Identification and Characterization of *Pseudomonas syringae* Type III Effectors that Alter Auxin Responses

by

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Abstract

Plant hormones act in a complex network where their pathways regulate and interact to control different mechanisms, such as development and stress responses. This crosstalk between hormones can be exploited by pathogens to suppress plant defense responses and thereby increase pathogen growth.

*Pseudomonas syringae* pathogenicity is reliant on a Type III secretion system (TTSS) that acts as a specialized injection apparatus to deliver virulence proteins, known as type III effectors (TTEs), into the plant cell cytosol. In my work, I have screened hormone inducible promoter::GUS transgenic *Arabidopsis thaliana* lines against a *P. syringae* TTE library in order to identify TTEs involved in the perturbation of hormone signaling *in planta*. Through this screen I identified two *P. syringae* TTEs, HopAK1 and HopAL1, both belonging to the same bacterial strain *P. syringae* pv. *maculicola* ES4326. I found that HopAK1 can sensitize *A. thaliana* plants to auxin. On the other hand, HopAL1 activates auxin signaling. Monitoring of auxin signaling was done using transgenic *DR5*::GUS plants. Both TTEs render the plant susceptible to bacterial infection, highlighting a potential relationship between increased auxin signaling and virulence.
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Abbreviations

ABA - Abscisic acid

ABCB - ATP-binding cassette transporter

ARF - Auxin response factor

AuxREs - Auxin response elements

BAK1 - BRI1-associated receptor kinase

BIRK1 - BAK1-interacting receptor-like kinase 1

BKK1 - See SERK4

BTH - Benzo(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester

CaMV - Cauliflower mosaic virus

CC-NB-LRR - Coil-coiled- nucleotide binding Leucine-rich repeat domain

Cfu - Colony forming unit

COI1 - Coronatine insensitive

Cub - C-terminus of ubiquitin

CUL3 - Cullin 3

DAMP - Damage-associated molecular patterns

DEPC - Diethylpyrocarbonate
DEX - Dexamethasone

DMSO - Dimethyl sulfoxide

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic acid

EF1-α - Elongation factor α

ER - Endoplasmic reticulum

ERF1 - Ethylene response factor 1

EV - Empty vector

FLS2 - Flagellin-sensing 2

GAP - GTPase activating protein

GEF - Guanine nucleotide exchange factor

GUS - β-glucuronidase

HA - Hemagglutinin

HP - Hypothetical protein

Hpt - hours post inoculation

HR - Hypersensitive response

ISC1 - Isochorismate synthase 1
JA - Jasmonic acid

JAZ - Jasmonate ZIM domain

KO - Knock-out

LiCl - Lithium chloride

LRR domain - leucine-rich repeat domain

MAMP - Microbe-associated molecular patterns

MPKs - Mitogen-activated protein kinases

MS - Murashige and Skoog media

MUG - 4-Methylumbelliferyl-β-D-glucuronide

NAA - 1-naphthaleneacetic acid

NB - nucleotide binding

NPR1 - non-expressor of PR genes 1

NS - Nuclear localization signal

Nub - N-terminus of ubiquitin

OCP3 - Overexpressor of cationic peroxidase 3

OD - Optical density

OLG - Oligolacturonide
PAMP - Pathogen-associated molecular patterns

PDF1.2 - Plant defensin 1.2

PEPR1 - PEP receptor 1

PILS - PIN-like auxin carriers

PR - Pathogenesis-related

PR1 - Pathogenesis-related 1

PROPEP1 - Peptide protein 1

PRR - Pattern recognition receptors

R protein - Resistance protein

RboD - Respiratory burst oxidase homolog D

RIN4 - Rpm1 interacting protein 4

RPM1 - Resistance to P. syringae maculicola 1

RPS2 - Resistance to P. syringae 2

RT - Room temperature

SA - Salicylic acid

SABP2 - Salicylic acid binding protein 2

SAR - Systemic acquired resistance
SERK4 - Somatic embryogenesis receptor-like kinase 4

SID2 - SA induction deficient 2

TIR1 - Transport inhibitor response 1

TIR-NB-LRR - Toll interleukin 1 receptor-nucleotide binding leucine-rich repeat domain

TTEs - Type three effectors

TTSS - Type three secretion system

WRKY70 - WRKY protein 70

X-gluc - 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
Chapter 1

General Introduction
1.1 Plant immunity

Plants face numerous environmental stresses, including biotic (bacteria, fungi, viruses, herbivores, and nematodes) and abiotic agents (cold, salt, drought and heat) that are constantly threatening their survival and fitness. Plants have evolved various methods to avoid being affected by these agents, many of which are tightly controlled in order to appropriately allocate resources under stress situations (Pajerowska-Mukhtar et al., 2012; Atkinson and Urwin, 2012). This is especially important since uncontrolled activation of stress responses can lead to detrimental effects on plant growth (Glazebrook, 2005). Unlike animals, plants lack an adaptive immune system involving specialized immune cells, and instead each cell must rely on their innate immune responses to counter infections. Determining the complex molecular mechanisms underlying activation of defense responses continues to be a major challenge in the field of plant-microbe interactions and is crucial for development of effective and durable disease-control strategies.

1.2 Plant defense and elicitors

The complexity of the plant response to biotic stress is evident in its layered defense system. The first layer consists of preformed mechanical barriers, like the cell wall and cuticle, as well as antimicrobial molecules that are constitutively produced (Hatsugai and Hara-Nishimura, 2010). In addition, plants have inducible defences that
can be activated by a variety of small molecules such as salicylic acid (SA) as well as pathogen associated features (Halim et al., 2009). Pathogen-associated molecules can be recognized by plant receptors and act as elicitors that trigger plant defence responses (Nicaise et al., 2009; Bittel and Robatzek, 2007; Hahn, 1996). Four main classes of elicitors have been described (McCann et al., 2012): microbe-associated molecular patterns (MAMP) or pathogen-associated molecular patterns (PAMP), damage-associated molecular patterns or DAMPs, toxin and pore-forming molecules and pathogen effectors recognized by plant resistance (R) proteins (McCann et al., 2012). R proteins are the product of the R genes and have the ability to recognize specific pathogenic effectors (Friedman and Baker, 2007; Belkhadir et al., 2004).

Perception of MAMPs relies on a specific class of receptors termed pattern recognition receptors (PRR) (Nicaise et al., 2009; Bittel and Robatzek, 2007; Zipfel, 2009). A well-studied MAMP is the flagellin protein, a major component of bacterial flagella that is highly conserved among species (Felix et al., 1999; Chinchilla et al., 2006). flg22 is a 22-amino acid oligopeptide in the N-terminus of flagellin, which is perceived through leucine-rich repeat (LRR) domains of the LRR-kinase flagellin-sensing 2 (FLS2) protein (Felix et al., 1999; Naito et al., 2007; Zhao and Qi, 2008). FLS2 has an extracellular 28 LRR motif, a transmembrane domain and a Ser/Thr kinase cytoplasmatic domain (Nicaise et al., 2009). It has been demonstrated that flg22 binds directly to FLS2 (Sun et al., 2012; Albert and Felix, 2010; Chinchilla et al., 2006). The LRR sites 9-15 of FLS2 were demonstrated to influence binding to flg22 but the direct binding site remains unknown (Nicaise et al., 2009; Ali and Reddy, 2008; Dunning et al., 2007). In the absence of flagella or flg22, FLS2 interacts with Botrytis-induced Kinase 1.
Almost immediately upon perception of flg22, FLS2 associates with the LRR receptor kinase, BRI1-associated receptor kinase (BAK1) and the somatic embryogenesis receptor-like kinase 4 (SERK4) also known as BKK1. These associations provoke the phosphorylation of BIK1, BAK1 and FLS2, which in turn leads to BIK1 disassociation from the FLS2 complex (Boller and Felix, 2009; Asai et al., 2002; Sun et al., 2012). The LRR-kinase BAK1-linteracting Receptor-like Kinase 1 (BIRK1) negatively regulates multiple defense signaling pathways, including FLS2 pathways (Gao et al., 2009). When plants are not under pathogen invasion, BIRK1 and BAK1/SERKs protein complex was proposed to be guarded by two or more hypothetical R proteins. These R proteins are activated after BIRK1 has been turned off, probably due to phosphorylation by BAK1 (Wang et al., 2011). One of the involved R proteins is a TIR-LRR class that activates the PAD4-dependent pathway. The second R protein activates the SOBIR1-dependent pathway (Gao et al., 2009; Wang et al., 2011). Some early downstream signaling events for FLS2 include a Ca^{2+}-associated membrane depolarization, the activation of calcium-dependent protein kinases and mitogen-activated kinases and oxidative processes mediated by respiratory Burst oxidase homolog D (Rbo D) (Felix et al., 1999; Asai et al., 2002; Zhang et al., 2007, 2012; Boller and Felix, 2009; Jeworutzki et al., 2010).

A second category of molecules that can induce plant immunity is damage-associated molecular patterns or DAMPs (Krol et al., 2010; McCann et al., 2012). For example, many pathogens use hydrolase enzymes to break down plant physical barriers, including the cell wall, resulting in a variety of oligosaccharides and peptides. Some of these breakdown products, such as oligolacturonide (OLG) molecules, can be
recognized by plants as signals to induce their immune response against pathogen invasion. In another example, the elicitor AtPep1, the C-terminal portion of a cytoplasmic precursor of peptide protein 1 (PROPEP1), is recognized by PEP receptor 1 (PEPR1) in a fashion that resembles the association between flg22 and FLS2. PEPR1 is strongly induced in response to cell wall degradation, wounding, jasmonic acid (JA) and ethylene (Zipfel, 2009; Huffaker and Ryan, 2007; Krol et al., 2010; Yamaguchi et al., 2006; Huffaker et al., 2006).

The third class of pathogen elicitors are toxins and pore-forming molecules, like fumonisin B1, a fungal toxin, which triggers cell death in plant cells (McCann et al., 2012; Li et al., 2008; Asai et al., 2000). Finally, the fourth class of immunity elicitors are pathogen effector proteins that can be recognized by R proteins. This topic will be developed in more detail below. Overall, the pathogen elicitor recognition systems described above highlight the intricate interplay between pathogens and their hosts which has resulted in an evolutionary arms race whereby pathogens must evolve effective ways to inhibit plant immunity in order to grow and cause disease but at the same time avoid recognition by an ever-evolving host surveillance system. A model for the study of this arms race has been the Pseudomonas syringae-Arabidopsis thaliana pathosystem which is the focus of this PhD thesis.

1.2.1 Pseudomonas syringae and plant immunity.

P. syringae is a rod-shaped Gram-negative bacterium with a polar flagella, collectively associated with important plant diseases in a broad range of hosts (Rohmer
et al., 2004; Troisfontaines and Cornelis, 2005). It was one of the first pathogens demonstrated to infect *A. thaliana* (Katagiri et al., 2002). *P. syringae* enters host tissue through wounds and stomata. In susceptible plants, plants that fail in deploy defense responses (Yang et al., 1997), the bacteria rapidly increase in number and produce water-soaked patches on leaves during the first stage of infection and chlorosis/necrosis at later stages (Preston, 2004; Katagiri et al., 2002; Park, 2005; Lewis et al., 2009).

The pathogenicity of *P. syringae* depends on a needle-like Type Three Secretion System (TTSS) (Fig. 1-1). The TTSS acts as a specialized injection apparatus that delivers virulence proteins, known as Type Three Effectors (TTEs), into the plant cell cytosol (Galan and Collmer, 1999). TTEs possess a translocation signal at their N-terminus which is required and sufficient for TTSS translocation of most TTEs. However, some TTEs also require TTSS chaperone proteins to mediate their passage from the bacterial space to the host cytoplasm (Lloyd et al., 2002; Stebbins and Galán, 2001; Arnold et al., 2009; Yang et al., 2010; Wang et al., 2011). One of the major functions of phytopathogenic TTEs is to block plant immunity (Grant et al., 2006; Block et al., 2008; Innes, 2003; Lewis et al., 2009). Although little is known about the mechanistic functions of these effectors, they are vital to pathogens for a successful infection such that mutations that interfere with TTSS function render bacteria non-pathogenic (De Wit, 2007; Baker, 1997; Goel et al., 2008; Innes, 2003).

### 1.2.2 Type three effectors suppress plant immunity.

A number of TTEs have been demonstrated to influence the plant immune response either directly or indirectly by targeting systems such as vesicle trafficking,
RNA processing and plant hormone signaling pathways; the latter is discussed in section 1.4 (Lewis et al., 2009).

HopAl1 and HopAB1 (formerly AvrPtoB) are examples of *P. syringae* TTEs that directly target the plant immune system. HopAl1 irreversibly dephosphorylates phosphothreonine residues thereby inactivating mitogen-activated protein kinases (MPKs) (Zhang et al., 2007; Li et al., 2007). MPKs play an important role in plant defense as exemplified by the roles that MPK3 and MPK6 play in immunity against fungal and bacterial pathogens (Shan et al., 2007; Pitzschke et al., 2009). Some events downstream of MPK signaling include reinforcement of the cell wall, transcriptional activation of MAMP-responsive genes and the oxidative burst (Beckers et al., 2009). Both MPK3 and MPK6 are direct targets of HopAl1. The inhibition of MPKs suppresses the reinforcement of the cell wall and transcriptional activation of MAMP-responsive genes, thus leading to a successful infection (Zhang et al., 2007; Li et al., 2007). HopAB1, an E3 ubiquitin ligase, can also suppress plant immunity by ubiquitination of the PRR proteins FLS2 and the EF-Tu receptor (EFR) resulting in their degradation (Göhre et al., 2008; Gimenez-Ibanez et al., 2009; Gohre et al., 2008). HopAB1 also interacts with BAK1, possibly to interfere with FLS2 binding (Cohn and Martin, 2005; Lewis et al., 2009).

Another TTE from *P. syringae*, HopM1, leads to the degradation of MIN7, a protein of the ADP ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) family involved in vesicle trafficking. Mutant plants for MIN7 have increased susceptibility to bacterial infection, suggesting that AtMIN7-mediated vesicle trafficking
Figure 1-1. Type III Effectors and plant immunity. Schematic depicting TTS effectors (TTE, red dots) as both elicitors and suppressors of plant defense. A- Pattern recognition receptors (PRR) recognize PAMPs of the bacterium and trigger PTI responses such as callose deposition. TTEs can block this initial line of defense to cause disease. B- NB LRR R (nucleotide binding-leucine-rich repeat domain resistance) proteins can recognize TTEs to trigger ETI (effector-triggered immunity) responses such as HR and oxidative burst. C- TTEs may affect vesicular trafficking, transcription and alter hormone pathways to suppress PTI (PAMP-triggered immunity) and/or ETI in order to increase virulence.
is involved in plant immunity (Block et al., 2008; Boller and He, 2009; Nomura et al., 2011).

In the continual arms race between pathogen and host, some TTEs or their activities are recognized by resistance (R) proteins, products of the R genes, subsequently triggering R protein-mediated defenses (Lewis et al., 2010). R protein-mediated defenses often culminate in a localized cell death response known as a hypersensitive response (HR) which is believed to rapidly isolate infected cells from the healthy tissue (Park, 2005; Heath, 2000; Glazebrook, 2005; Katagiri and Tsuda, 2010).

The majority of the R proteins are characterized by the presence of a leucine-rich repeat domain (LRR) and a nucleotide binding domain (NB). These (NB-LRR) R proteins can be divided into two subgroups depending on the structure of their N-terminus; the toll interleukin 1 receptor (TIR-NB-LRR) and the coiled-coil (CC-NB-LRR) (Fig. 1-2). It has been demonstrated that the LRR domain is a crucial component for TTE specificity (Zhang et al., 2012; Bonardi et al., 2011; Caplan et al., 2008). Currently, the guard model is widely accepted for the R protein's role in recognition of bacterial effectors. This model, based on the gene-for-gene interaction model described by Flor in the 1940's, proposes that R proteins monitor TTE targets and that the recognition of a TTE-induced modification leads to the activation of the R protein and the subsequent triggering of downstream defense responses (Van der Hoorn and Kamoun, 2008; Dangl and Jones, 2001; Biezen and Jones, 1998). Two variations of the model may apply. The R protein may bind constitutively to the guardee or it may bind to its guardee only after the guardee interacts with the bacterial effector (Biezen and Jones, 1998; Dangl and Jones, 2001; van der Hoorn and Kamoun, 2008).
An example of the guard hypothesis is the recognition of AvrRpm1 by the A. thaliana CC-NB-LRR R protein resistance to P. syringae maculicola 1 (RPM1). RPM1 is required to associate with RPM1 interacting protein 4 (RIN4) in order to recognize AvrRpm1 (Mackey et al., 2002). In addition, RIN4 interacts with AvrRpm1 and it has been proposed that RIN4 is “guarded” by RPM1 (Mackey et al., 2003; Axtell and Staskawicz 2003; Gao et al., 2011; Zhang et al., 2012). Upon interaction with AvrRpm1, RIN4 is phosphorylated on T166 by the A. thaliana RIN4-interacting receptor-like kinase (RIPK) leading to the activation of RPM1 (Gao et al., 2011; Chung et al., 2011; Liu et al., 2011).

In addition to AvrRpm1, RIN4 is also targeted by three sequence unrelated TTEs; AvrB, AvrRpt2 and HopF2. AvrB also induces RIN4 phosphorylation and is recognized by RPM1 (Mackey et al., 2002) AvrRpt2 is a protease that cleaves RIN4 leading to the activation of a distinct R protein called resistance to P. syringae 2 (RPS2) (Mackey et al., 2003). HopF2 ribosylates RIN4 and requires RIN4 to promote P. syringae virulence (Wilton et al., 2010; Wang et al., 2010).

Other groups have developed extensions of the guard model to explain the modus operandi of R proteins. One of these is the decoy model (Van der Hoorn and Kamoun, 2008). This model states that a decoy protein interacts with the recognized effector protein and is necessary for effector recognition by the R protein; however, the decoy is not directly involved in defense in the absence of the R protein. Unlike the guard model, the decoy does not play any role in pathogen fitness (Van der Hoorn and Kamoun, 2008). RIN4 is unlikely to be a decoy since it has been demonstrated to negatively regulate basal resistance and is a virulence target of the TTE HopF2 (Kim et
al., 2005; Wilton et al., 2010). Nevertheless, the decoy model may apply to other systems since the mode of action of most effector proteins and their cognate resistance mechanisms remain to be determined.

1.3 Hormones in plant immunity

One of the key mechanisms protecting plants against pathogens involves phytohormones (Fig. 1-3), which are structurally unrelated small molecules that regulate plant physiology and development at relatively low concentrations (Bari and Jones, 2009). Virtually every aspect of plant development is under hormonal control (Nemhauser et al., 2006; Santner et al., 2009; Santner and Estelle, 2009) (Table 1-1). In addition, responses to biotic stresses are also regulated by phytohormones (Pieterse et al., 2009; Spoel and Dong, 2008a; Truman et al., 2010). SA, JA and ethylene were the first hormones identified as key players in plant defense responses (Truman et al., 2010; Glazebrook, 2005) (Fig. 1-3). Recently, abscisic acid (ABA), gibberellin, brassinosteroids and auxin have also been demonstrated to contribute to resistance responses against pathogens (de Torres-Zabala et al., 2007; Navarro et al., 2008; Kazan and Manners, 2009; Wang, 2012; Belkhadir et al., 2012; Albrecht et al., 2012) (Fig. 1-3). Crosstalk between different hormonal signalling pathways can increase their regulatory potential and allow plants to face a variety of environmental challenges through synergistic or antagonistic interactions (Robert-Seilaniantz et al., 2007; Nemhauser et al., 2006; Verhage et al., 2010). This suggests that the balance between hormone signalling pathways is crucial for determining the outcome of plant-pathogen interactions (Navarro et al., 2008; Staswick, 2009; Bari and Jones, 2009).
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<th>Physiological effect</th>
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<td>Embryo development.</td>
<td>Woodward and Bartel, 2005</td>
<td>Promotes growth of <em>A. tumefaciens</em> and <em>P. syringae</em>. Auxin mutants compromise resistance to <em>Botrytis cinerea</em> and <em>Plectosphaerella cucumerina</em>.</td>
<td>Spoel and Dong, 2008</td>
<td>AvrRpt2</td>
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<td>Seed dormancy.</td>
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<td>Defense against necrotrophic pathogens and herbivore insects.</td>
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<td>Bari and Jones, 2009 AvrPto HopAB2</td>
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<td>Stomata closing.</td>
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<td>Drought and high salinity stress responses.</td>
<td>Milborrow, 2001 Wasilewska et al., 2008</td>
<td>Negative regulation of plant defense against <em>B. cinerea</em>, <em>Erwinia chrisantemi</em>, <em>P. syringae</em>.</td>
<td>Bari and Jones, 2009 Torres-Zabala et al., 2007</td>
<td>HopAB2</td>
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Figure 1-2. NB-LRR protein domain organization. The two major families of plant NB-LRR resistance proteins are characterized based on their N-terminal domain. A- TIR-NB-LRR class. TIR-amino-terminal domain homologous to the Toll and interleukin 1 receptors. B- CC-NB-LRR. CC-amino-terminal domain is an α-helical coiled-coil domain. N-terminus: amino terminus; C-terminus: Carboxyl terminus. TIR: Toll/interleukin-1 receptor-like domain; CC: coiled-coil domain; NB: nucleotide binding site; LRR: leucine-rich repeat
Figure 1-3. Major plant hormone structures. UIPAC names are indicated.
SA is a plant hormone that is involved in plant defense and establishment of systemic acquired resistance (SAR) and is considered the main plant hormone involved in defenses against biotrophic and hemibiotrophic (biotrophic life style in the first stages of infection and necrotrophic at later stages) pathogens like *P. syringae* (Halim et al., 2009; Vlot et al., 2009; Loake and Grant, 2007). The idea that SA was involved in resistance against pathogens was first suggested by White in 1979, where he attributed the resistance-conferring properties to SA’s negative charge (White, 1979). Later, in 1990, two different groups identified the accumulation of SA at the site of infection and linked this accumulation to the induction of the pathogenesis related (PR) genes, suggesting that SA is an endogenous defense signal and could be the mobile signal for SAR response (Malamy et al., 1990; Métraux et al., 1990). Malamy et al. (1990) found that SA increased in uninfected tobacco leaves 48 hours after treatment with TMV. In a different work Metraux et al. (1990), followed 14C-labeled SA inoculated in lower leaves of cucumber plants and recovered 0.4% of the labeled SA in upper leaves. All these observations suggested that it was SA itself the mobile signal in SAR.

Since then, many studies have shown that SA triggers defense responses to biotrophic and hemibiotrophic pathogens (Vlot et al., 2009; Malamy et al., 1990; Fragnière et al., 2011; Loake and Grant, 2007).

In 2007, using chromatography-mass spectrometry and mutant analysis, Park et al. showed that MeSA is a mobile signal during SAR. This is because SAR requires functional salicylic acid binding protein 2 (SABP2), which has esterase activity, in systemic tissue and inhibition of this activity in infected tissue. SABP2 allows the conversion from biologically inactive MeSA to active SA. They also found that MeSA
increased in infected tissue and later in phloem exudate of primary infected leaves and in systemic leaves (Park et al., 2007). A year later Vlot et al. (2008) published a summary of other suggested SAR signals; lipids, peptide and vasculature associated signaling (Vlot et al., 2008).

Although the receptor for SA had been elusive for years, recently, Wu et al. (2012) have presented non-expressor of PR Genes 1 (NPR1) as the SA receptor. NPR1 is a well-studied key component of SA signaling that acts downstream of SA accumulation in SA-signaling. It encodes for a transcriptional coregulator containing ankyrin repeats (Wu et al., 2012; Cao et al., 1997). SA and its analog benzo (1,2,3) thiadiazole-7-carbothioc acid S-methyl ester (BTH), bind specifically to NPR1 and this interaction is mediated by copper in A. thaliana (Wu et al., 2012). In another study, Fu et al. (2012) have presented NPR3 and NPR4 as the receptors for SA during immune responses. They found that NPR3 and NPR4 bind to SA with different strengths. NPR3 and NPR4 seem to act as Cullin 3 (CUL3) adaptors for the degradation of NPR1 through the interaction with NPR1 Bric-a-Brac, Tramtrack, Broad-complex (BTB) domain. Yeast two hybrid and pull-down assays have shown that NPR3-NPR1 interaction occurs only in the presence of SA. Conversely, NPR4-NPR1 interaction occurs in the absence of SA. SA directly binds to both NPR3 and NPR4 with different affinities to differentially influence NPR1 activity (Fu et al., 2012).

The differences between the experimental data in these two works may provide a clue about which could be the *bona fide* receptor for SA. Fu et al. (2012) showed that NPR4 has a strong affinity for SA without the requirement of a metal catalyzer and NPR3 also showed affinity but it was lower than in the case of NPR4. Wu et al. (2012) found that
the NPR1 interaction with SA requires copper and occurs with a much lower affinity than
the interaction between NPR4 and SA. However, it is higher than the NPR3-SA
interaction. These interactions were tested by different methods by both authors. Fu et
al. showed that the interaction occurs in vitro as well as in vivo by pull-down assay and
yeast two hybrid system. On the other hand, Wu et al. only presented in vitro data. A
competitive assay between NPR1, NPR3 and NPR4 could possibly shed more light on
whether one or all three are the true receptors. It is likely that SA binds to all three NPR
proteins to modulate a proper SA response.

Previously, it has been shown that NPR1 exists as a multimer in the cytoplasm
(Dong, 2004; Robert-Seilaniantz et al., 2010). A redox change in the cytoplasm caused
by SA accumulation induces NPR1 dissociation to monomers. This leads to
relocalization of NPR1 into the nucleus and binding to transcription factors to activate
SA responsive genes (Robert-Seilaniantz et al., 2010; Vlot et al., 2009; Maier et al.,
2011; Dong, 2004).

Although both SA and JA are involved in pathogen resistance, it has been
suggested that SA has an antagonistic relationship with JA, which plays an important
role in defense against a broad spectrum of necrotrophic pathogens (Avanci et al.,
2010). Interestingly, NPR1 has been suggested to be a crosstalk point of SA-JA
antagonism (Koornneef et al., 2008; Navarro et al., 2008; Robert-Seilaniantz et al.,
2010; Spoel et al., 2003; Leon-Reyes et al., 2009). Cytosolic NPR1 can suppress
expression of plant defensin 1.2 (PDF1.2), a typical JA-response gene (Spoel et al.,
2003). There is also evidence that overexpression of cationic peroxidase 3 (OCP3), a
gene that encodes a transcription factor repressed during the response to infection by
necrotrophic pathogens, modulates the cytosolic function of NPR1, though the mechanism of this regulation remains unclear (Ramírez et al., 2010). Furthermore, the suppression of other JA-responsive genes may also be regulated by NPR1 (Li et al., 2004, 2006). WRKY protein 70 (WRKY70) is also suggested to contribute to the interplay between SA and JA. WRKY70 is a transcription factor that activates defense genes associated with SA but downregulates JA-dependent defense responses (Li et al., 2004). In the work by Li et al., they found that WRKY70 was induced based on an A. thaliana library infected with Erwinia carotovora. It was also found that WRKY70 expression is upregulated by SA and possibly acts downstream NPR1. Upregulation of WRKY70 promotes activation of SA-dependent genes such as PR2 and PR5 whereas JA treatment downregulates WRKY70 expression. The lowered levels of WRKY70 lead to expression of JA-dependent genes (Li et al., 2004).

Another point of crosstalk that has been predicted between SA and JA is MYC2 (myelocytomatosis 2). JA-responsive genes require the transcription factor MYC2 to initiate transcription (Laurie-Berry et al., 2006). In the absence of JA, MYC2 is associated with a repressor complex including jasmonate ZIM domain (JAZ) containing proteins, NINJA and TPL (Robert-Seilaniantz et al., 2010). The repressive complex can be targeted by the coronatine insensitive (COI1) F-box protein and SCF (SCF\textsuperscript{COI1}) to form JAZ-SCF\textsuperscript{COI1} that leads to the degradation of JAZ by the 26S proteasome, allowing MYC2 to transcribe JA-responsive genes (Pauwels et al., 2010; Robert-Seilaniantz et al., 2010). MYC2 positively regulates defense responses against herbivores and is necessary for the suppression of SA during P. syringae infection (Laurie-Berry et al., 2006; Robert-Seilaniantz et al., 2010).
Ethylene also acts together with JA to control development and defense responses. Ethylene is a plant gaseous hormone involved in several developmental processes like germination, senescence, abscission of flowers and leaf and fruit ripening (Kieber, 1997; Stepanova and Alonso, 2005). The role of ethylene in disease resistance against necrotrophs and its interaction with other hormones in plant defense has been widely studied (Dong, 1998; van Loon et al., 2006; Nandi et al., 2003; Lorenzo et al., 2003; Anderson et al., 2004). Ethylene response factor 1 (ERF1), which encodes a transcription factor that responds to pathogen invasion (Lorenzo et al., 2003; Solano et al., 1998), is rapidly activated by application of JA or ethylene, but both signaling pathways are required to be intact, suggesting that it requires synergistic inputs from both hormone pathways (Leon-Reyes et al., 2009, 2010). Indeed, studies show that a number of genes regulated by both ethylene and JA are regulated by ERF1 (Lorenzo et al., 2003; Guo and Ecker, 2004; Solano et al., 1998; Leon-Reyes et al., 2009; Anderson et al., 2004).

In contrast to the synergistic relationship between JA and ethylene, JA/ethylene and ABA appear to function antagonistically in fungal defense (Anderson et al., 2004). ABA is a hormone involved in developmental processes such as seed dormancy, responses to drought, high salinity and cold conditions stresses (Mohr and Cahill, 2007; Fujita et al., 2005). The ABA auxotrophic mutant aba2-1 has substantially higher expression of JA-ethylene regulated defense genes and enhanced resistance against necrotrophic fungal pathogens suggesting that ABA negatively regulates immunity against these pathogens (Anderson et al., 2004).
The extensive crosstalk between defense hormone signalling pathways hints at the complexity of their interactions. Although this has been relatively well characterized for SA, JA, and ethylene, hormones such as auxin (discussed in section 1.5) and ABA with well known roles in development and/or abiotic stress, are also emerging as important regulators of immunity through synergistic or antagonistic crosstalk with defense signaling pathways.

1.4 *P. syringae* and plant hormones in plant immunity

*P. syringae* TTEs can hijack hormone pathways and crosstalk as an indirect way of targeting the immune system. For example, using microarray data it was found that AvrPto1 and HopAB2 stimulated ethylene production genes in tomato and further experiments using ethylene-deficient lines showed that both TTE enhances disease related symptoms (Cohn and Martin, 2005). In addition, AvrRpt2 which is a cysteine protease that can directly affect plant immunity by manipulating RIN4, has also been implicated in modulating auxin levels to promote virulence (Innes, 2003; Chen et al., 2007). In Chen et al. (2007), it was found that AvrRpt2 overexpressing lines showed increased auxin sensitivity phenotypes and that auxin production was enhanced. These changes were accompanied by increased bacterial growth.

Microarray analysis found that HopAB2 and HopAM1 (formerly AvrPpiB) can alter ABA signaling in order to enhance pathogen growth (De Torres-Zabala et al., 2007).
1.4.1 Plant defense and hormone crosstalk.

Hormonal crosstalk is a term widely used to describe interference (positive or negative) of one hormone pathway with another hormone. Chandler (2009) provides a good description of hormone crosstalk, describing three different types of crosstalk, direct, indirect and co-regulation. In direct crosstalk, different hormones pathways converge at an “integrator”, which acts as a common element to regulate a given response. Indirect crosstalk is where a hormone signaling pathway is regulated by another hormone signaling pathway. This regulation can occur at the level of hormone biosynthesis, transport or degradation. The third type of crosstalk, co-regulation, occurs when a process is affected by two or more hormones and the outcome is due to the contribution of these independent pathways (Chandler, 2009).

ABA is a hormone involved in developmental processes such as seed dormancy, stress responses to drought, high salinity and cold conditions (Mohr and Cahill, 2007; Fujita et al., 2005). More recently numerous groups have demonstrated a role for this hormone in biotic stress responses. For example, it was reported that P. syringae upregulates the ABA pathway to suppress defense-induced plant transcriptional responses (De Torres-Zabala et al., 2007; Goel et al., 2008). This is likely due to the antagonism that exists between ABA and SA signaling pathways. Yasuda et al. (2008) have demonstrated in tomato that the resistance afforded by the SA synthetic analog, BTH, can be reversed by application of ABA. This work also showed that ABA application can significantly reduce expression of the SA biosynthetic gene, isochorismate synthase 1 (ISC1) also known as SA induction deficient 2 (SID2) as well
as the SA-induced gene *PR1* expression (Yasuda et al., 2008). This SA-ABA antagonism has also been demonstrated in several plants species including *Nicotiana benthamiana*, *Orzya sativa*, and *Solanum lycopersicum* (Cao et al., 2011; Mohr and Cahill, 2007; De Vleesschauwer et al., 2010; Jiang et al., 2010; Mosher et al., 2010; Yasuda et al., 2008).

Interestingly, depending on the stage of infection, the relationship between SA and ABA can also be synergistic. The synergistic interaction between these two hormones occurs mainly at the leaf surface during the pre-invasive stage of bacterial infection. Pathogenic bacteria invade the leaf tissue through wounds or natural openings called stomata (Katagiri et al., 2002; Preston, 2004). ABA is known to regulate stomata closure during drought stress (Atkinson and Urwin, 2012; Wasilewska et al., 2008; McAdam et al., 2011; Le et al., 2009; Nakashima et al., 2009) and it has been suggested that this mechanism is also used to prevent bacterial entry into leaf tissue (Cao et al., 2011). Mutant studies suggest that infection of *P. syringae* can significantly suppress stomatal closure by SA. NPR1, which has been suggested to be a crosstalk point between SA and JA, also seems to be a crosstalk point between SA and ABA for stomatal closure (Mang et al., 2012; Zeng and He, 2010).

The evidence suggesting that alteration of plant hormone is an active strategy by pathogens is constantly growing, but work on direct hormone-pathway hijacking by *P. syringae* is scarce. Mechanistic analysis of hormone responses due to single pathogen effectors will provide valuable insights into the evolutionary relationship between pathogens and their hosts.
1.5 Auxin and plant immunity

Several reports have shown how pathogens alter the normal auxin homeostasis during infection. *Botrytis cinerea* is a necrotrophic fungal pathogen that infects a wide range of host plants. Llorente et al. (2008) showed that infection of *A. thaliana* with *B. cinerea* leads to repression of numerous AUX/IAA and ARFs-encoding genes, as well as the repression of the auxin receptor-encoding gene TIR1 (Llorente et al., 2008; Kazan and Manners, 2009). Additionally, a report from Gonzales-Lamothe et al. (2012) showed that infection with this fungal pathogen leads to an increase of the auxin conjugate IAA-Asp after infection. IAA-Asp promotes pathogen growth in infected plants by regulating transcription of virulence genes in vivo (González-Lamothe et al., 2012).

1.5.1 Regulation of auxin.

Auxin is the generic name given to an important group of phytohormones. Indole-3 acetic-acid or IAA (Fig. 1-3), the predominant form of auxin in plants, has a well-documented ability to regulate many aspects of plant development (Teale et al., 2008, 2006; Woodward and Bartel, 2005; Paciorek and Friml, 2006), including cell division, expansion and differentiation (Yamada, 1993; Teale et al., 2006). It controls primary root growth, lateral root formation as well as apical meristem development and vascular differentiation (Lohmann et al., 2010; Tatematsu et al., 2004; Aloni et al., 2003). Although IAA is a type of auxin, the terms auxin and IAA will be used interchangeably in this study.
Auxin is ubiquitously distributed in the plant, but its effects depend on tissue and developmental stage (Lau et al., 2008; Christian et al., 2008). Finding the main components of the IAA biosynthesis pathway presented a challenge for researchers and it was only recently that the main regulators of IAA synthesis were elucidated (Fig. 1-4). Mashiguchi et al. (2011) demonstrated that the indole-3-pyruvic acid pathway (IPA) is the major biosynthesis pathway for IAA in A. thaliana. They propose that the YUCCA protein family acts cooperatively with the Tryptophan Aminotransferase of A. thaliana family (TAA) to regulate IAA production (Mashiguchi et al., 2011; Strader and Bartel, 2008; Woodward and Bartel, 2005).

In A. thaliana, auxin signalling is characterized by three main families of proteins; the F-box auxin receptors transport inhibitor response 1/auxin signaling F-Box protein 1-3 (TIR1/AFB1-3), the negative regulatory proteins Aux/IAA, and transcription factors called auxin response factors (ARFs) (Fig. 1-5). Aux/IAA family members are nuclear localized and short-lived proteins that include 29 members in A. thaliana (Kalluri et al., 2007; Stewart and Nemhauser, 2010). At low concentrations of auxin (Fig. 1-6), Aux/IAA dimers associate with two ARFs via Aux/IAA domain III/IV (Fig. 1-6). The Aux/IAA domain I is an active repression domain that is transferable and dominant over activation domains of the ARF proteins (Tiwari et al., 2004) (Fig. 1-5). Activation of auxin signalling (Fig. 1-6) is initiated when IAA binds to the TIR1/AFB1-3 family of auxin receptors. These F-box receptors are associated with the SCF-class E3 ubiquitin ligase. SCFs have a characteristic subunit structure, consisting of a SKP1 protein, a Cullin, an F-box protein and RBX1. SKP1 links the F-box protein to the Cullin, which in turn interacts with RBX1 (Kepinski and Leyser, 2005; Lau et al., 2008; Robert-Seilaniantz et
al., 2011). The RBX1–Cullin dimer catalyses the transfer of activated ubiquitin from a ubiquitin-conjugating enzyme to the target protein, in this case, the Aux/IAA proteins (Kepinski and Leyser, 2005; Parry et al., 2009). IAA binds to the F-box TIR1 acting as a molecular glue to bring the F-box and Aux-IAA proteins together. Domain II of the Aux/IAA proteins is required for rapid degradation by the 26S proteosome (Dreher et al., 2006). Once Aux/IAA proteins are degraded active ARF transcriptional activators are released.

Twenty three ARFs are known in A. thaliana (Paponov et al., 2009) (Fig. 1-5). Once the ARFs are released, they bind to the promoter region of auxin-responsive genes (Ulmasov et al., 1997a; Okushima et al., 2005). The promoters of these genes contain specific sequences called auxin response elements (AuxREs) characterized by a TGTCTC sequence that is recognized by ARFs. The TGTCTC sequence is repeated in tandem in the synthetic auxin-responsive promoter DR5 (Ulmasov et al., 1997).

Auxin is a weak acid and as such requires the presence of carriers to move through the plasma membrane (Zazímalová et al., 2010; Swarup and Péret, 2012). This mobility is made possible by a combination of efflux and influx transporters. The efflux transporters are the PIN family, the PIN-like auxin carriers (PILS) and the ATP-binding cassette superfamily B (ABCB) of transporters. PINs are gradient driven transporters with eight
Figure 1-4. Biosynthesis pathways for Indole-3-acetic acid (IAA) in plants. Indole-3-acetaldoxime (IAOx), indole-3-pyruvic acid (IPA) indole-acetamide (IAM) TAM: tryptamine. IAN: indole-3-acetonitrile. IAAId: indole-3-acetaldehyde. TAA1: tryptophan aminotransferase of Arabidopsis 1. CYP79B3: cytochrome P450, family 79, subfamily B, polypeptide 3. AMI1: amidase-like protein 1. Known biosynthetic enzymes are indicated (Zhao, 2010; Mashiguchi et al., 2011).
Figure 1-5. Aux/IAA and ARF protein domain organization. A- Aux/IAAs. Four major domains are present in all members. Domain I (yellow box) functions as a strong transcriptional repressor (Tiwari et al., 2004), while domain II (red box) acts as a destabilization motif, and alterations to this region result in extended protein lifespans (Zenser et al., 2001; Ouellet et al., 2001). Domains III and IV in Aux/IAA are similar to domains III and IV (blue boxes) present in the C-terminus of the ARF proteins and mediate protein-protein interactions between the two proteins (Tiwari et al., 2004; Reed, 2001; Zenser et al., 2001). B- ARF protein domains. At the N-terminus ARFs have a DNA binding domain (green box) that binds to Auxin Response Elements (AuxRE) present in the promoter regions of auxin-regulated genes. The middle region is variable, and defines the ARF as either a repressor or activator (Reed, 2001; Paponov et al., 2009; Ulmasov et al., 1997).
family members in *A. thaliana* (Křeček et al., 2009). The PIN family of proteins can be classified into two groups: the PIN1-type proteins (PIN1, 2, 3, 4, and 7) that localize to the plasma membrane and the PIN5-type proteins (PIN5, 6, and 8), that localize to the endoplasmic reticulum (ER) (Blakeslee et al., 2007; Vieten et al., 2005; Křeček et al., 2009). Only the PIN5-type proteins have demonstrated roles in the regulation of auxin homeostasis (Křeček et al., 2009; Vieten et al., 2005), since PIN1-types appear to have redundant functions and no visible phenotype if only one member of the group is knocked out (Křeček et al., 2009; Peer et al., 2010; Huang et al., 2010). PILS have been recently described and have been suggested to be regulating auxin homeostasis (Feraru et al., 2012; Swarup and Péret, 2012). ABCBs are ATP-driven transporters that seem to function when auxin gradients are low or when auxin needs to move against its gradient (Kim et al., 2010; Swarup and Péret, 2012; Zázímalová et al., 2010). Some ABCB transporters, like the case of ABCB4, can act as efflux carriers when auxin concentrations are high but turn to influx carriers when auxin concentrations are decreased in the cell (Yang and Murphy, 2009; Zázímalová et al., 2010). Other than the ABCB, there is one more studied family of auxin influx carriers, the H⁺-symporters AUX/LAXes proteins (Kleine-Vehn et al., 2006; Zázímalová et al., 2010; Swarup and Péret, 2012). In *A. thaliana* there are 4 highly conserved members, *AUX1* and the AUX1-like, *LAX1, LAX2* and *LAX3* (Parry et al., 2001). AUX1 has been shown to play an important role in the gravitropic response and it is expressed in gravity-perceiving tissue. Other AUX/ LAXes are not related to gravitropism perception (Swarup and Péret, 2012; Péret et al., 2012).
Figure 1-6. Schematic depicting auxin signaling. At lower concentrations of IAA, the repressor proteins Aux/IAA form a dimer complex with transcription factors (ARF) inhibiting auxin-responsive gene expression. When IAA levels increase, IAA molecules bind to the TIR1 receptor which is part of the F-box E3 ligase complex, SCF$^{\text{TIR1}}$. This complex ubiquitinates the Aux/IAA proteins and targets them for degradation (De Smet et al., 2011; Quint and Gray, 2006; Li et al., 2009).
Besides the above described transporters, there is a possibility that other anion transporters could also contribute in auxin mobility (Zazímalová et al., 2010).

1.5.2 Auxin crosstalk with plant immunity.

Auxin is a participant in the complex regulatory network involved in plant development. More recently, there is an emerging role for IAA in plant defense against *P. syringae*, where IAA antagonistic interactions with other hormones are used to increase pathogen fitness and suppress host defense response. Glickman et al. (1998) revealed that auxin production is a common feature of most pathovars of *P. syringae*. Fifty six strains of *P. syringae* produced auxin in various concentrations when they were incubated with tryptophan, an auxin precursor. *Pseudomonas* pathovars were also examined for the presence of *iaaH, iaaM* and/or *iaaL*, which encode enzymes involved in IAA synthesis. Forty five pathovars have at least one of these IAA biosynthetic genes. Furthermore, 8 strains were able to produce high concentrations of IAA in the absence of tryptophan, suggesting that auxin production is an integral part of *P. syringae* viability (Glickmann et al., 1998; Gardan et al., 1992). The authors speculate that IAA production by these strains may inhibit plant defense mechanisms.

It has been postulated that the advantage of manipulating auxin signaling by bacteria lies in the antagonism between auxin and SA. Microarray data show that SA suppresses auxin related genes when plants are treated with BTH, an SA analog (Wang et al., 2007). Moreover, bacterial growth is decreased in the auxin insensitive *A. thaliana* mutant *axr2-1* suggesting that auxin signaling promotes pathogen susceptibility (Wang et al., 2007; Kazan and Manners, 2009; Mutka et al., 2013). Increasing auxin levels
promotes stomatal opening (Dietrich et al., 2001; Chen et al., 2007) and upregulates ethylene signaling which could lead to pathogen-related tissue damage (Arteca and Arteca, 2008; Hansen and Grossmann, 2000; Broekaert et al., 2006). Currently, there is only one example of a TTE, AvrRpt2 which manipulates the auxin pathway. Chen et al., (2007) showed that expressing AvrRpt2 in the A thaliana mutant rps2 which lacks the RPS2 gene, that encodes the cognate R protein for AvrRpt2, exhibited elevated levels of IAA and enhanced susceptibility against P. syringae. Furthermore, these plants also show increased lateral root number and longer primary root. These phenotypes are reminiscent of an altered auxin physiology, thus indicating that AvrRpt2 can manipulate auxin accumulation (Deng and Huang, 1999; Kazan and Manners, 2009; Spaepen and Vanderleyden, 2011). However, the mechanism by which AvrRpt2 alters auxin responses is not yet understood.

1.5 Thesis overview

The objective of my work was to identify P. syringae TTEs that alter auxin signaling and to analyze their potential role in the hijacking of A. thaliana defense responses. In order to address this objective, I started this project by identifying TTE that manipulate hormone pathways using a high-throughput system that I developed by modifying a liquid assay described in Schreiber et al. (2008). A. thaliana transgenic plants with the uidA reporter gene (encoding β-glucuronidase: GUS) downstream of a hormone-responsible promoter were infected with A. tumefaciens able to deliver
individual *P. syringae* TTEs. Changes in GUS activity with respect to the control were monitored to isolate TTE candidates for further characterization.

Two auxin-manipulating TTE candidates, HopAK1 and HopAL1 were identified, from *P. syringae* pv. maculicola ES4326. I found that HopAK1 confers hypersensitivity to exogenously applied auxin, while HopAL1 activates auxin signaling when expressed in *A. thaliana*. Furthermore, transgenic *A. thaliana* expressing HopAK1 or HopAL1 showed enhanced susceptibility against *P. syringae*. Finally, I have attempted to identify potential interactors for HopAK1 by yeast two-hybrid analysis.

I hypothesize that the TTEs, HopAK1 and HopAL1, can manipulate the auxin pathway to increase *P. syringae* virulence.
Chapter 2

Materials and Methods
2.1 Plant material and bacterial strains

All seed lines described are in the Columbia (Col-0) background of Arabidopsis thaliana.

Seeds for soil-grown plants were sterilized prior to sowing by exposing them to chlorine gas (Cl\textsubscript{2}) for 3.5 hours. Seeds were sown on standard Promix BX soil (Premiere Horticulture, Ref Hill, PA, USA). Stratification was done at 4°C for 2 days in the absence of light. Plants were grown under fluorescent light (100 μE/m\textsuperscript{2}/s) with a 9-hour light period and a day/night temperature of 22°C/18°C.

2.1.1 Plasmid constructs.

2.1.1.1 Plasmids for plant transformation.

In all cases the full length TTE coding regions were cloned with an ATG start codon and a C-terminal hemagglutinin (HA) tag under the control of a dexamethasone inducible promoter (DEX). The constructs were introduced into A. tumefaciens strain GV3101 through electroporation for plant transformation.

a) HopAK1.

A 1622 bp fragment corresponding to full length CDS with an in-frame HA tag were amplified by PCR using HopPmaH forward and reverse primers (Table 2-1). Bacterial extract was used as PCR template. The resulting product was inserted into the pBD vector (Lewis et al. 2008) with the restriction endonucleases \textit{XhoI} and \textit{SpeI}. This construct was confirmed by sequencing.
b) HopAL1. 

A 1393 bp fragment corresponding to full length CDS was amplified by PCR using HopPmaK forward and reverse primers (Table 2-1). Bacterial extract was used as a PCR template. The resulting product was inserted into the pBD vector (Lewis et al. 2008) with the restriction endonucleases XhoI and SpeI. This construct was confirmed by sequencing.

2.1.1.2 Plasmids for split-ubiquitin yeast two-hybrid analysis.

HopAK1.

Full length HopAK1 CDS was amplified from HopPmaH/pBD vector by PCR using HopAK1/pBT-3N forward and reverse primers, and Pfu taq polymerase (Fermentas). Primers incorporated SfiI restriction sites into the final product for cloning into the yeast bait vector pPBT3N. This resulting new vector, HopAK1/pPBT3N, was amplified in Escherichia coli and verified for sequence and frame prior to transformation into yeast.

2.1.2 A. thaliana transformation.

A. thaliana transformations were performed using the floral dip method according to Clough and Bent, (1998). In all cases the DR5::GUS (DR5::uidA) transgenic plant (Ulmasov et al., 1997) was used as background to generate transgenic A. thaliana plants expressing each effector (HopAK1 and HopAL1). HopAK1 and HopAL1 were
cloned into the pDB vector with an ATG start codon and in-frame with the C-terminal hemagglutinin (HA) tag. The constructs were sequenced and transformed into *A. tumefaciens* GV3101. A 250 mL culture of the *A. tumefaciens* strain GV3101 containing the desired binary plasmid was grown for 2 days at 28°C with shaking (200-250 rpm). Cells were pelleted at 4,000 rpm for 10 min. The cell pellet was resuspended in a 5% sucrose solution containing 0.05% Silwet L-77. Plant inflorescences were dipped into the cell suspension for approximately 40 sec. Selection of transformed plants was done by spraying 1/1000 v/v of BASTA (Bayer Inc.) in soil or 1.8g/1L of Biapholos (SIGMA) on Murashige and Skoog (MS) agar plates (0.5X MS salts (SIGMA), 1.5% (w/v) sucrose (BIOSHOP), pH 5.7 , 0.8% (w/v) agar (BIOSHOP)). Two independent single insertion lines for HopAK1 (H1 and H2) and HopAL1 (K1 and K10) were obtained. Expression of TTEs was verified by western blot analysis.

### 2.1.3 Bacterial strains.

The following bacterial strains were used: *P. syringae* pv. maculicola ES4326, *P. syringae* pv. maculicola ES4326/ΔhrcC (Schreiber et al., 2012), *P. syringae* pv. tomato DC3000 (Cuppels, 1986), *P. syringae* pv. tomato DC3000/ΔhrcC (Yuan and He, 1996) and *P. syringae* pv. maculicola M6ΔE (Rohmer et al., 2003). All *P. syringae*. pv. maculicola strains are resistant to streptomycin and kanamycin; *P. syringae* pv. tomato DC3000 is resistant to rifampicin.
2.2 Histochemical detection of GUS and GUS quantification assays

2.2.1 Histochemical GUS detection.

Two independent transgenic lines expressing HopAK1, H1 and H2, and 2 independent transgenic lines expressing HopAL1, K1 and K10, were used for all experiments. Four week-old transgenic HopAK1/DR5::GUS plants were grown under short day conditions and sprayed with 30μM DEX. Four week-old plant leaves of HopAK1/DR5::GUS and 4 week-old whole plants of HopAL1/DR5::GUS were harvested for histochemical detection of β-glucuronidase (GUS) activity. They were vacuum infiltrated with GUS buffer (100 mM sodium phosphate buffer pH 7.2, 10 mM EDTA, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, (X-gluc; X-Gluc Direct. UK) supplemented with 0.05 mM potassium ferrocyanide and 0.05 mM potassium ferricyanide (SIGMA)) and incubated at 37°C for 16 hours. The enzymatic reaction was terminated by fixing the tissue in ethanol:acetic acid (3:1, v/v) overnight at room temperature. Samples were rinsed twice in 70% (v/v) ethanol and once in water for 30 minutes each.

Tissues were then immersed in a glycerol:water solution (3:1 v/v) overnight before mounting on slides.
2.2.2 GUS activity assay.

For the GUS activity quantification assay I modified the GUS fluorometric assay of Uchimiya et al. (1990). GUS activity was normalized to seedling number and protein concentration for seedlings in the liquid assay screen and for mature leaf tissue, respectively. Protein concentration was quantified using Bradford assays.

GUS quantification for mature leaves was done as follows: 50-100 mg of tissue was ground with 100 μl of 10X extraction buffer (50mM NaCl₂, 10mM EDTA, 0.1% Triton X-100, 0.1% sarkosyl, and 10 mM 2-mercaptoethanol). The homogenate was centrifuged at 12,000 rpm for 5 minutes at 4°C and 80 μl of the supernatant was transferred to a new eppendorf tube. 170 μl of extraction buffer (1X) was added to the tube containing the supernatant and 50 μl of each sample was transferred to one well of a 96-well plate. 50 μl of freshly prepared 4-Methylumbelliferyl-β-D-glucuronide (MUG) (3.5 mg of MUG in 10 ml of 1X extraction buffer) was added to each well. The plate was incubated at 37°C for 16 hours. Fluorescence spectrophotometer (BMG Labtech POLARstar OPTIMA plate reader, Fisher Scientific (UK)) was used for readings at 355 nm excitation and 520 nm emission. Each sample was prepared in triplicate and each treatment was repeated three times for all experiments.

50 μl of each biological replicate sample was kept for Bradford assay to determine protein concentration.
2.3 Auxin sensitivity assay and Flg22 assay

2.3.1 Auxin treatment.

IAA used in this study was obtained from Sigma (Sigma, St. Louis, MO, USA) and was dissolved in DMSO (dimethyl sulfoxide, Bioshop) and stored at -20°C. Dexamethasone (DEX) (SIGMA) was also dissolved in DMSO. Four week-old HopAK1/DR5::GUS plants and empty vector (EV)/DR5::GUS plants were sprayed with 30 μM DEX 48 hours prior to the assay. As a control (mock treatment), both HopAK1/DR5::GUS and EV/DR5::GUS plants were sprayed with water containing 0.006% DMSO. Fully expanded leaves of ten DEX-treated transgenic HopAK1 and EV plants and ten mock-treated plants were cut and soaked in solutions containing IAA (SIGMA-ALDRICH) at 0, 0.1, 1, 10, 50 or 100 μM. Samples were taken at the specified time points for GUS analysis (0, 6 and 8 hours). For the flg22 assay, fully expanded leaves were cut and soaked in solutions containing 0 or 10 μM IAA with or without 100 nM flg22. Samples were taken for gene expression analysis 8 hours after treatment.

2.4. Auxin measurements

Extraction and purification of IAA were performed by solid-phase extraction. The stable isotope-labeled compound D$_2$-IAA (Sigma-Aldrich, Oakville, ON, Canada) was used as an internal standard (Jikumaru et al., 2004). Four week-old transgenic HopAK1/DR5::GUS and EV/DR5::GUS plants were sprayed with 30μM DEX or water + 0.006% DMSO. After 48 hours, 50-100 mg of leaves were harvested and frozen for total endogenous IAA extraction. Samples were ground and mixed with 500 μl of 80% (v/v)
methanol containing 1% (v/v) acetic acid and an internal standard. The mixture was incubated at 4°C overnight. Subsequently, the samples were centrifuged at 14,000 ×g for 10 min at 4°C, and the pellet was washed with 80% (v/v) methanol containing 1% (v/v) acetic acid. A combination of supernatant extracts was vacuum-dried and applied to a pre-equilibrated Oasis HLB column cartridge (30 mg, 1 ml, Waters). After washing with 1 ml of 1% (v/v) acetic acid in water, IAA was eluted with 1 ml of 1% (v/v) acetic acid in methanol. The eluting materials were vacuum-dried to obtain extracts in 1% (v/v) acetic acid (water), and applied to a pre-equilibrated Oasis MCX column cartridge (30 mg / 1 ml water). After washing the MCX cartridges with 1 ml of 1% (v/v) acetic acid in water, the sample was added. The MCX cartridges were further washed (2X) with 1 ml of 1% (v/v) acetic acid in water. The final elution was done with 1% acetic acid in methanol and kept at -20°C until measurements were taken. Samples were loaded into the LC mass spectrophotometer following previously established methods (Preston et al., 2009).

2.5 Root growth assays

In order to identify the optimal (non-toxic) DEX concentration to induce gene expression, HopAK1/DR5::GUS seeds were sown on 0.5X MS agar media with 1.5% (w/v) sucrose, at pH 5.7 and supplemented with various DEX concentrations. Concentrations of 0.025 μM and 0.1 μM DEX were found to be optimal for lines H1 and H2, respectively. Expression of the gene was analyzed by western blot. HopAK1 seedlings were sown in MS plates with or without DEX, stratified for 4 days and allowed
to grow for 6 days under continuous light at 22°C. Seedlings were then transferred to plates containing NAA (naphthalene-1-acetic acid) (SIGMA), a stable auxin analog, at different concentrations (0, 0.01, 0.1 and 1 μM) and allowed to grow for 4 days. Measurements of primary root length after transferring to NAA plates were taken using ImageJ (Abràmoff et al., 2004). Lateral root numbers were counted using a dissection microscope.

### 2.6 RNA extraction, cDNA synthesis and qPCR

Total RNA was extracted from aerial tissues of 21 day-old plants using TRIZOL (Invitrogen) and treated with RNase-free Deoxyribonuclease I (Invitrogen) according to manufacturer’s instructions. The amount of RNA was then quantified based on absorbance at 260 and 280 nm. Oligo d(T)-primed cDNA was synthesized from 5 μg of total RNA using SuperScript II (Invitrogen) according to the manufacturer’s protocol. About 100 ng of cDNA template was used for Quantitative real time PCR (qPCR) reactions containing gene-specific primers at a concentration of 100 ng/μl. qPCR was conducted using a Bio-Rad Chromo4 real-time PCR detector using Power SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer’s instructions. Thermal cycling conditions consisted of 3 min at 95°C and 55 cycles of 15 sec at 95°C, 25 sec at 58°C, and 20 sec at 72°C. The results were analyzed using the Opticon Monitor version 3.1 (Bio-Rad). Transcript levels were normalized to the expression of EF1-α (elongation factor 1-α) measured in the same samples. Primer sequences are provided in Table 2-1.
qPCR data was analyzed following the ΔCt method (relative quantification normalized to a reference gene) described in the Bio-Rad qPCR manual.

2.7 Bacterial growth assays

HopAK1/DR5::GUS transgenic leaves were pressure-infiltrated with *P. syringae* resuspended to an optical density of OD<sub>600</sub>: 0.0002 (2x10<sup>-4</sup> cfu/ml). Eight plants per treatment per line were used. Four leaf disks (1 cm<sup>2</sup>) were harvested per plant at each time point and were ground in 10 mM MgCl<sub>2</sub>. Bacteria-containing leaf extract were plated on KB plates with the corresponding antibiotic and cycloheximide and allowed to grow overnight. Colonies were counted the following day.
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2.8 SDS-PAGE and western blot

50 mg of leaf tissue was ground in protein extraction buffer (per 200 ml: 4 ml of 1M Tris pH 7.5, 2 ml of 5M NaCl₂, 400 μl of 0.5M DTT and 2 μl of Nonidept P-40, topped with ddH₂O). Samples were heated to 99°C and loaded in a 12% SDS-PAGE gel for protein separation. Proteins were then transferred to a nitrocellulose membrane and detected with HA antibodies (Roche) by chemiluminiscence (Amersham Bioscience).

2.9 Split-ubiquitin yeast two-hybrid assay

The library was constructed using *A. thaliana* plants that were infected with *P. syringae* pv. tomato DC3000, *P. syringae* pv. tomato DC3000 / AvrRpm1, *P. syringae* pv. tomato DC3000/ΔhrcC or mock infiltration. Leaf samples were collected at 4 and 6 hours after infection for total RNA extraction. Pooled RNA was quantified by spectrophotometer, and used for library construction (Lewis et al., 2012).

Split-ubiquitin yeast two-hybrid analysis was conducted as reported in Iyer et al., 2005. HopAK1 construct was expressed under the weak CYC1 promoter in the pBT3-N vector (section 2.1.1.2). AP-4 yeasts carrying the bait construct were transformed using the PEG/LiAc method. Briefly, yeast carrying the bait construct were subcultered in 300 μl of yeast peptone dextrose adenine (YPDA) media at 28°C for 16 hours to and OD₆₀₀: 4.5. Yeast was inoculated in a 300 μl YPDA media to an OD₆₀₀: 0.1 and incubated at 28°C for 3-4 hours. Culture was then washed twice with sterile water and resuspended in 1.5 ml before transformation. Transformations were done
using 1 μg of cDNA library, 200 μL of yeast cells, 600 μL of PEG/LiAc (50% PEG, 120 mM LiAc, 10μL 10 mg/mL boiled salmon sperm DNA) by the heat shock method at 42°C for 45 min. Interacting colonies were identified by growth on SD-LeuTrpHis + 3-AT and selected based on size (bigger colonies were screened). Prey plasmids were extracted for identification. Yeast was grown overnight in drop-out media/synthetical defined bases (SD) without tryptophan for 2 days. Yeast was picked up and place in an eppendorf tube and resuspended in yeast lysis buffer (2.5M LiCl, 50 mM Tris-HCl pH 8, 4% Triton X-100 and 62.5mM EDTA pH 8). Small glass beads were added and samples were vortexed vigorously for a minimum of one minute. Samples were centrifuged at 14,000g for 5 minutes at RT. The supernatant was transferred to a new tube and mixed with 600 μl of 95% ice-cold ethanol followed by centrifugation at 14000g for 10 minutes at 4°C. Pellets were washed with 70% ethanol and dried at RT before being resuspended in 20 μl of DEPC-treated water. The resulting plasmids were transformed using electroporation into E. coli DH5α. Plasmids of selected prey candidates were co-transformed with HopAK1 as bait for confirmation.
Chapter 3

High-Throughput Screen for Type III Effectors that Alter Plant Hormone Signaling
3.1 Introduction

Plant immunity is controlled by complex signalling networks that influence the type and amplitude of defenses deployed. These events are influenced by local and systemic signals mediated by plant hormones (Robert-Seilaniantz et al., 2010; Tsuda et al., 2009). Plant hormones influence almost every aspect of plant life, from development to adaptive responses to biotic and abiotic stress (Chandler, 2009; Donnell et al., 2003, Zhao, 2010; Overvoorde et al., 2010; Park et al., 2007; Bari and Jones, 2009).

It is a complex network of interconnected signalling pathways that allows plant hormones to regulate such a variety of plant responses. This crosstalk between hormone pathways can be exploited by pathogens to avoid plant immune responses and to benefit pathogen growth (Spoel and Dong, 2008; Robert-Seilaniantz et al., 2010).

The plant hormones, SA, JA and ethylene are well-known for their role in regulating plant defense (Halim et al., 2006; Reymond and Farmer, 1998; Loake and Grant, 2007; Li et al., 2004). It has been well documented that SA regulates defense responses against biotrophic pathogens (Spoel et al., 2007; Spoel and Dong, 2008; Cohn and Martin, 2005; Robson et al., 2010) and acts antagonistically to JA/ethylene (Vlot et al., 2009). Other major hormones, such as ABA and auxin have also been recently implicated in modulating pathogen resistance responses, however their role in defense is not completely understood at the molecular level (Navarro et al., 2006, 2008; de Torres-Zabala et al., 2007; Truman et al., 2010; Koornneef et al., 2008; Asselbergh et al., 2008).
*P. syringae* is a Gram-negative bacterium associated with important plant diseases and its virulence is highly dependent on the TTSS and TTEs that it translocates (Rohmer et al., 2004; Troisfontaines and Cornelis, 2005). The numbers of TTEs delivered by each *P. syringae* strain varies. For example, the *P. syringae* pv. tomato DC3000 has a minimum of 36 TTEs (Innes, 2003, 2004), whereas *P. syringae* pv. maculicola ES4326 has at least 32 (*Pseudomonas syringae* Genome Resources; http://www.pseudomonas-syringae.org/). Although there is limited knowledge of the role of these effectors, some of their functions have been studied and a few TTEs have been shown to alter hormones responses (Chen et al., 2007; de Torres-Zabala et al., 2007). For examples, Cohn and Martin (2005) showed that the TTEs AvrPto (formerly AvrPto1) and HopAB2 (former AvrPtoB) increase disease symptoms in tomato leaves by stimulating ethylene production (Cohn and Martin, 2005). Navarro et al. (2006) showed that HopAB2 modifies the host ABA signalling pathway to suppress plant defenses. Moreover, it has been shown that the TTE, HopAM1, enhances pathogen growth and virulence by manipulating host ABA responses (Goel et al 2008). These studies highlight the effect that TTEs have on hormonal signaling pathways to promote their virulence. However, to date no direct interaction between a TTEs and a hormone signalling component has been reported.

The aim of this part of my work was to identify the TTEs that manipulate hormone signalling to increase virulence. To that end I developed a high-throughput liquid assay using standard 96-well plates to identify TTEs that can influence hormone responsive gene expression.
Using this approach, I identified several TTEs from \textit{P. syringae} that appear to activate auxin and ABA signalling (using reporter genes) as well as TTEs that attenuate SA signaling. The results from this assay forms the foundation for the follow-up experiments presented in later chapters of this thesis.

3.2 Results

3.2.1 Establishing a high-throughput system for transient expression of TTEs in \textit{A. thaliana} seedlings.

\textit{A. tumefaciens} has the unique ability to transfer its own DNA across kingdoms into a eukaryotic host cell where it can integrate into the plant genome (Hooykaas and Beijersbergen, 1994; Tzfira and Citovsky, 2006; de la Riva et al., 1998). It has been widely used for generation of stable transformants in many plant species through either tissue culture or floral dip as well as for transient expression in \textit{N. benthamiana} through leaf infiltration (Tzfira and Citovsky, 2006; de la Riva et al., 1998). In \textit{A. thaliana}, floral dip and tissue culture methods are commonly used for transformation, but transient expression has been challenging due to limited efficiency and consistency (Clough and Bent, 1998; Tsuda et al., 2012; Kim et al., 2009; Opabode, 2006).

I modified a liquid assay used for chemical screening in \textit{A. thaliana} that has been previously described by Schreiber et al. (2008) for transient expression of individual TTEs using \textit{Agrobacterium}-mediated transformation. To optimize the efficiency of the transient expression in seedlings, two different \textit{A. tumefaciens} strains were tested
(GV3101 and EHA105) with varying incubation periods. For this experiment, 8-10/well sterilized A. thaliana seeds were incubated in MS liquid media (0.5X MS; 0.002 M MES pH 5.8) in a 96-well plate for 5 days. A. tumefaciens (OD$_{600}$: 0.1) carrying individual HA-tagged TTEs were inoculated in the media together with acetosyringone (50 µg/ml) and samples were taken 24 hours after infection for western blot analysis. Acetosyringone was previously shown to enhance A. tumefaciens-mediated plant transformation (Sheikholeslam and Weeks, 1987; Baker et al., 2005; Dion et al., 1995).

Figure 3-1 shows a western blot analysis of the two A. tumefaciens strains tested carrying the HA-tagged genes for HopZ1a$_{PsyA2}$ and HopZ1c$_{PmaES4326}$. Fifteen A. thaliana ecotype Columbia seedlings were pooled together and homogenized for protein extraction of HA-tagged TTEs. From the results I found that both strains showed no difference in the protein expression of TTEs at 24 hours after treatment with A. tumefaciens. However, A. tumefaciens GV3101 showed higher transformation efficiency compared with A. tumefaciens EHA105 based on intensity of the bands. Thus, GV3101 was used for all subsequent experiments. Since Western blot could not detect expression in individual seedlings, GUS assay was used to test for the transformation efficiency of individual A. thaliana seedlings in our liquid assay. A. tumefaciens GV3101 was transformed with pBI121, a vector containing the reporter gene β-glucuronidase (GUS) gene, uidA, under control of cauliflower mosaic virus (CaMV) 35S promoter. Eight to ten wild type Col-0 seedlings were incubated with A. tumefaciens GV3101, and stained in GUS buffer 24 hours after infection. Using these conditions, I found that 21.18% and 26.44% of the seedlings were transformed in two independent experiments.
as indicated by the presence of GUS staining (Fig. 3-2). Thus ~25% of seedlings can be detectably transformed in our transient transformation liquid assay.

3.2.2 Characterization of transgenic plants expressing hormone responsive reporter constructs.

Recent advances have greatly enriched our knowledge of the molecular basis of phytohormone perceptions and signaling pathways. Functional analysis of the corresponding hormone responsive genes has led to the identification of reliable markers for hormonal responses in planta. For this study, I obtained various promoter – GUS reporter systems that have been established to be responsive to specific hormone treatments. The transgenic plants obtained were: auxin-responsive DR5::GUS and aux/IAA1::GUS, JA/ethylene-responsive PDF1.2::GUS, ABA-responsive RAB18::GUS, and SA-responsive PR1::GUS responding to SA (Table 3-1). DR5 is a highly active auxin-response synthetic element composed of multiple repeats of the auxin response element (TGTCTC) found in the promoters of auxin inducible genes (Ulmasov et al., 1997, 1995). Aux/IAA1 is an early auxin-response gene, frequently used as an auxin inducible marker (Yang et al., 2004; Park et al., 2002; Abel et al., 1994). ABA accumulation was monitored by using RAB18::GUS lines. RAB18 is an ABA response gene involved in stress responses (Park et al., 2008; Jeannette et al., 1999). PR1 (Pathogenesis-related 1) is a defense-related gene that is expressed in response to SA (Abramovitch et al., 2006). Finally PDF1.2 is a defense-related gene against
Figure 3-1. Comparison of TTEs expression in *A. thaliana* seedlings transiently transfected with *A. tumefaciens* strains GV3101 and EHA105. Seedlings were incubated with 2 different strains of *A. tumefaciens* carrying the HA-tagged TTEs HopZ1a or HopZ1c. Samples were taken 24 hours after infection for western blot analysis using anti-HA antibodies. HopZ1a: 42.1 kDa and HopZ1c: 30 kDa (arrows). Ponceau staining was used as a loading control. Molecular weight marker (kDa) are indicated.
necrotrophs that is regulated by both, JA and ethylene (Penninckx et al., 1998). To test if these marker genes were induced by the appropriate hormones in the liquid assay, each transgenic line was grown in the 96-well plate for 5 days and treated with the corresponding hormone at concentrations shown in Figure 3-3 for 24 hours. The GUS expression pattern was compared relative to non-treated samples. As shown in Figure 3-3, in all cases the basal level of GUS expression in treated seedlings increased after treatment with the corresponding hormones relative to the control.

3.2.3 High-throughput screening for TTEs that influence hormone marker gene expression.

The manipulation of hormone pathways was monitored in A. thaliana plants (8-10 seeds/well, 8 well/TTE) using the GUS reporter constructs described above (Table 3-1). Individual TTEs (Table 3-2) were cloned into the DEX-inducible pDB vector with a C-terminal HA-epitope tag (Aoyama and Chua, 1997) and transformed into A. tumefaciens GV3101 and used for transient expression assays.

The TTEs tested are described in Table 3-2. Sterilized seeds were grown in a 96-well plate (8-10 seeds/well) in MS liquid medium (0.5X MS; 0.002 M MES pH5.8) under continuous light for 5 days at 22°C. For transient expression of individual TTEs, seedlings were infected with A. tumefaciens GV3101 carrying TTE-expression constructs at a concentration of OD$_{600}$ : 0.1. Acetosyringone (50 μg/ml) was added to the growth media at the time of the infection to enhance the transformation efficiency of A. tumefaciens (Dion et al., 1995). The expression of an individual TTE was induced by
Figure 3-2. Transformation of *A. thaliana* seedlings with the 35S::GUS vector, pBI121. *A. thaliana* seedlings ecotype Col-0, were incubated in MS media with *A. tumefaciens* GV3101 carrying the 35S::GUS vector, pBI121. Twenty four hours after incubation seedlings were stained to check for GUS activity. Seedlings incubated with *A. tumefaciens* GV3101 alone were used as negative control. The rate of transformation was determined to be ~25%.
**Figure 3-3.** Hormone treatment of transgenic hormone-responsive promoter-GUS lines. (+): GUS staining 24 hours after hormone treatment. (-): GUS staining 24 hours after mock (water) treatment. DR5::GUS and Aux/IAA1::GUS are auxin responsive. PDF1.2::GUS is JA responsive (the JA derivative MeJA was used to activate PDF1.2 expression). RAB18::GUS is ABA responsive. PR1::GUS is SA responsive. Hormone concentrations used are indicated in µM.
Figure 3-4. Schematic of the high-throughput screening. Eight to ten transgenic seedlings with hormone-specific promoter::GUS were sown per well in a 96 well plate. Five day old seedlings were incubated in MS media together with *A. tumefaciens* GV3101 carrying individual TTEs. One specific TTE had 8 replicates per plate. Forty eight and 72 hours after DEX- treatment, seedlings were incubated with GUS buffer and later fixed. Seedlings showing increased GUS staining were considered as positive hits, whereas non-stained or weakly stained seedlings were considered negative.
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<td>ABA</td>
<td><em>RAB18</em></td>
<td>unpublished</td>
<td>AT5G66400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Ghassemian and McCourt)</td>
<td></td>
</tr>
<tr>
<td>JA</td>
<td><em>PDF1.2</em></td>
<td>(Anderson et al., 2004)</td>
<td>AT5G44420</td>
</tr>
<tr>
<td>SA</td>
<td><em>PR1</em></td>
<td>(Cao et al., 1994)</td>
<td>AT2G14610</td>
</tr>
</tbody>
</table>

Table 3-1. List of hormone responsive GUS-reporter transgenic seeds used in this study
Table 3-2. TTE library cloned in *A. tumefaciens* used for screening

<table>
<thead>
<tr>
<th>TTE (new name)</th>
<th>TTE (old name)</th>
<th>Ps. Strain</th>
<th>MW (kDa)</th>
<th>Vector</th>
<th>Agro str.</th>
<th>Stock #</th>
</tr>
</thead>
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<td>pBD</td>
<td>GV3101</td>
<td>1645</td>
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</tr>
<tr>
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<td>41.9</td>
<td>pBD</td>
<td>GV3101</td>
<td>1648</td>
<td></td>
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<td>Pma ES4326</td>
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<td>pBD</td>
<td>GV3101</td>
<td>1683</td>
</tr>
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<td>1660</td>
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<td>GV3101</td>
<td>1710</td>
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<td>Pto DC3000</td>
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<td>1711</td>
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<td>GV3101</td>
<td>1712</td>
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<td>HopPtoA2</td>
<td>Pto DC3000</td>
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<td>1713</td>
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<tr>
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<td>HopPtoH</td>
<td>Pto DC3000</td>
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<td>pBD</td>
<td>GV3101</td>
<td>1715</td>
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<tr>
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<td>Pma ES4326</td>
<td>38.48</td>
<td>pBD</td>
<td>GV3101</td>
<td>1692</td>
</tr>
<tr>
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<td>pBD</td>
<td>GV3101</td>
<td>1683</td>
</tr>
<tr>
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<td>AvrPpiB</td>
<td>Pto DC3000</td>
<td>31.31</td>
<td>pBD</td>
<td>GV3101</td>
<td>1709</td>
</tr>
</tbody>
</table>
applying 30 μM DEX to the liquid medium 6 hours post-infection. Seedlings from 3 replicates of the same experiment were each stained in GUS buffer at 24, 48 and 72 hours after DEX induction. Samples were fixed using fixing buffer (acetic glacial acid and ethanol (1:1)) (Fig. 3-4). *A. tumefaciens* GV3101 carrying an empty vector (EV) was used as a negative control and treatment of 0.5 μM IAA, 50 μM ABA, 2 μM JA or 100 μM SA were conducted as a positive control to check for GUS staining in the correspondent hormone-promoter::GUS (*uidA*) line (Fig. 3-3). Since each TTE is fused with an HA tag, induction of the effectors was confirmed by western blotting using anti-HA antibodies (Fig. 3-5).

3.2.3.1 TTEs that alter auxin signaling.

Auxin responses were monitored by the use of transgenic seedlings expressing DR5::GUS or *Aux/IAA1::GUS* (Lee et al., 2009).

Twenty six TTEs were screened, mainly from *P. syringae* pv. tomato DC3000 and *P. syringae* pv. maculicola ES4326, and monitored for changes in expression of those two reporters, which would be indicative of an altered hormone response. Samples were collected at 24, 48 and 72 hours after DEX induction and stained in GUS buffer. Seedlings that stained more than the basal staining seen in the empty vector were considered as positive (Table 3-3, Fig. 3-6 and Fig. 3-7).

Both transgenic seedlings incubated with *A. tumefaciens* carrying EV showed GUS staining at the tip of the cotyledon and the roots (Fig. 3-6). Similar results were observed for untreated seedlings, indicating that this staining is due to basal levels of
Figure 3-5. Representative western blot on *A. thaliana* Col-0 seedlings transfected with TTEs carried by *A. tumefaciens*. Five day old *A. thaliana* Col-0 seedlings were incubated with *A. tumefaciens* carrying individual HA-tagged TTEs and expression was activated by DEX treatment. Twenty four hours after incubation samples were taken (n=35-40) for protein extraction. Molecular weight markers are indicated (kDa). Ponceau staining was used as loading control. HopAA1-2: 51.14 kDa, HopPtoC: 28.99 kDa, AvrPto1: 18.18 kDa, HopAL1: 33.64, HopH1:24.32 kDa (arrows).
Table 3-3. List of TTEs that activated hormone responsive reporter genes in the screen.

<table>
<thead>
<tr>
<th>TTE (New name)</th>
<th>TTE (Old name)</th>
<th>Ps. Strain</th>
<th>Hormone affected</th>
<th>Known function</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>AvrPto1</td>
<td>AvrPtoA1</td>
<td>Pma ES4326</td>
<td>SA</td>
<td>auxin</td>
<td>ABA</td>
</tr>
<tr>
<td>HopAB3-1</td>
<td>HopPmaL</td>
<td>Pma ES4326</td>
<td>ABA</td>
<td>interacts with Pto</td>
<td></td>
</tr>
<tr>
<td>HopAK1</td>
<td>HopPmaH</td>
<td>Pma ES4326</td>
<td>SA</td>
<td>auxin</td>
<td>putative pectin lyase</td>
</tr>
<tr>
<td>HopAL1</td>
<td>HopPmaK</td>
<td>Pma ES4326</td>
<td>auxin</td>
<td>unknown function</td>
<td></td>
</tr>
<tr>
<td>HopJ1</td>
<td>HopPmaJ</td>
<td>Pma ES4326</td>
<td>auxin</td>
<td>ABA</td>
<td>unknown function</td>
</tr>
<tr>
<td>HopX1</td>
<td>AvrPphE</td>
<td>Pma ES4326</td>
<td>auxin</td>
<td>ABA</td>
<td>putative N-glyconase</td>
</tr>
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<td>HopZ1c</td>
<td>HopPmaD</td>
<td>Pma ES4326</td>
<td>SA</td>
<td>auxin</td>
<td>ABA</td>
</tr>
<tr>
<td>HopZ1a</td>
<td>Psy A2</td>
<td>SA</td>
<td>Acetyltransferase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HopC1</td>
<td>HopPtoC</td>
<td>Pto DC3000</td>
<td>SA</td>
<td>cysteine -type endopeptidase</td>
<td></td>
</tr>
<tr>
<td>HopAA1-2</td>
<td>AvrPtoA2</td>
<td>Pto DC3000</td>
<td>SA</td>
<td>ABA</td>
<td>unknown function</td>
</tr>
<tr>
<td>HopAM1</td>
<td>AvrPpiB</td>
<td>Pto DC3000</td>
<td>SA</td>
<td>auxin</td>
<td>ABA</td>
</tr>
<tr>
<td>HopF2</td>
<td>HopPtoF</td>
<td>Pto DC3000</td>
<td>SA</td>
<td>Interact with. RIN4</td>
<td></td>
</tr>
<tr>
<td>HopH1</td>
<td>HopPtoH</td>
<td>Pto DC3000</td>
<td>SA</td>
<td>unknown function</td>
<td></td>
</tr>
<tr>
<td>HopN1</td>
<td>HopPtoN</td>
<td>Pto DC3000</td>
<td>SA</td>
<td>cysteine protease</td>
<td></td>
</tr>
<tr>
<td>HopP1</td>
<td>HopPtoP</td>
<td>Pto DC3000</td>
<td>SA</td>
<td>ABA</td>
<td>Lytic glycolysate, helper protein</td>
</tr>
</tbody>
</table>

Note: TTEs were affected in their ability to down regulate SA signaling and to up regulate auxin and ABA signaling.
Figure 3-6. TTEs causing increased expression of DR5::GUS in seedlings. A) Five day old seedlings were infected with *A. tumefaciens* GV3101 carrying individual TTEs in the DEX-inducible vector pDB. Treatment with 0.5 μM IAA was used as a positive control. Mock: DMSO treated seedling were used as a negative control. Samples for GUS staining were taken at B) 48 hours and C) 72 hours after DEX induction. Percentages indicate percentage of stained seedlings. Seven biological replicates were done.
Figure 3-7. TTEs causing increased expression of Aux/IAA1::GUS in seedlings. A) Five day old seedlings were infected with A. tumefaciens GV3101 carrying individual TTEs in DEX-inducible vector pDB. Treatment with 0.5 μM of IAA was used as GUS staining positive control. Mock: DMSO-treated seedling were used as negative control. Samples for GUS staining were taken at B) 48 hours and C) 72 hours after DEX induction. Percentages indicate percentage of stained seedlings. Seven biological replicates were done.
auxin which is common in young seedlings (Péret et al., 2012). Thus, this pattern of staining was considered as background. As shown in Figure 3-6, inoculation of *A. tumefaciens* carrying 7 TTEs (HopZ1c<sub>PmaES4326</sub>, HopAK1<sub>PmaES4326</sub>, HopAL1<sub>PmaES4326</sub>, HopX1<sub>PmaES4326</sub>, HopJ1<sub>PmaES4326</sub>, HopAM1<sub>PtoDC3000</sub> and HopAA1-1<sub>PmaES4326</sub>) induced GUS expression more than control in DR5::GUS transgenic seedlings, showing increased ubiquitous staining throughout the seedling when compared to EV controls.

Using the Aux/IAA1::GUS transgenic line, 4 TTEs (HopZ1c<sub>PmaES4326</sub>, HopAK1<sub>PmaES4326</sub>, HopJ1<sub>PmaES4326</sub> and HopX1<sub>PmaES4326</sub>) were identified to induce GUS expression (Fig. 3-7). These 4 candidate TTEs were also identified using DR5::GUS transgenic seedlings (Fig. 3-6), confirming the validity of the liquid assay.

### 3.2.3.2 TTEs that alter ABA signaling.

ABA is a hormone involved in many plant processes, such as seed germination and development as well as in stress regulation against drought, cold and salinity (Mohr and Cahill, 2003, 2007). Its role in plant defense against biotrophs has been studied recently, and it has been suggested that ABA could suppress SA signalling (Mohr and Cahill, 2007; Cao et al., 2011).

I screened for changes in the RAB18 expression pattern resembling RAB18::GUS seedlings treated with ABA (Fig. 3-3). Eight TTEs (HopZ1c<sub>PmaES4326</sub>, HopJ1<sub>PmaES4326</sub>, HopX1<sub>PmaES4326</sub>, HopAM1<sub>PtoDC3000</sub>, HopAA1-2<sub>PtoDC3000</sub>, HopAB3-1<sub>PmaES4326</sub>, HopP1<sub>PtoDC3000</sub> and AvrPto1<sub>PmaES4326</sub>) induced GUS expression more than EV control at 24, 48 and 72 hours after DEX induction (Fig. 3-8). HopAA1-2<sub>PtoDC3000</sub>,
**Figure 3-8.** TTEs that showed increased expression of RAB18::GUS in seedlings. A) Five day old seedlings were infected with *A. tumefaciens* GV3101 carrying individual TTE in DEX-inducible vector pDB. Treatment with 50 μM of ABA was used for staining positive control. Untreated: DMSO-treated seedling as negative control. Samples for GUS staining were taken at B) 48 hours and C) 72 hours after DEX induction. Percentages indicate percentage of stained seedlings. Five biological replicates were done.
HopAB3-2_pmaES4326, HopP1_PtoDC3000 and HopZ1c_pmaES4326 showed higher intensity of the staining than the other TTEs despite showing less than 25% of stained seedlings.

### 3.2.3.3 TTEs that alter SA signaling.

SA is a plant hormone whose role in defense is well established (Halim et al., 2006; Vlot et al., 2009; Reymond and Farmer, 1998). It has been shown that SA acts as a positive regulator of plant defense against biotrophs and a negative regulator against necrotrophs (Wang et al., 2007; Vlot et al., 2009; Li, 2004; Robert-Seilaniantz et al., 2010). Because SA levels are elevated upon bacterial pathogen infection, it is in the best interest of a bacterial pathogen to counter with mechanisms to suppress SA responses (Vlot et al., 2009). Therefore, and unlike the rest of the other screens, I was interested in finding TTEs that can downregulate SA responses, which could, in turn, lead to increased virulence in the host.

Seventy two hours after A. tumefaciens and DEX treatment, A. thaliana seedlings were treated with 100 µM SA to stimulate PR1::GUS expression. Seedlings were incubated for additional 48 hours and samples were taken for GUS staining. SA-treated seedlings treated by A_tumefaciens carrying EV showed considerably high GUS staining in cotyledons and veins. Nine candidate TTEs (HopZ1c_pmaES4326, HopAK1_pmaES4326, HopN1_PtoDC3000, HopAM1_PtoDC3000, HopC1_PtoDC3000, HopF2_PtoDC3000, HopP1_PtoDC3000, AvrPto1_pmaES4326 and HopZ1a_PsyA2) downregulated GUS induction and showed decreased staining compared to EV controls, suggesting that these TTEs can downregulate SA signalling (Fig. 3-9).
Figure 3-9. TTEs showing decreased expression of PR1::GUS. A) Five day old seedlings were infected with A. tumefaciens GV3101 carrying individual TTEs in the DEX-inducible vector pDB. TTEs expression was induced six hours after infection by application of 20 μM DEX. One hundred μM of SA was induced to every well 72 hours after DEX induction. Samples were taken at B) 48 hours following SA application. Untreated seedlings (DMSO) did not receive SA application to control for basal expression of PR1. Representative picture are presented. Percentage indicates percentage of seedlings with no significant staining. Four biological replicates were done.
3.2.3.4 TTEs that alter JA/ethylene signaling.

JA and ethylene often act synergistically in their signalling pathways and are likely to be exploited by bacterial pathogens due to the antagonism between JA/ethylene and SA (Navarro et al., 2008; Traw and Bergelson, 2003; Cohn and Martin, 2005; Mersmann et al., 2010). In this screen I used PDF1.2::GUS A. thaliana transgenic plants to identify TTEs that could upregulate JA/ethylene signalling. No TTEs induced GUS staining at 48 or at 72 hours after induction under these experimental conditions (data not shown).

3.3 Discussion

Phytohormonal crosstalk is a mechanism that helps plants modulate disease and resistance depending on the lifestyle of the invading pathogen. Traditionally, plant-pathogen interaction research has focused on the responses mediated by SA and/or JA/ethylene, but it is now clear that other hormones, such as auxin and ABA, play an important role in defense responses. Studies have reported that individual TTEs can interfere with different hormone signaling pathways to either increase pathogen virulence or promote pathogen growth, such as AvrRpt2, AvrB1 and HopAM1. AvrRpt2 can upregulate and down-regulate auxin and SA signaling pathway, respectively (Chen et al., 2007; Cui et al., 2010; Goel et al., 2008). AvrB1 is able to induce the activity of an ethylene response promoter RAP2.6 with the help of JA/ethylene signaling pathways (He et al., 2004; Chen et al., 2009). Finally HopAM1 is reported to increase ABA
sensitivity in water-stressed plants in order to enhance its virulence at different stages of the infection (Goel et al., 2008).

To understand how TTEs interact with hormone pathways \textit{in planta}, I performed a screen using transgenic \textit{A. thaliana} plants containing hormone-inducible-GUS reporter genes which I confirmed to be responsive to hormone application in the liquid assay that I used for screening. I monitored changes in expression, reminiscent of hormonal treatments following infection with \textit{A. tumefaciens} carrying individual DEX-inducible TTEs, to identify the TTE candidates capable of modulating different hormone signaling pathways. Table 3-2 is a summary of all the TTEs used for this screen and Table 3-3 lists the candidate TTEs obtained from this screen.

Inconsistency was seen in the GUS staining process where some seedlings showed exceptionally high levels of GUS expression while the other seedlings in the same treatment showed no expression of GUS at all. The phenomena that only a small fraction of the seedlings showed high GUS expression after \textit{A. tumefaciens}-mediated transient expression could be explained by the low compatibility of the \textit{A. thaliana} / \textit{A. tumefaciens} system and correlates with the 25% transformation efficiency that I observed using a 35S::GUS reporter construct (Zipfel et al., 2006; Fig. 3-2).

In the case of JA, I did not identify any TTEs affecting PDF1.2::GUS expression in this screen. Although there is a possibility that none of the TTEs tested in this study upregulate the JA/ET signaling pathway, this may also be explained by other reasons; such as ABA/JA antagonism and/or experimental conditions. It has been reported that JA/ethylene signaling has an antagonistic relationship with ABA (Anderson et al., 2004; Adie et al., 2007). For example, the transcription factor AtMYC2 can upregulate ABA-
inducible genes and suppress certain JA-dependent genes (Mauch-Mani and Mauch, 2005). It was found that disruption of AtMYC2 resulted in increased transcription of JA/ethylene responsive genes (Anderson et al., 2004). In this screening system, a substantial amount of basal activation of ABA was observed in untreated samples, which indicate that ABA signaling is activated without any treatment in this liquid assay. This elevated ABA level might have masked the possible effect of TTEs that can manipulate JA/ethylene signaling due to ABA antagonism. Consequently, the current system might have not been able to detect alteration in JA/ethylene signaling caused by the TTEs.

PDF1.2::GUS expression was observed when the transgenic seedlings were treated with JA. This observation suggests that there may be a threshold level of JA signaling required to overcome an antagonistic effect. This could be evaluated using co-application of various JA and ABA concentrations.

It is interesting that HopZ1c<sub>PmaES4326</sub> seems to affect all hormones pathways tested except for the JA pathway. HopZ1c<sub>PmaES4326</sub> upregulated ABA and auxin signaling and downregulated SA signaling, consistent with the known antagonism between ABA/auxin and SA (Bari and Jones, 2009; Wang et al., 2007). In addition to HopZ1c<sub>PmaES4326</sub>, several other TTEs also affected multiple hormone signaling pathways indicating their possible involvement with hormone signaling crosstalk. For example, HopAK1<sub>PmaES4326</sub> was able to increase auxin signaling and decrease SA signaling, in agreement with the antagonistic interaction of these hormones proposed by Wang et al. (2007), where exogenous application of auxin could suppress SA-mediated response and enhance disease symptoms by <i>P. syringae</i> (Wang et al., 2007). Similarly,
HopAM1$_{PtoDC3000}$ and AvrPto1$_{PmaES4326}$ could upregulate ABA signaling while downregulating SA signaling. In accordance with these results, Goel et al. (2008) showed that HopAM1 can enhance ABA responses in A. thaliana. The antagonism between ABA and SA has been suggested previously (Yasuda et al., 2008; Mosher et al., 2010) and could be the strategy used by P. syringae carrying these TTEs to counteract defense responses. On the other hand, HopJ1$_{PmaES4326}$ and HopX1$_{PmaES4326}$ were capable of manipulating both ABA and auxin signaling, but not SA. It is possible that these effectors are contributing to virulence through a way that does not involve downregulating SA responses. It has been suggested that manipulation of auxin could lead to changes in physiology, such as cell wall loosening and leakage of nutrients, that could lead to increased virulence (Chen et al., 2007).

The liquid transient transformation assay system described in this chapter was a quick and effective method to identify candidate TTEs that putatively modulate hormone signaling. Even though the mechanism of how the TTEs in Table 3-3 manipulate their respective hormone pathways remains to be determined, the screening data does identify TTEs that can manipulate hormone signaling inside host cells. In the subsequent chapters I will attempt to address how 2 of the candidate TTEs; HopAK1$_{PmaES4326}$ and HopAL1$_{PmaES4326}$ affect auxin signaling and virulence. HopAK1$_{PmaES4326}$ is a good candidate to study the crosstalk between auxin and SA since the antagonism between these two hormones is reflected in the GUS staining pattern. It is also a highly conserved TTE in several strains of P. syringae and is therefore likely to play an important role in virulence (Heath O’Brien personal communication, Kvitko et al., 2007, Guttman et al., 2002). HopAL1$_{PmaES4326}$ caused accumulation of auxin only in the
DR5::GUS transgenic lines and no other hormone pathway seems to be upregulated, which may represent a more directed manipulation of auxin signaling. Moreover HopAL1_PmaES4326, is found in only two strains of *P. syringae* and may represent a more specialized strategy to manipulate hormone signaling (Heath O'Brien, personal communication).

Another interesting observation from the screen is that *P. syringae* pv. maculicola ES4326 TTEs mainly activate auxin and ABA signaling whereas TTEs belonging to *P. syringae* pv. tomato DC3000 preferentially downregulate SA. These findings might highlight different infection strategies by these two *P. syringae* strains. Looking for differentially activated hormone-related genes in a comparative microarray analysis after infection with each strain could be useful to determine if regulation of different hormone pathways is important for virulence.
Chapter 4

Characterization of HopAK1$_{PmaES4326}$

and HopAL1$_{PmaES4326}$
4.1 Introduction.

Auxin signaling can promote plant pathogenesis in diverse ways. Several reports have exposed the antagonistic relationship between SA and auxin signaling (Zhang et al., 2007; Iglesias et al., 2011; Truman et al., 2010; Wang et al., 2007). Auxin also mediates pathogen-induced tissue damage promoting disease phenotypes (Chen et al., 2007; Fu and Wang, 2011; Wang et al., 2007). Auxin treatment enhances symptoms in *A. thaliana* infected with *P. syringae* pv. tomato DC3000. Plants treated with auxin also support higher bacterial growth respect controls (Chen et al., 2007). Another benefit of increased endogenous auxin to pathogen is the promotion of stomatal opening, which can facilitate bacterial colonization (Merritt et al., 2001; Tanaka et al., 2006).

Evidence that TTEs can alter hormone responses is increasing rapidly. For instance, AvrPtoB increases endogenous ABA levels in host plants, presumably to promote *P. syringae* virulence (De Torres-Zabala et al., 2007). Expression of the TTE AvrRpt2 in *A. thaliana* mutants lacking RPS2, the resistance gene that recognizes this TTE, showed elevated levels of IAA and increased susceptibility to *P. syringae* infection indicating the negative impact of IAA on pathogen resistance (Chen et al., 2007). These transgenic seedlings also show phenotypes reminiscent of an altered auxin physiology presumably due to high levels of auxin accumulation. The phenotypes include increased lateral root formation, higher fresh weight, defects in gravitropism and less pronounced apical hooks when grown in the dark compared with wild type plants (Chen et al., 2007).

In a screen to identify TTEs that alter auxin signaling, I found 2 effectors that altered the expression pattern of the auxin reporter DR5::GUS in a manner similar to
exogenous auxin treatment. The two effectors are the \textit{P. syringae} pv. maculicola ES4326 TTEs HopAK1 (formerly HopPmaH) and HopAL1 (formerly HopPmaK). HopAK1 sensitized \textit{A. thaliana} to auxin, while HopAL1 showed enhanced auxin signaling. Both effectors increased susceptibility to bacterial infection.

4.2 Results

4.2.1 HopAK1\textsubscript{PmaES4326}

4.2.1.1 Plants expressing HopAK1 are hypersensitive to auxin.

As a follow up confirmation of the GUS staining results (Chapter 3), GUS quantification was measured in DR5::GUS seedlings incubated with \textit{A. tumefaciens} carrying HopAK1. Results showed a correlation between GUS staining and quantification (Fig. 4-1).

To analyze the effects of HopAK1 in auxin signaling, transgenic \textit{A. thaliana} expressing HopAK1 were generated in a Col-0/DR5::GUS background. Transformation was done using the full length coding region of HopAK1 cloned with a start codon and a C-terminal hemagglutinin (HA) tag under the control of the DEX inducible promoter. Expression of the TTE was confirmed by Western blot (Fig. 4-2).

In order to confirm that HopAK1 alters auxin signaling as observed in the high throughput screen, 2 independent HopAK1-expressing DR5::GUS/Col-0 (referred to as DR5::GUS) \textit{A. thaliana} lines, H1 and H2, 4-5 week old were assessed for GUS accumulation. 0-15\% of the leaves expressing HopAK1 showed more GUS staining
Figure 4-1 Fluorometric analysis for GUS quantification in seedlings transfected with HopAK1. Five-day old *A. thaliana* DR5::GUS seedlings were incubated with *A. tumefaciens* GV3101 carrying HopAK1 in the DEX-inducible vector pDB. Seedlings transfected with EV were used as negative control. Samples for GUS quantification were taken at 48 hours after DEX induction. (n=30). Error bars represent standard error. Star (*) indicates significant difference from No DEX treatment. P<0.05. Experiment was repeated twice with similar results. Representative graph is shown.
Figure 4-2. Western blot on HopAK1 expressing plants. Four-week old transgenic plants were sprayed with 30 µM DEX. Samples for TTE expression analysis were taken at 0, 6, 24 and 48 hours post treatment (hpt) (DEX (+) or water+ DMSO (-)). Experiment was done with two independent lines; A: H1 and B: H2. Loading control for protein was monitored using ponceau staining (P).
Figure 4-3. GUS staining in HopAK1 expressing plants. Two HopAK1 transgenic DR5::GUS lines (H1 and H2) were assayed for GUS staining after DEX treatment. 30 µM DEX (+) or water + DMSO(-) was applied to 5-week old plants. Leaves of 5 plants were soaked in GUS staining buffer for 16 hours and destained overnight. Samples were taken at the indicated times after DEX treatment. No significant difference in GUS staining between treatments could be observed. Insert numbers represent proportion of stained leaves to the total number of leaves assayed.
when compared to the control (no DEX-treatment) (Fig. 4-3). However, the results were inconsistent over several replicate experiments. One reason for this discrepancy could be that the growth conditions and growth stage of plants used in the high throughput liquid assay were substantially different from that of the transgenic soil-grown plants. Also, unlike the mature leaves investigated in the transgenic plants, young seedlings from the liquid assay would be expected to produce large amounts of auxin (Mattsson et al., 2003).

Given these differences, it is possible that transgenic expression of HopAK1 was not sufficient to increase auxin signaling to a detectable level. Moreover, it is possible that the presence of A. tumefaciens in the high throughput liquid assay also contributed to alteration of auxin signaling. Indeed, Chen et al. (2007) showed that transgenic plants expressing AvrRpt2 could significantly increase endogenous auxin production and this production was greater when infiltrated with P. syringae pv. tomato DC3000. Their results suggested that AvrRpt2 promotes auxin production during infection. In line with these observations, I hypothesized that the TTE HopAK1 sensitizes A. thaliana to auxin.

In order to test this hypothesis, 4-week old A. thaliana HopAK1/DR5::GUS (referred to as HopAK1) plants were exposed to different concentrations of auxin and assayed for DR5::GUS expression. Plants were sprayed with water + 0.006% DMSO or 30μM DEX to induce HopAK1 48 hours before leaves were harvested. Leaves were then soaked in solutions containing IAA at different concentrations. Subsequent GUS staining revealed only a weak induction of the GUS reporter in control plants (no DEX-treatment) after treatment with 10 and 100 μM IAA (Fig. 4-4). On the other hand the staining increased dramatically at both IAA concentrations in two independent DEX-
treated HopAK1 transgenic lines compared to control plants transformed with empty pBD vector. This difference was most apparent 8 hours after IAA-treatment (Fig. 4-4). Results were confirmed with GUS quantification (Fig. 4-5). This result supports the hypothesis that HopAK1 hypersensitizes plants to auxin.

Activation of auxin signaling was also monitored in HopAK1 transgenic plants by quantifying the expression of Aux/IAA1, an auxin responsive marker gene, using RT-qPCR. First, to determine if DEX treatment (expression of HopAK1) alone can alter Aux/IAA1 levels, 4 week old HopAK1/DR5::GUS plants were sprayed with 30 μM DEX or water+ 0.006% DMSO and samples were harvested 48 hours after treatment. Quantification of Aux/IAA1 expression indicated no significant difference between plants with and without DEX treatment. Also, no difference was observed between HopAK1-expressing plants and EV-transformed plants. These data suggest that the expression of HopAK1 alone does not affect Aux/IAA1 expression levels (Fig. 4-6).

Next, I tested whether HopAK1 expression can hypersensitize plants to 10 μM IAA similar to what was observed with the DR5::GUS reporter. Four-week old plants were sprayed with 30 μM DEX and treated with 10 μM IAA 48 hour later. Samples were harvested at 0 and 8 hours after IAA treatment. Aux/IAA1 expression was significantly up-regulated in transgenic plants expressing HopAK1 compared with non-expressing control plants 8 hours after treatment (Fig. 4-6). These results support the hypothesis that expression of HopAK1 can sensitize A. thaliana plants to auxin.

Classically, auxin mutant plants have been identified using root growth phenotypes since auxin regulates cellular division and expansion during root formation (Overvoorde et al., 2010). A. thaliana seedlings exposed to high concentrations of auxin
Figure 4-4. GUS staining in HopAK1 expressing DR5::GUS transgenic plants. Four week old plants were treated with DEX (+DEX) or water + DMSO (-DEX) 48 hours prior to treatment with IAA. Leaves were soaked in a solution containing the indicated concentration of IAA. Samples for staining were taken at 0, 6 and 8 hours post IAA treatment (h). H1 and H2 are 2 independent HopAK1 lines. Staining is more evident for both lines at 8 hours with 10 µM IAA treatment and this point was used for further studies. Experiment was repeated three times with similar results. Representative images are shown.
Figure 4-5. Fluorometric analysis for GUS quantification in HopAK1 expressing DR5::GUS transgenic plants. Plants were treated with DEX (+DEX) or water + DMSO (-DEX) 48 hours prior to treatment with IAA. Leaves were soaked in a solution containing the indicated concentration of IAA. Samples for staining were taken at 8 hours post IAA treatment. Experiment was done twice with similar results and representative graphs are shown. Bars represent standard error. Star (*) indicates significant difference from corresponding control. P<0.05 (t-test). A- H1 line and B- H2 line.
Figure 4-6. Aux/IAA1 expression in HopAK1 DR5::GUS transgenic plants. Plants were treated with 30 µM DEX or water (control). These 2 treatments were divided into 2 subgroups. A set of DEX treated (+DEX) and control + DMSO (-DEX) plants were treated with IAA 10 µM and the other set was treated with water. Samples were taken 8 hours after IAA treatment. Two leaves per plant from a total of 3 plants per treatment were used for total RNA extraction. Pooled RNA was used to prepare cDNA to conduct RT-qPCR. EF1α was used as reference gene for normalization. Bars represent fold change of Aux/IAA1 expression relative to time 0 of IAA treatment. There is a considerable upregulation of Aux/IAA1 in plants expressing HopAK1 after IAA treatment. Experiment was done twice with similar results and representative graphs are shown. Bars represent standard error. Star indicates significant difference from corresponding control. P<0.05 (t-test). A- H1 line and B- H2 line.
have increased lateral root number and shorten root length (Pernisová et al., 2009; Overvoorde et al., 2010). This phenotype is also observed in auxin hypersensitive mutants, such as *afb4* and *sax1* (Greenham et al., 2011; Ephritikhine et al., 1999). To monitor physiological impact of HopAK1 on auxin signaling, these phenotypes were analyzed in transgenic plants expressing HopAK1.

First, seeds from the 2 independent transgenic lines expressing HopAK1/DR5::GUS (H1 and H2) were sown on MS agar plates containing different concentrations of DEX to identify the ideal, non-toxic DEX concentration for both lines. It was established that the concentration of DEX to use in further experiments were 0.1 μM for the EV and H1 lines and 0.025 μM for the H2 line and non-transformed DR5::GUS plants (Fig.4-7).

HopAK1 transgenic seedlings were grown in MS/DEX plates for 6 days and then transferred to plates containing various concentrations of NAA, a stable auxin analog (Savaldi-Goldstein et al., 2008). Seedlings were allowed to grow for 4 days and measurements of root length and lateral root number are summarized in Figures 4-8 and 4-9, respectively.

Both independent lines expressing HopAK1 tended to have a shorter root growth at NAA concentrations of 0.01μM. Lateral root number also increased in HopAK1-expressing seedlings grown in 0.1μM NAA. No significant differences in root length or lateral root number were observed in EV plants. These observations in root growth support the notion that HopAK1 causes auxin hypersensitivity in *A. thaliana*. 
4.2.1.2 Enhanced auxin response after IAA treatment in HopAK1 transgenic plants is not due to increased endogenous IAA levels.

Endogenous auxin in HopAK1 transgenic plants was measured to determine if the increased sensitivity to auxin was due to an increased level of endogenous auxin. Free auxin was measured at 2 different growth stages in transgenic plants: 6 day-old and 4 week-old plants. Results for seedlings as well as for mature tissue showed that endogenous auxin in HopAK1 expressing plants were equivalent to or lower than non-expressing control plants (Fig. 4-10). Auxin levels did not change after DEX-treatment in EV control plants. These data indicate that activation of auxin signaling by HopAK1 is not due to increased endogenous auxin levels.

4.2.1.3 HopAK1 transgenic plants show increased susceptibility to bacterial infection.

Since an activation of auxin signaling correlates with enhanced pathogen growth (Chen et al., 2007; Wang et al., 2007) and given that HopAK1 can upregulate auxin signaling, I investigated whether HopAK1 transgenic plants were more susceptible to bacterial infection compared to controls. Transgenic DR5::GUS plants transformed with HopAK1 were pressure-infiltrated with *P. syringae* pv. maculicola M6ΔE, *P. syringae* pv. maculicola/ΔhrcC, *P. syringae* pv. maculicola ES4326, *P. syringae* pv. tomato DC3000 or *P. syringae* pv. tomato DC3000/ΔhrcC at a concentration of 2x10⁴ and their growth was quantified at 0 and 72 hours post-infection. *Pma* M6ΔE is derived from *Pma* M6 that lacks the plasmid carrying AvrRpm1 and HopX1 (formerly AvrPhE) (Rohmer et al.,...
Figure 4-7. DEX tolerance in DR5::GUS, HopAK1 and EV seedlings. Five week old DR5::GUS and HopAK1 or EV transgenic seedlings were grown on plates containing different concentrations of DEX (0, 0.025, 0.5 and 1 µM). Red boxes indicate the DEX concentration chosen for seedling assays. Experiment was done twice. Representative images are shown. EV represents transgenic DR5::GUS carrying the empty pBD vector.
**Figure 4-8.** Primary root length in HopAK1 seedlings. HopAK1 or EV seedlings were grown in MS plates containing DEX for six days. Seedlings were then transferred to MS plate containing the indicated concentrations of the IAA analog, NAA, and allowed to grow for 4 days. Root length was measured by subtracting the initial root length at time of transfer to IAA plates from the final length after 4 days of incubation. (n=15). Error bars represent standard error. Star (*) indicates significant difference from control. P<0.05 (t-test). Experiment was done at least three times with similar results and representative graphs are shown. Averages, standard error and P values are indicated A: HopAK1 line H1, B: HopAK1 line H2, C: DR5::GUS containing empty vector pDB vector.
**Figure 4-9.** Lateral root number in HopAK1 seedlings. HopAK1 seedlings were grown on MS plates containing DEX for six days. Then seedlings were then transferred to MS plate containing the indicated concentrations of NAA and allowed to grow for 4 days. Lateral root number was counted. (n=15). Error bars represent standard error. Star (*) indicates significant difference from control. P<0.05 (t-test). Experiment was repeated at least three times with similar results and representative graphs are shown. Averages, standard error and p-values are indicated. A: HopAK1 line H1, B: HopAK1 line H2, C: DR5::GUS carrying empty pDB vector.
Figure 4-10. Endogenous IAA levels in HopAK1 plants. HopAK1 or empty vector plants and seedlings were treated with DEX (+DEX) or water + DMSO (-DEX). A, C, E: Three mature leaves per plant from a total of 3 plants were used to extract endogenous IAA. B, D, and F: 6 day old seedlings were used to extract endogenous IAA. 100 seedlings were used per sample, 3 biological replicates were done for error calculation in all experiments except B where only one biological replicate with 100 seedlings was done. Bars represent standard deviation. Star (*) indicates significant difference from no DEX treated plants (-DEX). (P<0.05). Experiment was done twice with similar results and representative graphs are shown. A and B: HopAK1 line H1. C and D: HopAK1 line H2. E and F: DR5::GUS carrying empty pDB vector.
2004). ΔhrcC mutants of *P. syringae* pv. maculicola ES4326 and *P. syringae* pv. tomato DC3000 lack the injectosome structure of the TTSS and are unable to release TTEs into the host cell space (He, 1998). Two independent lines of HopAK1-expressing 4 to 5 week old plants were infected with each strain of *P. syringae* and immediately sprayed with 30 μM DEX or water + 0.006% DMSO. All experiments were repeated at least twice for both lines, except the infections with *P. syringae* pv. tomato DC3000 which were done once. Bacterial growth increased significantly in DEX-treated plants compared to water-treated controls for all the tested *P. syringae* strains. Only the wild type strain of *P. syringae* pv. maculicola ES4326 showed a slight (non-significant) decreased growth in one of the independent HopAK1 transgenic lines (H1), in one out of two experiments. This provides strong evidence that expression of HopAK1 promotes bacterial growth and increases susceptibility to infection in *A. thaliana* (Fig. 4-11. Fig. A1-1, Fig A1-2).

4.2.1.4 *PR1* is downregulated in HopAK1-expressing plants.

Previous studies have suggested that high levels of auxin can suppress SA accumulation (Wang et al., 2007; Chen et al., 2007). To determine whether HopAK1 suppresses SA signaling as a result of the manipulation of auxin signaling, expression of the SA-responsive marker gene, *pathogenesis-related protein 1 (PR1)* was analyzed in HopAK1 expressing transgenic plants.

Expression of HopAK1 was induced by DEX 48 hours before SA treatment (1 mM) by soil-drenching. 24 hours after SA treatment, *PR1* expression was significantly reduced in plants expressing HopAK1 compared with non-DEX treated plants, suggesting that HopAK1 can suppress SA signaling (Fig. 4-12).
4.2.1.5 HopAK1/axr2-1 crosses show increased bacterial growth.

To analyze if activation of auxin signaling correlates with the observed increased susceptibility to *P. syringae* infection, bacterial growth was studied in an auxin insensitive *A. thaliana* mutant, *axr2-1*, expressing HopAK1.

HopAK1 plants were crossed with the auxin insensitive mutant, *axr2-1*. F2 plants were assayed for bacterial growth. *axr2-1* is a dominant gain of function allele for the AUX/IAA gene, IAA7/AXR2, which controls gravitational growth in seedlings (Mai et al., 2011). The mutation presents a single amino acid change (Pro to Ser at codon 87) in the conserved domain II of IAA7/AXR2. This change stabilizes the AUX/IAA protein IAA7/AXR2, and thereby decreases auxin responses (Timpte et al., 1994; Nagpal et al., 2000; Mai et al., 2011). Four week old plants were pressure-infiltrated with *P. syringae* pv. maculicola ES4326/ΔhrcC at a concentration of 2x10⁴ cfu/ml² and bacterial growth was quantified at 0 and 72 hours post-infection. Surprisingly, impairment of auxin signaling did not alter the ability of HopAK1 to enhance bacterial growth (Fig. 4-13), suggesting three possibilities: 1- that the auxin pathway is not necessary for HopAK1 function to promote bacterial virulence; 2- that HopAK1 might be acting downstream IAA7/AXR2 and 3- that HopAK1 has multiple mechanisms to promote virulence and auxin manipulation is only one of them. It is important to point out that this experiment was done only once, and repetitions are needed to confirm reproducibility.

![Graph A) Line H1 (day 0)](image)

![Graph B) Line H1 (day 3)](image)
Figure 4-11. Bacterial growth of *P. syringae* in HopAK1 transgenic plants. HopAK1 plants were pressure infiltrated with bacteria at a concentration of $2 \times 10^4$ cfu/ml. Samples were taken 3 days after infiltration and prepared for bacterial count. ES4326: *P. syringae* pv. maculicola ES4326, ES4326/$\Delta hrcC$: *P. syringae* pv. maculicola ES4326/$\Delta hrcC$, DC3000: *P. syringae* pv. tomato DC3000, DC3000/$\Delta hrcC$: *P. syringae* pv. tomato DC3000/$\Delta hrcC$. M6$\Delta E$: *P. syringae* pv. maculicola M6$\Delta E$. Bars indicate standard error. Star (*) indicates significant differences. (P>0.05). Experiments were done at least twice with similar results and representative graphs for data collected on day 3 are shown. A: HopAK1 line H1 day 0, B: HopAK1 line H1 day 3, C: HopAK1 line H2 day 0 D: HopAK1 day 3 and E: empty vector (EV).
**Figure 4-12.** *PR1* expression in HopAK1 plants. HopAK1 plants were treated with or without DEX and 48 hours later plants were treated with 1mM SA through soil drenching. Samples were taken for total RNA extraction 24 hours later. Two leaves per plant from a total of 3 plants were pooled to extract total RNA. *EF1α* was used as reference gene for RT-qPCR and Tubulin for RT-PCR. Experiments were done at least twice with similar results and representative graphs are shown. Bars represent standard error. Star (*) indicates significant differences. (P<0.05). hpt: hours after treatment. A,B: RT-qPCR. Values represents *PR1* expression relative to no DEX treatment (-DEX). C, D: RT-PCR. A, C: HopAK1 line H1. B, D: HopAK1 line H2.
Figure 4-13. Bacterial growth of *P. syringae* in HopAK1/axr2-1 plants. HopAK1 line H2 was crossed with the auxin insensitive mutant axr2-1 and two independent lines were obtained (#1 and #2). HopAK1/axr2-1 plants were pressure infiltrated with *P. syringae* pv. maculicola ES4326/ΔhrcC at a concentration of 2x10⁴ cfu/ml and sprayed with DEX (+) or water + DMSO (-). Samples were taken at 0 and 72 hours post infection for colonies count. Experiment was done once. Bars indicate standard deviation. Stars (*) represent significant changes compared with no DEX treatment. (P<0.05). (1): HopAK1/axr2-1 line #1. (2): HopAK1/axr2-1 line #2.
Figure 4-14. Aux/IAA1 expression in flg22 treated HopAK1 transgenic plants. HopAK1 plants were treated with DEX or water + DMSO (control) 48 prior flg22 treatment. Leaves were soaked in a solution containing 10 µM IAA with or without 100 nM flg22. Eight hours after treatment 6 leaves were pool to extract total RNA for cDNA preparation. qRT-PCR was performed. Values represent fold change in Aux/IAA1 expression relative to time 0 after IAA treatment. EF1α was use as reference gene for normalization. Experiment was done twice with similar results and representative graph is shown. Bars are standard error. Stars (*) represents significant differences. (P<0.05). A: HopAK1 line H1. B: HopAK1 line H2.
4.2.1.6 Flg22 treatment down-regulates auxin signaling in HopAK1 transgenic plants.

flg22, a bacterial elicitor consisting of a 22 amino acid sequence found in the N-terminus of flagellin (Naito et al., 2007), has been shown to quickly induce miR393, which depletes the auxin receptor TIR1 mRNAs, resulting in reduced auxin signalling (Navarro et al., 2006, 2008). To determine whether HopAK1 acts upstream or downstream TIR1, HopAK1 expressing plants were simultaneously treated with auxin to activate auxin signaling and flg22 to downregulate TIR1 expression.

If HopAK1 stimulates the auxin pathway downstream of TIR1, plants would be expected to show upregulation of the auxin signaling marker Aux/IAA1. On the other hand, if HopAK1 activates signaling at or upstream TIR1, a downregulation of Aux/IAA1 marker gene expression would be expected. Upon Flg22 treatment, Aux/IAA1 expression is down-regulated in plants expressing HopAK1 (Fig. 4-14). This result suggests that HopAK1 requires TIR1 to sensitize plants to auxin. Combined with the results that HopAK1 does not induce auxin accumulation (Fig. 4-10), the manipulation of the auxin signaling pathway may occur at a different level, such as auxin transport.

4.2.2 HopAL1<sub>PmaES4326</sub>

4.2.2.1 HopAL1 plants show increased auxin signaling.

As a follow up confirmation of the GUS staining results, GUS quantification was measured in DR5::GUS seedlings incubated with A. tumefaciens carrying HopAL1. Results showed a correlation between GUS staining and quantification (Fig. 4-15).
To confirm the results from the initial seedling screen in mature leaf tissue, two independent, homozygous transgenic *A. thaliana* lines expressing HopAL1 were created using DR5::GUS/Col-0 as background (referred to as HopAL1). Expression of HopAL1 was confirmed by western blot and auxin signaling activation was analyzed (Fig. 4-16).

Similar to the analysis for HopAK1, 4 week old HopAL1 plants were sprayed with 30 µM DEX to induce HopAL1 expression and incubated in GUS staining buffer to visualize the activation of the auxin signalling pathway. Samples were taken at 0, 24 and 48 hours after DEX treatment. Expression of HopAL1 activated DR5::GUS at both 24 and 48 hours, as visualized by strong GUS staining (Fig. 4-17). The staining was more intense at 48 hours, possibly due to increased HopAL1 expression. GUS staining was initially observed around the edges of leaves which spread inwards after 48 hours. The pattern of staining likely correlates with auxin accumulation as the edges of leaves are zones of auxin production (Swarup and Péret, 2012). Together, the data indicate that HopAL1 expression in transgenic plants activates the DR5 promoter, and unlike HopAK1, it does not require external auxin application in mature plants.

### 4.2.2.2 HopAL1 transgenic plants show increased susceptibility to bacterial infection.

The activation of auxin signaling suggested that HopAL1 might enhance susceptibility to bacterial infection, similar to HopAK1, albeit by a different mechanism. To test this, I performed a bacterial growth assay in 4 to 5 week old HopAL1-expressing
Figure 4-15 Fluorometric analysis for GUS quantification in seedlings transfected with HopAL1. Five day old *A. thaliana* DR5::GUS seedlings were incubated with *A. tumefaciens* GV3101 carrying HopAL1 in the DEX-inducible vector pDB. Seedlings transfected with EV were used as negative control. Samples for GUS quantification were taken at 48 hours after DEX induction. (n=30). Error bars represent standard error. Star (*) indicates significant difference from No DEX treatment. P<0.05. Experiment was repeated twice with similar results. Representative graph is shown.
**Figure 4-16.** Western blot of transgenic HopAL1 plants. HopAL1 plants were treated with DEX or water (control) and samples for protein were taken at the indicated time points after DEX (+) or water + DMSO (-) treatment. HA antibodies were used for detection of epitope tagged HopAL1. Loading control for protein was monitored using ponceau staining. Experiment was repeated twice and a representative image is shown.
Figure 4-17. GUS staining in HopAL1 transgenic plants. Two independent lines for HopAL1 plants (K1 and K10) were sprayed with 30 µM DEX (+DEX) or water + DMSO (-DEX). Whole plants were taken for GUS staining at 0, 24 and 48 hours after DEX treatment. Experiment was repeated three times with similar results. A: HopAL1 line K1, B: HopAL1 line K10.
Figure 4-18. Bacterial growth of *P. syringae* pv. maculicola ES4326 in HopAL1 plants. Two independent lines for HopAL1 transgenic plants (K1 and K10) were pressure infiltrated with *P. syringae* pv. maculicola ES4326/ΔhrcC at a concentration of 2x10⁴ cfu/ml. 24 hours prior to infiltration, plants were sprayed with 30 µM DEX (+DEX) or water + DMSO (-DEX). Samples were taken at 0 and 3 day for colony count. Experiment was repeated twice with similar results and representative experiments are shown. Bars are standard deviation. Star (*) represents significant differences. (P<0.05). A: HopAL1 line K1. B: HopAL1 line K10.
plants pressure-infiltrated with the TTSS-deficient *P. syringae* pv. maculicola ES4326/ΔhrcC at a concentration of 2×10⁴ cfu/ml. DEX and water + 0.006% DMSO (control) were applied immediately after bacterial infiltration to induce HopAL1 expression and samples were taken for bacterial quantification at 0 and 72 hours after infiltration (Fig. 4-18). Bacterial growth increased significantly in both independent transgenic lines when compared to control (water + 0.006% DMSO -treated). This indicates that HopAL1 confers susceptibility to bacterial infection when expressed transgenically in *A. thaliana*.

### 4.3 Discussion

Phytohormone crosstalk allows plants to regulate defense responses by controlling the expression of defense-related genes according to the type of invading pathogen (Koornneef and Pieterse, 2008). Conversely, it has been suggested that pathogens have evolved to manipulate plant hormones as a strategy for circumventing plant defenses (De Torres-Zabala et al., 2007).

In this chapter, I have shown that HopAK1 expression in mature plants can sensitize mature leaves to external treatment using concentrations of auxin that would not normally induce significant auxin-responsive gene expression in mature plants. These results suggest that auxin hypersensitivity may represent a bacterial virulence strategy to downregulate host defenses using the TTE HopAK1.

Auxin treatment, however, was not required in young seedlings in the liquid assay of the initial screen. A possible explanation for this difference is that young seedlings produce more auxin than mature plants due to their developmental stage.
This higher level of endogenous auxin in seedlings may have worked like an external treatment of auxin, thus allowing the GUS reporter gene expression (activation of auxin signaling) by HopAK1 in the liquid assay. Another possibility is that *A. tumefaciens*, which was used to deliver the TTE in the liquid assay, may contribute to the activation of auxin signaling by HopAK1. Chen et al. (2007) reported a similar observation when measuring auxin accumulation following infection with *P. syringae*. In *rps2* plants infected with *P. syringae* pv. tomato DC3000 overexpressing AvrRpt2, auxin levels were significantly higher than those in wild type *P. syringae* pv. tomato DC3000-infected and uninfected control plants. This increased auxin accumulation was not observed when AvrRpt2 was constitutively expressed in *rps2* plants, whose auxin levels were similar to non-transformed, uninfected *rps2* control plants. This indicates that the presence of the bacteria was required to induce auxin accumulation by the TTE AvrRpt2.

In HopAK1 transgenic plants, the observed activation of the auxin signaling was also reflected in their root growth. Numerous studies have shown that auxin accumulation leads to increased lateral root formation and shortening in primary root (Jiang and Feldman, 2010; Overvoorde et al., 2010; Kim et al., 2001; Xie et al., 2000). HopAK1 seedlings show such auxin-related root phenotypes compared to control plants. To assess if the activation of the auxin signaling was due to increased amount of auxin itself, endogenous IAA levels were measured in mature leaves as well as young seedlings. No significant differences were observed in HopAK1 plants compared to control plants. The data suggests that activation of auxin signaling by HopAK1 is not related to an increase in endogenous auxin levels.
Navarro et al. (2006) showed that flg22 repressed auxin signaling through TIR1 expression by upregulating miR393. This repression was still observed in HopAK1-expressing plants indicating that activation of auxin signaling by HopAK1 might occur upstream of TIR1. Although the expression of the auxin signaling marker was significantly lower in HopAK1-expressing plants treated with IAA and flg22 compared with expressing plants treated only with IAA, the increased auxin signaling in HopAK1-expressing plants can still be observed when comparing to non-expressing plants (No DEX treated) (Fig.4-14). TIR1 is the major auxin receptor, but there are five others, AFB 1 to 5 (Dharmasiri et al., 2005; Parry et al., 2009; Teale et al., 2006; Robert-Seilaniantz et al., 2010). Although these other auxin receptors do not respond to flg22 as efficiently as TIR1 (Navarro et al., 2006), there is a possibility that the AFB receptors might also be regulating the signaling pathway where HopAK1 is involved. Another possibility is that HopAK1 might be affecting the transportation of auxin to influence the levels of this hormone only in the infected cell. Alternatively, HopAK1 could act downstream of TIR1 but requires TIR1 to perceive auxin. This could explain the activation of auxin signaling without any alteration in endogenous auxin levels by HopAK1 expression.

To test the biological effect of activation of auxin signaling, pathogen growth was analyzed using transgenic plants expressing HopAK1 with different *P. syringae* strains and their TTSS mutants (ΔhrcC). ΔhrcC mutants have a mutation in the gene that encodes an outer membrane protein that is part of the TTSS (Deng and Huang, 1999; Schreiber et al., 2012). This mutation prevents the bacteria from delivering TTEs into the host cell. The ΔhrcC mutation compromises bacterial growth allowing detection of minimal changes to colony counts. In my work, I found that HopAK1 transgenic plants
were more susceptible to all the tested strains compare to controls, indicating that this TTE is a virulence protein that can promote *P. syringae* growth.

SA is a widely studied plant hormone that activates defense responses against biotrophic pathogens and *PR1* is a molecular marker for SA signaling activation that is upregulated after various pathogen infections (Vlot et al., 2009). Transgenic plants expressing HopAK1 showed decreased *PR1* expression compared to the control after SA treatment. This downregulation of the SA pathway is supported by the initial screening where *PR1::GUS* lines transfected with HopAK1 significantly decreased the staining levels. These findings indicate that expression of HopAK1 can suppress SA signaling, which is crucial for biotrophic pathogen resistance. Supporting this idea, Wang et al. (2007) have suggested that the activation of auxin signaling could downregulate SA signaling. In turn, this downregulation could lead to diminished defense responses. Further analysis will be required to address whether SA-suppression is due to auxin-upregulation by HopAK1.

In an attempt to address the role of auxin-upregulation in HopAK1 virulence activity, HopAK1-transgenic plants were crossed with the auxin insensitive mutant *axr2-1*. Assuming that HopAK1 mediates an activation of the auxin signaling, and that this activation is responsible for increased bacterial growth through antagonism with SA signaling, I would expect that in an auxin insensitive plant, HopAK1 virulence functions would be lost. However, this was not the case. Promotion of bacterial growth by HopAK1 was similar in wild-type and *axr2-1 A. thaliana* plants. This result indicates that HopAK1 might have multiple mechanisms to promote bacterial virulence, one of which
is activating auxin signaling. Another possibility is that auxin activation by HopAK1 occurs downstream of AXR2, but this is very unlikely since HopAK1 appears to work upstream of TIR1 in the flg22 analysis, and one of the HopAK1 targets pulled from the yeast two hybrid assay is the auxin transporter PIN4 (details will be discussed in Chapter 5).

HopAK1_{ES4326} has homologous proteins in several \textit{P. syringae} strains. It is 100% identical in its nucleotide sequence to a TTE in \textit{P. syringae} pv. maculicola YM7930. It is also 71% similar to another TTE in \textit{P. syringae} pv. tomato DC3000 (Heath O'Brian, personal communication, Kvitko et al., 2007; Guttman et al., 2002). Protein sequence analysis of HopAK1_{ES4326} shows a putative pectate lyase in its C-terminus (Guttman et al., 2002). This domain is shared with all the \textit{P. syringae} homologs. The N-terminal region of HopAK1 is more variable and contains a series of deletions compared to the homologs in \textit{P. syringae} pv. tomato DC3000. Pectate lyases are involved in the maceration and soft rotting of plant tissue and it is responsible for the eliminative cleavage of pectate, yielding oligosaccharides with 4-deoxy-alpha-D-mann-4-enuronosyl groups at their non-reducing ends (Wing et al., 1990). Enzymes with this activity are present in both plants and pathogens. Some pathogens utilize this type of enzymes to degrade cell walls to facilitate invasion (Marin-Rodriguez et al., 2002). This function would appear logical with an effector that remains outside the host cell, however, HopAK1_{ES4326} was shown to be translocated into the cells by Guttman et al. (2002). Despite this report, HopAK1 homologs from other strains such as \textit{P. syringae} pv. tomato DC3000, do not appear to be injected into the intracellular space (Grant et al., 2006; Guttman et al., 2002). Some studies have shown that HopAK1 from \textit{P. syringae}
pv. tomato DC3000 is a harpin that facilitates the TTSS function of delivering TTEs (Kvitko et al., 2007). Harpins are a class of bacterial proteins that seem to facilitate effector translocation and have been termed “helpers”. These helper proteins have been suggested to exert their activity in the apoplastic space (Kvitko et al., 2007; Büttner and He, 2009). Although harpins tend to function outside host cells, there is an example of a harpin-function TTEs that is effectively translocated within the cell space, HopP1 (Oh et al., 2007). It has been suggested that HopP1<sub>DC3000</sub> is translocated but it is biologically active outside the host cell space; however, the mechanism for this dual localization has not been determined. In the case of HopAK1, not all HopAK1 homologs have been tested for translocation. It is possible that HopAK1<sub>ES4326</sub> has evolved to be translocated and obtained new functions. On the other hand, it might also be the case that HopAK1<sub>ES4326</sub> is an ancestral form of the TTE and that the HopAK1<sub>DC3000</sub> has lost the ability to be translocated. It would be interesting to investigate the translocation of multiple HopAK1 family members. To test for translocation, the C-terminus of AvrRpt2, an area responsible for induction of the HR response in <i>A. thaliana</i>, could be fused to the N-terminus of several HopAK1 homologs and examined for delivery by <i>P. syringae</i> (Guttman et al., 2002). Observation of HR would be an indication of translocation. In addition, fusion of the various HopAK1 family members with a fluorescent marker such as YFP could be used to analyze its localization, helping to understand where different HopAK1 members may act.

HopAL1 from <i>P. syringae</i> pv. maculicola ES4326, unlike HopAK1, is not widely represented in other <i>P. syringae</i> strains. Its sequence is identical to a TTE found in <i>P.
syringae pv. maculicola YM7930 (O’Brien et al., 2011). Its function is unknown and no significant similarities to known motifs or domains were found in several protein databases. However, using PHYRE (http://www.sbg.bio.ic.ac.uk/~phyre), a secondary structure analysis program, I was able to find that HopAL1 is slightly similar to the viral RNA polymerases (SCOP code: c2po4A) and other nucleotide related proteins. Furthermore, a BLAST search for nucleotide sequences suggested a weak similarity with SMC1, a chromosome maintenance protein. Interestingly, all of these proteins are able to bind to DNA, suggesting that HopAL1 may bind to nucleotides. Preliminary analysis of signaling motif prediction using CELLO localization predictor (http://cello.life.nctu.edu.tw) suggests that HopAL1 has a nuclear localization signal (Fig. 4-19). If this is the case, then HopAL1 would likely be in the nuclei of the host cell.. Mutational analysis could confirm the veracity of the nuclear signal and it could also help to understand what residues are important for nuclear localization, virulence and activation of auxin signaling.

BLAST analysis also showed that HopAL1 is similar to a few other hypothetical bacterial proteins (Fig. 4-19). Interestingly, all these proteins lack the N-terminus area which contains the potential translocation signal present in HopAL1. Based on this, it might be speculated that HopAL1 and these hypothetical proteins share a common ancestral protein. In the case of HopAL1_{ES4326}, this bacterial factor could have acquired a translocation signal becoming a TTE secreted into the host cell. Functional and evolutionary analysis of HopAL1 and all the homologous hypothetical proteins from P. syringae could shed light about this issue. I also found that HopAL1, unlike HopAK1, can activate the DR5 promoter without applying IAA. More studies are required to
understand if this auxin signaling activation is at the signaling or at the biosynthesis level. This will be addressed by measuring endogenous levels of IAA and by analyzing expression of auxin markers in HopAL1 transgenic plants. It will also be interesting to study auxin-related phenotypes, like root growth in these transgenic plants.
**Figure 4-19.** Alignment of HopAL1 with other related protein sequences found using BLAST. A: relative position of the related sequences with respect to HopAL1 (labeled with red letters). Red area in HopAL1 indicates estimated position of nuclear localization signal (NLS) found using CELLO. Numbers indicate amino acids in HopAL1. HP: hypothetical protein. B: Related sequences were aligned using Clustal W. The black-shaded areas are identical residues.
In this study, I also found that HopAL1 renders the plant susceptible to \textit{P. syringae} pv. maculicola ES4326/\textit{ΔhrcC}. It would be interesting to see how this growth promoting activity is related to the auxin accumulation observed in the GUS lines.

The potential DNA binding activity of HopAL1 and potential nuclear localization and the increased auxin signaling in transgenic plants suggest that this TTE could act as a transcription factor to increase either auxin biosynthesis, for example by inducing the expression of auxin biosynthesis enzymes. Analysis for DNA binding activity and yeast two hybrid analysis for identification of potential interactors can be useful to determine the role of HopAL1. Collectively, these studies will help to clarify if increased virulence is directly related to auxin manipulation. It would also be interesting to analyze whether transgenic plants expressing HopAL1 can confer the same susceptibility to other non-maculicola \textit{P. syringae} strains. Also, a transcription profiling can help to identify differentially regulated genes that are auxin and or defense related in HopAL1 transgenic plants.

HopAK1 and HopAL1 are two different TTEs that belong to the same pathovar of \textit{P. syringae}. I found that both TTEs can activate auxin signaling but in different ways. HopAK1 seems to sensitize the plants to auxin while HopAL1 activates auxin signaling on its own. These two TTEs were studied separately for their virulence effects and enhanced auxin responses in constitutively expressing transgenic plants. However, under natural conditions, the bacteria would likely inject both TTEs together and it is possible that they potentiate each other’s function. It is possible that HopAL1 stimulates auxin accumulation and that HopAK1 can potentiate HopAL1-induced auxin accumulation by enhancing auxin sensitivity, thereby triggering an enhanced auxin
response which may be effective at suppressing SA signaling and immunity. It remains to be elucidated if decreased SA signaling mediated by HopAK1 is due to the activation of the auxin pathway or if it is auxin-independent. It will be of interest to co-express both HopAK1 and HopAL1, in the same plant or bacteria to address if they can cooperatively promote bacterial virulence. Moreover, auxin phenotypes would likely be accentuated in the presence of both TTEs. Finally, uncovering interactors of these TTEs will shed light on their mechanism of action (see Chapter 5).
Chapter 5

Identification of HopAK1_{PmaES4326} Interacting Proteins
5.1 Introduction

In order to disturb plant homeostasis and increase their fitness, gram negative bacteria utilize TTEs that are delivered to the host cell space (McCann and Guttman, 2008; Büttner and He, 2009; Galan and Collmer, 1999; Crabill et al., 2010). Several interactions between a variety of plant cell proteins, such as RIN4, and TTEs have been documented in the last few years (Lewis et al., 2010; Bogdanove and Martin, 2000). These TTEs interactions also take place with plant hormone signaling (Chen et al., 2007; de Torres-Zabala et al., 2007). In many cases, these interactions lead to alterations in the hormone signaling pathways (Wang et al., 2007; Zhang et al., 2007; de Torres-Zabala et al., 2007; Chen et al., 2007; Navarro et al., 2006). For example, It was shown by Cui et al. (2010) that AvrB directly interacts with RAR1, enabling AvrB to associate with HSP90 and MPK4 in vivo, consequently promoting MPK4 kinase activation. This, in turn, perturbs hormone signaling pathways, such as those of JA and SA (Cui et al., 2010).

TTEs also perturb transcriptional regulation. For example, it was shown by transcriptional profiling that AvrPto1 and HopAB2 enhance the expression of many ethylene biosynthesis genes. In the same study the authors reported that the necrosis associated with disease symptoms is related to the effector's ability to increase ethylene production in the infected tissue (Cohn and Martin, 2005). HopAB2 has also been shown to induce the expression of NCED3, which encodes a key biosynthetic enzyme for ABA. It was suggested that AvrPtoB manipulates ABA signalling through alteration of NCED3 expression (De Torres-Zabala et al., 2007). In another study, Goel et al. (2008)
found that HopAM1 can enhance ABA responses to suppress defense responses through ABA-SA antagonism in *A. thaliana*.

Another hormone signaling that may be targeted is the auxin signaling pathway. Chen et al. (2007) demonstrated that AvrRpt2 is able to induce the accumulation of auxin in *rps2* plants and that this induction was even greater in the presence of *P. syringae pv. tomato* DC3000. The increased levels of endogenous auxin correlated with increased susceptibility to the bacteria (Chen et al., 2007). The authors proposed that activation of auxin signaling is a strategy used by *P. syringae* to diminish SA responses to increase virulence (Chen et al., 2007; Wang et al., 2007; Chandler, 2009; Navarro et al., 2006; Ghanashyam and Jain, 2009). These studies indicated that the manipulation of hormonal signaling is one of the functions of TTEs to suppress host defense responses.

Despite the fact that these studies strongly support the notion that TTEs alter host hormonal signaling, no hormone pathway component has been reported as a direct target for TTEs. To understand the molecular mechanism behind such hormonal signaling alterations, it is crucial to find the host targets of TTEs. Thus, in order to identify the targets of HopAK1, I have conducted split-ubiquitin yeast two-hybrid analysis using an *A. thaliana* library.
5.2 Results

5.2.1 Split-ubiquitin yeast two-hybrid screen to identify the host targets of HopAK1.

The split-ubiquitin yeast two-hybrid system is a modified version of the traditional yeast two-hybrid system where bait and prey are attached to the plasma membrane where the interaction takes place (Snider et al., 2010; Iyer et al., 2005). This system involves fusing N- or C-terminal halves of ubiquitin to 2 interacting proteins, which are membrane bound. Upon interaction of the proteins, the 2 halves of ubiquitin are brought together, and a transcription factor fused to one of the proteins is cleaved by ubiquitin specific proteases and released. The free transcription factor then enters the nucleus and activates transcription of reporter genes (Iyer et al., 2005). Some advantages of the split-ubiquitin system over the traditional method is that there is no need of reconstitution of transcription factor in the nucleus, which may lead to false positives due to transcriptional activation domains present in some bait proteins. There is also a minimized steric hindrance due to the use of small ubiquitin halves attached to the linker (Stagljar et al., 1998; Iyer et al., 2005).

HopAK1 from *P. syringae* pv. maculicola ES4326 was used as a bait (see Chapter 2) for the split-ubiquitin yeast two-hybrid system. An *A. thaliana* cDNA library used was generated from RNA extracted from 4-5 week old *A. thaliana* (Col-0) plants uninfected and infected with *P. syringae* pv. tomato DC3000, a non-virulent strain lacking the TTSS apparatus; *P. syringae* pv. tomato DC3000 ΔhrcC; and an avirulent strain, *P. syringae* pv. tomato DC3000 expressing AvrRpm1. The extraction of RNA and
bacterial infection were carried out by Dr. Mike Wilton, Ms. Corinna Felsenteiner and collaborators in the Desveaux laboratory. The RNA was sent to Norclone Biotech Laboratories, Ontario to prepare the cDNA library.

Instability or degradation of the bait could lead to self-activation of the transcriptional reporter signal. If the self-activation is strong enough that yeast growth with the empty vector or the NubG fusion protein (negative controls) is observed, the medium needs to be supplemented with the appropriate concentration of 3-amino-1,2,4-triazole (3-AT) (Iyer et al., 2005). 3-AT competes with the HIS3 gene product thus increasing the stringency of selection and preventing self-activation (Iyer et al., 2005). Several trials identified 2.5 mM 3-AT as the optimum concentration to prevent self-activation of the reporter gene; thus, this concentration was used for the screen. Control results are shown in Figure 5-1. Two negative controls were used; a co-transformation of the bait plasmid with an empty prey and a co-transformation with a mutated form of Nub (N-terminus of ubiquitin), pFur4-NubG. NubG, has no affinity for Cub (C-terminus of ubiquitin) and pFur4 is a yeast plasma membrane protein (Iyer et al., 2005). The positive control was a co-transformation of the bait with pFur4-Nubi, which has a strong affinity with Cub. The screen with HopAK1 yielded a total of 220 interacting yeast colonies (See Chapter 2, section 2.9).

Plasmids were extracted for all 220 hits and re-transformed into E. coli DH5α for identification and further confirmation. The obtained prey plasmids from E. coli DH5α were sequenced to identify potential HopAK1 interactors (Table 5-1). The sequences of the positive interactions were analyzed to assess if their sequences were in frame and cloned into the prey vector in the correct orientation. Positive hits that did not fit these
requirements were excluded from the potential interactor list. From the remaining list, consisting of eight possible interactors, I selected UNK (At4G21215.2) for further studies, which was the most represented interactor in the yeast two hybrid that was not considered a sticky protein, as was the case of NPL4 (At2G47970). NPL4 is considered sticky not only because it occurs in 38% of the hits, but also because it was highly represented in other unrelated screens (personal communication with Kimberley Chin and Jennifer Lewis). Thus, NPL4 was excluded from further analysis. The other candidate that was selected for further studies was PIN4 (At2g01420), an auxin-efflux transporter (Ganguly et al., 2010; Křeček et al., 2009; Ljung et al., 2005; Marquès-Bueno et al., 2011). This was the only auxin-related protein identified in the screen.

Candidates were re-transformed into the prey vector for confirmation (Fig. 5-2). cDNAs in the library are often truncated during the process of the library construction. Thus, the potential interactors obtained from the screen were also likely truncated proteins (lengths of the sequences are indicated in Table 5-1). Therefore, confirmation of the interaction using full length proteins is necessary to determine true interaction. The full length coding sequences of UNK and PIN4 were cloned into the same prey vector used for the initial screen. Interactions remain to be confirmed.

5.2.2 Analysis of A. thaliana T-DNA insertion lines.

T-DNA insertion lines for UNK and PIN4 (UNK: SALK 007070, SALK 040059, PIN4: GK-593F01-021827 and GK-296G09-015495), were obtained from Arabidopsis Biological Resource Center (ABRC), to analyze whether these genes were involved in
resistance to *P. syringae*. According to T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress), the T-DNA insertions disrupt exons of the UNK and PIN4 genes. Neither line presents differences in morphology when compared with the wild type plants under standard growth conditions (data not shown).

Four to 5 week old UNK (Salk_007070C) and PIN4 (GK-593F01-021827) T-DNA insertion lines were pressure-infiltrated with the TTSS-deficient *P. syringae* pv. maculicola ES4326/ΔhrcC (Deng and Huang, 1999). Although these T-DNA lines were not analyzed in terms of expression of the target genes, homozygosity was assessed by analyzing the presence or absence of the T-DNA insertion and full length gene by PCR (Fig 5-3).
Figure 5-1. Split-ubiquitin yeast two-hybrid controls. Pfur4-NubI and Pfur4-NubG are cloned in the prey vector pPR3N and fused to half of the ubiquitin protein. Pfur4-NubI (positive control) and Pfur4-NubG (negative control), carrying the N-terminal half of ubiquitin, were co-transformed with HopAK1 cloned in the bait vector pBTN3 which carries the C-terminal half of ubiquitin. The empty vector pPR3N (EV) was used as an additional negative control for checking 3-AT suppression of growth. A 2.5 mM concentration of 3-AT was selected for the screen.
**Table 5-1. HopAK1 interactor list.**

<table>
<thead>
<tr>
<th>Name</th>
<th>AGI</th>
<th>RL (TP)</th>
<th>TL (TP)</th>
<th>In-frame</th>
<th>%</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPL4</td>
<td>AT2G47970</td>
<td>88-1071</td>
<td>1242</td>
<td>yes</td>
<td>38.01</td>
<td>protein degradation</td>
</tr>
<tr>
<td>Unknown</td>
<td>AT4G21215</td>
<td>593-978</td>
<td>1305</td>
<td>yes</td>
<td>11.11</td>
<td>unknown function</td>
</tr>
<tr>
<td>APG1</td>
<td>AT3G63410</td>
<td>151-641 (157-1012)</td>
<td>2401</td>
<td>Yes</td>
<td>3.5</td>
<td>methylation step of plastoquinone synthesis</td>
</tr>
<tr>
<td>emb1990</td>
<td>AT3G07430</td>
<td>77-615</td>
<td>1006</td>
<td>Yes</td>
<td>2.9</td>
<td>embryonic development ending in seed dormancy</td>
</tr>
<tr>
<td>ATTDT</td>
<td>AT5G47560</td>
<td>761-1181</td>
<td>2267</td>
<td>Yes</td>
<td>1.75</td>
<td>malate/fumarate transporter</td>
</tr>
<tr>
<td>PIN4</td>
<td>AT2G01420</td>
<td>1138-1649</td>
<td>2353</td>
<td>Yes</td>
<td>1.17</td>
<td>auxin transporter</td>
</tr>
<tr>
<td>LHCA4</td>
<td>AT3G47470.1</td>
<td>334-1083</td>
<td>1266</td>
<td>Yes</td>
<td>2.34</td>
<td>chlorophyll a/b-binding protein</td>
</tr>
</tbody>
</table>

**Note:** RL: Length of the gene region obtained during the screen. TL: total length of the gene. %: percentage of obtained colonies during screen. Interactors selected for further studies are highlighted. NPL4: nuclear pore localization protein. APG1: albino or pale green mutant 1. ATTDT: *Arabidopsis thaliana* tonoplastic dicarboxilate transporter. PIN4: PIN-formed 4. LHCA4: light harvesting complex A 4.
at a concentration of $2 \times 10^4$ cfu/ml. At day 3 after infection, bacterial growth in both insertion lines, was between one and one and a half log higher compared to Col-0 control plants (Fig. 5-4). The experiment was repeated twice with similar results. These preliminary data showed increased susceptibility of T-DNA insertion lines to *P. syringae* infection.

### 5.3 Discussion

Split-ubiquitin yeast two-hybrid assay was performed to identify potential interactors of HopAK1. In this screen, HopAK1 was used as bait against a cDNA library prepared from infected and uninfected *A. thaliana* leaf tissues. The use of infected plants to prepare the cDNA library may allow the detection of interactors that are only expressed during an infection process.

Two possible interactors, UNK (At4G21215.2) and PIN4 (At2g01420) were selected for further analysis. UNK is a small protein of 199 amino acids (21.41 kDa) with unknown function. Preliminary sequence analysis did not identify any conserved domain.

PIN4 is an auxin-efflux transporter that belongs to the PIN1-type subfamily of the PIN family of auxin transporters (Paponov et al., 2005; Křeček et al., 2009). It is a member of a family of transmembrane proteins that determines auxin polar transport.
**Figure 5-2.** Preliminary confirmation of UNK and PIN4 as HopAK1 interactors. UNK-NubI and PIN4-NubI were re-transformed with HopAK1-Cub and plated on selection media (leu-trp SD-media, 2.5 mM 3-AT). Both candidates showed high levels of interaction with HopAK1. Pfur4-NubI is a positive control and Pfur4-NubG is a negative control for the selection media.
Figure 5-3. Genotyping of progeny for T-DNA insertion lines of PIN4 (Salk_007070C and Salk_040059) and UNK (GK_593F01-021827). Numbers indicate individuals. Higher panel indicates PCR using gene specific primers and lower panel indicates PCR using T-DNA primers. PIN4 :1839 bp and UNK:600 bp.
Figure 5-4. Growth of *P. syringae* in PIN4 (GK-593F01-021827) and UNK (Salk_007070C) T-DNA insertion lines. Plants were pressure infiltrated with *P. syringae* pv. maculicola ES4326/ΔhrcC. Samples were taken at 0 and 3 days after infection to quantify growth. Bars indicates standard deviation. Stars (*) represent significant differences with respect to WT Col-0. (P<0.05). Experiment was done twice with similar results and representative experiments are shown.
which includes PIN1, 2, 3 and 7. The PIN1-type family have been reported to have redundant functions (Vieten et al., 2005) but other studies have shown that individual loss-of-function mutants present their own phenotypes (Huang et al., 2010; Friml et al., 2002a; Paciorek and Friml, 2006). For instance, PIN3 is involved in lateral root generation and mutants defective in PIN3 presents defects in differential growth (Friml et al., 2002b). PIN4 appears to be an auxin sink generator and a regulator of auxin homeostasis. PIN4 loss of function seedlings show changes in auxin distribution and patterning changes in cell division and cell determination (Peer et al., 2004; Friml et al., 2002a). In the embryo, PIN4 is localized in the precursor cells for the root meristem (Peer et al., 2004; Friml et al., 2002a). *A. thaliana* microarray data indicate that PIN4 is expressed in the whole seedling and in all leaf types of mature plants (Winter et al., 2007 Arabidopsis eFP browser, BAR). It is slightly down regulated during pathogen infection with *Botrytis cinerea, P. syringae* pv. tomato DC3000 and *P. syringae* pv. maculicola ES4326 (0.66 log, 0.63 log and 0.69 log respectively) (Winter et al., 2007 Arabidopsis eFP browser, BAR), suggesting that PIN4 may be involved in pathogen resistance.

The obtained homozygous lines for PIN4 and UNK did not show any auxin-related morphological phenotypes in the rosette leaves, such as curled leaves and reduced apical dominance (preliminary observation). However they showed increased susceptibility to bacterial growth (Fig. 5-4). Although the HopAK1/UNK and HopAK1/PIN4 interactions need to be explored in greater detail, I propose several possible mechanisms of action below.
Knock-out lines of an effector’s interactors can provide information about their roles in plant immunity and how effectors manipulate their target to promote infection. If the interactor is a negative regulator of defense responses, the KO lines will likely show increased defense responses and the effector likely activates or stabilizes its function. Activation of such negative regulator should suppress resistance and associated responses such as *PR1* expression. On the other hand, if the host interactor is a positive regulator for defense responses, the KO lines will likely show increased susceptibility to infection and the effector likely inactivates or destabilizes such targets to suppress immunity. Preliminary analysis using T-DNA insertion lines for both *UNK* and *PIN4* showed enhanced susceptibility, suggesting that they are positive regulators for pathogen resistance. The defense related phenotypes of HopAK1 transgenic plants, such as increased bacterial growth and suppression of *PR1* expression described in chapter 4 suggest that HopAK1 may negatively regulate UNK and/or PIN4 to suppress defense responses. This may include decreased protein levels in HopAK1 expressing plants. It will be interesting to test if UNK and/or PIN4 protein levels are altered using western blot analyses of HopAK1 transgenic plants. There is also the possibility that HopAK1 modifies the protein to suppress its function. HopAK1-induced post-translational modifications of UNK and/or PIN4 proteins, such as cleavage, can also be monitored by western blot analyses of these proteins in HopAK1 transgenic plants. Microarray analyses of *unk* and *pin4* KO plants may highlight which branches of plant immunity are affected by these 2 genes; for example SA signaling using *PR1* gene expression.
Bacterial growth analysis showed that PIN4 T-DNA insertion lines are more susceptible to *P. syringae* infection than wild type plants/control plants. Other than the microarray data obtained from the Arabidopsis eFP browser, there is no indication that PIN4 is involved in defense responses; thus, the observation of increased bacterial growth in the T-DNA insertion lines in this study could potentially be a novel phenotype associated with PIN4 loss-of-function plants, although this will have to be confirmed with additional T-DNA insertion lines.

It has been shown that the endogenous auxin levels dramatically decrease as leaves expand, but whole plants still retain the ability to synthesize auxin *de novo* (Ljung et al., 2001). Mature leaves are capable of *de novo* synthesis of auxin, which is eventually transported out from the source cell towards the forming shoot apical meristem in peas (Jager et al., 2007). One way that HopAK1 may function to downregulate defense responses is through inactivation or reduction of PIN4 activity, to prevent or reduce auxin transport outside the cell. The accumulation of auxin in cells resulting from PIN4 inactivation could account for the sensitization of auxin signaling observed in transgenic plants expressing HopAK1. If this is correct, analyzing auxin signaling markers in the KO lines would show increased expression. Moreover analysis of defense markers such as PR1, would address if the downregulation of the defense responses in *pin4* KO lines can be attributed to decreased PIN4 levels. As mentioned above, another point to analyze would be the expression of PIN4 protein in HopAK1 transgenic plants. If PIN4 is degraded or inactivated by HopAK1, HopAK1 expressing plants may show reduced levels of PIN4.
Determining the enzymatic function of HopAK1 will allow predictions on the type of interaction that it has with its target/s. Structural analysis of HopAK1 may help to understand the mode-of-action of HopAK1 on UNK and PIN4. For example, finding the domains involved in the interaction and creating mutations at key amino acids for the interaction may help to elucidate the nature of the association between these 2 proteins and how this affects to virulence and auxin signaling. Similar to HopAB1 (former AvrPtoB) (Xiao et al., 2007; Göhre et al., 2008; Gimenez-Ibanez et al., 2009), HopAK1 may also have more than one target and multiple functions. Analysis of the interaction between HopAK1 and its targets as well as the role/s the target will help to understand if defense suppression by HopAK1 is auxin-dependent or -independent.

Analysis of TTEs targets is critical to the understanding of how bacterial effectors act. In this chapter, I have shown 2 potential HopAK1 interactors, UNK and PIN4. UNK, is a protein for which no function has been described yet, thus it may provide the opportunity for novel findings in pathogen defense signaling. Another interactor, PIN4, could be the first hormone signaling component that directly interacts with a TTE. Although much remains to be done, both candidates present interesting and challenging questions to pursue.
Chapter 6

Final Discussion
The plant hormone network is an essential pillar of plant life that controls development and stress responses (Nemhauser et al., 2006; Qu and Zhao, 2011; Depuydt and Hardtke, 2011). This network is also an attractive target for hijacking by a variety of pathogens (O'Donnell et al., 2003; Robert-Seilaniantz et al., 2007; Mohr and Cahill, 2007). Although plant hormones like SA and JA/ethylene are the most studied hormones in plant defense, in recent years, many studies suggest that hormones that were originally thought to be only involved in development and abiotic stress responses, like ABA, brassinosteroid and auxin, are also involved in defense (De Torres-Zabala et al., 2007; Navarro et al., 2006; Wang et al., 2007; Wang, 2012).

When I initiated my work, I set the goal of finding TTEs from the bacterium *P. syringae* that were able to hijack plant hormone signaling and alter hormonal crosstalk. For this purpose, a high throughput screen was designed. This screen was designed to analyze TTE impacts on specific hormone signaling pathways in *A. thaliana* seedlings by assessing changes in hormone responsive reporter systems. After the screen, I chose to work with 2 TTEs that altered the auxin signaling pathway; HopAK1 and HopAL1.

Both TTEs appear to regulate auxin signaling differently. HopAL1 can activate auxin signaling in mature plants on its own whereas HopAK1 sensitizes mature plants to exogenously applied auxin. Much remains to be done to understand the mechanism by which both of these TTEs alter auxin signaling. Is it by direct or indirect interaction between TTEs and auxin signaling components? Is the increased auxin response antagonizing the SA pathway and providing advantages to the pathogen? Several works have reported on the ability of SA signalling to downregulate auxin signaling...
responses (Iglesias et al., 2011; Wang et al., 2007; Stewart and Nemhauser, 2010). In addition, other works have suggested the possibility of downregulation of SA responses by auxin signaling (Chen et al., 2007; Kazan & Manners, 2009). However, to this day, and to my best knowledge, only one experimental evidence demonstrates that SA signaling is downregulated by auxin. Wang et al., first treated plants with SA to activate expression of PR1, a SA marker, and followed by treating with an auxin analog NAA. This resulted in PR1 been downregulated with respect to the control (no NAA treatment) (Wang et al., 2007).

HopAK1 is a TTE widely found in other *P. syringae* strains. It would be interesting to carry out comparative studies between different alleles of HopAK1 regarding its function in virulence and auxin manipulation.

Like other TTEs, HopAK1 may exert several functions and its multiple functions may increase pathogen virulence (Fig. 6-1). I have found 2 possible targets for HopAK1 using the split-ubiquitin yeast two-hybrid system; however, *in planta* interaction still remains to be validated. Assuming that HopAK1 has multiple functions and its multi-functionality is achieved by multiple targets, it could be expected that; 1. HopAK1 utilizes both interactors to activate auxin signaling or 2. HopAK1 utilizes only one of them to activate auxin signaling and another one to increase its virulence in an auxin independent manner. A third possibility is that neither of these targets is used to manipulate auxin signaling.

One of the interactors of HopAK1 is PIN4, an auxin efflux transporter (Blakeslee et al., 2007; Peer et al., 2004; Paponov et al., 2005). PIN4 is already known to be
involved with auxin and it is a strong candidate as a HopAK1 target for regulating auxin sensitivity (Křeček et al., 2009). The other interactor is a protein whose function is unknown which I have named UNK. UNK does not have any documented functions in auxin signaling nor defense responses. Studying auxin signaling responses, such as changes to auxin-related markers, auxin accumulation or auxin-related developmental phenotypes, in UNK KO plants can help to determine if it is involved in auxin signaling.

The idea of HopAK1 having both, auxin-dependent and independent functions is supported by the preliminary data using the auxin insensitive mutant axr2-1 (Fig. 4-13). The results showed increased bacterial growth in axr2 expressing HopAK1, suggesting that HopAK1 can promote bacterial growth even when auxin signaling is impaired.

There are several examples in the literature for P. syringae TTEs that exert multiple functions. One of them is HopAB2 (former AvrPtoB). This TTE was shown to interact directly with BAK1 and FLS2. BAK1 is a LRR receptor-like kinase involved in brassinosteroid signaling and in PTI (Yang et al., 2011; Xiang et al., 2011) and FLS2 is a LRR receptor-like kinase that recognizes bacterial flagellin, and plays an important role in PTI (Ali and Reddy, 2008; Sun et al., 2012). It has been known that BAK1 and FLS2 interact to activate PTI (Xiang et al., 2011). HopAB2 suppresses PAMP immunity by disrupting this interaction (Gohre et al., 2008; Gimenez-Ibanez et al., 2009). The E3 ligase domain contained in HopAB2 in its C-terminus specifically targets FEN kinase, which is involved in PRF-mediated defenses (Xiang et al., 2008; Lewis et al., 2009; Mackey et al., 2002). An additional function of HopAB2 is the suppression of the transcription of miR393b, a PAMP inducible miRNA also involved in defense. Regarding hormone signaling, HopAB2 can upregulate ABA, which contributes to disease
susceptibility (Navarro et al., 2008; de Torres-Zabala et al., 2007). Another example of a TTE with multiple functions is AvrRpt2. It has been shown that AvrRpt2 interacts with RIN4 which is monitored by two R proteins, RPM1 and RPS2, and consequently suppresses defense responses (Mackey et al., 2003). AvrRpt2 also induces auxin accumulation in *A. thaliana* although the mechanism was not described and this accumulation enhanced bacterial growth (Chen et al., 2007). In a later publication it was shown that AvrRpt2 could promote de protein turn-over of the auxin signaling negative regulators Aux/IAA and that the cys protease activity of the TTE was necessary for this function (Cui et al., 2013).

How is auxin signaling affected by HopAK1? There are several possibilities. HopAK1 could increase auxin biosynthesis itself, however, this is very unlikely since no significant difference in free endogenous IAA was observed between HopAK1 expressing plants and controls. Thus, one possibility is alterations of signaling pathway downstream of auxin biosynthesis. HopAK1 could increase TIR1 receptor affinity for auxin or affect the SCF<sup>TIR1</sup> complex affinity for the Aux/IAA negative regulator proteins. Another possibility is that HopAK1 could decrease the affinity of the Aux/IAA for the ARF proteins by changing the structure of this interaction or by competing with IAA/Aux for binding with ARFs (Fig. 1-6). If one of these is the case, then UNK can be the interactor leading to such changes based in the growth assay data in T-DNA insertion lines. Alternatively, since PIN4 is a transporter of auxin, altering auxin transport is another possible mechanism for HopAK1 to activate auxin signaling in the infected cells. Analysis of endogenous levels of auxin in PIN4 KO plants and examining how HopAK1
affects PIN4 function, may help clarify the mechanism by which HopAK1 alters auxin signaling. For example, PIN4 needs to be phosphorylated in order to be distributed in the plasma membrane (Huang et al., 2010; Dai et al., 2012). HopAK1 could be impeding the phosphorylation of PIN4 avoiding the transport of auxin outside the cell. In vitro protein phosphatase activity assay could be used to determine if PIN4 phosphorylation in altered in the presence of HopAK1.

The other TTE that was identified to manipulate auxin signaling in this study was HopAL1. HopAL1 activates auxin signaling when expressed in mature A. thaliana tissues without exogenous treatment of auxin. Preliminary examination of the HopAL1 sequence suggests the presence of a potential nuclear localization signal (See Chapter 4, Fig.4-19). I hypothesize that HopAL1 may function as a transcription factor for regulating auxin biosynthesis and thereby affect auxin signaling. Chromatin immunoprecipitation (ChiP) can be used to identify the DNA regions that interact with HopAL1 which can be confirmed by electrophoretic mobility shift DNA-binding assay.

It is intriguing to speculate that HopAK1 and HopAL1 could act in a coordinated fashion to manipulate auxin signaling, opening the possibility for studying coordinated attacks with more than one TTE. It has been previously suggested that TTE may act in a cooperative way (Galán, 2009). A review by Galan (Galán, 2009) suggested that pathogenic effectors coordinate their activity by being delivered in a “precise temporal and spatial manner”. An interesting example is the Salmonella TTSS effector SptP. SptP is a GTPase activating protein (GAP) for several Rho-GTPase family members, involved in regulating actin dynamics in mammalian cells (Fu and Galan, 1999). It was first thought that this effector disrupted actin filaments of host cells, but further studies
found that it actually helps to recover cell homeostasis (Galan and Collmer, 1999).
Among *Salmonella* effectors, there are several Rho-GTPase members that cause an alteration in the cytoskeleton structure but it was observed that this alteration was rapidly reversed. The GAP activity of SptP results in downregulation of the actin-cytoskeleton rearrangements stimulated by other bacterial effectors, such as SopE (Galan and Collmer, 1999). Thus, HopAK1 and HopAL1 may be acting cooperatively, in a manner similar to SptP with other *Salmonella* effectors to increase virulence. This would provide the first example of cooperation between multiple *P. syringae* TTEs in order to enhance virulence. In such a case, determining which TTE acts first and whether one TTE influences (“leads”) the attack more than others, will help to develop countermeasures against such TTEs in an agricultural setting.

Based on my findings, I propose a model for the cooperative interaction between HopAK1 and HopAL1 regarding their regulation of the auxin pathway (Fig. 6-2). In this model, HopAL1 would be transported to the nucleus to activate genes related to auxin biosynthesis. Since the natural infection with *P. syringae* will initially affect a few cells, this auxin accumulation will not reach the auxin maxima needed for a visible auxin response. However, this increased auxin accumulation will be enough for HopAK1 to activate and potentiate auxin signaling favoring the pathogen. This could benefit the pathogen by downregulating SA defense responses through potential SA-auxin antagonism.

Alternatively, HopAL1 could be interfering with the conjugation of IAA, probably by altering GH3 function, an auxin conjugation enzyme (Bajguz et al., 2009), to make more IAA available in the media for HopAK1 to activate auxin signaling. More evidence
for HopAL1 function is needed to make a conclusion on how does it affect auxin signaling.

In this study, a high throughput system to identify TTEs that potentially alter hormone signaling was established. Using this system, several TTEs were identified to manipulate SA, ABA and auxin signaling. For detailed analysis, I chose 2 TTEs, HopAK1 and HopAL1, that affect auxin signaling and found that HopAK1 likely sensitizes plants to auxin, whereas HopAL1 potentially influences accumulation of auxin. Putative host interactors for HopAK1 were identified by yeast two hybrid system. Further detailed studies on the interaction between these interactors and the TTEs will provide significant insight on manipulation of hormonal signaling by TTEs. Although further research is required to clarify some of these unanswered questions, this study represents an important step in elucidating a full picture of how TTEs affect plant hormone signaling pathways for increased virulence.
Figure 6-1. The dual role of *P. syringae* pv. maculicola ES4326 HopAK1 in *A. thaliana* disease development. A novel proposed role for HopAK1 is that it sensitizes the plant to low concentrations of auxin in order to increase bacterial fitness. The source of auxin may be promoted directly or indirectly by *P. syringae*. Additionally HopAK1 could promote *P. syringae* growth in plants via an auxin-independent mechanism.
Figure 6-2. Proposed model of cooperation between *P. syringae* pv. maculicola ES4326 HopAK1 and HopAL1 to increase auxin signaling. During *P. syringae* infection, TTEs are delivered into the plant intracellular space. HopAL1 is then transported to the nucleus where it regulates the expression of auxin biosynthesis genes, increasing auxin production and accumulation in the cell. A second TTE, HopAK1, sensitizes the plant cell to low amounts of auxin. The combined effect of these 2 TTEs lead to activation of auxin signaling that in turn downregulates the plant defense response. Small circles in the model represent secreted TTEs.
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