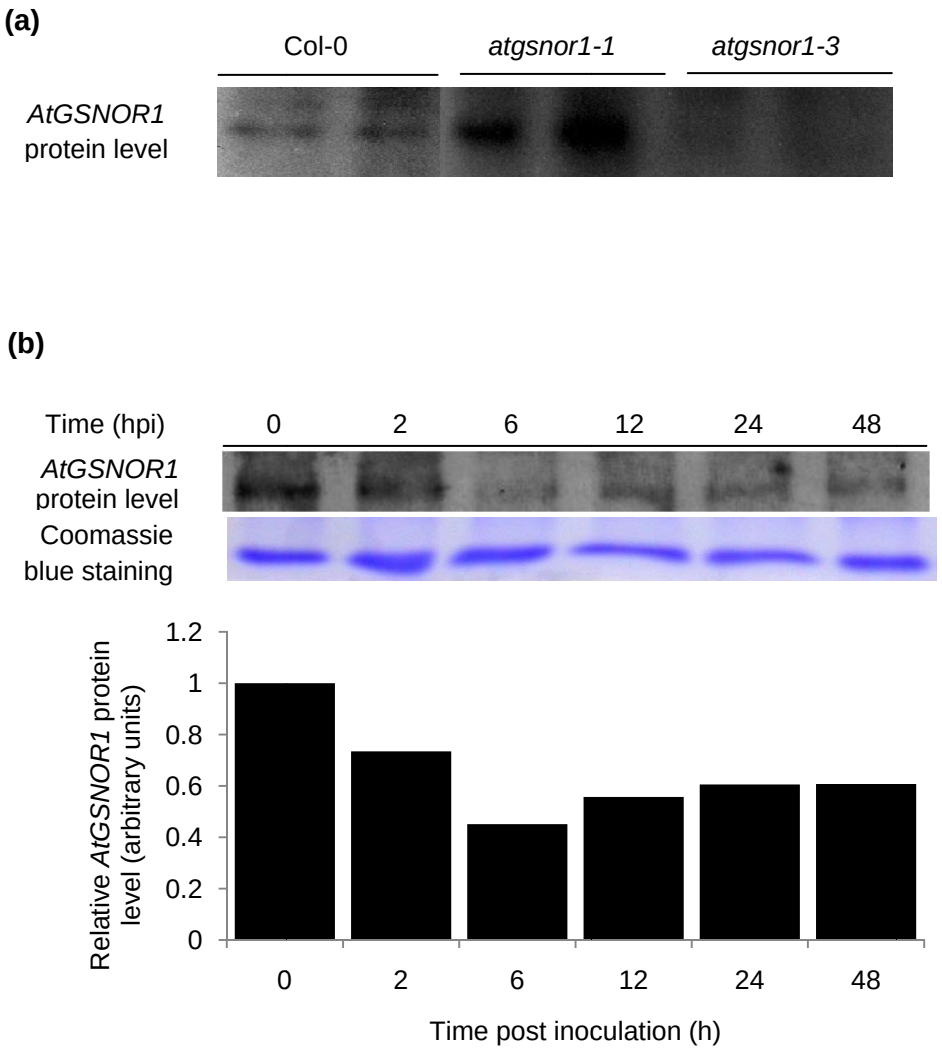



**Figure 3.9 In-gel staining for *AtGSNOR1* activity**

Col-0 was inoculated with *Pst*DC3000 (a) and *Pst*DC3000 *hrcC* (b). Infiltrated leaves were sampled at the time points indicated and protein extracts (10µg protein) were subjected to electrophoresis on 7.5% native-PAGE. Equal protein loading was confirmed by Coomassie blue staining. Gel products were quantified by densitometry. Results represent the mean and standard deviation of at least two biological replicates.

To check the protein level, proteins were extracted from infected plants. For each sample, a total of 10 µg protein was fractionated on SDS-PAGE and detected on western blots by using anti-GSNOR polyclonal antibody. The antibody recognized a band of 41kDa in leaf extracts from wild-type plant which is induced in *AtGSNOR1* T-DNA insertion line (*atgsnor1-1*) (Feechan et al., 2005) and absent in immunoblots with *atgsnor* loss-of-function mutant (*atgsnor1-3*) (Feechan et al., 2005) (Fig 3.10a). The protein level in wild-type plant following *Pst*DC3000 infection was consistent with the level of mRNA and relative enzyme activity which were determined earlier (Fig 3.10b). As expected, a change in the protein level was less rapid compared to the changes in mRNA levels. Thus, protein levels did not decrease until 6 hpi. While in *Pst*DC3000 *hrcC* infected plants, no major difference was observed in the *AtGSNOR1* protein levels (Fig 3.10c).



**(c)**

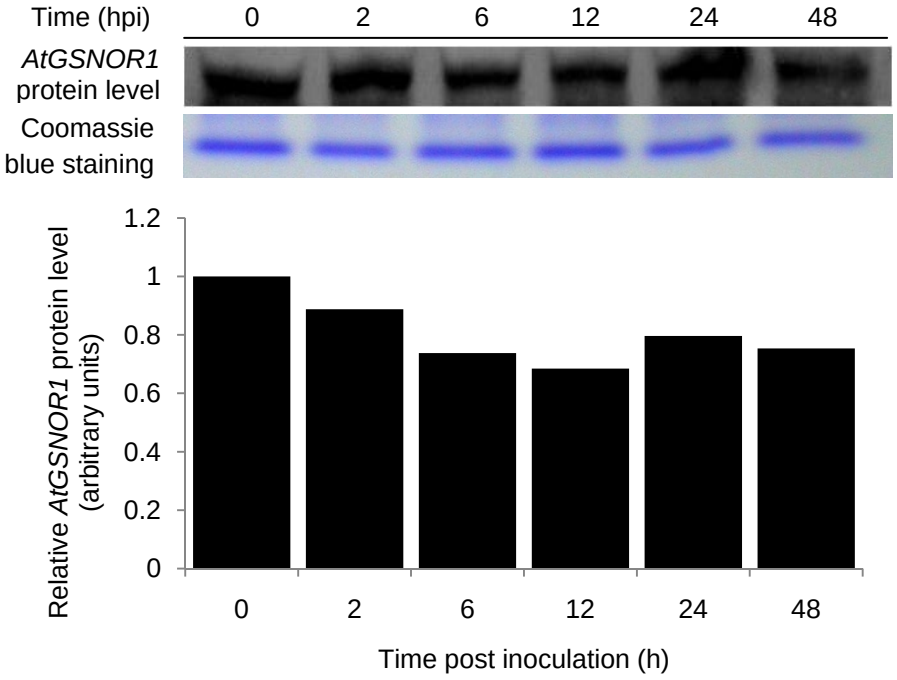



**Figure 3.10 *AtGSNOR1* proteins accumulation**

Total protein was isolated from Col-0, *atgsnor1-1 and atgsnor1-3* in the absence of pathogen (a). Total protein was isolated from leaves infected with *Pst*DC3000 (b) or *Pst*DC3000 *hrcC* (c) at indicated time points. The protein (10 µg) was separated on 7.5% SDS-PAGE gels and analyzed using anti-GSNOR antibody. Equal protein loading was confirmed by Coomassie blue staining (lower panel). Gel products were quantified by densitometry. Due to difficulties in obtaining a clear blot with anti-GSNOR antibody, results only represent a single blot for each experiment.

### 3.10 Discussion

As reported in the previous studies, higher SNO levels promote disease susceptibility by shutting down the normal function defence signalling components in plants, for example the master regulator SA-induced defence, *NPR1* (Tada et al., 2008). In order to elucidate if a virulent bacterial pathogen promotes pathogenesis by increasing the total intracellular SNO levels, we measured SNO content at different time points during early and later stages of pathogen infection. From the analysis, SNO levels barely change following inoculation with *Pst*DC3000 and *Pst*DC3000 *hrcC* within 24 hours. After 24 hours, the level starts to rise up gradually and are maintained in an induced state for up to seven days post inoculation with both strains suggested that the engagement of SNOs during plant-pathogen interaction was not evident until 24 hours.

This data supported the hypothesis that pathogen manipulates intracellular SNO levels to aid pathogenesis. This is true for both virulent *Pst*DC3000 and *Pst*DC3000 *hrcC* mutant, in which the latter is defective in TTSS. However, the effect was stronger in virulent pathogen suggesting that TTSS enhanced the increase of the intracellular SNO during infection to promote disease susceptibility. A similar observation was reported in *atgsnor1-3*, a T-DNA insertion line resulting in a loss-of-function mutation of *AtGSNOR1* which conveys increased susceptibility to bacterial pathogens (Feechan et al., 2005). Inoculation with the avirulent pathogen, *Pst*DC3000 (*avrB*) was reported to cause a modest increase of SNO levels in wild-type plant whereas in *atgsnor1-3*, the levels increased substantially indicating that in both cases, attempted pathogen infection triggers a pronounced accumulation of SNOs in host cells. Elevated SNO levels detected in *atgsnor1-3* upon avirulent pathogen challenge and in wild-type plant following virulent pathogen infection are associated with enhanced disease susceptibility.

The initial rise of NO as well SNO levels following both avirulent and virulent pathogen infection could be part of a host response to PAMPs (Delledonne et al., 1998). These general elicitors are the first microbial components that come into contact with host surface and triggered a series of signalling events that eventually activate a basal defence mechanism to halt pathogen growth (Nurnberger et al., 2004). However, as the infection process progressed, NO contributes to disease resistance in incompatible interaction through its immediate regulatory action in conjunction with ROIs during the establishment of the HR. Conversely, virulent

pathogen engages the host in compatible interaction where SNO levels are manipulated to promote virulence through S-nitrosylation of key regulator of plant defence.

As an important enzyme in controlling SNO homeostasis in animals and plant system, GSNOR is indispensable for providing protection from nitrosative stress (Liu et al., 2001). From this study, *AtGSNOR1* is found to be constitutively expressed in all organs in *Arabidopsis* indicating the general importance of this gene. GSNOR is also highly expressed and conserved in all other living organisms ranging from bacteria to humans (Liu et al., 2001). *AtGSNOR1* function in *Arabidopsis* comprises several physiological roles related to NO homeostasis. In addition to its original ascribed functions as a type III alcohol dehydrogenase and GSNO reductase (Uotila and Koivusalo, 1979; Sakamoto et al., 2002; Feechan et al., 2005), Lee et al. (2008) reported that *Arabidopsis HOT5* (sensitive to hot temperature) also encodes for GSNOR with a prominent role in thermotolerance, plant development and fertility, which further confirms the importance of GSNOR in modulating nitrosative stress. Fascinatingly, a recently discovered *Arabidopsis* gene *Paraquat Resistant 2* (*PAR2*) also encodes for GSNOR by acting downstream of superoxide to regulate cell death through modulation of intracellular NO levels (Chen et al., 2009). Furthermore, loss-of-function mutation of *AtGSNOR1* resulted in dwarf plants with bushy phenotypes, lower seed production, reduced fertility (Feechan et al., 2005, unpublished data) and enhanced susceptibility towards pathogen infection (Feechan et al., 2005). Together, these results imply that *AtGSNOR1* function is required to maintain normal growth and development and also in response to environmental stresses.

A previous study has revealed that *AtGSNOR1* is required for non-host resistance expressed against fungal and bacterial pathogens (Feechan et al., 2005). In conjunction with the previous study, we showed that *AtGSNOR1* expression is induced during non-host interaction with *Bgt* and *Psph*. The temporal pattern is similar to *NHO1* induction observed in response to *Psph* infection (Kang et al., 2003). *NHO1* is a gene from *Arabidopsis* that encodes glycerol kinase which is required for both general and specific resistance and is targeted by bacterial pathogens for parasitism (Lu et al., 2001; Kang et al., 2003; Li et al., 2005). The increased resistance to *Pst*DC3000 in *NHO1* overexpression plants (Kang et al., 2003) and *atgsnor1-1* mutant with increased levels of *AtGSNOR1* (Feechan at al., 2005) suggests an important role of these genes in disease resistance or parasitism. However, in contrast to host resistance, non-host resistance

operates under less understood mechanism (Mysore and Ryu, 2004). In this case, the recognition events that lead to induction of *AtGSNOR1* expression during non-host interaction, is not clear.

Plants respond to wounding by activating a set of defensive genes, such as phenylalanine ammonia lyase (*PAL*), proteinase inhibitor II genes of potato and tomato as well as wound inducible gene (*wun1*) from potato (Sanchez-Serrano et al., 1987; Logemann et al., 1989). Most of these genes play some role in wound healing and the prevention of subsequent pathogen invasion (Bowles, 1990; Conrath et al., 2001). There were a few conflicting reports on *AtGSNOR1* response to wounding stress. In this study, it was found that *AtGSNOR1* is induced in response to wounding. In contrast, Diaz et al (2003) reported the down-regulation of *AtGSNOR1* expression by mechanical wounding which is supported by publicly available microarray data from AtGenExpress Visualization Tool (TAIR, http://www.arabidopsis.org/index.jsp). This difference could be attributed to the different stages of plant growth and different growth condition used for the wounding treatment. These two aspects are found to be very crucial in stress related experiments (Kus et al., 2002; Rusterucci et al., 2007). In our long day (20ºC, 16 hours light and 8 hours dark) growth chamber, 6 weeks old plants already have undergone transition from vegetative to reproductive stage of development. Instead of using 4-6 weeks old plants, we strictly used 4 weeks old plants that have not yet entered the reproductive phase. In the publicly available microarray data, Harter et al. (2007) used seedlings as plant materials thus generating data that showed minor suppression of *AtGSNOR1* during the first 30 minutes followed by increasing levels of *AtGSNOR1* expression for up to 12 hours before it went slightly down at 24 hours, comparable to *AtGSNOR1* expression profiled in this study. Hence, it most likely that the expression of *AtGSNOR1* is up-regulated by wounding based on the reports from earlier studies carried out in different plant species that *GSNOR/FDH* expression is induced upon wounding (Kato-Naguchi, 2001; Barroso et al., 2006).

The suppression of host immune mechanism, including basal resistance, HR and *R* gene-mediated resistance as well as non-host resistance are major virulence strategies for pathogenic bacteria of plants. Most of these pathogenic bacteria depend on specific effector proteins to increase their virulence in host tissues. In *Pst*DC3000 alone, more than 40 distinct TTEs are delivered into host cells with abilities to manipulate host cellular processes for their benefit (Lewis et al., 2009; Cunnac et al., 2009). For example, AvrPto and AvrPtoB from *Pst*DC3000

can suppress basal resistance in *Arabidopsis* (Hauck et al., 2003; de Torres, 2006) and at the same time possess the ability to suppress HR and block *R* gene-mediated resistance in non-host plants (Abramovitch et al., 2003; Kang et al., 2004). NHO1, a key player in non-host resistance is also subjected to an active suppression from unknown TTEs from *Pst*DC3000 (Kang et al., 2003). Suppression of host defences is also well described for animal bacterial pathogens (reviewed in Galan and Collmer, 1999). However, the vast majority of host targets remain unknown and the mechanisms by which effector proteins subvert host immune response are poorly understood at the molecular level.

In this study, we identified *AtGSNOR1* as a virulence target for *Pst*DC3000. The transient suppression of *AtGSNOR1* expression in the early stage of infection relies on the secretion of TTEs via the TTSS because inoculation with *Pst*DC3000 defective in TTSS was unable to suppress the transcript accumulation in wild-type plants. This TTSS-dependent suppression is also observed at the protein level where the denitrosylase activity of *AtGSNOR1* is down-regulated. Conversely, coronatine which is another virulence factor in *Pst*DC3000 only play a minor role in the suppression of *AtGSNOR1* expression. Therefore, it is tempting to speculate that at least one of the secreted effectors could be targeting *AtGSNOR1* either directly by acting on its transcriptional and translational machinery or indirectly through modification of cofactors that might involve in regulating *AtGSNOR1* expression. This finding is also consistent with data that shows *AtGSNOR1* expression significantly reduces *Pst*DC3000 growth in planta (Feechan et al., 2005).

It is striking that *Pst*DC3000 carrying the *avrB* gene reactivated *AtGSNOR1* expression. Therefore, *R* gene–mediated resistance abolished the suppression of *AtGSNOR1* by *Pst*DC300. Even though *R* gene-mediated resistance was only reduced rather than abolished in the *atgsnor1-3* mutant, these data collectively suggest that *AtGSNOR1* defines an important component of the defence system.

## Conclusion

SNOs confer negative regulatory action when present excessively as described in this study and previous studies done by Feechan et al., (2005), Tada et al., 2008) and Wang et al., (2009) where the elevated level was associated with enhanced disease susceptibility. The pronounced levels of SNOs observed during *Pst*DC3000 infection were attributable to the TTSS since *Pst*DC3000 *hrcC* only caused a moderate increase in SNO levels. SNO levels are controlled by *AtGSNOR1* which is ubiquitously expressed in *Arabidopsis* and also plays a significant role in plant development and defence responses. As an important component in the plant immune system, *AtGSNOR1* is required for all modes of disease resistance in plants and our data implies this enzyme is targeted for parasitism. Consistent with this, *AtGSNOR1* expression is transiently suppressed by virulent *Pst*DC3000 and the suppression is also dependent on a functional TTSS.

On the other hand, *AtGSNOR1* expression is induced in response to wounding, avirulent and non-host pathogens; implying that *AtGSNOR1* is an inducible defence gene that is being deployed by the host for defence. *Pst*DC3000 actively suppressed *AtGSNOR1* expression via its TTSS suggesting that one or more TTEs could be targeting *AtGSNOR1* as a means to weaken the host immune system. The next important step is to explore the molecular mechanism underlying the manipulation of *AtGSNOR1* gene function in response to invading pathogens.

## 4. Dissecting the manipulation of *AtGSNOR1* gene expression by TTEs

### 4.1 Introduction

Plant disease is the exception rather than the rule because plants are naturally resistant to pathogen attack through a combination of constitutive and induced defences involving complex changes in gene expression. These responses are regulated by a complex network of intracellular signalling molecules and transcriptional regulators. It is crucial for plants to give a rapid response to a microbial attack and transcriptional regulation of defence related genes is considered to be vital to induced disease resistance in higher plants (Rushton and Somssich, 1998; Riechmann et al., 2000; Singh et al., 2002). There is a temporal and spatial hierarchy for defence gene activation, with primary-response genes exhibiting rapid and localized activation at the site of attempted attack, whereas other genes undergo slower activation (Zhu et al., 1996; Eulgem et al., 1999). A large proportion of these genes plays a role in the regulation of other genes and code for transcription factors.

Transcriptional activation of a defence gene is initiated by signal perception from elicitors in plant cells. These elicitors can be described as general or non-specific, which include glycoproteins, small peptides and oligosaccharides; while specific elicitors are designated for *avr* gene products that are delivered into host cells (Montesano et al., 2003). Following elicitor signal perception, a web of signal transduction networks commence, leading towards activation or de novo biosynthesis of transcription factors. These regulatory proteins will bind to specific DNA sites outside of protein-encoding regions, known as *cis*-acting elements and modulate the rate of transcription in order to maintain an appropriate mRNA level in cells. Hence, delineation of the *cis*-acting elements and *trans*-acting factors underlying the activation or suppression of defence-related genes in plants will provide the basis for characterizing molecular mechanism involved in the deployment of transcription-dependent defences.

Predominantly, it is understood that gene expression is controlled at the level of steady-state transcript accumulation. Transcript abundance in the cells is determined by its rate of synthesis and rate of decay (Khodursky and Bernstein, 2003; Meyer et al., 2004) in which the initiation of transcription is a primary mode of regulating a large number of genes, reflected by the vast amount of transcription factors in plants and other eukaryotes (Singh, 1998). Other genes are regulated solely by modulating the rate of mRNA decay and in many

occasions, both factors are taken into account simultaneously (Green et al., 1993; Carey and Smale, 2001; Wang et al., 2002; Khodursky and Bernstein, 2003).

Towards the elucidation of molecular mechanisms underlying the transcriptional control of *AtGSNOR1*, a computer search into plant promoter and transcription factor database was utilized to predict the transcriptional regulatory elements within the *AtGSNOR1* promoter. Further *in vivo* characterization of the *AtGSNOR1* promoter was carried out using 5' deletion analysis. From this study, it was shown previously that suppression of *AtGSNOR1* was dependent on TTSS because *Pst*DC3000 *hrcC* which is defective in TTSS lose the ability to suppress *AtGSNOR1* during infection. In the light of this finding, it is tempting to speculate that at least one of the secreted TTEs could be targeting *AtGSNOR1* to suppress its activity. To check this hypothesis, *AtGSNOR1* expression was studied in transgenic lines exhibiting conditional expression of a given effector.

### 4.2 Chemical-inducible gene expression system

One of the proven strategies for the identification of effector targets has been *in planta* expression. This method was predominantly useful in identifying weakly or transiently interacting partners of effectors and may improve the understanding of the mechanisms underlying plant-pathogen interactions. Bacterial effectors have been effectively localized to the plant nucleus and plasma membrane (Deslandes et al., 2003; Shan et al., 2000; Nimchuk et al., 2000) and the potential targets are expected to co-localize together with their interacting effectors. The *in planta* expression of TTEs was mostly undertaken through the application of chemical-inducible systems, because constitutive expression of some effectors causes toxic or lethal effects on plants, thereby blocking regeneration. Despite a few isolated cases describing the limitations of this system (Kang et al., 1999; Berger et al., 1992), it is still widely utilized for its flexibility as it allows the selective induction of gene expression and the quiescent status of the transgene in the absence of inducers which largely avoids the lethality or toxicity problems.

The artificial chemical-inducible systems in plants are generally based on de-repression (Gatz and Lenk, 1998), inactivation (Weinmann et al., 1994) or activation of the target gene (Aoyama and Chua, 1997; Zhuo and Chua, 2000) in which the latter are the most commonly used inducible expression systems in plants. In this study, *AtGSNOR1* expression was analyzed in transgenic lines expressing effector proteins from *Pst*DC3000 under a

dexamethasone or estradiol-based chemical-inducible promoter which are based on the same principle; the activation of the target gene. The regulation is based on the retention of GR-cellular protein (including heat-shock protein 90, HSP90) complex in the cytosol as a monomer in the absence of hormone ligand. Association of ligand with the hormone-binding domain leads to the release of associated cellular protein from the receptor (for example HSP90) followed by receptor dimerization and translocation into the nucleus to bind to target DNA (Aoyama and Chua, 1997; Zuo et al., 2000).

The dexamethasone-inducible system was developed by Aoyama and Chua (1997), taking advantage of the regulatory mechanism of vertebrate steroid hormone receptors which consists of a chimeric protein composed of the DNA-binding domain of the yeast transcription factor GAL4 (G), the transactivating domain of the herpes viral protein VP16 (V) and the receptor domain of the rat glucocorticoid receptor (G; GR) designated as GVG (Fig 6.1). Several years later, the same group developed a similar inducible system using estradiol (Zuo et al., 2000). This time the chimeric transcription activator, designated as XVE, was assembled by fusion of the DNA-binding domain of the bacterial repressor LexA (X), the transactivating domain of VP16 (V) and the regulatory region of human estrogen receptor (E; ER) (Fig 6.2).

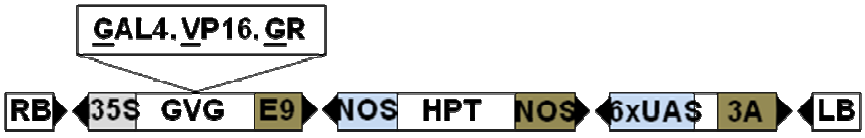



**Figure 4.1 Schematic diagram of the GVG vector.**

GVG expression systems in dex-inducible binary vector pTA7002 used for the construction of effectors transgenic plants. Only fragments between right border (**RB**) and left order (**LB**) were shown. The construct contains the CaMV *35S* promoter (**35S**), **GVG** fragment, the poly(A) addition sequence of the pea ribulose biphosphate carboxylase small subunit rbcS-E9 (**E9**), nopaline synthase promoter (**NOS**; blue box), hygromycin phosphotransferase II coding sequences (**HPT**), NOS terminator (**NOS**; green box), six copies of the GAL4 UAS (**6XUAS**) and rbcS-3A (**3A**) (Adapted from Aoyama and Chua, 1997).

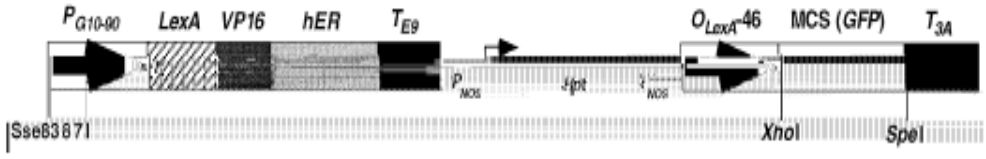



**Figure 4.2 Schematic diagram of the XVE vector.**

The vector contains $\mathbf{P_{G10\text{-}90}}$, a synthetic promoter controlling XVE; **LexA**, **VP16** and **hER** fragments constitute XVE; $\mathbf{T_{E9}}$, rbcS E9; $\mathbf{P_{NOS}}$; **HPT**; $\mathbf{T_{NOS}}$; $\mathbf{O_{LexA}}$, 8 copies of the LexA operator sequence; **-46**, the -46 35S minimal promoter; **MCS**, multiple cloning sites for target genes; $\mathbf{T_{3A}}$; rcbs 3A (Adapted from Zuo et al., 2000).

### 4.3 Analysis of common regulatory elements in the *AtGSNOR1* promoter

There are various publicly available algorithm and bioinformatics tools that have been developed to facilitate in silico analysis of plant promoter sequences, for example TRANSFAC, PLACE, AGRIS, PlantCARE and JASPAR (Wingender et al., 2000; Hiro et al., 1999; Duvuluri et al., 2003; Lescot et al., 2002; Sandelin et al., 2004). These computer approaches estimate the probability of occurrence of short DNA motifs based on random sampling or statistical modelling of a background distribution (Priest et al., 2009).

The regulatory elements in the *AtGSNOR1* promoter were analyzed using the online databases PLACE and PlantCARE. It was found that most of the identified putative elements were redundant between databases (same element being recognized more than once) or within the database itself and sometimes represented variation in the binding sites. Nonetheless, the search revealed several putative *cis*-acting elements related to environmental stresses including disease resistance and developmental cues (Fig 4.3). These elements were found as a cluster or a single motif and were spread over the whole length of the putative promoter region. The putative transcription start site was located at 71 bp upstream the ATG translation start codon. The presence of a few TATA and CAAT boxes in *AtGSNOR1* promoter region are reflective of typical eukaryotic promoters and both are required for initiation of the basal transcription complex (Zhu et al., 1995; Smale and Kadogaga, 2003).

As seen in many other defence-related genes such as *PR-1*, *AtGSNOR1* contains several putative W-boxes, a group of pathogen-inducible *cis*-acting elements that act as a binding site for members of the WRKY family of transcription factors. The term WRKY was derived from its domain which is defined by the conserved amino acid sequence WRKYGQK and a novel zinc-finger-llike motif at the N-terminal end. A group of three W-box elements are present in the *AtGSNOR1* promoter region between -531 and -361, while several others are present as a single motif at a various position in the promoter.

Another likely candidate present in the *AtGSNOR1* promoter is the ACGTA motif or G-box of the ABA-responsive element (ABRE) (Marcotte et al., 1989), which is located at position -398. ACGT sequence is known to be recognized by plant basic region/leucine zipper motif (bZIP) that regulate processes including pathogen defence, light and stress signalling and several developmental events (Jakoby et al., 2002). The G-box was previously detected in the *Arabidopsis alcohol dehydrogenase* (*Adh)* gene (Chang and Meyerowitz, 1986) and is bound in vivo by a protein factor (Ferl and Laughner, 1989), suggesting the functional significance of this motif in transcriptional activation or repression of *AtGSNOR1* promoter. The involvement of ABA or JA in mediating *AtGSNOR1* responses is further justified by the recurring MYB/MYC recognition sites, *cis*-acting elements well-known to be involved in plant development and JA/ABA signalling (Abe et al., 1997; Yang and Klessig, 1996; Anderson et al., 2004). There are only a few members of MYB/MYC family involved in plant defence responses. For example in *Arabidopsis*, *AtMYC2* is known to play a role in JA signalling and functions as negative regulator of plant defence genes (Boter et al., 2004) while a R2R3 MYB factor was reported to be induced by pathogens via a jasmonate-dependent pathway (Mengiste et al., 2003).

A repeated CT motif or pyrimidine-rich repeat, was among other significant motifs present in the *AtGSNOR1* promoter within the 5' untranslated leader regions (UTRs), 60 bp from translation start site. CT dinucleotide repeats have been shown to play an enhancer role and increase promoter activity in several plant genes as well as viruses (Bolle et al., 1994; Chen et al., 1996; Pauli et al., 2004). A previous study claimed that *AtGSNOR1* is up-regulated by light (Barroso et al., 2006) but there was no experimental data presented. Our results demonstrated that light-responsive elements (LRE) such as GATA boxes, also known as I-boxes and GT-boxes elements (Hiratsuka and Chua, 1997) are over represented throughout the *AtGSNOR1* promoter. Parallel to the aforementioned study, these data suggest that transcriptional regulation of *AtGSNOR1* is light-dependent. The occurence of AAAG or the reverse CTTT motifs add to the growing list of putative *cis*-acting elements in the

*AtGSNOR1* promoter. These elements are the binding sites for one finger (Dof) domain proteins, a plant-plant specific zinc finger transcription factor (Yanasigawa, 2004) that function as a transcriptional activator or a repressor involved in diverse physiological processes including light-regulated gene expression and disease resistance (Yanagisawa and Sheen, 1998; Gutterson and Reuber, 2004). In order to establish the putative pathogen responsive regulatory elements required for regulation of the *AtGSNOR1* promoter, a number of 5' end point deletions were generated and tested.

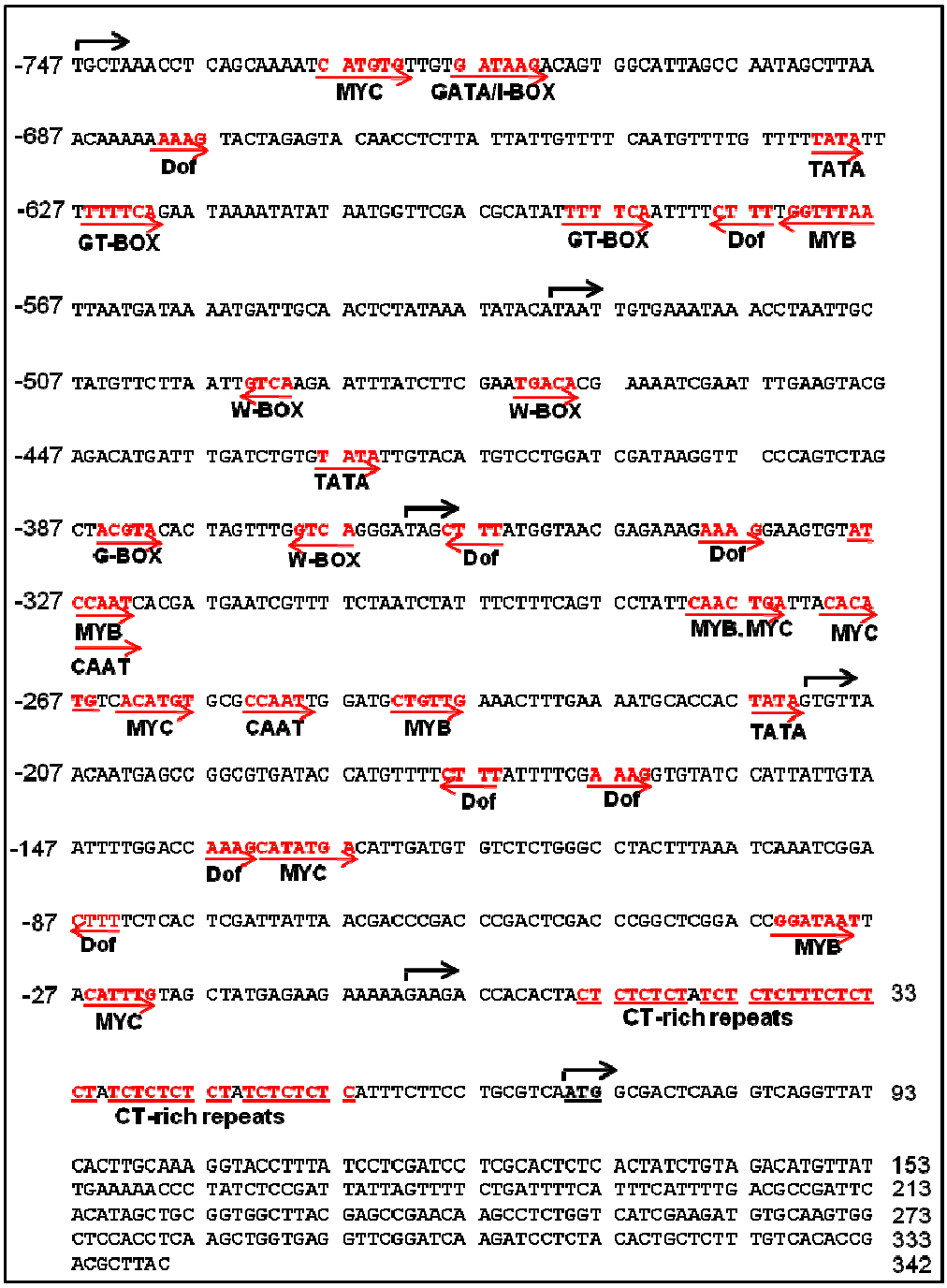



**Figure 4.3 *Cis*-element analysis of the promoter sequences of *AtGSNOR1*.**

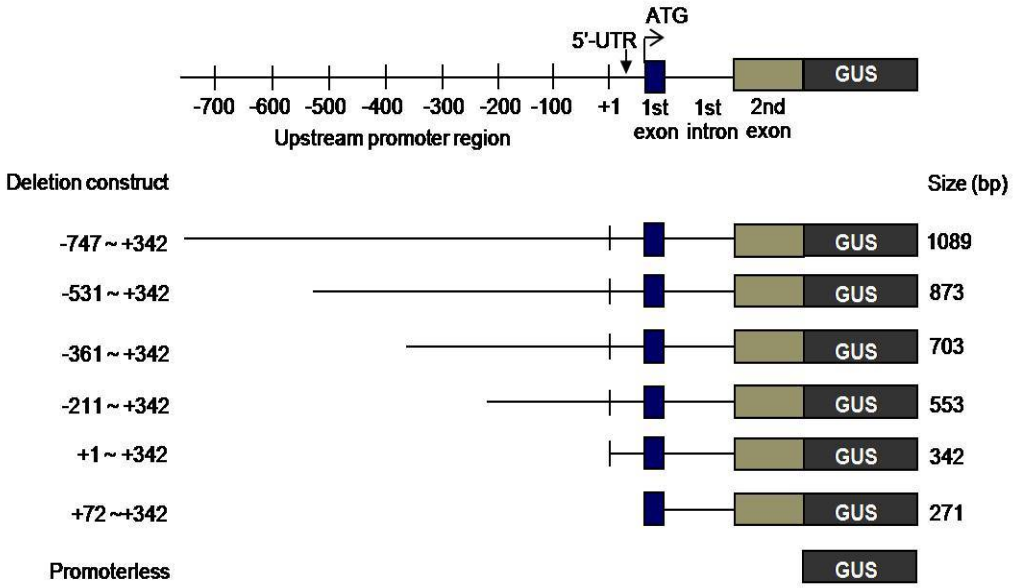
The letters in red indicate putative *cis*-elements sequence, as indicated. Forward and reverse red arrows represented the position of the elements on positive or negative strand respectively. The individual deletion constructs were represented by the forward black arrow. The numbers shown at the left and right end indicated the distance to the start codon.

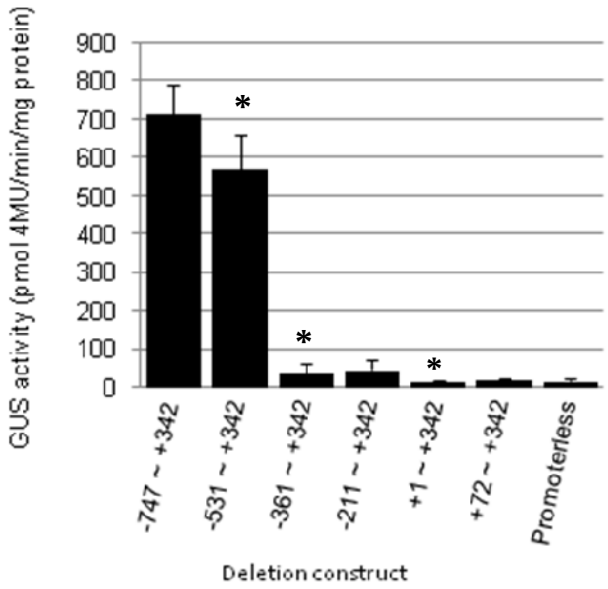
### 4.4 Analysis of relative promoter strength by fluorometric GUS assay

A promoter region may contain both enhancer and repressor elements. To probe which part of the full length promoter is required for the strong basal *AtGSNOR1* expression, a series of 5' deletion mutants constructs (Fig 4.4a) driving the expression of GUS reporter gene were generated and transformed into *Arabidopsis* Col-0. Control plants containing a promoterless GUS gene construct were also generated. *Arabidopsis* plants containing homozygous recombinant transgene were subjected to fluorometric analysis for GUS activity. The levels of GUS activity in leaves were assayed quantitatively in seven independently transformed *Arabidopsis* plants from T3 generation for each deletion constructs. Despite variation in GUS activity among individual plants from the same gene construct, which was most likely a result of position effect of transgene insertion (Dean et al., 1988; Peach and Velten, 1991), significant difference in GUS activity were observed among plants transformed with different deletion constructs (statistical significance in this experiment is confirmed by student t-test, $p<0.05$).

Plants harbouring the full length *AtGSNOR1* construct displayed the highest expression level (Figure 4.4b). Deleting the promoter from -747 to -531 showed a minor but significant reduction in promoter activity with only 20% decrease. Thus, the elements in this region might not be necessary for *AtGSNOR1* specific activity. Further deletions of the promoter to -361 significantly reduced promoter activity to 60% when compared to the level of activity in plants with an undeleted promoter, suggesting that elements in the region from -531 to -361 bp were required for *AtGSNOR1* specific activity and might enhance the promoter activity. This region contains three copies of W-box elements, with the first two boxes located in close proximity; as well as one TATA-box element. Deletion to position -211, which contains several MYB/MYC elements, two Dof binding sites and one CAAT and TATA boxes did not further reduced the promoter activity indicating that the elements present in this region are not important for the minimal promoter activity.

Deletion of 211-bp sequences (+1 to +324) just upstream of the transcription start site resulted in statistically significant reduction of GUS expression to background levels. Similarly, deletion of the entire promoter (position +72) resulted in expression indistinguishable from background levels. This also suggests that the high GUS activity displayed by the full length construct was solely conferred by the promoter region and not influenced by the presence of exons and intron. The promoterless construct only exhibited background GUS expression as expected.



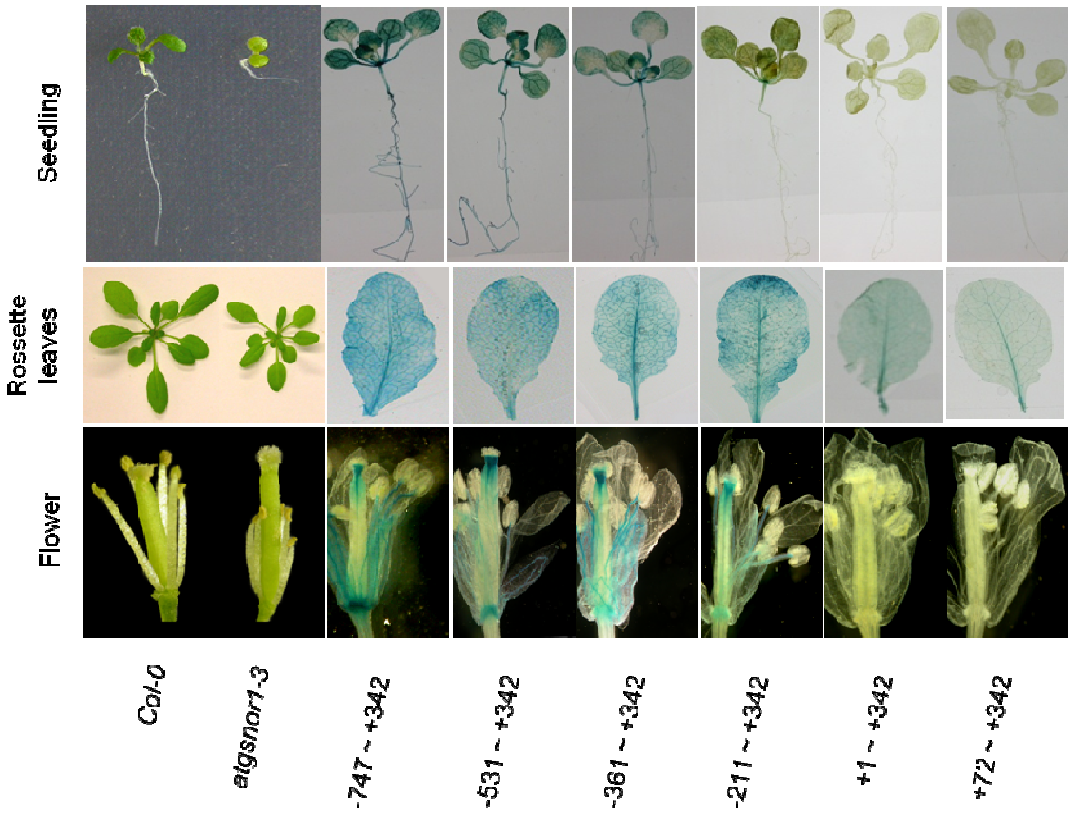



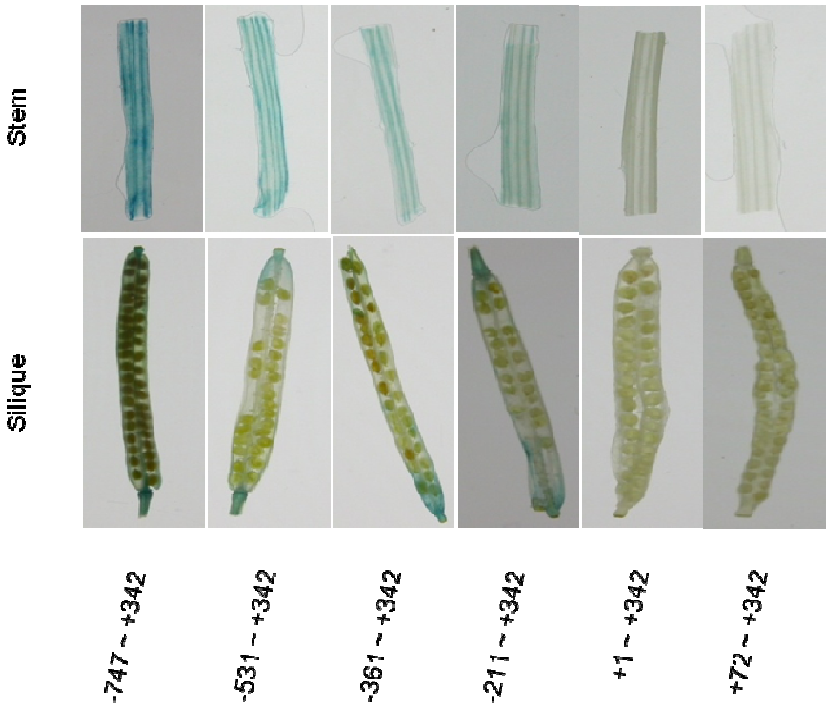

**4.4 Deletion analysis of the *AtGSNOR1* promoter**

(a) Schematic diagram of *AtGSNOR1::GUS* 5' deletion constructs. (b) Fluorometric GUS analysis of transgenic *Arabidopsis* plants carrying different *AtGSNOR1::GUS* deletion fragments. Results are mean of measurements from at least ten independent transgenic lines carrying a single copy of the given construct. Asterisks indicate statistically significant ($p<0.05$) decrease in GUS activity. Error bars indicate standard deviation (SD).

### 4.5 Histochemical analysis of *AtGSNOR1::GUS* promoter deletion constructs during plant development

Histochemical GUS staining of 14-day old seedlings, rosette leaves, flowers, stems and siliques revealed differential expression patterns in each deletion as observed in the quantitative analysis. Five independent transgenic lines were assayed and all had the same overall pattern of expression for each organ tested. As discussed previously, the undeleted promoter construct conveyed intense GUS activity throughout the reproductive and vegetative organs (Fig 4.5). In this analysis, constitutive expression gradually reduced with the deletion series. However, despite low promoter activity as measured quantitatively at position -361 and -211, GUS expression can still be clearly observed in all tissues. Similar to the fluorometric analysis, deletion to position +1 and +72 resulted in the absence of GUS expression in all organs tested.






**4.5 Histochemical analysis of *AtGSNOR1::GUS* deletion constructs**

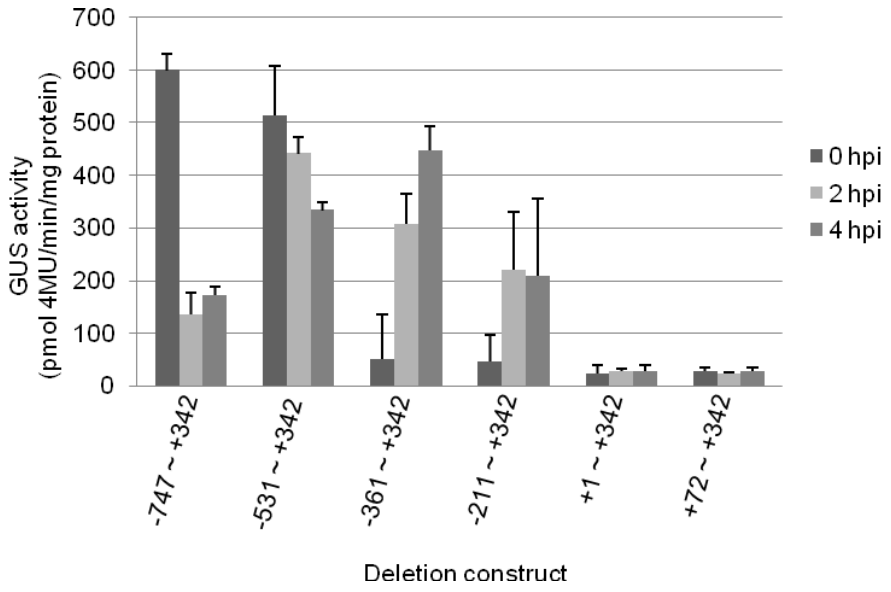
Seedlings, rosette leaves, flowers, stems and siliques were harvested from healthy Col-0 plant carrying full length *AtGSNOR1::GUS* and promoter deletion constructs. Samples were stained for GUS activity and observed under a light microscope.

### 4.6 Identification of a pathogen-responsive promoter region in *AtGSNOR1*

To identify promoter regions necessary for the transcriptional suppression or activation in response to pathogens, transgenic plants containing the deleted promoters were challenged with *Pst*DC3000 and subjected to GUS fluorometric analysis. GUS activity was monitored at the early time points (2 and 4 hpi) where the suppression of *AtGSNOR1* transcript is strongest (Fig 4.6). As discussed previously (section 4.4), the promoter construct containing 873 bp (-531 to +342) only showed a minor reduction in GUS activity in the absence of pathogen. Surprisingly, while the full length construct showed a huge drop in GUS activity following *Pst*DC3000 inoculation, the 873 bp construct lose the suppression.

Instead, the 837 bp construct showed elevated GUS activities at 2 and 4 hpi suggesting the presence of positive regulatory elements. These results also conclude that the deleted fragment in the region between -747 and -531 harbours the elements mandatory for the suppression of *AtGSNOR1* in response to *Pst*DC3000. In this case, MYC or GT-1 elements would be the most likely candidates for negative regulatory elements sites since both have been reported to negatively modulate gene transcription in response to pathogen challenge (Laurie-Berry et al., 2006; Buchel et al., 1999).

Interestingly, the deleted promoter from position -361 and -211 bp displayed increasing GUS activities at both time points following *Pst*DC3000 infection, despite the minimal GUS activities exhibited by both constructs in the absence of pathogen. When compared to the full length construct, the 703 bp construct (-361 to +342) displayed higher induction in GUS activity than the 553 bp construct (-211 to +342). This result suggests that this region of the promoter is dispensable for basal gene expression and no longer responsive to pathogen suppression, instead it contains elements conferring inducibility in response to pathogen. Thus, positive regulatory elements that may enhance the promoter activity in response to pathogen infection could be positioned in the region between -531 and -211 which include a few W-boxes, MYB and Dof binding sites. It is possible that these elements might act singly or additively since progressive deletion resulted in gradually decreasing value of GUS activity in response to pathogen infection. A change in promoter activity was not detected following deletion from position +1 and +72 due to complete removal of the promoter sequence which resulted in very low reporter gene activity indistinguishable from background activity.




**4.6 Differential expression of *AtGSNOR1::GUS* constructs in transgenic *Arabidopsis* plant inoculated with *Pst*DC3000.**

Fluorometric GUS analysis of transgenic *Arabidopsis* plants carrying different *AtGSNOR1::GUS* deletion fragments. The GUS activity at each time point was the average measured from three independent transgenic lines carrying a single copy of the given transgene. Error bars indicate standard deviation (SD).

**4.7 Role of W-box in mediating *AtGSNOR1* expression in response to pathogens.**

From previous analysis in this study, we have found that W-boxes elements in the promoter might constitute negative regulatory elements responsible for the suppression of *AtGSNOR1* expression in response to pathogen infection. Through the available resources from The *Arabidopsis* Information Resource (TAIR; http://www.arabidopsis.org/), several WRKY mutants in Col-0 background were obtained from SALK and tested for their expression profile in response to pathogen infection. As described in the previous chapter, *AtGSNOR1* expression was transiently suppressed following *Pst*DC3000 at an early stage of infection and the expression resumes within 24 hpi. A mutant with enhanced or opposite profile will be of interest in this study and further detailed analysis will be carried out to elucidate the nature of the interaction between the identified WRKY transcription factor and its cognate *cis*-acting elements in *AtGSNOR1* promoter.

Transcription factor WRKY70 is involved in the modulation of SA- and JA-mediated signalling events during plant responses to pathogen (Li et al., 2004; Li et al, 2006). WRKY70 has been shown to positively influence disease resistance, for example overexpression of *AtWRKY70* leads to constitutive *PR* gene expression (Li et al., 2004) and functional WRKY70 is required to confer disease resistance against wide range of pathogens including bacteria, fungus and oomycetes (Li et al., 2004; Li et al., 2006; Knoth et al., 2007). However, there was no change of *AtGSNOR1* transcript levels in a *wrky70* mutant during the early hours following pathogen challenge, suggesting the requirement of WRKY70 as a negative regulator for *AtGSNOR1* expression in response to pathogen infection (Fig 4.7). This is supported by the fact that apart from being a positive regulator, WRKY70 can also negatively regulates plant defence genes as reported in Li et al. (2004).

*AtGSNOR1* expression in the *wrky58* mutant was not detected in the absence of pathogen challenge (Fig 4.7). Based on this observation, WRKY58 could be responsible for the normal function of *AtGSNOR1* under developmental condition consistent with the stunted growth phenotype of the *wrky58* mutant (Wang et al., 2006), resembling the *atgsnor1* loss-of-function mutant (Feechan et al., 2005). After challenge by pathogen, the transcript level was suppressed at 2 hpi followed by a considerable increase at 6 hpi and remains at induced state for up to 48 hpi almost similar to the wild type Col-0 plant. In this case, *AtGSNOR1* response to pathogen was not altered in the *wrky58* mutant background.

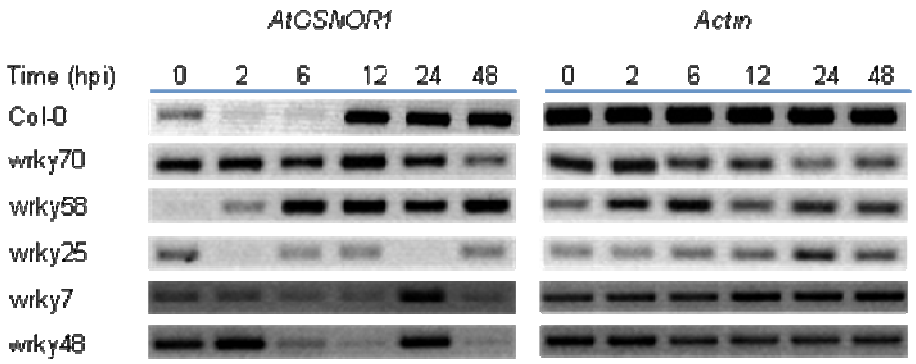



**Figure 4.7 Expression profile of *AtGSNOR1* in different WRKY mutant background.**

*wrky* mutants were inoculated with *Pst*DC3000. RNA was isolated at indicated time points for RT-PCR and *Actin-2* was analyzed as a control.

In *wrky25* mutant, *AtGSNOR1* transcript was absent at 2 hpi and 24 hpi (Fig 4.7). Moreover, compared to the wild type plant the expression of *AtGSNOR1* at 6, 12 and 48 hpi appeared to be very weak. In contrast to the previous study by Zheng et al. (2007), WRKY25 is thought to play a role as a positive regulator in *AtGSNOR1* response to pathogen and is required for the induced *AtGSNOR1* expression at the later stages of the infection. In addition, WRKY25 was also implicated with heat tolerance because overexpression of *WRKY25* enhanced thermotolerence (Li et al., 2009). The parallel function of *AtGSNOR1*/*HOT5* as a regulator of thermotolerance (Lee et al., 2008) further supported the potential involvement of WRKY25 in regulating *AtGSNOR1* during plant development and biotic stress.

*AtGSNOR1* expression in *wrky7* and *wrky48* exhibit a similar expression profile with no suppression at 2 hpi and induced transcript levels at 24 hpi, while at other time points *AtGSNOR1* transcripts were suppressed (Fig 4.7). The expression profile in the mutants' background was roughly the opposite match of wild type Col-0 profile, apart from the unexpected increase at 24 hpi. These results imply that there could be a complex network linking WRKY7, WRKY48 and *AtGSNOR1*.

### 4.8 Transgenic expression of effector proteins suppressed *AtGSNOR1* expression

In the light of the findings that *AtGSNOR1* suppression is TTSS-dependent, it is tempting to speculate that at least one of the secreted TTEs could be targeting *AtGSNOR1* to suppress its activity. To check this hypothesis, *AtGSNOR1* expression was studied in transgenic lines exhibiting conditional expression of a given effector. A total of four stably transformed transgenic plants expressing different effector proteins in different backgrounds (Table 2.1, Chapter 2) at four weeks old were sprayed once with dex or estradiol. Leaf samples were collected at indicated times and *AtGSNOR1* expression were analyzed using RT-PCR (Fig 4.8). In the transgenic line expressing the AvrPto effector protein, there was no significant changes in *AtGSNOR1* transcript accumulation after dex treatment at all time points tested. Similarly, in the transgenic line expressing HopAI1 the *AtGSNOR1* transcript level did not change over time. Interestingly, in the transgenic line expressing HopAM1, *AtGSNOR1* transcripts disappeared after 6 hours post treatment (hpt) with dex but re-appeared at 12 hpt and remained stable for up to 24 hpt.

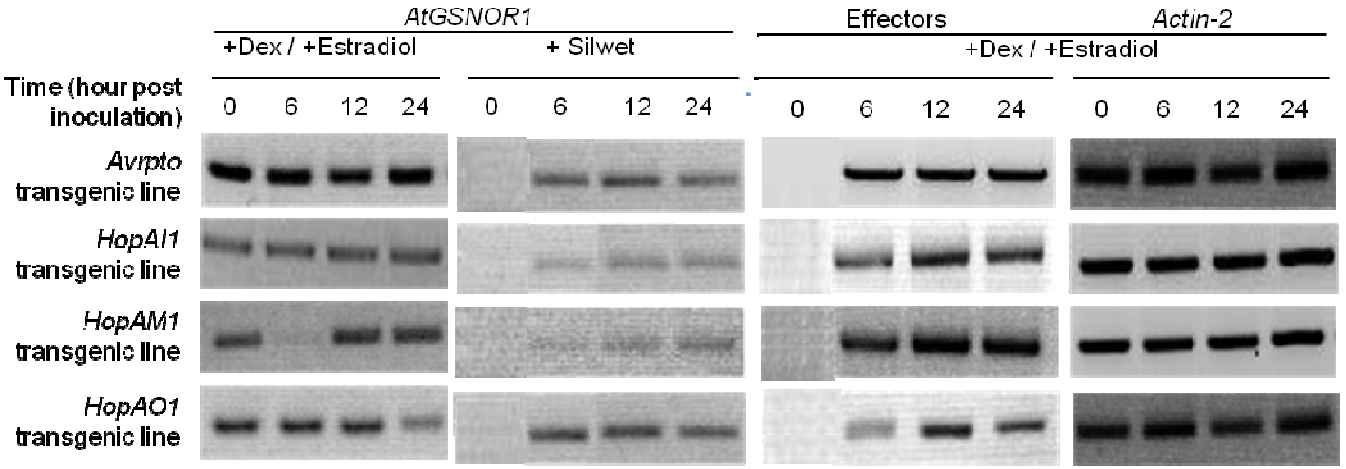



**Figure 4.8 Expression profile of *AtGSNOR1* in effector transgenic lines**

All transgenic plants were sprayed with a specific inducer (dex/estradiol) to induce transgene expression or silwet as a control. HopAI1, estradiol; AvrPto, HopAM1 and HopAO1, dex. Leaves were collected at indicated times and subjected to RT-PCR analysis. *Actin-2* was used as a loading control.

The RT-PCR data obtained from the three transgenic lines were reproducible across at least two independent experiments. In the transgenic line expressing HopAO1, the *AtGSNOR1* transcript levels did not vary from time 0 within the first 12 hpt but the transcript was suppressed at 24 hpt. However, this effect was failed to be accurately reproduced.

In a control experiment, the transgenic plants were mock treated with distilled water containing silwet, a surfactant that acts as a wetting agent. *AtGSNOR1* transcript levels remained stable after treatment with silwet within 24 hour period. In another control experiment, the induced level of the effector proteins following dex treatment was shown. Transcripts corresponding to all effectors were not detected in these transgenic lines before dex treatment. AvrPto expression level was induced from 6 hours and remains at the induced level for up to 24 hours. However, the induction of HopAI1 following estradiol treatment along with HopAM1 and HopAO1 expression following dex treatment was only moderate at 6 hours. Stronger induction was seen in all transgenic lines at 12 hpt which last until 24 hpt.

### 4.9 Discussion

To elucidate the molecular basis of *AtGSNOR1* gene expression, its genomic organization and promoter activity were analyzed in this study. Several 5' deletion mutants were fused to the GUS reporter gene and their expression tested in *Arabidopsis* plants. This approach revealed the organ and cell type specificity of the *AtGSNOR1* promoter during plant development and in response to bacterial infection. Expression of *AtGSNOR1* in all tissues and organs is consistent with the role of *AtGSNOR1* in plant development and plant defence. A dramatic drop in GUS activity was observed in all organs by deleting the promoter region from -531 to -361 indicating that positive regulatory elements might be located between -747 and -361.

It was found that the deleted fragment in the region between -747 and -531 harbouring MYC and GT-1 putative elements were mandatory for the suppression of *AtGSNOR1* in response to *Pst*DC3000. Meanwhile, positive pathogen-responsive elements might reside in the promoter region between -531 and -211 since this region no longer conferred suppression, instead the promoter activity increase in response to pathogen infection. The putative *cis*-elements in this region include a few W-boxes, MYB and Dof binding sites and they might act additively. Deletion in the region between +1 and +72 resulted in very low reporter gene

activity similar to the background activity and could not be further stimulated by pathogen challenge.

The presence of two adjacent W-box elements suggested that they may function in the *AtGSNOR1* promoter because spacing between transcription factor binding sites can significantly affect their strength in activating gene expression. As reported by Eulgem et al. (1999) and Yu et al., (2001), an individual contribution of W-box to elicitor-induced gene expression was low compared to a group of several W-boxes situated closely together. Two closely positioned GT-boxes were also identified in the critical region of the promoter which is required for the suppression by *Pst*DC3000, implying the likely involvement of GT-1 transcription factors as transcriptional repressors. A previous study showed that *AtGSNOR1* was repressed by JA and unresponsive to ABA, but the results were inconclusive (Diaz et al., 2003). The occurrence of several ABA/JA responsive elements such as MYB/MYC suggested that *AtGSNOR1* might be responsive to both JA and ABA that could leads to increased susceptibility. JA-mediated signalling was thought to antagonize defence activation exerted through SA-mediated signalling (Glazebrook et al., 2003) and similarly, ABA increases susceptibility by counteracting SA-dependent defences (de Torres-Zabala et al., 2007). Meanwhile, *AtGSNOR1* was shown to be regulated by light (Barroso et al., 2006) and through this present study, several light-responsive elements were identified in the promoter region which further support the previous report. The presence of CT repeats in the 5' UTR was thought to maintain the minimal promoter activity because their deletion reduced promoter activity to a background level.

Following the finding of a putative W-box in the *AtGSNOR1* promoter, several WRKY transcription factor candidates were tested for their potential engagement in transcriptional regulation of *AtGSNOR1*. The *AtGSNOR1* expression profile was examined in WRKY mutant backgrounds and a few conclusions were drawn from this analysis. WRKY70 was found to be a putative negative regulator, despite its positive role reported in previous studies (Li et al., 2004; Li et al., 2006; Knoth et al., 2007). A knock out mutant of WRKY58 did not have any significant effect on *AtGSNOR1* expression while WRKY25 was considered as putative positive regulator, which also contradicts its originally described function as a negative regulator (Zheng et al., 2007). *AtGSNOR1* expression in *wrky7* and *wrky48* exhibited complex profiles and it is difficult to make any deduction without further detailed analysis.

*AtGSNOR1* was proposed to be the target of *Pst*DC3000 effector proteins delivered through TTSS. Its expression was transiently suppressed in response to *Pst*DC3000 infection but adequately maintained in response to *Pst*DC3000 *hrcC*. To further verify this hypothesis, *AtGSNOR1* expression was determined in several independent transgenic *Arabidopsis* plants expressing different effector proteins. These effector proteins include AvrPto, HopAM1, HopAO1 and HopAI1. These transgenic lines were constructed using chemical-inducible systems which were developed by Zuo et al. (1993) and Aoyama and Chua (1997). The individual contribution of these effector proteins to promote virulence *in planta* were reported in detail in the previous studies (Hauck et al., 2003; Goel et al., 2007; Underwood et al., 2007; Li et al., 2005).

AvrPto was initially identified by its ability to induce ETI in tomato plants carrying a canonical R proteins of the NBS-LRR family, Pto and Prf (Chang et al., 2000). Conspiciously, ectopic overexpression of AvrPto in *Arabidopsis* restored growth of *Pst*DC3000 *hrcC* to almost wild-type levels, suppressed the expression of genes encoding defence and secreted cell-wall proteins, as well as inhibiting callose deposition induced by *Pst*DC3000 *hrcC* (Hauck et al., 2003). Together with several other findings on AvrPto (Li et al., 2005; He et al., 2006), these data suggested that AvrPto targets early PTI signalling components to promote virulence. *AtGSNOR1* was suppressed during the early stage of infection by *Pst*DC3000, hinting at a possbile connection to PTI signalling. Surprisingly, despite the strong virulence feaure of AvrPto when expressed *in planta*, there was no reduction observed in *AtGSNOR1* transcript levels in AvrPto transgenics plant after dex treatment. Nonetheless, it is possible that *AtGSNOR1* might still be involve in PTI signalling but through a different pathway that is not targeted by AvrPto.

HopAI1 is an effector protein with phosphothreonine lyase activity which belongs to an effector family widely conserved in both animal and plant pathogenic bacteria (Li et al., 2005; Zhang et al., 2007). HopAI1 suppressed flg22-induced immunity through direct dephosphorylation of MPK3 and MPK6 (Zhang et al., 2007) while *in planta* expression of HopAI1 suppressed *NHO1* expression, enhanced disease susceptibiltiy to *Pst*DC3000 as well promoting nonpathogenic bacterial growth, a characteristic similar to AvrPto (Li et al., 2005; Hauck et al., 2003). *AtGSNOR1* was shown earlier to be an important component of non-host resistance. However, similar to AvrPto, a high level of expression of HopAI1 *in planta* did not suppress *AtGSNOR1*.

From this experiment, HopAM1 is the only effector that was able to suppress *AtGSNOR1*. HopAM1, formerly known as AvrPpiB encodes protein of unknown function (Counoyer et al, 1995) and its expression in *Arabidopsis* strongly but transiently suppressed *AtGSNOR1*. The ABA response was thought to contribute to the virulence effect of HopAM1 (Goel et a., 2008). In fact, ABA has been shown to suppress defence responses and ABA–deficient mutants are more resistant to pathogens (Anderson et al., 2004; Mohr and Cahill, 2003). Moreover, *Pst*DC3000 manipulates ABA production and ABA responses to suppress defence responses and this phenomenon is dependent on TTSS (de Torres-Zabala, 2007). In parallel with an earlier findings in this study that there are several ABA-responsive elments present in *AtGSNOR1* promoter, this data also suggests that the type III effector HopAM1 might targets *AtGSNOR1* expression through the manipulation of ABA response to promote virulence.

HopAO1 was initially known as HopPtoD2, a modular proteins remisniscent of TTEs from animal (Kaniga et al., 1996). HopAO1 functions inside plant cells to suppress nonhost HR, PTI and ETI by targeting a step downstream or independent of MAPK activation (Espinosa et al., 2003; Bretz et al., 2003; Underwood et al., 2007; Guo et al., 2009). It has been shown to contribute to virulence as a *Pst*DC3000 mutant lacking HopAO1 was reduced in virulence in *Arabidopsis* (Espinosa et al., 2003; Bretz et al., 2003). However, *in planta* expression of *hopAO1* only cause a minor decrease in *AtGSNOR1* transcript accumulation at later time during infection process which does not correspond to the suppression effect delivered by the *Pst*DC3000 observed at the early stage.

**Conclusion**

*AtGSNOR1* is transcriptionally regulated in response to *Pst*DC3000. WRKY, GT-1 and MYB/MYC transcription factor might contribute to the transcriptional regulation of *AtGSNOR1* and to support this hypothesis, a few putative WRKY transcription factors have been identified. In addition to the possible involvement of the transcription factors, *AtGSNOR1* expression in response to *Pst*DC3000 may also dependent on ABA, JA and SA. Suppression by HopAM1 corroborates the idea that ABA plays an important role in mediating the transcriptional down-regulation of *AtGSNOR1* in response to infection.

## 5. General discussion

Nitric oxide (NO) was originally conceived as a freely diffusible second messenger that positively conveys signals through activation of guanylate cyclase and negatively influences cellular processes by contributing to toxicity through reaction with superoxide. At present, accumulating evidence indicates that modification of protein thiols via S-nitrosylation is part of the main principle behind the regulation of protein function by nitric oxide. This study further justified the important role of S-nitrosylation in disease resistance during the interaction between *Pseudomonas syringae* and *Arabidopsis*. Detailed analyses were also carried out to probe if *AtGSNOR1* might constitute a target of *Pseudomonas syringae* effector proteins and the cognate mechanism that underlies the manipulation of *AtGSNOR1*. Finally, experiments were done to identify the specific effector protein(s) that target *AtGSNOR1*.

### 5.1 Type III secretion-dependent induction of S-nitrosothiol levels in *Arabidopsis* during pathogenesis

S-nitrosylated proteins have been successfully identified in *Arabidopsis* and conspicuously, a majority of these proteins have already been identified as substrates for S-nitrosylation in animals, suggesting the common feature of NO-regulated protein function in plants and animals (Lindermayr et al., 2005). At an appropriate physiological condition, NO is constitutively delivered to the tissue from enzymatic and non-enzymatic sources as a ubiquitous signalling messenger, thus explains the presence of basal levels of SNO in plants and animals. Although it has not yet been possible to identify a plant enzyme exhibiting NOS-like activity, a body of evidence has been presented supporting the presence of L-arginine dependent nitric oxide production in plants (Corpas et al., 2009).

Pathogen inoculation was found to induce further protein S-nitrosylation in *Arabidopsis*. Similar level of SNO was detected at very early stage of pathogen infection following the inoculation of both virulent *Pst*DC3000 and TTSS-defective *Pst*DC3000 *hrcC* mutant suggesting that the engagement of SNOs in the very early stage of this plant-pathogen interaction are not influenced by effector proteins. However, there was a significant difference as the infection process progresses where *Pst*DC3000 caused a major increase in SNO levels after one day of infection compared to the *Pst*DC3000 *hrcC* mutant. Despite the

fact that an initial burst of NO is thought to be essential to confer immunity from attempted pathogen invasion, SNO synthesis and turnover seems to provide an alternative regulatory mechanism independent from NO biosynthesis. S-nitrosylation and denitrosylation are tightly regulated processes and certainly, SNOs are stored within intracellular substrates until they are required for the rapid response to stress signals (Gaston et al., 2003). Indeed, increased protein S-nitrosylation has been implicated with several cases of increased susceptibility through covalent modification of cysteine residue of several key components of plant defence signalling (Tada et al., 2008; Wang et al., 2009; Lindermayr et al., 2010). S-nitrosylation of *NPR1*, a master regulator of SA-mediated defence signalling, and *AtSABP3* are two examples of how pathogens disrupt the normal function of proteins to cause disease in plants.

Interestingly, *Pst*DC3000 *hrcC* mutant infected plants that lack typical disease symptoms only show a modest increase of SNO levels after a few days, suggesting that TTSS enhanced the increase of the intracellular SNO during infection to promote disease susceptibility. TTSS is essential for virulence of phytopathogenic bacteria in susceptible hosts. *Pseudomonas syringae* TTSS mutants, such as *Pst*DC3000 *hrcC* typically exhibit lack of growth in plant tissues (Kloek et al., 2000; Nomura et al., 2006), demonstrating the absolute requirement for type III secretion in pathogenesis. TTEs secreted through TTSS utilize various biochemical activities, including protein modification, transcriptional regulation, and hormone mimicry to control host cell function (reviewed in Grant et al., 2006; Cunha et al., 2007). In this case, the TTEs possibly exert their virulence function by increasing the rate of SNO synthesis or most likely by increasing the rate of SNO turnover through inhibition of a denitrosylase enzyme.

The accumulating evidence suggests that pathogens of animals and plants are exploiting SNO levels to increase pathogen susceptibility (Akaike and Maeda, 2000; Tada et al., 2008; Wang et al., 2009; Atochina-Vasserman et al., 2009; Husain et al., 2010). While there have been quite a number of reports on negative effect of S-nitrosylation on animal defence systems (Stamler et al., 2001; Ricciardolo et al., 2004; Ascenzi et al., 2005; Zaki et al., 2005; Wei et al., 2010 ), only a few cases have been reported in plants as mentioned previously. Having said that, many potential targets are awaiting discovery since the majority of the S-nitrosylation targets identified in *Arabidopsis* are already well known S-nitrosylation targets in animals.

**5.2 *Pst*DC3000 targets *AtGSNOR1* through TTSS to manipulate SNO level in plants during pathogenesis.**

In cells, interaction between NO and glutathione (GSH), a major cellular antioxidant resulted in rapid formation of S-nitrosoglutathione (GSNO), a stable and mobile molecule that can serve as a reservoir of NO bioactivity in vivo. Both NO and GSNO regulate a broad spectrum of cellular proteins and functions with a differential specificity S-nitrosylation (Foster et al., 2009). *Arabidopsis* GSNO reductase 1 (*AtGSNOR1*) is the enzyme responsible for the GSNO turnover as well as the turnover of S-nitrosylated proteins in vivo. This enzyme was initially known as *Arabidopsis* glutathione-dependent formaldehyde dehydrogenase (Martinez et al., 1996) that plays a role in glutathione-dependent formaldehyde oxidation before it was discovered to partially regulate nitrosothiol hemeostasis by reducing endogenous GSNO, hence it was renamed as *AtGSNOR1* (Sakamoto et al., 2002).

Later, Diaz et al. (2003) claimed that *AtGSNOR1* was associated with plant defence and in a separate study, loss-of-function mutation of *AtGSNOR1* resulted in increased SNO levels and disabling plant defence response, making the plant more susceptible to diseases (Feechan et al, 2005). As a denitosylase, *AtGSNOR1* is an important molecule that controls not only the cellular levels of GSNO but also the levels of S-nitrosylated proteins, thus it is central to the immune system. Additionally, *AtGSNOR1* is also required to maintain fertility, organ development, thermotolerence and cell death regulation through modulation of protein SNOs (Lee et al., 2008; Chen et al., 2009). In conjunction, the expression of *AtGSNOR1* is found to be constitutively expressed in all organs in Arabidopsis indicating the general importance of this gene. GSNOR expression is also present ubiquitously in mammals and together with various NOS isoforms as well as other proteins, GSNOR plays a very important role in regulating nitrosative stress (Foster et al., 2009).

As a vital component of plant defence response, *AtGSNOR1* was found to be induced by wounding stress, avirulent and non-host pathogen. Non-host and avirulent pathogens activate similar defence signalling components in a complex manner which is largely dependent on the TTSS (Mishina and Zeier, 2007). A strong induction profile of *AtGSNOR1* in response to non-host pathogen is similar to *NHO1*, a gene encoding glycerol kinase that is required for both general and specific resistance in response to bacterial attack (Kang et al., 2003) suggesting the similar requirement for *AtGSNOR1* in plant disease resistance. Wounding stress only causes a transient increase in *AtGSNOR1* transcript accumulation at a very early

stage of pathogen infection whereas infection by *Pst*DC3000 carrying avirulent gene reactivated *AtGSNOR1* expression indicating that *R* gene-mediated resistance masked the suppression of *AtGSNOR1* by a virulent *Pst*DC3000.

Despite remarkable progresses in the study of plant resistance to pathogens, little is known about the molecular basis of plant susceptibility to virulent pathogens. It is understood that key step in pathogenesis appear to be the suppression of host defences including basal defences, *R* gene-mediated resistance and non-host resistance. Defence suppression is collectively mediated by effector proteins which are secreted through type III secretion system and by coronatine, a bacterial toxin that structurally and functionally mimics methyl jasmonate.

Infection with *Pst*DC3000 but not *Pst*DC3000 *hrcC* transiently suppresses *AtGSNOR1* during the early stage and in conjunction with the initial finding that *Pst*DC3000 infection increase SNO levels, it is intriguing to say that *Pst*DC3000 suppresses denitrosylase function of *AtGSNOR1* to increase the SNO levels. This is supported by a study done by Loake group (Feechan et al., 2005) inferring that loss-of-function mutation of *AtGSNOR1* resulted in increase basal SNO levels. Moreover, both *AtGSNOR1* suppression and SNO augmentation were shown to be TTSS-dependent, indicating that the virulence effect exerted on *AtGSNOR1* by *Pst*DC3000 is delivered by at least one of the TTEs secreted through the TTSS either by directly targeting *AtGSNOR1* transcription machinery and translational machinery or indirectly through modification of cofactros that might be involved in regulating *AtGSNOR1* expression. On the other hand, phytotoxin coronatine does not play a role in the suppression of *AtGSNOR1* expression. Coronatine is known to induce JA pathway and functions as suppressor of SA-mediated defence responses during pathogenesis (Brooks et al., 2005; Cui et al., 2005). Yet, as happens with most bacterial phytotoxins, coronatine does not seem to be essential for pathogenicity by all strains of *Pseudomonas* bacteria and in fact, coronatine production did not enhance the growth of *Pst*DC3000 *hrcC in planta* or its ability to cause typical disease symptoms (Penaloza-Vazquez et al., 2000).

### 5.3 Transcriptional regulation of *AtGSNOR1* in response to *Pst*DC3000 infection

Transcriptional regulation of specific defence-related genes is a vital and major part of plant defence response to microbial pathogens. There are several crucial regions in *AtGSNOR1* promoter that have been found to influence the transcriptional regulation of the gene in response to *Pst*DC3000 infection. Most importantly, the region from -531 to -211 bp was required for the induction of GUS activity while the region from -747 to -531 was required for the suppression of the activity in response to pathogen infection. Meanwhile, computational analysis of *AtGSNOR1* promoter revealed the presence of several putative cis-elements including a GT-box, W-box, MYC/MYB and Dof binding sites that could be responsible for the regulation of *AtGSNOR1* expression by *Pst*DC3000 infection (a simplified schematic representation of transcriptional regulation of *AtGSNOR1* in response to pathogen infection is provided in Fig 5.1).

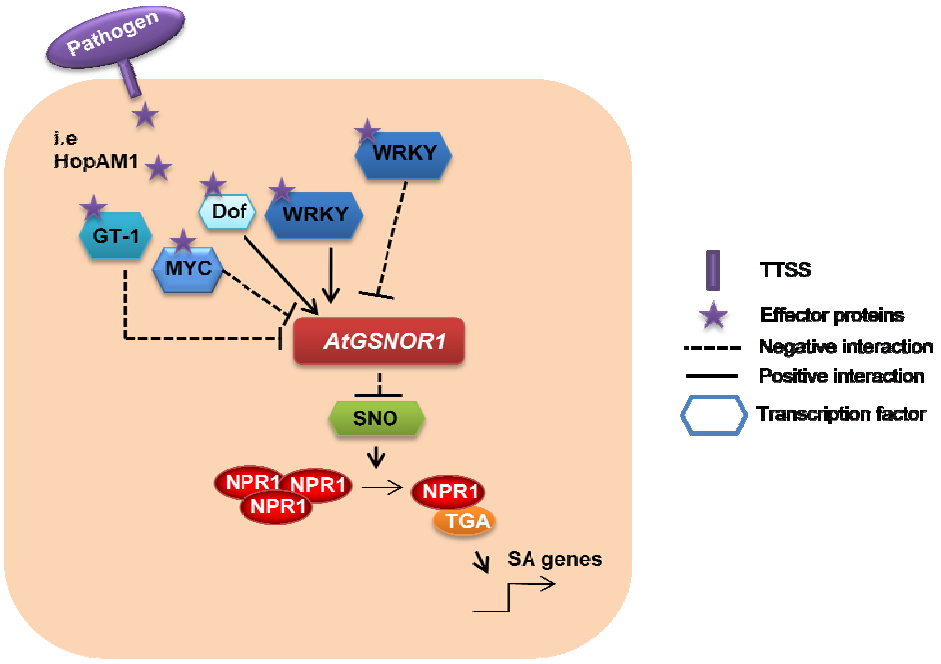



**Figure 5.1 Simplified schematic representation of transcriptional regulation of *AtGSNOR1* in response to pathogen infection**

Effector proteins for example HopAM1 are delivered by *Pst*DC3000 through TTSS during infection process. These effector proteins will interact with the transcription factors (WRKYs, Dof, GT or MYC) to positively or negatively influence *AtGSNOR1* expression. In the absence of pathogen, *AtGSNOR1* acts as a denitrosylase to control the level of SNOs. SNO is required for *NPR1* oligomer-monomer exchange to activate the expression of SA-dependent genes (Modified from Pieterse et al., 2009).

Two GT-boxes, the binding sites for GT-1 transcription factor were found in the critical region of the promoter which is required for the suppression by *Pst*DC3000. GT-1 factor can have a positive or negative effect on transcription (Park et al., 2004) and in this case, it is likely that the GT-1 transcription factor play a role as a transcriptional repressor. MYC/MYB are JA/ABA responsive elements and their presence further indicated that *AtGSNOR1* is responsive to JA (Diaz et al., 2003) and might be responsive to ABA that could leads to increased susceptibility. In fact, *Pst*DC3000 has been shown to induce a rise in ABA content which is TTSS-dependent and full virulence is only achieved when the ABA biosynthetic pathway is intact (de Torres-Zabala et al., 2007).

Finally, the presence of two W-boxes suggested the involvement of WRKY transcription factor, a class of DNA binding protein that play a diverse role in plant defence response. WRKY transcription factors constitute a large family that includes positive and negative regulators. Indeed, a few putative WRKY transcription factors have been identified that might play a role in transcriptional regulation of *AtGSNOR1*.

### 5.4 Type III effector proteins target denitrosylase activity of *AtGSNOR1* for virulence

It was shown earlier that *Pst*DC3000 transiently suppresses the denitrosylase activity of *AtGSNOR1* to increase the level of SNOs in plant cells and that this is TTSS-dependent. In conjunction, a few type III effector proteins were selected and tested for their suppressive effect on *AtGSNOR1* expression during *Pst*DC3000 infection. Effector protein HopAM1 showed the ability to suppress *AtGSNOR1* when expressed *in planta.* HopAM1 is a protein of unknown function and its conditional expression in *Arabidopsis* suppresses basal defence (Goel et al., 2008). Based on the same study, HopAM1 is thought to convey its virulence effects on *AtGSNOR1* through ABA response. The presence of several ABA responsive elements in *AtGSNOR1* promoter further supports the idea that HopAM1 targets *AtGSNOR1* expression through the manipulation of ABA response to promote virulence.

### 5.5 Future works

Probing deeper, the results in this thesis also provide a strong foundation for future works in DNA-protein or protein-protein interaction and more detail analysis on S-nitrosylation status during plant-pathogen interaction.

Following the identification of several putative cis-acting elements and a few related transcription factors, in depth analysis of the interaction between the transcription factors and their cognate genomic binding sites will definitely enhance our understanding on *AtGSNOR1* gene regulation. For example, two W-boxes have been found in the critical region of *AtGSNOR1* promoter and in parallel, several putative WRKY transcription factors were also identified. In order to confirm whether the putative W-boxes are indeed the functional binding sites for the WRKY transcription factors, a pull-down assay will be carried out using a glutathione (GST)-tagged WRKY protein as a 'bait" to capture its putative binding partner, a W-box elements or termed as 'prey'. The immobilized bait protein will be incubated with a cell lysate from *Pst*DC3000 infected plants and the interactors are selectively eluted for analysis by Western blot.

Based on the idea that effector protein delivered by *Pst*DC3000 suppresses *AtGSNOR1* to increase SNO levels in order to promote virulence, SNO levels are expected to be high in the effector transgenic line. SNO measurement will be done using gas phase chemiluminescence to further confirm this idea.

HopAM1 was found as the putative effector protein that might suppress *AtGSNOR1* expression. To further confirm this interaction in vivo, dex-induced and uninduced HopAM1 transgenic leaves will be subjected to immunoprecipitation with anti-AtGSNOR1 antibodies.

### 5.6 Conclusion

S-nitrosylation has now become one of the important themes in plant science. With increasing number of defence-related targets being identified in plants, this redox-based post-translational modification is indeed vital to maintain normal protein function during plant-pathogen interaction. The body of evidence presented here proposes that *AtGSNOR1* is a target of *Pst*DC3000 effector proteins. Through transcriptional regulation of its denitrosylase activity, virulent *Pst*DC3000 deflect SNO homeostasis towards its benefit which is to promote virulent and increase susceptibility. The knowledge gained from this study might be invaluable in order to further understand the role of S-nitrosylation in plant disease resistance.

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