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Molecular and Biochemical Signaling Underlying Arabidopsis-Bacterial/Virus/Fungal Interactions

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MOLECULAR AND BIOCHEMICAL SIGNALING UNDERLYING ARABIDOPSIS-BACTERIAL/VIRUS/FUNGAL INTERACTIONS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in the
College of Agriculture at the
University of Kentucky

By
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2016

ABSTRACT OF DISSERTATION

MOLECULAR AND BIOCHEMICAL SIGNALING UNDERLYING ARABIDOPSIS-BACTERIAL/VIRUS/FUNGAL INTERACTIONS

Systemic acquired resistance (SAR) is a form of inducible defense response triggered upon localized infection that confers broad-spectrum disease resistance against secondary infections. Several factors are known to regulate SAR and these include phenolic phytohormone salicylic acid (SA), phosphorylated sugar glycerol-3-phosphate (G3P), and dicarboxylic acid azelaic acid (AzA). This study evaluated a role for free radicals nitric oxide (NO) and reactive oxygen species (ROS) in SAR. Normal accumulation of both NO and ROS was required for normal SAR and mutations preventing NO/ROS accumulation and/or biosynthesis compromised SAR. A role for NO and ROS was further established using pharmacological approaches. Notably, both NO and ROS conferred SAR in a concentration dependent manner. This was further established using genetic mutants that accumulated high levels of NO. NO/ROS acted upstream of G3P and in parallel to SA. Collectively, these results suggest that NO and ROS are essential components of the SAR pathway.

Key words: Systemic acquired resistance, Azelaic acid, Glycerol-3-phosphate, Nitric oxide, Reactive oxygen species

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Introduction

1.1 General Introduction

Most plant species are subject to diseases depending on the surrounding conditions, which could be either infectious (biotic) or non-infectious (abiotic). There are several biotic agents that cause infectious diseases by reproducing and spreading on the host such as fungi, bacteria, viruses, nematodes and parasitic higher plants. On the other hand, temperature, light, moisture, inadequate oxygen, air pollution, and nutritional deficiencies are examples of non-biotic agents. Plants have developed various sophisticated mechanisms to enhance their defenses (Britannica 2016). In addition, all these abiotic diseases can make plants susceptible or more susceptible for pathogen infection. For a disease to occur, three conditions must be met:

1. The host plant is susceptible. Also, the plant must be in a developmental stage that is susceptible to infection by the disease agent.
2. The active pathogen is virulent to cause the disease. Also, the pathogen must be in the stage that can cause infection of the host plant.
3. The environment is suitable for the pathogen to cause plant disease.

There is no biotic disease unless all 3 legs of the plant disease triangle are present at the same time. Disease control strategies are based on breaking a leg of the triangle (Scholthof 2007).

1.2 Disease Resistance in Plants

1.2.1 Basal Disease Resistance

Basal resistance or innate immunity is the first line of inducible defenses that protect plants against pathogens. Plants have structural barriers that limit pathogen attachment and the first observable barrier is the outer waxy cuticle (Kachroo and Kachroo, 2009). The other type of barriers include chemical defense within the plant. Antimicrobial

compounds can be produced as part of normal plant growth and development or by transcriptional activation of some biosynthetic pathways as a result of microbial attacks (Bednarek and Osbourn 2009). For example, avenacin, a triterpene glycoside is produced in oat roots when challenged by the fungal pathogen of oat, *Gaeumannomyces graminis* var. *avenae* (Morrissey and Osbourn 1999). Moreover, several studies suggest that jasmonic acid and other plant defense signaling molecules trigger indole glucosinolate accumulation (Sasaki-Sekimoto et al., 2005).

1.2.2 Induction of Plant Immunity

Jones and Dangl (2006) proposed a four-phased zigzag model, which illustrates the current view of the plant immune system. In phase 1, recognition and perception of microbial/pathogen-associated molecular patterns (MAMPs/PAMPs) by trans-membrane pattern recognition receptors (PRRs) to provide the first layer of plant innate immunity named PAMP-triggered immunity (PTI). In phase 2, pathogen effectors overcome PTI resulting in effector-triggered susceptibility (ETS). In phase 3, *R*-gene products alleviate the effects of effectors activating the effector-triggered immunity (ETI), which is usually accompanied by hypersensitive cell death (HR) to limit pathogen growth at the infection site. In phase 4, pathogens avoid ETI by producing new advanced effectors that suppress ETI. Along with that, plants could develop new R specificities triggering ETI again leading to a competition between invasion and resistance.

The gene for gene resistance hypothesis states that if the pathogen, which has an avirulence (*avr*) gene, challenges a host plant with the corresponding disease resistance (R) gene, the pathogen fails to cause disease for the plant. As a result, the plant will be resistant to that pathogen, and the pathogen is defined as an avirulent in an incompatible interaction. On the other hand, compatible interaction occurs when a virulent pathogen infects a susceptible host plant (Tao et al., 2003). Pathogenic bacteria can produce 15-30 effectors per strain into host cells using a type III secretion system (TTSS) to suppress PTI (Jones and Dangl 2006). These effectors change basal defense functions by

suppressing papilla formation (*AvrPto1*, *AvrE1*, *AvrRpm1*, and *AvrRpt2*), altering hormonal responses (*AvrB1*, and *AvrRpt2*), and suppression of cell death (*AvrRpm1, AvrRpt2, AvrB2* (Grant et al., 2006). The host plasma membrane associated protein RIN4 (RPM1-interacting protein4) is the key player that is targeted by many bacterial effectors and a known RPS2 interactor. In the absence of pathogen infection, PTI is negatively regulated by RIN4. *AvrB* and AvrRpm1 target RIN4 by inducing the expression of RIPK (RIN4-Interacting receptor-like Protein Kinase) that interacts with and phosphorylates RIN4, and this phosphorylation is recognized by the R protein RPM1 to trigger ETI. In addition, *AvrB* and *AvrRpm1* may target RIPK to enhance RIN4 phosphorylation and to block PTI in the absence of RPM1. On the other hand, *AvrRpt2* cleaves RIN4 producing nonmembrane-tethered RIN4 fragments that suppress PTI more efficiently than non-cleaved RIN4, and this RIN4 proteolysis or cleavage is recognized by RPS2, which is a membrane-associated disease resistance protein of low abundance, to trigger ETI (Deslandes and Rivas 2012). Additionally, RIN4 is targeted by HopF2 (T3E from *Pseudomonas syringae*), which functions at the plasma membrane leading to an interference with *AvrRpt2*-RIN4 cleavage (Robert-Seilaniantz et al., 2006, Deslandes and Rivas 2012). *AvrPto* is targeted to the plasma membrane to interact with the Ser/Thr protein kinase (Pto) and the nucleotide-binding (NB) and leucine-rich repeat (LRR) protein (Prf) leading to RIN4 degradation and PTI activation (Deslandes and Rivas 2012). RPS4 is a well-characterized *R* gene that confers resistance against *Pseudomonas syringae* pv. *tomato* strain DC3000 expressing *avrRps4* (*Pst-avrRps4*) (Narusaka et al., 2009).

1.2.2.1 *Pseudomonas syringae*

Pseudomonas syringae belongs to Gram-negative bacteria that causes bacterial speck disease of tomato (Pedley and Martin 2003). Several strains of *Pseudomonas syringae* are able to infect Arabidopsis (Katagiri et al., 2002). The bacterial effector proteins manipulate host cell processes to enhance pathogen invasion. Recognition of these effectors by host proteins triggers disease resistance, but evasion elicits susceptibility. The Arabidopsis-Pseudomonas pathosystem has been studied widely, from the leaf

surface colonization, pathogen entry, release of effectors through type III secretion system (T3SS), and cell death (Hirano and Upper 2000, Melotto et al., 2006, Lindeberg et al., 2009). The epithet pathovar (pv.) is used to distinguish between bacteria within the same species but with different pathogenic abilities, and there are more than 40 different pathovars are described until now (Hirano and Upper, 2000). *P. syringae* is a hemibiotrohic pathogen, which infects the host through wounds or open stomata and multiplies in the intercellular spaces. Most stages of the life cycle occur in living host cells. Later, host cells die and the infected tissues become necrotic (Glazebrook, 2005). Several *P. syringae* strains of the pathovars *tomato*, *maculicola*, *pisi*, and *atropurpurea* were known to infect Arabidopsis (Crute et al., 1994). The compatible interaction occurs after infection with virulent pathogens that overcome basal resistance such as *P. syringae* pv. *tomato* DC3000 (*Pst*) and *P. syringae* pv. *maculicola* ES4326 (*Psm*). The incompatible interaction was studied after the discovery of the bacterial avirulence genes like *avrRpt2* and *avrRpm1*, and their corresponding plant resistance genes *RPS2* and *RPM1*, respectively (Dangl et al., 1992). The non-host resistance occurs in response to infection with *P. syringae* pv. *glycinea* (*Psg*) and *P. syringae* pv. *phaseolicola* (*Psp*), which can not cause disease in Arabidopsis plants (Mishina and Zeier, 2007).

1.2.2.2 *Colletotrichum higginsianum*

Colletotrichum higginsianum is a hemibiotrophic fungus that causes anthracnose disease of *Arabidopsis thaliana*, whereas anthracnose lesions develop on the leaves, petioles, and stems of turnip, mustard, and Chinese cabbage (Higgins 1917). Upon *C. higginsianum* infection, Columbia (Col-0) ecotype displays fungal growth, and disease symptoms similar to those on other cruciferous plants inoculated under the same conditions (Narusaka et al., 2004). The Colletotrichum-Arabidopsis interaction is considered an important model pathosystem to study the molecular basis of fungal pathogenicity and host defense responses. This pathogen establishes an intimate intracellular contact with host cells then starts to kill them in advance during colonization, and feed on the dead tissues (Schulze-Lefert and Panstruga 2003). Colletotrichum fungi enter and multiply within host cells by developing a series of specialized infection structures, such as germ

tubes, appressoria, intercellular hyphae, and secondary necrotrophic hyphae (Perfect et al. 1999). It has been concluded that glycerol-3-phosphate (G3P) is an important component for basal defense against *Colletotrichum higginsianum* (Chanda et al., 2008).

1.2.2.3 Turnip crinkle virus (TCV)

Turnip crinkle virus (TCV) is a plant pathogenic virus that belongs to Tombusviridae family. TCV is a single-stranded, positive-sense RNA virus that was isolated from turnip (*Brassica campestris* ssp. *rapa*). Also, it can infect different types of plant species such as *Nicotiana benthamiana* and *Arabidopsis thaliana* (Qu and Morris 1997). Host resistance against TCV is dependent on SA but independent of NPR1, JA or ethylene (Kachroo et al., 2000). Most ecotypes of Arabidopsis plants are susceptible to TCV, where it spreads systemically resulting in a crinkled leaf and drooping bolt appearance (Dempsey et al., 1997; Kachroo et al., 2000). A resistant line was isolated from the Dijon (Di) ecotype designated as Di-17 (Dempsey et al., 1997). Upon TCV infection, Di-17 plants display hypersensitive response (HR), express several defense genes, and accumulate salicylic acid (SA) (Dempsey et al., 1997; Kachroo et al., 2000). In Arabidopsis, the *HRT* (HR to TCV) dominant gene encodes a protein that shows homology to the coiled coil (CC) motif at the N-terminal of nucleotide binding site (NBS)-leucine rich repeat (LRR) class of resistance (R) genes. Plants lacking HRT suppresses local defense responses, such as HR development, defense gene expression, and SA accumulation in response to TCV infection. However, *HRT* alone is not sufficient to confer TCV resistance, whereas transgenic Col-0 plants expressing *HRT* are susceptible to TCV although these plants develop HR upon TCV inoculation (Cooley et al., 2000). A second gene, named *rrt* (regulates resistance to TCV), is required to regulate resistance against TCV (Chandra-Shekara et al., 2004).

1.2.2.4 Tobacco mosaic virus (TMV)

Tobacco mosaic virus (TMV) is the first discovered and purified virus. TMV can infect different hosts such as tobacco, tomato, and other solanaceous plants. TMV is a positive-

sense single stranded RNA virus that belongs to Tobamovirus genus (Scholthof, 2000). Local cell death called hypersensitive response (HR) develops against TMV on the inoculated leaves of tobacco carrying the *N* resistance gene causing necrotic lesion formation. HR precedes systemic Acquired Resistance development throughout the whole plant (Ross, 1961; Mittler et al., 1996). However, necrotic lesions formation is not necessarily required for TMV resistance (Mittler et al., 1996). The TMV/N gene interaction leads to an onset of cell death, which includes the oxidative burst and loss of plasma membrane integrity (Doke and Ohashi, 1988). As well, the TMV/N gene interaction, which displays HR and resistance, occurs effectively only at temperatures below 27°C (Weststeijn, 1981). In the early phase of the TMV/N gene interaction, a rapid cell collapse and death occurs, leading to the elimination of infected cells and the generation of signals that move into living neighboring cells. In the second phase, infected cells that survived initially due to lower levels of the viral elicitor die in response to the combination of newly synthesized elicitor and externally generated signals (Wright et al., 2000).

1.3 Plant Signaling Networks

Full understanding of the mechanisms by which plants perceive environmental stresses, and the accompanying signal transduction pathways involved in activating the corresponding responses is crucial for developing better management strategies. The induced signal transduction pathways involve an activation of secondary messengers, which target the proteins and transcription factors involved in cellular protection by activating protein phosphorylation cascades. The same stress factor can trigger several signaling mechanisms at different times and subcompartments for different outputs (Guo et al., 2002). Cross talk is the cooperative or antagonistic interference of different signaling pathways, which activated by the same or different stresses. The linear branches of the signaling networks are part of more complicated networks with an overlapped cross talk among many branches (Knight and Knight 2001). For instance, abscisic acid may represent a point of interaction between different signaling pathways, where it is crucial to mediate both biotic and abiotic stresses (de Torres-Zabala et al.,

2007).

1.3.1 Systemic Acquired Resistance (SAR)

Systemic Acquired Resistance (SAR) is one form of defenses where plants show resistance against a wide range of pathogens (Durrant and Dong 2004). SAR occurs in distal plant parts after localized infection by avirulent pathogen or a chemical treatment that induces a signal transduction pathway that involves many factors, which lead to the expression of defense-related proteins and resistance against a wide spectrum of pathogens (Ryals et al., 1996). Many factors that contribute to SAR have been discovered such as salicylic acid (SA) (Durrant and Dong 2004), methyl salicylic acid (MeSA) (Park et al., 2007), the intact cuticle which is important to initiate SAR (Xia et al., 2009, 2010, 2012), azelaic acid (Yu et al., 2013), auxin (Truman et al., 2010), DIR1 (defective in induced resistance) which is a protein that shows homology to the lipid transfer protein (LTP) family (Maldonado et al., 2002), glycerol-3-phosphate (G3P) (Chanda et al., 2011), pipecolic acid (Návarová et al., 2012), nitric oxide (NO)/Reactive Oxygen Species (ROS) (Wang et al., 2014), and galactolipids (Gao et al., 2014). As well, SAR can be transferred to the next generation via modifications in the chromatin structure (Luna et al., 2012, Slaughter et al., 2012).

1.3.1.1 The Roles of Different Signals in SAR

SA is an essential component of SAR, and all the identified chemical inducers can't induce SAR in SA mutants. During SAR, SA accumulation in the primary infected and/or systemic tissues is increased but SA accumulation alone is not enough to induce SAR (Cameron et al., 1999). Pathogen inoculation increases SA levels in the local leaves and also in the distal tissues but to a lower extent (Chanda et al., 2011). Glycerol-3-phosphate (G3P) application is involved in the induction of SAR (Chanda et al., 2011). In plants, G3P can be generated via two reactions, one through glycerol phosphorylation by glycerol kinase (GK) (GLI1) and/or dihydroxyacetone phosphate (DHAP) reduction by G3P dehydrogenase (G3PDH) (GLY1) (Kachroo et al., 2004, Chanda et al., 2011). G3P-

induced SAR is dependent on Defective In Induced Resistance1 (DIR1) and Azelaic Acid Induced1 (AZI1) proteins, which interact with each other and with the bioactive G3P-derivative forming a complex that translocate to the distal tissues to induce SAR (Chanda et al., 2011, Yu et al., 2013). Azelaic acid (AzA) was supposed to induce SAR by priming plants to accumulate SA (Jung et al., 2009). Yu et al., (2013) suggested that AzA induces SAR by promoting G3P accumulation by upregulating expression of the G3P biosynthetic genes GLY1 and GLI1. Also, some (<7%) of this AzA translocate to the distal tissuses in the form of derivatized conjugates (Yu et al., 2013). As well, Yu et al., (2013) showed pathogen infection releases unsaturated fatty acids (FAs) from membrane lipids, and AzA can be generated by hydrolysis of these free fatty acids. Nitric oxide (NO) and reactive oxygen species (ROS act as inducers of SAR in a concentration dependent manner meaning that low or high NO/ROS can compromise SAR. ROS function additively to generate azelaic acid (AzA) by the chemical breakage of C9 of C18 fatty acids, which induces production of glycerol-3-phosphate (G3P) (Wang et al., 2014). In contrast, Attaran et al., (2009) showed that MeSA production induced by *Pseudomonas syringae* is dependent on the JA pathway but that JA biosynthesis and its downstream signaling components are not required for SAR. As well, digalactosyldiacylglycerol (DGDG) plays a vital role in SAR by contributing to NO and salicylic acid biosynthesis. On the other hand, monogalactosyldiacylglycerol (MGDG) regulates azelaic acid (AzA) and glycerol-3-phosphate (G3P) biosynthesis (Gao et al., 2014). Moreover, The non-protein amino acid Pipecolic acid (Pip), which is a product of lysine catabolism, is an important regulator of basal resistance and SAR by positively regulating SA biosynthesis. Pipecolic acid accumulates in inoculated local leaves, and in distal leaves of Arabidopsis thaliana. Mutant AGD2-LIKE DEFENSE RESPONSE PROTEIN1 (ALD1), which lacks Pipecolic acid production, is compromised in both local resistance and SAR (Návarová et al., 2012).

1.3.1.2 External Factors affecting SAR

There are many factors and conditions that control SAR such as induction time of infection, light intensity, environmental conditions, age of the plant at the infection time

and pathogen induction by using the appropriate strain of the bacterial pathogen (Liu et al., 2011). Age related resistance improves resistance against avirulent pathogens where old plants show decreased pathogen infection in comparison with younger plants (Rusterucci et al., 2005, Develey-Rivière and Galiana 2007). Chloroplasts link light and pathogen resistance, whereas chloroplasts are the light harvesting centers in plant cells, and the site of SA biosynthesis and reactive oxygen species (ROS) production (Karpinski et al., 2003). As a result, this link is proposed as a possible cross-talk between photoreceptors and the other components of the resistant signaling pathways. For example, light-dependent resistance against Turnip crinkle virus (TCV) requires cryptochrome 2 and phototropin 2 (Jeong et al., 2010). Also, HR development and local resistance against *Pst avrRpt2* are dependent on phytochromes A and B (Genoud et al., 2002). As well, phytochromes A and B are required for SAR, whereas *phyAphyB* mutant plants show compromised SA accumulation, *PR-1* and FMO1 (Flavin Monooxygenase1) expressions in the distal leaves (Griebel and Zeier, 2008). Furthermore, high light intensity induces photooxidative stress that triggers several defense responses, such as ROS production, programmed cell death, *PR-1* expression, and enhanced resistance against pathogen infection (Muhlenbock et al., 2008). Furthermore, high light intensity induces SAR in the absence of systemic SA accumulation (Zeier et al., 2004). This light-dependent, and SA-independent pathway might be controlled by FMO1, whereas its local expression is mediated in an SA-independent manner, and its systemic expression is phytochrome dependent (Mishina and Zeier, 2006; Griebel and Zeier, 2008). The intensity and the duration of light have significant roles in defense responses mediated by SA. Salicylic acid is a key-signaling component of different types of resistance within plants such as basal resistance (PTI), R-mediated resistance (ETI), and systemic acquired resistance (SAR) (Zeier et al., 2004, Loake and Grant 2007). The strength of defense responses correlates with the number of light exposure after infection, whereas the longer the light exposure period, the stronger the SAR (Griebel and Zeier, 2008). Continuous darkness after pathogen infection suppresses local defense responses, such as HR formation, *PR-1* expression, SA accumulation, and favors the pathogen growth (Zeier et al., 2004; Chandra-Shekara et al., 2006). Also, darkness abolishes SAR and the systemic accumulation of SA (Zeier et al., 2004). Methyl salicylate plays a vital role in inducing

SAR signaling in plants, which had little light exposure after the primary treatment before entering the dark (Liu et al., 2011). MeSA is not required for SAR if *bsmt1* mutant plants, which lack a MeSA synthesizing benzoic acid/salicylic acid methyl transferase 1, were inoculated in the morning (AM), which allows extended time exposure. In contrast, late primary inoculations (PM) require MeSA. As well, AM inoculations of Col-0 wild-type (Wt) plants induced a stronger SAR than was detected in Wt plants inoculated at PM, or AM -inoculated *bsmt1* mutant plants suggesting that MeSA is required only for PM-inoculated plants, and maximal robust of SAR in AM-inoculated plants (Liu et al., 2011). AM inoculations partially restored SAR in *med4*, which lacks MeSA esterase activity, *dir1*, and *gly1*. On the other hand, AM inoculations failed to restore SAR in *fmo1* mutant plants providing an evidence of the linkage between light and other SAR signals (Liu et al., 2011).

1.3.1.3 SAR in nature

Plants in nature could develop induced resistance mechanisms against future pathogen attack, and without the help of these resistance mechanisms, we could see severe disease symptoms everywhere not only in specific areas where there are virulent pathogens that can break resistance. This defense priming confers protection against a wide range of virulent pathogens. Furthermore, this induced priming is a cost-efficient defense strategy under unfavorable environmental conditions (Ahmad et al., 2010). In the meantime, for triggering SAR, plants need to be activated by an avirulent pathogen that can elicit ETI, which is required for SAR where virulent pathogen causes PTI and severe local infection without triggering SAR. As well, non-host pathogens cannot induce SAR and cannot infect the plants. Natural constitutive SAR in the field is not guaranteed where SAR is induced only for a certain time and if the pathogen causes infection after that time, the plant exhibits severe symptoms. For example, 0.5mM BTH prime *Capsicum annuum (Ca) pathogenesis-related protein 4 (PR4)* gene (*CaPR4*) of pepper (Capsicum annuum) for 20 days and induce SAR against *Xanthomonas axonopodis* (Yi et al., 2012).

1.3.1.4 Priming

Priming, using small concentrations of chemicals, which is an additional feature of SAR, improves yield and overcomes diseases. In addition, priming is an essential to sustain agricultural productions particularly in view of growing world population. Furthermore, use of primed plants to prevent spread of disease will significantly reduce use of fungicides and pesticides that lead to severe environmental consequences. Plant defense and development are antagonistic processes and induction of plant defense often counteracts plant growth and yield. However, plants primed for defense are able to counter the biotic challenges just as effectively without negatively impacting growth or yield. Priming is "battle ready" status wherein plants are able to induce strong defense when challenged by a pathogen but is not associated with accumulation of defense compounds prior to the attack. For instance, controlled spray with low dose of chemical inducers of defense can help plants attain a primed state against microbial pathogens, herbivore insects or even abiotic stresses (Ahmad et al., 2010). Recent studies have shown that priming can enhance both local as well as systemic defenses. Interestingly, the primed status of plants is inheritable across at least one generation (Luna et al., 2012).

Plant diseases have a major negative impact on yield. Traditional methods employed to control diseases include plant breeding and chemical sprays. These become less effective over time as pathogens adapt and/or become tolerant to chemical treatments. Moreover, chemical sprays have a pronounced damaging effect on our environment. Control of plant diseases based on plants innate immune response is a safer and more effective way to combat plant diseases. The primed status of plants allows growers to mount a durable resistance response against diseases without causing any developmental phenotypes. Thus, mechanism underlying priming and characterization of components involved in priming will likely open up alternate ways to engineering broad-spectrum disease resistance in plants.

1.3.1.5 Fitness and Defense in SAR

There are different methods to fight plant diseases and we need to choose among those strategies. First, by using chemical inducers at considerable concentrations that will confer resistance against pathogens with nominal fitness costs if there is no infection compared with untreated plants. Second, the use of resistant transgenic plants, but the possible risks on humans and animal health should be put into consideration. As well, pathogens can break resistance after some time. Third, the use of fungicides and pesticides can control the diseases significantly, but have serious implications for human and environmental welfare. As a result, the use of chemical inducers to induce SAR is studied to check the possibility of using this method for disease resistance, and the most studied ones are the functional analogs of salicylic acid, BTH and 2,6-dichloroisonicotinic acid (INA) (Maleck et al., 2000). High concentrations of BTH or INA exhibit signs of phytotoxicity independent of the defense response (Louws et al., 2001). BTH is used in Europe to control powdery mildew of wheat and barley (Görlach et al., 1996). Induction of SAR was successful in controlling the plant diseases without high fitness costs. The *npr1* mutation compromise SAR without any effect on fitness under growth chamber conditions, but decreased fitness in the field. Constitutive activation of SAR by *cpr1* (constitutive expressor of *PR1* genes), *cpr5*, and *cpr6* negatively affect fitness in the field. This means that SAR is costly in the absence of infection but it provides protection under field conditions and this protection is affected by environmental conditions (Heidel et al., 2004). For instance, *Peronospora parasitica*, and *Albugo candida* favor high humidity environment, and low humidity limits their pathogenicity and virulence to *Arabidopsis thaliana* (Holub et al., 1994, Holub et al., 1995). Also, induction of direct defense using high concentrations of BTH, which accompanied by activated *PR1* expression even before pathogen infection, shows slightly higher levels of resistance than primed plants, which got induced by small concentrations of BTH. Moreover, high chemical concentrations that triggers direct defense involve higher costs on plant growth, which analyzed by quantifying relative growth rate and seed production, than low chemical concentrations that induce priming. As well, primed plants display the highest growth rates and seed production under conditions of disease

pressure (van Hulten et al., 2006). Also, elicitation of SAR is not giving the plant a full protection against enemies. For example, activation of young cotton seedlings was activated with BTH is not stopping the oviposition of whiteflies *Bemisia tabaci* (Gennadius) or the feeding of bollworms *Helicoverpa armigera* (Hübner) although this activation is inducing the activities of pathogenesis related (PR) proteins suggesting that SAR is not efficient against phytophagous insects (Inbar et al., 2001).

1.3.2 Salicylic acid (SA) and Defense

Salicylic acid (SA) and many phytohormones are involved in several plant processes, which regulate development and stress responses. For instance, both jasmonic acid (JA) and ethylene are important components of the defense-signaling pathway against necrotrophic pathogens, such as *Botrytis cinerea*. On the other hand, SA is the key factor of the signaling pathway that confers resistance against biotrophic pathogens, such as *Pseudomonas syringae* (Anderson et al., 2004). Salicylic acid is a monohydroxy benzoic acid and a natural phenolic compound existing in all plant species (Raskin et al., 1990). It was thought initially that SA is a systemic signal that generated in the local infected tissues and transported via the phloem to confer systemic acquired resistance in distal tissues due to the significant induction of SA in distal leaves after challenging the local leaves with pathogens (Vlot et al., 2009). In addition, transgenic tobacco and Arabidopsis expressing *nahG* gene, the bacterial salicylate hydroxylase that encodes an enzyme that converts SA to catechol, are defective in SAR and *PR* proteins accumulation (Delaney et al., 1994). However, Vernooij et al. (1994) reported that the grafted *nahG* plants into wild-type tobacco, which lack SA accumulation, were able to confer normal SAR when challenged with TMV. In plants, SA is synthesized via the shikimic acid pathway either by isochorismate synthase (ICS), or by phenylalanine ammonia lyase (PAL). As well, most of SA is further converted into SA-O-β-glucoside (SAG) by SA glucosyltransferase (SAGT) (Vlot et al., 2009). Although SA was suggested to be a mobile signal during SAR (Shulaev et al., 1995; Mölders et al., 1996), grafting studies showed that SA is not transported from local to distal leaves but that its systemic accumulation, which is triggered by perception of mobile signals in distal leaves, is critical for SAR (Vernooij et al., 1994; Shah, 2009).

1.3.2.1 Non-expresser of Pathogenesis-related proteins (NPR1) gene

The Arabidopsis Non-expresser of Pathogenesis-related proteins (NPR1) gene controls SAR where *npr1* mutant fails to confer SAR in response of pathogen and several inducing treatments. Also, *npr1* mutant abrogates SA mediated induction of PR, where it exhibited a low PR1 expression after pathogen and SA treatment, suggesting that NPR1 is a positive regulator of the SA pathway (Cao et al., 1997). NPR1 exists as an oligomer where NPR1 monomers are connected through intermolecular disulfide bridges. Upon activation of defense responses within the plant, salicylic acid will be elevated to change the redox status conditions that reduce disulphide bonds of two cysteine residues Cys82 and Cys216 by THIOREDOXIN (TRX)-H5 and TRX-H3 within NPR1 oligomers in the cytoplasm allowing the release of NPR1 monomers that accumulates in the nucleus. On the other hand, S-nitrosylation of Cys156 facilitates disulphide linkage formation between NPR1 monomers leading to the reverse formation of NPR1 oligomer, which may maintain NPR1 homeostasis (Mou et al., 2003, Tada et al., 2008, Spoel and Loake 2011). The nuclear NPR1 protein interacts with several members of the TGA transcription factors and thus activates the expression of defense gene expression (Tada et al., 2008).

Objectives

1. Determine the role of NO and ROS in SAR by deciphering the interrelationships among the well-known chemicals.
2. Determine the cross talk between salicylic acid (SA) and nitric oxide (NO) – glycerol-3-phosphate (G3P) signaling pathways in SAR.
3. Determine the role of light in SAR.

CHAPTER 2
Materials and Methods

2.1 Plant growth conditions

Arabidopsis thaliana seeds were sown on steam sterilized soil and stratified overnight at 4°C. The plants were grown in MTPS 144 (Conviron, Winnipeg, MN, Canada) walk-in chambers at 22°C, 65% relative humidity and 14 h photoperiod. These chambers were equipped with cool white fluorescent bulbs (Sylvania, F096/841/XP /ECO). The photon flux density of the daytime period was 106.9 μmol m^{-2} s^{-1} (measured with a digital light meter, Phytotronic Inc, MO). The transplanted seedlings were covered with transparent plastic domes for 2-3 days to allow seedlings to adapt to the new soil. For blue light treatments, light was filtered through blue Roscolene filters (Vincent Lighting Systems) and the spectrum of the filtered light was measured using spectroradiometer (Jeong et al., 2010).

2.2 Plant treatments

Glycerol-3-phosphate (G3P) (100 μM; Sigma-Aldrich, MO, USA), salicylic acid (SA) (500 μM, pH 7.0; Sigma-Aldrich, MO, USA), 2-(N,N-Diethylamino)-diazenolate-2-oxide (DETA-NONOate) (100 μM; ENZO, USA), the nitrous oxide donor SULFO-NONOate (100 μM; ENZO, USA) and hydrogen peroxide (H_2O_2) (500 μM; Sigma-Aldrich, MO, USA) were prepared in pure sterile water. Azelaic acid (100 μM; Sigma-Aldrich, MO, USA) and pipecolic acid (1 mM; Sigma-Aldrich, MO, USA) were first dissolved in 200 μl of methanol and then diluted with pure sterile water. All these chemicals were injected into Arabidopsis plants with 1 ml needleless syringe to study SAR. SA was sprayed onto plants for some *PR* gene expression studies.

2.3 Pathogen infections

2.3.1 *Pseudomonas syringae* Pv. Tomato

2.3.1.1 Inoculum preparation: *Pseudomonas syringae* DC 3000 or *Pseudomonas syringae* expressing *avrRpt2* were prepared by inoculating a single bacterial colony in 10 mL of King's B medium (1.5 g K_2HPO_4, 1.5 g $MgSO_4.7H_2O$, 20 g tryptone, 10 mL glycerol, 15 g agar in 1 L of water, pH adjusted to 7.5 with 1 N HCl) containing the antibiotics kanamycin (50 µg/ mL; Sigma-Aldrich, MO, USA) and rifampicin (25 µg/ ml; Sigma-Aldrich, MO, USA). After overnight incubation on a shaker at 29°C in the dark, the cells were harvested by centrifugation at 3000 rpm for 10 min and the pellet was suspended in 10 mM $MgCl_2$. The cell density was measured at A_{600} with a spectrophotometer (Biomate type; Thermo Electron Corporation, England).

2.3.1.2 Inoculation: For SAR, the primary leaves were inoculated with $MgCl_2$ or the *P. syringae* bacteria at 10^7 colony forming unit (CFU)/ml. 24-48 h later, the distal leaves were inoculated with virulent bacteria (10^5 CFU/ml). Leaf discs (4 mm) were harvested from the distal leaves at 0 and 3 dpi with a cork borer. The leaf discs were ground and homogenized in 10 mM $MgCl_2$ and the undiluted (0 dpi) or the 10^3 fold diluted (3 dpi) homogenates were plated on King's B agar plates to be incubated at 29°C for 2 days in the dark. Then, the bacterial colonies were counted with a colony counter (Scienceware, Bel-Art Product, USA). For quantification of different metabolites such as G3P and azelaic acid, the leaves were inoculated with *avrRpt2* bacteria (10^6 CFU/ml).

2.3.2 *Colletotrichum higginsianum*

Colletotrichum higginsianum Sacc. Isolate IMI 349063 (provided by CABI Bioscience) was grown on oatmeal agar (Difco, NJ, USA) for 7 days at 22°C. Six-week-old Arabidopsis plants were used for spot inoculations with 10^6 conidia/mL. For spot inoculations, 10 µL of inoculum suspension was used to inoculate 3-4 leaves per Arabidopsis plant. The inoculated plants were transferred to a PGV36 Conviron walk-in

chamber and covered with a plastic dome to keep high humidity that favors pathogen growth. Disease symptoms were scored at 11 dpi with a digital Vernier caliper (Fischer scientific, PA, USA) to measure lesion size in inoculated leaves. The experiment included 40 to 50 plants and was repeated twice.

2.3.3 Turnip Crinkle Virus (TCV)

The 6 µg of linearized pT7TCV66 (*Sma* I) was used to synthesize TCV RNA using T7 RNA polymerase (NEB, USA), 5x transcription buffer (Promega, USA), 0.1M DTT, rNTPs (10 mM each), RNase inhibitor (Promega, USA), and DEPC. After incubation at 37°C for 60 min, phenol: chloroform: isoamyl alcohol (25:24:1) was used to purify TCV RNA that was subsequently precipitated by isopropanol. Viral RNAs were resuspended in DEPC-treated water and used for viral infections. A total of 10 ng of infection solution consisting of 2x infection buffer containing bentonite, DEPC water and TCV transcript was inoculated to three leaves of Arabidopsis plants on the outside cuticle with a surface-sterilized glass rod. Plants were incubated in a growth chamber permitted for infected plants at 14500-lux light intensity.

2.3.4 Tobacco Mosaic Virus (TMV)

Fully expanded leaves of 4-5 week-old plants were infected with tobacco mosaic virus (TMV, strain U1). Carborundum was evenly applied to the surface of tobacco leaves. 200 µl of TMV, at a concentration of 1 µg/ml in 10 mM HEPES buffer pH 7.0, was rubbed onto the leaves with a sterilized glass rod. Control plants were treated with HEPES buffer and Carborundum only (Park et al., 2007). TMV-resistant (*NN*) and TMV-susceptible (*nn*) Xanthi-nc tobacco plants (*Nicotiana tabacum*) were used.

2.4 NO staining

For NO staining, the adaxial sides of leaves were infiltrated with 4 µM of 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM DA) (Life technologies,

Oregon, USA) and incubated in the dark for 5 min. Afterwards, leaves were observed under an Olympus FV1000 laser-scanning confocal microscope with a 488 nm laser.

2.5 DNA extraction

The leaf samples were harvested and frozen in liquid nitrogen and ground with pestles (Fisher Scientific, PA, USA). The homogenized tissue was suspended in 150 µl of DNA extraction buffer containing 200 mM Tris-HCl pH 8.0, 25 mM EDTA, 1% SDS and 250 mM NaCl. Then, 100 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the extract and centrifuged for 10 min at 12,000 rpm, and the supernatant was precipitated with 100 µL of isopropanol. The samples were centrifuged for 10 min at 12,000 rpm, and the DNA pellet was air dried and suspended in 50-75 µL Tris:EDTA (10mM:1mM, pH 8.0) or sterile water.

2.6 RNA extraction and northern analysis

2.6.1 RNA extraction

Arabidopsis leaf samples (100 mg) were frozen in liquid nitrogen and extracted with 1000 µL Trizol reagent (Invitrogen, CA, USA). To this, 200 µL of chloroform was added. The homogenates were shaken several times and centrifuged at room temperature 10 min at 12,000 rpm. The supernatants were precipitated with 0.5 mL isopropanol. The precipitate was washed with 75% ethanol (diluted with DEPC), air-dried and suspended in 15-30 µL of DEPC-treated water. RNA was quantified by spectrophotometer (A_{260}) and ~ 7-10 µg total RNA was mixed with the loading mixture (39 µg/mL ethidium bromide, 0.39 X MOPS, 13.7% formaldehyde and 39% formamide and 2 µL of loading dye. The loading dye was composed of 50% glycerol, 1mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol. The RNA was electrophoresed on a 1.5% agarose gel containing 3% formaldehyde and 1X MOPS. MOPS buffer was prepared by adding 41.8 g MOPS, 6.8 g NaOAc, and 0.38 g EDTA, adjusted to pH 7.0, and sterile water was added to 1 L.

2.6.2 Northern analysis

The electrophoresed RNA gel was washed with 2xSSC and blotted onto Hybond^{TM}-NX nylon membrane by the capillary transfer of RNA from the electrophoresis gel to the blotting membrane (Amersham Biosciences, NJ, USA). After overnight wet-transfer, RNA was fixed under UV for 0.9 min in a CL-1000 ultraviolet cross-linker (115V, 0.7Amps, Model CL-1000; UVP, Upland, CA, USA). The membrane was washed in 2xSSC and dried at 65°C for at least 20 min. The membrane was hybridized in sodium phosphate buffer (200 mM, pH 7.0) containing sheared salmon sperm DNA (100 µg/mL), 7% SDS and 1.25 mM EDTA.

2.6.3 cDNA synthesis

Total RNA samples (3-7µg) were denatured at 65°C and annealed with 1 µL oligo dT_{17} solution (0.63 µg/ µl). The reaction mixture was supplemented with 1 µL reverse transcriptase (200 U/µL, Invitrogen, USA), 1 µL RNAase inhibitor (40 U/µL, Invitrogen, USA), 0.5 mM dNTPs and 10 mM DTT and incubated at 42°C for 1 h. The reaction was stopped by incubating the tubes at 65°C for 15 min and subsequently used for RT-PCR.

2.6.4 Synthesis of probe and hybridization

The DNA fragment was amplified from cDNA of wild-type plants with specific primers. The gel-purified DNA fragment was denatured at 90°C in a water bath for 5 min, kept on ice for 5 min and mixed with 1 µL Klenow enzyme (NEB, 2000 U/mL), 2 µL 10 x BSA and 10 µL of labeling mixture [containing hexanucleotide primers, 10 mM of Deoxyadenosine triphosphate (dATP), Deoxyguanosine triphosphate (dGTP) and Deoxythymidine triphosphate (dTTP), and 25 µCi α-^{32}P-dCTP (Perkin Elmer, USA)]. The reaction was incubated at 37°C for 1 h and the probe was purified by a MicroSpin G-50 Sephadex column (GE Healthcare). The labeled DNA was denatured by 14 µL 2 N NaOH for 15 min, neutralized with 13 µL of 1 M Tris pH 7.5 for 15 min and added to the

hybridization buffer. Hybridization was carried out overnight at 60 °C in a hybridization oven (Labnet International Inc.). The hybridized membrane was washed at 60°C twice with 2x SSC, 0.5% SDS and once with 1 x SSC, 0.1% SDS solutions. The membrane was exposed on a Storage Phosphor Screen (Amersham Biosciences) overnight and scanned on a Typhoon 9400 Variable Mode Imager (GE Healthcare). ImageQuant TL V2005 software was used to quantify signal intensity.

2.7 Real-time Quantitative PCR

Real-time quantitative PCR (RT-qPCR) was done on 96-well PCR plates with a Fast Real-Time PCR system PRISM 7900HT instrument (Applied Biosystems, CAUSA). The reaction was carried out with the Power SYBR® Green PCR Master Mix (2 x) reagent kit (Applied Biosystems, CA-USA). The cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min, and ended by a dissociation stage with a temperature regime of 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec. Each 20 µl reaction included 5 µl of cDNA, 10 µl SYBR® Green PCR Master Mix (2 x) and 0.2 µM each of forward and reverse primers, which were designed according to real-time PCR conditions. Three replicas of each sample were used to obtain standard curves. Analysis of both the target gene and the endogenous control gene (Actin) was done on the same plate to avoid plate-to-plate variations. Cycle threshold (Ct) values were automatically calculated by the SDS 2.3 software (PRISM 7900HT) with the default baseline setting (cycles 3-15). Expression of all tested genes was calculated with the relative comparative Ct method ($\Delta\Delta$Ct = normalized Ct as ΔCt – calibrator, where ΔCt = Ct of target gene – Ct of Actin, and calibrator = median of ΔCt), with Actin as the reference gene for normalization. The relative level of gene expression was then converted into fold-difference relative to the calibrator as $2^{-\Delta\Delta Ct}$.

2.8 Protein extraction and Western blot analysis

2.8.1 Protein extraction

Fresh plant tissue samples (0.1 g) were extracted in 200-300 µL protein extraction buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, and 1 x protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO-USA). The extract was centrifuged twice at 12000 rpm for 10 min at 4°C. Bio-Rad protein assay (Bio-Rad, CA-USA) was used to measure protein concentration.

2.8.2 Western blot analysis

For SDS-PAGE gel, 10-100 µg protein samples were mixed with 3x SDS loading buffer (3.0 mL H_2O, 1.2 mL 1 M Tris-HCl pH 6.8, 2.4 mL glycerol, 0.48 SDS, 60 µL 10% bromophenol blue and 1.5 mL β–mercaptoethanol). The samples were boiled at 100ºC in a water bath for 5-10 minutes. The samples were run on a SDS-PAGE minigel (6x9 cm) at 100V in 1x running buffer (14.4 g glycine, 3 g Tris-base, 1 L H_2O) until the bromophenol blue reached the bottom of the gel. For protein transferring, PVDF membrane (Immun-Blot, Bio-Rad) was first dipped in methanol, and other materials were pre-wet in 1x transferring buffer (3 g Tris-base, 15 g glycine and 1L H_2O), and the materials were packed in the transferring case in the following order: sponge, Whatman paper, PVDF membrane, protein gel, Whatman paper, sponge). The transfer was run at 400 mA for 1-2 h on ice with the frozen Bio-Rad mini-gel box electrotransfer. After transfer, PVDF membranes were stained with Ponceau-S solution (40% methanol, 15% acetic acid, 0.25% Ponceau-S). The excess stain was removed by washing the membrane with deionized water for 30 seconds. The membrane was photographed to show the amount of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) protein in different samples. The membrane was distained by rinsing in cold tap water for 2-3 minutes. The membrane was first blocked in 10 mL 5-8% non-fat dry milk dissolved in 1x TBST buffer (5 mM Tris-base, 20 mM NaCl, pH 7.4, 0.1% Tween 20) for 30-45 minutes on a shaker. After blocking, the primary antibody was added and incubated on a

shaker for 2-4 h. The membrane was washed 3-4 times for 10 min with 1x TBST buffer, and then the secondary antibody was added and incubated on a shaker for 2-4 h. The membrane was washed 3-4 times for 10 min with 1x TBST buffer. The membranes were developed with an ECL kit (1 mL/membrane) (Super-Signal, Thermo Scientific) and exposed to autoradiography film (Santa Cruz Biotechnology, USA), or developed by alkaline phosphatase-based color detection.

2.9 Collection of phloem exudates for different purposes

Arabidopsis leaves (3 leaves/plant) were inoculated with *P. syringae* containing *avrRpt2* (10^6 CFU/mL), or with $MgCl_2$ as a negative control. Twelve hours later, leaf petioles were excised, surface-sterilized in 50% ethanol, rinsed twice in sterile 1 mM EDTA and submerged in 1 mM EDTA containing 100 μg/mL ampicillin. The phloem exudates were collected after 48 h of incubation in a growth chamber and infiltrated into WT and mutant plants to study signal generation/perception of mutant plants. For SAR experiments, *Pseudomonas syringae* DC 3000 was inoculated in the distal leaves 48 h after infiltration of exudates. For G3P quantification, exudates collected from wild-type and mutant plants were freeze dried, rehydrated in 0.75 ml of pure sterile water, and purified on a 0.45 μm nylon columns (Corning Inc., USA). The extracts were run on PA1 columns and ion chromatography (ICS-3000, Dionex Inc., USA) was conducted. The quantification of G3P was based on the peak areas of the standard G3P sample (Sigma, USA) and internal standard 2- deoxyglucose using High-performance liquid chromatography (HPLC).

2.10 Extraction and quantification of azeliac acid

Two mL of 3% H_2SO_4 in methanol were aliquoted in 13 mm x 100 mm test tubes. Leaf tissue samples (0.1 g) were fully immersed in the test tube. 50 ng of suberic acid were added to all test tubes. The test tubes were incubated at 80°C till ~0.5 mL remained. The tubes were left to cool down for 5-10 min. To each tube was added 1 mL of n-hexane (Sigma, USA), and then the tubes were vortexed briefly and left to stand in the rack for 5-10 min. The upper phase was placed into glass inserts and transferred to GC glass vials to

be analyzed by GC-MS.

2.11 Extraction and quantification of Salicylic acid and Salicylic acid glucoside (SAG)

For SA and SAG extraction, 0.3 g of $MgCl_2$-, or *avrRpt2*- (10^6 CFU/mL) inoculated leaves were collected and analyzed using with an Agilent 1100 (Agilent Technologies, Palo Alto, CA, USA). Detection was done with diode-array and fluorescence-array detectors using on a Novapak C18 column (Waters, Milford, MA, USA). Sample extraction and analysis was carried out in collaboration with Dr. Duroy Navarre (USDA-ARS, Prosser, Washington).

2.12 Extraction and quantification of free radicals

Electron spin resonance spectrometry (ESRS) was used to quantify free radicals generated in response to $MgCl_2$ and *Pst avrRpt2* in Arabidopsis plants. The different free radicals were detected at 12 hpi in local tissues and at 24 hpi in distal tissues. 0.1 g of plant tissues was extracted with α-(4-Pyridyl *N*-oxide)-*N*-*tert*-butylnitrone (POBN) (50 mM in 20 mM HEPES buffer pH 7; Sigma-Aldrich, USA), which detects hydroxyl and carbon-centered radicals, or with 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO), which detects superoxide anion radicals (50 mM in 20 mM HEPES buffer pH 7; Sigma-Aldrich, USA). Extract (10 µL) was transferred to a graduated capillary tube, sealed with glue, and then analyzed by ESRS.

Table 2.1. Seed materials used in the study.

Sl No.	Mutants and transgenic seeds	References
1	Columbia-0 (Col-0)	Kachroo et al. (2003)
2	Dijon (Di-17)	Kachroo et al. (2000)
3	Nossen (Nö)	Kachroo et al. (2001)
4	*gly1-1* (Col-0)	Miquel (1998), Kachroo et al. (2004)
5	*gli1* (*nho1*) (Col-0)	Kang et al. (2003), Kachroo et al. (2005)
6	*gly1-1 gli1*(Col-0)	Chanda et al. (2011)
7	*sid2-1* (Col-0)	Wildermuth et al. (2001)
8	*dir1* (Col-0)	Arabidopsis Biological Resource Center (ABRC)
9	*rbohD* (Col-0)	Wang et al. (2014)
10	*rbohF* (Col-0)	Wang et al. (2014)
11	*nox1* (Col-0)	Wang et al. (2014)
12	*gsnor1-3* (Col-0)	Wang et al. (2014)
13	*attrx5-1* (Col-0)	Wang et al. (2014)
14	*attrx5-2* (Col-0)	Wang et al. (2014)
15	*noa 1 nia2* (Col-0)	Mandal et al. (2012); Wang et al., 2014
16	*npr1-1* (Col-0)	Kindly provided by Prof. Xinnian Dong
17	*med4* (Col-0)	Kindly provided by Prof. Dan Klessig
18	*pad4* (Col-0)	Jirage et al. (1999)
19	*npr3* (Col-0)	Kindly provided by Prof. Xinnian Dong
20	*npr4* (Col-0)	Kindly provided by Prof. Xinnian Dong
21	*npr3 npr4* (Col-0)	Kindly provided by Prof. Xinnian Dong
22	*wrky18* (Col-0)	Kindly provided by Prof. Xin Li
23	*tga256* (Col-0)	Kindly provided by Prof. Xin Li
24	*bsmt1* (Col-0)	Kindly provided by Prof. Dan Klessig
25	*azi1-1* (Col-0)	Yu et al., 2013
26	*fmo1* (Col-0)	Arabidopsis Biological Resource Center (ABRC)
27	*ssi2* (Nö)	Kachroo et al. (2001)
28	*Nicotiana tabacum*	Kindly provided by Prof. Dan Klessig

CHAPTER 3

Role of Nitric oxide and Reactive Oxygen Species in Systemic Acquired Resistance

3.1 Introduction

Plants are subjected to a wide range of biotic and abiotic stresses, which reduces crop yields. Understanding different defense signaling pathways within plants can improve our understanding to develop new strategies to enhance plant resistance. Systemic immunity is one form of defense response where plants show an increased resistance against secondary infections. In this chapter, I will describe a form of systemic immunity called systemic acquired resistance (SAR), and its relation to nitric oxide (NO) and reactive oxygen free radicals.

3.1.1 Nitric Oxide (NO) and defense

Nitric oxide (NO) is a small free radical that exists as a gas, and is soluble in water and lipids. NO is known to play significant signaling roles in mammalian systems (Neill et al., 2003). NO is synthesized in mammalian cells by NO synthase (NOS) enzyme, which catalyzes the NADPH-dependent oxidation of arginine to form NO (Delledonne et al., 1998). NO is involved in several plant physiological processes such as, germination, stomatal closure, and cross talk with plant hormones (Delledonne et al., 1998; Durner et al., 1998; Lamattina et al., 2003; Besson-Bard et al., 2008). Recently, NO was shown to play important roles as a signaling component in defenses against biotic and abiotic stresses (Neill et al., 2002). Induction and interaction of NO with reactive oxygen species (ROS) in response to biotic and abiotic stresses plays a vital role in plant defense (Durner et al., 1998; Beligni and Lamattina 1999; Neill et al., 2002). A balance between ROS and NO is crucial since excess NO or ROS can have damaging effects on plants (Hausladen et al., 1998).

In plants, NO biosynthesis occurs via nitrate reductase (NR) and NITRIC OXIDE ASSOCIATED1 (NOA1)–catalyzed reactions (Guo et al., 2003; Besson-Bard et al.,

2008). NR is a cytosolic enzyme that catalyzes NAD(P)H-dependent reduction of nitrate to nitrite. NR is encoded by two genes in Arabidopsis, *Nitrate reductase 1 (NIA1)* and *2* (Besson-Bard et al., 2008; Moreau et al., 2008). NOA1 was thought to function similar to the mammalian NO synthases (Guo et al., 2003). Recently, Moreau et al. (2008) showed that NOA1 has GTPase activity. As well, oleic acid (18:1), which is synthesized within chloroplast nucleoids, regulates NOA1 stability and NO biosynthesis. Reductions in 18:1 levels lead to increased NOA1 levels, which increase NO levels (Mandal et al., 2012).

NO has significant roles in various signal transduction pathways. The free radical NO reacts rapidly with glutathione (GSH) in an O_2-dependent reaction to form S-nitrosoglutathione (GSNO). Consequently, GSNO can function as a stable reservoir for NO. The reaction of NO with thiol group of cysteine residues in proteins is called S-nitrosylation, which can alter protein stability, activity, and/or localization. As a result, S-nitrosylation is considered one of the key regulatory processes in NO-mediated signaling (Yu et al., 2014). A balance between nitrosylation and denitrosylation must be achieved to maintain the signaling within the cell. Two enzymatic systems are involved in denitrosylation and they are important in ameliorating the nitrosative stress resulting from the increased GSNO levels (Benhar et al., 2010). The first enzyme is the thioredoxin/thioredoxin reductase (Trx/TrxR) that functions as an oxidoreductase to catalyze NADPH to NADP+ to convert the oxidized thioredoxin (Trx-S_2) to reduced thioredoxin (Trx-$(SH)_2$) (Benhar et al., 2009). The second enzyme system is S-nitrosoglutathione Reductase (GSNOR), which reduces GSNO to glutathione disulphide (GSSG) and ammonia (NH3) in a NADH dependent reaction (Sakamoto et al., 2002). Mutant *gsnor1* in Arabidopsis displays severe developmental phenotypes such as delayed seed germination, reduced plant growth, loss of apical dominance, and increased numbers of highly branched shoots (Holzmeister et al., 2011). In addition, a mutation in GSNOR disables R-mediated, basal and non-host disease resistance (Feechan et al., 2005). GSNOR is also involved in plant-herbivore defense in tobacco by regulating the accumulation of jasmonic acid and ethylene (Wünsche et al., 2011).

3.1.2 Reactive Oxygen Species (ROS) and Defense

A free radical is any species being able to exist independently with one or more unpaired electrons (Halliwell 1991). Different environmental stresses excite unreactive molecular oxygen O_2 to form reactive free radicals (Polidoros et al., 2005), which can cause oxidative damage of proteins, lipids, carbohydrates and other cellular metabolites. Reactive oxygen species (ROS) accumulate in response to biotic and abiotic stresses, and play a vital role in signal transduction events (Moller et al., 2007). Pathogen infection triggers rapid accumulation of ROS in a process named oxidative burst (Grant and Loake 2000). The different types of ROS are singlet oxygen (O_2^1), which is produced as a result of O_2 excitation, superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and a hydroxyl radical (OH^-), which result from the transfer of one, two or three electrons to O_2, respectively. In addition, ROS production and scavenging occurs in a number of cellular compartments (Table 3.1) (Mittler 2002).

NADPH oxidases, which are localized into the plasma membrane, catalyze the production of superoxide radicals in animals and plants (Sagi and Fluhr 2006). Plant NADPH oxidases are known as respiratory burst oxidases (Rboh) due to the closest functional homology to mammalian NADPH oxidases (Torres and Dangl 2005). There are ten Rboh genes in Arabidopsis that are expressed in a tissue specific manner (Table 3.2) (Sagi and Fluhr 2006). Both RbohD and RbohF are required for ROS production in Arabidopsis in response to *Pseudomonas syringae* and *Peronospora parasitica* (Torres et al., 2002). Silencing of *NbrbohA* and *NbrbohB* genes lead to reduced production of ROS and severe susceptibility against *Phytophthora infestans* (Yoshioka et al., 2003).

Reactive oxygen species have the ability to react with lipids, proteins and DNA. Also, ROS such as H_2O_2 can diffuse through the biological membranes to participate in systemic responses (D'Autréaux and Toledano 2007). The signaling role of H_2O_2 includes the control of hypersensitive response and induction of the expression of genes that encode glutathione-S-transferase and glutathione peroxidase (Levine et al., 1994).

Mechanism of Production	Localization	Reactive Oxygen Intermediates
Photosynthesis electron transport and PSI or II	Chloroplast	O_2^-
Respiration electron transport	Mitochondria	O_2^-
Glycolate oxidase	Peroxisomes	H_2O_2
Excited chlorophyll	Chloroplast	O_2^1
NADPH oxidase	Plasma membrane	O_2^-
Fatty acid β-oxidation	Peroxisomes	H_2O_2
Oxalate oxidase	Apoplast	H_2O_2
Xanthine oxidase	Peroxisomes	O_2^-
Peroxidases, Mn^{2+} and NADH	Cell wall	H_2O_2, O_2^-
Amine oxidase	Apoplast	H_2O_2

Table 3.1: Production mechanisms of reactive oxygen intermediates in plants (Mittler 2002)

Rboh	Tissue Specificity
A	Root, elongation zone
B	Root, elongation zone
C	Root, elongation zone
D	All plant parts
E	Cell suspension, root, and seeds
F	All plant parts
G	Root, elongation zone
H	Stamens, pollen
I	Root, elongation zone
J	Stamens, pollen

Table 3.2: Different Rboh and their tissue specificity in Arabidopsis (Sagi and Fluhr 2006)

3.2 Results

3.2.1 Exogenous application of an NO donor confers SAR

Arabidopsis *Pst*-inoculated leaves were treated with the NO sensitive dye, 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM DA;Balcerczyk et al., 2005) at 12 h post inoculation (hpi) and compared to mock-inoculated plants. Confocal microscopy of *Pst*-infected leaves showed increased NO accumulation, which was detected as green fluorescence at 12 hpi (Figure 3.1). To determine if NO accumulation played a role in SAR, I infiltrated wild type (Wt) plants (ecotype Col-0) with $MgCl_2$, *Pst avrRpt2*, the NO donor 2-(N,N-Diethylamino)-diazenolate-2-oxide (DETA-NONOate), and the nitrous oxide donor SULFO-NONOate. The distal leaves of all plants were then challenged with a virulent strain of *Pst* (DC3000) and the bacterial growth was quantified at 0 and 3 dpi. The results showed that DETA-NONOate application significantly reduced the growth of *Pst* DC3000, suggesting that NO might play a role in SAR (Figure 3.2).

3.2.2 NO induces SAR in a dose dependent manner

It is well known that NO functions in a dose dependent manner in mammalian systems (Wink et al., 2011). I therefore tested SAR in response to different NO concentrations. As shown in Figure 3.3, SAR was progressively stronger in plants infiltrated with increasing concentrations of DETA-NONOate, and a concentration of 100 µM DETA-NONOate conferred strong SAR. However, higher concentrations of DETA-NONOate did not confer SAR, suggesting that NO acts in a dose dependent manner. This result was also strengthened by evaluating SAR in genetic mutants (NO overproducer:; *nox1, S*-nitrosoglutathione reductase:; *gsnor1*, and THIOREDOXIN-H5:; *trx5*) that accumulated elevated levels of NO (Figure 3.4). All these NO over-accumulating mutants showed compromised SAR (Figures 3.5A, 3.5B, 3.5C). Moreover, exogenous application of

DETA-NONOate could not confer SAR in the *nox1*, *gsnor1, attrx5-1* and *attrx5-2* mutant plants (Figure 3.6A, 3.6B). The NO accumulation correlated well with the increased levels of NO associated protein 1 (AtNOA1) in the *nox1* and *gsnor1* mutant plants. As shown before, the *ssi2* (Suppressor of SA Insensitivity) mutant that accumulates constitutive NO accumulated higher levels of NOA1 (Figure 3.7).

3.2.3 Compromised SAR in *noa1nia2* is not associated with salicylic acid (SA) biosynthesis or response

SA is an essential component of SAR that was reported to function downstream of NO (Durner et al., 1998). I assessed relationship between SA and NO by assaying *PR-1* levels in Wt plants in response to SULFO-NONOate or DETA-NONOate treatments. Treatments with 100 µL of SULFO- or DETA-NONOate did not induce *PR-1* expression in comparison to *Pst-avrRpt2* infection (Figure 3.8A). This suggests that SULFO- or DETA-NONOate likely does not induce SAR by activating the SA pathway. Moreover, *noa1 nia2* plants were able to induce Wt-like *PR-1* expression in response to SA (Figure 3.8B). Together, these results suggest that compromised SAR in *noa1 nia2* plants was not associated with their SA responsiveness, and that SA does not function downstream of NO in the SAR pathway.

3.2.4 Compromised local resistance in NO-accumulating mutants

In this part, I investigated the effect of high NO on local defense responses in *ssi2, nox1* and *gsnor1* mutants. Arabidopsis leaves were inoculated with a virulent strain of *Pst* (DC3000) and/or an avirulent strain of *Pst avrRpt2,* then, the bacterial growth was monitored at 3 dpi. Interestingly, unlike *ssi2*, which shows constitutive resistance against virulent bacterial pathogen (Kachroo et al., 2005), both *nox1* and *gsnor1* showed compromised basal resistance (Figure 3.9A). Also, *nox1* plants showed compromised R-mediated resistance in comparison with Col-0 (Figure 3.9B). These differences could

either be because of different levels of NO accumulation in the *ssi2, nox1,* and *gsnor1* plants, or variable NO localization in these mutant backgrounds.

To test the effect of high NO on *PR* expression, Wt and mutant Arabidopsis plants were analyzed in response to *Pst avrRpt2* and SA. The results showed a reduced *PR-1* and *PR-2* expression in *gsnor1* in response to *Pst avrRpt2* and SA, in comparison to Col-0 plants (Figures 3.10A-D). Also, Figure 3.10 (panel E) shows reduced *PR-1* expression in *attrx5-1, attrx5-2* and *nox1* in response to *Pst avrRpt2* in comparison to Col-0. Data presented in Figures 3.11, 3.12, showed reduced accumulation of SA glucoside levels in the local leaves of *avrRpt2* -infected *gsnor1* (Figure 3.11)*, attrx5-1, attrx5-2* and *nox1* (Figure 3.12), and reduced accumulation of SAG in distal leaves in case of *gsnor1* (Figures 3.11B, 3.12B). In addition, *attrx5-1, attrx5-2* and *nox1* displayed a reduced accumulation of SA in the local leaves (Figure 3.12A). As well, *nox1* showed a reduced accumulation of SA in the distal leaves (Figure 3.12A).

These findings prompted us to investigate if exogenous SA can recover SAR in NO-accumulating mutants by pre-infiltrating Col-0, *gsnor1* and *nox1* with SA, and water to serve as a negative control. The distal leaves of all plants were then inoculated by a virulent strain of *Pst* (DC3000) and the bacterial growth was monitored at 0 and 3 dpi. The results showed that SA could not confer SAR in *gsnor1* and *nox1* mutants in comparison with Wt (Figure 3.13). I next assayed if high NO affect SAR signal generation or signal perception. For this, I collected petiole exudates (EX) from Wt and *gsnor1* plants that were pre-infiltrated with either $MgCl_2$ (EX_{MgCl2}) or *Pst avrRpt2* ($EX_{avrRpt2}$) and infiltrated these into a fresh set of Wt and *gsnor1* plants (Figure 3.14). The distal leaves of all plants were inoculated with *Pst* DC3000 24 h later, and growth of *Pst* DC3000 was monitored at 0 and 3 dpi (Figure 3.14). Both $^{Col\text{-}0}EX_{avrRpt2}$ and $^{gsnor1}EX_{avrRpt2}$ were able to confer SAR in Wt but not in *gsnor1* plants (Figure 3.14). This result suggested that the impaired SAR in *gsnor1* plants was associated with their inability to perceive the SAR inducing signal(s).

Next, I tested if the increased NO levels in *nox1* enhanced resistance to *Colletotrichum higginsianum*. Col-0, and *nox1* were infected by spot inoculations of 10^6 spores/ml. The inoculated leaves were scored for disease symptoms at 11 dpi. The *nox1* plants appeared more resistant to *C. higginsianum* than the wild type plants (Figure 3.15A, 3.15B). These results suggested that high NO levels in *nox1* plants might be associated with the increased resistance to *C. higginsianum.* This further prompted me to check basal resistance of *nox1* against Turnip crinkle virus (TCV) (Figure 3.16A-C). TCV was inoculated on resistant ecotype Di-17, susceptible ecotype Col-0, and *nox1* plants. Local (3dpi) and systemic (14 dpi) samples were analyzed for TCV levels by detecting the presence of the viral coat protein (CP). Both Col-0, and *nox1* showed susceptibility to TCV and accumulated Wt (Col-0)-like levels of CP in the local and systemic tissues (Figure 3.16A, 316B). The unexpected accumulation of TCV-CP in the resistant Di-17 could be a result of using old growing plants but this accumulation is still lower than that was observed in Col-0, and *nox1*. This result suggests that constitutive accumulation of NO might not contribute to viral resistance.

3.2.5 NO acts upstream of azelaic acid (AzA) and glycerol-3-phosphate (G3P)

Since the dicarboxylic acid AzA functions upstream of G3P to induce SAR (Yu et al., 2013), it was necessary to study the relation of NO with both AzA and G3P. Col-0 and *noa1nia2* were pre-infiltrated with AzA, and 0.01% methanol to serve as a negative control to assay SAR. The distal leaves of all plants were then inoculated by a virulent strain of *Pst* (DC3000) and the bacterial growth was monitored at 0 and 3 dpi. The results presented here show that AzA was able to confer SAR in *noa1 nia2* (Figure 3.17). Also, AzA levels were checked in mock ($MgCl_2$) and avirulent pathogen-inoculated (*avrRpt2*) local leaves of Col-0 and *noa1 nia2* plants 24 h after inoculation, and the data showed reduced accumulation of AzA in the local leaves of *noa1 nia2* (Figure 3.18). Next, G3P levels were analyzed in petiole exudates collected from mock- and *avrRpt2*-inoculated Col-0 and *noa1 nia2* leaves that were sampled 24 h after inoculations. Results presented in Figure (3.19), show reduced G3P levels in *noa1 nia2* plants. Collectively, these results

suggest that NO likely functions upstream of AzA/G3P.

3.2.6 ROS is required for SAR in a dose dependent manner

Since NO and ROS co-regulate a number of processes (Grant and Loake, 2000), I next assessed if ROS induced SAR and its dose-response relationship. First, Wt plants were pre-infiltrated with water or 5-2000 μM H_2O_2. Then, the distal leaves of all plants were inoculated by *Pst* DC3000 and bacterial growth was investigated at 0 and 3 dpi. Plants pre-infiltrated with 500 μM H_2O_2, but not water, showed significantly reduced bacterial growth, and this was comparable to growth of *Pst* DC3000 in plants pre-infected with *Pst avrRpt2* (Figure 3.20). As with NO, pre-infiltration of lower concentration of H_2O_2 (5 μM) and higher concentration (2 mM) were less effective in inducing SAR (Figure 3.20). This suggested that, like NO, H_2O_2 was a potent inducer of SAR in Wt plants, and that H_2O_2-triggered SAR was concentration dependent.

To test whether the H_2O_2 induced SAR was biologically relevant, I assayed SAR in ROS mutants (respiratory burst oxidase homologs, *rboh*), which are defective in ROS production (Torres et al., 2002; Sagi and Fluhr, 2006). There are ten Rboh genes in Arabidopsis that are expressed in different cellular compartments but only two of these (*RBOHD* and *RBOHF*) are expressed throughout the whole plant (Sagi and Fluhr, 2006). I first evaluated the expression of *RBOHD* and *RBOHF*, in $MgCl_2$- and *avrRpt2*-infiltrated plants. In comparison to mock-infiltrated plants, *avrRpt2*-infected plants showed induction of *RBOHD* and *RBOHF* expressions (Figure 3.21). Next, I evaluated SAR response in *rbohD* and *rbohF* mutants, and found that both *rbohD* and *rbohF* were defective in SAR (Figure 3.22). Next, I assayed basal and R-mediated resistance in *rbohD* and *rbohF* plants. Arabidopsis leaves were inoculated with a virulent *Pst* (DC3000) or an avirulent strain of *Pst avrRpt2,* and the bacterial growth was monitored at 3 dpi. Both *rbohF* and *rbohD* showed normal basal resistance against virulent bacterial pathogen (Figure 3.23A), and normal R-mediated resistance against *avrRpt2* (Figure 3.23B) in comparison with Col-0. These findings suggest that RBOHD and RBOHF are

specifically required only for SAR. It is possible that RBOHD and RBOHF might act redundantly in basal and R-mediated resistance against *P. syringae*.

To quantify the difference in free radicals production between mock-infiltration and *Pst avrRpt2* infection in Wt plants, I collaborated with Dr. M.B. Shine to monitor ROS levels (superoxide radicals) using electron spin resonance spectrometry (ESRS). As expected, pathogen infection induced accumulation of ROS in local and distal tissues of Wt plants at 12 and 24 h post infection respectively (Figure 3.24). We monitored ROS levels in *rboh* mutants to determine if their compromised SAR correlated with the defect in ROS levels. Quantification of free radicals in local and distal tissues of *rbohD* plants showed significantly reduced levels of free radicals trapped by α-(4-Pyridyl *N*-oxide)-*N*-*tert*-butylnitrone (POBN) in comparison with Wt (Figure 3.25). This in turn correlated with their compromised SAR. The *Pst avrRpt2*-inoculated *rboh* mutants also showed a defect in the accumulation of superoxide anion radicals, detected by 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide (EMPO) spin trap (Figure 3.26). To determine the relationship between NO and ROS during SAR, ROS levels were monitored in *noa1 nia2* and *gsnor1* plants. Interestingly, similar to *rbohD* mutants, pathogen infected *noa1 nia2* plants also accumulated reduced levels of the different free radicals (Figures 3.25, 3.26). This result suggested that the NOA1- and NIA2-derived NO was essential for inducing ROS production in response to pathogen infection, and that ROS likely functioned downstream of NO. In accordance with this suggestion, exogenous local application of ROS could recover SAR in *noa1 nia2* plants (Wang et al., 2014), whereas DETA-NONOate did not confer SAR on the *rboh* mutants (Figure 3.27).

3.2.7 Compromised SAR in ROS mutants is not associated with salicylic acid (SA) biosynthesis or response

Earlier results had suggested that compromised SAR in *noa1 nia2* plants was not related to SA, and that SA does not function downstream of NO in the SAR pathway (Figure 3.9). To test if this was also the case for *rboh* mutants, I analyzed *PR* gene expression levels in *rboh* plants. Pathogen inoculation induced similar *PR-1* expression in the infected and

distal tissues of Col-0 and both *rboh* mutants (Figure 3.28A). Furthermore, the *rboh* mutants induced similar levels of *PR-1* in response to SA as Wt (Figure 3.28B). Together, these results suggested that the defective SAR in the *rboh* mutants was not associated with a defect in the SA pathway. Moreover, water or H_2O_2 treatments did not induce *PR-1* expression in local and distal leaves of Wt, *rbohD, rbohF,* and *noa1nia2* (Figure 3.28C-F). The SA non-responsive mutant *npr1-1* was used as control in this experiment. Furthermore, localized application of SA also failed to induce SAR in *rbohD* and *rbohF* plants (Figure 3.29). These results suggest that H_2O_2 does not induce SAR by activating the SA pathway and that NO-ROS and SA comprise two distinct branches of the SAR pathway.

3.2.8 ROS act upstream of azelaic acid (AzA) and glycerol-3-phosphate (G3P)

To check the relation of ROS with both AzA and G3P, Col-0 and ROS mutant plants were pre-infiltrated with AzA (Figure 3.30), and G3P + *Pst-avrRpt2* (Figure 3.31) to assay SAR. As expected, both AzA and G3P + *Pst-avrRpt2* confered SAR in *rbohD* and *rbohF* plants, suggesting that AzA acts downstream of ROS.

3.2.9 Signal generation and perception in ROS mutants

Next, I assayed signal generation or signal perception in ROS mutants. For this, I collected EX from Wt, *rbohD* (Figure 3.32A), and *rbohF* (Figure 3.32B) plants that were pre-infiltrated with either $MgCl_2$ (EX_{MgCl2}) or *Pst-avrRpt2* ($EX_{avrRpt2}$) and infiltrated these into a fresh set of Wt and mutant plants (Figure 3.34). Then, the distal leaves of all plants were challenged with *Pst* DC3000 24 h later, and growth of *Pst* DC3000 was monitored at 0 and 3 dpi. All of $^{Col\text{-}0}EX_{avrRpt2}$, $^{rbohD}EX_{avrRpt2}$ and $^{rbohF}EX_{avrRpt2}$ were able to confer SAR in Wt but not in ROS mutant plants. This result suggested that the impaired SAR in ROS mutant plants was associated with their inability to perceive the SAR inducing signal(s).

3.3 Discussion

The plant immune system depends on several layers of constitutive and inducible defenses (Thordal-Christensen, 2003), and recruits metabolites that function in various subcellular compartments (Bednarek and Osbourn, 2009). Systemic acquired resistance (SAR) is a well-known response of plants to biotic stress (Shah and Zeier, 2014; Gao et al., 2015). In this chapter, I have characterized the role of NO and ROS in plant defense. I show that NO and ROS are key players in SAR signaling.

Basal levels of SA is required for AzA- and G3P-mediated SAR (Yu et al., 2013), while NO has been reported to function upstream of SA (Durner et al., 1998). Furthermore, NO levels can be regulated by oleic acid (18:1) (Mandal et al., 2012), which acts as a precursor for AzA and induces G3P biosynthesis (Yu et al., 2013). This regulation occurs via the association of 18:1 with the NO Associated (NOA1) protein (Mandal et al., 2012). This suggests a possible link between NO and C18 FA→AzA→G3P mediated SAR. Consistent with these results, NOA1 protein accumulates in both local and distal tissues in response to *Pst-avrRpt2* infection (Wang et al., 2014). In this study, after screening of a time-course analysis of NO levels with the NO sensitive dye (DAF-FM DA; Balcerczyk et al., 2005), an increase of NO accumulation was found by confocal microscopy of *Pst*-infected leaves. Also, the NO donor (DETA-NONOate) significantly reduced the growth of *Pst* DC3000 in SAR experiments. Moreover, *noa1nia2* double mutant, which is compromised in pathogen-responsive NO accumulation, shows compromised SAR (Wang et al., 2014). Together, these findings suggest that NO serves as a signal in SAR pathway. NO functions in a dose-dependent manner in mammalian systems (Wink et al., 2011). Similarly, higher or lower doses of NO do not induce SAR. This result was further ascertained by assaying SAR in NO-accumulating mutants (NO overproducer; *nox1, S*-nitrosoglutathione reductase; *gsnor1*, and THIOREDOXIN-H5; *trx5*). All these over-accumulating NO mutants were compromised in SAR. Moreover, exogenous application of DETA-NONOate was unable to confer SAR in the *nox1*, *gsnor1, attrx5-1* and *attrx5-2* mutant plants. In contrast to the compromised SAR of *gsnor1* plants, antisense downregulation of *GSNOR1* was reported to confer increased disease resistance

(Rustérucci et al., 2007). This result is thought to be due to partial reduction of GSNOR1 activity in the silenced plants compared with the complete loss-of-function in the knockout plants (Espunya et al., 2012). This in turn is consistent with our data, which showed that NO confers SAR in a concentration dependent manner.

SA is an essential component of SAR that functions downstream of NO (Durner et al., 1998). I investigated the involvement of NO in activating the SA signaling pathway. Data presented here show that NO donor (DETA-NONOate) treatment did not induce *PR-1* expression compared to pathogen infection. Moreover, *noa1 nia2* plants were able to induce as much *PR-1* expression as Wt plants in response to SA. Furthermore, my results showed that exogenous SA could not confer normal SAR in a NO-deficient mutant *noa1nia2* plants and in NO-accumulating mutants *gsnor1* and *nox1*. Together, these results suggested that DETA-NONOate does not induce SAR by activating the SA pathway, and that compromised SAR in *noa1 nia2* plants was not associated with SA, and that SA does not function downstream of NO in the SAR pathway.

Double mutations in the NOA1 NIA1/2 block the constitutive NO biosynthesis and the constitutive defense signaling in *ssi2* (Suppressor of SA Insensitivity) mutant plants (Mandal et al., 2012). Like the *ssi2* mutation, mutations in *NOX1* or *GSNOR1* also result in constitutive NO accumulation (Wang et al., 2014). This correlated well with increased levels of NOA1 protein in *nox1* and *gsnor1* mutant plants compared to Wt plants (El-Shetehy et al., 2015). Unlike *ssi2*, which shows constitutive resistance against virulent bacterial pathogen (Kachroo et al., 2005), the *gsnor1* and *nox1* plants showed compromised basal resistance. Also, *nox1* showed compromised R-mediated resistance in comparison with Col-0. These differences could be either due to different levels of NO accumulation or to variable NO levels in different subcompartments of the cell (El-Shetehy et al., 2015). Lower *PR* expressions in NO-accumulating mutants in response to *Pst avrRpt2* and SA than with Col-0, and the reduced accumulation of the SA and SA glucoside levels in local and distal leaves in response to *Pst- avrRpt2* infection suggested that high NO levels might downregulate the expression of genes that encode the enzymes

involved in SA biosynthesis. These findings are consistent with previous findings that SA-binding protein 3 (SABP3), which shows a high affinity for SA and expresses carbonic anhydrase (CA) activity can be S-nitrosylated *in vivo* during later stages of plant immune function (Slaymaker et al., 2002; Wang et al., 2009). CA activity is also thought to be required for lipid biosynthesis (Hoang & Chapman, 2002) and lipid molecules are involved in plant immunity and their functions have been linked to SA signaling (Kachroo et al., 2001). Furthermore, the TGACG motif binding factor 1 (TGA1), a basic leucine zipper (bZIP) protein that is essential for SA-dependent gene expression, can be S-nitrosylated at Cys260 and Cys266 (Lindermayr et al., 2010). Also, $^{gsnor1}EX_{avrRpt2}$ was able to confer SAR in Wt but not in *gsnor1* plants. This result suggest that the impaired SAR in *gsnor1* plants is associated with their inability to recognize the SAR inducing signal(s). Together, these data might suggest that high NO could compromise resistance by downregulating the signaling roles of several SAR signals. Since dicarboxylic acid AzA functions upstream of G3P to induce SAR (Yu et al., 2013), it was necessary to study the relation of NO with both AzA and G3P. The data showed less accumulation of AzA and G3P in *noa1nia2* in response to *Pst- avrRpt2* infection. The reduced G3P levels in *noa1nia2* plants could be associated with a defect in AzA accumulation in the local leaves. Collectively, these results suggest that there is a linear connection between NO and AzA/G3P and that NO functions upstream of AzA/G3P.

I next studied the relation between the increased NO levels and the disease resistance to *C. higginsianum,* and TCV. The data presented here showed that *nox1* plants were more resistant to the hemibiotrophic fungal pathogen *C. higginsianum* than wild type plants. These results agreed with Wang and Higgins, (2005), who reported that exogenous application of NO (100 µL) delayed spore germination of *C. coccoides* (Wang and Higgins, 2005). As well, a rapid accumulation of NO has been reported in tobacco leaves (Foissner et al., 2000) and potato tubers (Yamamoto et al., 2003) in response to the fungal elicitor cryptogein and an elicitor from *Phytophthora infestans*, respectively. On the other hand, both Col-0, and *nox1* were equally susceptible to TCV.

Many reports demonstrate that ROS play crucial signaling roles within plants (Mittler, 2002; Moller et al., 2007). Since NO is connected to ROS in defensive responses (Grant and Loake, 2000), I next assessed if ROS induces SAR and if there is a dose-response relationship. Results showed that both *rbohD* and *rbohF* mutant plants were defective for SAR. As well, compared to mock-infiltrated plants, *avrRpt2*-infected plants showed comparable induction of RBOHD and RBOHF expression. This result is consistent with Sewelam et al. (2013), who showed that RBOHD was induced by *Pseudomonas syringae*. Also, data here showed that quantification of the different free radicals in local and distal tissues of *rboh* plants showed significantly reduced levels of free radicals trapped by POBN in comparison with Wt, which correlated with their compromised SAR. These findings suggested that, like NO, H_2O_2 was a potent inducer of SAR in Wt plants, and that H_2O_2-triggered SAR was concentration dependent. The normal basal and R-mediated resistance against *P. syringae* could be because both RBOHD and RBOHF are required only for SAR and not for basal and R-mediated resistance against the bacterial pathogen. Furthermore, $^{\text{Col-0}}EX_{avrRpt2}$, $^{rbohD}EX_{avrRpt2}$ and $^{rbohF}EX_{avrRpt2}$ were able to confer SAR in Wt but not in ROS mutant plants. This result suggested that the impaired SAR in ROS mutant plants was associated with their inability to perceive the SAR inducing signal(s). Together, these data might suggest that low ROS might downregulate the signaling pathway of SAR.

In terms of the relationship between NO and ROS during SAR, our results showed that pathogen infected *noa1nia2* plants also accumulated reduced levels of the different free radicals. This suggests that the NOA1- and NIA2-derived NO is important for inducing ROS production in response to pathogen infection, and that ROS likely functioned downstream of NO. Supporting this suggestion, exogenous local application of ROS could recover SAR in *noa1 nia2* plants (Wang et al., 2014), whereas DETA-NONOate did not confer SAR on the *rboh* mutants. Additionally, pathogen infection did not show NO accumulation in *rbohF* plants, and localized H_2O_2 treatment induced the NOA1 protein in both local and distal tissues. This suggested that NO and ROS are interdependent on each other during SAR, and operate in a feedback loop. This result agrees with the previous finding that the function of the NADPH oxidase, the respiratory

burst oxidase homolog D (RBOHD), not RBOHF, is modulated by S-nitrosylation (Yun et al., 2011). Thus, a balance must be maintained between the activation and inhibitory responses of the different signaling components to allow the optimal induction of SAR. The compromised SAR in *noa1 nia2* plants was not related to SA, and SA does not function downstream of NO in the SAR pathway.

The next goal was to decipher the relation between ROS and SA. Data here elucidated that *rboh* mutants induced as much *PR-1* in response to *Pst-avrRpt2* and SA as Wt did. Also, H_2O_2 treatments did not induce *PR-1* expression in Wt, *rbohD, rbohF* and *noa1nia2*. Furthermore, localized application of SA was also unable to induce SAR in *rbohD* and *rbohF* plants. Together, these results suggest that the defective SAR in the *rboh* mutants was not associated with a defect in the SA pathway and that NO-ROS and SA compose two distinct branches of the SAR pathway.

As a further step, it was necessary to check the relation of ROS with both AzA and G3P. Our data showed that both AzA and G3P conferred SAR in *rbohD* and *rbohF* plants. This suggests a linear connection between NO/ROS and G3P in SAR and a likely function of NO/ROS upstream of AzA/G3P. Consistent with this assumption, Wang et al., (2014) reported that exogenous G3P conferred SAR on *noa1 nia2*. Additionally, exogenous NO application did not induce SAR in *gli1* mutant plants (G3P-deficient mutant).

Results from (Wang et al., 2014) showed that NO/ROS facilitate the chemical breakage of the C9 double bond in the C18 Fas to form AzA. They assayed the conversion of C18 fatty acids (18:1,18:2 or 18:3) to AzA or its intermediate 9-oxononanoic acid (ONA) using *in vitro* assays. They analyzed the compounds resulting from incubation of C18 fatty acid with different chemicals, which generate NO and ROS (Mao et al., 1995; Bruchey and Gonzalez-Lima, 2008). Exogenous DETA-NONOate or H_2O_2 had no effect on these Fatty acids by themselves, whereas, compounds that generate superoxide anion and singlet oxygen radicals were effective on C18 fatty acids. These results suggested that the different free radicals might act additively to generate AzA or its precursor ONA.

In conclusion, this study shows that NO/ROS accumulation serves as one of the early events in SAR establishment, which feeds into the AzA/G3P-dependent pathway.

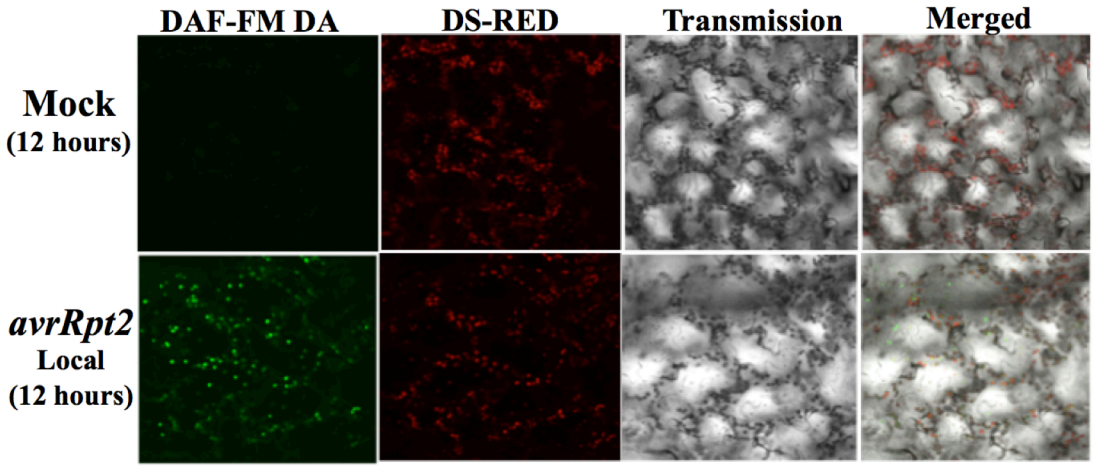

Figure 3.1. Confocal micrographs showing pathogen induced NO accumulation in Col-0 at 12 hr post-inoculation. The leaves were infiltrated with $MgCl_2$ (mock) or *Pst-avrRpt2*, and leaves were stained with DAF-FM DA and analyzed using confocal microscopy. DS-RED was used to check autoflourescence of chloroplasts. Scale bar, 10 µm. The experiment was performed twice with similar results.

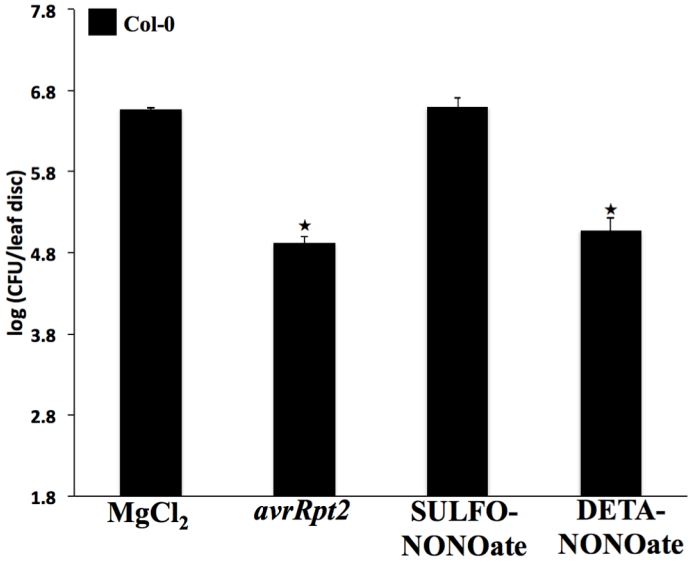

Figure 3.2. SAR response in distal leaves of Wt Col-0 plants treated locally with $MgCl_2$, avirulent pathogen (*avrRpt2*), SULFO-NONOate (100 µM) and DETA-NONOate (100 µM)). The virulent pathogen (DC3000) was inoculated 24 hr after local treatments. Error bars indicate SD (n = 4). The experiment was performed twice with similar results.

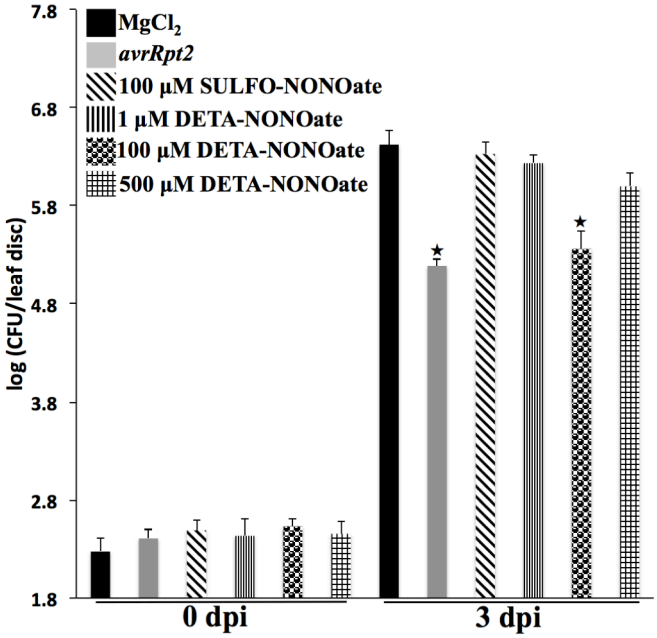

Figure 3.3. SAR response in distal leaves of Wt Col-0 plants treated locally with $MgCl_2$, avirulent pathogen (*avrRpt2*), SULFO-NONOate (100 µM) and different concentrations of DETA-NONOate (1–500 µM). The virulent pathogen (DC3000) was inoculated 24 hr after local treatments. Error bars indicate SD (n = 4).

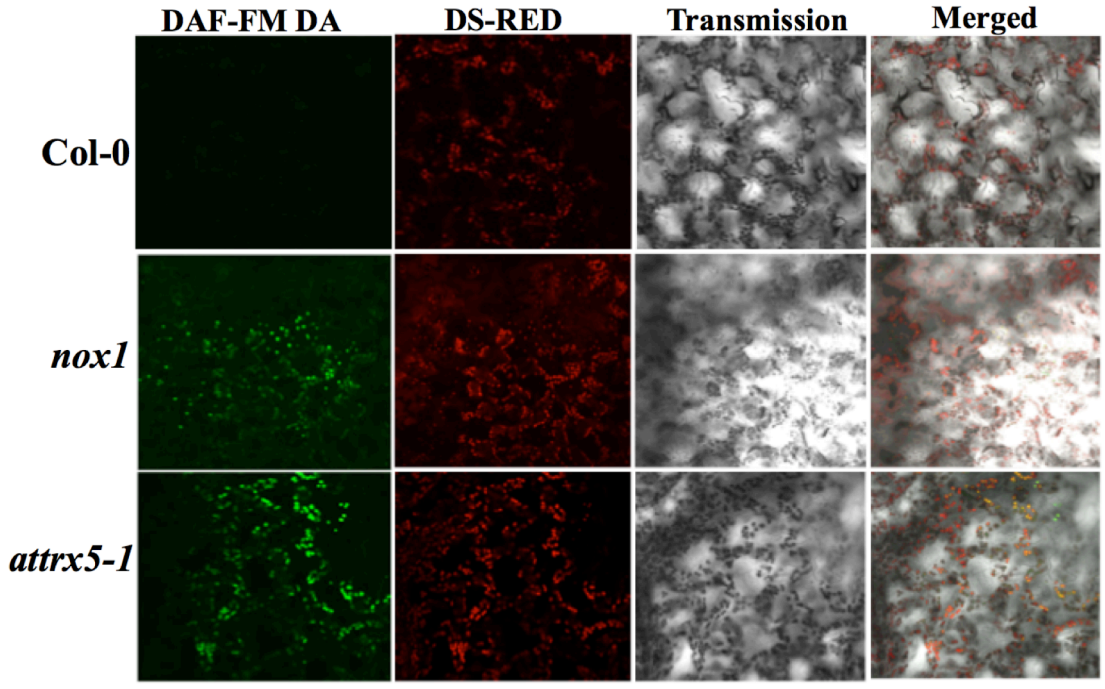

Figure 3.4. Confocal micrographs showing basal constitutive NO accumulation in Col-0, *nox1* and *attrx5-1* plants. The leaves were stained with DAF-FM DA and analyzed using confocal microscopy. DS-RED was used to check autoflourescence of chloroplasts. Scale bar, 10 mm.

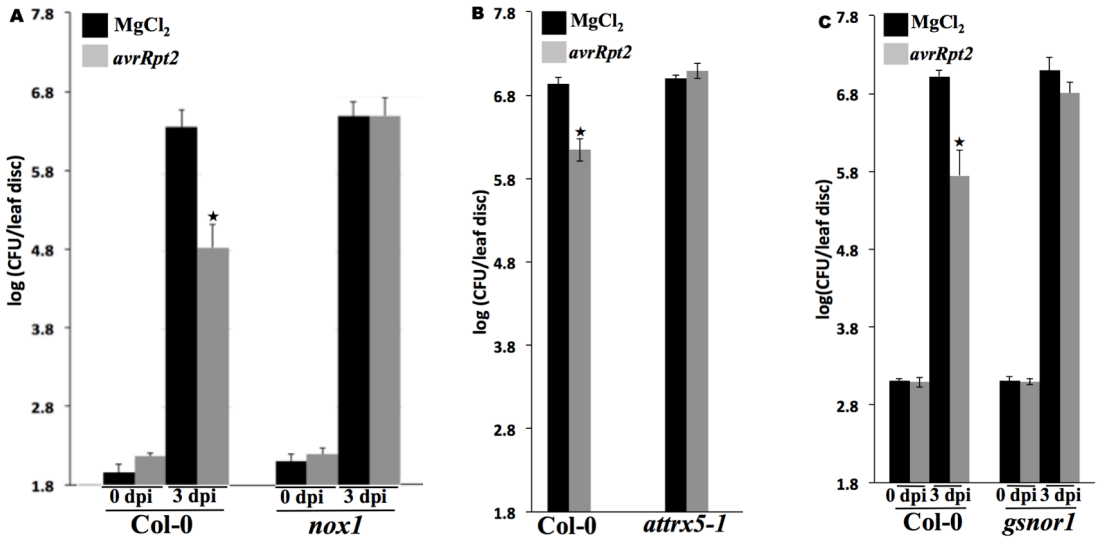

Figure 3.5. SAR response in distal leaves of *nox1* (A), *attrx5-1* (B), and *gsnor1* (C) plants treated locally with $MgCl_2$ or avirulent pathogen (*avrRpt2*) in comparison with Col-0 (Wt). The virulent pathogen (DC3000) was inoculated 24 hr after local treatments. Error bars indicate SD (n = 4). The experiment was performed twice with similar results.

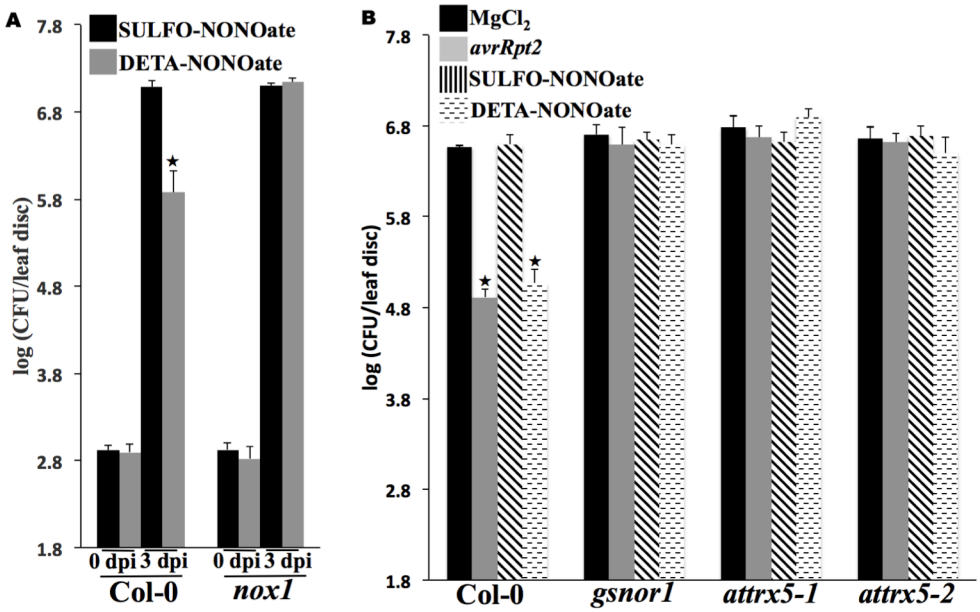

Figure 3.6. SAR response in distal leaves of *nox1* (A), *attrx5-1, attrx5-2* and *gsnor1* (B) plants treated locally with SULFO-NONOate, and DETA-NONOate (100 µM each) in comparison with Col-0 (Wt). The virulent pathogen (DC3000) was inoculated 24 hr after local treatments. Error bars indicate SD (n = 4). The experiment was performed twice with similar results.

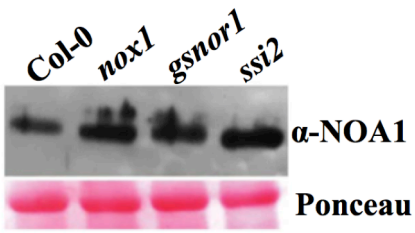

Figure 3.7. Protein immunoblot showing NOA1 levels in indicated genotypes. Ponceau-S staining of the immunoblot was used as the loading control. The experiment was performed twice with similar results.

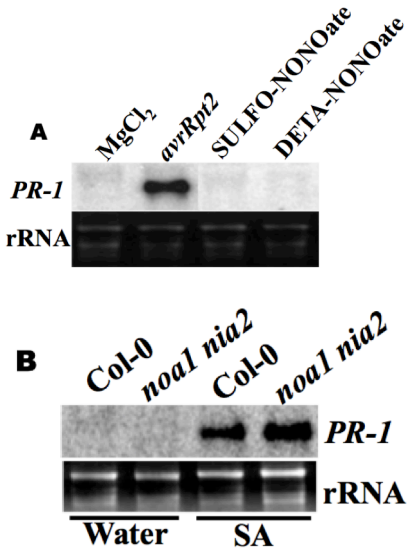

Figure 3.8. RNA gel blots showing transcript levels of *PR-1* showing its relation with NO.
(A) *PR-1* levels in SULFO-NONOate and DETA-NONOate infiltrated leaves of Col-0 in comparison with *Pseudomonas syringae* pv *tomato* (*Pst*)-infected leaves. Leaves were sampled 24 or 48 hr after treatments.
(B) *PR-1* levels in Col-0 and *noa1nia2* leaves in response to SA. Leaves were spayed with 500 µM SA and sampled 24 hr after treatments.
(A-B) Ethidium bromide staining of rRNA was used as loading control.

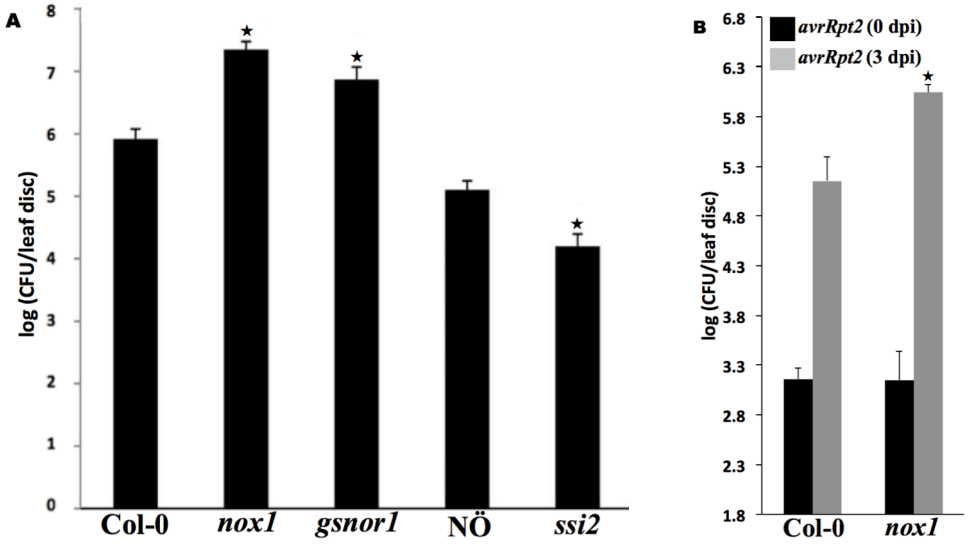

Figure 3.9. Pathogen resistance in NO-accumulating mutants. The experiment was performed twice with similar results.
(A) Growth of virulent *Pseudomonas syringae* DC3000 bacteria on indicated genotypes. The error bars indicate SD (n=4). Asterisks indicate data statistically significant different from wild-type (Columbia, Col-0; Nössen, NÖ $P<0.003$).
(B) Growth of avirulent *avrRpt2* bacteria on *nox1*. The error bars indicate SD (n=4). Asterisks indicate data statistically significant different from wild-type (Columbia, Col-0; $P<0.003$).

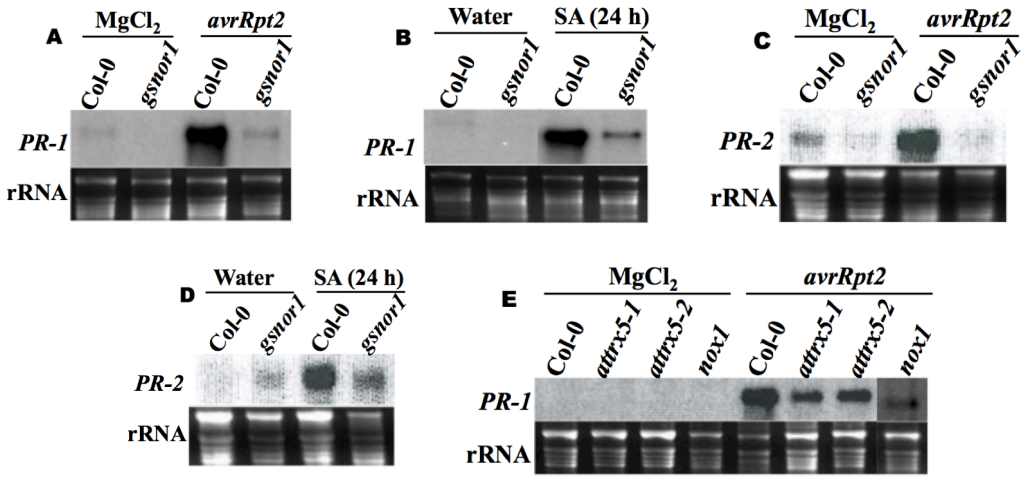

Figure 3.10. RNA gel blots showing transcript levels of *PR* expression in NO-accumulating mutants.

(A) *PR-1* levels in Col-0 and *gsnor1* leaves infiltrated with *Pst- avrRpt2*.
(B) *PR-1* levels in Col-0 and *gsnor1* leaves in response to SA. Leaves were spayed with 500 μM SA and sampled 24 hr after treatments.
(C) *PR-2* levels in Col-0 and *gsnor1* leaves infiltrated with *Pst-avrRpt*$_2$.
(D) *PR-2* levels in Col-0 and *gsnor1* leaves in response to SA. Leaves were spayed with 500 μM SA and sampled 24 hr after treatments.
(E) *PR-1* levels in Col-0, *attrx5-1, attrx5-2* and *nox1* leaves infiltrated with *Pst-avrRpt2*.
(A-E) Ethidium bromide staining of rRNA was used as loading control and leaves were sampled 24 hr after treatments. The experiment was performed twice with similar results.

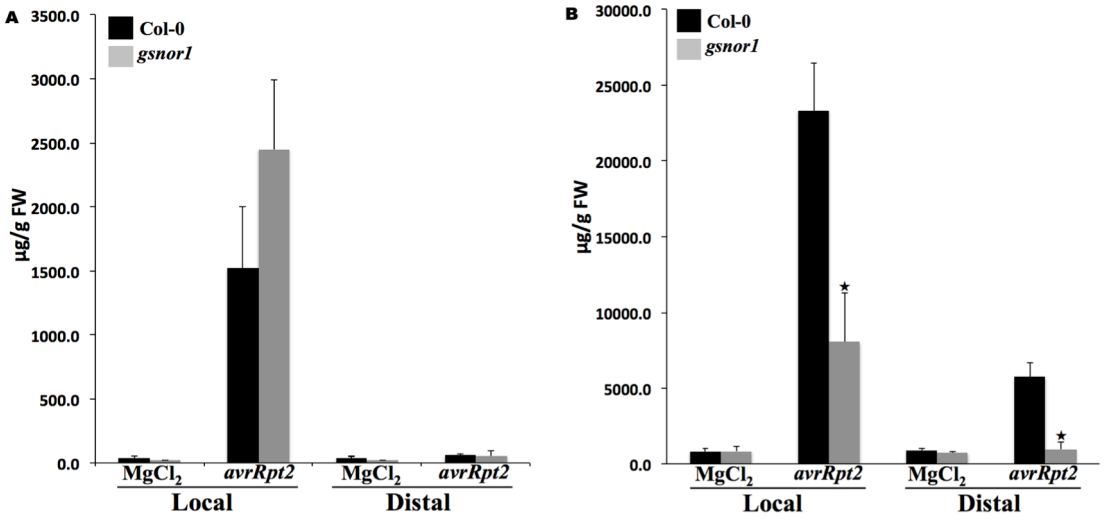

Figure 3.11. SA (A) and SAG (B) levels in mock- ($MgCl_2$) or avirulent pathogen-inoculated (*avrRpt2*) local and distal leaves of Col-0 and *gsnor1* plants 48 hr after inoculation. Asterisks denote significant differences compared with $MgCl_2$-treated plants (t test, $p < 0.05$). Error bars indicate SD (n = 4).

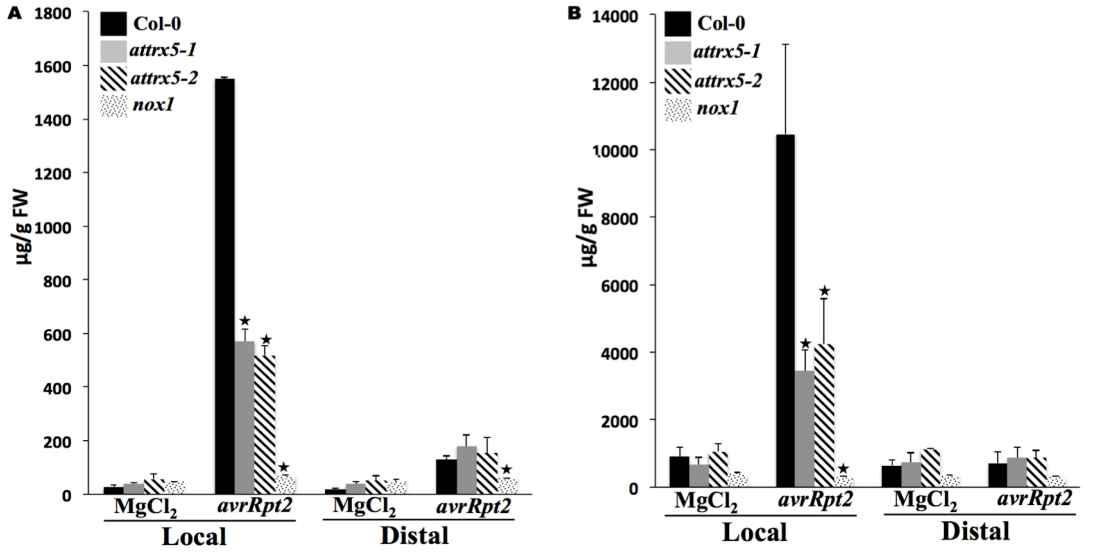

Figure 3.12. SA (A) and SAG (B) levels in mock- ($MgCl_2$) or avirulent pathogen-inoculated (*avrRpt2*) local and distal leaves of Col-0, *attrx5-1, attrx5-2* and *nox1* plants 48 hr after inoculation. Asterisks denote significant differences compared with $MgCl_2$-treated plants (t test, $p < 0.05$). Error bars indicate SD (n = 4).

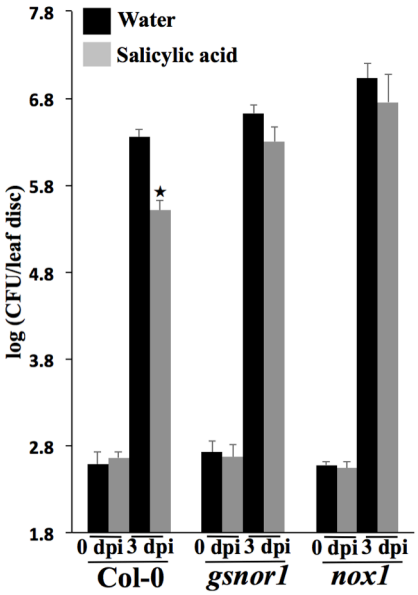

Figure 3.13. SAR response in distal leaves of *gsnor1* and *nox1* plants treated locally with water and SA (500 µM) in comparison with Col-0 (Wt). The virulent pathogen (DC3000) was inoculated 24 hr after local treatments. Error bars indicate SD (n = 4).

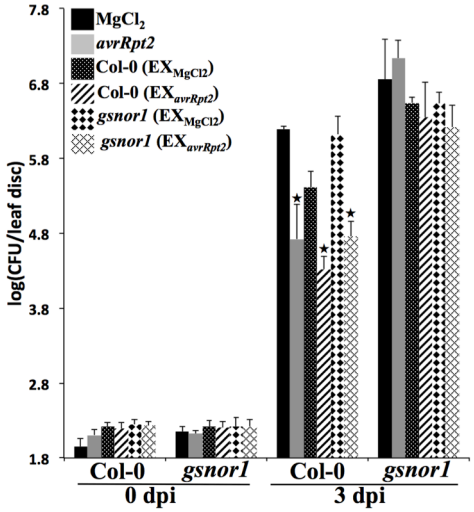

Figure 3.14. SAR response in Col-0 and *gsnor1* plants infiltrated with petiole exudates collected from Col-0 or *gsnor1* plants that were treated either with $MgCl_2$ (EX_{MgCl2}) or *avrRpt2* ($EX_{avrRpt2}$). The distal leaves were inoculated with virulent pathogen at 48 h after infiltration of primary leaves. Error bars indicate SD (n = 4). The experiment was performed twice with similar results.

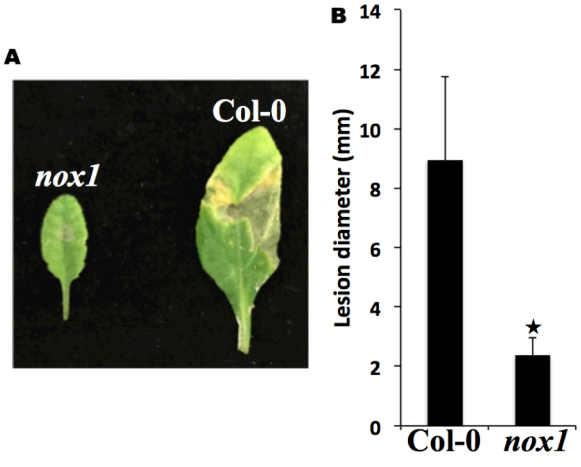

Figure 3.15. Pathogen response in *Colletotrichum higginsianum*-inoculated *nox1* plants. The experiment was performed twice with similar results.
(A) Disease symptoms on Col-0, and *nox1* plants inoculated with 10 µl of 10^6 spores/ml of *C. higginsianum*.
(B) Lesion size in spot-inoculated plants. The lesions size was measured from 20 to 30 independent leaves at 11 dpi. Asterisks indicate data statistically significant from that of control (Col-0; $P<0.05$).

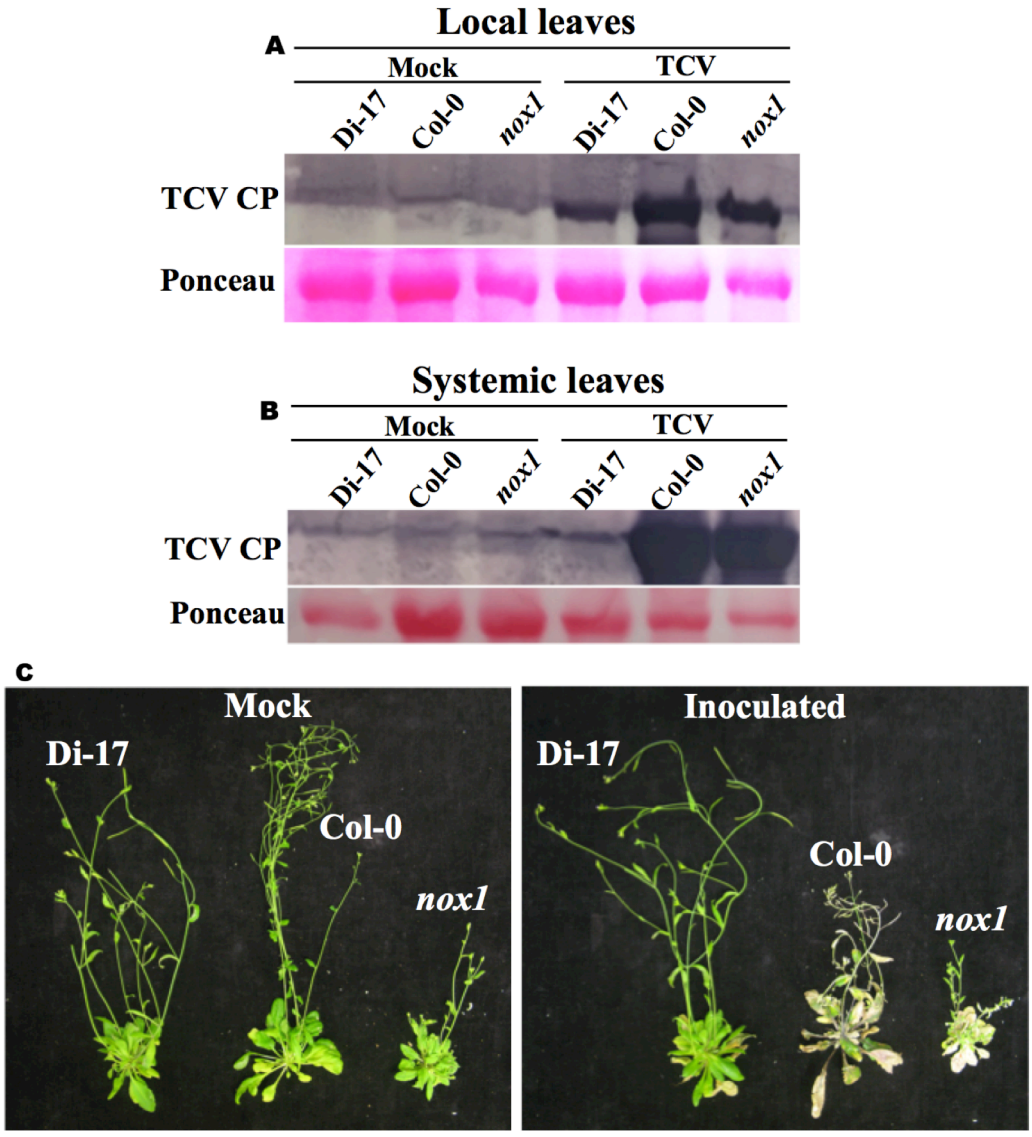

Figure 3.16. Pathogen response in TCV-inoculated *nox1* plants. The experiment was performed twice with similar results.

(A) Immunoblot showing levels of TCV coat protein (CP) in total proteins extracted from local tissues of inoculated Col-0 and *nox1*.
(B) Immunoblot showing levels of TCV coat protein (CP) in total proteins extracted from systemic tissues of inoculated Col-0 and *nox1*.
(C) Symptoms in Di-17, Col-0, and *nox1* at 14 days after TCV inoculation.

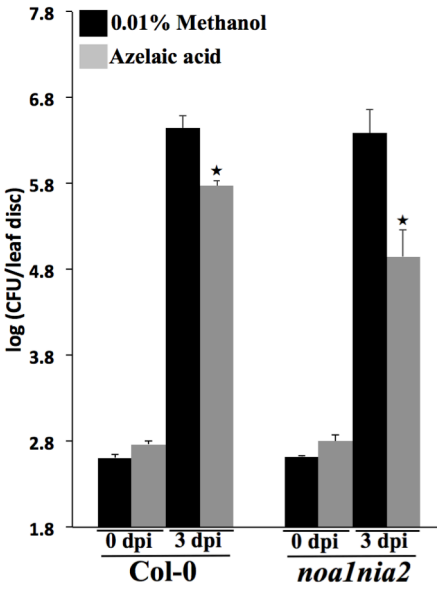

Figure 3.17. SAR response in distal leaves of Col-0, and *noa1 nia2* plants treated locally with 0.01% methanol, and azelaic acid (100 µM). The virulent pathogen (DC3000) was inoculated 48 hr after local treatments. Error bars indicate SD.

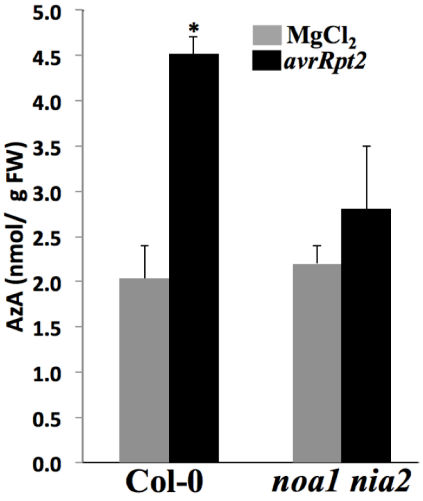

Figure 3.18. AzA levels (per gram fresh weight [FW]) in Col-0 and *noa1 nia2* leaves 24 hr after mock and *avrRpt2* inoculation. Error bars indicate SD (n = 3).

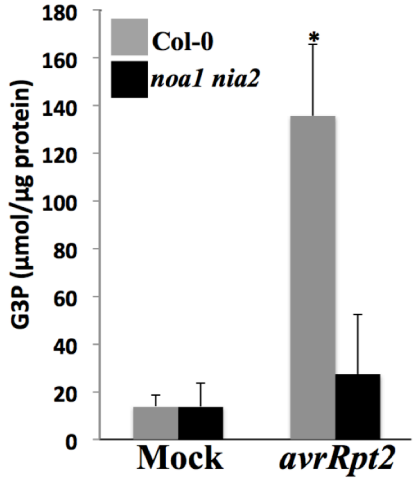

Figure 3.19. G3P levels in petiole exudates collected from mock- and *avrRpt2*-inoculated Col-0 and *noa1nia2* plants. Leaves were sampled 24 hr after inoculations. Error bars indicate SD (n = 3).

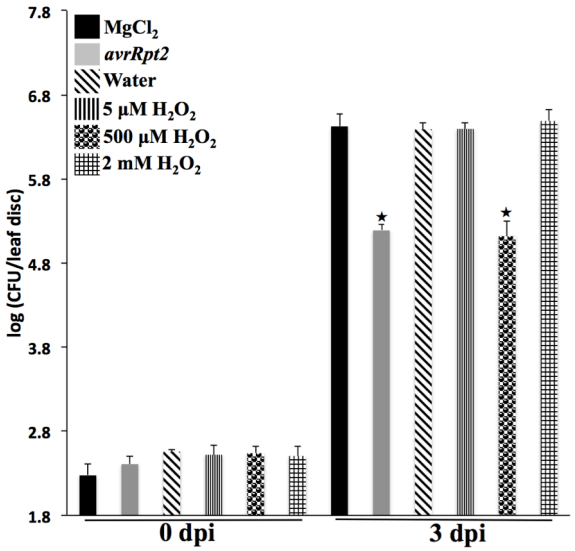

Figure 3.20. SAR response in distal leaves of Wt Col-0 plants treated locally with $MgCl_2$, avirulent pathogen (*avrRpt2*), and different concentrations of H_2O_2. The virulent pathogen (DC3000) was inoculated 24 hr after local treatments.

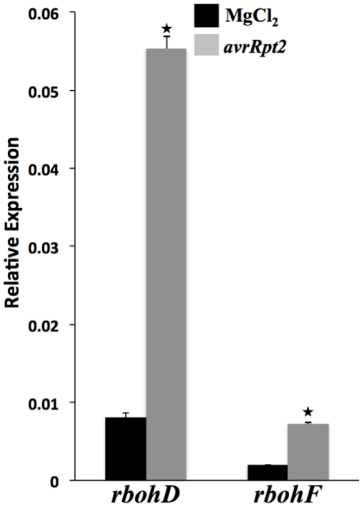

Figure 3.21. Quantitative RT-PCR analysis showing relative expression levels of *RBOHD and RBOHF* in mock ($MgCl_2$)- and *avrRpt2*-inoculated local leaves of Col-0 (Wt) plants. The experiment was performed twice with similar results.

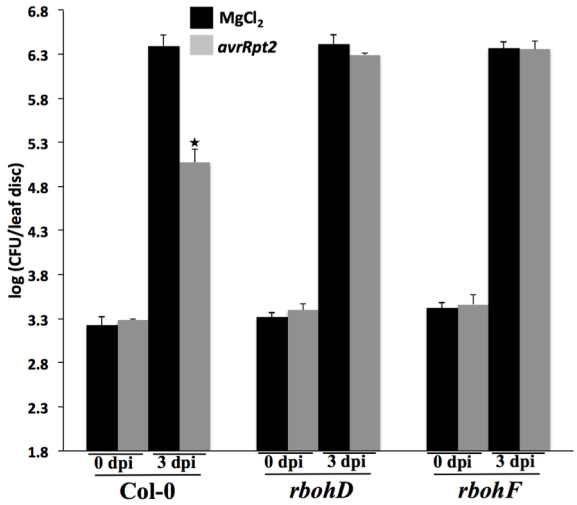

Figure 3.22. SAR response in distal leaves of Wt Col-0, *rbohD* and *rbohF* plants treated locally with $MgCl_2$, and the avirulent pathogen (*avrRpt2*). The virulent pathogen (DC3000) was inoculated 24 hr after local treatments. The experiment was performed twice with similar results.

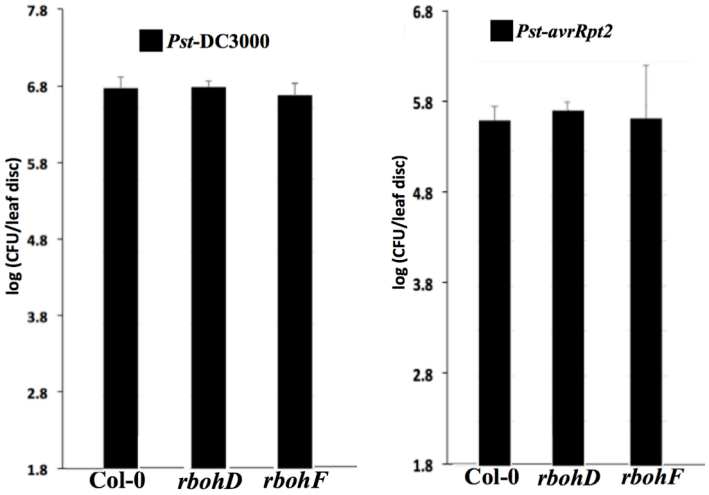

Figure 3.23. Pathogen resistance in ROS mutants. Growth of virulent *Pseudomonas syringae* DC3000 bacteria (A), and avirulent *avrRpt2* bacteria (B) on indicated genotypes. (A-B) The error bars indicate SD (n=4). The experiment was performed twice with similar results.

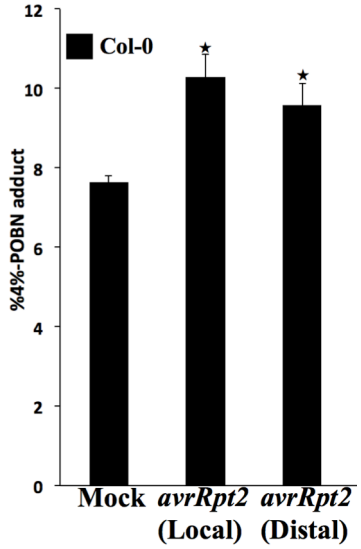

Figure 3.24. Relative levels of POBN adduct in local and distal tissues of *avrRpt2* inoculated Col-0 plants at 12 hpi and 24 hpi respectively. The experiment was performed twice with similar results.

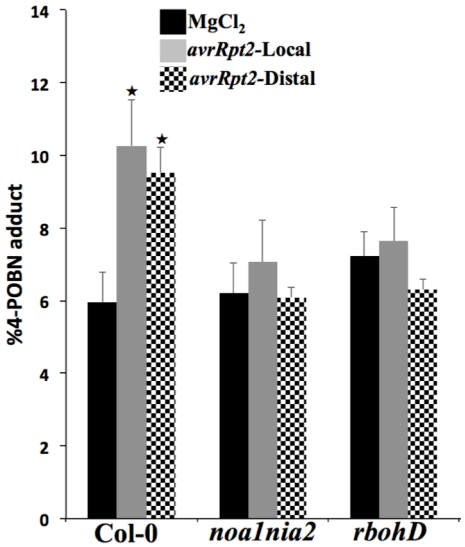

Figure 3.25. Relative levels of POBN adduct in local and distal tissues of *avrRpt2* inoculated Col-0, *noa1nia2* and *rbohD* plants at 12 hpi and 24 hpi respectively.

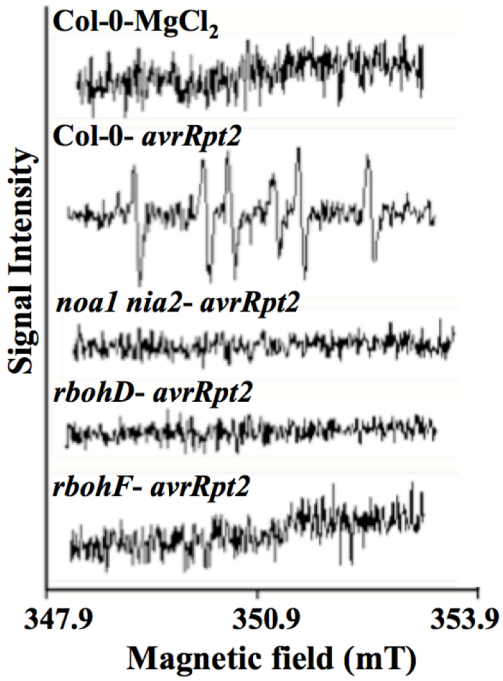

Figure 3.26. ESRS spectra showing superoxide anion radical levels in distal leaves of mock- and *avrRpt2* -inoculated Col-0, *rbohD, rbohF* and *noa1nia2* plants. The leaves were sampled at 24 hpi and EMPO was used as the spin trap.

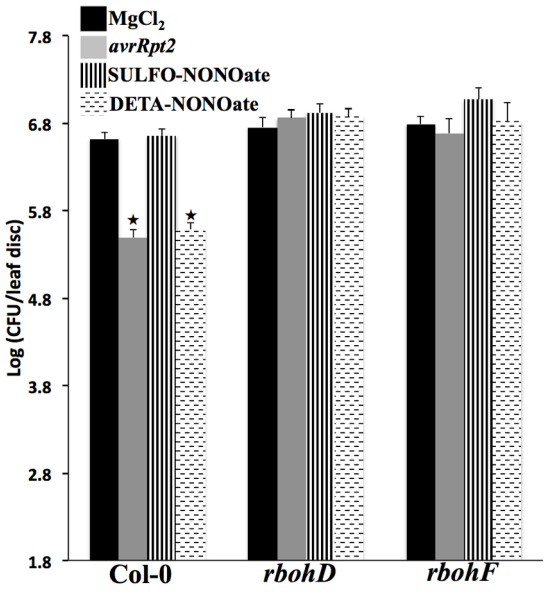

Figure 3.27. SAR response in distal leaves of *rbohD* and *rbohF* plants treated locally with SULFO-NONOate, and DETA-NONOate (100 µM each) in comparison with Col-0 (Wt). The virulent pathogen (DC3000) was inoculated 24 hr after local treatments. Error bars indicate SD (n = 4).

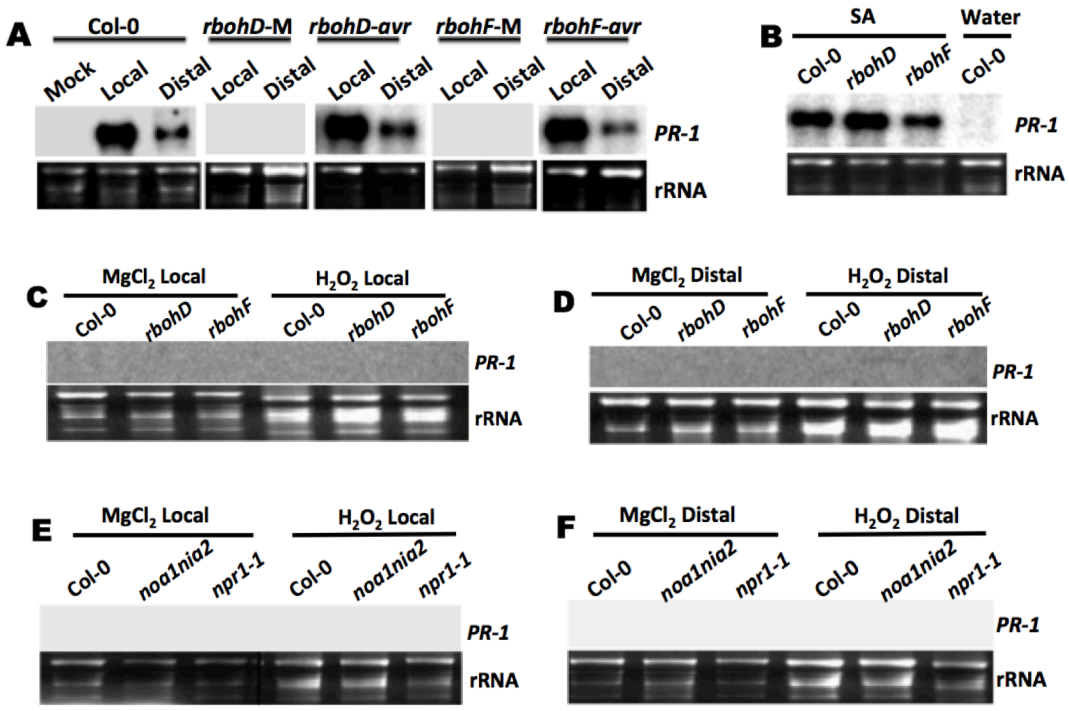

Figure 3.28. RNA gel blots showing transcript levels of *PR-1* showing the relation of SA pathway with ROS. The experiment was performed twice with similar results.

(A) *PR-1* levels in local and distal leaves of Col-0, *rbohD* and *rbohF* in response to (*Pst*)- *avrRpt2*. Leaves were sampled 24 hr after treatments.

(B) *PR-1* levels in Col-0, *rbohD* and *rbohF* local leaves in response to SA. Leaves were spayed with 500 μM SA and sampled 24 hr after treatments.

(C-D) *PR-1* levels in local (C) and distal (D) leaves of Col-0, *rbohD* and *rbohF* plants infiltrated either with water or H_2O_2. Leaves were sampled 24 hr after treatments.

(E-F) *PR-1* levels in local (C) and distal (D) leaves of Col-0, *noa1nia2* and *npr1-1* plants infiltrated either with water or H_2O_2. Leaves were sampled 24 hr after treatments.

(A-F) Ethidium bromide staining of rRNA was used as loading control.

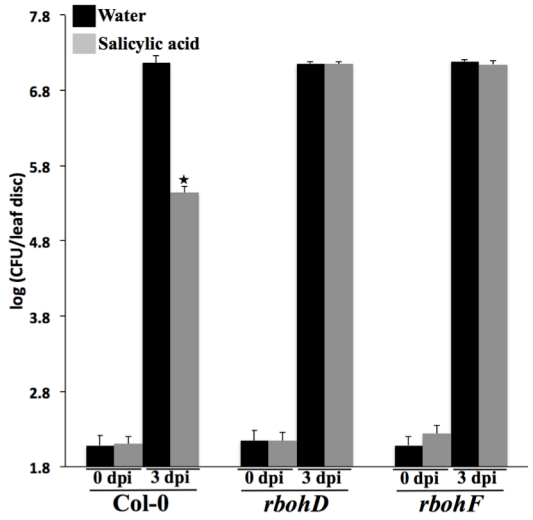

Figure 3.29. SAR response in distal leaves of *rbohD* and *rbohF* plants treated locally with water and SA (500 µM each) in comparison with Col-0 (Wt). The virulent pathogen (DC3000) was inoculated 24 hr after local treatments. Error bars indicate SD (n = 4). The experiment was performed twice with similar results.

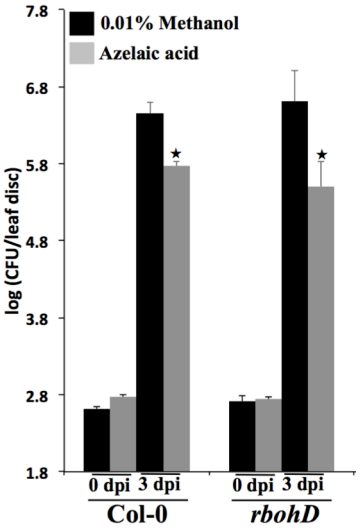

Figure 3.30. SAR response in distal leaves of Col-0, and *rbohD* plants treated locally with 0.01% methanol, and azelaic acid (100 µM). The virulent pathogen (DC3000) was inoculated 48 hr after local treatments. Error bars indicate SD. Asterisks denote significant differences with mock-treated plants (t test, $p < 0.05$). The experiment was performed twice with similar results.

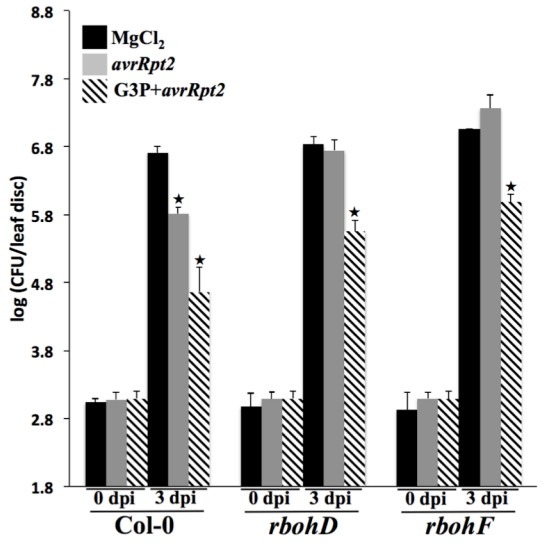

Figure 3.31. SAR response in distal leaves of Col-0, *rbohD*, and *rbohF* plants treated locally with $MgCl_2$, avirulent pathogen (*avrRpt2*), and *avrRpt2*+G3P (100 µM). The virulent pathogen (DC3000) was inoculated 48 hr after local treatments. Error bars indicate SD. Asterisks denote significant differences with mock-treated plants (t test, $p < 0.05$). The experiment was performed twice with similar results.

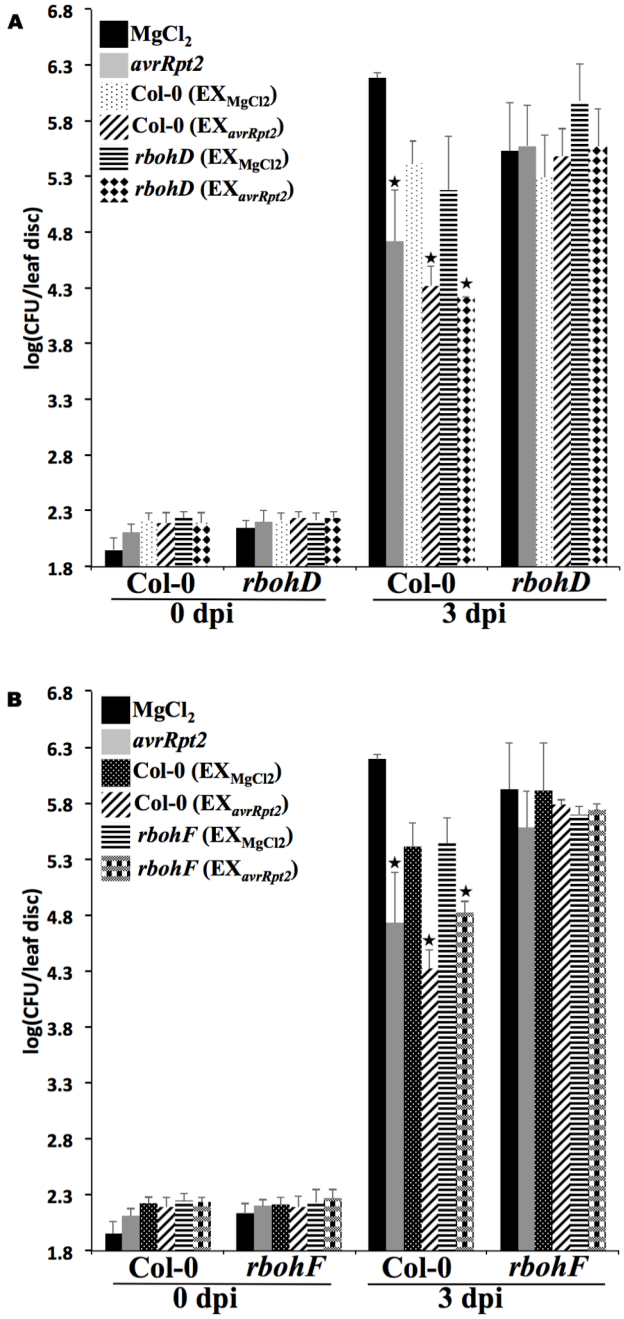

Figure 3.32. SAR response in Col-0, *rbohD* (A) and *rbohF* (B) plants infiltrated with petiole exudates collected from Col-0 or *rbohD/rbohF* plants that were treated either with $MgCl_2$ (EX_{MgCl2}) or *avrRpt2* ($EX_{avrRpt2}$). The distal leaves were inoculated with virulent pathogen at 48h after infiltration of primary leaves. Error bars indicate SD (n = 4). The experiment was performed twice with similar results.

CHAPTER 4

Cross Talk between Salicylic acid (SA) and Azelaic acid (AzA) – Glycerol-3-Phosphate (G3P) Signaling Pathways in Systemic Acquired Resistance (SAR)

4.1 Introduction

A gene-for-gene interaction between plant and pathogen involves direct or indirect interaction between plant resistance protein (R) and the pathogen encoded avirulence (Avr) effector. This recognition event often results in accumulation of SA, nitric oxide (NO), reactive oxygen species (ROS), and induces expression of defense genes including *Pathogenesis Related 1* (*PR1*) (Greenberg and Yao, 2004). SA is synthesized either via the isochorismate synthase (ICS), or phenylalanine ammonia lyase (PAL)-catalyzed steps of the shikimic acid pathway. Both ICS- and PAL-derived branches utilize chorismate as a common precursor (Singh et al., 2013). The Arabidopsis genome encodes four *PAL* isoforms (Huang et al., 2010), and two isoforms of ICS, of which ICS1 (SID2) accounts for ~95 % of pathogen-induced SA (Garcion et al., 2008). A mutation in ICS1 or PAL isoforms impairs SAR (Wildermuth et al., 2001; Jung et al., 2009; Huang et al., 2010), suggesting that SA synthesized by both ICS and SID2 is critical for SAR (Gao et al., 2015).

Salicylic acid is synthesized in the chloroplast, then exported to the cytosol via Enhanced Disease Susceptibility 5 (EDS5), which is located in the chloroplast envelope and shows homology to MATE (Multidrug and Toxin Extrusion) transporter family of proteins (Nawrath et al., 2002; Serrano et al., 2013). A mutation in *EDS5* blocks SA biosynthesis within the chloroplast due to the negative feedback regulation of ICS1 by SA (Serrano et al., 2013). In addition to EDS5, a number of other proteins including EDS1 (Enhanced Disease Susceptibility 1), PAD4 (Phytoalexin- Deficient 4) and NDR1 (Non-race-specific Disease Resistance 1) also contribute to pathogen induced SA accumulation and SAR (Gao et al., 2015). EDS1 and PAD4 proteins show homology to eukaryotic lipases (Jirage et al., 1999). EDS1 interacts with PAD4 in both cytosol and nucleus, and with another

lipase-like protein SAG101 (Senescence Associate Gene 101) in the nucleus. EDS1, PAD4, and SAG101 function together and/or independently in pathogen defense (Zhu et al., 2011).

SA is converted into a number of biologically inactive derivatives, including 2-*O*-β- D-glucose (SAG), SA glucose ester (SGE), methyl SA (MeSA) (Pierpoint, 1994; Dempsey et al., 2011). Methyl SA (MeSA) is volatile and accumulates in the infected and distal leaves in response to pathogen infections. The biosynthesis of MeSA is catalyzed by SA methyltransferases (SAMT/BSMT), and methyl esterase (MES) catalyzes reversible conversion of MeSA to SA (Chen et al., 2003; Koo et al., 2007). The SA-binding protein2 (SABP2) has MES activity and is required for SAR establishment (Kumar and Klessig, 2003). Arabidopsis carries multiple MES and BSMT homologs and among these AtMES9 (MED4) and AtBSMT1 are known to be required for SAR (Liu et al., 2011). Interestingly, SAR phenotype in *bsmt1* and *med4* can be restored by prolonged exposure to light (Liu et al., 2011). The biological role of MeSA transport to distal tissues remains unclear particularly in view of the fact that SA is also transported to the distal tissues (Lim et al., 2016).

NPR1 (Nonexpresser of *PR* genes 1) is a key regulator of the SA signaling pathway (Cao et al., 1994) that operates downstream of SA. NPR1 contains two conserved domains; BTB (Bric-a-brac, Tramtrack, Broad-complex) domain and ankryin repeat domain (Zhang et al., 1999).). Under basal conditions NPR1 forms an inactive oligomer in the cytosol to prevent undue defense activation. Upon pathogen infection or SA treatment, NPR1 oligomer is reduced to monomers to be translocated into the nucleus and regulate defense gene expression (Mou et al., 2003). NO-mediated *S*-nitrosylation also promotes NPR1 localization to nucleus (Lindermayr et al., 2010). NPR1 undergoes phosphorylation and ubiquitination and turnover of NPR1 is essential for NPR1-mediated responses (Spoel et al., 2009; Pintard et al., 2004). The *Arabidopsis* genome contains five paralogs of NPR1 (Liu et al., 2005). NPR1 interacts with NPR3 and NPR4, which are part of the Cullin 3 E3 ligase complex that mediate NPR1 degradation in an SA-

dependent manner. NPR3 and NPR4 bind SA although NPR4 has higher binding affinity for SA than NPR3 (Fu et al., 2012). In infected tissues, high SA levels facilitate NPR3 and NPR1 interaction leading to degradation of NPR1, allowing cell death and ETI to occur. This is consistent with the observation that NPR1 suppresses programmed cell death during ETI (Rate and Greenberg, 2001). In contrast, low SA accumulation in distal tissues are thought to interrupt NPR1–NPR4 interaction allowing NPR1 accumulation, which promotes cell survival and SA-mediated resistance. Reduced SA levels in distal tissues are throught to be insuffucient to facilitate NPR3–NPR1 interaction (Fu et al., 2012). NPR1 also interacts with several TGA transcription factors, which belong to basic leucine zipper (bZIP) family of proteins (Zhou et al., 2000). Like NPR1, the *tga2 tga5 tga6* triple mutant is unresponsive to SA and compromised in SAR (Zhang et al., 2003).

In this chapter, I analyzed relationship between the SA- and AzA/G3P-dependent pathways.

4.2 Results and Discussion

4.2.1 Cross talk between AzA/G3P-dependent pathway and components of the SA pathway

To determine cross-talk between SA and AzA/G3P pathways, I assayed AzA/G3P-mediated SAR on SA mutants. In the beginning, I investigated SAR in *med4-1* mutant, which is deficient in MeSA esterase activity and impaired in SAR (Liu et al., 2011). Col-0, and *med4* leaves were pre-infiltrated with AzA or 0.01% methanol (mock) and the distal leaves of these plants were inoculated with virulent pathogen 48 h post treatments (Figure 4.1A). For G3P-mediated SAR, Col-0, and *med4* leaves were pre-infiltrated with $MgCl_2$, *Pst-avrRpt2*, G3P, and G3P+ *Pst-avrRpt2* (Figure 4.1B) and 24 h later inoculated with virulent pathogen. The bacterial growth of virulent pathogen (DC3000) was monitored at 0 and 3 dpi. Interestingly, AzA reproducibly conferred partial SAR on *med4* plants and G3P + *Pst-avrRpt2* conferred a more robust SAR on *med4* compared to AzA

treatment. These results suggested that there might be cross talk between SA and AzA/G3P branches of the SAR pathway.

Phytoalexin- deficient4 (PAD4) and SA function in a positive signal-amplification loop to activate SA-mediated defenses (Jirage et al., 1999). I next assayed AzA/G3P-mediated SAR on *pad4* mutant plants. The local leaves of Col-0 and *pad4* plants were infiltrated with AzA or G3P (with or without *Pst-avrRpt2*) and 48 h later distal leaves were inoculated with virulent bacteria. Interestingly, neither AzA nor G3P were able to confer SAR on *pad4* plants (Figure 4.2A, 4.2B). I next evaluated G3P-mediated SAR on the SA biosynthetic mutant *sid2*. Consistent with prior results (Chanda et a., 2011), G3P + *Pst-avrRpt2* was unable to confer SAR on *sid2* (Figure 4.2C). These results suggest an absolute requirement for PAD4 and SA in AzA/G3P-mediated SAR.

4.2.2 Relationship between G3P-mediated SA and NPR1

We recently showed that NO acts upstream of AzA/G3P (Wang et al., 2014), and since NO also nitrosylates NPR1, I evaluated AzA/G3P-mediated SAR on *npr1* mutant plants. Since NPR3, NPR4, WRKY and TGA factors operate together with NPR1, I also evaluated G3P-mediated SAR on *npr3*, *npr4, npr3 npr4, wrky18,* and *tga2/5/6* mutants. The local leaves of Col-0, and mutant plants were pre-infiltrated with AzA or 0.01% methanol followed by inoculation of distal leaves with virulent bacteria 48 h post local treatments. For G3P-mediated SAR, the local leaves were infiltrated with $MgCl_2$, *Pst-avrRpt2*, G3P or G3P+ *Pst-avrRpt2* followed by inoculation of virulent bacteria 24 h post local treatments. Notably, both AzA and G3P + *Pst-avrRpt2* were able to confer a partial SAR on *npr1-1* plants (Figure 4.3A, 4.3B). Likewise, G3P + *Pst-avrRpt2* also conferred a partial SAR on *npr3*, *npr4, npr3 npr4* (Figure 4.3C)*, wrky18,* and *tga2/5/6* (Figure 4.3D) plants.

4.2.3 Conclusions

Earlier studies have shown that exogenous applications of AzA or G3P do not induce SA accumulation (Chanda et al., 2011; Yu et al., 2013) and that the SA and AzA/G3P branches of the SAR pathways operate in parallel (Chanda et al., 2011; Yu et al., 2013; Wang et al., 2014; Gao et al., 2014). For example, neither G3P nor AzA can confer SAR in SA-deficient mutant, *sid2* and conversely, exogenous SA does not confer SAR on G3P-deficient mutants, *gly1* and *gli1* (Chanda et al., 2011; Yu et al., 2013; Wang et al., 2014). My results show that G3P is also unable to restore SAR in plants mutated in PAD4, an important component of the SA pathway (Gao et al., 2015). However, both AzA and G3P were able to confer partial SAR on *med4*, *npr1*, *npr3*, *npr4, tga2/5/6* and *wrky18* plants. These observations suggest that SA biosynthesis and certain components like PAD4 are absolutely essential for AzA and G3P-mediated SAR while a requirement for NPR1, TGA and MED4 can be partially compensated by increasing the levels of AzA or G3P. Notably pathogen induced SA levels in *pad4* plants, although lower than in wild-type, are significantly higher than the mock-inocualted plants (Glazebrook et al., 1997). This suggests an important role for PAD4 in SAR, which might be independent of SA (Figure 4.4).

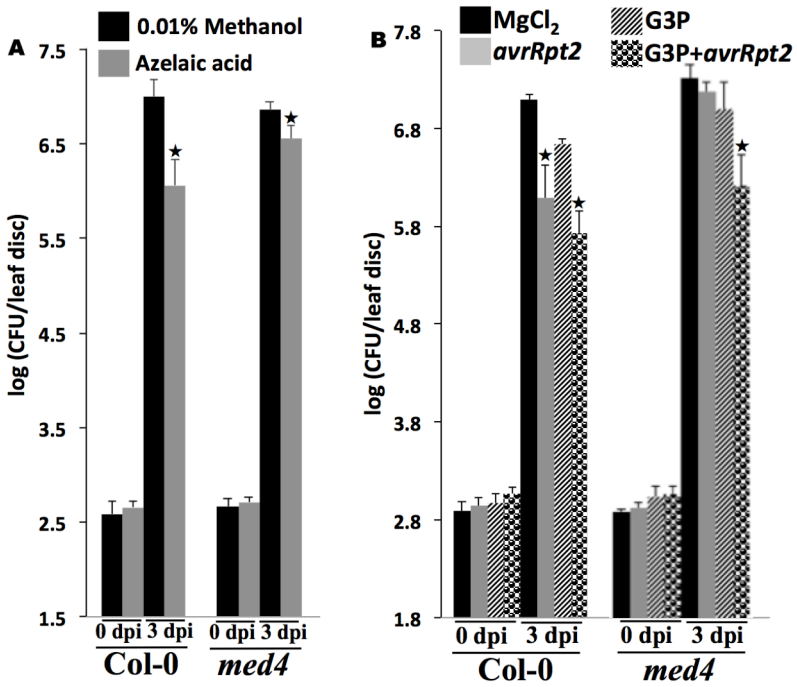

Figure 4.1. SAR in *med4* mutant plants. Error bars indicate SD. The experiment was performed twice with similar results.

(A) SAR response in distal leaves of *med4* mutant plants treated locally with 0.01% methanol, and azelaic acid (100 µM) in comparison with Col-0 (Wt) plants.
(B) SAR response in distal leaves of *med4* plants treated locally with $MgCl_2$, avirulent pathogen (*avrRpt2*), G3P (100 µM) and *avrRpt2*+G3P in comparison with Col-0 (Wt) plants.
(A-B) The virulent pathogen (DC3000) was inoculated 48 hr after local treatments.

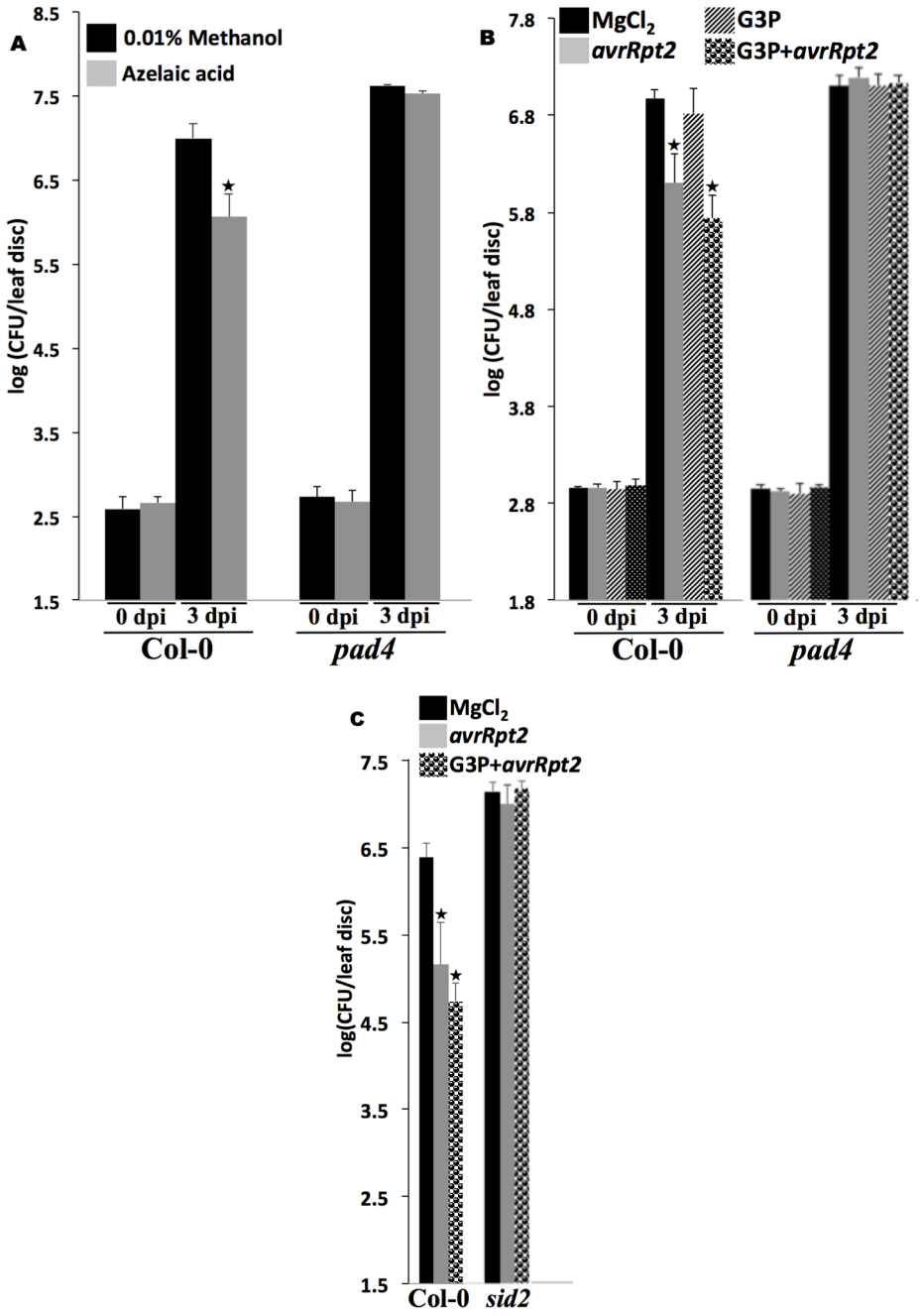

Figure 4.2. SAR in *pad4* mutant plants. Error bars indicate SD. The experiment was performed twice with similar results. The virulent pathogen (DC3000) was inoculated 48 hr after local treatments.

(A) SAR response in distal leaves of *pad4* mutant plants treated locally with 0.01% methanol, and azelaic acid (100 µM) in comparison with Col-0 (Wt) plants.

(B) SAR response in distal leaves of *pad4* mutant plants treated locally with $MgCl_2$, avirulent pathogen (*avrRpt2*), G3P (100 µM) and *avrRpt2*+G3P in comparison with Col-0 (Wt) plants.

(C) SAR response in distal leaves of *sid2* mutant plants treated locally with $MgCl_2$, avirulent pathogen (*avrRpt2*), and *avrRpt2*+G3P in comparison with Col-0 (Wt) plants.

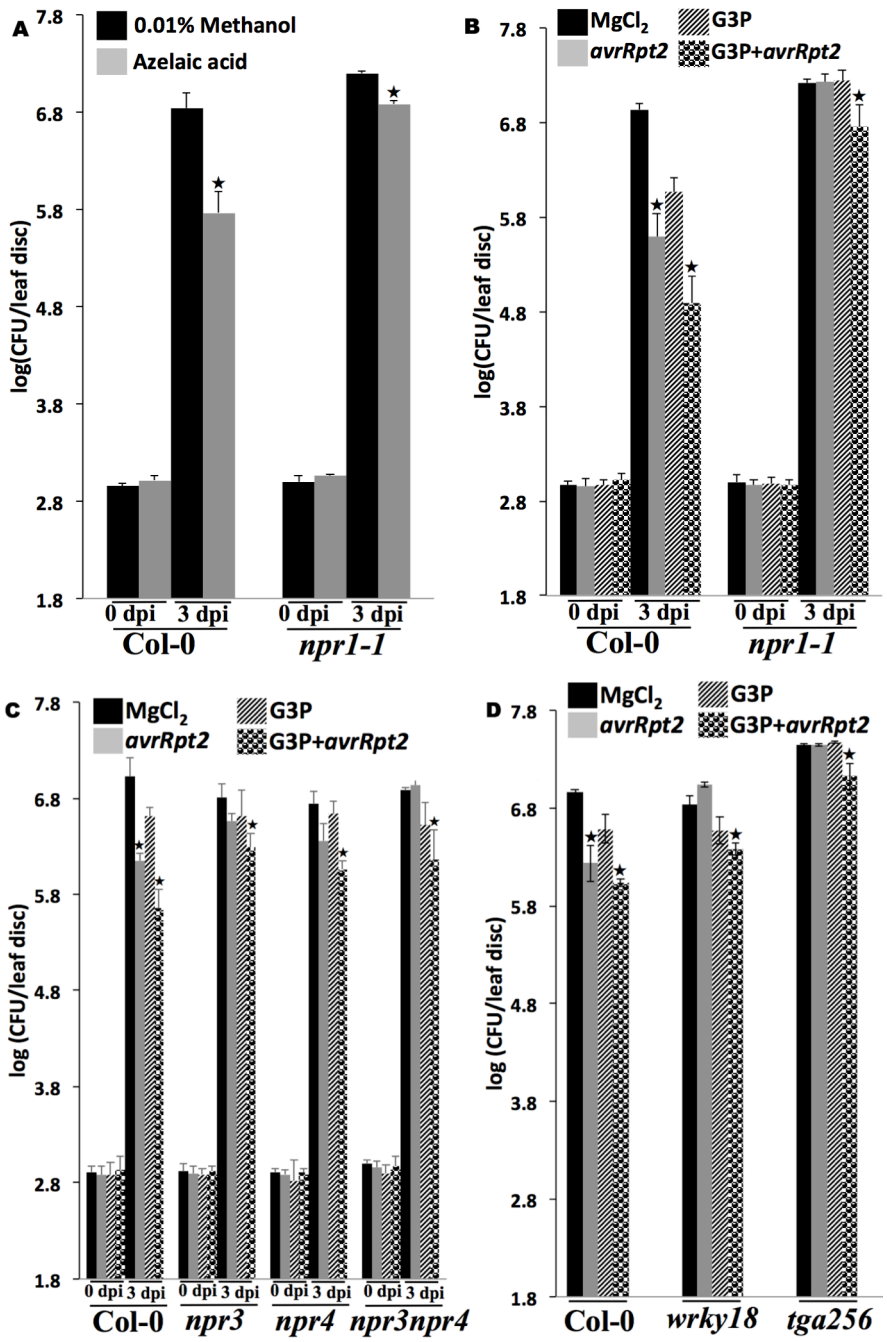

Figure 4.3. SAR in genetic mutants of downstream signaling components of SA pathway. Error bars indicate SD. The experiment was performed twice with similar results.

(A) SAR response in distal leaves of *npr1-1* mutant plants treated locally with 0.01% methanol, and azelaic acid (100 µM) in comparison with Col-0 (Wt) plants.
(B-D) SAR response in distal leaves of *npr1-1* (B), *npr3*, *npr4*, *npr3npr4* (C), *wrky18* and *tga256* (D) mutant plants treated locally with $MgCl_2$, avirulent pathogen (*avrRpt2*), G3P (100 µM) and *avrRpt2*+G3P in comparison with Col-0 (Wt) plants.
(A-D) The virulent pathogen (DC3000) was inoculated 48 hr after local treatments.

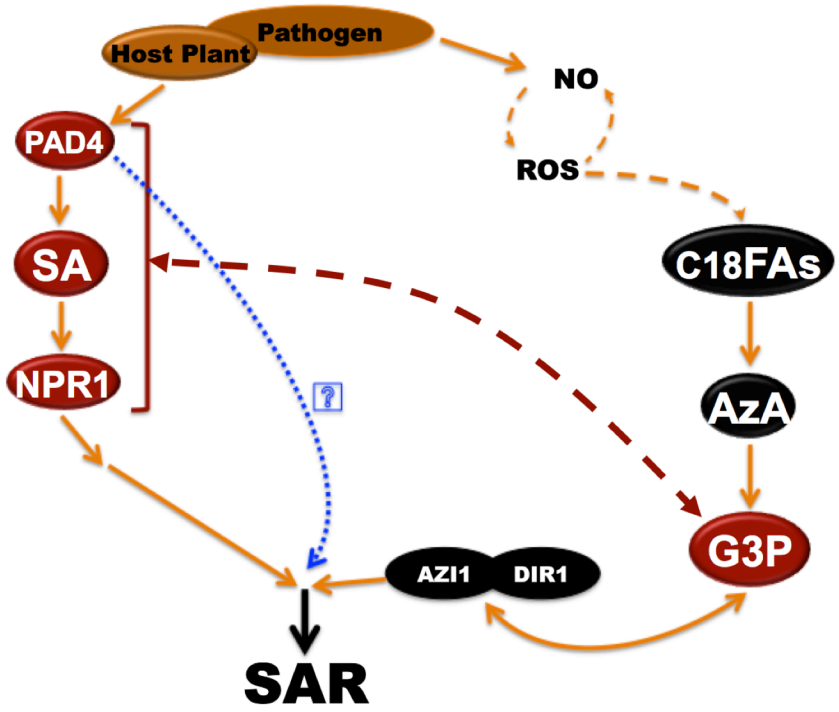

Figure 4.4. Proposed Model for SAR. Inoculation of avirulent pathogen triggers the accumulation of salicylic acid (SA) and nitric oxide (NO). NO acts upstream and in a feed back loop with reactive oxygen species (ROS), which act in an additive manner to catalyze the oxidation of double bond at C9 on C18 unsaturated fatty acids (C18FAs) to generate AzA (azelaic acid), which induces G3P (glycerol-3-phosphate) biosynthesis. G3P synthesis is dependent on the cytosolic lipid transfer proteins DIR1 and AZI1, and operates in a feedback loop with them. Pathogen induces PAD4 (Phytoalexin Deficient 4), which is critical for SAR development by triggering the lipid basal signal in the presence of SA. The relation of PAD4 with SAR development remains to be clarified. SA biosynthesis and certain components like PAD4 are absolutely essential for SAR, whereas a requirement for NPR1 (Nonexpresser of *PR* genes 1) can be partially compensated by increasing the levels of AzA or G3P.

CHAPTER 5

Role of Light in Systemic Acquired Resistance

5.1 Introduction

Plant diseases have a major negative impact on yield. Traditional methods employed to control diseases include plant breeding and chemical sprays. These become less effective over time as pathogens adapt and/or become tolerant to chemical treatments. Moreover, chemical sprays can have a pronounced damaging effect on our environment. Control of plant diseases based on plants innate immune response is a safer and more effective way to combat plant diseases. Among several factors regulating plant defense, light is well known to play an important role (Roberts and Paul, 2006). For example, hypersensitive cell death (HR) triggered against bacterial and viral pathogens in tobacco and Arabidopsis is dependent on light (Zeier et al., 2004; Chandra-Shekara et al., 2006). Likewise, production and perception of SA in Arabidopsis is dependent on light (Genoud et al., 2002; Zeier et al., 2004).

Solar radiation is the source of most visible light energy on earth. The natural visible light spectrum is composed of several wavelengths ranging from 350 nm (violet) to 750 nm (red, far red). The relative levels of these wavelengths vary depending on the time of the day. Plants being sessile have to particularly modify their growth and development to optimize utilization of ambient light. Light provides energy, and metabolic precursors, which are required for the production of defense metabolites. Also, light also acts as a signal to regulate plant growth, development, and physiology (Gyula et al., 2003). Light is perceived and transduced into cellular responses by different photoreceptor families. There are three classes of photoreceptors; one class called phytochromes (PHY) that detect light in the red/far-red (600–700 nm) range, and two classes of blue light photoreceptors called cryptochromes (CRY) and phototropins (PHOT) are activated by blue wavelength (320–500 nm) (Casal, 2000). Plant photoreceptors mediate circadian rhythm, growth, development and defense. Thus, elucidation of the components involved

in the light related defense signaling pathways may provide an overview into a bigger signaling network through which plants regulate a wide variety of responses.

5.2 Results and Discussion

5.2.1 The effect of AM versus PM inoculations on SAR

SAR is compromised if plants are kept in dark subsequent to primary infection (Zeier et al., 2004). As well, a role for MeSA in SAR has been associated with light; MeSA is required for SAR only when plants receive no light before the start of night cycle (Liu et al., 2011). To test how various other SAR mutants behaved under variable light conditions, I followed light conditions described by Liu et al. (2011) to assay SAR (Figure 5.1A, 5.1B). Briefly, plant were either inoculated such that they received no (PM) or 6-8 (AM) hours of light before the night cycle. Mutants assayed in this study included, *gly1 and gli1* (G3P deficient; Chanda et al., 2011), *bsmt1* (MeSA deficient; Liu et al., 2010), *dir1* and *azi1* (lacking functional LTPs; Maldonado et al., 2002; Jung et al., 2009), and *fmo1* (defective in flavin-dependent monooxygenases 1; Bartsch et al., 2006).

Notably, wild-type plants inoculated at AM showed a stronger SAR compared to plants inoculated at PM (Liu et al., 2011). However, all mutants showed compromised SAR regardless of the time-frame of the primary inoculation. This is in contrast to Liu et al. (2011), who showed SAR in *med4*, *dir1* and *gly1* plants when these were inoculated at AM. However, the *fmo1* mutant behaved in a similar manner between this study and Liu et al., (2011). These results suggest that conditions other than the duration of the light cycle after primary inoculation might be responsible for positive SAR reported by Liu et al., (2011) for *med4*, *dir1* and *gly1* plants.

5.2.2 Exposure to blue-light compromises SAR

The blue-light photoreceptors, CRY2 and PHOT2, are required for the stability of the R protein HRT (HR to TCV) and plants exposed to blue-light show enhanced susceptibility to TCV (Jeong et al., 2010). To determine if blue-light played a similar role in SAR, I assayed SAR in Wt (Col-0) plants that were exposed to blue-light after pathogen inoculations. Plants were first infiltrated with $MgCl_2$, and *Pst avrRpt2* followed by inoculation of the distal leaves with virulent *Pst* (DC3000). The plants were kept in closed light proof containers and sealed on the top with filters that allowed white or blue-light (Roscolene Filters). The blue-light chamber allowed ~7 μmol m^{-1} s^{-1} light (Jeong et al., 2010). Notably, plants exposed to blue-light showed compromised SAR in comparison to plants that were exposed to white light (transparent filters) (Figure 5.2A). Analysis of AzA levels and *PR-1* expression in these plants showed that plants exposed to blue-light accumulated reduced levels of AzA and *PR-1* gene (Figures 5.2B, 5.2C). To test if blue-light had a similar effect on other plants, I assayed SAR in tobacco plants exposed to white or blue-light. TMV-resistant (*NN*) were first inoculated with HEPES buffer (mock) or TMV and the distal leaves of these plants were subsequently challenged with TMV after 2 days of primary inoculations. The HR lesions and viral replication was analyzed at 3 dpi of second inoculation. The plants exposed to blue-light showed bigger and more lesions compared to plants kept under white light (Figure 5.3 A, B, C, D). Also, plants kept under blue-light showed increased levels of TMV in their distal leaves (Figure 5.3 E). Together, these results suggested that blue-light compromises SAR, likely by interfering with both SA and AzA pathways. More work is required to further characterize this effect and to ensure that blue-light mediates a specific effect on SAR.

5.2.3 AzA and Pip confer SAR in tobacco

To further characterize the role of SA, G3P, AzA and Pip in SAR I assayed their ability to confer SAR in tobacco plants inoculated with TMV (Figure 5.4). Plants were locally infiltrated the water, G3P, AzA and Pip and 2 days later distal leaves were inoculated with TMV. The control plants were inoculated locally with HEPES buffer or TMV. As

expected, the average diameter and number of HR lesions on distal leaves of plants inoculated locally with TMV were less than those infiltrated with HEPES buffer (Figure 5.4A, 5.4B, 5.4C, 5.4D). Notably, both AzA and Pip conferred SAR as evidenced by decreased number and size of HR lesions, as well as TMV replication in their distal leaves (Figure 5.4A, 5.4B, 5.4C, 5.4D, 5.4E). As shown before (Chanda et al., 2011), G3P treatments were unable to confer SAR, and it is possible that tobacco plants express G3P specific phosphatases that render G3P treatments ineffective.

Next, I assayed ability of AzA and Pip to confer SAR on TMV-susceptible (*nn*) Xanthi-nc tobacco plants (Figure 5.5). These plants lack the resistance protein N and therefore do not show any visible HR lesions. Intriguingly, only local treatement of Pip, but not AzA, was able to reduce TMV replication on distal leaves (Figure 5.5B). Moreover, plants infiltrated with Pip showed higher levels of *PR1* in their distal leaves compared to plants infiltrated locally with water, G3P or AzA (Figure 5.5C). These results correlate with the observation that exogenous Pip can stimulate SA biosynthesis (Návarová et al. 2012) whereas AzA does not (Yu et al., 2013). Inability of AzA to confer SAR in the absence of N protein will require further analysis.

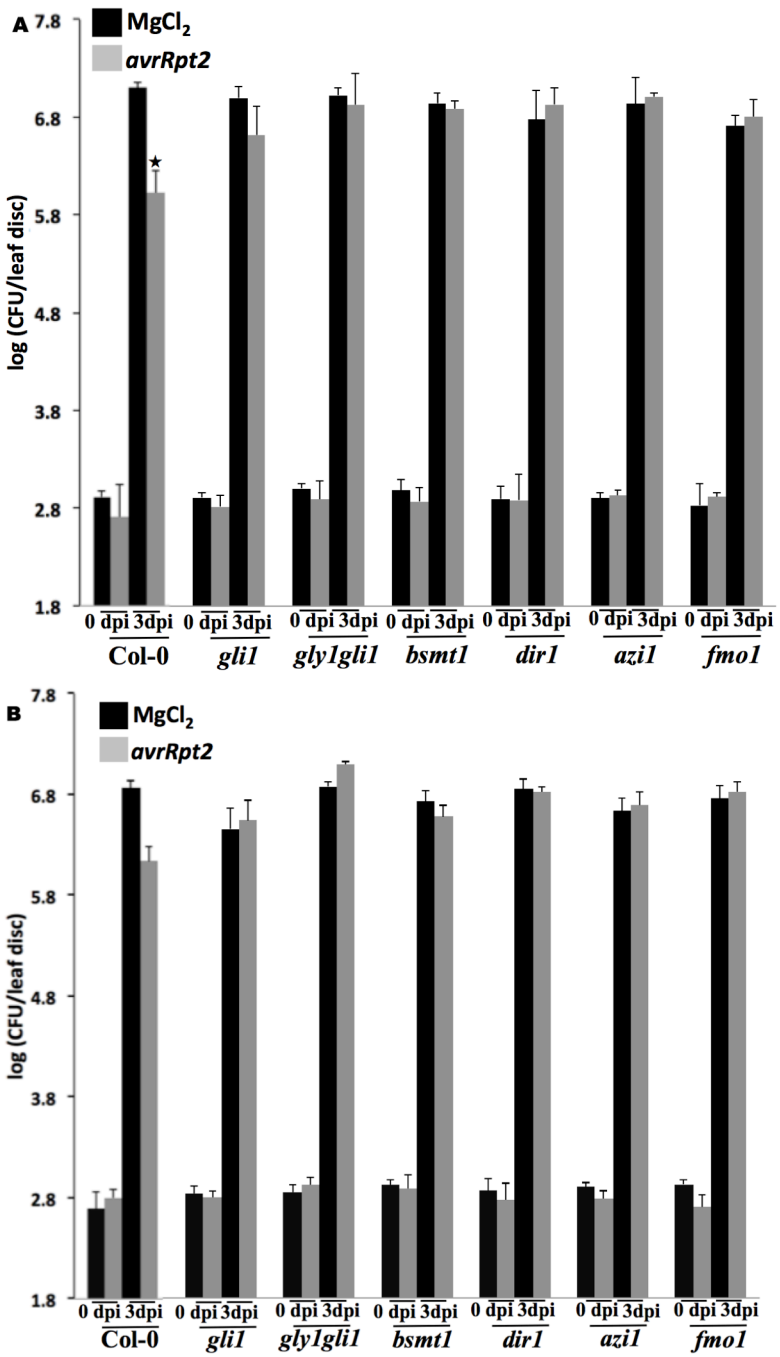

Figure 5.1. SAR response at AM or PM in distal leaves of *gli1*, *gly1gli1, bsmt1*, *dir1, azi1* and *fmo1* plants treated locally with $MgCl_2$ or avirulent pathogen (*avrRpt2*) in comparison with Col-o (Wt). The virulent pathogen (DC3000) was inoculated 48 hr after local treatments. Error bars indicate SD (n = 4).

(A) AM inoculations: plants kept in light immediately after inoculations allowing extended light exposure for the plants before entering the dark cycle.

(B) PM inoculations: plants kept in dark immediately after inoculations for 6-9 h before being exposed to light again.

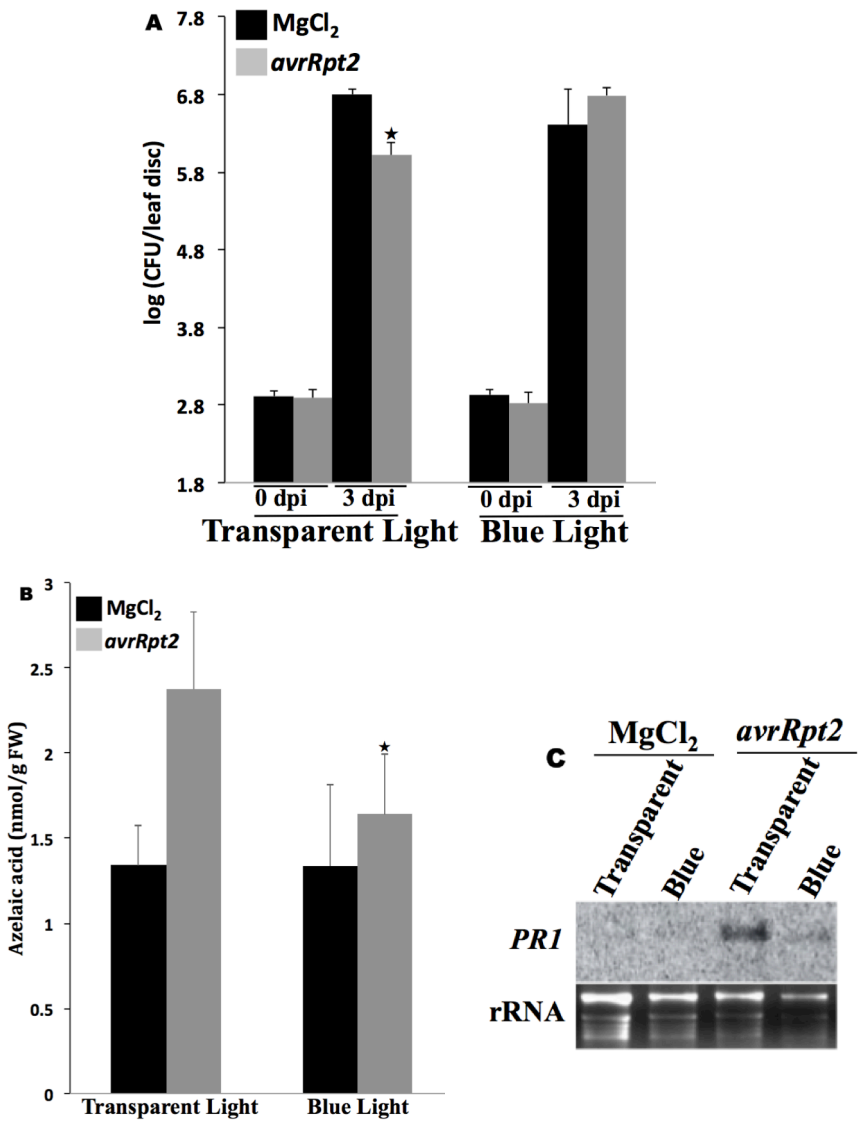

Figure 5.2. The effects of blue light spectra in Arabidopsis plants.

(A) SAR response in distal leaves of Col-0 plants treated locally with $MgCl_2$ or avirulent pathogen (*avrRpt2*) under blue light spectra. The virulent pathogen (DC3000) was inoculated 48 hr after local treatments. Error bars indicate SD (n = 4).

(B) AzA levels (per gram fresh weight) in Col-o (Wt) plants under blue light spectra 24 hr after mock and *avrRpt2* inoculation.

(C) *PR-1* levels in Col-0 leaves infiltrated with *Pst- avrRpt2* under blue light spectra.

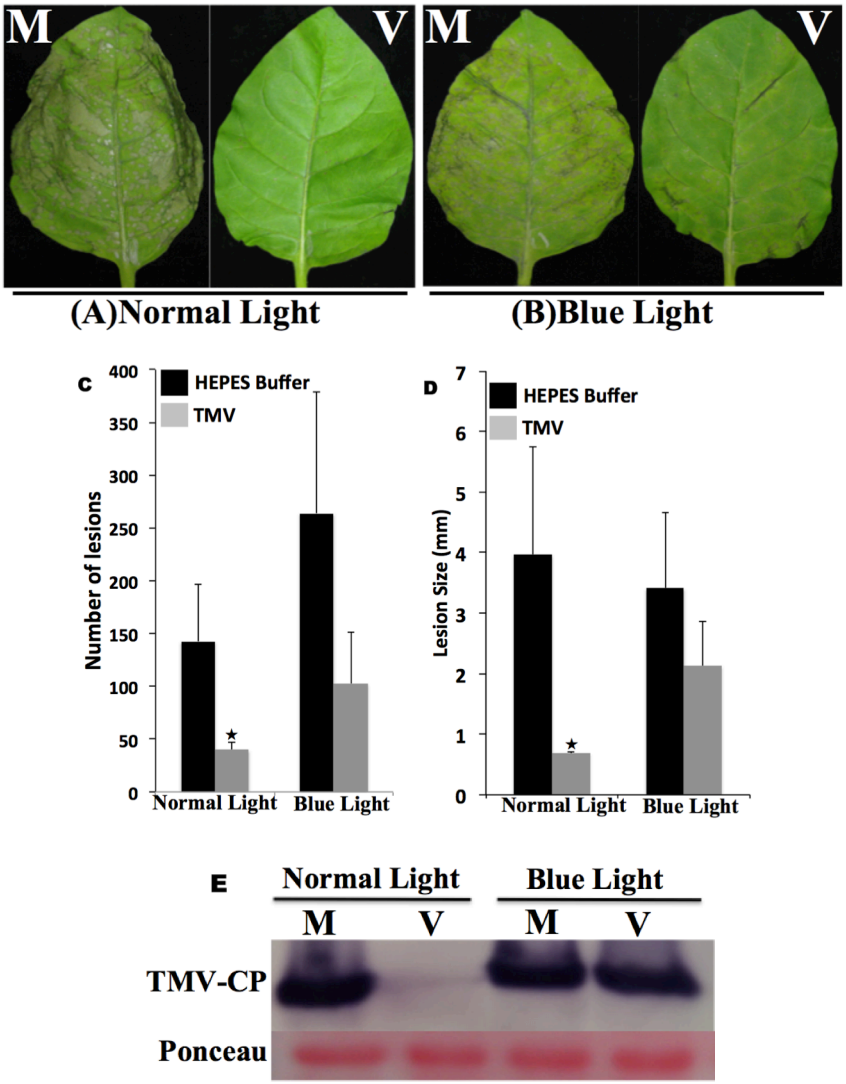

Figure 5.3. SAR response in distal leaves of Tobacco Mosaic Virus (TMV)-resistant (*NN*) Xanthi-nc tobacco plants (*Nicotiana tabacum*) treated locally with of mock (HEPES buffer) and TMV under blue light conditions. TMV was inoculated 48 hr after local treatments. Error bars indicate SD (n = 4). (M refers to the plants that were pre-infiltrated at local leaves with HEPES buffer, and V refers to the plants that were pre-infiltrated at local leaves with TMV).
(A-B) Typical morphological phenotypes of TMV-inoculated distal leaves of mock (HEPES buffer)- and TMV-inoculated *NN* tobacco plants under normal (A) and blue **(B)** light conditions.
(C-D) Number of lesions (C) and lesion size (D) in TMV-inoculated distal leaves of resistant tobacco plants (*Nicotiana tabacum*) treated locally with mock (HEPES buffer) and TMV under blue light conditions.
(E) Protein immunoblot showing spread and replication of TMV in TMV-inoculated distal leaves of resistant tobacco plants (*Nicotiana tabacum*) treated locally with mock (HEPES buffer) or TMV under blue-light. Ponceau-S staining of the immunoblot was used as the loading control.

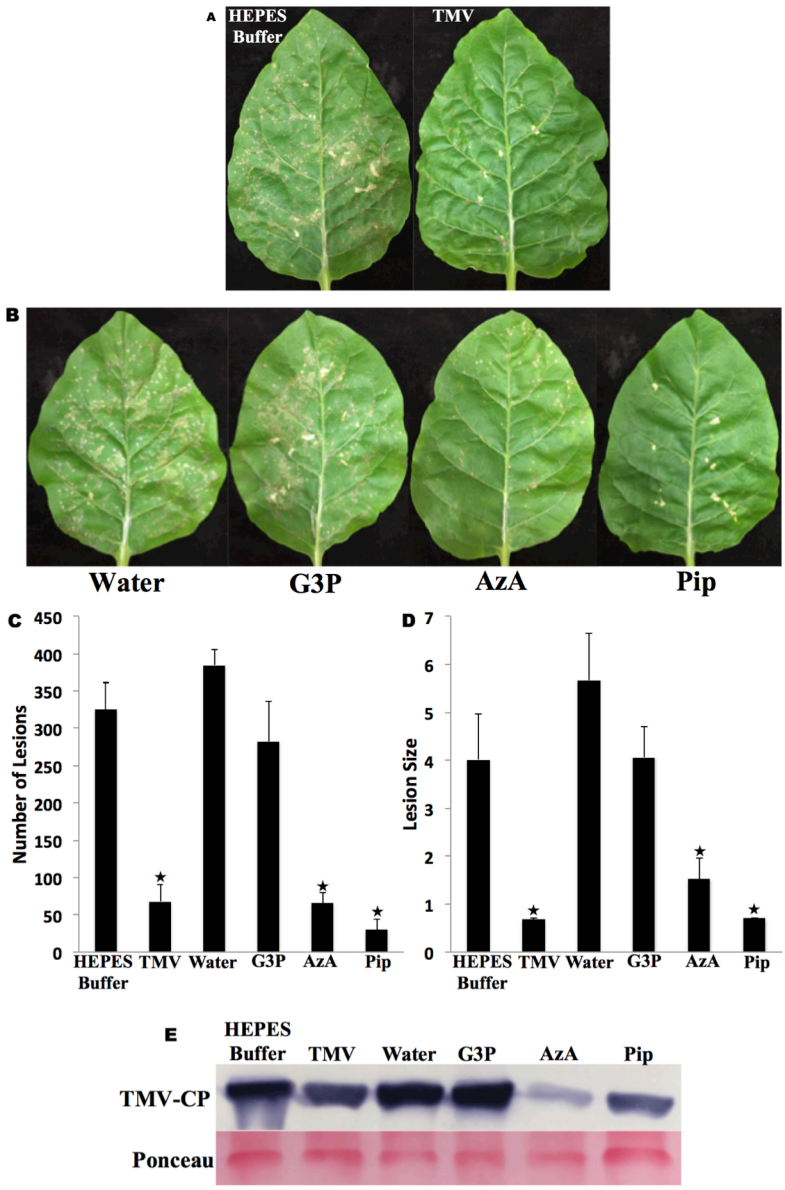

Figure 5.4. SAR response in distal leaves of Tobacco Mosaic Virus (TMV)-resistant (*NN*) Xanthi-nc tobacco plants (*Nicotiana tabacum*) treated locally with different treatments. TMV was inoculated 48 hr after local treatments. Error bars indicate SD (n = 4).

(A) Typical morphological phenotypes of TMV-inoculated distal leaves of mock (HEPES buffer)- and TMV-inoculated *NN* tobacco plants.

(B) Typical morphological phenotypes of TMV-inoculated distal leaves of mock (Water)-, G3P-, AzA-, and Pip-inoculated *NN* tobacco plants.

(C-D) Number of lesions (C) and lesion size (D) in TMV-inoculated distal leaves of resistant tobacco plants (*Nicotiana tabacum*) treated locally with different treatments.

(E) Protein immunoblot showing spread and replication of TMV in TMV-inoculated distal leaves of resistant tobacco (*Nicotiana tabacum*) plants treated locally with different treatments. Ponceau-S staining of the immunoblot was used as the loading control.

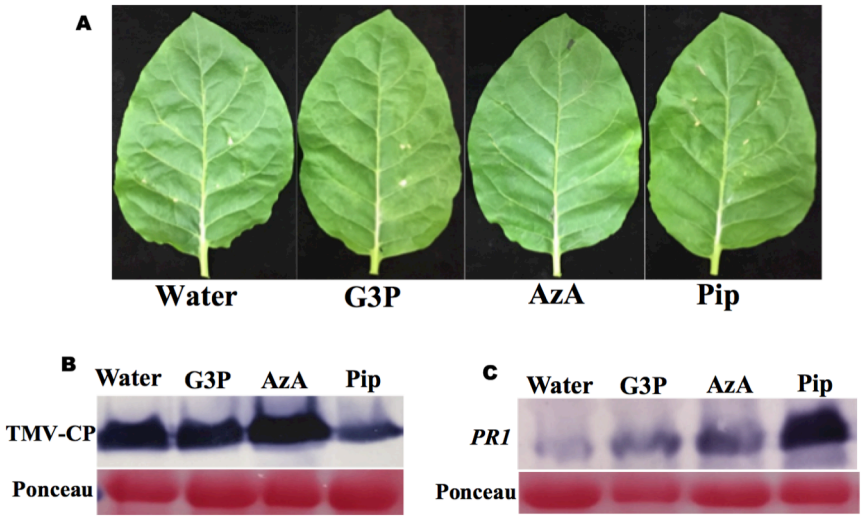

Figure 5.5. SAR response in distal leaves of Tobacco Mosaic Virus (TMV)-susceptible (*nn*) Xanthi-nc tobacco plants (*Nicotiana tabacum*) treated locally with different treatments. TMV was inoculated 48 hr after local treatments.

(A) Typical morphological phenotypes of TMV-inoculated distal leaves of mock (Water)- and different chemicals (G3P, AzA, Pip)-infiltrated *nn* tobacco plants.
(B) Protein immunoblot showing spread and replication of TMV in TMV-inoculated distal leaves of *nn* tobacco plants (*Nicotiana tabacum*) treated locally with different treatments.
(C) Protein immunoblot showing *PR1*-expression in TMV-inoculated distal leaves of *nn* tobacco plants (*Nicotiana tabacum*) treated locally with different treatments.
(B-C) Ponceau-S staining of the immunoblot was used as the loading control.

CHAPTER 6
Future Prospects

- Dose-dependence relationship between NO and ROS levels and SAR remains unclear. Detailed characterization of inhibitory effects of higher concentrations of NO and ROS at biochemical and molecular levels will be useful. It will also be useful to analyze role of other RBOH members in SAR.

- More experiments are required to characteize cross talk between the SA-NPR1 and AzA/G3P branches of the SAR pathway and should provide useful leads.

- Role of blue-light in SAR will require more careful analysis using LED chambers, and detailed characterization of chemical signals and proteins involved in SAR. In addition, it will be useful to assay SAR in mutants that are defective in photoreceptors and various steps of light photomorphogenesis.

APPENDIX
LIST OF ABBREVIATIONS

Acronym/ abbreviation	Expansion
L/mL/ µL	Liter/ milliliter/ microliter
M/mM/ µM	Molar/millimolar/ micromolar
g/mg/ µg/ng	Gram/ milligram/ microgram/ nanogram
h/min/sec	Hours/minutes/seconds
Rh	Relative humidity
^{o}C	Degrees centigrade
ALD1	AGD2-like defense response protein 1
AM	Ante meridiem
AzA	Azelaic acid
AZI1	Azelaic acid induced 1
BSA	Bovine serum albumin
BSMT1	Benzoic acid/salicylic acid methyl transferase 1
BTB	Bric-a-brac, Tramtrack, Broad-complex domain
BTH	Benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester
$CaCl_2$	Calcium chroride
CC	Coiled coil
CFU	Colony forming unit
dATP	Deoxyribo adenosine triphosphate
dCTP	Deoxyribo cytosine triphosphate
DETA-NONOate	2-(N,N-Diethylamino)-diazenolate-2-oxide
DEPC	Diethyl pyrocarbonate
DGDG	Digalactosyldiacylglycerol
DHAP	Dihydroxyacetone phosphate
DIR1	Defective in induced resistance 1
DNA	Deoxyribonucleic acid
dNTP	Deoxyribo nucleic triphosphate
DMSO	Dimethyl sulfoxide
DPI	Days post inoculation
DPT	Days post treatment
DTT	Dithiothreitol
EDS1	Enhanced Disease Susceptibility 1
EDS5	Enhanced Disease Susceptibility 5
EDTA	Ethylene diamine tetraacetic acid
EtBr	Ethidium bromide
FA	Fatty acid
FMO1	Flavin-dependent monooxygenases 1
G3P	Glycerol-3-phosphate
G3Pdh	Glycerol-3-phosphate dehydrogenase
GC	Gas chromatography
GFP	Green fluorescent protein
GLI1/GK	Glycerol insensitive 1/ glycerol kinase

GLY1	Glycerol dependent 1
GSH	Glutathione
GSNO	S-nitrosoglutathione
GSNOR	S-nitrosoglutathione Reductase
GSSG	Glutathione disulphide
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
HRT	Hypersensitive response to TCV
JA	Jasmonic acid
K_2HPO_4	Potassium phosphate, dibasic
KH_2PO_4	Potassium phosphate, monobasic
KCl	Potassium chloride
KOH	Potassium hydroxide
LB	Luria-Bertani
LRR	Leucine rich repeat
LTP	Lipid transfer protein
MED4	Methyl esterase deficient 4
MES	Methyl esterase
MeSA	Methyl SA
$MgCl_2$	Magnesium chloride
MGDG	Monogalactosyldiacylglycerol
MOPS	3-(N-morpholino) propanesulfonic acid
MS	Murashige and Skoog
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
Na_2HPO_4	Sodium hydrogen phosphate
NaN_3	Sodium azide
NBS	Nucleotide binding site
NDR1	Non-race-specific Disease Resistance 1
NH_3	Ammonia
NO	Nitric oxide
NOA1	NO associated protein 1
NOX1	Nitric oxide overproducer 1
NPR1	Nonexpresser of *PR* genes 1
O_2^1	Singlet oxygen
O_2^-	Superoxide radical
OH^-	Hydroxyl radical
PAD4	Phytoalexin- Deficient 4
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFD	Photon flux density
Pip	Pipecolic acid
PM	Post meridiem
PR-1	Pathogenesis related 1

R	Resistant or resistance
rrt	regulates resistance to TCV
RBOH	Respiratory burst oxidase homology
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SAG	Salicylic acid glucoside
SAG101	Senescence Associate Gene 101
SAMT/BSMT	SA methyltransferases
SAR	Systemic acquired resistance
SID2	Salicylic acid insensitive 1
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SGE	SA glucose ester
SSC	Sodium chloride, sodium citrate
SULFO-NONOate	Nitrous oxide donor
TBE	Tris-borate/ EDTA electrophoresis buffer
TCV	Turnip crinkle virus
TE	TRIS-EDTA
TMV	Tobacco mosaic virus
TRIS	Hydroxymethyl Aminomethane
TRX	Thioredoxin
WT	Wild-type
18:1	Oleic acid
18:2	Linoleic acid
18:3	Linolenic acid

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VITA

Mohamed El-Shetehy

Education

- 2011- present: PhD student, Department of Plant Pathology, University of Kentucky, Lexington, Kentucky (GPA: 3.927)
- 2003-2009: MS (Microbiology), College of Science, Tanta University, Tanta, Egypt
- 2003: BS (Microbiology), College of Science, Tanta University, Tanta, Egypt

Awards, Honors and Recognition

- The 2014 Cell Reports paper was featured on Global Medical Discovery (https://globalmedicaldiscovery.com), a highly selective online site that highlights only 0.1% of key scientific discoveries

- The 2014 Cell Reports paper was featured on UKNOW as well as UKAgNews (http://news.ca.uky.edu/article/kachroos'-labs-make-discovery-plant-disease-resistance)

- Travel Award by University of Kentucky to attend The American Phytopathological Society (APS)- The Canadian Phytopathological Society (CPS) Annual Meeting- Minneapolis, Minnesota, August 9-13, 2014

- John S. Niederhauser Student Travel Award to attend APS-CPS Joint Meeting- Minneapolis, Minnesota, August 9-13, 2014

- Travel Award by University of Kentucky to attend the 21st International Symposium on Plant Lipids- Guelph, Ontario, Canada- July 6-11, 2014

- Travel Award by the American Society of Plant Biologists (ASPB) Midwestern Section (MS) to attend MS-ASPB Annual meeting- Columbus, Ohio, March 22-23, 2014

- Awarded Four year fellowship by the Egyptian Government to support PhD research at University of Kentucky, April 2011-April 2015

- Awarded "Ideal Student Prize" in BS (Out of 100 students)

- Scored first rank in BS (Out of 100 students), based on which I was offered permanent job as a demonstrator, Tanta University.

- One of 30 biology students (Out of 1000) who were offered admission to the medical program, Tanta University. Not pursued since I was interested in agriculture-based research.

Memberships

• American Society for Plant Biologists
• American Phytopathological Society
• International Society of Plant-Microbe Interactions

Synergistic Activities

- Member, Association of Plant Pathology Scholars (APPS), 2011-present
- As an APPS member I am involved in organizing social and educational annual departmental picnic, the graduate student retreat, and the annual extension department luncheon and seminars
- As a demonstrator and assistant lecturer in Botany department, College of Science, Tanta University, Tanta, Egypt, I participated in organizing supervising several scientific trips to pharmaceutical companies and factories
- Manuscript reviews: reviewed manuscripts for Molecular Plant Pathology and BMC Plant Biology

Publications

1. **Mohamed El-Shetehy**, Caixia Wang, M.B. Shine, Keshun Yu, Duroy Navarre, David Wendehenne, Aardra Kachroo, and Pradeep Kachroo (2015). Nitric oxide and reactive oxygen species are required for systemic acquired resistance in plants. ***Plant Signaling & Behavior*** 10(9):e998544.
   DOI: http://dx.doi.org/10.1080/15592324.2014.998544

2. Caixia Wang, **Mohamed El-Shetehy**, M.B. Shine, Keshun Yu, Duroy Navarre, David Wendehenne, Aardra Kachroo, and Pradeep Kachroo (2014). Free Radicals Mediate Systemic Acquired Resistance. ***Cell Reports*** 7:348–355
   DOI: http://dx.doi.org/10.1016/j.celrep.2014.03.032

PRESENTATIONS

Invited Oral Presentations

1. American Society of Plant Biologists Midwestern Section Annual meeting- Ohio State University, Columbus, Ohio, March 22-23, 2014- Chemical inducers of systemic acquired resistance in plants.
2. The 2014 Annual Meeting of the Southern Section of the American Society of Plant Biologists (SS-ASPB)- University of Kentucky, Lexington, Kentucky, March 29-31, 2014- Chemical inducers of systemic acquired resistance in plants.
3. 21st International Symposium on Plant Lipids 2014 (ISPL2014)- University of Guelph in the city of Guelph, Ontario, Canada- July 6-11, 2014- Chemical inducers of systemic acquired resistance in plants
4. The American Phytopathological Society- The Canadian Phytopathological Society Annual Meeting (APS-CPS Joint Meeting)- Minneapolis, Minnesota- August 9-13, 2014- Chemical inducers of systemic acquired resistance in plants.

Poster Presentations

Infectious Disease research Day 2015, Lexington, Kentucky-October 22, 2015: Mohamed El-Shetehy, Caixia Wang, M B Shine, Keshun Yu, Aardra Kachroo, and Pradeep Kachroo, Nitric oxide and reactive oxygen species are required for systemic acquired resistance in plants

Workshops Attended

1. **August 2-5, 2009:** Basic Molecular Biology: organized by Medical Technology Center for Research and Services, Alexandria University, Alexandria, Egypt
2. **March 7-11, 2009:** Biodiversity of genera and species of fungi which deteriorate food materials with special attention to toxigenic, and human, animal and plant pathogenic species: organized by Assiut University Mycological Centre (AUMC), Assiut, Egypt
3. **March 8-13, 2008:** Biodiversity of Ascomycota with special attention to the genera and species of medical, agricultural and biotechnological significance: organized by AUMC, Assiut, Egypt
4. **March 17-22, 2007:** Biodiversity and identification of Fusarium species with special attention to toxigenic, and human, animal and plant pathogenic species: organized by AUMC, Assiut, Egypt
5. **March 18-23, 2006:** Biodiversity of indoor and outdoor air-borne fungi and their role in human, animal, and plant diseases and in food deterioration: organized by AUMC, Assiut, Egypt