THE ROLE OF SALICYLIC ACID RECEPTORS NPR1 AND NPR3/4 IN PAMP-TRIGGERED IMMUNITY AND EFFECTOR-TRIGGERED IMMUNITY

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THE ROLE OF SALICYLIC ACID RECEPTORS NPR1 AND NPR3/4 IN PAMP-TRIGGERED IMMUNITY AND EFFECTOR-TRIGGERED IMMUNITY

submitted by Ana Radojicic in partial fulfillment of the requirements for

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Abstract

Plants are constantly exposed to different pathogens in their environment and they have evolved complex defense strategies to avoid potential disease. The two general layers of plant defense are pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is activated upon recognition of PAMPs by plant's pattern recognition receptors (PRRs) localized on plasma membrane, while ETI is triggered when the presence of bacterial effector proteins is detected by intracellular plant nucleotide-binding leucine-rich repeat (NLR) proteins. Salicylic acid (SA) is a major regulator of plant immunity. A new model of SA signaling in *Arabidopsis thaliana* was recently established. Non-expressor of Pathogenesis-Related (*PR*) genes 1 (NPR1), NPR3 and NPR4 are SA receptors that have opposite roles in transcriptional regulation of defense-related genes. NPR1 functions as a transcriptional co-activator, while NPR3 and NPR4 are redundant transcriptional co-repressors of defense-related genes. Binding of SA activates NPR1, while it blocks transcriptional repression activity of NPR3 and NPR4. Although general function of these proteins in plant defense is understood, their requirement for different regulatory pathways in plant immunity is not entirely explored. In this thesis I analyze how NPR1 and NPR3/4 are involved in regulation of PTI response, and ETI response mediated by different NLR proteins. Infection assays with different strains of *Pseudomonas syringae* pv. *tomato* DC3000 revealed that PTI is regulated through both NPR1- and NPR3/4-dependent signaling, and that basal levels of SA contribute to PTI and AvrRPS4- or AvrRpt2-triggered ETI. The knock-out mutant *npr1-1* and a dominant gain-of-function mutant allele *npr4-4D* fully suppress expression of *PR1* and resistance to *H.a.* Noco2, but partially suppress dwarf morphology and constitutive *PR2* expression of autoimmune

snc1 mutant. Similarly, *npr1-8* and *npr4-4D* partially suppress autoimmune phenotype of *mkk1/2* mutants. Results of ion-leakage assay suggest that SA serves as a negative regulator of AvrRpt2-triggered hypersensitive response through NPR1 and NPR4-mediated signaling. My results support the new model of SA-dependent signaling, confirming that NPR1 and NPR3/4 function in parallel pathways. Although SA is the major regulator of plant defense, not all aspects of immune response rely entirely on SA receptors NPR1 and NPR3/4, confirming existence of SA-independent signaling.

Lay Summary

My thesis provides better understanding on how plant defense against pathogens is regulated in model plant *Arabidopsis thaliana*. I demonstrate how receptors of salicylic acid, one of the major plant hormones involved in plant defense against pathogens, influence different layers of plant immunity and whether they are partially or fully required for different signaling pathways. My results provide significant support for the newly and recently established model of salicylic acid signaling that explains how genes important for plant immunity are activated in response to salicylic accumulation upon pathogen attack. Better understanding of plant immunity regulation paves way for potential applications in the field of crop protection, since it provides useful background for possible design of plants that are resistant to devastating diseases.

Preface

The research project presented in this thesis was designed by Dr. Yuelin Zhang and I conducted the majority of the experiments under his supervision.

The cross between *snc1* and *npr1-1 npr4-4D* was performed by Dr. Yuli Ding from Dr. Yuelin Zhang's lab. The cross between *mkk1/2* and *npr1-8 npr4-4D* was performed by Dr. Tongjun Sun from Dr. Yuelin Zhang's lab. I was provided with the seeds (F2 generation) from these crosses and I did the genotyping by PCR to obtain the following mutants: *snc1 npr4-4D*, *snc1 npr1-1 npr4-4D*, *mkk1/2 npr1-8*, *mkk1/2 npr4-4D*, *mkk1/2 npr1-8 npr4-4D*. I conducted RNA isolation and qRT-PCR analysis for combined mutants of *snc1*, *npr1-1* and *npr4-4D* together with Dr. Yuli Ding. I performed all infection assays, with occasional technical support from Dr. Yuli Ding and Hainan Tian from Dr. Yuelin Zhang's lab. I conducted qRT-PCR for *mkk1/2*, *npr1-8* and *npr4-4D* mutant combinations, I carried out all statistical analysis of the data and made all the figures presented in this thesis.

The work presented in paragraph 3.1.2.4 was published in the following review article: Radojičić, A., Li, X., & Zhang, Y. (2018) Salicylic acid: A double-edged sword for programed cell death in plants. *Frontiers in Plant Science*, 9:1133.

Dr. Yuelin Zhang designed the experiments, I conducted ion leakage assays, analyzed the data, made figures, and all the authors wrote the manuscript.

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List of Abbreviations

ATP	Adenosine triphosphate
avr	Avirulence
BAK1	Brassinosteroid Insensitive 1 (BRI1)-Associated Receptor Kinase
BTB/POZ	Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger
BTH	Benzothiadiazole
CC	Coiled-coil
CC-NB-LRR	Coiled-coil - Nucleotide-binding - Leucine-rich Repeat
CDK	Ca^{2+}-dependent kinase
CERK1	Chitin Elicitor Receptor Kinase 1
CNL	Coiled-coil - Nucleotide-binding - Leucine-rich Repeat
CRCK3	Calmodulin-binding Receptor-like Cytoplasmic Kinase 3
DAMP	Danger-associated molecular pattern
EDS1/5	Enhanced Disease Susceptibility 1/5
EF-Tu	Elongation Factor Thermo unstable
EFR	EF-Tu Receptor
ETI	Effector-triggered immunity
FLS2	Flagellin-Sensitive 2
H.a. Noco2	*Hyaloperonospora arabidopsidis* Noco2
HR	Hypersensitive response
ICS1 (ICS2)	Isochorismate Synthase 1 (2)
INA	2, 6 - dichloroisonicotinic acid

LRR	Leucine-rich repeat
MAPK	Mitogen-Activated Protein Kinase
MATE	Multidrug and Toxin Extrusion
MKK	Mitogen-Activated Protein Kinase (MAPK) Kinase
mkk1/2	*mkk1 mkk2*
NB	Nucleotide-binding
NDR1	Non-race-Specific Disease Resistance 1
NLR	Nucleotide-binding Leucine-rich Repeat protein
NPR1 (NPR3/4)	Nonexpresser of *PR* genes 1 (3/4)
PAD4	Phytoalexin Deficient 4
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated molecular pattern
PAT1	Protein Associated with Topoisomerase II 1
PBS3	AvrPphB Susceptible 3
PRR	Pattern-recognition receptor
Psm ES4326	*Pseudomonas syringae* pv. *maculicola* ES4326
Pst DC3000	*Pseudomonas syringae* pv. *tomato* D3000
PTI	Pathogen-associated molecular pattern (PAMP)-triggered immunity
PR genes/proteins	Pathogenesis-related genes/proteins
R genes/proteins	Resistance genes/proteins
RIN4	Resistance to *P. syringae* pv. *maculicola* (RPM1)-Interacting Protein
RLK	Receptor-like kinase
RLP	Receptor-like protein

RPS	Resistance to *Pseudomonas syringae*
ROS	Reactive oxygen species
RPM1	Resistance to *Pseudomonas syringae* pv. *maculicola* 1
RPP13-Nd	Resistance to *Peronospora parasitica* 13, Accession Nd-1
RPP8	Resistance to *Peronospora parasitica* 8
SA	Salicylic acid
SAG	Salicylic acid glucoside
SAG101	Senescence-Associated Gene 101
SAR	Systemic acquired resistance
sid2	*salicylic acid induction deficient 2*
SNC1	Suppressor of *npr1-1*, Constitutive 1
SUMM2	Suppressor of *mkk1 mkk2*
TIR	Toll/Interleukin 1 receptor
TIR-NB-LRR	Toll/Interleukin 1 receptor - Nucleotide binding - Leucine Rich Repeat
TLR	Toll-like receptor
TNL	Toll/Interleukin 1 receptor - Nucleotide binding - Leucine Rich Repeat
WT	wild type

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Chapter 1: Introduction

1.1 Plant immunity

Plants are constantly challenged by different pathogenic organisms present in their environment. Although plants do not possess specialized immune cells and complex immune system like mammals do, they have developed very effective defense mechanisms in order to combat pathogen attacks. To emphasize how effective plant defense is we say that 'most plants are healthy most of the time'. In other words, even though humans are focusing on plant diseases because of the great economical losses they can cause in agriculture, from plants' perspective diseases are actually rare events. Plants are able to defend themselves from the majority of different pathogens they encounter, and it usually takes a specific pathogen strain to attack a given host species and establish the disease. Nevertheless, when these rare events occur, they can have devastating consequences on society. The most famous example is the disease called late potato blight caused by the oomycete pathogen *Phytophthora infestans.* This devastating potato disease caused starvation of a million people and emigration of more than a million from Ireland, which is remembered as the Great Irish Potato Famine (Donnelly, 2001). Understanding how pathogens and plants interact with each other and how plant immunity is regulated helps us develop new ways to protect plants that are of great value for our society.

Plant defense responses are initiated both locally and systemically as a result of successful detection of pathogens and activation of downstream signal transduction, and all these steps are tightly regulated. Two general conceptual layers of innate immunity are recognized in plants: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and Effector-triggered immunity (ETI) (Jones & Dangl, 2006).

1.1.1 PAMP-triggered immunity

PAMP-triggered immunity (PTI) is the first layer of active defense in plants. It is initiated upon the recognition of PAMPs by pattern recognition receptors (PRRs) located on the plant plasma membrane. PAMPs are molecules that are common for certain groups of microorganisms. Well known examples are bacterial protein flagellin, and the major structural component of fungal cell walls, chitin (Felix *et al*., 1999; Miya *et al*., 2007). Since some of these molecules are found on microorganisms regardless of their pathogenicity, they are also referred to as microbe associated molecular patterns (MAMPs) (Mackey & McFall, 2006). PAMPs contain highly conserved motives that are sufficient to trigger PTI response. Synthetic peptides widely used in research as elicitors of PTI are flg22, a conserved 22-amino-acid polypeptide located on the N-terminal domain of bacterial flagellin, and elf18/elf26, the N-terminus peptides of bacterial Elongation factor Tu (EF-Tu) (Felix *et al*., 1999; Kunze *et al*., 2004). In addition to PAMPs, plants can also detect molecules resulting from the damage caused by pathogen-secreted enzymes called damage associated molecular patterns (DAMPs). DAMPs could be fragments of the cell wall, cutin monomers or small peptides secreted by plants, and they can also trigger plant defense responses (Boller & Felix, 2009; Yamaguchi & Huffaker, 2011).

PRRs are often transmembrane proteins and they can be divided into two groups: receptor-like kinases (RLKs) and receptor-like proteins (RLPs). RLKs have extracellular leucine-rich repeats domain (LRR), a transmembrane domain and an intracellular kinase domain. Unlike RLKs, RLPs do not have the kinase domain; hence they have to interact with other kinases in order to activate downstream signaling (Zipfel, 2014). It is interesting that plant PRRs share structural similarity with mammalian Toll-like receptors (TLRs) that are involved in innate immunity (Akira, 2003). Flagellin-sensing 2 (FLS2) is the RLK that can detect the presence of

flg22, while EF-Tu is recognized by the RLK named Elongation factor receptor (EFR) (Gómez-Gómez & Boller, 2000; Chinchilla *et al.*, 2006; Zipfel *et al*., 2006**)**. FLS2 and EFR have structural similarities and they both associate with the co-receptor Brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1) to initiate the downstream signaling (Roux *et al.*, 2011). On the other hand, Chitin elicitor receptor kinase 1 (CERK1) that binds chitin has extracellular LysM domain instead of LRR domain (Miya *et al*., 2007; Wan *et al.*, 2008, Petutschnig *et al.*, 2010). As there are various bacterial, fungal and oomycete molecules of different chemical structures that can serve as PAMPs, additional PAMP – PRR pairs need to be identified and characterized.

Despite the diversity of PAMPs and PRRs, the defense responses triggered by different PAMPs are more or less uniform. Immediate responses which happen within a few minutes after the PAMP detection include oxidative burst and rapid Ca^{2+} influx from the apoplast. The consequent intracellular increase in concentration of Ca^{2+} ions can lead to activation of Ca^{2+}-dependent protein kinases (CDKs), or activation of other voltage-dependent ion channels (Bigeard *et al.*, 2015). Reactive oxygen species (ROS) can act directly as antimicrobial agents, and indirectly as signaling molecules (Apel & Hirt, 2004). Furthermore, alongside with the CDKs, activation of Mitogen-activated protein kinase (MAPK) cascade results in changes in protein phosphorylation and gene expression (Benschop *et al.* 2007, Bigeard *et al.*, 2015, Withers & Dong, 2017). The expression of around 1000 genes is upregulated upon recognition of flg22, including Resistance genes (*R* genes), RLKs, transcription factors, antimicrobial substances, and genes involved in signaling transduction; among around 200 downregulated genes are the ones involved in plant growth and development (Zipfel *et al*., 2004). Different PAMPs often regulate expression of a similar set of genes, showing that PTI response is common

to different PRRs (Boller & Felix, 2009). Another typical PTI response is callose deposition between the plasma membrane and the cell wall, which is a putative physical barrier for the invading pathogens. All these defense mechanisms activated by the recognition of PAMPs act as the first line of active protection from colonization and proliferation of pathogenic microorganisms in plant tissues.

1.1.2 Effector-triggered immunity

In order to successfully attack plant tissues, pathogens have evolved mechanisms to interfere with plant's defense signaling pathways and to suppress the PTI response. They manage this by producing proteins called effectors and delivering them into the plant cells. These effectors interfere with different regulators of PTI in the host cell and consequently, they weaken plant's ability to protect itself from the ongoing pathogen attack. However, in response to effectors, plants have evolved Resistance proteins (R proteins) that can detect the presence of the effectors and trigger ETI (Jones & Dangl, 2006).

ETI is a rapid defense response that is significantly stronger than PTI. Activation of ETI typically results in the accumulation of plant hormone salicylic acid (SA), expression of pathogenesis related genes (*PR* genes), ROS production and hypersensitive response (HR) (Jones & Dangl, 2006). HR is the classical outcome of ETI and it represents rapid programed cell death at the site of the infection. The purpose of these local necrotic lesions most likely is to block proliferation of the pathogen and its further colonization of surrounding tissues (Greenberg *et al.*, 1994). Therefore, it is not surprising that HR is an effective mechanism only against biotrophic and hemibiotrophic, and not the necrotrophic pathogens (Govrin *et al*., 2000). A different

pathway which relies on plant hormones ethylene and jasmonic acid is responsible for the protection against necrotrophs (Glazebrook, 2005).

In order to activate ETI, R proteins need to detect the pathogen effectors. There are a few examples of R proteins interacting directly with the effectors, such as the rice R protein Pi-ta directly binding the *Magnaporthe grisea* effector AVR-pita, and the *Arabidopsis thaliana* R protein Resistant to *Ralstonia solanacearum* 1 (RRS1) binding the *R. solanacearum* effector PopR2 (Jia *et al*., 2000; Deslandes *et al*., 2003). This receptor-ligand type of recognition is one way to address the "gene-for-gene" model in which plant resistance genes encoding R proteins have their corresponding pathogen avirulence (*avr*) genes that encode the effector proteins (Flor, 1971). However, there are more examples where R proteins recognize effectors indirectly, by sensing their activities such as modifications or degradation of target proteins inside the host cell. This indirect recognition of an effector by the cognate R protein is explained through the Guard model (Dangl & Jones, 2001). This model suggests that different R proteins can guard one host protein (called target or "guardee"), and these R proteins are activated upon interaction between different pathogen effectors and the target protein. According to this model, the target protein is supposed to have a role in the positive regulation of plant immunity or some other cell function (Dangl & Jones, 2001). A well-studied example for this model is *Arabidopsis* RPM-interacting protein 4 (RIN4) which is guarded by R proteins Resistance to *Pseudomonas syringae* pv. *maculicola* 1 (RPM1) and Resistance to *Pseudomonas syringae* 2 (RPS2). RPM1 can detect phosphorylation of RIN4 induced by *P. syringae* effectors AvrRpm1 and AvrB, while RPS2 can sense the cleavage of RIN4 by effector AvrRpt2 (Mackey *et al.*, 2002; Belkhadir *et al*., 2004; Mackey *et al*., 2003; Liu *et at*., 2012). The Guard model was further expanded to a slightly more complex Decoy model, which suggests the existence of protein "decoys" or "baits" that are false

targets of pathogen effectors (Van der Hoorn & Kamoun, 2008). These decoys can indirectly activate ETI through interaction with R proteins, but the decoys themselves have lost their function in plant immunity. Instead, they have evolved to become "traps" for effectors and serve only as sensors of the pathogen attack (Van der Hoorn & Kamoun, 2008). Tomato protein Pto is one of the putative decoys. It interacts with R protein Prf and is targeted by *P. syringae* effectors AvrPto and AvrPtoB (Van der Hoorn & Kamoun, 2008; Khan *et al*., 2016). This example along with several others requires stronger experimental evidence in order to be reclassified from the Guard to Decoy model.

The majority of R proteins belong to the group of nucleotide-binding leucine-rich repeat (NB-LRR or NLR) proteins. There are 182 NLRs in *A. thaliana* and they can be classified into two major groups: TIR-NB-LRR (TNL) proteins, that have N-terminal Toll/Interleukin 1 receptor (TIR) domain, and CC-NB-LRR (CNL) proteins, which have a coiled-coil (CC) N-terminal domain (Meyers *et al*., 1999; Meyers *et al.*, 2003; Sarris *et al.*, 2016). These two N-terminal domains are responsible for the activation of signaling by forming homo- or heterodimers of NLRs and interacting with the downstream signaling components (Takken & Goverse, 2012, Kapos *et al.*, 2019). The NB domain binds ATP and uses energy from ATP hydrolysis to change the conformation of NLR and turn it into the active state (Van Ooijen *et al*., 2008). The C-terminal LRR domain functions as a sensor of the effector's presence in the host cell and, together with TIR/CC domain, it can also be involved in auto-inhibition of NLR activity in the absence of effectors (Takken & Goverse, 2012, Hu *et al*., 2013, Kapos *et al.*, 2019). Although there is a diversity of NLRs that deviate from the CC/TIR-NB-LRR structure as the most prominent one (Li *et al.*, 2015), for the purpose of this research, I will focus only on the two major groups: TNL and CNL proteins.

Different components are required for signaling pathways downstream of TNLs and CNLs (Aarts *et al*., 1998). Enhanced Disease Susceptibility 1 (EDS1) is the central regulator of defense responses activated by TNL proteins such as Resistance to *P. syringae* 4 (RPS4) and Suppressor of *npr1-1* constitutive 1 (SNC1) (Aarts *et al*., 1998; Zhang *al*., 2003b). EDS1 shares homology with eukaryotic lipases, but whether it has a function of a lipase in the plant immunity and the exact mechanism of its activity is not yet clear (Falk *et al*., 1999). So far, we know that EDS1 forms complexes with Phytoalexin Deficient 4 (PAD4) and Senescence-Associated Gene 101 (SAG101), and that these two proteins are important for EDS1 activity in ETI (Parker *et al*., 1996; Falk *et al*., 1999; Feys *et al*., 2001; Feys *et al*., 2005; Cui *et al.*, 2017).

On the other hand, Non-race-specific Disease Resistance 1 (NDR1) is an integrin-like membrane protein that functions as a core signaling component downstream of CNL proteins RPM1, RPS2 and RPS5 (Century *et al*., 1995; Aarts *et al*., 1998, Coppinger *et al*., 2004). The RPM1 and RPS2 dependency on NDR1 is in line with the observed interaction between NDR1 and plasma membrane-localized RIN4 (Day *et al.*, 2006). However, CNL proteins Resistance to *P. parasitica* 8 (RPP8), Resistance to *P. parasitica* 13, Accession Nd-1 (RPP13-Nd) and Hypersensitive Response to Turnip Crinkle Virus (HRT) are NDR1-independent, therefore we can no longer view NDR1 as a hallmark of CNL signaling (McDowell *et al*., 2000; Bittner-Eddy & Beynon, 2001; Chandra-Shekara *et al.*, 2004). The mechanism of NDR1 activity is not fully established, but the data suggests that NDR1 is regulating cell wall-plasma membrane adhesions and it contributes to both PTI and ETI response (Knepper *et al.*, 2011).

1.2 Salicylic acid in plant immunity

Salicylic acid is a plant hormone that is involved in regulating a variety of physiological processes in plants - from germination and vegetative growth, thermogenesis, photosynthesis, flower formation, senescence, to regulation of responses to abiotic stresses such as drought, heavy metal tolerance, heat and osmotic stress (Rivas-San Vicente & Plasencia, 2011). Nevertheless, the best-defined physiological role of SA is the regulation of plant immunity. There are several lines of evidence that SA is required for plant defense against pathogens. It has been demonstrated that SA accumulates in both local (infected) and systemic tissues after the pathogen infection (Métraux *et al*., 1990; Malamy *et al.*, 1990). Moreover, SA was recognized as an essential component of systemic acquired resistance (SAR), a long-lasting resistance to a broad spectrum of pathogens that is established on systemic level (whole plant) upon signal transduction from the local site of infection (Lawton *et al.*, 1995). Inability to activate SAR and increased susceptibility to pathogen infection were demonstrated in transgenic plants carrying *Pseudomonas putida* gene *nahG* (Delaney *et al.,* 1994). This gene encodes a salicylate hydroxylase that degrades SA (Gaffney *et al.,* 1993). Likewise, mutants defective in SA biosynthesis or perception are highly susceptible to pathogens, while exogenous application of SA or its chemical analogues 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) restores the resistance (Durrant & Dong, 2004; Görlach *et al*., 1996; Métraux *et al*., 1991). All this information has led to decades of further extensive research on SA biosynthesis, signaling and perception with reference to plant-pathogen interactions, but some questions are still waiting to be resolved.

1.2.1 SA biosynthesis

SA is a phenolic compound that is produced through two biosynthetic pathways: the phenylalanine ammonia-lyase (PAL) pathway and the isochorismate synthase (ICS) pathway. The ICS pathway is considered to be of great importance for plant immunity, since the majority (approximately 90%) of pathogen-induced SA in *Arabidopsis* is produced through this pathway (Wildermuth *et al.,* 2001). The PAL pathway seems to be less significant for plant defense in *Arabidopsis*, although there is evidence of its contribution. The quadruple knockout mutant *pal1 pal2 pal3 pal4* that has the activity of PAL enzymes reduced to 10% shows pathogen-induced SA accumulation reduced by 50% and a slight but noticeable effect on resistance to *P. syringae* DC3000 (Huang *et al*., 2010). Moreover, research in soybean indicates that PAL pathway might be as important for plant immunity in other species as the ICS pathway is (Shine *et al*., 2016).

One of the mutants defective in pathogen-induced SA biosynthesis named *salicylic acid induction deficient2* (*sid2*) carries a null mutation in the *ICS1* gene and this mutant exhibits reduced resistance to biotrophic pathogens (Nawrath *et al*, 1999; Wildermuth *et al.,* 2001). Another gene involved in SA biosynthesis through ICS pathway is *ICS2*, though its contribution to overall SA accumulation is small and it is not yet clear what the specific role of this gene is (Garcion *et al.,* 2008). Additional mutant deficient in pathogen-induced SA accumulation is *sid1*/*enhanced disease susceptibility* (*eds5)* (Nawrath *et al.,* 1999). EDS5 localizes to the chloroplast membrane and it is a member of the multidrug and toxin extrusion (MATE) transporter family. It is essential for SA accumulation induced by biotic and abiotic stress (UV light) and it was previously thought to serve as a transporter to move SA from chloroplasts to the cytoplasm (Nawrath *et al.,* 2002; Serrano *et al.,* 2013). Serrano *et al*. (2013) suggested a model in which SA accumulating inside the chloroplasts of *eds5* plants inhibits ICS1 through unknown

mechanism, resulting in block of SA biosynthesis. However, the steps of SA biosynthesis through ICS pathway and a more precise function of EDS5 in that process were recently described by Rekhter *et al*. (2019a). Chloroplast-localized ICS1 converts chorismate to isochorismate, which is then exported to the cytoplasm by the membrane transporter EDS5. Consuming this isochorismate and glutamate from the cytoplasm, AvrPphB Susceptible 3 (PBS3) catalyzes synthesis of isochorismate-9-glutamate. Isochorismate-9-glutamate is an unstable intermediate, thus it spontaneously decomposes and generates SA and enolpyruvyl-N-glutamate (Warren *et al*., 1999; Rekhter *et al.*, 2019a).

SA exists in the plant in free form and glucose-conjugated form, known as SA glucoside (SAG). SAG serves as a storage of SA and it is reversibly transported and stored inside the vacuole (Dean *et al*., 2005). Mutants defective in pathogen-induced accumulation of SA such as *sid2* and *eds5* are still able to produce a small amount of both free and conjugated SA (Nawrath and Métraux, 1999). It remains unclear whether and to what extent these basal levels of SA are involved in plant defense.

1.2.2 SA signaling

The major regulator of SA-dependent immune response is Nonexpresser of *PR* genes 1 (NPR1). NPR1 encodes a receptor for SA that positively regulates SA-induced *PR* gene expression (Cao *et al*., 1994; Delaney *et al*., 1995; Wu *et al*., 2012; Manohar *et al.*, 2015). It contains two structural domains with functions in protein-protein interactions: an ankyrin repeat domain and a BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) domain (Cao *et al*., 1997; Boyle *et al.*, 2009). These domains are important for interactions with bZIP transcription factors TGA2, TGA5 and TGA6 (TGA2/5/6) which are also

involved in positive regulation of the transcription of *PR* genes and resistance to pathogens (Zhang *et al*., 1999; Després *et al*., 2000; Zhang *et al*., 2003a). *NPR1* has two close paralogs, *NPR3* and *NPR4*, that are, in contrast to *NPR1*, negative regulators of SA-mediated immunity (Zhang *et al*., 2006; Fu *et al*., 2012). NPR3 and NPR4 function redundantly as SA receptors and they also interact directly with TGA2/5/6 transcription factors (Zhang *et al*., 2006; Fu *et al*., 2012). Until recently, it was believed that NPR3 and NPR4 were regulating plant immunity through control of NPR1 degradation, acting as adaptors for Cullin 3 ubiquitin E3 ligase (Fu *et al*., 2012). This model proposes that NPR4, that has lower affinity for SA than NPR3, in conditions of low SA cannot interact with NPR1, so the NPR1 is not degraded. On the other hand, when SA is accumulated to high levels upon the pathogen infection, NPR3 binds SA and that enables it to interact with NPR1 and tag it for degradation by the proteasome (Fu *et al*., 2012). However, this model is not supported by genetic evidence that NPR3 and NPR4 function redundantly in negative regulation of defense responses (Zhang *et al*., 2006). A new model established by Ding *et al*. (2018) places NPR3/4 in a pathway independent of NPR1, where they function as co-repressors of defense-related gene expression (**Figure 1.1**). When levels of SA are low, NPR3/4 repress *PR* gene expression. After pathogen attack, SA accumulates to high levels and binds to NPR3/4, which inhibits their transcriptional repression activity, and at the same time, SA binds to NPR1 to promote *PR* gene expression.

A dominant gain-of-function mutant *npr4-4D* is incapable of binding SA, which results in constitutive repression of defense-related gene expression (Ding *et al*., 2018). As *NPR3* and *NPR4* are redundant genes, I am using *npr4-4D* mutant line in this research to mimic a dysfunctional NPR3/4 branch of defense related signaling.

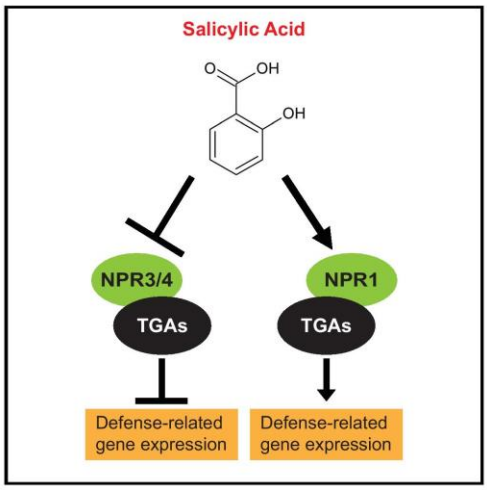

Figure 1.1 The model of SA-dependent regulation of defense-related gene expression SA receptors NPR1 and NPR3/4 regulate defense-related gene expression through interaction with TGA transcription factors. SA binding activates NPR1, the positive regulator of defense-related gene expression. NPR3 and NPR4 are co-repressors of defense-related genes, and SA blocks their transcriptional repression activity (adapted from Ding *et al.*, 2018).

1.2.3 SA as regulator of HR

The rapid programed cell death that occurs at the infection site, known as the HR, often appears as a result of ETI. The purpose of this defense mechanism is to block biotrophic pathogen advancement to surrounding tissues by limiting the resources available for pathogen growth and proliferation. The tight control of HR must exist to avoid potential spreading of cell death outside of the limited area surrounding the infection site. Although SA accumulation is linked to ETI response, there is no simple answer to the question how SA regulates HR. There is evidence of SA being both a positive and a negative regulator of HR, but the exact mechanism is not yet clear.

Lesion-mimic mutants are known for their phenotype of spontaneous cell deaths. Some of the lesion mimic mutants such as *lesion simulating disease 6* (*lsd6*), *lsd7*, *accelerated cell death 6* (*acd6)* and *acd11* accumulate high levels of SA. When accumulation of SA in these

mutants is blocked by *nahG*, the lesion-mimic phenotype is gone (Weymann *et al.*, 1995; Rate *et al.*, 1999; Brodersen *et al*., 2005). Exogenous application of low SA amounts on *lsd1* leads to the enhanced lesion phenotype (Dietrich *et al.*, 1994). This data supports the hypothesis that SA is promoting HR response. However, *defense, no death 1* (*dnd1*) and *suppressor of npr1-1, constitutive* (*snc1*) are examples of mutants with high SA accumulation and constitutively activated defense, but no obvious HR lesions, implying that SA alone is not enough to trigger HR (Yu *et al.*, 1998; Li *et al*., 2001). Furthermore, *lsd2* and *lsd4* exhibit the lesion-mimic phenotype regardless of *nahG*, pointing that in this case, lesion-mimic phenotype is independent of SA accumulation (Dietrich *et al*., 1994; Hunt *et al*., 1997). Exogenous application of SA can also have a negative impact on HR activation. Devadas & Raina (2002) reported that pre-treatment of Col-0 plants with SA blocks HR activated by *P. syringae* pv. *maculicola (Psm)* ES4326 carrying *avrRpm1*.

SA receptor NPR1 is also suggested to have a negative role in HR regulation. *npr1-1* mutant shows higher cell death compared to the wild-type after infection with *Psm* ES4326 *avrRpm1*, while the *NPR1* overexpression line exhibits suppressed HR (Rate & Greenberg, 2001). Moreover, *aberrant growth and death 2* (*agd2*) mutant has attenuated AvrRpm1-induced HR, while *agd2 npr1-1* has HR restored to the wild-type level (Rate & Greenberg, 2001). Regarding the two other SA receptors, knockout mutant *npr3 npr4* treated with *Psm* ES4326 *avrRpt2* has attenuated HR, while in *npr3 npr4 npr1* mutant HR is restored, further supporting that NPR1-mediated signaling is involved in negative regulation of HR, while the role of NPR3/4 in HR control is not fully clear (Fu *et al*., 2012).

1.3 SNC1-mediated ETI response

SNC1 (Suppressor of *npr1-1*, Constitutive 1) is used in my thesis project as a representative of a TNL group of R proteins. A dominant mutant allele *snc1* was identified from a suppressor screen in the *npr1-1* background (Li *et al.*, 2001). The *snc1* is a gain-of-function mutant which contains a Glu522Lys substitution in the linker between NB and LRR domains (Zhang *et al.*, 2003). SNC1 protein that carries this mutation is not as prone to ubiquitination as the wild type protein is, so the turnover of the SNC1 protein is decreased, resulting in SNC1 accumulation and constitutively activated defense response (Cheng *et al*., 2011). *snc1* plants have dwarf phenotype and curly leaves, which is probably linked with the accumulation of high levels of SA (Bowling, 1997; Li et al., 2001). *snc1* mutant accumulates 15 x higher levels of SA compared to the wild type, while *snc1 npr1-1* accumulates 21 x more SA than then the wild type, supporting the claim that NPR1 is also involved in the negative feedback regulation of pathogen-induced SA accumulation (Li *et al.*, 2001). Although there are autoimmune mutants that accumulate SA and exhibit spontaneous lesions that resemble the HR, the *snc1* autoimmune phenotype does not include spontaneous cell death, implying that high levels of SA are not sufficient to trigger hypersensitive response (Li *et al*., 2001). Besides the dwarf morphology, the *snc1* plants also exhibit constitutive expression of *PR1* and *PR2* and resistance to virulent bacterial pathogen *P. syringae* pv. *maculicola* ES4326 and oomycete pathogen *Hyaloperonospora arabidopsidis* Noco2 (Li *et al.*, 2001). The *snc1* mutant phenotype can be fully suppressed by inactivation of *EDS1* or *PAD4*, but not *NDR1*, indicating that SNC1 signals like a typical TNL protein (Li *et al*., 2001; Zhang *et al.*, 2003).

Constitutive expression of *PR1* in *snc1* is regulated through both NPR1-dependent and NPR1-independent pathways, while expression of *PR2* seems to be independent of NPR1. When

nahG is introduced into *snc1* background, constitutive *PR2* expression is completely abolished, indicating that SA is required for *PR2* expression. Interestingly, *PR2* expression could not be fully suppressed in the *snc1 eds5-3* mutant (Li *et al.*, 2001; Zhang, Zhang *et al.*, 2003). One proposed explanation is that catechol, a product of SA degradation by salicylate hydroxylase, suppresses *PR2* expression (Van Wees & Glazebrook, 2003). Although *eds5-3* plants are defective in pathogen-induced SA accumulation, they can still accumulate low levels of SA and those levels are slightly higher than in *nahG* transgenic plants (Nawrath and Métraux, 1999), suggesting an alternative hypothesis that SA accumulated independently of EDS5 is responsible for *PR2* expression (Zhang *et al.*, 2003). If this hypothesis was correct, it would imply that basal levels of SA play a critical role in defense responses. Hence, it is important to analyze the roles of basal levels of SA in plant defense.

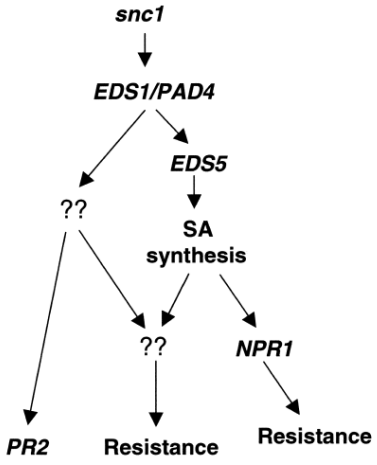

Figure 1.2 The proposed model of *snc1*-mediated signaling
Defense responses activated in *snc1* plants require *EDS1* and *PAD4*, and they are partially dependent on SA. The SA-dependent pathway consists of both NPR1-dependnet and NPR1-independnet pathway to trigger the resistance. *PR2* is activated through SA-independent pathway (Zhang *et al*., 2003)

As illustrated in the model proposed by Zhang *et al*., (2003) (**Figure 1.2**), immune response mediated by SNC1 is partially dependent on SA, and both NPR1-dependent and NPR1-independent pathways contribute to the autoimmune phenotype. It is not clear whether NPR4 plays a role in defense pathways downstream of SNC1 and weather it is a major regulator of above mentioned NPR1-independent pathway. Analysis of the *snc1 npr1-1 npr4-4D* triple mutant should elucidate the contribution of the two SA receptors in defense pathways downstream of TNL protein SNC1.

1.4 SUMM2-mediated ETI response

Mitogen-activated protein kinase (MAPK/MPK) cascades are important three-kinase signaling modules in eukaryotes. They transduce signals from the upstream receptors to the downstream targets and therefore regulate cellular responses to different intracellular and extracellular signals (Meng & Zhang, 2013). MAPKs are at the lowest level of the cascade, phosphorylating target proteins. In mammals, they are also called the Extracellular signal-related kinases ERK (Chang & Karin, 2001). MAPK activity is regulated by the upstream MAPK kinases (MAPKK/MKK/M2K) also called MAP and ERK kinases (MEK), while the MAPKKs are regulated by the third level kinase, MAPKK kinase (MAPKKK/MKKK/M3K or MEKK) (Meng & Zhang, 2013). MAPK cascades are involved in regulation of plant growth and development, and responses to both biotic and abiotic stress (Suarez Rodriguez *et al*., 2010; Sinha *et al.*, 2011; Meng & Zhang, 2013; Xu & Zhang, 2015).

MAPK cascades play important regulatory role in plant immunity. When PAMPs are recognized by PRRs on the plasma membrane, downstream MAPK are activated as one of the earliest responses and they result in phosphorylation of different regulators of defense-related

genes (Asai *et al*., 2002; Bethke *et al*., 2012; Meng & Zhang, 2013). The best-studied MAP kinases are MPK3, MPK4 and MPK6, but other MAP kinases are also involved in the regulation of plant immunity (Nuhse *et al*., 2000; Asai *et al.*, 2002; Tsuda *et al.*, 2013; Nitta *et al.*, 2014).

The MEKK1 - MKK1/MKK2 - MPK4 cascade is required for the regulation of many flg22-induced genes and it is characterized as a positive regulator of basal resistance against pathogens (Zhang *et al.*, 2012; Dit Frey *et al.*, 2014). MKK1 and MKK2 are two partially redundant MAP kinase kinases that function upstream of MPK4 and they phosphorylate MPK4 to activate it (Gao *et al.*, 2008; Ichimura *et al.*, 2006; Mészáros *et al*., 2006; Qiu *et al*., 2008; Suarez-Rodriguez *et al*., 2006). *mkk1 mkk2* (*mkk1/2*) double mutant has extreme dwarf morphology with premature senescence of cotyledons and first true leaves, and it also shows constitutive *PR* gene expression and increased resistance to virulent pathogens *Pst* DC3000 and *H.a.* Noco2 (Gao *et al.*, 2008; Qiu *et al.*, 2008). SA levels are elevated in *mkk1/2* mutants, but *sid2* can only partially suppress *mkk1/2* phenotype, suggesting that *mkk1/2* immune response is partially dependent on SA (Qiu *et al.*, 2008). MEKK1-MKK1/MKK2-MPK4 cascade negatively regulates defense responses mediated by CNL protein Suppressor of *mkk1 mkk2* 2 (SUMM2). *summ2* mutant allele can fully suppress *mkk1/2* autoimmune phenotype (Zhang *et al*., 2012). *P. syringae* effector HopAI1 was found to inhibit the kinase activity of MPK4, resulting in the activation of SUMM2 (Zhang *et al.*, 2012). Although MPK4 controls the activity of SUMM2, these two proteins do not interact directly. Instead, SUMM2 senses the changes in phosphorylation of the MPK4 substrates calmodulin-binding receptor-like cytoplasmic Kinase 3 (CRCK3) and Protein associated with topoisomerase II 1 (PAT1) (Roux *et al*., 2015; Zhang *et al*., 2017). From the perspective of the Guard hypothesis, we can say that SUMM2 guards MEKK1-MKK1/2-MPK4 cascade and that it is activated when the cascade is disrupted by pathogen activity.

1.5 Research goals

Existence of structurally similar SA receptors functioning in an opposite manner gives us the idea of how SA-mediated signaling is tightly controlled. Until recently, NPR1 was considered to be a major regulator of SA-dependent responses, while NPR3 and NPR4 were thought to control NPR1 stability. Now with a better insight into the function of NPR3/4 we can try to fill in the gaps in our understanding of how SA is involved in different signaling pathways of plant immunity and how NPR3 and NPR4 regulate immunity alongside NPR1. Since SA accumulates during both PTI and ETI, it would be interesting to define the role of different SA receptors in each layer of plant immunity. Furthermore, SA reportedly plays a significant role in the regulation of HR, but the mechanism of that regulation is not clear. The objectives of my thesis are to:

1) Determine whether NPR1 and NPR3/4 are involved in the regulation of PTI and ETI;

2) Establish whether NPR1 and NPR3/4 are required for the responses downstream of both CNL and TNL R proteins;

3) Investigate how NPR1and NPR3/4 are involved in the SA-mediated regulation of HR;

4) Explore whether basal levels of SA contribute to PTI and ETI.

Chapter 2: Methods

2.1 Plant material and growth conditions

In this research, *Arabidopsis thaliana* Columbia ecotype (Col-0) was used as the wild-type. Previously published mutant lines are listed in the **Table 2.1** and they are all in the Col-0 background. Mutants *snc1 npr4-4D* and *snc1 npr1-1 npr4-4D* were generated from the cross of *snc1* with *npr1-1 npr4-4D*. Mutant lines *mkk1 mkk2 npr1-8, mkk1 mkk2 npr4-4D* and *mkk1 mkk2 npr1-8 npr4-4D* were generated in the cross of *mkk1 mkk2* with *npr1-8* (GK-016D02) *npr4-4D*. The primers used for the genotyping by Polymerase Chain Reaction (PCR) are listed in **Table 2.2**.

Mutant name	Reference
eds1	(Parker *et al.*, 1996)
eds5-3	(Nawrath *et al.*, 1999)
mkk1 mkk2	(Gao *et al.*, 2008)
npr1-1	(Cao *et al.*, 1994)
npr1-1 npr4-4D	(Ding *et al.*, 2018)
npr4-4D	(Ding *et al.*, 2018)
snc1	(Li *et al.*, 2001)
snc1 npr1-1	(Li *et al.*, 2001)

Table 2.1 The list of previously published mutants used for this research

Seeds were sterilized in 15% bleach and washed twice in distilled water. After sterilization, seeds were kept at 4°C/48h for stratification and exposed to light for 24h before sowing. Seedlings were grown on soil (Sunshine mix #4, Sun Gro Horticulture, Canada) at 23°C under the long day regime (16h light/8h dark). Plants used for bacterial infection assays and the ion-leakage assay were transplanted in soil after 2-3 weeks and grown at 23°C under short day regime (12h light/12h dark). Seedlings of genotypes Col-0, *snc1*, *snc1 npr1-1*, *snc1 npr4-4D* and

snc1 npr1-1 npr4-4D used for RNA extraction were grown under long day regime at 23°C on half-strength solid Murashige and Skoog (MS) medium (PhytoTech Labs, USA) containing 1% sucrose (Bio Basic, Canada) and 0.6% agar (Bio Basic, Canada) (pH = 5.7).

Mutant	Primer name	Sequence (5' – 3')
npr1-1	npr1-1-WT-F1	GAGGGGATATACGGTGCTAC
	npr1-1-mut-F2	GAGGGGATATACGGTGCTAT
	npr1-1-R1	AGCAGCGTCATCTTCAATTC
npr1-8 (GK-016D02)	GK-016D02(npr1)-F	AGAGGCACTTATTGGACGTTG
	GK-016D02(npr1)-R	GTGCTTCCGTTGGAAAAAGA
	GABI-KAT 8474	ATAATAACGCTGCGGACATCTACATTTT
npr4-4D	npr4-4D-WT-R	ATCGACTAGGTCAATACCAC
	npr4-4D-MT-R	ATCGACTAGGTCAATACCAT
	npr4-4D-F	GCTTCGTAACTATGTTGAGAAG
snc1	SNC1 XbaI NF	ATGAGGTGGAGTTCCCATCTG
	SNC1 XbaI NR	CATCCCATTTTGATTGCTGGAAAG
mkk1-1 (SALK_140054)	mkk1-1 T-DNA F	TCCCTCCTCTTGAGCAATCC
	mkk1-1 T-DNA R	CCGACATGTTGATTCTTCTG
	LBA-1	TGGTTCACGTAGTGGGCCATCG
mkk2-1 (SAIL_511_H01)	mkk2-1 T-DNA-F	CCATCAGTTCCTTTGCAGAG
	mkk2-1 T-DNA-R	GGTGTGAGTACCGTTATGAC
	SAIL-1F	CATTAAAAACGTCCGCAATGTG

Table 2.2 The list of primers used for genotyping by PCR

2.2 Bacterial infection assays

Infection assays were performed on four-week-old plants. Two leaves of each plant were infiltrated using plastic syringe with 10mM $MgCl_2$ bacterial suspensions of *Pst* DC3000 *hrcC*$^-$ (OD_{600} = 0.002), *Pst* DC3000 *avrRpt2* (OD_{600} = 0.0002), or *Pst* DC3000 *avrRrps4* (OD_{600} = 0.005). For protection assay induced by flg22, plants were pretreated with 1μM flg22 or distilled water (mock) 12h before the bacterial infection with *Pst* DC3000 (OD_{600} = 0.0005). One disc (R

= 6mm) was cut from each infiltrated leaf three days post inoculation. The cut tissue was ground and plated on solid LB broth (Miller) (Bio Basic, Canada). For the infection with *Pst* DC3000 *hrcC*$^{-}$, LB medium contained 100µg/mL rifampicin, and for infections with *Pst* DC3000 *avrRpt2* and *Pst* DC3000 *avrRps4* LB medium was supplemented with 100µg/mL rifampicin and 100µg/mL kanamycin. The number of colony forming units (CFU) was counted and bacterial growth was presented as the number of CFU per square centimeter or leaf surface [log(CFU/cm^2)]. As an inoculation control, bacterial growth was also measured for Day 0, when the leaf tissue was harvested immediately after inoculation. Each genotype was represented by 6 to 12 biological replicates, depending on bacterial strain used for infection, with each biological replicate containing two leaf discs from the same plant.

2.3 *H.a.* Noco2 infection assay

To analyze the resistance to *Hyaloperonospora parasitica* Noco2, two-week-old *A. thaliana* plants grown on soil were sprayed with spores at a concentration of 5 x 10^4 spores/mL of water and grown covered with a clear lid at 18°C and 12h light/12h dark photoperiod. Seven days post inoculation 4 - 5 seedlings were harvested as one biological replicate, placed into a tube with distilled water and vortexed thoroughly. The number of spores was counted using hemocytometer and presented as the number of spores per gram of fresh weight [spores (x 10^4) g^{-1}(fw)]. Each genotype was represented with four biological replicates. Statistical differences between the genotypes were analyzed using one-way ANOVA with post hoc Tukey HSD test ($p < 0.01$).

2.4 RNA isolation and quantitative RT-PCR

Ten-day-old plants (Col-0, *snc1*, *snc1 npr1-1*, *snc1 npr4-4D*, *snc1 npr1-1 npr4-4D*) grown on a half-strength MS medium and twenty-day-old plants (Col-0, *mkk1/2*, *mkk1/2 npr1-8*, *mkk1/2 npr4-4D*, *mkk1/2 npr1-8 npr4-4D*) grown on soil were used for RNA extraction with EZ-10 Spin Column Plant RNA Mini-Preps Kit (Biobasic, Canada). Instead of the lysis buffer provided in the kit, 500ml of NucleoZOL (Macherey-Nagel, Germany) per sample was used. The complementary DNA was obtained by reverse transcription using EasyScript Reverse Transcriptase (ABM, Canada). qPCR was performed using Takara SYBR Premix Ex (Clontech, USA), and primers used for this purpose are listed in the **Table 2.3**. Three independent samples represented each genotype. Expression level of *PR1* and *PR2* was normalized to the expression of ACTIN1. One-way ANOVA with post hoc Tukey HSD test ($p < 0.01$) was applied to analyze statistical differences between the genotypes.

Gene accession number	Primer name	Primer sequence 5' – 3'
AT2G14610	PR1 F-2	AGGCAACTGCAGACTCATAC
	PR1 R-2	TTGTTACACCTCACTTTGGC
AT3G57260	PR2-A	GCTTCCTTCTTCAACCACACAGC
	PR2-B	CGTTGATGTACCGGAATCTGAC
AT2G37620	ACTIN1-F	CGATGAAGCTCAATCCAAACGA
	ACTIN1-R	CAGAGTCGAGCACAATACCG

Table 2.3 The list of primers used for qRT-PCR

2.5 Ion-leakage assay

Leaves of four-week-old *A. thaliana* plants were infiltrated using a syringe with 10mM $MgCl_2$ (mock treatment) or *P. syringae* pv. *tomato* DC3000 *avrRpt2* (OD_{600} = 0.02). For each plant, two leaves were infiltrated, and one leaf disk was cut from each leaf immediately after infiltration. The leaf disks were subsequently washed twice in distilled water. Six leaf disks from three plants, representing one biological replicate, were transferred into a 50ml plastic tube containing 20ml of distilled water and electrical conductivity was measured at different time points after infiltration using VWR EC meter (Model 2052). Two-tailed t-test was performed for each time point between wild type (Col-0) and *eds5-3* plants ($p < 0.01$). One-way ANOVA with post hoc Tukey HSD test was performed for each time point to compare the conductivity of Col-0, *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* mutants ($p < 0.01$).

Chapter 3: The role of NPR1 and NPR3/4 in PTI and ETI

3.1 Results

3.1.1 The role of NPR1 and NPR3/4 in PTI

3.1.1.1 *npr1-1 npr4-4D* has impaired flg22-triggered protection against *Pst* DC3000

To test whether SA receptors NPR1 and NPR3/4 regulate PTI, I pre-treated Col-0, *npr1-1*, *npr4-4D*, *npr1-1 npr4-4D*, and *eds5-3* plants with flg22 to trigger the PTI response, and afterwards I challenged them with the virulent pathogen strain *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). I used *eds5-3* mutant to investigate whether basal levels of SA influence the PTI. The goal was to compare bacterial growth in *eds5-3*, a mutant impaired in pathogen-induced SA accumulation, and *npr1-1 npr4-4D*, a mutant defective in SA-perception. The results show that flg22-treated *npr1-1* and *npr4-4D* are more susceptible to *Pst* DC3000 than the Col-0 (**Figure 3.1**). This suggests that *npr1-1* and *npr4-4D* have reduced flg22-triggered PTI response. Furthermore, the double mutant *npr1-1 npr4-4D* shows even more bacterial growth than both *npr1-1* and *npr4-4D*, demonstrating the additive effect of these two mutant alleles. While there is no significant difference between any lines pre-treated with H_2O, pre-treatment with flg22 significantly reduced bacterial growth in Col-0, *npr1-1* and *npr4-4D* plants. On the other hand, *npr1-1 npr4-4D* pre-treated with H_2O or flg22 showed similar growth of *Pst* DC3000, implying that NPR1 and NPR3/4 are together required for the successful activation of PTI upon the recognition of flg22. There is no significant difference between *eds5-3* plants pre-treated with H_2O and flg22. Moreover, bacterial growth in *eds5-3* plants is comparable to that of *npr1-1 npr4-4D* plants. This finding suggests that the basal levels of SA accumulated in *eds5-3* have no effect on flg22-triggered PTI.

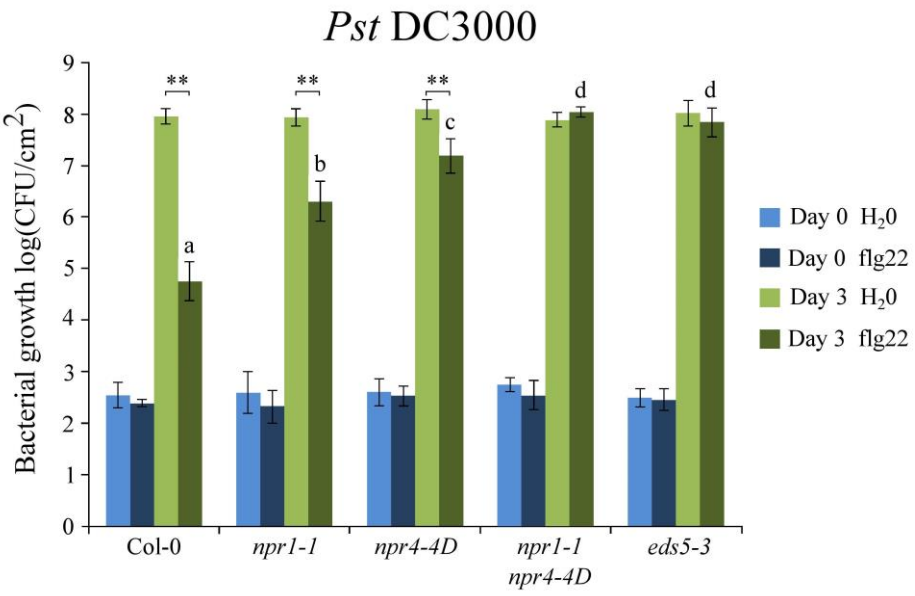

Figure 3.1 Growth of *Pst* DC3000 on Col-0 (WT), *npr1-1*, *npr4-4D*, *npr1-1 npr4-4D* and *eds5-3* pretreated with flg22/H_2O

Leaves of four-week-old plants were infiltrated with 1µM flg22/distilled H_2O 12h prior to the infection with bacterial suspension of *Pst* DC3000 (OD_{600}=0.0005). Bacterial growth is presented as the number of colony-forming units (CFU) per square centimeter of the leaf surface. Each bar represents mean ± SD of 8 - 10 biological replicates. Different letters (a, b, c, d) mark statistical differences between the tested genotypes on Day3 flg22. There are no statistically significant differences between the genotypes on Day 0 H_2O, Day 0 flg22 and Day3 H_2O (One-way ANOVA with post hoc Tukey HSD test, $p < 0.01$). Two-tailed t-test was performed to analyze differences between Day3 H_2O and Day3 flg22 (**$p < 0.01$).

3.1.1.2 *npr1-1 npr4-4D* mutant is more susceptible to *Pst* DC3000 *hrcC*$^-$ than *eds5-3*

To further analyze the requirement of NPR1 and NPR3/4 in PTI, I treated plants with *P. syringae* pv. *tomato* DC3000 *hrcC*$^-$ *(Pst* DC3000 *hrcC*$^-$*).* This strain carries a mutation in the gene encoding a structural component of bacterial Type III Secretion System (T3SS) (Roine *et al*., 1997). Because of dysfunctional T3SS, bacteria are incapable of delivering effectors into the plant cell, so the only way for plant to recognize the presence of the pathogen is through detection of PAMPs. Consequently, infection with *Pst* DC3000 *hrcC*$^-$ can only trigger the PTI, but not the ETI response, which makes it a good tool for PTI analysis.

The results presented in **Figure 3.2** show that *npr1-1*, *npr4-4D* and *eds5-3* support similar growth of *Pst* DC3000 *hrcC*⁻ as Col-0 (WT), while *npr1-1 npr4-4D* double mutant exhibits the highest bacterial growth. This data confirms previously reported additive effect of *npr1-1* and *npr4-4D* on plants susceptibility to this pathogen strain (Ding *et al.*, 2018). However, Ding *et al*. also reported that single mutants *npr1-1* and *npr4-4D* were significantly more susceptible than the wild type plants. Unlike in the **Figure 3.1**, there is a notable difference in bacterial growth between *npr1-1 npr4-4D* and *eds5-3*. The double mutant *npr1-1 npr4-4D* is more susceptible than *eds5-3*, pointing to a weaker PTI in *npr1-1 npr4-4D*. It seems that basal levels of SA accumulated in *eds5-3* mutant are sufficient to trigger PTI response, whereas *npr1-1npr4-4D* is incapable of responding to pathogen-induced SA accumulation.

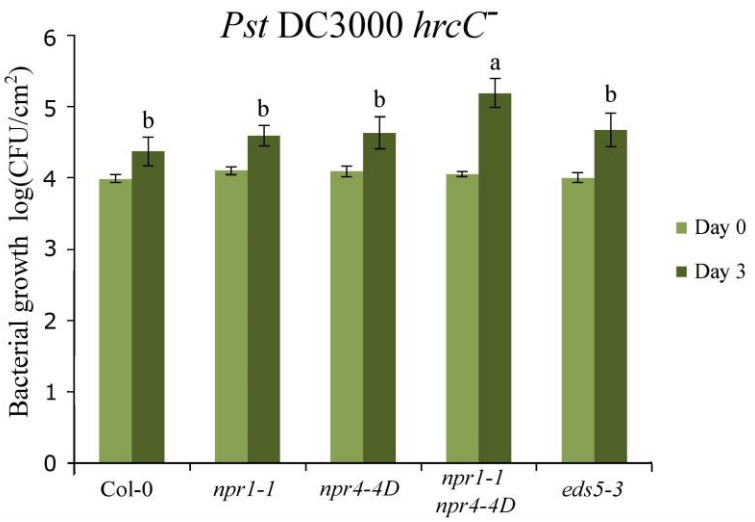

Figure 3.2 Growth of *Pst* DC3000 *hrcC*⁻ on Col-0 (WT), *npr1-1*, *npr4-4D*, *npr1-1 npr4-4D* and *eds5-3*
Leaves of four-week-old plants were infiltrated with bacterial suspension (OD_{600}=0.002). Bacterial growth is presented as the number of colony-forming units (CFU) per square centimeter of the leaf surface. Each bar represents mean ± SD of 6 biological replicates. Different letters (a, b) mark statistical differences between tested genotypes on Day 3 (One-way ANOVA with post hoc Tukey HSD test, $p < 0.01$). There is no significant difference between the genotypes on Day 0.

3.1.2 The role of NPR1 and NPR3/4 in ETI

To analyze the role of NPR1 and NPR3/4 in the regulation of ETI, I tested how defense responses mediated by two different TNL and CNL proteins are affected by *npr1-1* and *npr4-4D* mutant alleles, and how these mutations influence HR response triggered by an avirulent pathogen.

3.1.2.1 ETI activated by RPS4 or RPS2 is weaker in *npr1-1 npr4-4D* than in *eds5-3*

In this experiment, my goal was to analyze whether SA receptors NPR1 and NPR3/4 were equally important for ETI mediated by both TNL and CNL proteins, and whether basal levels of SA could contribute to ETI response. To activate the desired ETI pathway, I challenged the plants with bacterial strains carrying different effector proteins. *Pst* DC3000 *avrRps4* strain carries an avirulence gene, which encodes the effector protein AvrRps4. This effector triggers ETI response mediated by the *A. thaliana* protein RPS4, a TNL-type R protein (Gassmann *et al.*, 1999). On the other hand, *Pst* DC3000 *avrRpt2* carries an avirulence gene encoding the AvrRpt2 that is recognized by the CNL protein RPS2 (Mackey *et al*., 2003).

All tested mutants showed higher growth of *Pst* DC3000 *avrRps4* compared to Col-0 (**Figure 3.3**). The additive effect of *npr1-1* and *npr4-4D* on bacterial growth implies that NPR1 and NPR3/4 work in parallel pathways to regulate ETI triggered by RPS4. *npr1-1 npr4-4D* plants are more susceptible than *eds5-3*, suggesting that basal levels of SA that are accumulated independently of EDS5 have a role in TNL mediated ETI. As a positive control, I used *eds1*, because EDS1 protein is required for TNL-dependent signaling. The double mutant *npr1-1 npr4-4D* exhibits lower bacterial growth than *eds1*. This finding indicates that although SA receptors are important for TNL-mediated ETI, a SA-independent signaling reliant on EDS1 may exist.

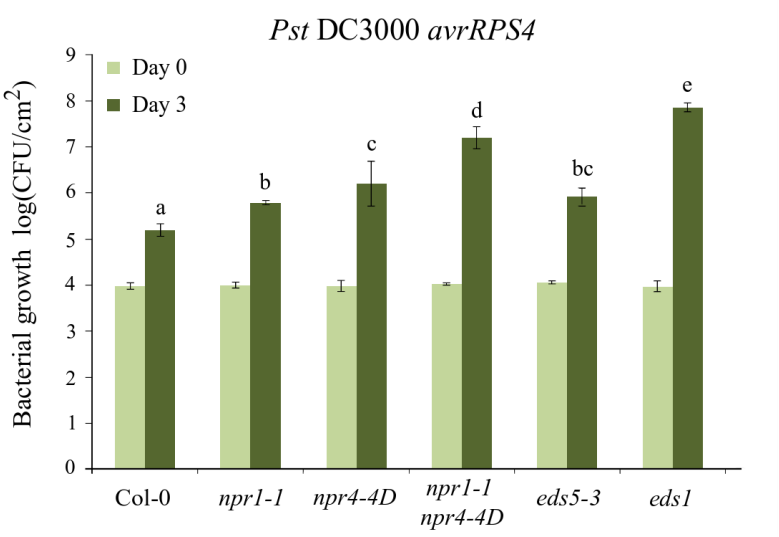

Figure 3.3 Growth of *Pst* DC3000 *avrRps4* on Col-0 (WT), *npr1-1*, *npr4-4D*, *npr1-1 npr4-4D*, *eds5-3*, and *eds1*
Leaves of four-week-old plants were infiltrated with bacterial suspension (OD_{600}=0.005). Bacterial growth is presented as the number of colony-forming units (CFU) per square centimeter of the leaf surface. Each bar represents mean ± SD of 8 - 10 biological replicates. Different letters (a, b, c, d, e) mark statistical differences between tested genotypes on Day 3 (One-way ANOVA with post hoc Tukey HSD test, $p < 0.01$). There is no significant difference between the genotypes on Day 0.

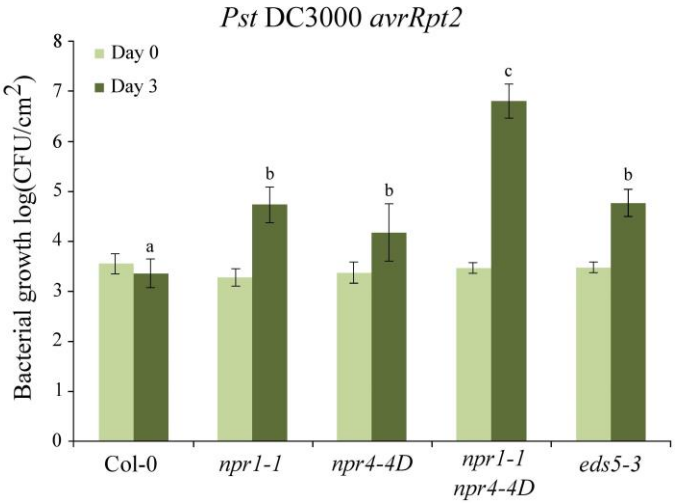

Figure 3.4 Growth of *Pst* DC3000 *avrRpt2* on Col-0 (WT), *npr1-1*, *npr4-4D*, *npr1-1 npr4-4D* and *eds5-3*
Leaves of four-week-old plants were infiltrated with bacterial suspension (OD_{600}=0.0002). Bacterial growth is presented as the number of colony-forming units (CFU) per square centimeter of the leaf surface. Each bar represents mean ± SD of 8 - 10 biological replicates. Different letters (a, b, c) mark statistical differences between tested genotypes on Day 3 (One-way ANOVA with post hoc Tukey HSD test, $p < 0.01$). There is no significant difference between the genotypes on Day 0.

The results of the infection with *Pst* DC3000 *avrRpt2* show that *npr1-1 npr4-4D* has more bacterial growth than the *npr1-1* and *npr4-4D* single mutants, demonstrating the additive effect of these two mutant alleles on bacterial growth (**Figure 3.4**). From this result, we propose that NPR1 or NPR3/4 pathway alone can trigger only weak ETI responses mediated by the CNL protein RPS2, but both pathways together are required for the activation of the full ETI. Furthermore, the susceptibility of *eds5-3* is comparable to that of *npr1-1* and *npr4-4D* plants and it is significantly lower than *npr1-1 npr4-4D*, suggesting that basal levels of SA might be able to bind to NPR1 and NPR3/4 and contribute to ETI mediated by CNL protein RPS2.

Collectively, these results suggest that the NPR1 and NPR3/4-dependent pathway function together in ETI activated by TNL protein RPS4 and CNL protein RPS2. Moreover, it seems likely that EDS5 independent SA is able to stimulate ETI by regulation of NPR1 and NPR3/4 activity.

3.1.2.2 *npr1-1* and *npr4-4D* partially suppress *snc1* autoimmune phenotype

To further analyze the dependence of TNL mediated immunity on NPR1 and NPR3/4, I tested whether NPR1 and NPR3/4 are required for the signaling downstream of TNL protein SNC1. For that purpose, I examined whether *npr1-1* and *npr4-4D* mutations could suppress the *snc1* autoimmune phenotype. From the cross between *snc1* and *npr1-1 npr4-4D*, I obtained two new mutant lines: *snc1 npr4-4D* and *snc1 npr1-1 npr4-4D*. As previously described by Li *et al*. (2001), *snc1* and *snc1 npr1-1* plants are dwarfed, with curly leaves, they have constitutively activated expression of *PR2*, and they exhibit strong resistance to oomycete pathogen *H.a.* Noco2.

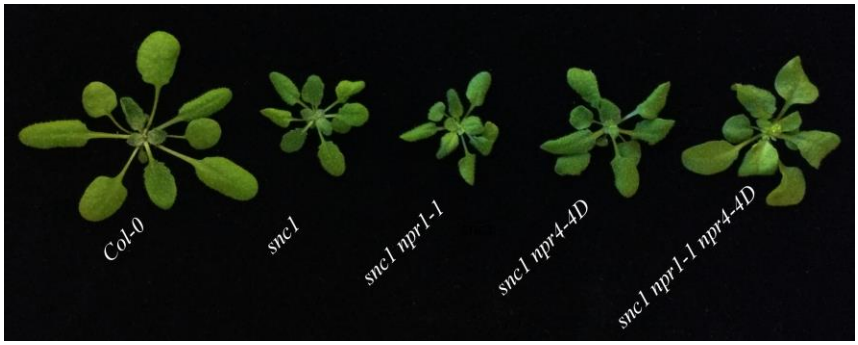

Figure 3.5 Morphology of Col-0 (WT), *snc1*, *snc1 npr1-1*, *snc1 npr4-4D* and *snc1 npr1-1 npr4-4D* plants
Four-week-old plants grown on soil at 23°C and 16h light / 8h dark photoperiod.

From the **Figure 3.5** we can see that *snc1 npr4-4D* plants are still slightly dwarfed, with the rosette larger than of *snc1* and *snc1 npr1-1*, but with wider, curly leaves. Furthermore, *snc1 npr4-4D* plants exhibit early flowering phenotype, similarly to *npr4-4D* plants (Ding *et al*., 2018). The triple mutant *snc1 npr1-1 npr4-4D* has a rosette that is still not comparable to the WT in size, with wide curly leaves that are a distinctive characteristic of autoimmune mutants. In addition to that, early flowering phenotype is present in *snc1 npr1-1 npr4-4D* plants similarly to *snc1 npr4-4D* mutant. The finding that *npr1-1* and *npr4-4D* can only partially suppress the *snc1* morphology phenotype and that *snc1 npr1-1 npr4-4D* plants are bigger than both *snc1 npr1-1* and *snc1 npr4-4D* plants implies that NPR1 and NPR3/4 together contribute to the dwarf morphology of *snc1*, but they are not fully responsible for all the characteristics of the *snc1* morphology.

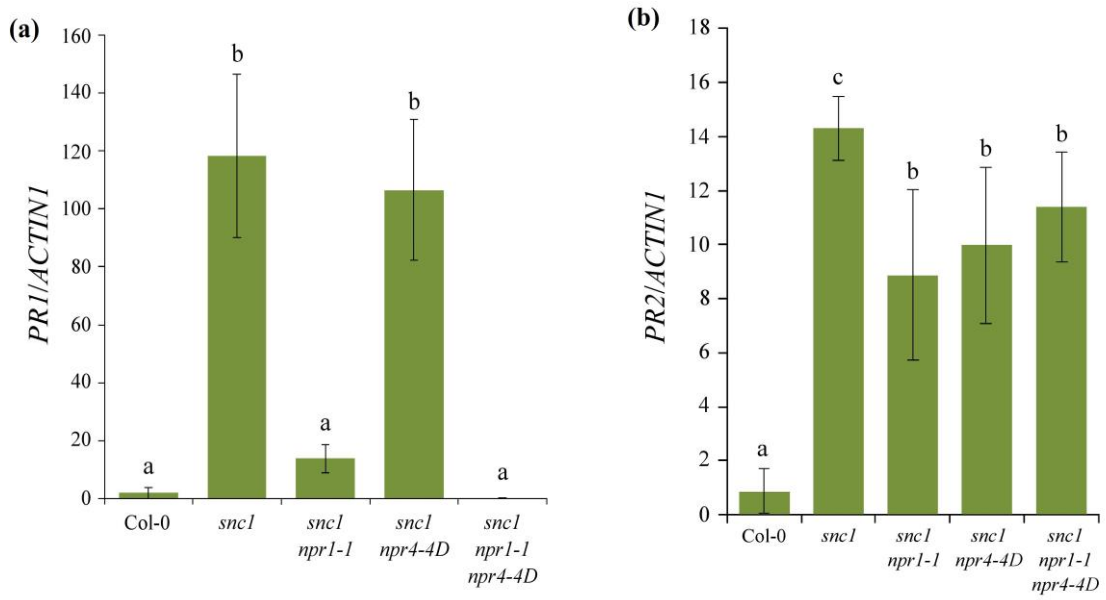

Figure 3.6 Relative expression of *PR1* and *PR2* in Col-0 (WT), *snc1*, *snc1 npr1-1*, *snc1 npr4-4D*, and *snc1 npr1-1 npr4-4D*

Twelve-day-old plants grown on solid half-strength MS medium were sampled for RNA extraction and consequent qRT-PCR analysis: **(a)** expression of *PR1* and **(b)** *PR2* normalized to the expression of *ACTIN1*. Each bar represents mean ± SD of three biological replicates. Different letters (a, b, c) mark significant differences between the groups (One-way ANOVA with post hoc Tukey HSD test, $p < 0.01$).

Next, I analyzed the expression of *PR1* and *PR2* in the mutants compared to WT. The qRT-PCR analysis shows that the *snc1 npr1-1* mutant has *PR1* transcript levels comparable to WT, while *snc1 npr4-4D* does not show significant suppression of constitutively expressed *PR1* compared to *snc1* (**Figure 3.6 (a)**). It seems that NPR3 and NPR4 as negative regulators have a weaker effect on *PR1* expression than the positive regulator NPR1. The triple mutant *snc1 npr1-1 npr4-4D* has *PR1* expression comparable to WT, implying that *npr1-1* and *npr4-4D* together fully suppress constitutively activated *PR1* expression in *snc1*. On the other hand, the results in the **Figure 3.6 (b)** show that *snc1 npr1*, *snc1 npr4-4D* and *snc1 npr1-1 npr4-4D* have partially suppressed *PR2* expression compared to *snc1*, but there is no significant difference among these

three mutants. This result suggests that *PR2* expression is not highly dependent on NPR1 and NPR3/4.

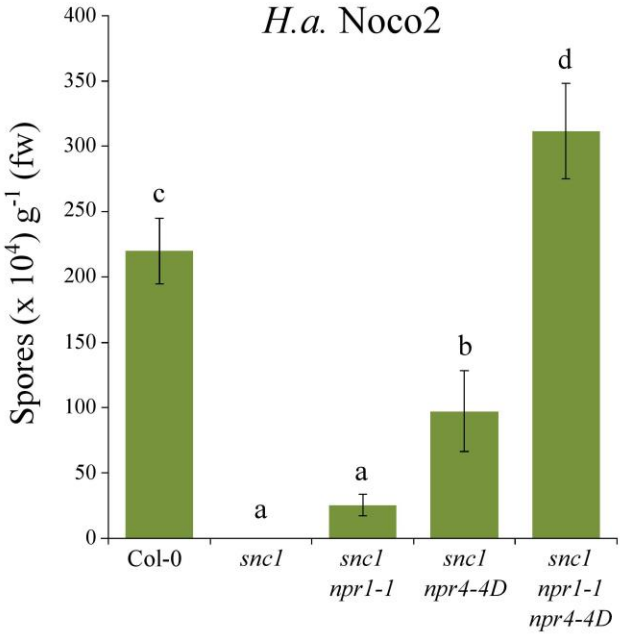

Figure 3.7 Growth of *H.a.* Noco2 on Col-0 (WT), *snc1*, *snc1 npr1-1*, *snc1 npr4-4D* and *snc1 npr1-1 npr4-4D*
Two-week-old plants were sprayed with *H.a.* Noco2 spores (5 x 10^4 spores/mL) and the number of spores per gram of fresh weight (fw) was determined 7 days after the treatment. Bars represent mean ± SD of 4 biological replicates. Statistical differences between the genotypes are determined by one-way ANOVA with post hoc Tukey HSD test ($p < 0.01$) and significant differences are labeled with different letters (a, b, c, d).

Finally, the results of infection with *H.a.* Noco2 demonstrate that *npr1-1* and *npr4-4D* alone cannot fully suppress the elevated resistance of *snc1* plants (**Figure 3.7**). *snc1 npr4-4D* mutant exhibits significant increase in number of spores, while *snc1 npr1-1* is as susceptible to *H.a.* Noco2 as *snc1*. However, in *snc1 npr1-1 npr4-4D* the number of spores exceeds the levels of Col-0, indicating that the two defense pathways specified by SA receptors NPR1 and NPR3/4 are both required for the full establishment of constitutively activated resistance of *snc1* plants to *H.a.* Noco2.

Some aspects of *snc1* autoimmunity, such as *PR1* expression and resistance to *H.a.* Noco2, fully rely on both NPR1 and NPR3/4, while the expression of *PR2* remains elevated regardless of the SA receptors. Furthermore, the morphological features of *snc1 npr1-1 npr4-4D* distinct from the WT plants imply that perception of SA by NPR1 and NPR3/4 is not the only factor that determines the *snc1* autoimmune morphology. Collectively, these results indicate that NPR1 and NPR3/4 are not fully responsible for the signaling pathway downstream of TNL protein SNC1.

3.1.2.3 Perception of SA by NPR1 and NPR4 is partially required for *mkk1/2* autoimmune phenotype

To analyze the involvement of NPR1 and NPR3/4 in CNL-activated ETI, I focused on defense responses triggered by SUMM2. For that purpose, I used *mkk1/2* mutant because it has constitutively activated SUMM2-mediated ETI. *mkk1/2* plants are extremely dwarfed, with elevated expression of *PR* genes and high resistance to *H.a.* Noco2. To investigate to what extent SA receptors are involved in the pathway downstream of SUMM2, I tested whether *npr1-8* and *npr4-4D* mutations could suppress the extreme autoimmune phenotype of *mkk1/2* plants. To obtain the necessary mutant lines, *npr1-8 npr4-4D* plants were crossed with *mkk1/2.*

As shown below (**Figure 3.8**), *mkk1/2 npr1-8* plants are slightly larger than *mkk1/2* and *mkk1/2 npr4-4D*, they have less curly leaves and they show bleaching in the center of the rosette. On the other hand, *mkk1/2 npr4-4D* plants have very similar phenotype as *mkk1/2* plants, but are slightly larger than *mkk1/2*. The quadruple mutant *mkk1/2 npr1-8 npr4-4D* is larger than both triple mutants, but its size and morphology are still not comparable to the WT and, similar to *mkk1/2 npr1-8* plants, there is bleaching of the tissue in the center of the rosette. Thus, *npr1-8*

and *npr4-4D* can only partially suppress the extreme dwarfism of *mkk1/2* plants, and once again, there is an additive effect of these two mutations on the suppression of dwarf phenotype.

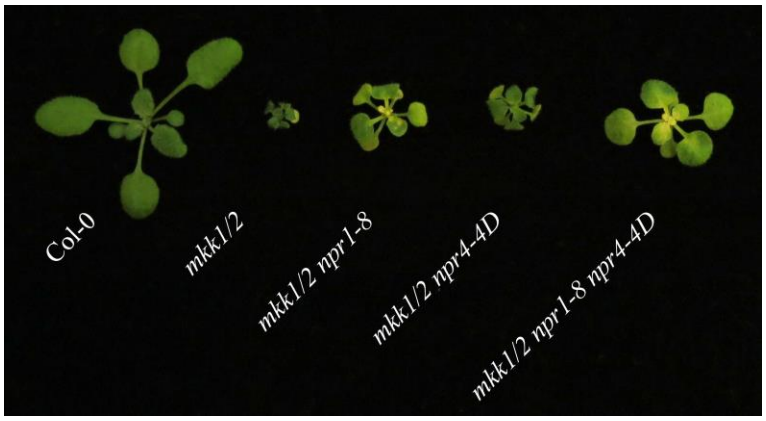

Figure 3.8 Morphology of Col-0 (WT), *mkk1/2*, *mkk1/2 npr1-8*, *mkk1/2 npr4-4D* and *mkk1/2 npr1-8 npr4-4D* plants
Four-week-old plants grown on soil at 23°C and 16h light / 8h dark photoperiod.

Since *mkk1/2* plants exhibit constitutively induced transcription of *PR* genes, I analyzed the expression of *PR1* and *PR2* in these mutants to see whether it is dependent on NPR1 and NPR3/4. The results of quantitative RT-PCR show that the triple mutant *mkk1/2 npr1-8* and the quadruple mutant *mkk1/2 npr1-8 npr4-4D* have the amount of *PR1* transcript comparable to Col-0 levels (**Figure 3.9 (a)**). Although we could not show statistically significant difference between *mkk1/2 npr1-8* and *mkk1/2 npr1-8 npr4-4D*, the quadruple mutant repeatedly showed lower *PR1* expression than the triple mutant did, and it was more similar to the Col-0 expression levels. Comparing the two triple mutants, there is a strong suppression of *PR1* in *mkk1/2 npr1-8*, while in *mkk1/2 npr4-4D* plants there is no suppression. Surprisingly, *mkk1/2 npr4-4D* exhibits higher expression of *PR1* compared to *mkk1/2*. This result suggests that positive regulatory role of

NPR1 plays a more significant role in the regulation of *PR1* expression compared to the negative regulators NPR3/4.

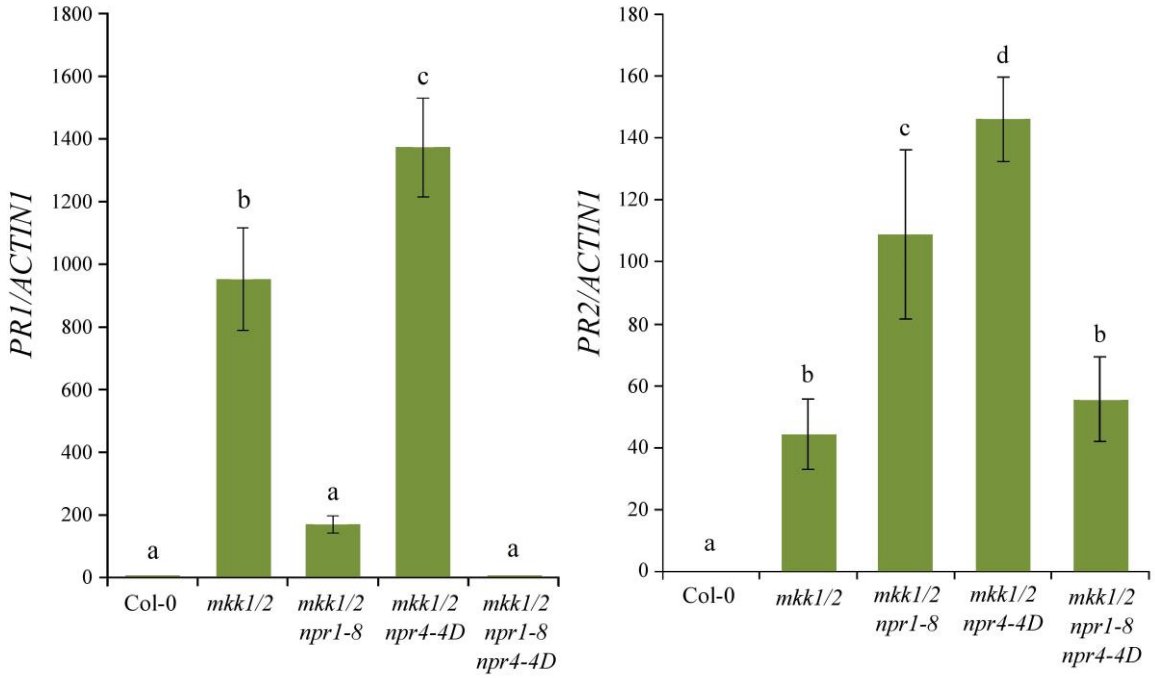

Figure 3.9 Relative expression of *PR1* and *PR2* in Col-0 (WT), *mkk1/2*, *mkk1/2 npr1-8*, *mkk1/2 npr4-4D* and *mkk1/2 npr1-8 npr4-4D*

Three-week-old plants grown on soil were sampled for RNA extraction and consequent qRT-PCR analysis: **(a)** expression of *PR1* and **(b)** *PR2* normalized to the expression of ACTIN1. Each bar represents mean ± SD of three biological replicates. Different letters (a, b, c) mark significant differences between the groups (One-way ANOVA with post hoc Tukey HSD test, $p < 0.01$).

With respect to the *PR2* gene (**Figure 3.9 (b)**), both *mkk1/2 npr1-8* and *mkk1/2 npr4-4D* show significantly higher expression compared to the *mkk1/2* and Col-0, suggesting that NPR1 and NPR3/4 negatively regulate *PR2* expression. In that case, we would expect to see even higher expression in the quadruple mutant, according to the model of Ding *et al.* (2018). However, *mkk1/2 npr1-8 npr4-4D* has transcript level similar to *mkk1/2*. *npr1-1* and *npr4-4D*

cannot suppress *PR2* expression in *mkk1/2*, but it is not clear how they are really affecting it and what the mechanism could be.

3.1.2.4 NPR1 and NPR3/4 negatively regulate AvrRpt2-induced HR

To further test the role of NPR1 and NPR3/4 in ETI response, I examined how these receptors regulate HR response. The ion-leakage assay was performed to quantify cell death in response to infection with the avirulent pathogen *Pst* DC3000 *avrRpt2* (**Figure 3.10 (a)**). This method is based on measuring electrical conductivity of the aqueous solution containing infected leaf tissue. Conductivity of the solution is proportional to the number of ions released from dead leaf cells. The results show that the conductivity of *npr1-1* and *npr4-4D* mutants is comparable to the wild-type, whereas the double mutant has notably higher conductivity, implying that NPR1 and NPR3/4 play a redundant role in the negative regulation of HR induced by the effector protein AvrRpt2. This finding suggests that SA is a negative regulator of HR. In order to confirm this, I tested the ion-leakage in *eds5-3* plants, which accumulate only basal levels of SA. The results show that *eds5-3* plants exhibit more cell death than Col-0 (**Figure 3.10 (b)**).

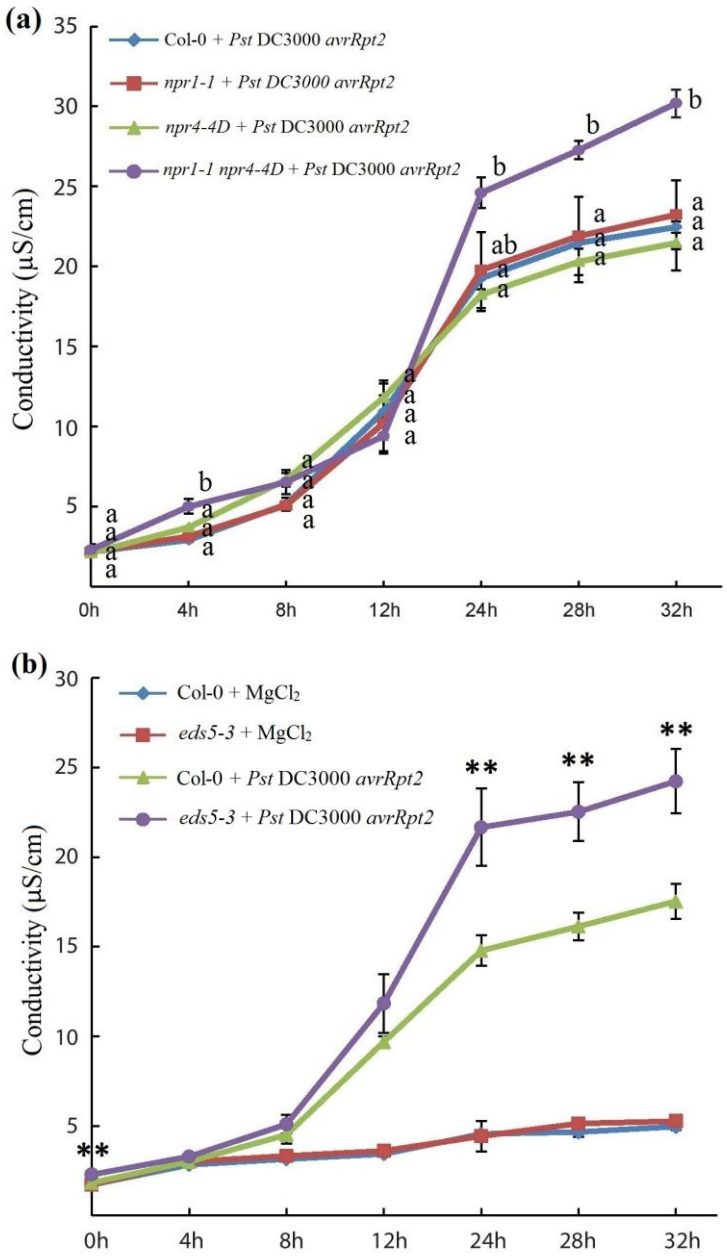

Figure 3.10 Analyses of ion leakage in Col-0 (WT), *eds5-3*, *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* plants treated with *Pst* DC3000 *avrRpt2*

Leaves of four-week-old plants were treated with *Pst* DC3000 *avrRpt2* (OD_{600} = 0.02) or mock (10mM $MgCl_2$). Electrical conductivity was measured at different time points post infiltration. Each data point on the graph represents the mean ± SD of three biological replicates. In (a), one-way ANOVA with post hoc Tukey HSD test was performed for each time point. Different letters (a,b) indicate statistically significant differences between the samples ($p < 0.01$). In (b), two-tailed t-test was performed for each time point between wild type (Col-0) and *eds5-3* plants treated with *Pst* DC3000 *avrRpt2* (**$p < 0.01$) (Radojičić *et al*., 2018).

3.2 Discussion

3.2.1 NPR1 and NPR3/4 are positive regulators of PTI and they are able to respond to basal levels of SA to enhance PTI

Infection assays performed with H2O/flg22 + *Pst* DC3000 (**Figure 3.1**) and *Pst* DC3000 *hrcC*$^{-}$ (**Figure 3.2**) suggest that PTI is regulated through NPR1 and NPR3/4-dependant signaling. In both experiments the double mutant *npr1-1 npr4-4D* exhibited lower resistance compared to the single mutants *npr1-1* and *npr4-4D*. The protection assay (**Figure 3.1**) clearly showed that *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* had reduced flg22-triggered protection against *Pst* DC3000, with double mutant being insensitive to flg22 treatment. This implies that NPR1- and NPR3/4-dependent signaling is important for flg22-triggered PTI. We see a similar trend in infection with *Pst* DC3000 *hrcC*$^{-}$. In line with the previously published results of Ding *et al*. (2018), the double mutant showed the highest bacterial growth, further supporting that both NPR1- and NPR3/4-dependant SA signaling is important for PTI response.

While both experiments show similar results in terms of NPR1 and NPR3/4 involvement in regulation of PTI, there is some difference regarding the potential role of basal SA levels. In the *Pst* DC3000 *hrcC*$^{-}$ infection assay (**Figure 3.2**), *eds5-3* plants showed 10 times lower bacterial growth compared to *npr1-1 npr4-4D*, suggesting that NPR1 and NPR3/4 in *eds5-3* mutant are able to bind basal SA and contribute to PTI response. However, the protection assay (**Figure 3.1**) showed no significant difference in bacterial growth between *eds5-3* and *npr1-1 npr4-4D*. A possible explanation for this discrepancy is the difference in virulence between the two bacterial strains. *Pst* DC3000 *hrcC*$^{-}$ is unable to interfere with plant defense, whereas *Pst* DC3000 delivers effector proteins that inhibit PTI, enabling bacterial proliferation to higher concentrations, which may overshadow the positive effect of basal SA on PTI in the observed

mutants. It is possible that for the same reason no significant differences in *Pst* DC3000 growth were observed between Col-0 and the mutants pretreated with H2O.

Previous studies have quantified the binding affinities of SA receptors for SA (Wu *et al*., 2012; Fu *et al*., 2012; Manohar *et al*., 2015; Ding *et al*., 2018). The binding affinity of NPR4 (K_d = 23.54 ± 2.743) is higher compared to NPR1 (K_d = 223.1 ± 38.85 nM) and NPR3 (K_d = 176.7 ± 28.31 nM). It was suggested that the affinities of all these three proteins may be lower than the molar concentration of basal free SA (~1.4 µM) (Ding *et al*., 2018). However, we cannot claim with confidence the molar concentration of SA inside Arabidopsis cells (especially in different subcellular compartments) and the *in vivo* from *in vitro* binding affinities of SA receptors could also be different. Nevertheless, defense related genes controlled by NPR1 and NPR3/4 are not upregulated in the absence of a pathogen, suggesting that SA receptors are not responding to basal SA (Ding *et al*., 2018). On the other hand, my data suggests that upon pathogen attack SA receptors could be responding to basal levels of SA. It is possible that certain protein modifications resulting from initial pathogen attack enable NPR1/3/4 to bind basal SA. It was previously shown that, as a result of redox changes induced by the pathogen-induced SA accumulation, NPR1 switches from oligomeric to monomeric form and it is translocated from the cytoplasm to the nucleus to regulate expression of defense related genes (Mou *et al*., 2003). However, the precise mechanism of this selective response to basal SA remains to be elucidated.

3.2.2 NPR1 and NPR4 regulate CNL- and TNL-mediated ETI and basal levels of SA are used to promote ETI response

Results of infection assays with *Pst* DC3000 carrying avirulence genes *avrRps4* (**Figure 3.3**) or *avrRpt2* (**Figure 3.4**) were similar regarding the role of NPR1 and NPR4 in regulation of

ETI. In both experiments the double mutant *npr1-1 npr4-4D* was notably more susceptible than single mutants *npr1-1* and *npr4-4D,* supporting the model in which NPR1 and NPR4 function in parallel pathways to positively regulate plant's defense.

When treated with *Pst* DC3000 *avrRps4*, *npr1-1 npr4-4D* plants exhibited approximately 100 times higher bacterial growth compared to Col-0 plants (**Figure 3.3**). However, *eds1* showed even higher bacterial growth, implying that despite the notable contribution of SA receptors to signaling downstream of RPS4, a SA-independent pathway also exists. This result supports the previously established model of Zhang *et al*. (2003), where signaling downstream of TNL protein SNC1 was fully dependent on EDS1, but partially dependent on EDS5 (**Figure 1.2**). In line with that model, *eds5-3* plants showed reduced growth of *Pst* DC3000 *avrRps4* compared to *eds1*. However, we also detected higher resistance of *eds5-3* compared to *npr1-1 npr4-4D,* which suggests that basal SA significantly contributes to RPS4-mediated defense response. Infection assay with *Pst* DC3000 *avrRpt2* further supported the positive regulatory role of NPR1 and NPR4 in ETI response mediated by CNL protein RPS2 (**Figure 3.4**). Once again, the *eds5-3* mutant exhibited considerably higher resistance compared *npr1-1 npr4-4D,* suggesting that basal levels of SA also contribute to RPS2-mediated defense response. Whether CNL-mediated ETI is partially or fully dependent on SA signaling remains to be determined.

The results of these two infection assays show that NPR1- and NPR4-dependent signaling pathways together regulate ETI activated by both RPS4 and RPS2, the representatives of TNL and CNL R proteins, respectively. While the upstream parts of the ETI signaling transduction vary, such as R proteins themselves (TNLs versus CNLs) and their proximate downstream signaling components (EDS1 versus NDR1), elements further downstream in the signaling

pathways share similar components, in this case NPR1 and NPR4, which regulate similar subsets of genes to regulate ETI (Ding *et al*., 2018).

Similar to the previously demonstrated role of basal SA in PTI response, results from infections with avirulent strains demonstrate the positive impact of basal SA on ETI response. One could argue that the differences observed between *eds5-3* and *npr1-1 npr4-4D* are not due to the effect of basal SA on ETI, since infection with avirulent pathogen does not activate ETI exclusively. However, PTI is a much weaker response than ETI and it is most likely disrupted by effectors which the plant was not able to detect. Thus, it can be assumed that the response we observed in infection experiments using *Pst* DC3000 *avrRpt2* and *Pst* DC3000 *avrRps4* is predominantly due to effects on ETI. Nevertheless, these results clearly demonstrate the significance of basal SA for the protection against these avirulent pathogen strains.

3.2.3 Perception of SA by NPR1 and NPR4 is partially required for SNC1-mediated ETI

Analysis of the combined mutants between *snc1*, *npr1-1* and *npr4-4D* showed that *npr1-1* and *npr4-4D* only partially suppress *snc1* autoimmune phenotype. The dwarf phenotype is not fully restored to wild-type size in *snc1 npr1-1 npr4-4D* plants, suggesting that there is NPR1- and NPR4-independent regulation of autoimmunity in *snc1* as indicated by the impaired growth (**Figure 3.5**). This result supports previously reported partial suppression of *snc1* growth by *eds5-3*, which already suggested the existence of SA-independent signaling downstream of SNC1 (Zhang *et al*., 2003). While the *snc1 npr4-4D* double mutant is larger compared to *snc1* plants, *snc1 npr1-1* is slightly smaller than *snc1*. This might be caused by the higher amount of SA accumulated in *snc1 npr1-1* plants (Li *et al*., 2001; Zhang *et al*., 2003). Interestingly, the joint effect of *npr1-1* and *npr4-4D* has a stronger positive impact on growth than *npr4-4D* alone,

indicated by the triple mutant being larger than *snc1 npr4-4D*. This result suggests that plant growth in *snc1* mutant is partially inhibited through activation of both NPR1- and NPR4-mediated pathways. It was previously reported that genes involved in auxin and gibberellic acid signaling transduction were downregulated when plants were treated with BTH (Wang *et al*., 2006; Wang *et al*., 2007). It was further shown that upon SA treatment different genes involved in photosynthesis were repressed (Ding *et al*., 2018). Moreover, dwarf phenotype of *constitutive expresser of pathogenesis related proteins 1 (cpr1), cpr6, cpr5,* and *dnd1* (mutants with elevated SA) is partially suppressed under conditions of high light, showing that impaired growth is at least partially caused by reduced photosynthetic performance of these mutants (Mateo *et al*., 2006). Thus, a likely scenario is that NPR1 and NPR4 respond to high levels of SA in *snc1* plants and suppress transcription of genes responsible for photosynthetic activity and growth hormone signaling, resulting in stunted plant growth. Nonetheless, SA-independent pathways that contribute to dwarfism in *snc1* plants remain to be characterized.

The constitutive expression of *PR1* is suppressed to wild-type levels in *snc1 npr1-1* and *snc1 npr1-1 npr4-4D* (**Figure 3.6 (a)**). However, *npr4-4D* alone did not significantly suppress the constitutive *PR1* expression of *snc1,* suggesting that NPR1 plays a major role in the regulation of *PR1* transcription. As NPR1 is the key regulator of *PR1* expression, thus it is not surprising that it has such a prominent impact on *PR1* expression during TNL-mediated ETI (Cao *et al*., 1994; Delaney *et al*., 1995; Rochon *et al*., 2006; Boyle *et al*., 2009). Although there are no statistically significant differences between Col-0, *snc1 npr1-1* and *snc1 npr1-1 npr-4D,* higher suppression of *PR1* transcription is consistently observed in the triple mutant compared to *snc1 npr1-1*, suggesting that NPR4 is also involved in the regulation of *PR1*. This result supports the fact that *PR1* expression is constitutively elevated in the *npr3-1 npr4-3* double knock out

mutant (Zhang *et al*., 2006). Therefore, we can conclude that *PR1* expression activated during SNC1-mediated ETI is fully dependent on NPR1- and NPR3/4-mediated signaling.

The constitutive expression of *PR2* is partially suppressed in *snc1 npr1-1*, *snc1 npr4-4D* and *snc1 npr1-1 npr4-4D*, however, there is no significant difference between the double and triple mutants (**Figure 3.6 (b)**). While results of Ding *et al*. (2018) showed that *PR2* expression induced by *Psm* ES4326 was fully suppressed in *npr1-1 npr4-4D* mutant compared to Col-0, in *snc1* background the same additive effect of *npr1-1* and *npr4-4D* mutant alleles was not observed. An explanation for this might be the difference in conditions under which *PR2* expression was analyzed. When plants are treated with the virulent pathogen such as *Psm* ES4326, *PR2* is upregulated as part of PTI response, while in *snc1* mutant it is part of TNL-mediated ETI. Interestingly, previously published data showed that elevated *PR2* expression in *snc1* was not suppressed by *eds5-3*, while it was suppressed in *snc1 nahG* plants (Li *et al*., 2001; Zhang *et al.*, 2003). One of the hypotheses proposed by Zhang *et al*. (2003) was that basal levels of SA were enough to cause *PR2* expression in *snc1 eds5-3*. My results showed that basal levels of SA *eds5-3* significantly contribute to TNL-mediated ETI triggered by AvrRps4 (Figure 3.3). If this hypothesis was true, we would expect to observe full suppression of *PR2* expression in *snc1 npr1-1 npr4-4D* mutant. However, the results presented in (**Figure 3.6 (b)**) show that *PR2* expression triggered by SNC1 is only partially dependent on SA receptors. Alternative hypothesis was that catechol, the product of NahG, inhibited constitutive *PR2* expression in *snc1 nahG* plants, instead of lack of SA (Zhang *et al*., 2003). Catechol was already shown to have negative impact on plant immunity (Van Wees *et al*., 2003). If the second hypothesis was true, unchanged *PR2* expression in *snc1 eds5-3* would suggest that *PR2* expression in *snc1* was SA-independent. In contrast, my results suggest that *PR2* expression is partially dependent on SA

receptors. To determine which hypothesis is true, a qRT-PCR analysis should be performed on *snc1 eds5-3* plants, to better quantify *PR2* expression and determine precisely whether there is suppression of transcription or not.

Infection assays using *H.a.* Noco2 clearly showed that perception of SA by both NPR1 and NPR4 is required for *snc1*-mediated resistance to this oomycete pathogen (**Figure 3.7**). The number of spores in *snc1 npr1-1 npr4-4D* even exceeds that of Col-0, emphasizing the importance of SA and its receptors for the defense. This result is supported by the previously demonstrated involvement of NPR1 and NPR3/4 in the protection against *H.a.* Noco2 (Ding *et al*., 2018). It was shown that *npr1* and *npr4-4D* mutants were more susceptible than wild-type plants. Moreover, these two mutant alleles had additive effect on susceptibility to *H.a.* Noco2, and my results demonstrate the similar trend (Ding *et al*., 2018). In conclusion, TNL mediated resistance to *H.a.* Noco2 in *snc1* plants is fully dependent on NPR1- and NPR4-mediated signaling.

While constitutive expression of *PR1* and high resistance to *H.a.* Noco2 are fully dependent on the NPR1 and NPR4 signaling pathways, dwarf morphology of *snc1* plants and constitutive expression of *PR2* are only partially dependent on these SA receptors. These results together imply that NPR1- and NPR3/4-dependent, as well as SA-independent signaling regulate ETI mediated by TNL protein SNC1.

3.2.4 SUMM2-mediated ETI in *mkk1/2* mutant partially requires perception of SA by NPR1 and NPR4

The combined mutants between *mkk1/2*, *npr1-8* and *npr4-4D* showed that perception of SA by its receptors NPR1 and NPR4 is only partially responsible for the *mkk1/2* dwarf

phenotype. Previously it was suggested that the *mkk1/2* autoimmune phenotype was partially dependent on SA, since *sid2* could not fully suppress it (Qiu *et al.*, 2008). It is unlikely that basal levels of SA in *sid2* mutant were responsible for the dwarfism, since disruption of SA perception in *mkk1/2 npr1-8 npr4-4D* was not sufficient to fully suppress the dwarfism. Interestingly, *npr1-8* and *npr4-4D* have different effect on *mkk1/2* phenotype. *mkk1/2 npr4-4D* plants look very similar to *mkk1/2* plants, except that they are slightly bigger. On the other hand, *npr1-8* seems to have a more dramatic effect on morphology of plants, since *mkk1/2 npr1-8* are bigger than *mkk1/2 npr4-4D* and the leaves are less curly. Another distinctive phenotype is bleaching in the center of the rosette in plants with *npr1-8* allele. From these results we can see that NPR1 seems to contribute more to the dwarf phenotype of *mkk1/2* plants than NPR4. Nonetheless, the joint effect of *npr1-8* and *npr4-4D* results in highest suppression of dwarfism of *mkk1/2*, indicating that NPR1- and NPR4-mediated signaling together contribute to its autoimmune phenotype. MAPK cascades regulate different physiological processes in plants, including growth and development (Suarez Rodriguez *et al*., 2010). Therefore, it is possible that *mkk1/2* also affects plant growth independently of SA receptors. However, that regulation would have to be dependent on SUMM2, since *summ2* fully suppresses extreme dwarfism of *mkk1/2*.

Regarding the constitutive expression of *PR1* in *mkk1/2*, my results showed that *mkk1/2 npr1-8 npr4-4D* have *PR1* expression suppressed to the levels of Col-0, suggesting that NPR1 and NPR4 are regulating expression of *PR1* in the pathway downstream of CNL protein SUMM2. Similar to the case of *snc1 npr1-1* (**Figure 3.6 (a)**), we observe a dramatic suppression of *PR1* expression by *npr1-8* mutant allele, implying that NPR1 is the major regulator of *PR1* expression. Surprisingly, *mkk1/2 npr4-4D* showed higher *PR1* expression than *mkk1/2*, which is hard to explain when we take into consideration that quadruple mutant has even lower expression

than *mkk1/2 npr1-8*. This result is likely caused by a technical mistake during experiment handling. Anyhow, perception of SA by NPR1 or NPR4 alone is not sufficient to fully suppress constitutive expression of *PR1* in SUMM2-mediated ETI response.

The regulation of *PR2* expression by NPR1 and NPR4 in *mkk1/2* is not very clear from these results (**Figure 3.9 (b)**). Elevated levels of *PR2* transcript in the triple mutants *mkk1/2 npr1-8* and *mkk1/2 npr4-4D* suggest that perception of SA by NPR1 and NPR4 negatively regulates *PR2* expression. Following the model of Ding *et al*. (**Figure 1.1**), we would expect to see even higher *PR2* expression in the quadruple mutant, however, that is not the case. Instead, the *PR2* expression in the quadruple mutant is similar to that of *mkk1/2*. Considering the fact that *summ2* fully suppresses constitutive expression of *PR2* in *mkk1/2* (Zhang *et al*., 2012), that suppression likely does not rely on SA receptors NPR1 and NPR3/4. Alternatively, the unexpected results in this experiment could be explained by possible technical issues. Furthermore, plants used for this experiment were grown on soil instead of MS medium, which likely contributed to the higher variability of results.

In summary, some aspects of *mkk1/2* autoimmune phenotype are regulated by NPR1- and NPR4-mediated signaling. Extreme dwarfism is only partially dependent on SA perception, while *PR1* expression fully relies on NPR1- and NPR4-dependent signaling. The contribution of these two signaling pathways to *PR2* expression needs to be further tested. Additional signaling components downstream of SUMM2 are likely responsible for *PR2* upregulation and dwarf phenotype of *mkk1/2* plants.

3.2.5 Perception of SA by NPR1 and NPR4 negatively regulates HR triggered by AvrRpt2

In *eds5-3* mutant plants, there is increased cell death induced by *Pst* DC3000 *avrRpt2* compared to the wild type Col-0 (**Figure 3.10 (a)**), suggesting that SA is a negative regulator of AvrRpt2-triggered HR and residual SA in *eds5-3* is not sufficient to suppress HR**.** The analysis of ion leakage in *npr1-1*, *npr4-4D* and *npr1-1 npr4-4D* showed that *npr1-1* and *npr4-4D* had an additive effect on cell death induced by *Pst* DC3000 *avrRpt2* (**Figure 3.10 (b)**), but *npr1-1* and *npr4-4D* alone are not sufficient to increase cell death. This result implies that perception of SA negatively regulates AvrRpt2-triggered HR. Previously NPR1 was suggested to function as a negative regulator of HR triggered by AvrRpm1 (Rate & Greenberg, 2001). NPR1 overexpression lead to decreased HR, while *npr1-1* mutant exhibited higher AvrRpm1-triggered HR compared to the wild-type. There was no significant difference between wild-type and *npr1-1* when plants were treated with *Psm* ES4326 *avrRpt2*, which is consistent with my results (**Figure 3.10 (b)**) (Rate & Greenberg, 2001).

My results show that *npr4-4D* together with *npr1-1* leads to increased cell death, while *npr4-4D* alone has conductivity similar to Col-0 and *npr1-1*. It is possibly that NPR1 and NPR4 regulate transcription of one or more negative regulators of HR, which could be explained through the SA signaling model from Ding *et al*. (2018) (**Figure 1.1**). Binding of SA activates NPR1 as the positive regulator of the HR suppressor. In parallel, NPR3 and NPR4 lose their transcriptional repression activity when they bind SA, so the transcription of HR suppressors is enabled. This is supported by previously published data showing that *npr3 npr4* knock-out mutant had attenuated HR compared to the wild-type (Fu *et al*., 2012). Two separate pathways

provide tighter control of HR, which is necessary for prevention of uncontrolled spreading of programmed cell death beyond the infection site, which would be detrimental for plant's fitness.

Chapter 4: Conclusions and future directions

Plants have tightly regulated defense responses to combat pathogens in their environment. This regulation is important to provide a rapid response to pathogen attack, but also to prevent activation of defense responses when they are not necessary. SA is a plant hormone that is a major regulator of plant immunity. While NPR3 and NPR4 were first thought to control the stability of SA receptor NPR1 (Fu *et al.*, 2012), a new model proposes that NPR1 is a transcriptional co-activator, and NPR3/4 are SA receptors that function as transcriptional co-repressors of defense-related genes (Ding *et al*., 2018). The objective of my thesis was to analyze the role of NPR1 and NPR3/4 in different signaling pathways of plant immunity, in light of the newly established model of SA-mediated signaling.

In this thesis I showed that NPR1 and NPR3/4 function as positive regulators of PTI response. Also, NPR1 and NPR3/4 most likely bind the residual amount of SA in *eds5-3* mutant during the pathogen attack and thus contribute to PTI and ETI. I further showed that ETI response mediated by TNL type of R proteins is partially regulated by NPR1 and NPR3/4. Both RPS4- and SNC1-mediated ETI consist of SA-dependent and SA-independent signaling pathways. Furthermore, CNL-mediated ETI response also relies on NPR1- and NPR3/4-dependent signaling, although it seems that CNL protein SUMM2 does not heavily rely on SA receptors, considering relatively weak suppression of *mkk1/2* dwarf phenotype by *npr1-8* and *npr4-4D*. A suppressor screen on *mkk1/2 npr1-8 npr4-4D* plant could potentially reveal the SA-independent signaling components downstream of SUMM2. Unfortunately, *H.a.* Noco2 infection assay with plants from *mkk1/2* x *npr1-8 npr4-4D* cross could not produce valuable data, because *mkk1/2 npr1-8* and *mkk1/2 npr1-8 npr4-4D* plants are not viable under conditions of high

humidity that are required for experiment. Therefore, it is unknown to what extent NPR1 and NPR3/4 are required for the high resistance of *mkk1/2* mutant. In the future, a different approach should be used on these mutants to evaluate their resistance to pathogens.

From my results we can conclude that NPR1 is the major regulator of *PR1* expression in ETI, while the mechanism of *PR2* transcriptional regulation by NPR1 and NPR3/4 is not yet clear. While SA receptors are positive regulators of *PR1* expression and resistance to *H.a.* Noco2 and selected *P. syringae* strains, they are negative regulators of HR triggered by bacterial effector AvrRpt2, which is another evidence of SA being a negative regulator of HR. Yet, we are not sure whether NPR1 and NPR3/4 always act as negative regulators, because control of HR by SA is likely complex and might include negative feedback mechanisms. It would be interesting to see how *npr1-1* and *npr4-4D* affect SA-dependent lesion phenotypes in lesion mimic mutants such as *lsd6, lsd7, acd6* and *acd11*.

My results show that low SA levels in *eds5-3* can significantly contribute to PTI and ETI, implying that although the amount of SA in *eds5-3* is very low, its effect on immunity in not to be neglected. NPR1 and NPR3/4 using basal levels of SA might enable plant to quickly respond to pathogen attack before SA accumulates to higher amounts, while the pathogen-induced SA accumulation further enhances defense response. It remains to be elucidated which factors during pathogen infection could influence the activity of SA receptors and ensure that immune response is not activated by the basal levels of SA in the absence of pathogens. Nevertheless, the cross between *eds5-3* and *npr1-1 npr4-4D* is the next step to confirm whether NPR1 and NPR3/4 are indeed influenced by the basal levels of SA. The triple mutant *eds5-3 npr1-1 npr4-4D* could be a better representative of mutants with abolished SA-dependent immunity. However, impaired SA accumulation is not the only effect that is evident in the *eds5-3* mutant. It is demonstrated that

EDS5 is required for the export of pipecolic acid from the chloroplast, the precursor of a putative SAR mobile signal N-hydroxy pipecolic acid (NHP) (Chen *et al*., 2018; Rekhter *et al*., 2019b). Since *eds5-3* has impaired biosynthesis of NHP, which is one of the crucial compounds in plant immunity, the results of infection assays on *eds5-3* plants are not solely caused by decreased SA levels, therefore we should reconsider using *eds5-3* as a negative control regarding abolished SA accumulation. A different mutant defective in SA biosynthesis like *sid2* would be a more reliable choice.

In conclusion, my results support the model of SA-dependent signaling proposed by Ding *et al*. (2018) and they demonstrate that NPR1 and NPR3/4 are important regulators of PTI response and ETI response downstream of both CNL and TNL R proteins. However, regulatory pathways independent of SA clearly exist. Further genetic analysis would help us characterize SA-independent regulators in signaling pathways downstream of NLR proteins.

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