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Screening of Novel Active Salicylic Acid Analogs and Identification of a Bacterial Effector Targeting Key Proteins Involved in Salicylic Acid-Mediated Defense

Ian Palmer

University of South Carolina - Columbia

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Screening of Novel Active Salicylic Acid Analogs and Identification of a Bacterial Effector
Targeting Key Proteins Involved in Salicylic Acid-Mediated Defense

by

Ian Palmer

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Accepted by:

Zhengqing Fu, Director of Thesis

Beth Krizek, Reader

Johannes Stratmann, Reader

Cheryl L. Addy, Vice Provost and Dean of the Graduate School

DEDICATION

I dedicate this work to my parents. Their love, support, and encouragement motivated me to keep getting out of bed and into the lab, even during the darkest of days, when I was feeling angry, frustrated, and isolated. I also dedicate this work to my grandmother, who is always curious about my research, and to whom I enjoy explaining my research as much as she enjoys hearing about it. I love all of you.

ACKNOWLEDGEMENTS

ABSTRACT

The master regulator of salicylic acid (SA)-mediated plant defense,
NPR1 (NONEXPRESSER OF PR GENES 1), and its paralogs NPR3 and NPR4 act as SA
receptors. After the perception of a pathogen, plant cells produce SA in the chloroplast.
In the presence of SA, NPR1 protein is reduced from oligomers to monomers, and
translocated into the nucleus. There, NPR1 binds to TGA and WRKY transcription factors
to induce expression of plant defense genes. EDS1 and PBS3 are two key proteins
involved in SA biosynthesis. Previous research has shown that several plant pathogens
produce SA hydroxylases. These pathogen-produced hydroxylases act to degrade SA,
preventing their host plant's cells from perceiving this important defense signal,
rendering the host susceptible to infection. Additionally, bacterial pathogens deliver
effectors into their host's cells via the type three secretion system. These effectors
target key defense proteins to subvert plant defense. Using a computational approach, a
list of salicylic acid analogs has been created. Several of these analogs can induce SA-
mediated defense and inhibit bacterial growth in *Arabidopsis*. These analogs, when
sprayed on *Arabidopsis*, can induce the accumulation of the master regulator of plant
defense NPR1. In a yeast two-hybrid system, these analogs can strengthen the
interactions between NPR proteins. I demonstrate that these analogs can induce the
expression of the defense marker gene *PR1* and induce PR1's accumulation. I hope to
test in future assays whether these analogs avoid degradation by pathogenic SA

hydroxylases. Additionally, I demonstrate that a bacterial effector secreted by

Pseudomonas syringae pv. tomato DC3000, HopAA1-2, interacts with EDS1 and PBS3,

causing a

reduction in the amount of these two proteins when transiently expressed in tobacco.

This interaction may be an attempt to subvert SA-mediated defense.

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CHAPTER 1

THE FUNCTION OF SALICYLIC ACID IN PLANT DEFENSE[1]

1.1 Introduction

Salicylic acid (SA) is well known as a precursor of aspirin, the active ingredient of which is acetylsalicylic acid. Aspirin is among the oldest, cheapest, and most widely used medicines in human history; it is broadly used as fever-reducer, pain-reliever, and anti-inflammatory medicine (Myers, 2007). Studies have shown that long-term use of aspirin may reduce the risk of stroke, cardiovascular disease, and heart attack. In addition, non-acetylated salicylate shows effectiveness in treating type II diabetes (Goldfine et al., 2013). In humans, aspirin irreversibly inhibits cyclooxygenase 1 (COX-1) (DeWitt et al., 1990), and modifies the enzymatic activity of COX-2, both of which catalyze the production of prostaglandin H2 from arachidonic acid, involved in inflammation, and thromboxane A2, involved in blood clotting (Preston et al., 1981; Smith, Garavito, & DeWitt, 1996). SA and its derivatives also inhibit IκB kinase (Yin, Yamamoto, & Gaynor, 1998), NF-κB (Kopp & Ghosh, 1994), and activate AMP-activated protein kinase (Hawley et al., 2012).

Plant immunity can be described as consisting of four phases, known as the zig-zag model (Jones & Dangl, 2006). First, pathogen associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) on the plant cell's surface. PAMPs are evolutionarily conserved molecules associated with pathogens such as flagellin, EF-Tu, and chitin (Eckardt, 2008; Gómez-Gómez & Boller, 2002; Zipfel et al., 2006). PAMP recognition results in PAMP-triggered immunity (PTI). PTI consists of

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an increase in cytosolic Ca^{2+} (Boller & Felix, 2009), oxidative burst (Lamb & Dixon, 1997), MAPK activation (Boudsocq et al., 2010), ethylene production (Tintor et al., 2013), stomatal closure , transcriptional reprogramming, SA accumulation (Mishina & Zeier, 2007), and callose deposition (Luna et al., 2011). This response is basal disease resistance against pathogens that can halt colonization. During the second phase of the zig-zag model, pathogens secrete effectors via the type three secretion system that can interfere with PTI, resulting in effector triggered susceptibility (ETS). Plants have evolved Resistance (R) proteins capable of specifically recognizing secreted effectors, resulting in effector-triggered immunity (ETI), as phase three. R proteins are nucleotide-binding leucine-rich repeat (NB-LRR) proteins that can respond to effectors from all classes of pathogens (Elmore, Lin, & Coaker, 2011). R proteins usually recognize effectors indirectly. They may act as accessory recognition proteins that detect effector modification of the effector's true virulence target, or act as decoys that mimic the effector's target (van der Hoorn & Kamoun, 2008). In phase four, pathogens either lose effector genes or acquire additional effector genes that can continue to suppress ETI and PTI. The loss of recognized effectors or the gain of novel effectors, causes selective pressure on the host to evolve new R proteins, resulting in ETI (Jones & Dangl, 2006) (See Figure 1.1).
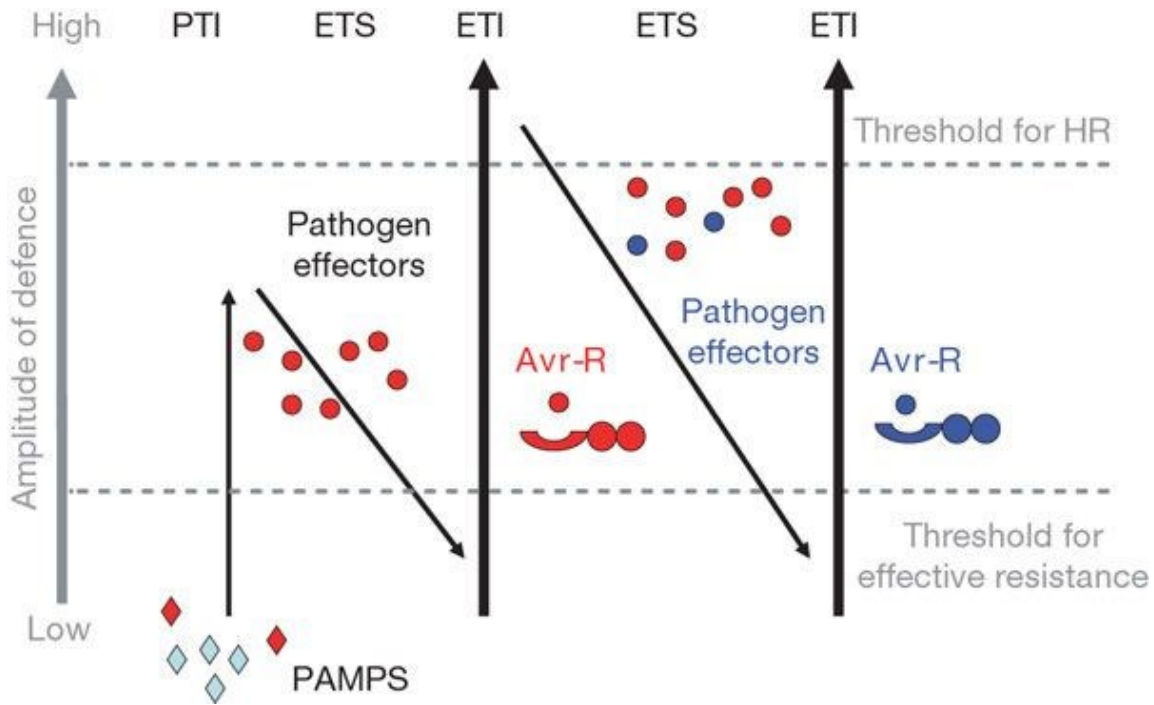
Figure 1.1. The zig-zag model of disease resistance and susceptibility. Phase 1, the plants detect PAMPs resulting in PTI. Phase 2, pathogens secrete effectors to inhibit PTI, resulting in ETS. Phase 3, plant cells recognize a secreted effector, resulting in ETI. Phase 4, the pathogen loses the red effector and gains the blue, allowing the pathogen to once again suppress resistance. Lastly, natural selection favors the evolution of new NB-LRR R proteins that can recognize the blue effector, resulting in ETI (From Jones & Dangl, 2006).

As one of the major plant hormones, SA plays a regulatory role in many physiological processes, such as seed germination, storage, and fruit maturity (Raskin, 1992). In addition, SA plays roles in regulating flowering development, sex differentiation, stomatal movement, and photoperiod. SA is both required and sufficient to induce a defense response against pathogens (Raskin, 1992). Transgenic plants overexpressing the *NahG* transgene from *Pseudomonas putida*, encoding SA-degrading hydroxylase, have been proven to be more susceptible to a variety of pathogens (Delaney et al., 1994).

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During pathogen infection, SA is synthesized in the chloroplast, primarily through the isochorismate pathway in *Arabidopsis*. Isochorismate synthases one and two (ICS1/2) are localized in the plastid, and ICS1 is responsible for the majority of SA accumulation in response to the presence of hemi- and biotrophic pathogens (Fragnière, 2011; Strawn, 2007). *Arabidopsis ics1* mutant plants are significantly reduced in SA level, and as a consequence, these mutants are more susceptible to pathogen infection. SA is an endogenous phytohormone, capable of inducing a potent systemic immune response known as systemic acquired resistance (SAR) (Vlot, Dempsey, & Klessig, 2009). SA is required for defense against biotrophic pathogens – tobacco and *Arabidopsis* plants lacking SA allow normally incompatible races of the bacterial pathogen *Pseudomonas syringae* to accumulate in their tissues (Delaney et al., 1994). SA binds to the master regulator of plant defense, NONEXPRESSOR OF PR GENES 1 (NPR1) (Wu, 2012), which acts as a transcriptional co-activator responsible for the transcriptional activation of SA-dependent genes (Rochon, Boyle, Wignes, Fobert, & Despres, 2006). NPR1 has been proposed to be the plant homolog of mammalian IκBα, due to the sequence conservation of their ankyrin-like repeats (Despres et al., 2003). Like NPR1, IκB proteins are responsible for regulating the transcription of NF-κB, which is responsible for triggering cellular responses to stress and pathogens (Baldwin Jr, 1996).

NPR1 is required for the expression of *PR* genes, which encode small proteins that may have antimicrobial properties. Induction of the expression of *PR1* is directly correlated with an increase of SA levels (Malamy, Carr, Klessig, & Raskin, 1990). The SA-dependent transcription of *PR1* is facilitated by the NPR1 enhanceosome (Rochon et al.,

2006) – a complex of NPR1 and a member of the TGA2 clade of bZIP transcription factors (Zhang, Tessaro, Lassner, & Li, 2003). By interacting with TGA2, NPR1, specifically its N-terminal BTB/POZ domain, represses TGA2's ability to silence *PR1* gene expression (Boyle et al., 2009). Further, NPR1 contains a transactivation domain, which activates the function of the enhanceosome (Rochon et al., 2006).

NPR1 paralogs NPR3 and NPR4 also function as SA receptors (Fu, 2012). These paralogs act as adaptor proteins for Cullin 3 E3 ubiquitin ligase, leading to the ubiquitination and degradation of NPR1, dependent on SA concentration – A high level of SA disrupts the interaction between NPR1 and NPR4, while promoting the interaction between NPR1 and NPR3, this creates a biphasic pattern of NPR1 level and defense response (Moreau, Tian, & Klessig, 2012). NPR3 and NPR4 are also known to form homo- and heterodimers, which has been proposed as a mechanism of auto-regulation (Fu, 2012). The formation of NPR3 and NPR4 homo- and heterodimers is strengthened by the presence of SA (Agriculture & Service, 2015; Fu, 2012).

In addition to inducing a local defense response, SA promotes systemic acquired resistance (SAR) after an invading pathogen is recognized (An & Mou, 2011). SAR protects the plant against further pathogen colonization by causing a systemic defense reaction including the production of pathogenesis related (PR) proteins, phytoalexins, and the strengthening of cell walls. SA is also responsible for regulating these later responses to pathogenic invasion (Lu, Greenberg, & Holuigue, 2016), and application of SA is sufficient to induce plant defense including SAR (Anand et al., 2008).

The SA-mediated plant defense pathway can be activated by exogenous application of SA, 2,6-dichloroisonicotinic acid (INA), or Benzothiadiazole (BTH) (Vernooij et al., 1995) (Leslie et al., 1996). Additionally, some synthetic compounds have been used in the past to elicit a defense response, protecting crops from disease. These synthetic compounds include 3-allyloxy-1,2-benzisothiazole-1,1-dioxide (Probenazole, PBZ), applied to *Oryza sativa* to prevent rice blast caused by *Magnaporthea grisea (Watanabe, 1977)*; the previously mentioned INA on *Cucumis sativus* and *Nicotiana tabacum* to prevent anthracnose (caused by *Colletotrichum lagenarium*) and Tobacco Mosaic Virus infection, respectively (Métraux et al., 1991) (Ward et al., 1991); N-cyanomethyl-2-chloroisonicotinamide (NCI) on *O. sativa* to induce defense against *Pyricularia oryzae*, a sexual morph of *M. oryzae* (Yoshida et al., 1990); and many others (Bektas & Eulgem, 2015).

1.2 Pathogen Strategies of Degrading SA

Unsurprisingly, due to the necessity of SA for defense induction, pathogens have evolved enzymes capable of degrading this key phytohormone. Bacterial members of the genera *Pseudomonas, Bacillus, Agrobacterium, Rhizobium, Sinorhizobium, Ralstonia,* and *Burkholderia* have genes encoding SA hydroxylases capable of metabolizing SA into less or inactive forms (Li et al., 2017). SA hydroxylases function typically by binding SA and NADH or NADPH, then binding molecular oxygen. The resulting products are catechol, H_2O, and CO_2 (You, Murray, Jollie, & Gunsalus, 1990). Ectopically expressing the bacterial SA hydroxylase gene, *NahG,* from *Pseudomonas putida* in *Arabidopsis*

suppresses the defense response against both bacterial and fungal pathogens, and abolishes SA accumulation after pathogen infection (Lawton et al., 1995).

Here, I present the results of a screen of 21 SA analogs. I demonstrate that by applying several of these analogs to *Arabidopsis* Col-0 plants, the accumulation of the master regulator of SA-mediated plant defense, NPR1, can be induced. I show that the application of these SA analogs results in the accumulation of defense protein PR1, and the induction of *PR1* expression. I demonstrate that these SA analogs can strengthen the protein-protein interactions between NPR1 paralogs NPR3 and NPR4 in a yeast two-hybrid system. I demonstrate that these analogs are effective in inhibiting bacterial growth, causing increased resistance against pathogen infection. I also demonstrate that a similar group of SA analogs that are functional in *Arabidopsis* are also capable of strengthening the interactions between NPR1 and NPR3 homologs in *Citrus sinensis*. Lastly, I will demonstrate that the bacterial effector HopAA1-2 from *Pseudomonas syringae* pv. tomato DC3000 interacts with EDS1 and PBS3, reducing the amount of these proteins present in the plant cell, and thereby potentially subverting SA-mediated defense.
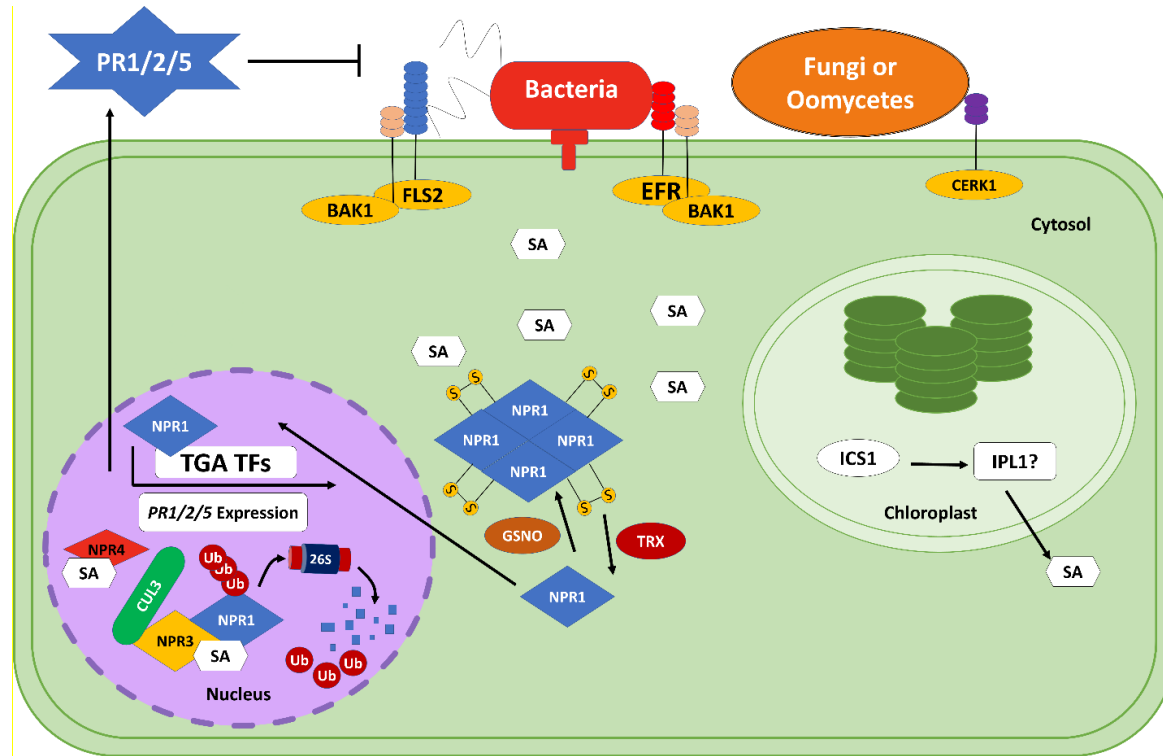
Figure 1.2. A model of SA-mediated plant defense. Biotrophic and hemi-biotrophic pathogens attempt to colonize plant tissue. After PRRs sense PAMPs, SA accumulates within the cell. SA is synthesized in the chloroplast by ICS1 and IPL1(?) through the isochorismate pathway. In the cytosol, NPR1 is reduced from oligomer to monomer, facilitated by thioredoxin (TRX). In the absence of SA, S-nitrosoglutathione (GSNO) facilitates NPR1's oligomerization. NPR1 monomer moves to the nucleus, where it interacts with TGA transcription factors to induce *PR1/2/5* expression. After synthesis, the PR proteins move to the apoplast, where they inhibit pathogen colonization. When SA accumulates to a high level, NPR3 interacts with CUL3 as an adaptor to ubiquitinate NPR1. NPR4 is present in the nucleus, but only acts as a CUL3 adaptor to ubiquitinate NPR1 when SA level is low. The main function of NPR3 and NPR4 is to maintain optimum level of NPR1 protein during plant defense response. After polyubiquitination, NPR1 is degraded within the nucleus by the 26S proteasome.

CHAPTER 2

MATERIALS AND METHODS

2.1 Yeast Two-hybrid (Y2H) Assays

Yeast strains were mated in YPDA media for 48 hr at 30 °C. Diploid yeast strains were plated on double dropout selective media. Colonies were selected, then grown for 48 h in liquid double dropout media at 30 °C. The resulting liquid culture was serially diluted to an OD600 value of 1.0, 0.1, and 0.01, then plated on quadruple synthetic dropout media with and without SA or SA analogs and incubated at 30 °C for 72 hr. CsNPR1 and CsNPR3 were cloned from *Citrus sinensis* Valencia into pDONR® 207 using the Gateway BP reaction. The Gateway LR reaction was used to generate pGADT7 and pGBKT7 yeast expression vectors containing CsNPR1 or CsNPR3. These vectors were transformed into yeast strains Y187 or AH109, respectively, then the yeast strains were mated and plated on synthetic quadruple dropout (QD) media with and without SA or SA analogs like the previously conducted Y2H assays.

2.2 SA Analog Spray Treatment

SA analogs were diluted in 50 mL sterile purified water to a final concentration of 1 mM. The SA analog solutions were sprayed using a Preval® Sprayer. The *Arabidopsis*

leaves were sprayed from multiple angles until the leaves were visibly wet to ensure

complete coverage. Between applications, the Preval® Sprayer was washed, and 15 mL

of sterile purified water was sprayed through to ensure no cross contamination of SA

analogs.

2.3 Immunoblotting

3-week-old *Arabidopsis thaliana* plants were sprayed with 1 mM SA or SA

analogs as above. Samples were collected 6 h after treatment for assaying NPR1

accumulation or 24 h after treatment for assaying PR1 accumulation. Composite

samples were taken consisting of one leaf each of a similar size and age from four

plants. Leaves were frozen in liquid nitrogen, then ground using a metal bead by

crushing for 2 min at 1200 RPM. Protein was extracted using 1x protein extraction

buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.2%

IGEPAL CA-630) with 1x protease inhibitor cocktail (Millipore Sigma), 10 mM DTT, 1 mM

PMSF, and 10 mM MG115. Protein samples assayed for NPR1 monomer and oligomer

were extracted using the same buffer without DTT. Samples were centrifuged at 15,000

x g for 30 min at 4 °C and the supernatant removed to a new tube. The centrifugation

was repeated twice. The protein concentration was determined by mixing 5 µL of

protein sample with 200 µL of 5x Bradford reagent (Bio-Rad) in a spectrophotometer

cuvette and filling to 1 mL with sterile deionized water. The samples were analyzed for

absorbance at 595 nm. Protein concentration was determined by comparing the

absorbance to a standard curve. 100 µg of protein were boiled for 10 min in 1x Laemmli

sample buffer (2% w/v SDS, 10% Glycerol, 60 mM Tris-HCL pH 6.8, 0.01% bromophenol

blue, 0.2% 2-mercaptoethanol), then samples were electrophoresed for 1 h at 120V.

Protein was transferred to a nitrocellulose membrane by transferring for 1 h at 100 V.

The membrane was incubated in 5% non-fat milk for 1 h at room temperature, then

incubated with anti-NPR1 or anti-PR1 antibody (Agrisera) overnight at 4 °C. The

membrane was washed three times for ten minutes in 1x PBST (0.1% Tween20), then

secondary antibody was added at a ratio of 1:5000 and incubated at room temperature

for 2 h. The membrane was washed as above, then incubated in Bio-Rad ECL substrate

for 5 min at room temperature. X-ray film was used to capture the resulting

chemiluminescence.

2.4 RT-qPCR

Three-week-old *A. thaliana* were sprayed with 1 mM SA or SA analogs as above,

and samples were collected after 24 h. Composite samples were collected consisting of

one leaf from ten biological replicates. Each leaf was of a similar size and age. Samples

were frozen immediately in liquid nitrogen and crushed using a Genogrinder at 1,200

RPM for 2 min. RNA was extracted using RNAzol® RT from Millipore Sigma per the

manufacturer's instructions. RNA concentration and purity were quantified

spectroscopically by measuring absorbance at 260 and 280 nm. qScript™ cDNA

SuperMix from QuantaBio was used to generate cDNA from 1 μg of the extracted RNA

according to the manufacturer's instructions. PerfeCTa SYBR® Green SuperMix from

QuantaBio was used to perform qPCR per the manufacturer's instructions. Relative

expression levels were calculated using the double-delta Ct method. The assays were performed with ten biological replicates and six technical replicates.

2.5 Co-immunoprecipitation

N. benthamiana plants were co-infiltrated with *Agrobacterium tumefaciens* strains containing the constructs *pK7FWG2-EDS1-GFP* or *pK7FWG2-PBS3-GFP* and *pLN462-HopAA1-2*-HA or *pLN462-EV*. The tobacco was infiltrated at OD_{600} 0.8. One large leaf was taken from three plants after 48 h. The plant tissue was frozen in liquid nitrogen and ground using a metal bead at 1200 RPM for 2 min. Protein was extracted using 1x protein extraction buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.2% IGEPAL CA-630) with 1x protease inhibitor cocktail (Millipore Sigma), 10 mM DTT, 1 mM PMSF, and 10 mM MG1151x added at a ratio of 1 µL/mg of sample weight. The samples were vortexed, and centrifuged for 30 min at 15,000 x g at 4 °C. The supernatants were collected in a new tube, and the centrifugation was repeated twice. GFP-Trap®_MA magnetic beads (Chromotek) were added to the protein samples according to the manufacturer's instructions. The samples were incubated with the beads for 1 h at 4 °C, then the beads were washed several times according to the manufacturer's instructions and resuspended in 100 µL of 1x Laemmli sample buffer (2% w/v SDS, 10% Glycerol, 60 mM Tris-HCL pH 6.8, 0.01% bromophenol blue, 0.2% 2-mercaptoethanol). The samples were boiled for 10 min, and the beads were removed using a magnetic strip. 2 µL of purified protein sample were loaded into two polyacrylamide gels along with 50 µg samples of un-purified protein from the same

sample as input. The samples were electrophoresed for 1 h at 120 V in 1x MOPS running buffer (50 mM Trizma® base (Sigma-Aldrich), 50 mM MOPS, 3 mM SDS, 1 mM EDTA) then transferred to nitrocellulose membranes in 1x Tris-bicine transfer buffer (20 mM Trizma® base (Sigma-Aldrich) and 25 mM bicine) for 1 h at 100 V, with the transfer apparatus on ice. The membranes were incubated for 1 h at room temperature in 5% non-fat milk, then incubated with either anti-GFP (Chromotek) or anti-HA (Roche) antibodies at 1:1000 dilution overnight at 4 °C. The membranes were washed with 1x PBST (0.1% Tween20) three times for 10 min at room temperature before being incubated with their respective secondary antibodies, at 1:5000 dilution. The membranes were washed again as above, then incubated for 5 min at room temperature in Bio-Rad ECL chemiluminescent substrate. X-ray film was used to capture the resulting chemiluminescence.

CHAPTER 3

SCREENING FOR ACTIVE SALICYLIC ACID ANALOGS

3.1 ChemMine Results

The SMILES string for SA, c1ccc(c(c1)C(=O)O)O, was used as input for ChemMine

Tools. This online suite of tools allows for comparing pairwise structural similarities

between compounds and provides ultra-fast structure similarity search algorithms.

ChemMine Tools also contains a Clustering Toolbox to group the mined chemicals based

on systematic structure and predicted activity (Backman, Cao, & Girke, 2011). This suite

of tools was used to find the 50 most similar compounds to SA, compiled into an excel

workbook. Candidate chemical compounds were then sorted by LogP value and

eliminated from the list based on predicted LogP value (See Table 1.1).
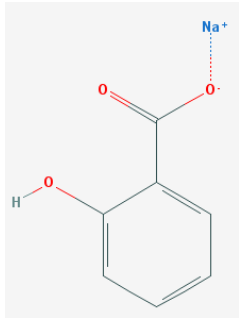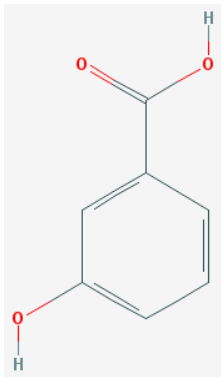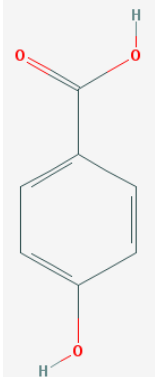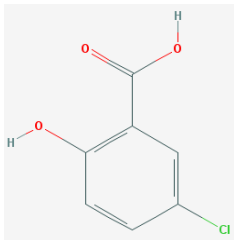
Of the list of 50 most similar compounds to SA, seven compounds were initially

selected, which I believed to be likely candidates. The initial seven compounds were

selected based on similarity to SA, solubility, availability, and price. These compounds

tested were 5-Chloro-2-hydroxybenzoic acid (5-C-2-HBA), 3,5-Dichlorosalicylic acid (3,5-DCSA),

3,6-Dichloro-2-pyridinecarboxylic acid (Clopyralid), 4-Hydroxy-6-methylnicotinic acid (4-H-6-

MNA), Methyl-4-aminobenzoate (Me-4-AB), Methyl salicylate (MeSA), and 6-Acetyl-2(3H)-

benzothiazolone (6-A-2(3H)-BTZ). 3-Hydroxybenzoic acid (3-HBA) and 4-Hydroxybenzoic acid

(HBA) were included as negative controls. SA, Acibenzolar-S-methyl (BTH), and 2,6-

15
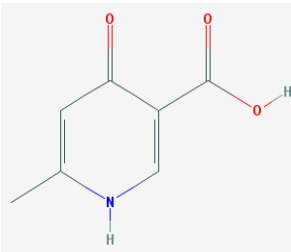
Dichloroisonicotinic acid (INA) were included as positive controls. The complete list of SA

analogs tested in this work can be found below in table 3.2

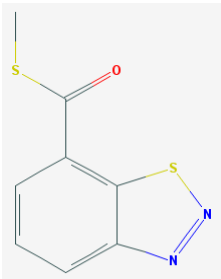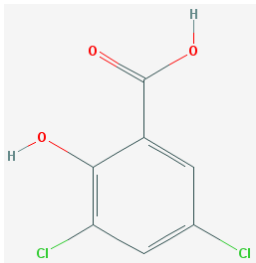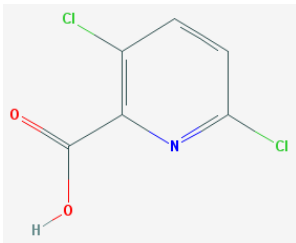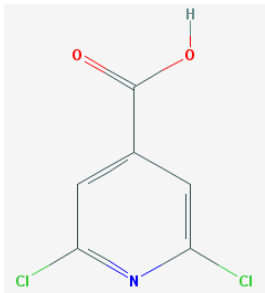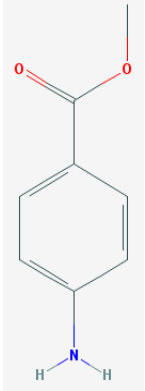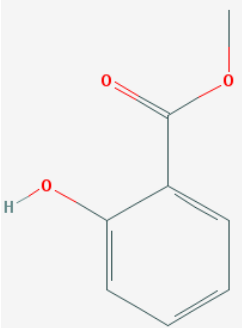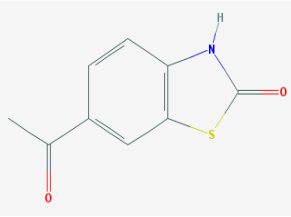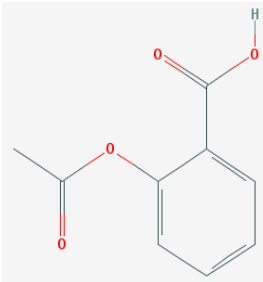| Table 3.1 List of Mined SA Analogs			
cid	Name	Molecular_weight	LogP
3469	2,5-DIHYDROXYBENZOIC ACID	1.53E+02	6.67E-01
9338	2,6-DIHYDROXYBENZOIC ACID	1.53E+02	6.67E-01
55251260	lithium 2,5-dihydroxybenzoate	1.60E+02	6.67E-01
1491	2,4-dihydroxybenzoic acid	1.53E+02	6.67E-01
23663423	Monosodium 2,4-dihydroxybenzoate	1.76E+02	6.67E-01
3418	fosfosal	2.17E+02	1.1109
11812	2-Hydroxyisophthalic acid	1.80E+02	1.3557
97257	2-Hydroxyterephthalic acid	1.80E+02	1.3557
6998	SALICYLALDEHYDE	1.22E+02	1.4218
67658	5-Fluorosalicylic acid	1.55E+02	1.4986
54675839	2,5-Dihydroxybenzoate	1.52E+02	1.5033
54712708	2,4-dihydroxybenzoate	1.52E+02	1.5033
53629521	62TEY51RR1	3.64E+02	1.6432
16682734	BISMUTH SUBSALICYLATE	3.63E+02	1.8035
8388	5-Iodosalicylic acid	2.63E+02	1.9641
72874	2-Hydroxy-4-iodobenzoic acid	2.63E+02	1.9641
4133	methyl salicylate	1.52E+02	2.0602
8375	2'-Hydroxyacetophenone	1.36E+02	2.1286
6738	3-Methylsalicylic acid	1.51E+02	2.1672
6973	5-Methylsalicylic acid	1.51E+02	2.1672
5788	4-METHYLSALICYLIC ACID	1.51E+02	2.1672
11279	2-HYDROXY-6-METHYLBENZOIC ACID	1.51E+02	2.1672
164578	4-Trifluoromethylsalicylic acid	2.05E+02	2.3783
8631	3,5-DIIODOSALICYLIC ACID	3.89E+02	2.4457
8365	Ethyl salicylate	1.66E+02	2.767
54683201	Copper disalicylate	3.38E+02	2.9625
54684589	Magnesium salicylate	2.99E+02	2.965
64738	Magnesium salicylate	2.99E+02	2.965
1.02E+08	Magan	2.99E+02	2.965
517068	CALCIUM SALICYLATE	3.14E+02	2.965
54684600	Calcium disalicylate	3.14E+02	2.965
1.32E+08	Magnesium salicylate	3.17E+02	3.1257
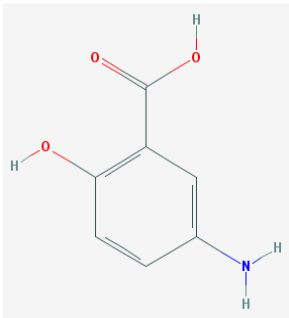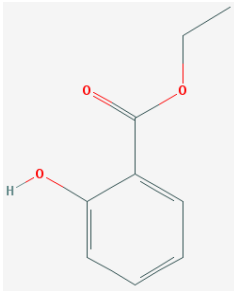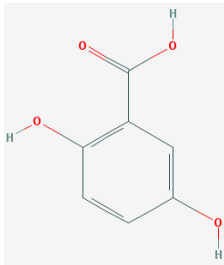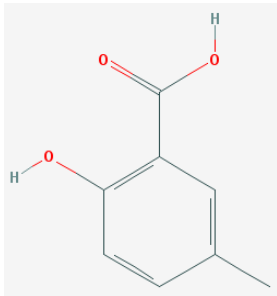
201887	2-Hydroxy-3-isopropylbenzoic acid	1.79E+02	3.5808
5282387	Magnesium salicylate tetrahydrate	3.71E+02	3.6078
54708862	Magnesium salicylate tetrahydrate	3.71E+02	3.6078
133124	Whitfield's ointment	2.58E+02	3.7803
6873	Isobutyl salicylate	1.94E+02	4.1806
16330	Butyl salicylate	1.94E+02	4.1806
50216	Prenyl salicylate	2.06E+02	4.276
16299	Amyl salicylate	2.08E+02	4.8874
6437473	trans-2-Hexenyl salicylate	2.20E+02	4.9828
5371102	cis-3-Hexenyl salicylate	2.20E+02	4.9828
103379	Benzoic acid, 2-hydroxy-, (3Z)-3-hexenyl ester	2.20E+02	4.9828
6021887	3-Hexenyl salicylate	2.20E+02	4.9828
22629	Hexylsalicylate	2.22E+02	5.5942
153705	3-Hexylsalicylic acid	2.21E+02	5.7012
196549	Tcp (antiseptic)	5.56E+02	6.2422
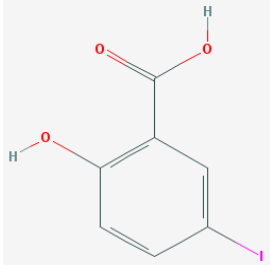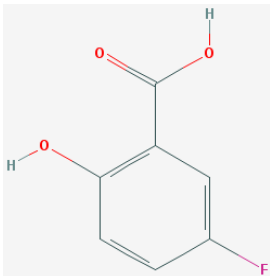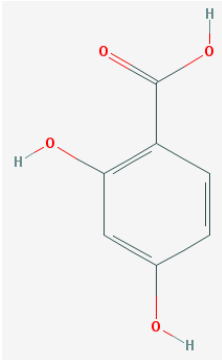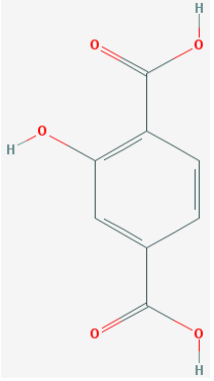
Table 3.2 List of Tested SA Analogs with Chemical Structures					
ID	Name	Abbv.	Structure	Formula	Mol. Weight
1	Sodium Salicylate	NaSA		$C_7H_5NaO_3$	160.104 g/mol
2	3-Hydroxybenzoic acid	3-HBA		$C_7H_6O_3$	138.122 g/mol
3	4-Hydroxybenzoic Acid	4-HBA		$C_7H_6O_3$	138.122 g/mol
4	5-Chloro-2-hydroxybenzoic acid	5-C-2-HBA		$C_7H_5ClO_3$	172.564 g/mol

5	Acibenzolar-S-methyl	BTH		$C_8H_6N_2OS_2$	210.269 g/mol
6	3,5-Dichlorosalicylic acid	3,5-DCSA		$C_7H_4Cl_2O_3$	207.006 g/mol
7	3,6-Dichloro-2-pyridinecarboxylic acid	Clopyralid		$C_6H_3Cl_2NO_2$	191.995 g/mol
8	2,6-Dichloroisonicotinic acid	INA		$C_6H_3Cl_2NO_2$	191.995 g/mol
9	4-Hydroxy-6-methylnicotinic acid	4-H-6-MNA		$C_7H_7NO_3$	153.137 g/mol

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10	Methyl-4-aminobenzoate	Me-4-AB		$C_8H_9NO_2$	151.165 g/mol
11	Methyl salicylate	MeSA		$C_8H_8O_3$	152.149 g/mol
12	6-Acetyl-2(3H)-benzothiazolone	6-A-2(3)H-BTZ		$C_9H_7NO_2S$	193.22 g/mol
13	Acetylsalicylic acid	AcSA		$C_9H_8O_4$	180.159 g/mol

14	5-Aminosalicylic acid	5-AminoSA		$C_7H_7NO_3$	183.137 g/mol
15	Ethyl salicylate	EtSA		$C_9H_{10}O_3$	166.167 g/mol
16	2,5-Dihydroxybenzoic acid	2,5-DHBA		$C_7H_6O_4$	154.121 g/mol
17	5-methylsalicylic acid	5-MeSA		$C_8H_8O_3$	152.149 g/mol

18	5-Iodosalicylic acid	5-I-SA		$C_7H_5IO_3$	264.018 g/mol
19	5-Fluoro-2-Hydroxybenzoic acid	5-F-2-HBA		$C_7H_5FO_3$	156.112 g/mol
20	2,4-Dihydroxybenzoic acid	2,4-DHBA		$C_7H_6O_4$	154.121 g/mol
21	2-Hydroxyterephthalic acid	2-HTPA		$C_8H_6O_5$	182.131 g/mol

3.2 Several Putative SA Analogs Increase the Strength of Interactions between NPR3/4 in Y2H

Due to the critical role that NPR1 paralogs NPR3 and NPR4 play in SA-mediated defense, I hypothesized that active SA analogs would increase the strength of the interactions between these proteins in a yeast two-hybrid system. Because the interaction between NPR1 and NPR3 is strengthened in response to SA and the interaction between NPR1 and NPR4 is disrupted by SA, I chose to examine the effects of SA analogs on the NPR3 and NPR4 interactions, which are strengthened by the presence of SA (Fu, 2012). By examining the interactions between NPR1 paralogs instead of NPR1 itself, I hoped to remove some ambiguity from my Y2H results, resulting from the SA analogs both strengthening and disrupting interactions between NPR1 and its paralogs in Y2H. Indeed, I observed that several SA analogs cause an increase in the number of yeast colonies that survive on quadruple dropout media. The number of surviving colonies treated with SA analogs can be compared to the number that grow when treated with sodium salicylate, appearing when diluted to OD_{600} 0.01. It is clear that 5-C-2-HBA and 3,5-DCSA consistently strengthen the protein-protein interactions in this Y2H hybrid system. Interestingly, BTH does not increase the strength of the interactions between NPR paralogs in this Y2H system, despite previous research showing that it is a potent SA analog (Friedrich et al., 1996). This may be because BTH has some negative effect on the growth of yeast or because BTH may only affect the protein-protein interactions involving NPR1. (See Figure 3.2).

Figure 3.1. Several SA analogs consistently strengthen the interactions between NPR proteins in a Y2H system. A. Interaction between NPR3 and NPR3. B. Interaction between NPR4 and NPR3. C. Interaction between NPR4 and NPR4. Yeast strains were incubated for 24 hours in double dropout liquid media before being washed in sterile deionized water, diluted, and plated on quadruple dropout agar media with or without 200 µM SA or SA analogs. Plates were incubated at 30 °C for 72 h. QD is quadruple dropout –Leu –Trp –His –Ade. DD is double dropout –Leu –Trp. The assay was repeated three times with similar results.

3.3 Several SA Analogs Induce NPR1 Accumulation

Next, to determine whether the SA analogs could induce the accumulation of NPR1, I treated wild type *Arabidopsis* with a 1 mM spray of SA analogs or SA, and compared the NPR1 protein levels, using untreated plants as a negative control. Previous research has shown that exogenous application of SA is sufficient to illicit a defense response, including the accumulation of NPR1. It was observed that BTH, INA, 5-C-2HBA, 3,5-DCSA, and 6-A-2(3)H-BTZ can induce NPR1 accumulation. NaSA can induce accumulation of both oligomer and monomer forms of NPR1. BTH and INA similarly can cause accumulation of monomer and oligomer forms of NPR1 above the level seen in non-treated plants or plants treated with 3- or 4-HBA. Interestingly, and 6-A-2(3)H-BTZ can also induce accumulation of oligomer and monomer forms of NPR1, despite being inactive in Y2H. 5-C-2-HBA and 3-5-DCSA, which were previously observed to be active in Y2H, can induce accumulation of the monomer form of NPR1, but not the oligomer (See Figure 3.2).

Figure 3.2. SA analog treatment induces accumulation of NPR1. 3-week-old *A. thaliana* were sprayed with 1 mM SA or SA analogs. Samples were collected 6 h after treatment. Composite samples were taken consisting of one leaf each of a similar size and age from four plants. 100 µg of protein was electrophoresed per sample. The membrane was incubated with anti-NPR1 antibody overnight at 4 °C. NT is non-treated. * indicates a non-specific band. The assay was repeated three times with similar results.

3.4 Substitutions on the Second and Fifth Carbon of SA May Lead to New SA Analog Discoveries

After considering the results I observed from previous experiments, I deduced that making substitutions to the second or fifth carbon of SA may be key to developing novel SA analogs that are functional but may resist degradation by bacterial pathogens. As a result of this conclusion, I refocused my work by returning to the list of likely SA analogs, and selected new SA analogs with substitutions on the second or fifth carbon (See Figure 3.3).
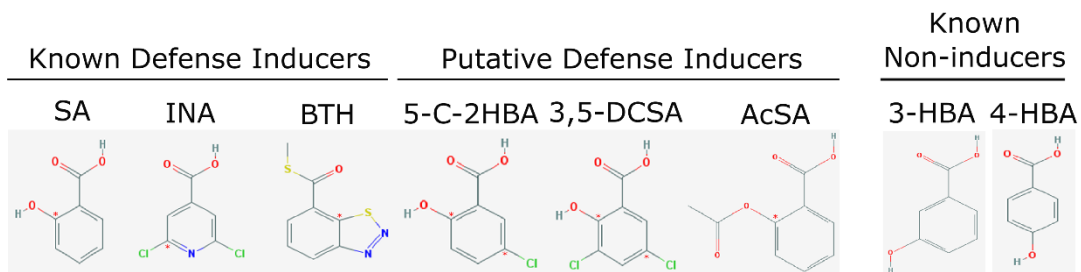
Figure 3.3 Comparison of known defense inducers and known non-inducers. Known inducers often have substitutions on carbon two and/or carbon five. Non-inducers have substitutions on carbon three or four. Substitutions on carbons two or five are indicated by a red asterisk.

3.5 Several Putative SA Analogs Increase the Strength of Interactions between NPR3/4 in Y2H

The Y2H assay was repeated using the new group of SA analogs. I hypothesized that using SA analogs with a 5- or 2-Carbon substitution would strongly enhance the interactions between NPR1 paralogs. Based on my observation, AcSA, 5-MeSA, and 5-F-2HBA appear to be active at a similar level as NaSA. Media treated with 5-AminoSA, EtSA, 5-I-SA, and 2,4-DHBA appear to increase the number of surviving yeast colonies

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above the non-treated group, although they cannot increase the strength of the interaction to the same level as NaSA (See figure 3.4).
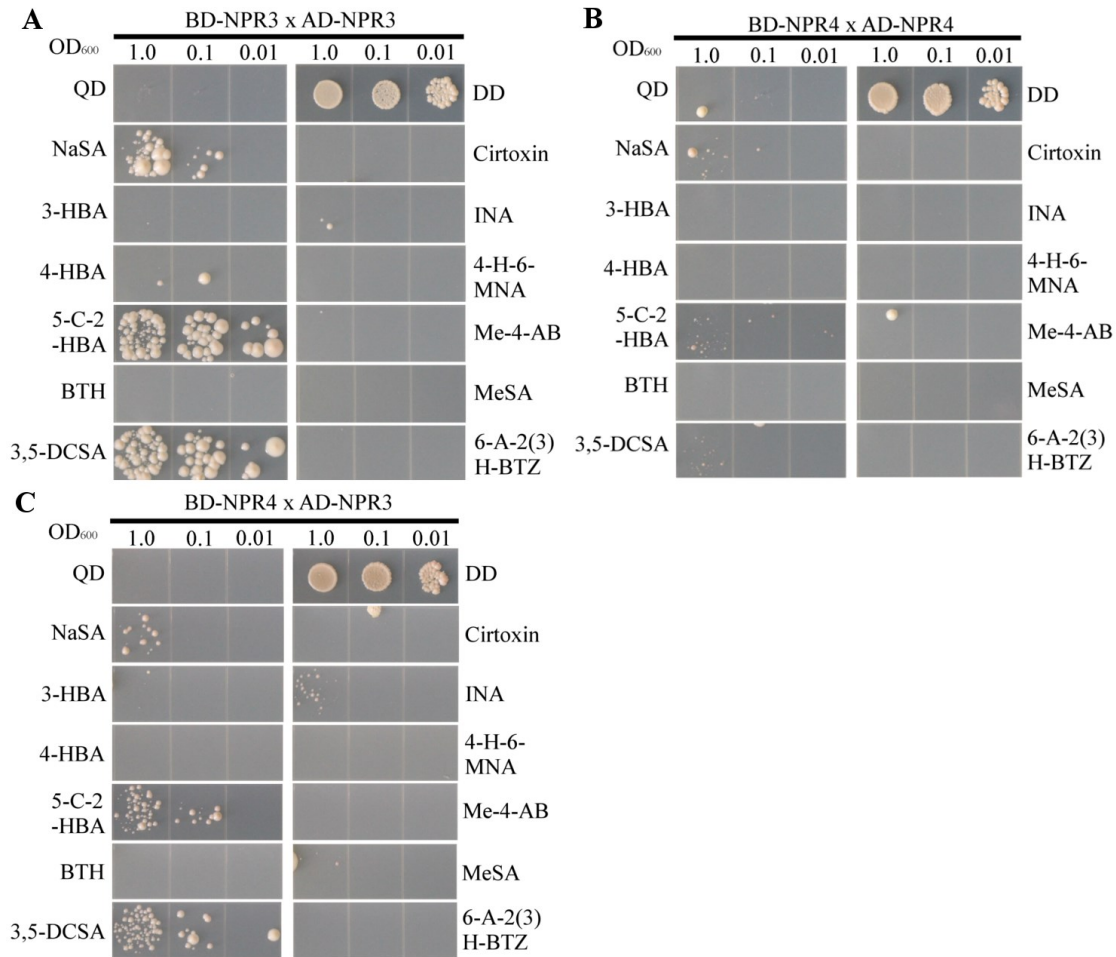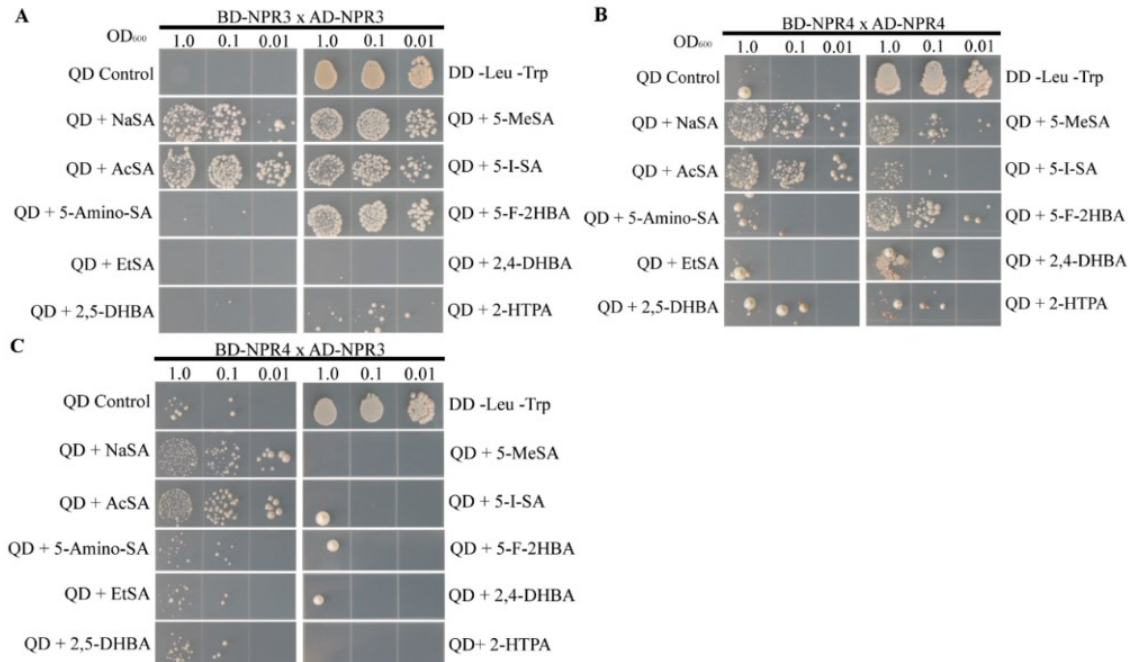

Figure 3.4. Several SA analogs consistently strengthen the interactions between NPR proteins in a Y2H system. A. Interaction between NPR3 and NPR3. B. Interaction between NPR4 and NPR3. C. Interaction between NPR4 and NPR4. Yeast strains were incubated for 24 hours in double dropout liquid media before being washed in sterile deionized water, diluted, and plated on quadruple dropout agar media with or without 200 μM SA or SA analogs. Plates were incubated at 30 °C for 72 h. QD is quadruple dropout –Leu –Trp –His –Ade. DD is double dropout –Leu –Trp.

3.6 Several SA Analogs Induce NPR1 Accumulation

I hypothesized that treatment with the new group of SA analogs would induceNPR1 to a similar level as NaSA. After using a 1 mM spray treatment, and immunoblotting to detect NPR1, I observed that that AcSA, 5-I-SA, 5-F-2-HBA, and 5-MeSA show a similar accumulation of the NPR1 protein, as compared to the accumulation observed using NaSA (See Figure 3.5).

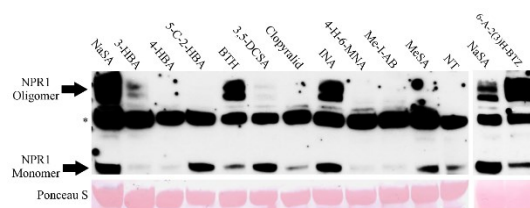Figure 3.5. SA analog treatment induces accumulation of NPR1. 3-week-old A.
thaliana were sprayed with 1 mM SA or SA analogs. Samples were collected 6 hpi.
Composite samples were taken consisting of one leaf each of a similar size and age
from four plants. 100 µg of protein was electrophoresed per sample. The membrane
was incubated with anti-NPR1 antibody overnight at 4 °C. NT is non-treated. The
assay was repeated three times with similar results.

3.7 Several SA Analogs Induce SAR

After observing that SA analogs could induce the accumulation of NPR1 *in
planta*, we were curious whether treatment with SA analogs could induce limit bacterial
growth. We observed that all but one SA analog, 2,5-DHBA could reduce the number of
CFU per leaf disc by at least one order of magnitude, when compared with non-treated
plants. Additionally, we observed no significant difference between the number of
bacteria found in the SA analog treated plants and the SA treated plants, again with the
exception of 2,5-DHBA (See Figure 3.6).
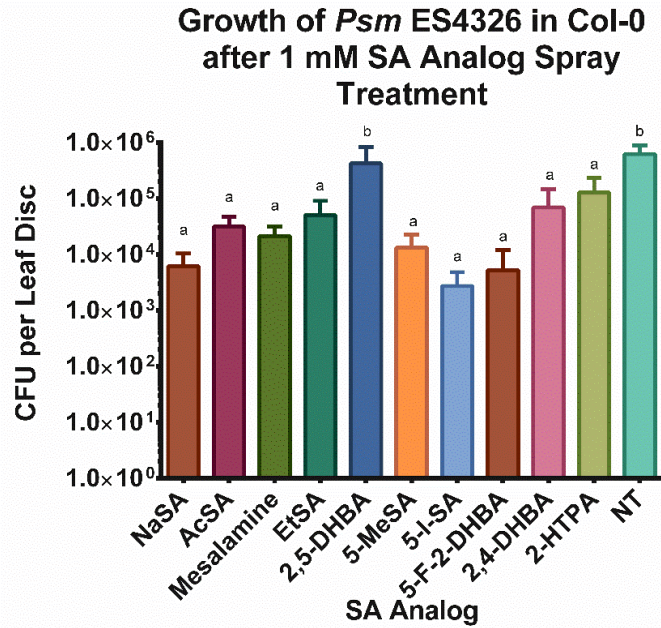
Figure 3.6. SA analog treatment reduces the amount of bacterial present in leaves of treated plants. 3-week old A. thaliana Col-0 were sprayed with 1 mM SA or SA analogs. After 24 h, two leaves each from three plants per treatment were infiltrated with *Pseudomonas syringae* pv. maculicula ES4326 at OD600 0.001 in 10 mM MgSO4. After 72 h, 2 discs were sampled from each leaf. Dunnett's multiple comparison test was used to generate groups of statistical significance. P≤0.05. NT is non-treated. The assay was performed twice with similar results.

3.8 SA analogs that induce NPR1 accumulation are inducers of PR1 protein accumulation

After observing that almost all SA analogs could inhibit pathogen growth, and that several analogs were potent inducers of NPR1 accumulation, I hypothesized that an increase in NPR1 protein must trigger the accumulation of PR1, a small peptide which is is known to inhibit the growth of bacterial pathogens. I sprayed Col-0 *Arabidopsis* with 1 mM SA or SA analogs, then collected leaf samples for western blotting after 24 h. I observed that AcSA induces the highest level of PR1 accumulation, even higher than the same concentration of NaSA. I observed that the other SA analogs could induce PR1

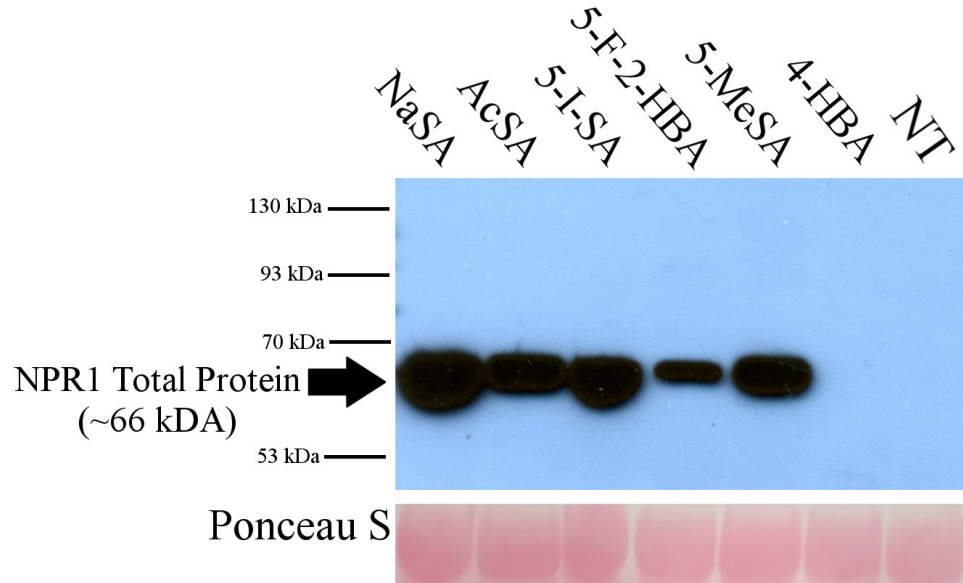accumulation, but at lower levels than NaSA or AcSA. (See figure 3.7).
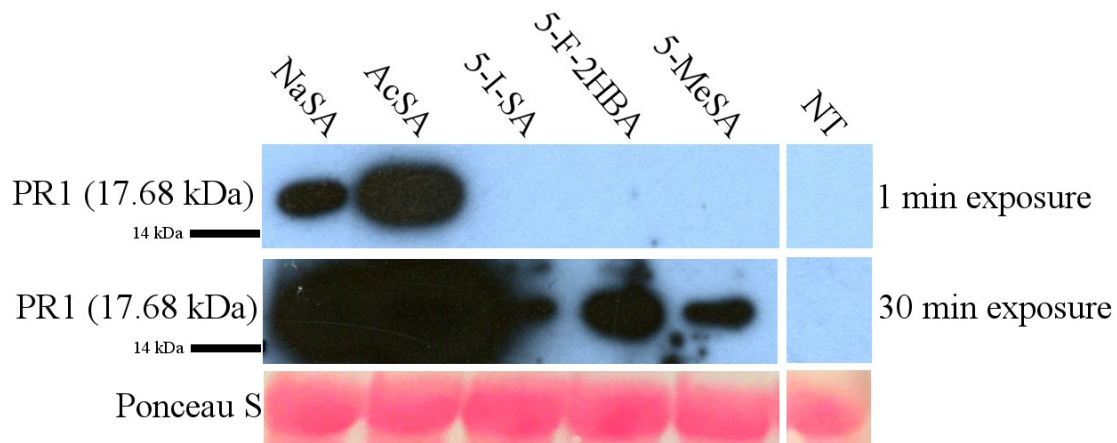
Figure 3.7. SA analog treatment induces accumulation of PR1. 3-week-old A. thaliana were sprayed with 1 mM SA or SA analogs. Samples were collected 24 hpi. Composite samples were taken consisting of two leaves each of a similar size and age from three plants. 50 µg of protein was electrophoresed per sample. The membrane was incubated with anti-PR1 antibody overnight at 4 °C. NT is non-treated. The assay was performed three times with similar results.

3.9. AcSA, 5-F-2-HBA, 5-I-SA, and 5-MeSA Induce *PR1* Expression

To confirm that *PR1* expression was induced by the SA analogs I sprayed

Arabidopsis as above and performed RT-qPCR to measure the expression level. *PR1* is

commonly used as a marker gene for defense induction. I observed that all four tested

analogs could induce *PR1* expression, in agreement with the level of PR1 I observed by

immunoblotting (See Figure 3.8).

Figure 3.8. Relative Normalized *PR1* Expression 24 h after 1 mM SA Analog Spray. Composite samples were made from five biological replicates. Samples were assayed using three technical replicates. Expression levels were calculated using the double-delta Ct method. Error bars represent standard error of measurement.

3.10 The interaction between CsNPR1 and CsNPR3 is strengthened by several SA analogs

I hypothesized that these SA analogs could be potent tools against the citrus greening pathogen, *Candidatus* liberibacter spp., which is known to produce an SA hydroxylase enzyme that functions to suppress plant defense (Li et al., 2017). I cloned the NPR1 and NPR3 homologs from *Citrus sinensis* Valencia and tested whether the SA analogs could also strengthen the interaction between citrus NPR proteins using Y2H

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(See Figure 3.9). I observed that NaSA, AcSA, 5-MeSA, 5-I-SA, 5-F-2-HBA, and 2-HTPA all

can strengthen the interaction between citrus NPR proteins in my Y2H system. This

finding it significant, because it suggests that the SA analogs I have tested using

Arabidopsis may also be effective for inducing a defense response in citrus. If these SA

analogs are active in citrus, then I speculate that they may be candidates for fighting the

citrus greening pathogen, because they may not be able to be degraded by the

pathogen's SA hydroxylase enzyme.
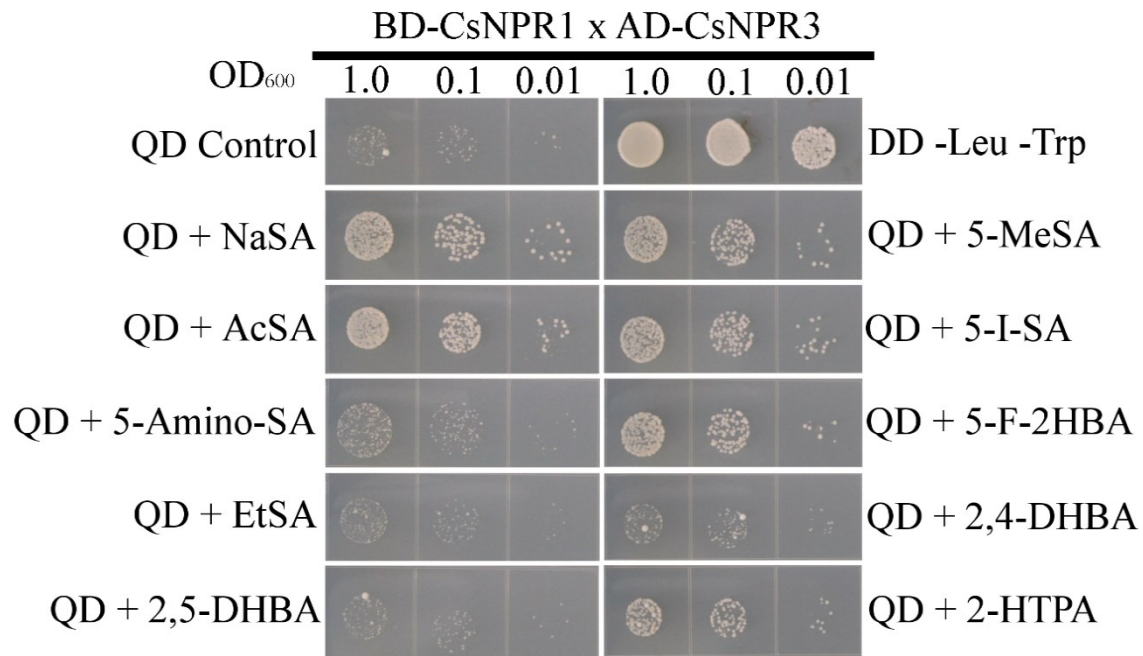
Figure 3.9. Several SA analogs consistently strengthen the interactions between citrus NPR1 and NPR3 proteins in a Y2H system. Yeast strains were incubated for 24 hours in double dropout liquid media before being washed in sterile deionized water, diluted, and plated on quadruple dropout agar media with or without 200 μM SA or SA analogs. Plates were incubated at 30 °C for 72 h. QD is quadruple dropout –Leu –Trp –His –Ade. DD is double dropout –Leu –Trp.

3.11 Results and Discussion

Acetylsalicylate, 5-Methylsalicylic acid, 5-fluoro-2-hydroxybenzoic acid, and 5-iodosalicylic acid are reliable inducers of plant defense. The data I've presented here suggests that these SA analogs would be worthy candidates for use against bacterial pathogens. Their ability to invoke a defense response from *Arabidopsis* and confer bacterial resistance are traits that warrant further investigation.

Previous research suggested that acetylsalicylate was effective against Tobacco Mosaic Virus in tobacco (White, 1979); however, there is little research into its use against bacterial pathogens. Acetylsalicylate's ability to induce defense is not entirely surprising when one considers that acetylsalicylic acid and SA also share a function in mammals. The ability for acetylsalicylate to induce a higher level of PR1 accumulation and *PR1* expression may be due to an increase in membrane permeability of that compound in relation to sodium salicylate. A compound's polar surface area can be used a measure of that compound's H-bonding potential, and therefore, its membrane penetration potential (van de Waterbeemd, Camenisch, Folkers, Chretien, & Raevsky, 1998). Acetylsalicylate has a slightly higher polar surface area at 63.6 $Å^2$ than sodium salicylate which is 60.4 $Å^2$ (Kim et al., 2016), which could make it slightly more bioavailable to the treated plant's cells.

5-fluoro-2-hydroxybenzoic acid and 5-iodosalicylic acid are likely inducers of plant defense, because of their structural similarity to SA. Usually, the chemical interaction between a protein and a small molecule is dictated by electrostatic forces --

H-bonding and Van der Walls forces, but halogen atoms can also generate intermolecular forces capable of stabilizing a protein complex that are similar to H-bonding in both strength and directionality (Parisini, Metrangolo, Pilati, Resnati, & Terraneo, 2011). This realization has enabled researchers to develop new halogen-substituted ligands that are more membrane permeable and have a longer biological half-life by avoiding the normal catabolic processes that normally degrade the drug (Parisini et al., 2011). For these reasons, 5-F-2HBA and 5-I-SA would make great candidates for use against pathogens that produce SA hydroxylase enzymes.

My research demonstrates that 5-methylsalicylic acid can induce NPR1 and PR1 accumulation, *PR1* expression, inhibit pathogen growth, and promote the interaction between NPR proteins. 5-MeSA differs from methyl salicylate (MeSA), which has a methyl group appended to the carboxyl group on carbon 1 of the aromatic ring, rather than the methyl substitution on carbon 5. Unlike methyl salicylate which is a volatile, wintergreen-scented compound that is a liquid at room temperature, 5-MeSA is a white, odorless compound that is solid at room temperature. 5-MeSA's use as a defense inducer warrants further research, because it is similar enough in structure to SA, but may be able to avoid degradation by bacterial SA hydroxylases due to its methyl group substitution on carbon 5.

Ensuring the security of our food supply is one of humanity's greatest challenges. Climate change is causing changes to occur in the suitability of certain areas to produce crops. Temperature changes cause decreases in crop yield and changes to the size or

region of insect pest ranges, allowing these insect vectors to carry plant pathogens to a wider area (Richard, L., & J., 2018). The spread of these insect vectors coupled with the spread of new plant diseases puts the security of the human food supply at risk. The development of new compounds to treat plant diseases is one method by which we can ensure the future security of our food supply.

CHAPTER 4

IDENTIFICATION OF A BACTERIAL EFFECTOR PROTEIN TARGETING EDS1 AND PBS3

4.1 Introduction

Plants and plant pathogens are locked in an evolutionary arms race to develop more advanced proteins to enhance or subvert plant defense, respectively. EDS1 is a positive regulator of basal resistance to biotrophic pathogens (Wiermer, Feys, & Parker, 2005), required by many *Arabidopsis* Toll interleukin receptor (TIR) – nucleotide binding (NB) – leucine rich repeat (LRR) class R proteins to activate ETI (Bart J. Feys, Moisan, Newman, & Parker, 2001). EDS1 is required for accumulation of SA in response to a pathogen (Parker et al., 1996), and it has been reported that the reduced levels of SA in *eds1* and *pad4* mutants results in increased susceptibility to pathogen infection (Falk et al., 1999; Jirage et al., 1999; Nawrath, Heck, Parinthawong, & Metraux, 2002). In addition to EDS1, PAD4 also serves as a regulator of basal plant immunity. EDS1 forms heterocomplexes with PAD4 in the nucleus and cytoplasm, which are required for HR and pathogen resistance. In addition to PAD4, EDS1 also interacts with SAG101 (SENESCENCE ASSOCIATED PROTEIN 101), which can form a ternary complex with EDS1 and PAD4, and plays a pivotal role in pathogen resistance (B. J. Feys et al., 2005; Wagner et al., 2013; Zhu et al., 2011)

PBS3 is a member of the GH3 family of acyl-adenylate/thioester-forming enzymes, which when mutated, causes SA to fail to accumulate, no induction of *PR1* defense gene, and increased pathogen susceptibility (Nobuta et al., 2007). EDS1, PBS3, and PAD4 proteins are critical to SA-mediated plant defense, and likely targets for pathogen effectors.

4.2 PBS3 and EDS1 Interaction with HopAA1-2 in Y2H

PBS3 and EDS1 were screened against a library of all *Pseudomonas syringae* pv. tomato DC3000 effectors in order to identify potential effector targets within the SA biosynthesis pathway. I hypothesized that effectors would likely target proteins in the pathway in order to subvert SA-mediated plant defense. I observed that HopAA1-2 interacts with two proteins involved in SA biosynthesis, EDS1 and PBS3, in our yeast two-hybrid system (See figure 4.1). This effector was chosen for further study, because it was found to target both EDS1 and PBS3, and relatively little is known about the function of this effector.
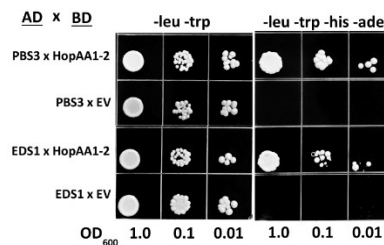
Figure 4.1. HopAA1-2 interacts with EDS1 and PBS3 in yeast two-hybrid assay. Strains were plated at OD_{600} = 1.0, 0.1, and 0.01 on DD and QD media. Photos were taken at 5 days post inoculation. This assay was repeated three times with similar results.

4.3 PBS3 and EDS1 Co-immunoprecipitate with HopAA1-2 Using Transient Expression in Tobacco

To further prove the interaction between HopAA1-2 and EDS1 and PBS3, *Agrobacterium* strains containing constructs encoding these genes under control of the 35S promoter were co-infiltrated into *Nicotiana benthamiana.* A co-immunoprecipitation assay was performed 24 hours after infiltration. I observed that PBS3-GFP and EDS1-GFP Co-immunoprecipitate with HopAA1-2-HA, verifying their interactions in a plant-based system (See figure 4.2).
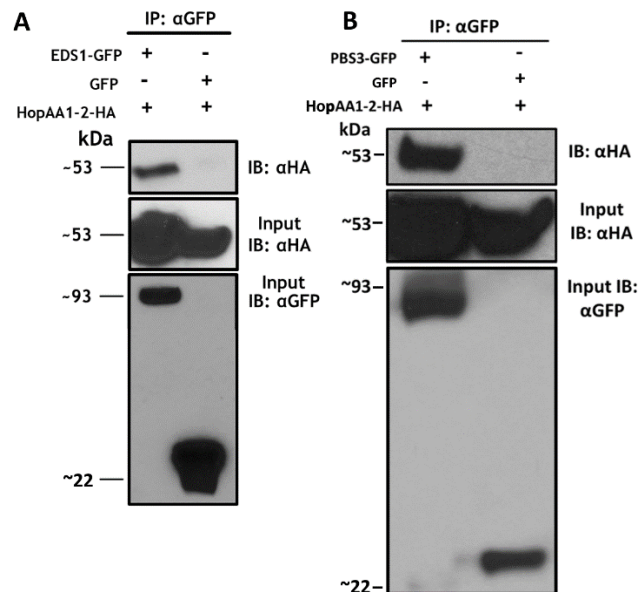
Figure 4.2. Co-IP assays of EDS1-GFP and PBS3-GFP and HopAA1-2 after transient expression in *N. benthamiana.* A. *35S:EDS1-GFP* or B. *35S:PBS3-GFP* and *EV-GFP* with *35S:HopAA1-2-HA Agro* strains were co-infiltrated into tobacco. Samples were taken from 3 biological replicates 48 hours post inoculation. Proteins were purified using anti-GFP beads, then electrophoresed, and probed with anti-GFP or anti-HA antibodies, respectively. This assay was repeated three times with similar results.

4.4 EDS1 and PBS3 Protein Levels Are Reduced When Co-expressed with HopAA1-2

After discovering the positive interactions between HopAA1-2 and PBS3 and EDS1, I

hypothesized that HopAA1-2 causes degradation of EDS1 and PBS3 as a result of the interaction.

I co-infiltrated *Agrobacterium* strains into tobacco containing *35S:HopAA1-2-HA* or an empty

vector and *35S:EDS1-FLAG* or *35S:PBS3-GFP*. I used a western blot to compare the levels of

EDS1-FLAG or PBS3-GFP in the tobacco plants infiltrated with *35S:HopAA1-2-HA* versus the

plants infiltrated with the empty vector. I observed that the plants infiltrated with the effector

had a much lower level of PBS3-GFP or EDS1-FLAG than the plants infiltrated with the empty

vector (See figure 4.3).
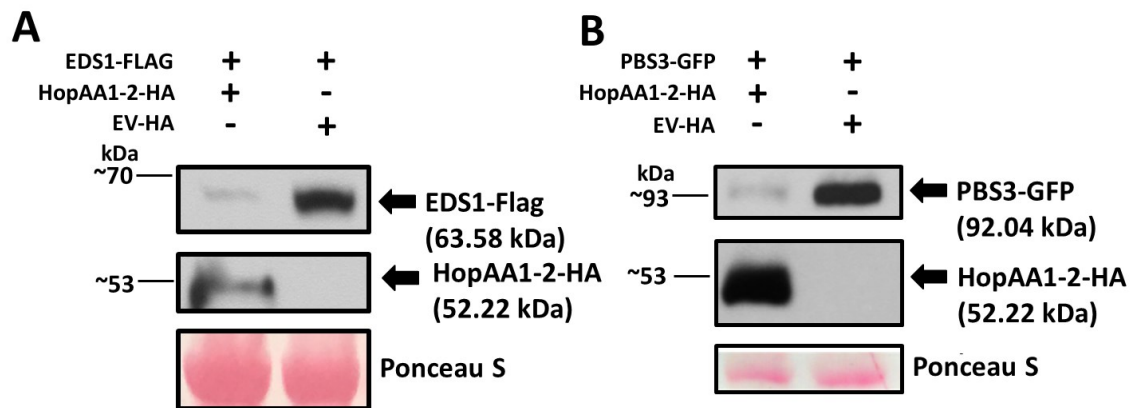
Figure 4.3. Immunoblotting assays of EDS1-GFP and PBS3-GFP and HopAA1-2 after transient expression in *N. benthamiana.* A. *35S:EDS1-FLAG* or B. *35S:PBS3-GFP* and *EV-GFP* with *35S:HopAA1-2-HA Agro* strains were co-infiltrated into tobacco. Samples were taken from 3 biological replicates 48 hours post inoculation. Proteins were probed with anti-GFP or anti-HA antibodies, respectively. These assays were performed three times with similar results.

4.5 Results and Discussion

I have demonstrated that the defense proteins EDS1 and PBS3 directly interact with the effector HopAA1-2, and that EDS1 and BS3 levels are reduced in plants co-infiltrated with HopAA1-2. I speculate that HopAA1-2 may degrade these plant defense proteins as part of a mechanism to subvert SA-mediated plant defense. HopAA1-2's function remains elusive, previous research has suggested that HopAA1-1 may act as a GTPase activating protein (GAP) (Munkvold, Russell, Kvitko, & Collmer, 2009); however, the paralogous GAP motif in HopAA1-2 differs significantly from HopAA1-1. A search of the NCBI's Conserved Domains Database failed to return any results.

Results obtained from the d*e novo* protein modeling software I-TASSER suggest that HopAA1-2 bears similarity to human Vinculin, a protein that is involved in terminating microfilaments at cell membranes (Geiger, Tokuyasu, Dutton, & Singer, 1980). HopAA1-2's function within the cytoskeleton in addition to its ability to interact with PBS3 and EDS1 remains plausible due the fact that effector genes are commonly shuffled around the bacterial genome, leading to duplication events, and the creation of hybrid effectors able to perform multiple subversive functions in the host. Further experiments are needed to determine whether HopAA1-2 plays a role in modifying the actin cytoskeleton to subvert plant defense, and whether it performs this function by acting on actin directly or indirectly.

Lastly, the purpose of EDS1's and PBS3's interaction with HopAA1-2 is unknown. Future experiments must be conducted to determine whether HopAA1-2 is degrading these proteins directly or is causing their degradation in an indirect manner.

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