The *Arabidopsis* NPR1 Protein Is a Receptor for the Plant Defense Hormone Salicylic Acid

By

Yue Wu, B.Sc.

A Thesis

submitted to the Centre for Biotechnology

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Brock University St. Catharines, ON

© Yue Wu, 2015
ABSTRACT

Systemic Acquired Resistance (SAR) is a type of plant systemic resistance occurring against a broad spectrum of pathogens. It can be activated in response to pathogen infection in the model plant Arabidopsis thaliana and many agriculturally important crops. Upon SAR activation, the infected plant undergoes transcriptional reprogramming, marked by the induction of a battery of defense genes, including Pathogenesis-related (PR) genes. Activation of the PR-1 gene serves as a molecular marker for the deployment of SAR. The accumulation of a defense hormone, salicylic acid (SA) is crucial for the infected plant to mount SAR. Increased cellular levels of SA lead to the downstream activation of the PR-1 gene, triggered by the combined action of the Non-expressor of Pathogenesis-related Gene 1 (NPR1) protein and the TGA II-clade transcription factor (namely TGA2). Despite the importance of SA, its receptor has remained elusive for decades.

In this study, we demonstrated that in Arabidopsis the NPR1 protein is a receptor for SA. SA physically binds to the C-terminal transactivation domain of NPR1. The two cysteines (Cys521 and Cys529), which are important for NPR1’s coactivator function, within this transactivation domain are critical for the binding of SA to NPR1. The interaction between SA and NPR1 requires a transition metal, copper, as a cofactor. Our results also suggested a conformational change in NPR1 upon SA binding, releasing the C-terminal transactivation domain from the N-terminal autoinhibitory BTB/POZ domain.
These results advance our understanding of the plant immune function, specifically related to the molecular mechanisms underlying SAR. The discovery of NPR1 as a SA receptor enables future chemical screening for small molecules that activate plant immune responses through their interaction with NPR1 or NPR1-like proteins in commercially important plants. This will help in identifying the next generation of non-biocidal pesticides.
DEDICATION

This thesis work is dedicated in memory of my beloved grandfather, Ming-De Guan, who has always been the role model for me. Although without opportunity to receive education in school, he never stopped self-educating. To his great capacity, he managed to learn mathematics, literature and calligraphy. His persistence and excellence finally qualified him for becoming an engineering budget analyst. At the age of fifty, he fulfilled his dream by getting accepted into university.

From him, I learned to be kind to others, to be responsible in work, and to respect knowledge. His influence on me will never fade away. I will continue this journey in science and be the extension of his dream.
ACKNOWLEDGEMENT

First and foremost, I would like to thank my supervisor Dr. Charles Després for his excellent guidance, assistance and encouragement during the past several years of my research. I came here without knowing what I can do, and what I would like to do. Dr. Després patiently helped me develop my interest in molecular biology, build my confidence in conducting scientific research and improve my writing skills. By working with Dr. Després, I found the beauty of science and also my career goal. Now I will leave with confidence and purpose. What he has done for me is life changing.

I would also like to express my gratitude to the members of my supervisory committee, Dr. Vincenzo De Luca and Dr. Tony Yan, for their patience and support during the course of my thesis.

I am grateful to our collaborators, Dr. Ian Brindle and Yong Wang, for their assistance with element measurement. Additionally, my work benefited from advices given by Dr. Fang Yu. This thesis work would not be feasible without their help.

Furthermore, I am indebted to my friends and colleagues at Brock University most notably Dr. Fang Yu, Yong Wang, Xiahezi Kuai and Wanxiangfu Tang for their support and accompany.

I also want to thank my parents, Xiaobao Wu and Hong Guan, my girlfriend, Rui Xiong, who have provided me with unconditional love, constant source of support and encouragement. It has been wonderful to have you in my life.
TABLE OF CONTENTS

ABSTRACT ..............................................................................................................................i
DEDICATION ............................................................................................................................iii
ACKNOWLEDGEMENT ...........................................................................................................iv
TABLE OF CONTENTS ..........................................................................................................v
LIST OF FIGURES ................................................................................................................x
LIST OF ABBREVIATIONS ....................................................................................................xi

CHAPTER 1 – INTRODUCTION ............................................................................................. 1
1.1 Outline ............................................................................................................................. 2

CHAPTER 2 – LITERATURE REVIEW .................................................................................. 3
2.1 Plant defenses against microbial pathogens ................................................................. 3
  2.1.1 Structural barriers .................................................................................................................. 4
  2.1.1.1 Plant cuticle ...................................................................................................................... 4
  2.1.1.2 Plant cell wall ................................................................................................................... 5
  2.1.2 Biochemical defenses ........................................................................................................ 7
  2.1.2.1 Antimicrobial chemicals ................................................................................................. 7
  2.1.2.1.1 Phytoanticipin – chemical barrier .............................................................................. 7
  2.1.2.1.2 Phytoalexin – inducible plant antibiotics .................................................................... 8
  2.1.2.2 Proteinaceous defenders ................................................................................................. 9
  2.1.3 Pathogen-associated molecular pattern-triggered immunity .................................. 10
  2.1.3.1 Common microbial molecular signatures that trigger PTI ..................................... 11
  2.1.3.2 Perception of PAMPs at the plasma membrane ......................................................... 12
2.1.3.3 Downstream signaling and physiological responses .......................... 14
2.1.4 Effector-triggered immunity .................................................................. 15
  2.1.4.1 Effectors ...................................................................................... 16
  2.1.4.2 Recognition of effectors by plant resistance genes ....................... 18
2.1.5 Hypersensitive response ....................................................................... 20
2.1.6 Systemic acquired resistance ............................................................... 23
  2.1.6.1 Biochemical markers ..................................................................... 24
  2.1.6.2 Long-distance systemic signal ...................................................... 25
  2.1.6.2.1 Salicylic acid is not the long-distance signal ............................. 26
  2.1.6.2.2 Methyl salicylate ..................................................................... 27
  2.1.6.2.3 Interplay of DIR1 with G3P ....................................................... 28
  2.1.6.2.4 Dehydroabietinal ..................................................................... 29
  2.1.6.2.5 Azelaic acid ............................................................................ 30
  2.1.6.2.6 Pipecolic acid .......................................................................... 31
  2.1.6.3 NPR1-mediated transcription reprogramming .............................. 32
  2.1.6.3.1 NPR1 is central to SA-induced SAR gene expression .............. 32
  2.1.6.3.2 NPR1 mediates SAR gene activation in the nucleus ............... 33
  2.1.6.3.2 NPR1 functions via interacting with TGA transcription factors ... 34
2.2 Defense hormone salicylic acid .............................................................. 37
  2.2.1 Biosynthesis and metabolism of salicylic acid ................................. 37
    2.2.1.1 The IC pathway ...................................................................... 38
    2.2.1.2 The PAL pathway ................................................................... 38
  2.2.2 Salicylic acid signaling in SAR ......................................................... 39
    2.2.2.1 Initiation of SAR signal ......................................................... 39
2.2.2.2 Salicylic acid induces physiological changes ......................... 40
2.2.2.3 Salicylic acid activates PR-1 gene expression ......................... 41
2.2.3 Perception of salicylic acid ..................................................... 44

2.3 Copper homeostasis influences plant immunity ......................... 46
2.3.1 Copper transport .............................................................. 46
2.3.1.1 Copper transporters ....................................................... 47
2.3.1.2 Nicotianamine-metal transporters .................................. 50
2.3.1.3 Copper transporters involved in plant immunity .................. 53
2.3.1.4 Copper chaperones ....................................................... 53
2.3.2 Regulation of copper transport .......................................... 55

CHAPTER 3 – The Arabidopsis NPR1 Protein Is a Receptor for the
Plant Defense Hormone Salicylic Acid ........................................... 57

3.1 ABSTRACT ........................................................................... 58
3.2 INTRODUCTION ..................................................................... 58
3.3 RESULTS .............................................................................. 61
3.3.1 NPR1 Binds Specifically to SA ............................................ 61
3.3.2 NPR1 Binds SA through Cys521/529 via the Transition Metal Copper. 63
3.3.3 The Conformation of NPR1 and Δ513 Are Altered by SA .......... 65
3.3.4 SA Disrupts the BTB/POZ-Transactivation Domain Interaction .... 67
3.3.5 The NPR1 BTB/POZ Inhibits the Transactivation Potential of Δ513 ... 69

3.4 DISCUSSION .......................................................................... 70
3.5 METHODS ............................................................................. 74
3.5.1 Protein Purification for Equilibrium Dialysis, ICP-MS, Scintillation
Proximity Assay, and Solid-Phase Binding ..................................... 74
3.5.2 Metal-Affinity Chromatography ......................................................... 75
3.5.3 Pull-Down Assays .................................................................................. 75
3.5.4 Plant Transcription and Two-Hybrid Assays ........................................... 76
3.5.5 Equilibrium Dialysis and Scintillation Proximity Assays ......................... 77
3.5.6 Solid-Phase Binding Assay ...................................................................... 78
3.5.7 Inductively Coupled Plasma-Mass Spectrometry ...................................... 79
3.5.8 Chromatography ................................................................................... 79
3.5.9 Quantitative RT-PCR ............................................................................ 80
3.5.10 Crosslinked Chromatin Chromatography—3C Method ............................ 81
3.5.11 Statistics .............................................................................................. 81

CHAPTER 4 – PRELIMINARY DATA ................................................................ 95

4.1 INTRODUCTION ....................................................................................... 95

4.2 Results ...................................................................................................... 96
4.2.1 Cu-NPR1 binding is dynamic. ................................................................. 96
4.2.2 Cu transport impacts Cu-NPR1 binding and PR-1 activation ................. 97
4.2.3 Cu binding induces disulfide bond formation ........................................ 98
4.2.4 Cu-NPR1 regulates YSL3 in a SA-independent manner .......................... 99

4.3 Discussion ............................................................................................... 99

4.4 Methods .................................................................................................. 101
4.4.1 Treatment of plants ............................................................................. 101
4.4.2 Immunoprecipitation .......................................................................... 101
4.4.3 In vitro NPR1-Cu binding assay ............................................................ 101
4.4.4 Leaf metal measurement ...................................................................... 102
4.4.5 Metal analysis by ICP-MS ................................................................... 102
4.4.6 Disulfide bond detection................................................................. 102
4.4.7 Gel filtration ................................................................................. 102
4.4.8 Quantitative RT-PCR .................................................................... 103

CHAPTER 5 – GENERAL DISCUSSION .................................................113
5.1 Other NPRs as SA receptors? ..........................................................113

REFERENCE .........................................................................................116
LIST OF FIGURES

Chapter 3

Figure 1. NPR1 Is a Specific SA Receptor ...........................................82
Figure 2. Cys521/529 and Copper Are Essential for SA Binding ............83
Figure 3. SA Causes a Change in the Conformation of NPR1 ...............84
Figure 4. NPR1 Binds SA to Relieve Sequestration of the Transactivating Domain by the BTB/POZ .................................................................86
Figure S1. NPR1 Is an SA Receptor, Related to the Introduction and to Figure 1 .............................................................................................................88
Figure S2. Multiple Alignment of NPR1-Related Proteins, Related to the Discussion ....................................................................................................90

Chapter 4

Figure 1. NPR1 binds to more Cu after SA treatment. .........................104
Figure 2. Δ513 binds Cu spontaneously. .................................................105
Figure 3. Cu enhances SA-induced PR-1 expression. .............................106
Figure 4. SA induces reallocation of Cu in leaves.................................107
Figure 5. YSL3 is required for the full-scale PR-1 activation. ...............108
Figure 6. Cu induces disulfide bonds in Δ513. ........................................109
Figure 7. Cu does not cause the deoligomerization of NPR1 ..............110
Figure 8. Cu regulates YSL3 expression. ..............................................111
Supplemental Figure 1. SA activates YSL3 expression. .......................112
LIST OF ABBREVIATIONS

acd11 – accelerated cell death 11
Ag – silver
ald1 – aberrant growth and death 2-like defense response protein 1
ATX1 – Antioxidant protein 1
Avr – avirulence
AzA - azelaic acid
BTB/POZ - Broad complex, Tramtrack, and Bric a-Brac/Pox virus and Zinc finger
BTH – benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester
bZIP – basic leucine zipper
CC – coiled-coil
CCH – Copper Chaperone
CCS – Copper Chaperone for SOD
Cd – cadmium
CDPK – calcium-dependent protein kinase
CEBiP – Chitin Elicitor-binding Protein
CERK1 – Chitin Elicitor Receptor Kinase 1
ChIP – Chromatin Immunoprecipitation
Co – cobalt
COPT – CTR-like copper transporter
COX17 – Cytochrome c Oxidase Copper Chaperone 17
CRR1 – Copper Response Regulator 1
CTR – copper transporter
Cu – copper
CUL3 – cullin3
Cys – cysteines
DA – Dehydroabietinal
DB – Gal4 DNA binding domain
dir1 – defective in induced resistance
DMA – 2’-deoxymugineic acid
DND1 – Defence Not Death
DTT – dithiothreitol
EDS – Enhanced Disease Susceptibility 5
EF-Tu – elongation factor Tu
EqD – equilibrium dialysis
ETI – Effector-triggered immunity
ETR1 – Ethylene Response 1
Fe – Iron
FLS2 – Flagellin Sensing 2
FMO1 – Flavin-dependent Monooxygenase 1
FRO – Ferric Reductase Oxidase
G3P – glycerol-3-phosphate
GFP – Green Fluorescent Protein
GRP7 – Glycine-rich Protein 7
GSNO – S-nitrosoglutathione
HCC1 – Homolog of the Copper Chaperone SCO1
HMA – Heavy Metal P-type ATPase
HR – hypersensitive response
ICP-MS – inductively coupled plasma-mass spectrometry
ICS1 – isochorismate synthase 1
INA – 2,6-dichloroisonicotinic acid
IPL – isochorismate pyruvate lyase
IκB – Inhibitor of Kappa-light-chain-enhancer of Activated B Cells
Kd – dissociation constant
LAC – laccases
LPS – lipopolysaccharide
LRR-RLK – leucine-rich repeats receptor-like kinase
LS7 – Linker Scan 7
lsd1 – lesions simulating disease 1
Lys – lysine
MAPK – Mitogen-activated protein kinases
MATE – multidrug and toxin extrusion
MeSA – methyl salicylate
Met – Methionine
NA – nicotianamine
NahG – salicylic acid hydroxylase
NB-LRR – nucleotide binding leucine-rich repeat
NF-κB – Nuclear Factor κB
NIM1 – Non-inducible Immunity 1
NPR1 – Non-expressor of Pathogenesis-related Gene 1
pad3 – phytoalexin deficient 3
PAL – phenylalanine ammonia-lyase
PAMP – pathogen-associated molecular pattern
Pb – lead
PCD – programmed cell death
PGN – peptidoglycan
Phe – Phenylalanine
Pip – Pipecolic acid
Pol II – polymerase II
PR – Pathogenesis-related
PRR – pattern-recognition receptor
PS – phytosiderophores
PTI – pathogen-associated molecular pattern-triggered Immunity
R – resistance
Rboh – respiratory burst oxidase homologs
RIN4 – RPM1 interacting protein 4
RLK – receptor-like kinase
RLP – receptor-like protein
ROS – reactive oxygen species
SA – salicylic acid
SABP2 – SA-binding Protein 2
SAI1 – Salicylic Acid-insensitive 1
SAMT1 – SA-methyltransferase 1
SAR – Systemic Acquired Resistance
SCO1 – Synthesis of Cytochrome c Oxidase
Ser – serine
sfd1 – suppressor of fatty acid desaturase deficiency 1
sid2 – SA induction-deficient 2
SOD – superoxide dismutase
SPA – scintillation proximity assay
SPL7 – SQUAMOSA Promoter Binding Protein-like 7
t-CA – trans-cinnamic acid
TGA - TGACG DNA-binding protein
TIR – Toll/interleukin-1
TM – transmembrane
TMV – Tobacco mosaic virus
TRX – thioredoxin
YSL – Yellow Stripe-like proteins
ZIP – Zinc-regulated transporters, Iron-regulated transporter-like Protein
CHAPTER 1 – INTRODUCTION

Plants have existed for hundreds of millions years on earth. They have become a major source of nutrients for bacteria, fungi, insects, animals and humans. As a result, they are constantly under attack by heterotrophic organisms. In order to combat hazardous microbial pathogens, unique defense mechanisms have evolved in plants.

The first line of defense is the structural barrier imposed by the waxy cuticle on the leaf surface, the thick cell wall of plant cells, and the glandular trichomes containing defense metabolites. This limits attachment, invasion and infection by most plant pathogens (Brown and Ogle, 1997).

Once the first line of defense is penetrated, the innate immune responses can be induced after invading pathogens are detected (Muthamilarasan and Prasad, 2013). Unlike humans, plants do not have a circulating immune system containing mobile cells that function as defenders. Instead, they acquire resistance against pathogen infection through the systemic biosynthesis of chemicals and proteins which are involved in self-defense in response to biotic stress.

SAR is the most agronomically relevant type of plant immunity. This long-lasting and broad-spectrum immune response can be activated upon infection by avirulent pathogen, and is marked by the activation of defense genes including the PR genes. The deployment of SAR is mediated by the defense hormone SA. Accumulation of SA at distal tissue leads to the activation of defense genes during SAR (Fu and Dong, 2013). Investigating
how SA is perceived and subsequently activates downstream signaling pathways will serve to further our understanding of the molecular mechanisms under which this plant immunity is achieved. The research in this thesis will facilitate the development of new effective and eco-friendly pesticides that function via inducing endogenous immune responses.

1.1 Outline

Chapter 2 is a literature review describing our current knowledge of plant defense mechanisms. It begins with a general description of the plant immune system, and then focuses on the molecular basis of SAR. Details in the NPR1-mediated SA signaling pathway during SAR are also discussed. Additionally, Cu homeostasis and its interplay with plant immunity are introduced in the end.

Chapter 3 is a published manuscript, which demonstrates that *Arabidopsis* NPR1 specifically binds to SA, and such NPR1-SA interaction uses a transition metal copper. SA-binding induces conformational change in NPR1, which enables its function as a transcriptional coactivator. These findings establish that NPR1 is a receptor for the defense hormone SA.

Chapter 4 presents our preliminary results, showing Cu homeostasis has impacts on SA-NPR1 signaling, and a potential new mechanism by which NPR1 regulates gene expression independent of SA.

Chapter 5 discusses the controversies in SA-perception, as well as the future direction of research on NPR1.
CHAPTER 2 – LITERATURE REVIEW

2.1 Plant defenses against microbial pathogens

Plants are targeted for infestation by a variety of microbes including fungi, bacteria, oomycetes, viruses and nematodes. These pathogens are classified into two categories, biotrophs and necrotrophs, based on their distinct parasitic lifestyle. Biotrophs keep their hosts alive to provide them with nutrients. Examples include the common garden fungus powdery mildew of rose (*Sphaerotheca pannosa*), which causes the leaves to gradually die and fall off, and a devastating bacterial pathogen of rice (*Xanthomonas oryzae*), which causes serious blight. In contrast, necrotrophs cause lethal damages by secreting toxins or tissue-degrading enzymes that trigger quick release of nutrients. The vine grape fungus, *Botrytis cinerea*, is a member of this category of pathogens (Freeman and Beattie, 2008; Glazebrook, 2005). Both biotrophic and necrotrophic pathogens could severely interfere with plant growth, development and even viability. Pathogenic microbes have been one of the major threats to agriculture. It was estimated that 10% to 20% of worldwide crop yield loss is due to diseases caused by phytopathogens (Oerke et al., 2012). Under this biotic selective pressure, plants have developed a sophisticated innate immune system to fend off microbial pathogens. Understanding the molecular mechanisms underlying disease resistance against phytopathogens has tremendous implications on future crop improvement.
2.1.1 Structural barriers

The establishment of a parasitic relationship between a pathogen and a host requires direct physical contact. Fungal pathogens (e.g., *Botrytis cinerea*) penetrate plant cells and acquire nutrients through hyphae, whereas bacterial pathogens (e.g., *Pseudomonas syringae*) live extracellularly where they modify host physiology to release nutrients (Freeman and Beattie, 2008; Vidaver and Lambrecht, 2004).

2.1.1.1 Plant cuticle

In order to keep pathogens outside, plants have evolved a robust structural barrier, namely the cuticle, on the outer surface. The plant cuticle consists of cutin (an inter-crosslinked polyester polymer) and waxes (complex mixtures of long-chain aliphatic compounds). The insoluble cutin is impregnated and covered by cuticular waxes, forming an extremely hydrophobic surface (Riederer and Müller, 2008). This cuticle layer is very successful in blocking the invasion of wound pathogens. Blue mold of citrus (*Penicillium digitatum*), as an example, is unable to penetrate the plant surface, but could gain entry into wounded fruits (Vilanova et al., 2013).

In addition to the physical separation of plant cells from microbes, the hydrophobicity of plant cuticle also plays a role in limiting pathogen infection. Bacterial and fungal pathogens spread in the field in the form of spores. The attachment of spores to the plant surface is critical to the initiation of pathogenesis. This step requires the presence of water. Surface moisture is also important for the germination of pathogen spores and the movement of pathogens. The hydrophobic cuticle layer minimizes the presence of surface
moisture and thus reduces the chance of spore attachment and germination (Brown and Ogle, 1997).

This structural barrier limits pathogen infection, rather than completely prevents pathogens from entering plants. During the long period of evolution, many microbial pathogens have developed different adaptations to overcome this barrier.

Although being covered by cuticle, plant cells are not completely isolated from their external environment. Vent-like structures, termed stomata, exist on plant leaves that permit photosynthesis through the exchange of CO$_2$ and O$_2$, as well as for the evaporation of water through transpiration. These natural-openings are targeted by some pathogens as a route of invasion (Melotto et al., 2008). *Puccinia graminis*, a pathogen of wheat, can form a specialized apparatus (apressorium) over stomata that permits the insert of fine hyphae into wheat leaves. Sub-stomatal vesicles can then be established within infected plants. Infection hyphae are developed from sub-stomatal vesicles, and spread within wheat leaves. Pathogens like *Botrytis cinerea* use a more drastic measure. They directly penetrate cuticle by pressing the pointy penetration peg into the plant surface (Brown, 1980).

**2.1.1.2 Plant cell wall**

When pathogens enter host plants through natural-openings, wounds or direct penetration, the cell wall is the other structural barrier for them to overcome before they reach cellular contents. The plant cell wall is a tough layer that surrounds the plant cell membrane, providing structural support
and tensile strength to plant cells. The main components of the plant cell wall are polysaccharides including cellulose (hemicellulose and pectin), phenolic compounds (such as ferulic and coumaric acids) and glycoproteins. The pectin matrix imbedded with crosslinked cellulose microfibrils, structural proteins and phenolic polymers separates plant cells from invading pathogens (Underwood, 2012). The constitution and thickness of the plant cell wall influence the plant susceptibility to pathogens. Although this has been speculated upon for decades, only recently has this been observed experimentally. Studies showed that modifying plant cell wall metabolism-associated genes results in altered host plant susceptibility to pathogens. Tomato plants suppressed for two plant cell wall hydrolase genes (endo-b-1,4-glucanases Cel1 and Cel2) are less susceptible to *Botrytis cinerea* than the wild type. Tomato fruits with reduced level of Polygalacturonases (pectin-hydrolyzing enzymes) exhibit enhanced resistance to *Geotrichum candidum* and *Rhizopus stolonifer*. Overexpression of Extensin-1, which encodes a cell wall structural glycoprotein, in *Arabidopsis* reduces the invasiveness of *Pseudomonas syringae* (Cantu et al., 2008). In order to break through this barrier, some pathogens, especially necrotrophs, have developed an array of cell wall-degrading enzymes (Kubicek et al., 2014).

Even though structural barrier is effective in limiting pathogenesis, it is not a perfect solution. More active and specialized measures (immune responses) are required for disease resistance against phytopathogens.
2.1.2 Biochemical defenses

In order to win a war, weapons are needed in addition to firm fortress walls. In the battle against microbial enemies, plants use chemicals and proteins as their weapons.

2.1.2.1 Antimicrobial chemicals

A large variety of plant-produced chemicals such as lactones, saponins, cyanogenic glucosides and terpenoids have antimicrobial activity (Brown and Ogle, 1997). These defense compounds are either preformed before challenge by pathogens, or produced after pathogen infection. The preformed chemicals are termed phytoanticipins, referring to “low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from preexisting constituents” (VanEtten et al., 1994). In contrast, phytoalexins are plant antimicrobial compounds that are synthesized de novo after pathogen infection (VanEtten et al., 1994).

2.1.2.1.1 Phytoanticipin – chemical barrier

Phytoanticipins (e.g., avenacin) are pre-synthesized and stored in preparation for potential pathogen attacks. Four structurally related avenacins have been found in young oat roots. These triterpenoids confer their antifungal activity by forming complexes with the sterols on the fungal cell plasma membrane. The complexes aggregate to cause pore formation and thus disrupts cell membrane integrity. The fungus *Gaeumannomyces graminis* var. *tritici* is known to be a severe threat to wheat and barley.
However, it is unable to infect oat roots due to its sensitivity to avenacins. The antifungal activity of avenacins was further confirmed by the report that the oat-infecting fungus, *G. graminis* var. *avenae*, carries a detoxification enzyme targeting the major avenacin, avenacin A-1 (Mert-Türk, 2006).

### 2.1.2.1.2 Phytoalexin – inducible plant antibiotics

Phytoalexins accumulate in plants after pathogen infection through the *de novo* expression of enzymes involved in their biosynthetic pathways. Camalexin, chemically known as 3-thiazol-2’-yl-indole, is one of the best-studied phytoalexins. While it was originally isolated from the leaves of the crucifer *Camelina sativa* infected with *Alternaria brassicae* (Glawischnig, 2007), studies were mainly done in the model plant *Arabidopsis thaliana*. Camalexin is produced in *Arabidopsis* leaves responding to challenge by bacteria, viruses, fungi and oomycetes, and overaccumulates at the site of infection. Camalexin also significantly inhibits the growth of some pathogens. This inhibitory effect is more obvious in fungi than in Gram-negative bacteria. Compared to the wild type control, the *Arabidopsis phytoalexin deficient 3* (*pad3*) mutant lacking an enzyme required for camalexin biosynthesis is more susceptible to *Alternaria brassicicola* and *Leptosphaeria maculans*. In contrast, the *pad3* mutant and the wild type control do not exhibit any difference in their sensitivity to *Pseudomonas syringae pv maculicula* (González-Lamothe et al., 2009; Schuhegger et al., 2007).
2.1.2.2 Proteinaceous defenders

The production of defense proteins is the other important aspect of plant biochemical defense. Since producing defense proteins requires energy and resources, the expression of most defense proteins is enhanced or induced only after the plant immune system is activated. These proteins have been termed as PR proteins. The inducible PR proteins were first discovered in tobacco leaves challenged by tobacco mosaic virus (van Loon and van Kammen, 1970). Numerous studies characterizing the PR proteins have been conducted in tobacco and many other plant species. Currently, the PR proteins with various functions are classified into 17 families (van Loon et al., 2006).

Some PR proteins are hydrolytic enzymes such as chitinases, glucanases and lysozymes. These chitinases and glucanases hydrolyze chitin and glucans respectively, which are the two major components of the fungal cell wall. Tomato plants that simultaneously express tobacco PR-2e (encoding glucanase) and PR-3d (encoding chitinase) have more resistance to Fusarium oxysporum f.sp. lycopersici than wild type control plants (Melchers and Stuiver, 2000). Unlike the fungal cell wall, the bacteria cell wall is composed of peptidoglycan (PGN), a polymer of cross-linked polysaccharide with amino acid side chains. Some members in the PR-8 gene family have been suggested to encode proteins with lysozyme activity. They exert antibacterial effect through catalyzing the hydrolysis of PGN (van Loon et al., 2006).
Defensin is another type of PR proteins (PR-12 family). They are small (~5 kDa, 45 to 54 amino acids) cysteine-rich (8 cysteine residues) proteins that possess strong fungicidal activity (Thomma et al., 2002). The exact molecular mechanisms of their anti-fungal activity still remain unclear. Several mechanisms have been suggested. The radish defensin (RsAFP2) has been shown to induce lethal cell membrane permeabilization in susceptible yeast and fungi. It has also been reported that RsAFP2 plays a role in the induction of reactive oxygen species (ROS) generation in Candida albicans. The elevated level of ROS leads to the disruption of fungal cell membrane integrity and the arrest of cell growth. Defensins can also influence ion channels. The alfalfa defensin (MsDef1) inhibits hyphal growth by blocking the L-type calcium channel signaling required for tip growth (Stotz et al., 2009).

The PR proteins also include thaumatin-like proteins, proteinase-inhibitors, proteases, peroxidase, ribonuclease-like proteins, lipid-transfer proteins, oxalate oxidase and other proteins with uncharacterized properties (van Loon et al., 2006).

2.1.3 Pathogen-associated molecular pattern-triggered immunity

Aside from the structural and chemical barriers, plants also use an innate immune system for self-defense. Plant innate immunity is marked by the local and/or systemic de novo biosynthesis of defense-related proteins and chemicals. The innate immune responses are only induced as a result of pathogen infection, and last for a certain period of time. The length of such induced immunity is pathogen-dependent (Jones and Dangl, 2006).
Pathogen-associated molecular pattern-triggered immunity (PTI) is a type of quick and transient immunity that allows plants to rapidly react to pathogen invasion. After the host plant cells recognize invading pathogens, a series of immune responses are triggered to stop pathogenesis (Bittel and Robatzek, 2007). This form of PTI can be triggered by non-pathogens as well. Both host and non-host microbes transcriptionally activate a similar set of host genes, and induce comparable physiological responses (Thilmony et al., 2006). This suggests that the induction of PTI depends on the recognition of the common molecular signatures shared by foreign microbes, rather than the specific identity that can be attributed to individual pathogens.

### 2.1.3.1 Common microbial molecular signatures that trigger PTI

The common signatures of microbes recognized by the plant immune system, termed pathogen-associated molecular patterns (PAMPs), are sufficient to elicit plant immune responses. To date, flagellin, harpins, elongation factor Tu (EF-Tu), PGN, lipopolysaccharide (LPS), cold shock protein, chitin, oomycete necrosis-inducing phytophthora proteins, cryptogein and fungal elicitin have been identified as PAMPs (Nicaise et al., 2009).

Flagellin is the building block of a bacterial motility organelle flagellum. The conserved regions of flagellin harbor active PAMP epitopes that can be perceived by most higher plants such as tobacco (*Nicotiana benthamiana* Domin), tomato (*Solanum lycopersicum* L.), rice (*Oryza sativa* L.) and the model plant *Arabidopsis thaliana* (Boller and Felix, 2009). The well-characterized epitope flg22 is a stretch of 22 amino acids in the N-terminus of flagellin. The synthetic flg22 peptide is sufficient to elicit PTI responses at
subnanomolar concentrations (Felix et al., 1999). In addition to flg22, the bacterial flagellin also contains other epitopes such as flgII-28, another conserved region in the N-terminus of flagellin. The induction of PTI by flgII-28 treatment has been observed in tomato and some other Solanaceae species (Cai et al., 2011).

The microbial cell wall PGNs are also PAMPs perceived and targeted by the plant immune system. Arabidopsis can sense the PGN fragments from both Gram-positive and Gram-negative bacteria (Gust et al., 2007). LPS, a major constituent of the outer membrane of gram-negative bacteria, is also a PAMP (Newman et al., 2007). The best-studied PAMP identified in the fungal cell wall is chitin. It can elicit PTI responses in rice and Arabidopsis (Shibuya and Minami, 2001).

2.1.3.2 Perception of PAMPs at the plasma membrane

PAMPs are perceived with high sensitivity by a large set of cell surface receptors, namely pattern-recognition receptors (PRRs). The currently known PRRs are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Zipfel, 2014).

A RLK consists of an extracellular ligand-binding domain, a single transmembrane domain and an intracellular kinase domain. The conformation of RLK changes upon ligand binding, which allows for the autophosphorylation of the kinase domain (Zhang et al., 2006a), and/or the phosphorylation of downstream signaling components that interact with the kinase domain (Wang et al., 2008). In such, an extracellular signal is transduced into a series of intracellular molecular events. The best-
characterized RLK involved in PAMP perception is the flg22 receptor in *Arabidopsis*, Flagellin Sensing 2 (FLS2). The *FLS2* gene locus was identified through genetic screening for *Arabidopsis* mutants that are insensitive to flg22. The *FLS2* gene encodes a ubiquitously expressed RLK that belongs to the subfamily XII of leucine-rich repeats receptor-like kinase (LRR-RLK) (Gómez-Gómez and Boller, 2000). The subsequent report of the specific interaction between flg22 and FLS2 indicates that the FLS2 protein is the *bona fide* receptor for flg22 (Chinchilla et al., 2006). The binding of flg22 to FLS2 induces the rapid formation of hetero-complexes between FLS2 and its signaling partner, Brassinosteroid-insensitive 1-associated Receptor Kinase 1 (BAK1), followed by the phosphorylation of their kinase domains (Schulze et al., 2010).

RLPs are overall structurally similar to RLKs, but lacking the intracellular domain. Therefore, it is considered that RLPs function through interacting with RLKs to enable the intracellular signaling (Zipfel, 2014). In rice, the perception of fungal chitin requires both a lysine motif (LysM)-containing RLP, Chitin Elicitor-binding Protein (CEBiP), and a RLK, Chitin Elicitor Receptor Kinase 1 (CERK1) (Kaku et al., 2006). CEBiPs dimerize upon chitin binding, and further form hetero-oligomeric complexes with the CERK1 to initiate the intracellular signaling (Hayafune et al., 2014).

RLKs and RLPs represent a large family of the plant proteins (610 RLKs and 56 RLPs in the *Arabidopsis* genome). Only a few have been functionally characterized. There are many orphan PAMPs awaiting to be paired with their PRR partners (Bittel and Robatzek, 2007; Zipfel, 2014).
2.1.3.3 Downstream signaling and physiological responses

Although the molecular mechanisms of the intracellular signaling during PTI remain largely unknown, some representative signaling events have been characterized.

Ion channels are typically influenced by various PAMPs. PAMP perception induces a rapid (in 30-40 s) increase in cytosolic calcium concentration. It is suggested that the cytosolic calcium spike is sensed by calmodulin, calcineurin B-like proteins and calcium-dependent protein kinases (CDPKs) to activate corresponding downstream signal pathways (Bittel and Robatzek, 2007).

Following calcium influxes is increased production of ROS by the plasma membrane-localized NADPH oxidases, respiratory burst oxidase homologs (Rbohs). This quick and transient event is known as oxidative burst, which has been used as an indicator of the induction of PTI in bioassays (Torres et al., 2002; Wu et al., 2014). Since the N-termini of Rbohs can be phosphorylated by CDPKs, it has been suggested that CDPKs impact ROS generation through regulating the phosphorylation state of Rbohs (Gao et al., 2013). The PAMP-induced, Rboh-produced ROS not only reinforce cell wall by catalyzing the cross-linking of polymers and proteins, but also act as signals to induce gene transcriptional reprogramming (Torres et al., 2006).

Mitogen-activated protein kinases (MAPKs) are also important components in the signaling network of PTI. In Arabidopsis, FLS2 simultaneously activates two antagonistic MAPK cascades that respectively have positive and negative effects on PTI signaling (Bittel and Robatzek,
This allows for the fine-tuning of defense responses elicited during PTI. Together with the CDPKs, MAPKs regulate transcriptional reprogramming via phosphorylating the players involved in the PTI signaling events such as enzymes and transcription factors (Meng and Zhang, 2013).

Other physiological responses during PTI include callose deposition, phosphatidic acid production, ubiquitination-mediated protein degradation and stomata closure (Macho and Zipfel, 2014). Extensive research is needed to advance our understanding of the molecular mechanism underlying the PTI responses.

### 2.1.4 Effector-triggered immunity

Effector-triggered immunity (ETI) is the second layer of the plant innate immunity. Unlike PTI, which is considered as basal defense that non-specifically reacts to microbes, ETI can only be triggered by microbes that carry a set of host-modifying molecules (namely effectors) recognizable by plants (Jones and Dangl, 2006). The recognition of such molecular identity by the host plant leads to both local and systemic immune responses. A rapid and localized programmed cell death (PCD), known as the hypersensitive response (HR), at the site of infection is usually associated with ETI (Morel and Dangl, 1997). This localized self-sacrifice of plant cells limits the growth and the spread of biotrophic and hemibiotrophic pathogens, since biotrophs and hemibiotrophs feed on living hosts. Concurrently, the non-infected tissue can launch SAR in preparation for potential pathogen attacks (Fu and Dong, 2013). Pathogens that induce ETI are called avirulent pathogens because
they activate the plant immune responses, rendering themselves less virulent. The effectors that trigger ETI are termed avirulence (Avr) proteins.

### 2.1.4.1 Effectors

As a result of the co-evolution of phytopathogens with plants, some pathogens have evolved the ability to modify the physiology of their hosts in favor of pathogenesis by secreting effectors with diverse functions. Even though the repertoire of known effectors is rapidly increasing due to the extensive number of bioinformatics studies in the last decades, our knowledge of the targets, function and dynamics of these effectors is still limited (Abramovitch et al., 2006a; Chisholm et al., 2006).

Since the PTI responses are detrimental to pathogens, suppressing PTI is an obvious direction of evolution for pathogens. Changing identity is one option to avoid being recognized by the plant immune system. However, this is very difficult and slow, because PTI targets the highly conserved signatures of pathogens, which are commonly mutation-intolerant due to their importance to pathogen survival and/or growth. Even though single amino acid polymorphism in flg22 has been found in some subspecies of *Xanthomonas campestris pv. campestris* to account for the variations in the recognition of these pathogens by their hosts (Sun et al., 2006), the PAMPs in most pathogens do not tolerate mutations (Nicaise et al., 2009).

An alternative way of suppressing basal defense is to interfere with the essential signaling pathways involved in PTI. Bacterial and fungal pathogens secrete a large number of effectors acting on the components involved in these pathways.
Effectors can influence both PRR biogenesis and degradation in order to reduce the protein level of PRRs in planta. PTI is initiated by the interaction between the PAMPs and the cell surface PRRs, followed by PRR endocytosis, as well as the transcriptional upregulation of a number of PRRs. This is to regenerate ligand-free PRRs on the cell surface, and also increase the sensitivity to a collection of PAMPs (Boller and Felix, 2009). The effector HopU1 from *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000) targets a RNA-binding protein, Glycine-rich Protein 7 (GRP7), for ADP-ribosylation in *Arabidopsis*. This modification interferes with the binding of GRP7 to the mRNAs encoding FLS2, an important process for the translation of FLS2 mRNAs. The resulting reduction in the biogenesis of FLS2 renders the host plant less resistant to *Pseudomonas syringae* (Jeong et al., 2011). Additionally, effectors such as AvrPtoB from *Pseudomonas syringae* promote the degradation of PRRs. AvrPtoB strongly interacts with tomato ubiquitin in the yeast-two-hybrid screening system (Abramovitch et al., 2006b). The C-terminus of AvrPtoB has E3 ligase activity (Abramovitch et al., 2006b) of marking PRRs such as FLS2 and CERK1 for ubiquitin-mediated, 26S proteasome-dependent degradation (Gimenez-Ibanez et al., 2009; Göhre et al., 2008).

Effectors can also directly inhibit the function of PRRs. The N-terminus of the above-mentioned AvrPtoB is an inhibitor of the cytoplasmic kinase domain of Bti9, the tomato ortholog of CERK1. The physical interaction between AvrPtoB and Bti9 abolishes the Bti9-mediated plant immune responses (Zeng et al., 2012). The central domain of AvrPtoB has binding
affinity for FLS2 and its signaling partner BAK1. The binding of AvrPtoB to these two PRRs disrupts the complexation of FLS2 with BAK1 (Shan et al., 2008). Similarly, the *Pseudomonas syringae* effector AvrPto directly binds to the kinase domain of FLS2, preventing FLS2 from phosphorylating its substrates (Xiang et al., 2011).

The signaling components downstream of PRRs are also targets of effectors. HopAI1 from *Pst* DC3000 exerts its inhibitory effect on PTI by modulating PAMPs-activated MAPK cascade. MPK3 and MPK6 in *Arabidopsis* are two direct targets of HopAI1. HopAI1 possesses a unique phosphothreonine lyase activity, which remove the phosphate group from a phosphorylated threonine residue. The dephosphorylation of MPK3/6 catalyzed by HopAI1 resulted in the inactivation of the MPK3/6-mediated signaling pathway (Zhang et al., 2007). Moreover, the release of defense proteins into the apoplastic area requires the eukaryotic vesicle trafficking system. Secretion of effector XopB is secreted by *Xanthomonas campestris pv. vesicatoria* suppresses PTI via the interruption of vesicle trafficking (Schulze et al., 2012).

**2.1.4.2 Recognition of effectors by plant resistance genes**

Effector-carrying pathogens are more virulent to the susceptible host plants, than those lacking effectors. To cope with the emergence of pathogenic effectors, plants have evolved a surveillance system monitoring the presence of harmful effectors (Jones and Dangl, 2006).

In the 1940s, the plant immunologist Flor proposed the “gene-for-gene” hypothesis to explain the pathogen-host relationship in disease resistance.
According to this theory, host resistance is conferred when a resistance (R) gene product in the host mediates the specific recognition of an Avr gene product in the avirulent pathogen. This pairwise interaction between the Avr gene and the R gene is considered to be sufficient to trigger immune responses in the resistant plant (Flor, 1971). This concept has guided research in field of modern plant immunity in the following decades. Successful cloning of the first Avr gene (Staskawicz et al., 1984) and the first R gene (Martin et al., 1993) in the 1980s strongly support this hypothesis.

Most R genes encode nucleotide binding leucine-rich repeat (NB-LRR) proteins composed of a N-terminal coiled-coil (CC) or Toll/interleukin-1 (TIR) domain, C-terminal LRRs, and an intervening NB domain. The straightforward logic is that NB-LRRs recognize Avr proteins via direct protein-protein interaction, which has been observed in the case of the flax R protein L6 (Dodds et al., 2006). However, the direct interaction between R and Avr proteins has rarely been demonstrated. Instead, reports on indirect recognition of Avr effectors by R proteins prevail in the literature. The Arabidopsis R protein RPM1 and the Avr protein AvrRpm1 from Pseudomonas syringae pv. maculicola represent a great example. AvrRpm1 has no binding affinity to RPM1, but physically interacts with RIN4 (RPM1 interacting protein 4), a positive regulator of plant immunity. The interaction between RIN4 and AvrRpm1 results in the hyperphosphorylation of RIN4, which can be sensed by RPM1 (Liu et al., 2011; Mackey et al., 2002). To conceptualize this indirect phenomenon, two of the pioneer scientists in the field of plant immunity proposed a “guard hypothesis” (Dangl and Jones, 2001). In this
theory, the integrity of the molecular targets of effectors, rather than effectors themselves, are monitored by R proteins in the host. The perturbation of a host target by effector(s) can be recognized by a corresponding R protein, which leads to the initiation of ETI (Jones and Dangl, 2006).

Many R proteins have been characterized in the last decades. Nonetheless, the exact molecular mechanisms of how these NB-LRRs are activated, how the NB-LRRs mediate signaling-transduction, and to whom the signal is transduced still remain elusive (Bonardi et al., 2012; Jones and Dangl, 2006).

2.1.5 Hypersensitive response

The induction of ETI is often associated with HR, a spatially confined cell death at and around the infection zone. This phenomenon has been described in some plant species such as wheat since the 1900s (Gibson, 1904; Marryat, 1907; Ward, 1902). This drastic but controlled action is highly correlated to the disease resistance against fungus. In 1915, Stakman subsequently defined this resistance phenotype as HR (Stakman, 1915). Later morphological studies revealed that HR shares similarities with the three most-studied types of cell death in the animal system. Interestingly, HR possesses most of the typical features of both the non-programmed cell death (mitochondrial swelling of oncosis) and the PCD (cytoplasmic shrinkage, chromatin condensation of apoptosis, and vacuolization of autophagy) (Mur et al., 2008). Therefore, whether HR is programmed has been debated for years. Nevertheless, it plays an indisputable role in limiting
pathogen growth by releasing chemicals and defense proteins to the extracellular environment.

The initiation of HR is due to the activation of the host R proteins by the pathogenic Avr proteins. This is supported by the report that AvrPtoB fails to elicit HR in tomato plants in the absence of the R protein Prf (Rathjen et al., 1999). Similarly, AvrRpm1 induces HR in a RIN4- and RPM1-dependent manner.

To date, our knowledge about the signaling events downstream of R protein activation is still limited. However, some important molecular components involved in HR have been characterized (Brodersen et al., 2005). Unlike the morphological studies, the molecular evidence suggests that HR is, at least to some degree, programmed. Genetic screening in Arabidopsis has been conducted to search for mutants that initiate either compromised or spontaneous HR after effector elicitation. An isolated mutant lesions simulating disease 1 (Lsd1) exhibited a phenotype of uncontrolled cell death when inoculated with HR-eliciting bacteria (Dietrich et al., 1997). This suggests that LSD1 protein plays a role in HR. Further studies revealed that LSD1 inhibited cell death by activating the expression of Cu/Zn superoxide dismutase (SOD) genes and thus reducing the accumulation of superoxide radicals (Kliebenstein et al., 1999). It has also been shown that LSD1 suppressed HR by disrupting the nuclear localization of the basic leucine zipper 10 (bZIP10) transcription factor (Kaminaka et al., 2006). Such evidence implies that LSD1 functions a critical cellular “switch” of HR.
Aside from proteins, chemical signals produced during HR have also been extensively studied. Calcium influx represents an important signal for HR initiation. In cowpea challenged by a rust fungus, sustained increases in cytoplasmic calcium concentration was observed during HR (Xu and Heath, 1998). Further, chemical blocking or knockout mutation of a calcium cyclic nucleotide gated channel encoded by Defence Not Death (DND1) abolishes HR in Arabidopsis (Ali et al., 2007; Clough et al., 2000).

ROS has also been suggested to be a positive signal in HR. Tobacco mosaic virus (TMV)-elicited HR can be effectively delayed by the infiltration of ROS scavengers such as SOD or catalase (Doke and Ohashi, 1988).

Defense hormone SA is another important HR signal. SA overaccumulates at the site of infection and acts as a positive regulator of HR. In TMV-challenged tobacco leaves, the concentration of SA in the HR zone is much higher than that in the HR-free area. A gradient is established in the lesion with the highest SA concentration in the center and the lowest at the edge (Enyedi et al., 1992). The high correlation between SA and HR implies a role of SA in HR. Although the application of exogenous SA did not induce cell death in intact plants, it strongly accelerates HR-like PCD in soybean suspension cells (Shirasu et al., 1997). This positive effect of SA on HR has been confirmed by the use of SA-deficient mutants. Two avirulent isolates of Peronospora parasitica fail to induce HR in several different SA-deficient Arabidopsis mutants, while TMV elicits delayed HR in SA-deficient tobacco (Mur et al., 1997; Nawrath and Métraux, 1999). Additionally, the spontaneous cell death mutants such as lsd6 and accelerated cell death 11
(acd11) have elevated levels of SA. Overexpression of the bacterial SA hydrolase gene nahG in lsd6 or acd11 knockout mutants eliminates free SA and fully suppresses PCD. This suppression of PCD can be reversed by the application of SA agonists such as 2,6-dichloroisonicotinic acid (INA) and benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Brodersen et al., 2002; Weymann et al., 1995). The interplay of SA and NPR1 also plays a role in HR. In contrast to SA, NPR1 is a negative regulator of HR (Rate and Greenberg, 2001). Despite the fact that SA and NPR1 are important to HR, very little is known about the mechanism by which they regulate HR.

Because of the lack of clear evidence, it is difficult to determine the sequential order and the causal link between the molecular events during HR. Therefore, whether HR is a real disease resistance response that plants utilize to fend off pathogens, or simply an extreme case of physiological hypersensitivity in response to pathogen infection, is still unclear.

### 2.1.6 Systemic acquired resistance

It was well documented already in the early 1900s that plants can acquire immunity from the first infection by a parasite. Such acquired immunity enables plants to resist reinfection (Chester, 1933). This phenomenon was termed as “systemic acquired resistance” by Ross in 1961, since whole tobacco plants acquire immunity from the localized TMV-inoculation and thus gain resistance to the subsequent reinfection (Ross, 1961). Typically, once activated, SAR can last for weeks to months, and confer resistance against a spectrum of pathogens in the host plant (Ward et
al., 1991). Concurrently, the plant is primed to a sensitized state, which allows for faster and stronger reactions to future pathogen infection (Conrath, 2011). Understanding the molecular mechanisms of SAR is of great interest to plant scientists, because SAR has practical value in engineering crop plants with enhanced disease resistance, as well in developing novel chemicals (plant activators) that activate the plant inherent immunity.

2.1.6.1 Biochemical markers

The characterization of SAR began with the search for biomarkers. In tobacco, the expression of a set of genes is highly correlated with SAR induction. These so-called SAR genes did not contain all the defense-related genes, but rather cover a subset of them. This particular gene expression profile serves as a fingerprint of SAR induction, and distinguishes SAR from other plant physiological responses (Ward et al., 1991). Highly similar SAR-induced gene expression patterns are found in other plant species such as tomato, wheat and the model plant Arabidopsis (Ryals et al., 1996). Most of these SAR genes encode PR proteins that are essential to the maintenance of disease resistance. In tobacco, the expression of at least nine families of PR proteins is strongly induced during SAR. The antimicrobial activity of these PR proteins has been demonstrated in vitro, and overexpression of some SAR genes confers resistance to several tobacco pathogens (Ryals et al., 1996). Taken together, a strong linkage is established between the induced expression of the SAR genes and the deployment of SAR. Coordinately, these genes are considered as biomarkers for SAR. In Arabidopsis, the PR-1 gene has been routinely used to monitor the manifestation of SAR. The molecular
mechanisms by which PR-1 expression is regulated have also been extensively studied for decades.

Specific changes in plant metabolism could also serve as a marker for SAR. For example, SA is tightly associated with SAR. After pathogen-inoculation, SA overaccumulates in both the infected tissue and the systemic tissue. Therefore, SA is suggested to be a SAR signal (Rasmussen et al., 1991; Shulaev et al., 1995). This is strongly supported by the genetic evidence. SA fails to accumulate after pathogen infection in either the nahG-transformed (NahG hereafter) Arabidopsis, or the SA induction-deficient 2 (sid2) mutants lacking a functional enzyme (isochorismate synthase 1, ICS1) that is critical to SA biosynthesis. The SA deficiency in NahG and sid2 mutants results in compromised SAR establishment and failed SAR gene activation (Friedrich et al., 1995; Wildermuth et al., 2001). Further, application of exogenous SA or SA agonists is sufficient to induce the SAR response and the expression of SAR genes (Uknes et al., 1992; Vernooij et al., 1995; Ward et al., 1991). These lines of evidence together indicate that SA accumulation is required for the deployment of SAR. Thus, SA is a valid chemical marker, considering its key role in SAR signaling.

Research on SA and marker genes in the last a few decades has revealed many essential regulatory components of SAR signaling pathways, including NPR1, TGA and WRKY transcription factors (Fu and Dong, 2013).

2.1.6.2 Long-distance systemic signal

The establishment of SAR requires a mobile signal that initiates from the site of infection, travels throughout the plant and triggers transcriptional
reprogramming in naïve tissues. This systemic signal is likely to be transmitted within the phloem. In *Arabidopsis*, the phloem sap collected from the primary pathogen-infected leaves is sufficient to activate SAR in the systemic tissue (Maldonado et al., 2002). Grafting experiments indicate that an intact phloem is critical for the transmission of the SAR signal. In tobacco, the removal the phloem tissue in the stem above the infected site blocks the spread of the SAR signal (Tuzun and Kuć, 1985). Therefore, phloem sap-enriched petiole exudates are a biochemical pool for the isolation of the long-distance signal for SAR induction.

**2.1.6.2.1 Salicylic acid is not the long-distance signal**

SA was originally thought to be the mobile signal because of its accumulation in the phloem sap of infected tobacco and cucumber, and its ability to regulate SAR (Métraux et al., 1990; Yalpani et al., 1991). It was also demonstrated that SA is largely synthesized in the pathogen-infected leaves, and is transported from the primary infected tissue to the systemic tissue. This translocated SA accounts for up to 70% of the increase in SA levels in the uninfected tobacco tissue, and 50% in the cucumber tissue (Molders et al., 1996; Shulaev et al., 1995).

However, a body of evidence suggests that SA is not the long-distance SAR signal. In cucumber, the systemic accumulation of SA and the expression of SAR genes are not affected by the removal of primary infected leaves before SA accumulates in the phloem (Rasmussen et al., 1991). In grafted tobacco plants, the establishment of SAR is successful in wild-type scions that are grafted onto the TMV-inoculated NahG rootstocks, whereas
the NahG scions grafted onto the wild-type rootstocks fail to launch SAR (Vernooij et al., 1994).

Therefore, although being indispensible for SAR, SA per se is not the long-distance signal that triggers SAR in the systemic tissue. Additionally, the de novo biosynthesis of SA in the uninfected tissue is required for SAR.

### 2.1.6.2.2 Methyl salicylate

In the TMV-inoculated tobacco plants, elevated levels of methyl salicylate (MeSA) are found in petiole exudates from infected leaves, as well as in the untreated tissues. This suggests that MeSA is a potential mobile SAR signal (Park et al., 2007). The tobacco SA-methyltransferase 1 (SAMT1) gene encodes a S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase that catalyzes the conversion of SA to MeSA. Conversely, the MeSA esterase, encoded by the SA-binding Protein 2 (SABP2) gene, converts MeSA back to SA (Dempsey and Klessig, 2012). Through a series of grafting experiments using SAMT1-silenced, SABP2-mutated and wild-type tobacco plants, it has been demonstrated that SAMT1 is required at the site of TMV infection to accumulate MeSA for systemic transport, while SABP2 is required in distal untreated tissues to hydrolyze MeSA into SA (Park et al., 2007). This suggests a role of MeSA as a long-distance SAR signal.

Although MeSA is required for SAR induction in tobacco and potato, whether it is a long-distance signal in Arabidopsis is debatable. Contradictory results have been reported in the studies on MeSA using the Arabidopsis mutant bsmt1 that lacks a MeSA-synthesizing benzoic acid/salicylic acid methyl transferase 1. Liu et al. (2010) reported that pathogen-treated bsmt1
launch weaker SAR than wild-type control plants. In contrast, *bsmt1* is totally SAR competent in response to pathogen inoculation (Attaran et al., 2009; Liu et al., 2010). This discrepancy may be due to the fact that additional factors such as light have an impact on MeSA-mediated SAR induction in *Arabidopsis* (Liu et al., 2011). MeSA seems to be important for SAR only when the pathogen inoculation is conducted close to the dark period.

2.1.6.2.3 **Interplay of DIR1 with G3P**

The *Arabidopsis defective in induced resistance* (*dir1*) was identified in a genetic screen for mutants that are unable to initiate SAR. The transport of the long-distance SAR signal is compromised in *dir1* mutants. The phloem sap-enriched petiole exudates collected from the pathogen-challenged wild-type leaves are sufficient to activate SAR in *dir1* plants, whereas wild-type plants do not respond to petiole exudates from *dir1* plants (Maldonado et al., 2002). Considering that the DIR1 protein possesses a consensus motif shared by lipid transfer proteins, DIR1 was hypothesized to be a chaperone of the systemic signal (Maldonado et al., 2002). This is corroborated by the expression of *DIR1* in the phloem sieve elements and companion cells, and the secretion of its gene product to the cell wall (Champigny et al., 2011). Additionally, trypsin-treated petiole exudates fail to induce SAR, indicating that proteinaceous factors are involved in transmission of the SAR signal (Chanda et al., 2011).

Evidence for lipid being involved in the long-distance communication came from studies on the *Arabidopsis* mutant, *suppressor of fatty acid desaturase deficiency 1* (*sfd1*) (Nandi et al., 2004). The *SFD1* gene encodes
a plastid-localized glycerol-3-phosphate (G3P) dehydrogenase that catalyzes the conversion of dihydroxyacetone phosphate to G3P. The defects in lipid composition renders *sfd1* SAR-defective after pathogen inoculation (Nandi et al., 2004). In the *gly1* mutant, which carries a mutation in *SFD1*, the SAR-inducing activity is restored by the co-infiltration of G3P with avirulent pathogen (Chanda et al., 2011). These findings suggest a role of G3P in the long-distance signaling.

Although evidence for the direct interaction between DIR1 and G3P or a G3P-dependent factor is lacking, they do function synergistically to induce SAR. Application of exogenous G3P alone does not induce SAR in wild-type *Arabidopsis*, nor does infiltration of DIR1 alone in a G3P-deficient mutant. However, co-infiltration of G3P and DIR1 is sufficient to activate SAR. Further, 14C-labeling studies showed that the translocation of DIR1 to distal tissues requires G3P, and also that the accumulation of G3P or its derivatives in distal tissues is DIR-dependent (Chanda et al., 2011). Therefore, the interplay of G3P with DIR1 is considered as a systemic signal for SAR.

**2.1.6.2.4 Dehydroabietinal**

Dehydroabietinal (DA), an abietane type diterpenoid, has been isolated from the petiole exudates collected from the *Arabidopsis* leaves treated with avirulent pathogen. When applied locally to *Arabidopsis* leaves, DA is rapidly transported throughout the plant, and accumulates systemically. Elevated levels of SA are found in both DA-treated and untreated distal tissues, suggesting that DA activates SAR by inducing systemic SA accumulation.
(Chaturvedi et al., 2012). This is supported by the failure of DA to induce SAR in NahG Arabidopsis plants.

Interestingly, the DA level in petiole exudates does not increase after pathogen inoculation. Instead, the properties of DA-associating molecules change in response to avirulent pathogen. DA is enriched in high molecular weight (HMW) complexes (>100 kD) in the petiole exudates collected from pathogen-treated leaves, but enriched in low molecular weight (LMW) complexes (<30 kD) in the petiole exudates from mock-treated leaves. The isolated HMW complexes, but the LMW complexes, are sufficient to induce SAR. Additionally, trypsin treatment abolishes the SAR-inducing activity of the HMW complexes (Chaturvedi et al., 2012).

Taken together, DA associates with HMW proteinaceous complexes to activate SAR by inducing SA accumulation.

2.1.6.2.5 Azelaic acid

Unlike the factors mentioned above, azelaic acid (AzA), a nine carbon dicarboxylic acid, is a defense-priming molecule that prepares plants for future pathogen infection. After pathogen inoculation, a significant increase in AzA concentration is found in petiole exudates collected from the inoculated Arabidopsis leaves. Heavy atom labeling studies showed that AzA translocated within the plant and distributed systemically. Thus, Aza was proposed to be long-distance signal involved in SAR activation (Jung et al., 2009). Surprisingly, although the AzA-treated plants have enhanced disease resistance, the application of exogenous AzA does not induce SA accumulation or PR-1 gene expression in the systemic tissue, suggesting that
the disease resistance conferred by AzA is a result of defense priming (Jung et al., 2009). Intriguingly, recent reports suggested that AzA is not an immune signal required by SAR, but rather a general marker of lipid peroxidation that is derived from galactolipids via free radical-catalyzed fragmentation (Návarová et al., 2012; Zoeller et al., 2012). Therefore, AzA is not a requirement for SAR establishment. It does, however, prime the plant for a more robust and rapid defense response to potential pathogen attacks.

### 2.1.6.2.6 Pipecolic acid

Pipecolic acid (Pip), a Lys metabolite, is also a defense-priming molecule. It substantially accumulates (a 7-fold increase compared to mock-treated control plants) in the petiole exudates from pathogen-inoculated *Arabidopsis* leaves, and translocates throughout the plant (Návarová et al., 2012). Pip is required for SAR deployment in distal tissues. SA accumulation and SAR deployment are completely abolished post-inoculation in the systemic tissue of the Pip-deficient mutant *ald1* (*aberrant growth and death 2-like defense response protein 1*) (Song et al., 2004). Exogenous Pip is sufficient to restore the ability of *ald1* to accumulate SA and establish SAR in distal tissues post-inoculation. In contrast, the local responses are only partially impacted by Pip, indicating that Pip exerts its SAR-inducing effect mainly in the systemic tissue (Návarová et al., 2012).

When applied to *Arabidopsis*, Pip does not induce SA accumulation in the absence of pathogen, but primes the plant to a state that allows for stronger defense responses. Compared to mock-treated control, plants that are pretreated with Pip exhibit increased SA accumulation and enhanced
resistance to pathogen infection (Návarová et al., 2012). This defense-priming activity of Pip requires the presence of Flavin-dependent Monooxygenase 1 (FMO1), a positive regulator of SAR (Mishina and Zeier, 2006). Pip-treated fmo1 mutant fail to accumulate SA in the systemic tissue and establish SAR post-inoculation. Interestingly, the expression of ALD1 and the accumulation of Pip in distal tissues are also compromised in the fmo1 mutant. A slight increase in Pip levels found in the systemic tissue of fmo1 mutant is presumably due to the translocation of Pip from the site of infection (Návarová et al., 2012), suggesting that Pip translocates from the infection tissue to the systemic tissue, where it induces its own biosynthesis in an FMO1-dependent manner. The induced de novo biosynthesis of Pip in distal tissues is critical to the subsequent SAR establishment (Návarová et al., 2012).

All these lines of evidence together suggest that the long-distance signal may not be a single chemical or protein. Instead, it may be orchestrated by a combination of different biochemical entities. The timing and place of their function may also vary according to the specific properties of individual signals.

### 2.1.6.3 NPR1-mediated transcription reprogramming

#### 2.1.6.3.1 NPR1 is central to SA-induced SAR gene expression

Since SA is indispensible for SAR, investigating the SA signaling pathway is of particular interest. In order to identify the important components involved in the SA-dependent signaling pathway, genetic
screenings were carried out in the early 1990s in search for *Arabidopsis* mutants that are insensitive to SA (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). Surprisingly, all these SA-insensitive mutants obtained from independent screenings carry recessive mutations in a single gene locus *NPR1/NIM1* (*Non-inducible Immunity*)/*SAI1* (*Salicylic Acid-insensitive*). Pathogen-inducible SAR phenotypes and expression of SAR genes were completely abolished in *npr1* mutants, indicating a critical role of NPR1 in regulating SAR. However, SA is still able to accumulate in *npr1* mutants post-inoculation, but to a slightly higher level than in wild-type control plants (Rasmussen et al., 1991). This suggests that NPR1 functions as a central regulator of SAR downstream of SA.

### 2.1.6.3.2 NPR1 mediates SAR gene activation in the nucleus

The *Arabidopsis* NPR1 protein consists of a plant-specific NPR1-like C-terminus, an N-terminal BTB/POZ (Broad complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) protein-protein interaction domain, and intervening ankyrin repeats (Rochon et al., 2006). Since the ankyrin repeats share significant homology to the mammalian transcription factor inhibitor IκB (Inhibitor of Kappa-light-chain-enhancer of Activated B Cells), it was originally postulated that NPR1 functions through interactions with transcription factors (Ryals et al., 1997). This is supported by the requirement of nuclear localization of NPR1 for the SA-induced expression of SAR genes. When NPR1 is sequestered in the cytoplasm, neither SA nor INA (SA agonist) induces the expression of marker gene *PR-1* (Kinkema et al., 2000).
Unlike IκB, which retains transcription factor NF-κB (Nuclear Factor κB) in the cytoplasm to suppress gene transcription, NPR1 is involved in gene activation or derepression in the nucleus. It has been reported that NPR1-GFP (Green Fluorescent Protein) fusion proteins exist mainly in the cytoplasm in the absence of SA, but move into the nucleus in the presence of SA. The cytosolic NPR1-GFP proteins oligomerize via intermolecular disulfide bonds at Cys82 and Cys216 residues. After INA treatment, a proportion of NPR1-GFP oligomer is reduced into bioactive monomers, which translocate into the nucleus and mediate the transcriptional activation of PR-1 (Mou et al., 2003). The redox status of NPR1 is maintained by the interplay of different reducing and oxidizing agents such as GSH/GSSG, S-nitrosoglutathione and thioredoxin (Mou et al., 2003; Tada et al., 2008). Thus, the NPR1 oligomer is in dynamic equilibrium with the monomers, depending on the redox environment in which NPR1 is localized. This is corroborated by the detection of cytosolic and nuclear NPR1 in unstimulated Arabidopsis leaf cells (Després et al., 2000).

2.1.6.3.2 NPR1 functions via interacting with TGA transcription factors

A subclass of transcription factors in the bZIP protein family (TGA2, TGA3, TGA5, TGA6) has been identified through yeast-two-hybrid screenings, which interacts with NPR1. They exhibit specific binding affinity for NPR1 both in the yeast system and in in vitro pull-down assays (Zhang et al., 1999). NPR1 differentially interacts with these TGA transcription factors. In yeast,
TGA2 and TGA3 interact strongly with NPR1, whereas TGA5 and TGA6 weakly affinity interact with NPR1 (Zhou et al., 2000).

Genetic studies have investigated the role of TGAs in the transcriptional activation of NPR1-dependent SAR genes. Knockout mutation of single TGA genes in Arabidopsis do not cause any change in phenotype because of the redundancy of these genes. However, a tga2/5/6 triple knockout mutant show compromised SAR deployment post-inoculation and failure in PR-1 gene activation after SA treatment (Zhang et al., 2003). This supports the hypothesis that the role of NPR1 in regulating SAR is exerted via recruiting TGA transcription factors.

TGA2/5/6 are negative regulators of the PR-1 gene, because PR-1 expression is found to be about 50 folds higher in the tga2/5/6 mutant than that in wild-type Arabidopsis, and overexpression of TGA2/5/6 does not transcriptionally activate PR-1 (Zhang et al., 2003). Further, TGA2 suppresses the activation of reporter gene in plant transcription assays (Rochon et al., 2006). In contrast, NPR1 harbours a cryptic transactivation domain within its C-terminus suggesting a role of NPR1 in gene activation. Surprisingly, NPR1 does not induce the expression of a reporter gene driven by the PR-1 promoter (Rochon et al., 2006). Further investigation revealed the synergistic effect of TGA2 and NPR1 on the transcriptional activation of PR-1. Neither TGA2 nor NPR1 alone induces the expression of reporter gene driven by the PR-1 promoter. However, TGA2 and NPR1 together activated the reporter gene expression in an SA-dependent manner (Rochon et al., 2006). This indicates that TGA2 and NPR1 form an enhanceosome on the PR-
promoter in the presence of SA, leading to PR-1 gene activation (Rochon et al., 2006). Gel filtration analysis suggests that the stoichiometry of this complex is likely to be two NPR1, two TGA2 and one DNA. It was further demonstrated using gel shift assays that the BTB/POZ domain of NPR1 interacts with the N-terminus of TGA2, and only allows for the binding of TGA2 dimer to the PR-1 promoter by excluding other forms of TGA2 from its cognate DNA sequence (Boyle et al., 2009; Rochon et al., 2006). The enhanceosome activates PR-1 gene expression possibly through the transactivation domain of NPR1. Critical to the transactivation activity are the Cys521 and Cys529 residuals in this domain. Substitution of these two Cys with serine (Ser) abolishes the function the transactivation domain (Rochon et al., 2006).

In addition to the above-mentioned TGAs, TGA1 and TGA4 are also reported to have binding affinity for NPR1 in SA-treated Arabidopsis, even though such interaction is not detected in yeast (Lindermayr et al., 2010; Zhou et al., 2000). This discrepancy is probably due to the difference in the redox status of TGA1 at Cys260 and Cys266 residuals. NPR1 interacts with the reduced form of TGA1, rather than the oxidized form of TGA1 in which Cys260 and Cys266 form an intramolecular disulfide bond (Lindermayr et al., 2010). Substitution of Cys260 and Cys266 with asparagine (Asn) and Ser respectively, enable the interaction between NPR1 and TGA1, both in yeast and in planta. The interaction of NPR1 with TGA1 significantly enhances the DNA binding activity of TGA1 to its cognate binding sites (Lindermayr et al., 2010).
Taken together, NPR1 functions as a transcriptional coactivator in concert with TGA transcription factors to mediate the transcriptional activation of SAR genes (Rochon et al., 2006).

2.2 Defense hormone salicylic acid

SA and other plant benzoic acids were once considered as secondary metabolites due to the lack of evidence showing their involvement in critical physiological processes (Dempsey et al., 2011). However, numerous studies since 1900s indicate that SA serves as a hormone involved in plant growth and development, as well as physiological responses to abiotic and biotic stresses (Fu and Dong, 2013; Vicente and Plasencia, 2011; Yuan and Lin, 2008). SA is generally considered as a defense hormone because of its essential role in plant defense against microbial pathogens. Extensive efforts have been devoted to investigation of SA biosynthetic and signaling pathways.

2.2.1 Biosynthesis and metabolism of salicylic acid

After pathogen infection, SA is synthesized in both the site of infection and the systemic tissue to initiate local and systemic immune responses, respectively. Two SA biosynthetic pathway have been identified so far, namely the isochorismate (IC) pathway and the phenylalanine ammonia-lyase (PAL) pathway. To date, our understanding of these two pathways is still limited due to the lack of genetic and/or biochemical evidence of certain reactions (Dempsey et al., 2011).
2.2.1.1 The IC pathway

The IC pathway and the PAL pathway both start in the plastids from the same precursor, chorismate. In Arabidopsis, ICS1 and ICS2 catalyze the conversion of chorismate to IC as the first step in the IC pathway (Wildermuth et al., 2001). SA is then synthesized from IC. The enzyme that catalyzes this reaction has not been genetically or biochemically characterized in plants. In some bacterial species, chorismate is used by ICS as a precursor to produce IC intermediate, which is then converted into SA and pyruvate by isochorismate pyruvate lyase (IPL) (Mercado-Blanco et al., 2001; Verberne et al., 1999). Plants may also possess such IPL activity. The gene that encodes such enzyme remains to be identified.

Pathogen inoculation only induces the expression of ICS1, not ICS2, indicating that ICS1 is the enzyme involved in the de novo SA biosynthesis in response to biotic stress (Wildermuth et al., 2001). Furthermore, in the pathogen-inoculated sid2 mutant, SA only accumulates to very low levels (about 5% of the SA levels in wild-type plants) (Wildermuth et al., 2001). Thus, SA is mainly synthesized through the IC pathway. After being produced, SA is exported from the chloroplast to the cytoplasm through a multidrug and toxin extrusion (MATE)-like transporter encoded by EDS5 (Enhanced Disease Susceptibility 5) (Serrano et al., 2013).

2.2.1.2 The PAL pathway

The PAL pathway is an alternative SA biosynthetic pathway. $^{13}$C-labeling studies suggest that SA is synthesized from Phenylalanine (Phe) via trans-cinnamic acid (t-CA) (Dempsey et al., 2011). This is supported by genetic
evidence that pathogen-induced SA accumulation is reduced in the PAL-silenced background, and biochemical evidence that the pathogen-inoculated tobacco, cucumber and *Arabidopsis* plants have reduced SA accumulation when treated with the PAL inhibitor 2-aminooindan-2-phosphonic acid (AIP) (Dempsey et al., 2011). Therefore, the PAL pathway also contributes to the SA biosynthesis induced by abiotic stress, but to less extent than the IC pathway.

In the PAL pathway, Phe is produced in the plastids from chorismate and then transported into the cytoplasm via an unknown mechanism. In the cytoplasm, PAL converts Phe into t-CA. Cytosolic t-CA is then used as a precursor to produce benzoic acid by either the CoA (Coenzyme A)-dependent or the CoA-independent non-oxidative pathway (Widhalm and Dudareva, 2015). In contrast, t-CA in the peroxisome is used to synthesize benzoic acid by the CoA-dependent β-oxidative pathway (Widhalm and Dudareva, 2015). All three pathways converge at benzoic acid. At the last step, SA is synthesized by the hydroxylation at ortho-position of benzoic acid. This reaction is catalyzed by a soluble P450 monooxygenase. Although this enzyme has been partially purified in tobacco, the corresponding gene has not yet been isolated (León et al., 1995).

### 2.2.2 Salicylic acid signaling in SAR

#### 2.2.2.1 Initiation of SAR signal

SA has been known to play an important role in many aspects of SAR. After the long-distance signal is perceived in the systemic tissue, SA
overaccumulates through translocation and de novo biosynthesis of SA. The induced SA biosynthesis requires the presence of FMO1, as well the translocated and locally-produced Pip. Translocated Pip induces its own biosynthesis in the systemic tissue in an FMO1-dependent manner. This induction of Pip biosynthesis also requires the presence of SA (Návarová et al., 2012). Therefore, it is suggested that ALD1, FMO1 and ICS1 are central players that are incorporated into the SAR signal amplification loop. The resulting amplified SA biosynthesis is critical to the initiation of SAR in the systemic tissue (Návarová et al., 2012).

2.2.2.2 Salicylic acid induces physiological changes

It has been suggested that pathogen-induced SA accumulation stimulates a biphasic change in cellular redox state, starting with an early systemic oxidative burst, followed by a reducing environment (e.g., high GSH/GSSG ratio) (Mou et al., 2003; Park et al., 1998a; Park et al., 1998b). Such dual role of SA in regulating cellular redox state has also been observed in studies on HR response and heavy metal stresses. In HR, SA induces Rboh-dependent oxidative burst (Yoshioka et al., 2001; Zhang et al., 2009) In contrast, SA alleviates the heavy metal-induced oxidative stress (Popova et al., 2009). Thus, SA has a significant impact on the redox homeostasis in plants. To cope with biotic and abiotic stresses, SA may dynamically adjust the cellular redox conditions for a certain type of molecular event.

The SA-induced physiological changes have been suggested to influence the redox state of NPR1 (Mou et al., 2003). After SA treatment, the nuclear fraction of NPR1-GFPs is enriched possibly due to the reduction of
intermolecular disulfide bonds at Cys82 and Cys216 residues (Mou et al., 2003). The cytosolic NPR1-GFP oligomers are disassembled into monomers when treated with the reducing agent dithiothreitol (DTT) or GSH/GSSG (3.0 mM/0.2 mM) (Mou et al., 2003). Additionally, thioredoxin (TRX) is also a redox mediator of NPR1. The expression of TRX-h5 is upregulated after pathogen inoculation. It has been shown that TRX-h5 interacts with NPR1, and catalyzes the deoligomerization of NPR1 (Mou et al., 2003). In contrast with these reducing agents, S-nitrosoglutathione (GSNO) promotes the oligomerization of NPR1 via S-nitrosylation on Cys residues (Tada et al., 2008). In the cell-free assays, less monomeric NPR1-GFPs are detected as the concentration of GSNO increases. This is consistent with in vivo results showing that the SA-induced monomerization and nuclear localization of NPR1 are impaired in the GSNO reductase knockout mutant atgsnor1-3 (Tada et al., 2008). Although GSNO negatively regulates SAR by promoting the oligomerization of NPR1, it is required for SAR establishment. The GSNO-mediated S-nitrosylation at Cys156 residue is critical to the protein homeostasis of NPR1 upon SA treatment. Substitution of the Cys156 residue with alanine results in the failure of detecting NPR1 (C156A) in SA-treated plants (Tada et al., 2008). This suggests that the S-nitrosylation at Cys156 enhances the stability of NPR1, possibly due to inhibiting NPR1 degradation (Mou et al., 2003; Tada et al., 2008).

### 2.2.2.3 Salicylic acid activates PR-1 gene expression

Not only does SA affect the cellular environment, it also impacts the transcriptional regulation of SAR genes. PR-1 has been used as a research
model for the investigation of transcriptional regulation of SAR genes. Gene transcription is largely dependent on the regulatory elements within promoters and their associated regulators (such as transcription factors). Since SA can induce the expression of PR-1, the cis-acting regulatory elements responsible for such SA-inducibility is likely to be present on the PR-1 promoter. Analysis of the PR-1 promoter using linker-scanning mutagenesis reveals both positive and negative elements (Lebel et al., 1998). The positive element in the Linker Scan 7 (LS7) region responds to the SA agonist INA, whereas the negative element in the LS5 region is constitutive. Both LS7 and LS5 contain the binding sequence for TGA transcription factors. Other studies confirm that TGA2 transcription factors bind to both of these two sites (Després et al., 2000).

The SA-induced PR-1 expression is NPR1-dependent. Although required for gene activation, NPR1 is not able to activate PR-1 in the absence of SA. In uninfected Arabidopsis plants, overexpression of NPR1 does not lead to elevated level of PR-1 expression, despite the presence of nuclear NPR1 (Després et al., 2000; Mou et al., 2003). Further, although NPR1 harbours a transactivation domain in the C-terminal, the transactivation activity is SA-dependent. Plant transcription assays show that the NPR1-DB (Gal4 DNA binding domain) fusion protein activates the expression of reporter gene in the SA-treated Arabidopsis leaves, while it does not in the untreated leaves (Rochon et al., 2006). Thus, SA is required to enable NPR1’s function as a transcriptional coactivator.
SA is likely to regulate the interaction of NPR1 with TGA2 in planta. Even though NPR1 interacts with TGA2 in an SA-independent manner in the yeast-two-hybrid system, it may not be the case in plants (Fan and Dong, 2002). Chromatin Immunoprecipitation (ChIP) results indicates that NPR1 and TGA2 are independently recruited to the PR-1 promoter in untreated plants. The interaction between NPR1 and TGA2 only occurs in the SA-treated leaves (Rochon et al., 2006). It is possible that NPR1 is associated with other DNA binding protein(s) on the PR-1 promoter, which prevents formation of NPR1-TGA2 complexes in the unstimulated tissue. Since nuclear NPR1 is constantly targeted by cullin3 (CUL3)-based E3 ligases for proteasomal degradation, free NPR1 and TGA2-bound NPR1 may be rapidly degraded (Mou et al., 2003). The NPR1 recruited to the PR-1 promoter may be, however, protected by its associated partners. This proportion of NPR1 may be in a “ready-to-go” state for SA-inducible activities (Rochon et al., 2006).

The presence of SA is also critical to the full-scale induction of SAR genes and SAR deployment. It has been reported that SA promotes the phosphorylation of NPR1 at two N-terminal residuals, Ser11 and Ser15. This phosphorylated form of NPR1 has enhanced binding affinity for the E3 ubiquitin ligase scaffold protein CUL3A, and hence undergoes faster ubiquitin-mediated proteasomal degradation. Surprisingly, this enhanced degradation of NPR1 is required for the full induction of SAR genes (Spoel et al., 2009). It has been suggested that NPR1 may be phosphorylated by a kinase attached to RNA polymerase II (Pol II) through mediating the assembly of the Pol II
initiation complex. After the initial transcriptional activation, the phosphorylated NPR1 is quickly degraded. In such, “exhausted” NPR1 is replaced by fresh NPR1 in order to prepare for the next round of transcription initiation (Mukhtar et al., 2009; Spoel et al., 2009).

2.2.3 Perception of salicylic acid

Ever since SA was demonstrated as an immune signal, extensive efforts have been devoted to the search for the SA receptor(s). An SA receptor should be able to specifically bind to SA, and activate downstream signaling events. Candidates that fulfill such requirements could be defined as a receptor for SA. Therefore, searching for SA-binding candidates is the first step in the identification of the SA receptor(s).

In the last decades, several SABPs have been identified using biochemical approaches. The first characterized SABP, a tobacco catalase, binds SA with low affinity. The apparent dissociation constant (Kd) is 14 μM (Chen and Klessig, 1991). The binding of SA inhibits the hydrogen peroxide (H₂O₂) breakdown. Additionally, SA analogs with higher activity of activating PR genes show stronger inhibition of the catalase activity, and also are more effective in competing with radioactive-labeled SA for SABP binding. Therefore, H₂O₂ was suggested to function as a secondary messenger downstream SA during SAR (Chen et al., 1993). However, later studies argued against this by showing that H₂O₂ does not induce PR-1 gene expression in the absence of SA, and that pathogen-induced SA accumulation does not cause any significant change in the overall catalase activity (Bi et al., 1995; Neuenschwander et al., 1995). Further investigation has revealed
that SA has an affinity for iron-containing enzymes due to its ability to chelate iron. Accordingly, enzymes such as aconitase, lipoxidase and peroxidase could also bind to SA (Rüffer et al., 1995). All these lines of evidence indicate that this SABP is not a SA receptor.

Some other SABPs have also been characterized, but none of them is a receptor for SA. For example, SABP2 isolated from tobacco is a high affinity (Kd = 90 nM) SABP that possesses MeSA esterase activity. It catalyzes the conversion of MeSA to SA, and SA in turn binds to SABP2 as a product inhibitor to inhibit this reaction (Dempsey and Klessig, 2012; Forouhar et al., 2005). SABP3 in tobacco is a chloroplast-localized carbonic anhydrase. Its binding affinity for SA is very low (Kd = 3.7 mM) (Slaymaker et al., 2002). SABP3 is unlikely to be a SA receptor. Although being synthesized in the chloroplast, SA has been known to exert its effect on PR gene activation and SAR establishment mainly in the cytoplasm and the nucleus (Fu and Dong, 2013). Furthermore, the de novo synthesized SA needs to be exported from the chloroplast via the MATE-like transporter EDS5. Characterization of the Arabidopsis eds5 mutant shows that SA is retained in the chloroplast, and hence renders eds5 SA-deficient (Serrano et al., 2013).

In addition to biochemical approaches, forward genetic screenings for SA-insensitive Arabidopsis mutants were also carried out in order to identify the SA receptor(s). The loss-of-function mutations in NPR1 alleles are responsible for the insensitivity of those mutants to SA. This suggests a potential role of NPR1 in SA sensing (Durrant and Dong, 2004; Ryals et al., 1996). In addition, it has been demonstrated that SA can activate the cellular
function of NPR1, as well as influence the interaction of NPR1 with its associated signaling components (Maier et al., 2011; Rochon et al., 2006). Both genetic and biochemical evidence indicate that NPR1 is a good candidate as a receptor for SA.

In spite of the critical role of SA in plant immunity, the mechanism by which SA is perceived in plants during SAR still remains to be elucidated. Further investigations are in demand to determine whether or not NPR1 is a SA receptor.

2.3 Copper homeostasis influences plant immunity

Emerging evidence suggests that Cu homeostasis plays a role in plant immunity (Binder et al., 2010; Chen et al., 2014; Liu et al., 2015; Yuan et al., 2010). Since NPR1 uses Cu as a cofactor to facilitate SA perception (Chapter 3), it is very likely that Cu homeostasis is also involved in SAR by controlling Cu delivery to NPR1.

2.3.1 Copper transport

Copper has been known as an important micronutrient for both animals and plants. It is involved in many aspects of physiological activities, serving as a cofactor or a scaffold for enzymes, transcription coactivators and other biologically important proteins (Festa and Thiele, 2011). However, excessive copper could be harmful owing to its redox potential. Cycling between Cu(II) and Cu(I) leads to the production of ROS (Schützendübel and Polle, 2002). Thus, the availability of copper ions must be and is tightly controlled within the cell, with approximately one free copper ion per cell (Rae et al., 1999).
This is achieved by: (i) the coordination of copper ions by proteins such as copper chaperones, metallothioneins, and also by small molecules like nicotianamine and glutathione (Cobbett and Goldsbrough, 2002; Curie et al., 2009; Pilon et al., 2006); in cooperation with (ii) the translocation by transporters in order to maintain the intracellular levels of copper (Peñarrubia et al., 2015).

2.3.1.1 Copper transporters

In nature, plants utilize two forms of Cu ions, Cu(II) and Cu(I). The uptake of Cu ions has different mechanisms. In an aerobic environment, Cu(II) is the most abundant form. The intake of Cu(II) by plants from soil has been suggested to be mediated by the ZIP (Zinc-regulated transporters, Iron-regulated transporter-like Proteins) family of metal transporters (Peñarrubia et al., 2015). The substrate promiscuity allows ZIPs to transport Zinc (Zn), Iron (Fe), Cu and possibly other metal ions. In Arabidopsis, the ZIP gene family contains 15 members. Among these ZIP genes, the expression of ZIP2 and ZIP4 correlates with the Cu levels in plants (Wintz et al., 2003). Microarray and Real-time PCR results showed that the expression level of ZIP2 and ZIP4 increases under Cu-deficiency conditions and decreases when Cu is sufficient. Further, the growth defect phenotype of yeast strain Δctr1, which is caused by the lack of high affinity Cu transport, is rescued by expressing either ZIP2 or ZIP4 (Wintz et al., 2003). Although these lines of evidence strongly suggest a role of ZIP2/4 in Cu(II) transport, this has not yet been proved in planta.
The transport of Cu(I) relies on a more sophisticated system. Eukaryotic cells from yeast, plants, and mammals, including humans, utilize a conserved family of copper transporter (CTR) proteins to mediate high-affinity Cu transport from the outside of the cell into the cytosol (Lee et al., 2002). CTR proteins oligomerize and assemble as trimers to form a channel-like permeation pathway within the membrane to transport copper across the phospholipid bilayer (Aller et al., 2004). The similar structure shared by the CTR family members consists of three putative transmembrane (TM1-3) regions, with the N-terminus located in the extracellular space and the C-terminus located in the cytosol (Puig et al., 2002). In the CTR trimer, three TM2 from each monomer are closely positioned in the center, forming the core channel. The Methionine (Met) residues of the conserved MXXXM motif on TM2 contribute directly to copper conduction through the pore by providing coordination sites for Cu(I) (De Feo et al., 2009). TM3 is structurally important to the CTR trimer. It is involved in the CTR oligomerization and the tight helix packing in the CTR trimer complex (Aller et al., 2004; De Feo et al., 2009). Most CTRs possess methionine-rich motifs (Mets motifs) in their extracellular N-terminus. Since the deletion of the Mets motifs reduce the activity of Cu(I) transport especially when Cu(I) is limited, the N-terminus has been suggested to capture Cu(I) for subsequent transport (Puig et al., 2002). After Cu(I) is transported across the phospholipid bilayer, it is held in the C-terminus by the cysteine-rich CXC motifs and ready for further transport (Xiao and Wedd, 2002). *Arabidopsis* carries six genes encoding CTR-like copper transporters (COPT1-6). COPT1,
COPT2 and COPT6 have been demonstrated to be plasma membrane transporters. They are responsible for the Cu(I) uptake in root tip/pollen, root elongation zone and vasculature of green tissues respectively (Jung et al., 2012; Perea-García et al., 2013; Sancenón et al., 2004). COPT5 is localized to the prevacuolar/vacuolar membrane, where it exports Cu(I) into cytosol (Garcia-Molina et al., 2011; Klaumann et al., 2011). The biological function of COPT3 and COPT4 is still not clear. Notably, the transport of Cu(I) has been suggested to be the main route of Cu uptake under Cu deficiency conditions (Peñarrubia et al., 2015). The efficiency of this type of Cu transport largely depends on the reduction of Cu(II) into Cu(I) at the biological membrane. Genetic and biochemical evidence suggests that this reaction is catalyzed by metalloreductases such as Arabidopsis Ferric Reductase Oxidase 4 (FRO4) and FRO5 (Bernal et al., 2012).

In addition to ZIPs and COPTs, Heavy Metal P-type ATPase (HMA) is another important type of Cu transporters. HMAs are key players that maintain metal homeostasis in most organisms. Members in the P_1B-type ATPase subgroups have been shown to transport various metals including Cu, silver (Ag), Zn, cobalt (Co), cadmium (Cd) and lead (Pb) ions with distinct selectivity (Williams and Mills, 2005). P_1B-type ATPases share a conserved structure that consists of eight TMs, an A-domain between TM4 and TM5, a P-domain and an N-domain between TM6 and TM7 (Argüello et al., 2007). The nucleotide of ATP binds to the N-domain, and the phosphate group is cleaved within the P-domain. This leads to the catalytic phosphorylation of the aspartic acid (D) in the conserved P-domain DKTGT sequence, and allows
for the insertion of the A-domain between the P- and N-domain. The resulting conformational changes in the TMs cause a drastic decrease in the binding affinity of metal to its binding motif CPX, which facilitates the metal moving across the membrane (Argüello et al., 2007; Bublitz et al., 2011). Five members of the eight Arabidopsis HMA s (HMA1-8) have been demonstrated to transport Cu. AtHMA5, AtHMA6/PAA1, AtHMA8/PAA2 and AtHMA7/RAN1 in the P_{1B-1}-type ATPase subgroup transport Cu(I)/Ag(I) at their distinct location within the cell. AtHMA7 are localized to the membrane of post-Golgi compartment, where it pumps Cu(I) inside and allows for the subsequent Cu-coordination in ethylene receptor Ethylene Response 1 (ETR1), a step required for ethylene-ETR1 binding (Hirayama et al., 1999). AtHMA6 is responsible for the Cu(I) delivery into the chloroplast, and AtHMA8 subsequently import Cu(I) into thylakoid to facilitate the maturation of plastocyanin, a critical Cu-binding protein involved in photosynthetic electron transfer (Boutigny et al., 2014). AtHMA5 is localized to the plasma membrane. It has a putative role in Cu detoxification by pumping Cu(I) out of the cell (Williams and Mills, 2005). AtHMA1 belongs to the P_{1B-4}-type ATPase subgroup whose members exhibit broader substrate specificity. It can transport Cu(II) in addition to the typical substrates such as Co(II) and Zn(II). It is also responsible for the Cu import into the chloroplast (Boutigny et al., 2014).

2.3.1.2 Nicotianamine-metal transporters

The above-mentioned transporters mobilize Cu by directly binding to Cu ions. In contrast, another type of plant metal transporters, namely Yellow
Stripe-like proteins (YSLs), transport Cu(II) which is complexed with nicotianamine (NA) or its derivatives phytosiderophores (PS) (Curie et al., 2009).

The plant metabolite NA is an endogenous metal chelator required for maintaining metal homeostasis in plants. In the Poaceae, NA can be further modified into PS, which are also metal chelators that facilitate Fe uptake from soil. However, NA is the final product in dicotyledonous and non-grass monocotyledonous plants, since neither PS-synthesizing gene nor PS has yet been found in these plant species. It has been demonstrated that NA is involved in the xylem/phloem transport of biologically important metals such as Fe(II), Fe(III), Cu(II), Ni(II) and Zn(II) (Curie et al., 2009). In the NA-free tomato mutant chloronerva, the root-to-shoot Cu transport is disrupted. The leaves suffer from Cu deficiency, while the roots accumulate Cu normally. The Cu deficiency in leaves is restored when the NA-free tomato plants are grafted onto the wild-type rootstocks, or when exogenous NA is applied to the NA-free mutants (Ling et al., 1999; Pich and Scholz, 1996; Pich et al., 1994). Both genetic and biochemical evidence suggests a critical role of NA in the intercellular and interorgan Cu transport.

The NA-dependent Cu(II) transport has been suggested to be mediated by the YSL transporters. The YSLs form a distinct subgroup in the oligopeptide transporter (OPT) family (Curie et al., 2009). The first characterized member in the YSL family is the maize Yellow Stripe 1 (ZmYS1) protein (Curie et al., 2001). Heterologous expression of ZmYS1 in the Cu-deficient yeast strain Δctr1 restores the growth defect only in the
presence of PS (mugineic acid) (Roberts et al., 2004). This is corroborated with the PS-Cu(II) transport activity of ZmYS1 observed in the two-electrode voltage clamp analysis using ZmYS1-expressing Xenopus laevis oocytes. 2’-deoxymugineic acid (DMA)-Cu(II) complex, but rather DMA alone, is transported by ZmYS1 (Schaaf et al., 2004). Other YSLs such as barley HvYS1 and rice OsYSL15 have also been shown to possess such PS-metal transport ability (Inoue et al., 2009; Murata et al., 2006). In non-grass plant species, YSLs are likely to transport NA-Cu(II). This is supported by the fact that AtYSL2 is able to complement the mutation of the yeast CTR1 Cu transporter only in the presence of NA (Didonato et al., 2004). Although direct evidence of YSLs transporting NA-Cu(II) is still lacking, the involvement of YSLs in Cu transport has been proved. AtYSL1 and AtYSL3, localized mainly to the plasma membrane of xylem parenchyma cells, are involved in the long-distance movement of Cu within the plant (Waters et al., 2006). Elevated Cu levels are found in the leaves of the AtYSL3-overexpressing plants (Chu et al., 2010). This suggests a role of AtYSL1/3 in the root-to-shoot Cu transport. Additionally, the loading of Cu into seeds also requires the presence of AtYSL1/3. Low Cu levels are found in the seeds of the ysl1ysl3 double knockout plants. This defect of Cu loading is partially rescued in the ysl1ysl3 scions grafted onto the wild-type rootstocks (Chu et al., 2010). Genetic and biochemical evidence strongly suggests that YSLs are important players involved in Cu movement in plants.
2.3.1.3 Copper transporters involved in plant immunity

Increasing evidence suggests that copper homeostasis plays a role in the host-pathogen interaction. The plant hormone jasmonate and ethylene are known to be involved in the immune responses to necrotrophic pathogens. Ethylene perception requires the coordination of Cu(II) in the ethylene receptor ETR1. The delivery of Cu to ETR1 is mediated by the Cu transporter AtHMA7/RAN1 (Binder et al., 2010).

Some pathogens can also take advantage of plant Cu homeostasis to enhance their virulence. In *Xanthomonas oryzae pv oryzae* (Xoo)-infected rice, the expression of a membrane localized protein XA13 is upregulated by transcriptional activator-like effectors secreted by Xoo. XA13, COPT1 and COPT5 are likely to form a trimeric complex that is responsible for the export of Cu from the xylem. The resulting decreases Cu levels within the xylem promotes the growth and the spread of Xoo (Yuan et al., 2010).

Recently, another Cu transporter has been associated with disease resistance. Chen et al. reported that the Arabidopsis YSL3 is involved in plant pathogen defense. The expression of YSL3 is induced by SA and pathogen inoculation in an NPR1-dependent manner. Pathology tests showed that *ysl3* knockout mutants exhibit enhanced susceptibility to *Pst* DC3000 compared to the wild-type control plants. This indicates that YSL3 plays a role in the SA-regulated immune response (Chen et al., 2014).

2.3.1.4 Copper chaperones

After Cu is transported across biological membranes by transporters, Cu chaperones deliver Cu to Cu-binding proteins. In *Arabidopsis*, Antioxidant
protein 1 (ATX1) and Copper Chaperone (CCH) are two homologs of the yeast Atx1 (Himeblau et al., 1998; Mira et al., 2001). They are responsible for the delivery of Cu(I) to copper transporter HMA5, in order to remove Cu from the cytosol. In yeast, Cytochrome c Oxidase Copper Chaperone 17 (COX17) delivers Cu(I) to two other chaperones, Synthesis of Cytochrome c Oxidase (SCO1) and COX11. SCO1 and COX11 then insert Cu(I) into the two Cu-binding sites (CuA and CuB) in cytochrome c oxidase (Robinson and Winge, 2010). The Arabidopsis COX17 has been found to be homologous to the yeast COX17 (Balandin and Castresana, 2002). SCO1 and COX11 also have their homologs in Arabidopsis, Homolog of the Copper Chaperone SCO1 (HCC1) and COX11 respectively (Carr et al., 2002; Steinebrunner et al., 2011). It is likely that the mechanism of metallation of cytochrome c oxidase is conserved in plants. Copper Chaperone for SOD (CCS) is responsible for the delivery of Cu to Cu/Zn SODs. Cu chaperones possess the conserved copper-binding site, MXCXXC. The two Cys are suggested to coordinate Cu(I) in trigonal or diagonal bonding. The methionine may have no role in copper transfer although it is highly conserved (Harrison et al., 1999; Pufahl et al., 1997). COX17 is an exception, which does not have the typical MXCXXC sequence, but rather uses CCXC as the copper-binding site. Cu delivery is realized through direct protein-protein interaction between a Cu chaperone and a target Cu-binding protein. Ligand exchange happens when the Cu ion on the chaperone is properly positioned and in close proximity with the Cu-binding site on the target protein (Harrison et al., 1999).
2.3.2 Regulation of copper transport

Since Cu is an important micronutrient for plants, efforts have been devoted to study how plants cope with Cu scarcity. Plants undergo transcriptional reprogramming under Cu-deficient conditions, including downregulation of Cu-binding proteins such as Cu/Zn SODs and laccases (LACs), and upregulation of Cu transporters (Bernal et al., 2012; Peñarrubia et al., 2015). In *Arabidopsis*, such Cu-related transcriptional responses are mediated by a Zn finger transcription factor, namely SQUAMOSA Promoter Binding Protein-like 7 (SPL7) (Bernal et al., 2012; Yamasaki et al., 2009). SPL7 is a transcriptional activator which exerts its activity through binding to the Cu-response element (GTAC motifs) in the promoters of Cu-responsive genes under Cu-deficient conditions. For example, the expression of COPT1/2 and ZIP2 were induced by Cu-depletion in an SPL7-dependent manner (Yamasaki et al., 2009). SPL7 could also indirectly downregulate Cu-responsive genes by controlling microRNA expression. MicroRNAs such as miR398 and miR408, which target Cu/Zn SOD1/2 and LAC3/12/13 mRNAs respectively for degradation, are upregulated by SPL7 under Cu-deficiency (Abdel-Ghany and Pilon, 2008; Yamasaki et al., 2009; Yamasaki et al., 2007). This is suggested to save Cu for other Cu-binding proteins that are essential for survival (e.g., plastocyanin).

SPL7 seems to have no biological role when Cu is sufficient. However, when Cu is scarce, SPL7 controls the flow of Cu to its final targets by enhancing active Cu transport and reducing the biosynthesis of non-essential cupric proteins. (Yamasaki et al., 2009). This suggests that Cu levels in
plants can be sensed by an as yet unknown mechanism. How SPL7 senses (or is activated by) Cu deficiency is still not clear. Several mechanisms have been proposed in the last decade. It was originally postulated that SPL7 is activated by the replacement of Cu(I) by Zn(II) in the Zn finger DNA binding domain (SBP). This is based on the fact that the SBP of Copper Response Regulator 1 (CRR1), an SPL7 ortholog in *Chlamydomonas reinhardtii*, is able to bind Cu(I) $10^5$-fold more tightly than Zn(II). The DNA binding activity of SBP is effectively inhibited in the presence of excessive Cu (Sommer et al., 2010). Further studies revealed that SPL7 is localized to the ER membrane through its operative transmembrane domain (TMD). Cu deficiency-induced ER stress results in the cleavage of TMD, enabling the nuclear localization of SPL7 (Garcia-Molina et al., 2014). Whether SPL7 senses Cu directly, or through interaction with other proteins requires further investigation.
CHAPTER 3 – The *Arabidopsis* NPR1 Protein Is a Receptor for the Plant Defense Hormone Salicylic Acid

**Contributions**

This manuscript was the product of my primary research project. I conducted the experiments pertaining to Figure 1, Figure 2: F to H, Figure 3, and Supplemental Figure S1. I was directly involved in the development of hypotheses, experimental design, data analysis and manuscript editing.

This manuscript was published in *Cell Reports* (2012), 1(6): 639-647.

**Yue Wu¹, Di Zhang¹, Jee Yan Chu¹, Patrick Boyle¹,³, Yong Wang², Ian D. Brindle², Vincenzo De Luca¹ and Charles Després¹**

¹Department of Biological Sciences, Brock University, 500 Glenridge Avenue, St. Catharines, Ontario, Canada L2S 3A1

²Department of Chemistry, Brock University, 500 Glenridge Avenue, St. Catharines, Ontario, Canada L2S 3A1

³Present address: Boyce Thompson Institute for Plant Research, Ithaca, NY 14853, USA

**Acknowledgement**

C.D. and V.D.L. are supported by the National Science and Engineering Research Council (NSERC) Discovery Grant and P.B. by the Ontario (OGS) and NSERC Graduate Scholarship (P.B.) programs. I.D.B. is supported by Environment Canada and the Ontario Ministry of the Environment under the
Canada-Ontario Agreement and the Best in Science programs, respectively. Lab equipment (C.D. and V.D.L) and the ICP-MS (I.D.B) were purchased with funds from the Canada Foundation for Innovation and the Ontario Innovation Trust. V.D.L. is a Tier 1 Canada Research Chair in Plant Biotechnology.

3.1 ABSTRACT

Salicylic acid (SA) is an essential hormone in plant immunity, but its receptor has remained elusive for decades. The transcriptional coregulator NPR1 is central to the activation of SA-dependent defense genes, and we previously found that Cys521 and Cys529 of *Arabidopsis* NPR1’s transactivation domain are critical for coactivator function. Here, we demonstrate that NPR1 directly binds SA, but not inactive structural analogs, with an affinity similar to that of other hormone-receptor interactions and consistent with *in vivo* *Arabidopsis* SA concentrations. Binding of SA occurs through Cys521/529 via the transition metal copper. Mechanistically, our results suggest that binding of SA causes a conformational change in NPR1 that is accompanied by the release of the C-terminal transactivation domain from the N-terminal autoinhibitory BTB/POZ domain. While NPR1 is already known as a link between the SA signaling molecule and defense-gene activation, we now show that NPR1 is the receptor for SA.

3.2 INTRODUCTION

Salicylic acid (SA) is an endogenous phytohormone in the deployment of systemic acquired resistance (SAR), a broad-spectrum and long-lasting immune response activated by avirulent pathogens (Vlot et al., 2009). Its
deployment is monitored through the marker gene *PR-1* (Ward et al., 1991), whose activation requires the recruitment of an SA-dependent transcriptional enhanceosome to its promoter (Rochon et al., 2006). The enhanceosome contains members of the TGA2 clade of bZIP transcription factors (Zhang et al., 2003) and the transcriptional coactivator NPR1 (Rochon et al., 2006), the central regulator of SAR and SA-dependent gene activation (Cao et al., 1997; Ryals et al., 1997). TGA2 is a transcriptional repressor and thus requires a coactivator to effect gene activation (Rochon et al., 2006). NPR1 provides a dual function in the enhanceosome. First, its N-terminal region contains a BTB/POZ domain that interacts with and negates the function of the TGA2 repression domain (Boyle et al., 2009). Second, NPR1 harbors in its C-terminal region a transactivation domain (Figure S1), which contains two cysteines (Cys521 and Cys529) required for the activating function of the enhanceosome (Rochon et al., 2006).

Endogenous NPR1 localizes to both the nucleus and the cytosol (Després et al., 2000) and nuclear localization is critical to activate *PR-1* (Kinkema et al., 2000). A fraction of the nuclear NPR1 population acts as a latent coactivator, which is recruited under noninducing conditions to the *PR-1* promoter (Rochon et al., 2006). There thus exists an uncharacterized mechanism by which the NPR1 transactivating domain remains occluded under noninducing conditions and gets unveiled during SA-dependent gene activation. Furthermore, although genetic analyses have revealed many genes involved in SA signaling (Vlot et al., 2009), the receptor responsible
for sensing SA and leading to direct or indirect NPR1 activation remains elusive.

While enzymes, such as catalase (Chen et al., 1993), peroxidase (Durner and Klessig, 1995), and methyl-salicylate esterase (Forouhar et al., 2005), have been shown to directly interact with SA, their proposed role in SAR has been controversial (Attaran et al., 2009; Bi et al., 1995; Kvaratskhelia et al., 1997; Neuenschwander et al., 1995). SA was originally portrayed as a catalase and peroxidase inhibitor, leading to the generation of H$_2$O$_2$ and the production of PR proteins (Chen et al., 1993). However, H$_2$O$_2$ was later shown not to be a second messenger acting downstream of SA (Bi et al., 1995; Neuenschwander et al., 1995), invalidating the role of catalase and peroxidase as SA receptors for PR gene activation. Whereas methyl-salicylate esterase has been shown to play a role in tobacco (Forouhar et al., 2005), it clearly has no role in SAR in Arabidopsis (Attaran et al., 2009). Most importantly, these enzymes are not classical transcription regulators and therefore, they are unlikely to regulate gene expression directly. Therefore, in Arabidopsis, the SA receptor leading to PR gene activation remains elusive. The simplest model linking gene activation with SA perception is one in which SA directly interacts with NPR1 to effect gene activation.

It has been more than a decade since NPR1 was identified to be the key regulator of SAR in Arabidopsis. However, no one has reported a direct interaction between the NPR1 protein and SA, excluding NPR1 as the receptor for SA. Here we show that NPR1 specifically interacts with SA and the synthetic SAR inducer benzo(1,2,3)thiadiazole-7-carbothioic acid S-
methyl ester (BTH). We demonstrate that NPR1 binds both SA and copper through Cys521/529. Removal of metals through chelation abolishes the binding of SA by NPR1, even in the presence of intact Cys521/529. We show that the NPR1 oligomers can be disassembled by a direct binding of SA, effected by a conformational change in the C-terminal transactivation domain, which leads to a decrease in the affinity of this domain for the N-terminal BTB/POZ of NPR1. Finally, we show that the BTB/POZ domain of NPR1 is inhibitory to the function of the C-terminal transactivation domain. Broadly, we critically reveal that the Arabidopsis NPR1 is the SA receptor linking SA perception and transcription activation. Furthermore, the mechanism put forth in this paper has implications in all fields studying small molecule-protein interaction as it divulges an unprecedented mode of binding through coordinated metals.

3.3 RESULTS

3.3.1 NPR1 Binds Specifically to SA

To test whether NPR1 can bind SA directly, we coupled NPR1 to a solid phase and incubated it with $[^{14}\text{C}]$SA, followed by washes to remove unbound ligands and the counting of bound ligands. This method did not yield a measurable equilibrium dissociation constant (Kd) (Figure 1A).

Since washes could re-equilibrate SA between the solid and mobile phases, we opted to use an equilibrium method that would avoid such a potential pitfall. Using equilibrium dialysis (EqD) (Piscitelli et al., 2010), we determined that NPR1 and $[^{14}\text{C}]$SA could interact with each other, the
amount of SA bound to NPR1 being close to 4-orders of magnitude above a no-protein experiment (Figure 1B). From these data, a low apparent Kd of 140 ± 10 nM was calculated (137 ± 13 nM using the saturation curve in Figure 1C). The data were best-fitted to a single-site-binding rectangular hyperbola (Figure 1C), indicating that SA binds to one class of binding sites in NPR1. The maximum binding (Bmax) was 0.96 ± 0.01 mol SA per mol NPR1. Figure 1D shows a Scatchard plot of the data.

We also tested which of the two domains (BTB/POZ or C-terminal transactivation domain [construct Δ513]) can directly interact with SA. The data demonstrated that the binding of Δ513 to SA (Kd = 1.49 ± 0.02 µM) is more than two orders of magnitude above that of the BTB/POZ (Kd = 597 ± 14 µM) (Figure 1B). The NPR1 Kd is comparable to the Kd found for other plant-hormone receptor-ligand interactions and is in accordance with the in vivo SA concentration in Arabidopsis (Table S1).

Homologous and heterologous competitive binding curves (Figures 1E and 1F) indicated that the structurally related inactive analogs (Figure 1G), i.e., catechol (Delaney et al., 1994), methyl-salicylate (Me-SA) (Attaran et al., 2009), 4-hydroxy benzoic acid (4-OH BA) (Bi et al., 1995), and 3-hydroxy benzoic acid (3-OH BA) (Conrath et al., 1995), did not interact with NPR1 with the same affinity as SA. In contrast, the structurally related active analogs of SA (Figure 1G), 4-chloro SA, 5-chloro SA, and 3,5-dichloro SA (Conrath et al., 1995), could bind NPR1 with a similar or slightly better affinity than SA (Figure 1F), consistent with their capacity to trigger PR-1 expression in Arabidopsis (Figure 1H). This excellent affinity, saturability, and
chemical specificity of NPR1 for SA support a model in which NPR1 is an SA receptor.

From these data, one can deduce that a hydroxyl group in ortho position to a free carboxylate on the aromatic ring are two structural elements required for binding to NPR1. This inference agrees with the result in Figure 1E, which shows NPR1 has a similar or slightly higher affinity for BTH than for SA. BTH, containing two sulfur atoms in positions geometrically equivalent to the oxygens in the carboxylate and hydroxyl group of SA (arrows on BTH; Figure 1G), is a synthetic SAR and PR-1 expression inducer (Görlach et al., 1996; Lawton et al., 1996) (Figure 1H). However, a look at 2,6-dichloroisonicotinic acid (INA) reveals that though it is similar to 3,5-dichloro SA, it lacks the hydroxyl group (arrow on INA; Figure 1G). Therefore INA, as predicted, could not bind NPR1 (Figure 1E) and it was a poor inducer of PR-1 expression in Arabidopsis (Figure 1H), 42 times less effective than an identical concentration of SA (300 µM) and ten times less effective than the weakest active SA analog, 4-chloro SA (Figure 1I). These data suggest that INA may activate PR-1 through a mechanism different from that of SA. The binding data in Figure 1 have been validated by a second equilibrium approach, scintillation proximity assay (SPA) (Figure S1).

3.3.2 NPR1 Binds SA through Cys521/529 via the Transition Metal Copper

Cys521/529 of NPR1 is required, along with SA treatment, for the activation of PR-1 in vivo and for the transactivating function of Δ513 and the full-length NPR1 (Rochon et al., 2006). Since SA can coordinate transition
metals through its oxygen atoms (Palanisami et al., 2006), we asked whether Δ513 could interact with a transition metal and whether this interaction would be dependent on Cys521/529. To do so, HA-tagged Δ513 was passed through an immobilized metal-affinity column bound to Ni$^{2+}$ (Ni-NTA) and eluted with imidazole. Despite the absence of a His-tag on Δ513, the protein interacted with the metal-bound matrix and was eluted with imidazole just like a His-tagged protein would (Figure 2B). Chelation of the Ni$^{2+}$ by EDTA abolished the recruitment to the NTA matrix (Figure 2C), demonstrating that the binding of this protein is metal dependent. The recruitment of this protein to the Ni-NTA matrix was also abolished when Cys521/529 were both mutated to serine residues (Δ513 S521/529) or if the protein was further deleted by 20 amino acids (Δ533), suggesting that Cys521/529 are critical to the transition-metal-binding activity of NPR1 (Figures 2D and 2E).

To confirm that SA-binding occurs through Cys521/529 and is metal dependent, we tested the capacity of both full-length NPR1 S521/529 and Δ513 S521/529, as well as wild-type Δ513 in the presence of EDTA, to interact with SA, using EqD (Figure 2F). Both metal chelation and the Cys521/529 mutations drastically reduced the SA binding to the C terminus of NPR1 by several orders of magnitude (Figure 2F). Using these data, an apparent Kd of 1.23 ± 0.3 mM for Δ513 S521/529, and ≥125 mM for Δ513 + EDTA, could be calculated. The results of Figure 2 support a model in which SA binds to NPR1 via Cys521/529 through the coordination of SA by a transition metal.
We next asked which of the transition metals (defined as d-block elements of the periodic table) most commonly found in living organisms might be associated with NPR1 in vivo. First, purified Strep-tagged Δ513 from *Escherichia coli* was analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) (Wang and Brindle, 2011) (Table S2). The data indicated that Δ513 associated preferentially with copper (Figure 2G), and that the mutations of Cys521/529 severely curtailed metal interaction. Second, full-length wild-type NPR1 was immunoprecipitated from *Arabidopsis* using anti-NPR1 antibodies before metal analysis by ICP-MS (Table S2). As a negative control, plants expressing a variant of full-length NPR1 lacking Cys521/529 were used (Rochon et al., 2006). The results (Figure 2H) were consistent with the observations made from *E. coli*-produced proteins in that NPR1 associated preferentially with copper and to a lesser extent with nickel. Mutations of Cys521/529 severely curtailed the capacity of NPR1 to interact with these metals. Contamination by manganese and zinc was present in *Arabidopsis* extracts, but their detection did not depend on Cys521/529.

### 3.3.3 The Conformation of NPR1 and Δ513 Are Altered by SA

To explore the effect of SA on the conformation of NPR1, we performed gel filtration experiments (Figure 3). In the absence of SA, NPR1 eluted in the void volume of a Sephacryl S300 column (Figure 3A). Upon treatment with SA, NPR1 redistributed to the included volume (Figure 3B) with a stoichiometry consistent with that of a dimer (Tables S3 and S4). Mutations of Cys521/529 or chelation by EDTA negated the NPR1 conformation change due to SA treatment (Figures 3C–3E), confirming the requirement for
Cys521/529 and a metal for SA interaction (Figure 2F). A chemical specificity test using catechol, 4-OH BA, and Me-SA indicated that these inactive structural analogs did not alter the conformation of NPR1 (Figures 3F–3H), consistent with their reduced capacity to interact with NPR1 (Figure 1E). Finally, treatment of NPR1 with the reducing agent DTT did not induce a redistribution of the protein to the included volume (Figure 3I), indicating that reducing conditions were not required or sufficient for the SA-induced NPR1-redistribution observed here. A typical Coomassie-stained gel of the void fraction revealed that NPR1 and NPR1 S521/529 were the major protein components of the void (Figure 3J), and thus the oligomers were unlikely to be due to the presence of contaminating *E. coli* proteins.

We then addressed whether NPR1-dependent oligomers are present on DNA *in vivo* by combining chromatin crosslinking, gel filtration, and qPCR (the 3C Method). The rationale was that, if an NPR1-dependent oligomer forms on the PR1 promoter *in vivo*, we should be able to detect the presence of PR1 by qPCR in the void fraction of an S300 after the chromatin has been crosslinked and sheared by sonication. Figure 3K indicates that in wild-type (WT) plants, such an oligomer formed on the *PR-1* promoter (in the region −734 to −833) in the absence of SA (water control), but not after SA treatment. Repeating the experiment using *npr1*-3 mutants demonstrated that this oligomer was NPR1 dependent. Treatment of wild-type *Arabidopsis* with the inactive SA analog, 4-OH BA, did not reduce the amount of NPR1-dependent oligomer. These *in vivo* data are consistent with the *in vitro* data of Figures 3A, 3B, and 3G. We could not use BTH treatment in the *in vitro*
chromatography due to its low solubility in water. However, performing the 3C method on plants treated with BTH revealed that, like SA, this active analog also reduced the amount of NPR1-dependent oligomer on the PR-1 promoter (Figure 3K). In contrast, INA, which did not interact with NPR1 in vitro and did not activate PR-1 to the same extent as SA or BTH (Figures 1E and 1H), did not affect the NPR1-dependent oligomer on the PR-1 promoter (Figure 3K). This result further suggests that INA may not be a functional analog of SA.

We also investigated the conformation of Δ513 by gel filtration. Before and after SA treatment, Δ513 was found in the included volume of a Sephacryl S100 column (Figures 3L and 3N). The stoichiometry of the untreated Δ513 was consistent with that of both a dimer and a trimer (Figures 3L and 3M), while the stoichiometry of the SA-dependent redistributed form of Δ513 was consistent with that of a dimer (Figures 3N and 3O; Tables S5 and S6). However, the elution volumes of the dimer in the untreated (58.78 ml) versus the SA-treated (57.18 ml) Δ513 were different and therefore indicated that these dimers may not have the same conformation. The elution volume of the SA-dependent dimer was closer to that of the theoretical dimer (57.26 ml).

### 3.3.4 SA Disrupts the BTB/POZ-Transactivation Domain Interaction

When tethered to the Gal4 DNA-binding domain (DB) in an in vivo plant transcription assay, the transactivation domain of NPR1 (construct Δ513) can activate transcription in the absence of SA treatment, but tethering of the full-length NPR1 did not (Rochon et al., 2006) (Figure 4A), suggesting the
presence of an autoinhibitory domain in NPR1. Since BTB/POZ domains can be autoinhibitory (Bardwell and Treisman, 1994; Espinás et al., 1999; Katsani et al., 1999; Pinte et al., 2004), we tested whether the NPR1 BTB/POZ can interact with the NPR1 transactivation domain. We first used an *Arabidopsis* plant two-hybrid system (Boyle et al., 2009; Rochon et al., 2006), where the BTB/POZ was fused to the DB (POZ:DB) and the Δ513 was fused to the VP16 transactivation domain (Δ513:TA) (Figure 4B). Here, the reporter gene was monitored through its mRNA as opposed to its enzyme activity, which provided a greater signal-to-noise ratio. BTB/POZ self-association (POZ:DB + POZ:TA) in the absence or presence of SA (Boyle et al., 2009) served as a positive control. The interaction between the BTB/POZ and Δ513 (POZ:DB + Δ513:TA) was observable in the absence of SA (significantly different from Gal4 DB, p < 0.05), but not after SA treatment (not significantly different from Gal4 DB, p > 0.05), indicating that SA disrupts the BTB/POZ-Δ513 association (Figure 4B).

We then tested the interaction *in vitro* in a pull-down assay. Because the BTB/POZ was eluted from the solid support with the competing ligand, desthiobiotin, but not with 1 mM SA (Figure 4C, left panel), we could conclude that SA, at the concentration tested, did not disrupt the Strep-tag/StrepTactin interaction. The pull-down indicated that the BTB/POZ interacted with Δ513, but that the interaction was disrupted by 1 mM SA (Figure 4C, right panel). No Δ513 could be further eluted by desthiobiotin, indicating that SA displaced all of the Δ513 from the solid phase (Figure 4C, right panel). As negative controls, first an unrelated protein (VLRSgt) (Hall
and De Luca, 2007) was shown not to interact with BTB/POZ (Figure 4D) and second Δ513 was shown not to interact with the solid support in the absence of BTB/POZ (Figure 4E). Together these data demonstrate that SA directly disrupts the BTB/POZ-Δ513 interaction, which is consistent with the conformation change of NPR1 and Δ513 brought about by SA (Figure 3).

3.3.5 The NPR1 BTB/POZ Inhibits the Transactivation Potential of Δ513

We next addressed whether the BTB/POZ could modulate the transcriptional properties of Δ513 (Figure 4F). When Δ513:DB was coexpressed in Arabidopsis leaves with the BTB/POZ (not fused to any foreign transcription-activation or DNA-binding domain), expression of the reporter gene in untreated cells was reduced to background levels. However, the transcription activity of Δ513 in SA-treated cells was unaffected by the BTB/POZ, consistent with the fact that these two proteins could only interact in the absence of SA (Figure 4B). In an in vivo plant repression assay, where the reporter gene is first activated by LexA:VP16 before testing for repression using a Gal4 DB fusion, the NPR1 BTB/POZ did not appear to repress the promoter back to basal (Gal4 DB) level (Figure 4G). These data revealed the autoinhibitory capacity of the BTB/POZ despite it not being an autonomous transcriptional repression domain. Therefore, in the absence of SA, the BTB/POZ must have masked the interface on the C-terminal transactivation domain required for its function.
3.4 DISCUSSION

Given the saturability by SA, the low Kd, and the chemical specificity of the SA-NPR1 interaction, which are hallmarks of a receptor, Arabidopsis NPR1 is a bona fide SA receptor (Figure 1). Several lines of evidence suggest that NPR1 plays the role of an SA receptor in vivo, in the signaling cascade leading to PR-1 activation. First, unlike some enzymes that have been shown to interact with SA (see Introduction) but do not control the PR-1 gene, NPR1 is clearly accepted as the key regulator of SAR deployment and PR-1 activation (Cao et al., 1997; Ryals et al., 1997). Second, mutation of the NPR1 gene abolishes the SA-signaling leading to SAR deployment and PR-1 activation, indicating that NPR1 acts downstream of SA and therefore is placed in a proper position in the signaling cascade for it to be an SA receptor (Cao et al., 1997; Ryals et al., 1997). Third, SA, its chlorinated derivatives (Conrath et al., 1995), and BTH act as strong PR-1 activators in vivo (Görlach et al., 1996; Lawton et al., 1996) (Figures 1H and 1I). In vitro, they directly interact with NPR1 (Figures 1E and 1F). Fourth, four nonfunctional analogs structurally related to SA, but that do not activate PR-1 in vivo (Attaran et al., 2009; Bi et al., 1995; Conrath et al., 1995; Delaney et al., 1994), do not interact with NPR1 in vitro, either (Figures 1E and 1F). Fifth, we have identified that Cys521/529 are required for the in vitro binding of SA to NPR1 (Figure 2F). In vivo, mutation of these cysteines abolishes PR-1 activation by SA (Rochon et al., 2006). Sixth, NPR1 requires copper for its interaction with SA in vitro and copper binds NPR1 through Cys521/529 (Figure 2G). In vivo, NPR1 is a copper-binding protein and mutations of
Cys521/529 abolishes copper binding (Figure 2H). Seventh, SA disrupts the interaction between the N-terminal BTB/POZ and the C-terminal transactivation domain of NPR1, both in vivo and in vitro (Figure 4). We believe that these in vivo/in vitro correlative evidences provide substantial support for a model in which NPR1 functions as an SA receptor in vivo.

Although the role of SA in plant immunity has been known for over two decades and that the NPR1 protein has been known to positively regulate SAR since 1997 (Cao et al., 1997; Ryals et al., 1997), it is only now that NPR1 emerges as an SA receptor. The failure of the nonequilibrium solid-phase experiment to estimate the Kd of the NPR1-SA pair (Figure 1A) is the likely explanation for why it took so long to acknowledge that NPR1 is an SA receptor and suggests that SA may re-equilibrate very fast with the mobile phase to produce a highly labile NPR1-SA intermediate. Therefore, biochemically, this lability would have made it difficult to detect an SA-NPR1 complex by nonequilibrium approaches, using a ligand bound to a solid support to purify a binding protein from crude extracts, or filter-binding assays. From a biological perspective, this fast exchange of SA on the NPR1 receptor coupled with a low Kd would result in highly effective in vivo sensing of SA, by allowing NPR1 to rapidly respond to fluctuations in SA concentrations. This is reminiscent of other sensing receptors, such as the Saccharomyces cerevisiae ZAP1 transcription factor (Bird et al., 2003). Unlike zinc fingers involved in DNA-binding, which bind zinc very tightly, ZAP1 has evolved a specialized and uncommon high-lability zinc finger to
sense zinc levels and modify its transcription activity based on intracellular zinc concentrations (Bird et al., 2003).

It has been shown that, in vivo and in untreated tissue, NPR1-GFP behaves as an oligomer held together by disulfide bridges (Mou et al., 2003). Cys521/529 were not identified as being required for the formation of this oligomer. Upon treatment with an inducer, conditions become reducing and the oligomer disassembles (Mou et al., 2003). Our data are consistent with these previous findings, with the exception that the oligomeric form of NPR1 that we studied did not require reducing conditions to disassemble as SA alone was sufficient to directly cause this structural change (Figures 3A, 3B, and 3I). Put together, our data and that of Mou et al. (2003) call for a refinement of the oligomer disassembly model. First, a buildup of SA would lead to reducing conditions inside the cell, which would lead to the reduction of disulfide bridges in NPR1, as proposed by Mou et al. (2003). However, according to our results, this is not sufficient to break apart the oligomer, since reducing conditions were not sufficient to drive the deoligomerization of the complex as observed by gel filtration (Figure 3I). Therefore, we propose a second step, in which SA would directly interact with Cys521/529 of NPR1 to cause the final disassembly of the oligomer (Figures 3A and 3B). This step would have been missed by Mou et al. (2003) since the SDS-PAGE, which was used to assess oligomer formation, cannot discriminate between a monomer and a higher-order structure held together by noncovalent interactions.
Cys521/529 are not universally conserved in NPR1 orthologs. However, metal interaction with proteins is not strictly limited to Cys. In fact, any amino acid harboring electronegative elements in its side chain can potentially participate in metal interaction (Xiao and Wedd, 2010). Figure S2 shows that such electronegative atom-bearing amino acids are found in the vicinity of the equivalent Cys521/529 position in NPR1 orthologs. Conservation of Cys521/529 is not necessarily expected in NPR1 orthologs, since different species exhibit different resting levels of SA. For instance, rice displays very high levels of SA in unchallenged conditions compared to Arabidopsis (Silverman et al., 1995). Therefore, it would be expected that rice NPR1 would have different binding or kinetic constraints with respect to SA interaction. We can then imagine that the SA-binding mechanism would be similar from species to species, i.e., through a metal cofactor, but that the interface would be variable and tailored to the specific requirements of a given species. The current study will be the catalyst that spurs investigations in the intricacies of the SA-NPR1 ortholog binding parameters. In support of NPR1 orthologs also being SA receptors, tobacco NPR1 has been shown to be responsive in yeast treated with SA, i.e., SA stimulates NPR1's capacity to activate the transcription of a reporter gene when tethered to DNA (Maier et al., 2011). These results, while suggestive of a binding between tobacco NPR1 and SA, cannot, at this time, be attributed to direct effects of SA on the protein, since no experiments were designed to test for a direct interaction, such as the equilibrium dialysis used in the current study.
Although most major phytohormone receptors have been identified (Table S1), SA remained one of the major small-molecule plant hormones without a known signal transduction receptor, until now. In *Arabidopsis*, direct binding of SA by the receptor, NPR1, reorganizes the conformation of an NPR1-dependent oligomer at the *PR-1* promoter and abolishes the interaction between the autoinhibitory N-terminal BTB/POZ domain and the C-terminal transactivation domain of NPR1 (Figure 4H). Thus, a clear mechanistic path is established between the sensing of SA by NPR1 and the unveiling of the NPR1 transcriptional activation domain, a prerequisite to *PR-1* gene activation.

### 3.5 METHODS

#### 3.5.1 Protein Purification for Equilibrium Dialysis, ICP-MS, Scintillation Proximity Assay, and Solid-Phase Binding

Proteins were expressed in *E. coli* as N-terminal fusions to the Strep-Tag according to standard protocols. Recombinant proteins were purified using 1 ml Strep-Tactin Superflow Plus columns (QIAGEN) according to the manufacturer's protocol. The Strep-Tactin buffer contained 50 mM sodium phosphate at pH 8.0 and 300 mM NaCl. For ICP-MS analyses, the buffer did not contain NaCl and used metal-free water. For equilibrium dialysis that contained EDTA, bound proteins were treated with 10 ml of 50 mM EDTA followed by 10 ml of 5 nM EDTA, prior to elution with a buffer containing 5 nM EDTA. Protein concentrations were measured by Bradford assays according to the manufacturer's instruction (Bio-Rad) using BSA as a
standard. For metal determination from proteins expressed in *Arabidopsis*, extracts from SA-treated plants were immunoprecipitated with an anti-NPR1 antibody (Rochon et al., 2006, Després et al., 2000). Protein concentrations were based on sulfur content determined by ICP-MS. For Figures S1B and S1C, NPR1 was cloned in pGEX-4T-1 as a BamH1/Not1 fragment and expressed as described above. NPR1-GST was purified using 1 ml GSTrap FF column (GE Health) and cleaved on-column using thrombin as described by the manufacturer (GE Health). The eluted NPR1 was purified by S300 gel chromatography and recovered from the void fraction.

### 3.5.2 Metal-Affinity Chromatography

Proteins were expressed in *E. coli* as N-terminal fusions to the HA-Tag according to standard protocols. Crude lysates were loaded on 1 ml HisTrap FF columns (GE Health) according to the manufacturer's protocol. The HisTrap buffer contained 50 mM HEPES at pH 7.5, 40 mM imidazole, and 150 mM NaCl. Where indicated, the HisTrap matrix was stripped of metal using 10 column-volume of 50 mM EDTA followed by 10 column-volume of 5 nM EDTA. Elution was performed in the HisTrap buffer supplemented with 1 M imidazole.

### 3.5.3 Pull-Down Assays

The BTB/POZ (amino acids 1-190 of NPR1) was expressed in *E. coli* as a C-terminal fusion to the Strep-Tag according to standard protocols. The Δ513 of NPR1 was expressed as an N-terminal fusion to the HA-Tag as described above. The VLRSgt (Hall and De Luca, 2007) was expressed in *E. coli* as an
N-terminal fusion to the GST-Tag according to standard protocols. The pull-down assay was performed in the Strep-Tactin buffer. The antibodies used for detecting the tags in the BTB/POZ-Strep was from QIAGEN (catalog # 34850) and those used for the tags in HA-Δ513 or the GST-VLRSgt were from Santa Cruz Biotechnology (catalog #: sc-7392 and sc-138).

3.5.4 Plant Transcription and Two-Hybrid Assays

*Arabidopsis thaliana* (Columbia) leaves were harvested from four-week-old plants grown at 21°C (day) and 18°C (night) with a ten-hour photoperiod and transferred to Petri dishes containing MS salts and micronutrients supplemented with B5 vitamins, 1% sucrose and 0.8% agar at a pH of 5.8. When required, filter-sterilized salicylic acid was added to the medium at a final concentration of 1mM. Coating of the gold particles and general procedures and preparation of the biolistic experiments were as per the manufacturer's instructions (Bio-Rad). After bombardment with reporter, effector, and internal standard plasmids, leaves were kept in the conditions described above for a period of 24 hr before assaying. Enzyme assays were performed using the Dual-Luciferase Reporter Assay system (Promega) following the manufacturer's instructions. Luminescence was measured on a Berthold Lumat LB9507 Luminometer (Bad Wildbad, Germany) and the data obtained represented the value of the reporter gene divided by the value of the internal standard and expressed as Relative Luciferase Units. To increase signal-to-noise ratio in some experiments, we performed qPCR to measure the amount of *Firefly* and *Renilla Luciferases* mRNA. The data was reported as Relative Expression and represented the value of the reporter mRNA.
divided by the value of the internal standard mRNA. The ratio obtained for Ga14 DB was assigned an arbitrary value of 1. One µg of each effector plasmid, 1 µg of the 5X UAS<sup>Gal4</sup>:Firefly luciferase reporter plasmid, and 0.1 µg of the CaMV35S:Renilla luciferase internal standard plasmid were mixed together and the mixture was used to coat beads. This amount of DNA was used to perform 5 bombardments. For repression assays, the 3X UAS<sup>Gal4</sup>:1X LexA DNA element:minimal promoter:Firefly luciferase reporter was used instead of the 5X UAS<sup>Gal4</sup>:Firefly luciferase reporter plasmid. Every bar in each graph represents five bombardments repeated five times on different days (n = 25). The constructs used contained a Gal4 DB or VP16 N-terminal fusion or no fusion at all.

### 3.5.5 Equilibrium Dialysis and Scintillation Proximity Assays

For equilibrium dialysis, two 500 µl chambers (A and B) were separated by a dialysis membrane with a cut-off of 3.5 kD. The buffer used in the system was the Strep-Tactin buffer. Radiolabeled SA (PerkinElmer, 50 mCi/mmol) was added in chamber A to a concentration of 10 µM SA, calculated based on the total volume of the system (A + B). Four µM of Δ513 protein or 0.8 µM of NPR1 protein were added to chamber B. The system was allowed to equilibrate at 4°C for 24 hr. Where indicated, EDTA was added to both chambers to a final concentration of 5 nM. After the 24 hr period, 100 µl from each chamber was removed and counted for 14<sup>Carbon</sup>, allowing for the calculation of SA concentration in each chamber. Given the dissociation reaction: Protein-SA<sub>complex</sub> ⇌ Protein<sub>free</sub> + SA<sub>free</sub> the dissociation constant Kd
equates: \( \text{[Protein}_{\text{free}}] \times \text{[SA}_{\text{free}}]/\text{[Protein-SA}_{\text{complex}}] \). The different species were computed as follows:

\[
\text{[SA}_{\text{free}}] = \text{[SA}_{\text{ChamberA}}];
\]

\[
\text{[Protein-SA}_{\text{complex}}] = \text{[SA}_{\text{ChamberB}}] - \text{[SA}_{\text{ChamberA}}];
\]

\[
\text{[Protein}_{\text{free}}] = \text{[Protein}_{\text{initial}}] - \text{[Protein-SA}_{\text{complex}}]
\]

For SPA (Figures S1D–S1G), radiolabeled SA and Strep-tagged NPR1 were incubated with 2mg of Streptavidin SPA beads (PerkinElmer) in the Strep-Tactin buffer for 24 hr at 4°C on a rotation wheel. Specific binding was calculated by subtracting total cpm from nonspecific cpm, which were counted by adding a 10-fold excess of cold SA.

For the saturation binding curves (Figures 1C, S1D, and S1E), 0.8 µM of NPR1 protein was incubated with a final concentration of 0.007-14 µM \(^{14}\text{C}\text{SA}\). The data was analyzed by non-linear regression using GraphPad PRISM 4 and fitted to a one-site-binding rectangular hyperbola. For homologous and heterologous competitive binding curves (Figures 1E, 1F, S1F, and S1G), 0.08 µM of NPR1 protein was incubated with a final concentration of 0.07 µM \(^{14}\text{C}\text{SA}\). Competitors were used at 0.1-100 times the concentration of hot ligand, except for BTH, INA, 5-CSA, 4-CSA, and 3,5-DCSA, which were used at 0.1-10 times the concentration of hot ligand, due to their low solubility in water.

3.5.6 Solid-Phase Binding Assay

Eight µM of purified Strep-tagged proteins were incubated with 100 µM radiolabeled SA and 50 µl of Strep-Tactin beads in 500 µl of Strep-Tactin buffer for 24 hr at 4°C on a rotation wheel. After five washes with the Strep-
Tactin buffer, the beads were added to 5 ml of scintillation cocktail and radioactivity was counted on a scintillation counter.

### 3.5.7 Inductively Coupled Plasma-Mass Spectrometry

Optimized ICP-MS determinations were performed in a fashion previously described (Wang and Brindle, 2011). Sulfur determinations were made using the Dynamic Reaction Cell ICP-MS with chemical resolution, facilitated by using oxygen to generate SO$^+$. ICP-MS intensities were converted to concentrations using calibration curves (Table S2. Protein concentrations were based on sulfur content. Proteins were hydrolysed in 68%–70% nitric acid for 40 min and then diluted 40 times in metal-free water before analysis. The StrepTactin buffer run through the FPLC and through an empty (protein-free) StrepTactin column served as a baseline for metal contamination. ICP-MS intensities of the baseline were subtracted from those of the protein extracts.

### 3.5.8 Chromatography

Strep-tagged purified proteins in a final volume of 2 ml were subjected to gel filtration analysis on the Sephacryl S100 HR or Sephacryl S300 HR packed in 50 cm long HR 16 columns (GE Health) and equilibrated with S300 chromatography buffer (50 mM HEPES, pH 7.4, 250 mM NaCl. Elutions, in 0.5 ml fractions, were performed in the same buffer at a flow rate of 0.8 ml/min. Where indicated, proteins were incubated with 1 mM SA, 1 mM catechol, 1 mM 4-hydroxy benzoic acid, or 1 mM methyl-salicylate at room temperature for 30 min prior to chromatography as described above with the
exception that the chromatography buffer was supplemented with 1 mM SA, 1 mM catechol, 1 mM 4-hydroxy benzoic acid, or 1 mM methyl-salicylate, respectively. In the case of the EDTA treatment, NPR1 was stripped of its metal by a 50 mM EDTA treatment of 30 min, followed by an incubation of 30 min with 1 mM SA prior to gel filtration. In this case, the chromatography buffer was supplemented with 1 mM SA.

3.5.9 Quantitative RT-PCR

Total RNA was extracted from leaves using the RNeasy plant mini kit (QIAGEN) according to the supplier's instructions. After treatment with DNase I (Invitrogen), first strand cDNA synthesis was generated using SuperScript II reverse transcriptase (Invitrogen), and the (dT)17VN oligo in the presence of 0.4 U Rnasin (Fisher Scientific). The newly synthesized cDNA was diluted 1/200 to reflect a concentration of 10 ng µL−1 input total RNA. RT-PCR was performed on a CFX96 spectrofluorometric thermal cycler (BioRad). Firefly luciferase values were normalized against Renilla Luciferase and PR-1 values against Ubiquitin5. The sequences of the primers are as follow:

FLucF (5′-AGGTGGCTCCCGCTGAATTG-3′),
FLucR (5′-CATCGTCTTTCCGTGCTCCA-3′),
RLucF (5′-GTGGTAAACCTGACGTTGTA-3′),
RLucR (5′-CTTGGCACCTTCAACAATAG-3′),
PR1F (5′-GCTCTTGTAGGTGCTCTTGTTC-3′),
PR1R (5′-AGTCTGCAGTTGCCTCTTAGTTGTTC-3′),
UBQ5-1 (5′-ACCTACGTTTTACCAAAAGGAGGATGTGAA-3′), and
UBQ5-2 (5’-AGCTTACAAAATTCCAAATAGAAATGCAG-3’).

3.5.10 Crosslinked Chromatin Chromatography—3C Method

Plant treatment, crosslinking, sonication, and crosslinking reversal were performed in the same way as for chromatin-immunoprecipitation (Rochon et al., 2006). Chromatography was as described under “Chromatography.” qPCR was performed with PR1 and Ubiquitin5 primers. PR-1 values were normalized against Ubiquitin5. The sequences of the primers are as follow:

PR1a(−734) (5’-GATCACCGATTGACATTGTA-3’),
PR1b(−833) (5’-GAACACAAAAGTAGATCGGT-3’),
UBQ5a (5’-GACGCTTCATCTCGTCC-3’), and
UBQ5b (5’-GTAAACGTAGGTGAGTCCA).

3.5.11 Statistics

All graph results relating to Relative Luciferase Units and mRNA Relative Expression are reported as mean ± 1 standard deviation (SD) of 25 independent experiments. Comparisons were performed using two-tailed paired Student's t test. *p < 0.05. In every graph where error bars are shown, “n” represents the number of biological replicates and results are reported as mean ± 1 standard deviation (SD).
**Figure 1.** NPR1 Is a Specific SA Receptor

**A and B** Assays in which no protein, NPR1, BTB/POZ (POZ), or Δ513 were tested for SA binding using (A) a solid-phase method or (B) equilibrium dialysis (EqD) (n = 3).

**C** Saturation binding of SA to NPR1 using EqD.

**D** Scatchard plot of the data in (C).

**E and F** Competitive binding curves for the SA-NPR1 interaction using EqD (n = 3). (See also Table S1 and Figure S1).

**G** Structures of the competitors.

**H** Quantitative RT-PCR. All treatments were for 12 hr (n = 3).

**I** Fold induction of PR-1 using data in (H).

(A, B, E, F, and H) Data are reported as mean ± 1 SD.
Figure 2. Cys521/529 and Copper Are Essential for SA Binding

(A) Sequence of Δ513 showing Cys521 and Cys529.
(B–E) Immunoblot of HA-tagged Δ513 (B and C), Δ533 (D), or Δ513 Ser521/529 (E) separated by Ni-NTA in the absence (B, D, E) or presence (C) of 50 mM EDTA. FT indicates flowthrough.
(F) Assays in which Δ513, Δ513 + EDTA, Δ513 Ser521/529, or NPR1 Ser521/529 were tested for [14C]SA-binding using EqD (n = 3).
(G and H) Concentrations of metals associated with (G) E. coli-produced Δ513 (WT) or Δ513 Ser521/529 (mut) or (H) Arabidopsis-produced NPR1 (WT) or NPR1 Ser521/529 (mut) (n = 2).

In (F)–(H), data are reported as mean ± 1 SD (see also Table S2).
Figure 3. SA Causes a Change in the Conformation of NPR1

(A–I) Immunoblot analysis of protein fractions from an S300 elution profile of (A and C) untreated, (B and D) SA-treated, (E) EDTA and SA-treated, (F) catechol-treated, (G) 4-OH BA-treated, (H) Me-SA-treated or (I) DTT-treated Strep-tagged NPR1 or (C and D) Strep-tagged NPR1 Ser521/529. See also Tables S3 and S4.

(J) Coomassie stain of 30 µg each of purified NPR1 and NPR1 Ser521/529.

(K) 3C Method showing the presence/absence of NPR1-dependent oligomer on the PR-1 promoter in vivo as a result of treatment with SA, BTH, 4-OH BA
and INA. Data were reported as the ratio of PR-1 over UBQ5 (n = 2). Data are reported as mean ± 1 SD.

**L and N** S100 gel filtration chromatogram showing the elution profile of purified Strep-tagged Δ513 (L) untreated or (N) treated with 1 mM SA.

**M and O** Immunoblots using an anti-Strep antibody against fractions from the chromatograms in (L) and (N), respectively. These correspond to the void volume and the predicted elution volumes of a theoretical Δ513 oligomer of 11 sub-units, tetramer, trimer, dimer, and monomer and a fraction located between a dimer and monomer (D–M). Each fraction contains 0.5 ml. See also Tables S5 and S6.
Figure 4. NPR1 Binds SA to Relieve Sequestration of the Transactivating Domain by the BTB/POZ

(A) In vivo transcription assays showing that Δ513:DB, but not NPR1:DB, can activate the transcription of a reporter gene in the absence of SA treatment (n = 25).

(B) In vivo plant two-hybrid assays showing that Δ513:DB can only interact with the BTB/POZ domain (POZ:DB) in the absence of SA (n = 25).

(C–E) Pull-down assay using (C and D) the Strep-Tagged BTB/POZ coupled to the StrepTactin solid-phase or (E) the empty StrepTactin solid-phase and (C and E) the Δ513 fused to the HA-Tag or (D) the VLRSgt protein fused to the GST-tag.

(F) In vivo transcription assays using Δ513:DB, alone or in complex with the BTB/POZ (POZ) not fused to any domain (n = 25).
(G) *In vivo* repression assays using the BTB/POZ domain (POZ:DB). Where indicated, the LexA DB fused to the viral protein 16 transactivation domain (LexA:VP16) was also transfected in order to activate the reporter gene (n = 25).

(H) Model of the SA-induced disruption of the BTB/POZ-Δ513 interaction. (A, B, F, and G) Data are reported as mean ± 1 SD. (B, F, and G) n.s. indicates nonsignificance at p = 0.05 and the asterisks indicate significance at p = 0.05.
Supplemental figures and tables

Figure S1. NPR1 Is an SA Receptor, Related to the Introduction and to Figure 1

(A) Schematics of the NPR1 structure. NPR1 contains a BTB/POZ (Broad Complex, Tramtrack, Bric-à-brac/Pox virus and Zinc finger) domain (Aravind and Koonin, 1999), ankyrin repeats, a transactivation domain (TAD) and a
nuclear localization signal (NLS). The two arrows indicate the starting amino acids of deletion constructs Δ513 and Δ533.

(B) Saturation binding of [\(^{14}\text{C}\)]SA to untagged NPR1 using equilibrium dialysis.

(C) Scatchard Plot of the data in (B).

(D) Saturation binding of [\(^{14}\text{C}\)]SA to Strep-tagged NPR1 using Scintillation Proximity Assay (for a recent use of the technique and a comparison with EqD, see Piscitelli et al., 2010). Like EqD, SPA also measures complex formation at equilibrium. However, unlike EqD, there is no physical barrier for the ligands to cross. In SPA, scintillation is distance-dependent and therefore, only the bound ligands can cause the SPA beads to emit light.

(E) Scatchard Plot of the data in (D).

(F and G) Homologous and heterologous competitive binding curves for the [\(^{14}\text{C}\)]SA-NPR1 interaction using Scintillation Proximity Assay.
Figure S2. Multiple Alignment of NPR1-Related Proteins, Related to the Discussion

Amino acid sequences corresponding to the C terminus of NPR1 proteins from Arabidopsis thaliana, Theobroma cacao, Gossypium hirstum (cotton), Nicotiana tabacum (tobacco), and Oryza sativa (rice) and of Arabidopsis thaliana BOP1 and BOP2 (NPR5 and 6), two NPR1 paralogs involved in development and independent of the SAR pathway. Shown in yellow are C521/529 of Arabidopsis NPR1. The equivalent of C529 is conserved in some members of this superfamily (exemplified by TcNPR1 and GhNPR1). A tyrosine replaces C529 in many NPR1s found in the GenBank database (exemplified by NtNPR1). Tyrosines contain a low pKa hydroxyl group in their side-chain, which could potentially engage in a coordination bond with a metal. A third group of NPR1 members do not contain an equivalent to C529 or a tyrosine (exemplified by OsNPR1). C521 does not appear to be conserved outside the genus Arabidopsis. However, aspartate (shown in green), an amino acid with electronegative atoms in its side-chain (a carboxylate) occurs in the surrounding of the C521-equivalent position (C521 and C529 in rice) of many NPR1s. These observations suggest that NPR1s found outside the Arabidopsis genus may also bind SA through metals. Interestingly, BOP1 and BOP2 contain natural built-in histidine-tags (shown in blue), a domain known to bind metals. This suggests that metal-binding may be a general feature of the NPR superfamily.
Table S1. Calculated Kd of Various Plant Hormone-Receptor Pairs, Related to Figure 1 and Discussion

The NPR1 Kd is comparable to the Kd found for other plant-hormone receptor-ligand interactions and is in accordance with the in vivo SA concentration in Arabidopsis of 0.36 µM (0.05 µg/g FW) reported in unstimulated Arabidopsis cells and 7.24 µM (1 µg/g FW) after challenge with an avirulent strain of Pseudomonas syringae (Summermatter et al., 1995).

<table>
<thead>
<tr>
<th>Plant hormone receptor</th>
<th>Kd (nM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abscisic acid-RCAR1/ABI2</td>
<td>64 ± 8</td>
<td>(Park et al., 2009; Ma et al., 2009; Shen et al., 2006)</td>
</tr>
<tr>
<td>Abscisic acid-CHLH</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Auxin (indole acetic acid)</td>
<td>20-80</td>
<td>(Kepinski and Leyser, 2005)</td>
</tr>
<tr>
<td>Cytokinin (trans-Zeatin)</td>
<td>3.9</td>
<td>(Romanov et al., 2006; Inoue et al., 2001)</td>
</tr>
<tr>
<td>Ethylene</td>
<td>Not determined</td>
<td>(Schaller and Bleecker, 1995)</td>
</tr>
<tr>
<td>Gibberellin (16,17-dihydro-GA4)</td>
<td>1400</td>
<td>(Ueguchi-Tanaka et al., 2005)</td>
</tr>
<tr>
<td>Brassinosteroid</td>
<td>10.8 ± 3.2</td>
<td>(Wang et al., 2001)</td>
</tr>
<tr>
<td>Jasmonate</td>
<td>Not determined with endogenous hormone</td>
<td>(Yan et al., 2009)</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>140 ± 10</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table S2. Slopes and Coefficients of Determination Governing the ICP-MS Calibration Curves of the Various Elements Studied in Figure 2G and 2H, Related to Figure 2G and 2H and Experimental Procedures

1Elements were detected under standard mode.
2Fe was detected under DRC mode with NH3.
3Sulfur was used to determine the protein concentration of wild-type Δ513 and Δ513 bearing cysteine-to-serine mutations at positions 521 and 529. Sulfur was detected under DRC mode with O2.
4cps (counts per second). The equation was calculated by Linear Thru Zero.
Table S3. Operational Parameters of the S300 Gel Filtration Column, Related to Figure 3 (A to I)

Vt (Total bed volume of the column) = 100.5 ml
Vo (Void volume of the column evaluated with Blue Dextran 2000) = 38 ml
Kav = (Ve - Vo)/(Vt - Vo)
The equation of the standard curve was: y = -0.257x + 0.8453 with an R² = 0.9742.

Table S4. Predicted and Observed Elution Volumes Establishing the Stoichiometry of NPR1 in 1 mM SA on the S300 Column, Related to Figure 3 (A to I)

In Figure 3b (NPR1 + SA panel), the highest amount of NPR1 found in the included volume was in fractions 90 and 95. Since the predicted fraction number for the NPR1 dimer is 94 (a number between 90 and 95), it would suggest that NPR1 exists as a dimer after SA treatment. In Figure 3 (A to I), fraction 55 starts at elution volume 37.1 ml and ends at 37.6 ml. Each subsequent fraction also contains 0.5 ml.
Table S5. Operational Parameters of the S100 Gel Filtration Column, Related to Figure 3 (L to O)

\[ V_t \text{ (Total bed volume of the column)} = 98.922 \text{ ml} \]
\[ V_o \text{ (Void volume of the column evaluated with Blue Dextran 2000)} = 33.75 \text{ ml} \]
\[ K_{av} = \frac{V_e - V_o}{V_t - V_o} \]

The equation of the standard curve was: \( y = -0.4677x + 0.9726 \) with an \( R^2 = 0.9884 \).

Table S6. Predicted and Observed Elution Volumes Establishing the Stoichiometry of \( \Delta 513 \) in 1 mM SA on the S100 Column, Related to Figure 3 (L to O)
CHAPTER 4 – PRELIMINARY DATA

4.1 INTRODUCTION

The plant defense hormone SA has been known for decades to mediate the deployment of SAR, a type of broad-spectrum and long-lasting immunity against microbial pathogens (Ryals et al., 1996). The SA-induced transcriptional reprogramming is a critical step for the initiation of SAR. This is marked by the transcriptional activation of a set of SAR-related genes including the marker gene PR-1. The Arabidopsis NPR1 protein is a central regulator of the SA signaling pathway (Ryals et al., 1996). SA induces the complexation of NPR1 and TGA2 transcription factors on the promoter of the PR-1 gene. Such complexes function as transcriptional activators that enhance PR-1 expression (Rochon et al., 2006). In the absence of NPR1, the SA-mediated expression of SAR genes is abolished. Since npr1 knockout mutants are insensitive to SA, NPR1 has been speculated to be involved in SA sensing for years (Durrant and Dong, 2004). Only recently has NPR1 been demonstrated as a receptor for SA. SA specifically binds to the C-terminal transactivation domain of NPR1 with high affinity (kd = 140 nM), and induces conformational changes of NPR1 upon binding. Intriguingly, the interaction of SA and NPR1 requires transition metal copper (Cu) (Chapter 3). This indicates a connection between SAR and Cu homeostasis. Players involved in Cu homeostasis may also be integrated components of SAR, because they could affect SA perception by controlling the availability of Cu to NPR1. This is supported by our preliminary results that NPR1-Cu binding is a dynamic process regulated by SA, and that Cu is a positive regulator of SAR. We also
show that Cu alone impacts NPR1’s function, which may regulate gene expression independent of SA.

### 4.2 Results

#### 4.2.1 Cu-NPR1 binding is dynamic.

Cys\(^{521/529}\) of NPR1 has been known to be critical for Cu coordination, which facilitates SA binding to NPR1 (Chapter 3). Therefore, SA perception by NPR1 could be regulated via the control of the Cu coordination on NPR1. This requires the binding of Cu to NPR1 to be dynamic. To test if this is true in plants, NPR1 was immunoprecipitated from SA-treated and non-treated Arabidopsis leaves using anti-NPR1 antibodies, and then subjected to metal analysis by ICP-MS. Background was corrected by using values obtained with the WT plants to subtract values obtained with the negative control plants expressing a variant of full-length NPR1 lacking Cys\(^{521/529}\) (Chapter 3). The data showed that more Cu was bound to NPR1 after SA treatment (Figure 1), indicating that the binding of Cu to NPR1 is a dynamic process, which can be positively regulated by SA.

We next confirmed *in vitro* that the C-terminus of NPR1 reversibly binds Cu. Our previous data (Chapter 3) showed that *E. coli*-produced Δ513 coordinates Cu through Cys\(^{521/529}\). We here stripped metal from Δ513, which was immobilized on StrepTactin beads, by EDTA treatment. The resulting metal-free Δ513 was incubated with Cu, followed by washing out unbound Cu and quantifying bound Cu using ICP-MS. Bound Cu was detected in the Cu-treated Δ513, but not in the Cu-treated Δ513 S\(^{521/529}\) and the deionized (DI)
water-treated negative controls (Figure 2). The data demonstrated that the binding of Cu to Δ513 is reversible in vitro, which supports a model of dynamic NPR1-Cu binding.

The dynamic binding between NPR1 and Cu is also corroborated with our real-time RT-PCR results showing that SA-induced PR-1 expression can be further enhanced when Cu is co-applied with SA (Figure 3A). In order to rule out the possibility that Cu enhances PR-1 expression by activating SA biosynthesis, we also monitored PR-1 expression in sid2 mutants. The PR-1 expression levels in sid2 mutants treated with SA together with Cu were higher than that in SA-treated sid2 mutants (Figure 3B). These results suggest that Cu is a positive regulator of PR-1 expression, possibly through binding to NPR1.

4.2.2 Cu transport impacts Cu-NPR1 binding and PR-1 activation

The NA-metal transporter YSL3 has been demonstrated to be a positive regulator of plant immunity, and its expression can be activated by SA (Chen et al., 2014) (Supplemental Figure 1). We tested whether YSL3 is involved in the delivery of Cu to NPR1. The data showed that NPR1 immunoprecipitated from SA-treated ysl3-1 knockout mutants did not contain higher levels of bound Cu than that immunoprecipitated from non-treated ysl3-1 mutants (Figure 1), indicating that SA failed to promote Cu-NPR1 binding in the absence of YSL3. This suggests that YSL3 mediates Cu loading on NPR1.

YSL3 mainly expresses in vascular parenchyma cells along the veins, where they are responsible for moving metal ions, especially Cu (Waters et al., 2006). We asked if SA increases the loading of Cu into leaves by
activating YSL3 gene expression. Leaves of non-treated and SA-treated WT, ysl3-1 and npr1-3 mutants were harvested for metal analysis. Interestingly, SA-treated leaves showed no significant change in Cu levels compared to non-treated leaves (Figure 4), suggests that SA promotes Cu-NPR1 binding by inducing reallocation, rather than loading, of Cu in leaves.

Consistent with its role in mediating Cu-NPR1 binding, YSL3 positively regulates PR-1 gene expression. We observed decreased levels of SA-induced PR-1 expression in ysl3-1 mutants compared to WT plants (Figure 5). This suggests that the full-scale induction of PR-1 requires the presence of YSL3.

4.2.3 Cu binding induces disulfide bond formation

It is unclear how Cu is coordinated in the C-terminus of NPR1. To get structural information on Cu-bound NPR1, we collaborate with Dr. Normand Brisson at Université de Montréal. Dr. Brisson’s lab has conducted NMR analysis of a part of the C-terminus of NPR1 with and without Cu-binding. Their preliminary results suggest a structural change on NPR1 upon Cu-binding, including the formation of disulfide bonds (data not shown). Meanwhile, we also observed the Cu-induced formation of disulfide bonds in Δ513 in vitro. Metal-free Δ513 was reduced by DTT and subsequently incubated with Cu, followed by immunoblot analysis in the absence of reducing agent. Cu alone was able to induce the formation of disulfide bridges between Δ513 dimers (Figure 6).

We next asked if such Cu-induced disulfide bridges facilitate the dimerization of NPR1. Gel filtration experiments were performed to explore the effect of Cu on the conformation of NPR1. Although disulfide bridges
could form due to Cu treatment, NPR1 remained in oligomeric form and eluted in the void (Figure 7). This indicates that Cu can induce a conformational change of NPR1, which is different from the change induced by SA.

**4.2.4 Cu-NPR1 regulates YSL3 in a SA-independent manner**

Since Cu causes distinct conformational change of NPR1, we addressed whether this form of NPR1 functions independent of SA. YSL3 gene is selected for the test of Cu- and NPR1-dependent gene regulation, because its expression is regulated by both Cu (Chen et al., 2011; Waters et al., 2006) and NPR1 (Chen et al., 2014) (Supplemental Figure 1). Intriguingly, Cu could weakly activate YSL3 expression 2 hrs after treatment in WT (Figure 8A), and also in SA-deficient sid2 mutants (Figure 8C) and NahG plants (Figure 8B), but failed to activate YSL3 expression in npr1-3 mutants (Figure 8D). This suggests that such Cu-induced YSL3 expression is SA-independent and NPR1-dependent.

**4.3 Discussion**

Our preliminary results suggest that YSL3 is required for the SA-induced Cu acquisition by NPR1. In the ysl3-1 mutants, SA failed to promote NPR1-Cu binding as it did in the wild-type plants (Figure 1). Interestingly, the negative values of the ysl3-1 group indicates that less background, possibly derived from non-specific Cu binding to the beads and/or the antibody, was found in the ysl3-1 mutants than in the npr1-3 mutants. Such reduced background is likely to be a result of altered Cu distribution caused by the knockout
mutation of the YSL3 gene. The abolishment of increase in Cu-bound NPR1 levels result in decreased PR-1 expression. Cu enhanced PR-1 expression when applied together with SA in both WT plants and SA-deficient sid2 mutants. This indicates that Cu positively regulate PR-1 expression, which is uncoupled from SA biosynthesis. Therefore, YSL3 functions to amplify the PR-1 gene activation by SA, likely through providing NPR1 with Cu. Taken together, the SA-induced YSL3 expression and Cu loading on NPR1 are likely to be positive feedback of the SA-NPR1 signaling pathway. It would be interesting to know whether other Cu transporters are also involved.

We also demonstrated that Cu could activate YSL3 expression independent of SA (Figure 8), possibly via inducing conformational change (e.g., disulfide bond formation) of NPR1. This suggests a novel mechanism by which NPR1 regulates gene expression, which is uncoupled from SA. However, the weak activation (~ 2 folds) suggests that YSL3 might not be a good target regulated under this mechanism. Transcriptome analysis by RNA sequencing would help in identifying target genes regulated by Cu and NPR1, yet independent of SA.

It still remains unknown whether a chaperone is responsible for the delivery of Cu to NPR1. It is possible that proteinaceous chaperones or Cu-binding metabolites are involved in this process. Proteomic and metabolic profiling could be useful to reveal how NPR1 acquires Cu after SA treatment.

Also, little is known so far about the interplay between Cu deficiency and SAR. It would be interesting to know whether SPL7 is also involved in SAR under Cu deficient conditions.
4.4 Methods

4.4.1 Treatment of plants

*Arabidopsis* plants were grown on soil under short-day condition for four weeks prior to treatment. Plants were treated by leaf spraying. The concentration of SA used for treatment is 300 µM, and the concentration of Cu (CuCl$_2$) used for treatment is 100 µM.

4.4.2 Immunoprecipitation

3g of leaves were ground in liquid nitrogen and then in the presence of 1.5ml of IP buffer (PBS pH7.4 supplemented with 0.5% of tween20) and 0.5ml of 1X EDTA-free proteinase inhibitors (Sigma, Cat# S8830). The obtained cell extract was subjected to sonication for 10s at an interval of 15s on ice. After centrifugation at 8000rpm under 4°C, 1ml of the supernatant was incubated with anti-NPR1 antibodies (Després et al., 2000; Rochon et al., 2006) immobilized to Protein A Magnetic Beads (GenScript, Cat# L00273) for 2hrs at room temperature, followed by washes that removes the unbound and elution of NPR1.

4.4.3 In vitro NPR1-Cu binding assay

Strep-tagged NPR1 was immobilized on StrepTactin beads, and incubated with EDTA (5mM) and DTT (500µM) for 15min. After washing out EDTA and DTT, NPR1 was incubated with Cu (300µM) or SA (500µM) + Cu (300µM) for 30min, followed by washes to remove unbound ligands. NPR1 was then eluted from the beads and subjected to sample preparation for metal analysis.
4.4.4 Leaf metal measurement

Five-week-old leaves were collected and surface washed by metal-free water. The harvested leaves were ground into powder in liquid nitrogen using a micro-pestle. The leaf powder was lyophilized overnight and weighed to record dry weight, followed by sample preparation for metal analysis.

4.4.5 Metal analysis by ICP-MS

Samples were hydrolyzed using 300ul of trace metal grade nitric acid (VWR, Cat# CANX0407-1), and heated at 94°C for 20min. The hydrolyzed samples are diluted to 10ml prior to metal analysis by ICP-MS.

4.4.6 Disulfide bond detection

Strep-tagged Δ513 was immobilized on StrepTactin column. Reduced, metal-free Δ513 was obtained by on-column washes using StrepTactin NP Buffer supplemented with EDTA (5mM) and DTT (500µM), followed by washes using NP Buffer to removing EDTA and DTT, and elution of Δ513. The eluted Δ513 was treated with MQ water, Cu (5µM), or Cu (5µM) + SA (300µM), followed by sample preparation for SDS-PAGE. MQ water-treated Δ513 was prepared in both reducing and non-reducing conditions, whereas Δ513 treated with other chemicals was prepared in non-reducing conditions. All samples were subjected to SDS-PAGE and immunoblot analysis.

4.4.7 Gel filtration

His-tagged NPR1 was purified using Ni-NTA columns (GE Healthcare) and treated with Cu (100µM), followed by size-exclusive chromatography
using S300 gel filtration column (GE Healthcare). Void and included fractions were obtained and subjected to SDS-PAGE and immunoblot analysis.

4.4.8 Quantitative RT-PCR

Total RNA was extracted from ~50mg of leaves using the Aurum™ Total RNA Mini Kit (Bio-rad, Cat# 732-6820) according to the supplier's instructions. After treatment with DNase I (New England Biolabs, Cat# M0303S), first strand cDNA synthesis was generated using iScript Reverse Transcription (Bio-rad, Cat# 170-8840). The newly synthesized cDNA was diluted 1/100 to reflect a concentration of 10 ng µL⁻¹ input total RNA. Real-time PCR was performed on a CFX96 spectrofluorometric thermal cycler (BioRad). The sequences of the primers are as follow:

YSL3F (5′- GTGGCGGCAATCTCGTTA-3′),
YSL3R (5′- CCATCGGTAATGGAACCCAAT-3′),
PR1F (5′-GCTCTTGTAGGTGCTCTTGTTCTTCC-3′),
PR1R (5′-AGTCTGCAGTTGCCTCTTAGTTGTTC-3′),
UBQ5-1 (5′-ACCTACGTITTACCAGAAAGAGGAGTTGAA-3′), and
UBQ5-2 (5′-AGCTTACAATTTCCCAATAGAAATGCAG-3′).
**Figure 1.** NPR1 binds to more Cu after SA treatment.

Bars represent concentrations of Cu associated with immunoprecipitated *Arabidopsis* NPR1. Background was corrected by using values obtained with the WT and *ysl3-1* mutant plants to subtract values obtained with the negative control (*npr1-3*). Values obtained with the SA- and non-treated plants were respectively corrected with values obtained with the SA- and non-treated negative controls. Data are reported as means ± SD of three independent biological replicates.
Bars represent concentrations of Cu associated with *E. coli*-produced Δ513 (WT) or Δ513 Ser$_{521/529}$ (Mut), which was first stripped of metal by EDTA treatment and subsequently incubated with Cu or DI water. Data are reported as means ± SD of three independent biological replicates. DI, deionized.
Figure 3. Cu enhances SA-induced PR-1 expression.

Quantitative RT-PCR analysis of PR-1 relative expression levels in a 6-hr time course in the leaves of WT plants and sid2 mutant treated with SA (300 µM) or SA (300 µM) + Cu (100 µM). Relative expression values were normalized to the internal reference gene UBQ5 using the ΔCT method. Data are reported as means ± SD of three independent biological replicates. NT, No Treatment.
Figure 4. SA induces reallocation of Cu in leaves.

Bars represent relative metal concentrations of WT plant, ysl3 and npr1 mutants. Results are given as percentage change in plants treated with SA (300 µM) compared to non-treated plants. Data are reported as means ± SD of four independent biological replicates. NT, No Treatment.
**Figure 5.** YSL3 is required for the full-scale *PR-1* activation.

Quantitative RT-PCR analysis of *PR-1* relative expression levels in a 12-hr time course in the leaves of WT plants and *ysl3* mutant treated with SA (300 µM). Relative expression values were normalized to the internal reference gene *UBQ5* using the ΔCT method. Data are reported as means ± SD of three independent biological replicates. NT, No Treatment.
Figure 6. Cu induces disulfide bonds in Δ513.

Purified Strep-tagged Δ513 (WT) or Δ513 Ser^{521/529} (Mut) was reduced and stripped of metal by treatment of EDTA (5 mM) and DTT (500 µM). The resulting reduced, metal-free protein was treated with MQ water, Cu (5 µM), or Cu (5 µM) + SA (300 µM), and analyzed by non-reducing SDS-PAGE and immunoblot using anti-NPR1 antibodies. As a control, water-treated protein was also analyzed by reducing SDS-PAGE and immunoblot. NR, non-reducing. R, reducing.
Figure 7. Cu does not cause the deoligomerization of NPR1.

Immunoblot analysis of protein fractions from an S300 elution profile of purified His-tagged NPR1 treated with Cu (100 µM).
Figure 8. Cu regulates YSL3 expression.

Quantitative RT-PCR analysis of YSL3 relative expression levels in the leaves of WT plants (A), NahG plants (B), sid2 (C) and npr1 (D) mutants treated with SA (300 µM) or SA (300 µM) + Cu (100 µM). Relative expression values were normalized to the internal reference gene UBQ5 using the ΔCT method. Data are reported as means ± SD of three independent biological replicates. NT, No Treatment.
Supplemental Figure 1. SA activates YSL3 expression.

Quantitative RT-PCR analysis of YSL3 relative expression levels in a 12-hr time course in the leaves of WT plants and npr1 mutant treated with SA (300 µM). Relative expression values were normalized to the internal reference gene UBQ5 using the ΔCT method. Data are reported as means ± SD of three independent biological replicates. NT, No Treatment.
CHAPTER 5 – GENERAL DISCUSSION

5.1 Other NPRs as SA receptors?

After our manuscript (Chapter 3) was accepted by *Cell Reports*, a paper was published in *Nature* reporting that NPR3 and NPR4 are SA receptors. In this paper, Fu et al. (2012) demonstrated that NPR3 and NPR4 bind to SA with low (Kd = 981 ± 409 nM) and high affinity (Kd = 46.2 ± 2.35 nM), respectively. In naïve cells, where SA concentration is low, NPR4 interacts with NPR1 and functions as a CUL3 E3-ligase substrate-adapter that targets NPR1 for proteasomal degradation. In stimulated cells, where SA overaccumulates, NPR4 dissociates from NPR1 after binding to SA. On the contrary, SA-binding allows NPR3 to interact with NPR1. Similar to NPR4, NPR3 could also target NPR1 for degradation. Interestingly, Fu et al. (2012) showed that NPR1 did not bind to SA, whereas we observed specific interaction of NPR1 to SA. This discrepancy is due to the different nature of binding assays and the metal-dependence of NPR1-SA binding. By using the same method (solid phase binding assay), we also found that NPR1 was unable to bind SA (Chapter 3). Since the washing steps in this method could drive the equilibrium against ligand-receptor binding, we adopted an alternative method (equilibrium dialysis) to confirm the results. By using this appropriate method, we demonstrated that NPR1 clearly binds to SA with high affinity and specificity. Intriguingly, we also discovered that the NPR1-SA binding requires transition metal Cu as a cofactor (Chapter 3). Since Fu et al. (2012) performed binding assays in the presence of EDTA, NPR1-SA interaction would not be observed under these conditions. Recently, Manohar
et al. (2014) confirmed the NPR1-SA interaction by using three independent methods, ending the controversy of whether or not NPR1 binds to SA.

NPR1 is a central regulator of SAR. It mediates the SA-induced transcriptional activation of a set of SAR genes (Ryals et al., 1996). This thesis work provides new insight into the mechanism under which NPR1 transduces SA signal. NPR1 specifically binds to SA and functions as a high affinity receptor. Upon SA-binding, NPR1 undergoes conformational change, which is a hallmark of ligand-receptor binding. This enables downstream signaling events (Chapter 3).

Unlike NPR1, NPR3 and NPR4 are negative regulators of SAR. Double knockout mutant npr3-1npr4-3 are more resistant to Pseudomonas syringae pv. maculicola ES4326 (Psm ES4326) and Hyaloperonospora parasitica Noco2 than wild-type control plants (Fu et al., 2012; Zhang et al., 2006b). However, results from pathology tests of single npr3 and npr4 mutants indicate they are not more resistant to Psm ES4326 than wild-type plants (Fu et al., 2012; Shi et al., 2013; Zhang et al., 2006b). This suggests a functional redundancy, rather than distinct biological roles of NPR3 and NPR4 at different SA levels. Additionally, SA-binding does not induce any conformational change on NPR3 or NPR4 (Fu et al., 2012). Therefore, their SA-regulated interaction with NPR1 may be influenced by the SA-induced conformational change on NPR1, instead of NPR3-SA and NPR4-SA binding. According to the model, NPR3 is responsible for the NPR1 degradation at high SA levels, while NPR4 is dissociated from NPR1. However, 4 hrs after SA treatment, NPR1 protein levels in single npr4, npr3 mutant and npr3npr4
double mutant show no significant difference from each other. 8 hrs after SA treatment, NPR1 accumulates even more in npr4 mutant than in npr3 and npr3npr4 mutants (Fu et al., 2012). These results do not support the proposed functions of NPR3 and NPR4 at high SA levels. The above-mentioned points combined with mediation of SA signaling by NPR1 alone suggest that NPR3 and NPR4 do not have typical characteristics of hormone receptors.

Taken together, we conclude that NPR1 is a bona fide receptor for SA. The NPR1-mediated SA signaling is at the core of the SAR signaling network. NPR3 and NPR4 participate through targeting NPR1 for proteasomal degradation in an SA-regulated manner (Kuai et al., 2015). With the SA receptor in hand, efforts could be put into the screening for small molecules that activate SAR through interactions with NPR1 or NPR1 orthologs in commercially important crops.
**REFERENCE**


Durner, J. and Klessig, D. F. (1995). Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense


Gust, A. A., Biswas, R., Lenz, H. D., Rauhut, T., Ranf, S., Kemmerling,


**Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J. and Ward, E.**


