Identification and functional analysis of *Pseudomonas syringae* pv. *actinidiae* effector-triggered immunity in *Nicotiana spp.* and *Arabidopsis thaliana.*

A thesis presented in partial fulfilment of the requirements for
the degree of Doctor of Philosophy in Plant Science
at Massey University, Manawatu, New Zealand.

Sera Choi

2017

Abstract

Pseudomonas syringae pv. *actinidiae* (*Psa)* is the causal agent of bacterial canker in commercially important cultivars of kiwifruit (*Actinidia delicosa and A. chinensis*) worldwide, including New Zealand. Like many gram-negative pathogens, *Psa* is expected to utilise type III effectors to promote virulence in host plants. In order to better understand *Psa* effector-triggered immunity and susceptibility, we aimed to investigate multiple molecular characteristics of *Psa* type III effectors and their recognition mechanisms in model plants, *Nicotiana* spp. and *Arabidopsis thaliana.*

Nicotiana tabacum and *N. benthamiana* are widely-used model plants for *Agrobacterium*-mediated transient expression (agroinfiltration) of effectors for functional characterization. Firstly, we screened multiple characteristics of effectors from two *Psa* strains, *Psa* NZ V13 and *Psa* NZ LV5. The former is a strongly virulent and the latter is a weakly virulent strain in kiwifruit. By using agroinfiltration in *Nicotiana* spp. to express individual effector proteins*,* we observed diverse subcellular localisation for *Psa* effectors. Additionally, we identified multiple *Psa* effectors that can trigger HR-like cell death (HCD) in both *N. tabacum* and *N. benthamiana.* Using virus-induced gene silencing (VIGS), we identified that some *Psa* effector-triggered HCD requires the immunity regulator *SGT1*, suggesting that the *Psa* effector-triggered HCD could be a result of immunity activation.

We focused on one *Psa* NZ V13 effector, HopZ5, which belongs to the YopJ-like acetyltransferase family. HopZ5 triggers hypersensitive response (HR) in *Arabidopsis* accession, Ct-1. Another Arabidopsis accession, Col-0, does not develop an HR but shows immunity in response to HopZ5. The gene that confers HopZ5-triggered HR in Ct-1 was identified as *SOBER1 (SUPPRESSOR OF AVRBST-ELICITED RESISTANCE 1)* by using recombinant inbred lines derived from two parental accessions, Ct-1 and Col-0. *SOBER1* is a known suppressor of *Xanthomonas* effector AvrBsT-triggered immunity. Interestingly, AvrBsT also belongs to YopJ family. Uniquely, SOBER1 specifically suppressed HCD triggered by several YopJ-like acetyltransferase effectors in *N. benthamiana*, including HopZ5 and HopZ3 from *Psa*. This suggests a common mechanism shared between a subset of YopJ-like acetyltransferase effectors is suppressed by SOBER1.

Finally, we identified one *Arabidopsis* accession, Ga-0, which carries a truncated *SOBER1* variant but does not develop an HR upon HopZ5 delivery. Using bulked- segregant analysis of an F_2 population derived from a cross between Ct-1 and Ga-0, we mapped the locus conferring HopZ5-recognition in Ct-1 to the upper arm of Chromosome 3.

Acknowledgements

First of all, I would like to thank my primary supervisor, Prof. Kee Hoon Sohn for great advices and scientific encouraging on me during my PhD. Delightful and enlightening discussions with him really helped me a lot to carry on my project. I feel grateful for his comments and supports throughout the degree. Also, I want to show my gratitude to our former senior postdoc Prof. Cecile Segonzac for being an excellent mentor in the lab. I appreciate her for sharing her knowledge and guiding us from her experiences not only in the lab, but also outside of the lab. I also want to thank my co-supervisors, Dr. Rosie Bradshaw, Dr. Janet Reid, Dr. Matt Templeton and Dr. Erik Rikkerink for their supports and help.

I also would like to thank all KSL members, in New Zealand and in Korea for being excellent companions for the long journey. I was so lucky to have such nice PhD students, Toby, Maxim and Jay as colleagues also as friends. I especially thank my project mate, Jay, for being a nicest mentor I've ever had and for his support. I really appreciate his enthusiasm on science which drove me also into the project.

I sincerely thank my partner, Maxim for encouraging me to continue whenever I face difficulties, for inspiring me by his enthusiasm into science and holding my hands always.

Lastly, I appreciate endless supports and help from my family. Even though we were far away from each other, my mother and sister always made me feel like I was with them. Without their help, I wouldn't have been able to achieve this journey. I also want to thank my little cat, Tori for being my late-night mate in New Zealand.

Table of contents

Chapter 1. General introduction

1.1 Introduction

Plant pathogens cause significant reduction in crop production. Plants are sessile, surrounded by potential pathogens. Consequently, plants have developed specialized innate immune systems to detect invading pathogens. In plants, cell surface-localized pattern recognition receptors (PRRs) recognize the common features of microbes, pathogen/microbe-associated molecular patterns (PAMPs) and activate innate immunity, which is called PAMP-triggered immunity (PTI). Successful pathogens overcome PTI by injecting effectors into host plant cells to inhibit PTI. On the other hand, plants have also developed a surveillance system to detect the presence of effectors and activate effector-triggered immunity (ETI). During ETI, disease resistance (R) proteins specifically detect effectors within a host cell and induce various immune responses to restrict pathogen growth. A co-evolutionary arms race between pathogen effectors and their corresponding intracellular immune receptors has shaped the highly diversified repertoire of plant immune receptors (Jones and Dangl, 2006).

The majority of plant intracellular immune receptors carry a centrally located NB-ARC (nucleotide binding shared by Apaf-1, R proteins and CED4) domain and a carboxyl-terminal leucine-rich repeat (LRR) domain. These nucleotide-binding and leucine-rich repeat receptors (NLRs) can recognize pathogen effectors directly (Jia et al., 2000; Lawrence et al., 1995) or indirectly via a guardee/decoy (Dangl and Jones, 2001). In the latter, an effector modifies the guardee of an NLR or a decoy of the true effector target and this modification is recognized by a corresponding NLR protein to induce defence signaling (Ade et al., 2007; Lewis et al., 2013; Axtell and Staskawicz, 2003). NLR proteins carry a variable amino-terminal region with either a CC (coiled coil) or TIR (Toll/interleukin-1 like receptor) domain that is generally involved in activating downstream defence signaling (Cui et al., 2015). Understanding how NLRs confer disease resistance to pathogens is important to

develop stable disease resistance in crops. *Pseudomonas syringae* pv. *actinidiae* (*Psa*) causes bacterial canker disease in kiwifruit plants. *Psa* NZ V-13 from recent outbreaks of bacterial canker is highly virulent in commercialized kiwifruit cultivars in New Zealand and worldwide, leading to a significant loss of productivity. In this study, I investigated the interaction between *Psa* NZ V-13 and *Arabidopsis thaliana* and *Nicotiana* spp. to better understand the genetic basis of *Psa*-induced defence mechanisms in this non-host plant.

1.2 *Pseudomonas syringae* pv. *actinidiae* in kiwifruit

Psa is a bacterial pathogen causing canker symptoms in commercial kiwifruit cultivars (e.g. *Actinidia chinensis* cv. Hort 16A). Symptoms of canker disease include dark spots on leaves, wilting and browning of flowers, and a number of shoots showing blackening and terminal wilting and die-back, but there is no direct effect on fruits (Everett et al., 2011). Unlike other staple agricultural crops, kiwifruit (*Actinidia* spp.) has been domesticated very recently; therefore, outbreaks of disease and causal pathogens have been clearly recorded.

The first kiwifruit canker disease caused by *Psa* was reported on *Actinidia deliciosa* cv. Hayward in Shizuoka, Japan in 1984 and *Psa* was first taxonomically classified as a new subspecies in 1989 (Serizawa et al., 1989). Until 2008, the presence of *Psa* was reported in Korea , China (Wang Z, 1992) and Italy (Scortichini, 1994) but was restricted to these respective geographical regions. In 2008, a global outbreak of *Psa* occurred in several countries and genomic analysis revealed that these particularly virulent strains from this outbreak were different from the earlier strains recorded in Japan, Korea and Italy (McCann et al., 2013). These aggressive strains from the 2008 outbreak were subsequently grouped together as '*Psa*-V' (McCann et al., 2013).

Since the 2008 outbreak of *Psa*-V originally identified in Italy, *Psa*-V has spread worldwide, including Chile (2010) and New Zealand (2010). *Psa*-V is classified as biovar 3 and it is clearly differentiated from foliar kiwifruit canker-causing Japanese (*Psa*-J, biovar 1) and Korean (*Psa*-K, biovar 2) strains (Vanneste et

al., 2013). In New Zealand, a new low-virulence strain (*Psa*-LV, biovar 4) was also reported in *Psa*-contaminated orchards. Based on whole genome sequence analysis, McCann et al showed that *Psa*-J, *Psa*-K, *Psa*-LV and *Psa*-V belong to very distinctive phylogenetic clades (McCann et al., 2013). These authors further suggested that these distinctive clades are the result of various gene-shifting processes derived from different source populations, hinting at the probability of new virulence emergence in *Psa* populations.

1.3 Plant innate immune system

1.3.1 PAMP-triggered immunity (PTI)

The immobility of plants renders them susceptible to numerous attacks by microbes. In addition to physical barriers such as a waxy cuticle and a rigid cell wall, plants have developed surveillance systems that detect common microbial elicitors resulting in restricted growth of potentially pathogenic microbes. These common elicitors are referred to as pathogen/microbe-associated molecular patterns (PAMPs) and recognized by corresponding pattern recognition receptors (PRRs). The immune response activated by the recognition of PAMPs to restrict microbial growth is termed PAMP-triggered immunity (PTI). PTI is the first line of plant innate immunity to protect themselves from various microbial invasions.

1.3.1.1 Pathogen-associated molecular patterns (PAMPs)

PAMPs, which are also known as MAMPs (microbe-associated molecular patterns), are well-conserved microbial features. One of the best characterized PAMPs is bacterial flagellin. Flagellin is one of the structural protein components of the bacterial flagellum, which is a structure that facilitates mobility and adhesion to host plants. It was shown that the 22 amino acid sequence at the N-terminus of flagellin (flg22) is sufficient to elicit defence responses in plants (Felix et al. 1999).

Flg22 is recognized by FLS2 (FLAGELLIN SENSING 2), a receptor-like kinase (RLK) in *Arabidopsis.* Interestingly, there is a distinct part of flagellin which is detected in certain solanaceous species including tomato, potato and pepper, called flgII-28 (Clarke et al., 2013). flgII-28 recognition is independent of FLS2, and recently the new PRR FLS3 was revealed to be a flgII-28 receptor from wild tomato relatives (Hind et al., 2016). Elongation factor Tu (EF-Tu) is another well-characterized PAMP. EF-Tu is involved in the bacterial translation of mRNA to produce proteins and is one of the most abundant proteins in bacteria (Jeppesen et al. 2005). Highly conserved peptides of 26 or 18 amino acids in the N-terminus of EF-Tu, elf26 or elf18, respectively, are attributed to elicitor activity (Kunze et al., 2004). EF-Tu is recognized by *Arabidopsis* EF-Tu Receptor (EFR), which also belongs to the RLK family. Interestingly, as in the case of flagellin, the EF-Tu detection system seems to be variable depending on plant species. In rice, elf18 could not elicit PTI responses but the central part of EF-Tu, termed EFa50 ranging from Lys176 to Gly225 could trigger PTI (Furukawa et al., 2014). It is certain that evolutionary pressure also drives the diversity of PAMPs and PRRs. Peptidoglycan (PGN) and lipopolysaccharides (LPS), which are extracellular structural components of prokaryotes, have also been reported to be PAMPs. PGN is a major component of the bacterial cell wall outside of the membrane and present in all bacteria. PGN acts as an elicitor of plant innate immunity in *Arabidopsis*, tomato and tobacco (Newman et al., 2013). In contrast to animal cells where PGN is recognized via minimal PGN motifs, plants recognize the PGN sugar backbone (Gust et al., 2007).

In comparison to bacterial PAMPs, fungal and oomycetal PAMPs are poorly characterized. In fungi, chitin was reported as a PAMP (Kaku et al., 2006). Chitin β-glucan which is cross-linked to chitin in fungi and to cellulose in oomycetes is reported as an elicitor of PTI and is recognized by beta-glucan binding protein (GBP) in soybean (Umemoto et al., 1997).

1.3.1.2 Pattern recognition receptors (PRRs) in plants

Plants have developed a cell surface defence system by using PRRs to detect the PAMPs of invading microbes. To date, several RLKs and receptor-like proteins (RLPs) that recognize bacterial and fungal PAMPs have been identified. The PRR FLS2 was identified by screening *Arabidopsis* mutants for loss of flg22-induced growth restriction (Gomez-Gomez & Boller, 2000). FLS2 carries an extracellular LRR domain, a transmembrane domain and an intracellular serine/threonine kinase domain. The LRR domain is important for high-affinity binding of flg22 (Gomez-Gomez et al. 2001). In *Arabidopsis*, FLS2 physically interacts with flg22 specifically and triggers PTI signalling (Chinchilla et al. 2006). The recognition of flagellin induces varying degrees of immune responses among tested plant species. In tomato, compared to flg22, a shorter peptide sequence flg15 elicits a higher level of defence response, but the level of defence for flg15 is reduced in *Arabidopsis* (Felix et al., 1999). In rice full-length flagellin can elicit stronger defence responses than flg22 (Takai et al. 2008). Recognition variation of flagellin also exists, as it has been shown that there is different recognition of flagellin variants in different strains of *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Sun et al. 2006).

Activation of FLS2 by binding of flagellin induces an association with another RLK, BRI1-associated receptor kinase (BAK1), that was originally known to be required for activation of brassinosteroid-activated signaling (Chinchilla et al., 2006). Subsequently, the cytoplasmic receptor kinase *Botrytis*-induced kinase 1(BIK1) dissociates from FLS2 once the FLS2-BAK1 dimer is formed (Chinchilla et al., 2007). Free BIK1 is thought to phosphorylate downstream components, leading to downstream PTI signaling. Mitogen activated protein kinase (MAPK) signaling is involved in downstream processes (Asai et al., 2002) but a direct link to cell surface-localized PRRs remains unknown.

While FLS2-flagellin recognition has been reported in various families of plants, EF-Tu sensing by EF-Tu Receptor (EFR) was only found in the *Brassicaceae* family (Zipfel et al., 2006). EFR is another example of an RLK, which carries an extracellular LRR domain and an intracellular serine/threonine kinase domain.

While perception of EF-Tu and flagellin is independent from each other, downstream signaling components are well-conserved (Zipfel et al., 2006). Treatment with flg22 or elf18 induces similar expression patterns of defence-related genes in *Arabidopsis* (Zipfel et al., 2006, 2004).

Chitin oligosaccharide elicitor-binding proteins (CEBiPs in rice) and chitin elicitor receptor kinase 1 (CERK1 in *Arabidopsis*) are known to perceive fungal chitin (Kaku et al., 2006). In plants, lysine-motif (LysM) proteins are known to be involved in perception of PGN. In *Arabidopsis*, two kinds of CEBiP-like proteins, LYM1 and LYM3, are also involved in perception of bacterial PGN (Shimizu et al., 2010). LYM1 (LysM-domain protein 1) and LYM3 are plasma membrane associated proteins that bind to PGN. In addition, chitin elicitor receptor kinase 1 (CERK1) that belongs to the class of LysM-receptor kinases is required for the PGN perception and signaling although there is no evidence of direct PGN binding (Willmann et al., 2011). In addition, two lysine-motif containing proteins, LYP4 and LYP6, in rice are reported to bind both fungal chitin and bacterial PGN, indicating that perception of different microbial PAMPs can be shared (B. Liu et al., 2012).

1.3.1.3 Signaling events in PTI

One of the earliest physiological responses of PTI is ion fluxes across the plasma membrane (Boller, 1995). The alkalinization of the growth medium caused by altered ion fluxes is observed in early PTI responses (Felix et al., 1999). A rapid increase in the cytosolic Ca^{2+} level in plant cells is also observed in a very early stage of PTI. In parsley cells, *Phytophthora sojae* elicitor, Pep-13 induces a rapid elevation of cytosolic Ca^{2+} concentration (Blume et al. 2000). Flg22 also induces a rapid Ca^{2+} increase in 2-3 minutes after treatment in *Arabidopsis*. However, PAMP-induced cytosolic Ca^{2+} levels vary depending on the type of PAMP used. For example, increase of cytosolic Ca^{2+} concentration with PGN was less than that for flg22 (Gust et al., 2007). The change of Ca^{2+} concentration can be detected by different kinds of sensors including calmodulin and calcium-dependent protein kinases (CDPKs), resulting in altered levels of defence gene expression. Production of reactive

oxygen species (ROS) such as O_2^- and H_2O_2 is also one of the early PTI responses triggered by various PAMPs (Torres, Jones, & Dangl, 2006). ROS production can act directly as an antimicrobial agent or in an indirect manner, contributing to cell wall crosslinking and serving as a signal for various defence responses (Vatansever et al., 2013).

MAPK signaling after the flg22-triggered dimerization of BAK1 and FLS2 in *Arabidopsis* is another early PTI response. The perception of PAMPs can activate two branches of the MAPK cascade in *Arabidopsis*: MEKK1/MEKKs-MKK4/5-MPK3/6 and MEKK1-MKK1/2-MPK4 (Tena et al. 2011). MAPK cascades have been shown to directly regulate defence gene expression by phosphorylation of transcription factors (Meng & Zhang, 2013).

As constant activation of defence responses costs a lot to plants in terms of allocating energy, negative regulation of PRRs in order to keep PTI silent in the absence of pathogen invasion is critical. To prevent the constitutive activation of FLS2 following flg22 perception, E3 ligases PUB12 and PUB13 that ubiquitinate FLS2 are activated by BAK1. Ubiquitinated FLS2 is subsequently degraded (Lu et al., 2011). Late PTI responses include callose deposition (Boller and Felix, 2009).

1.3.2 Effector triggered immunity (ETI)

Physical barriers and PTI provide plants a general protection to most microbial invasions. However, successful pathogens do overcome this first line of defence and cause disease. Successful pathogens deploy secreted effectors to suppress PTI and facilitate colonization of their host plants, establishing effector-triggered susceptibility (ETS). Individual examples of effector-driven ETS will be described in section 1.4. In order to detect PTI-evading successful pathogens, plants have evolved disease resistance (R) proteins that can recognize specific effectors delivered from these pathogens. Perception of the pathogen effectors triggers strong defence responses and restricts pathogen growth. This layer of the plant defence system is called effector-triggered immunity (ETI). ETI occurs in a more specific manner than PTI because it detects one or a few effectors rather than

widely conserved motifs. Interestingly, effectors have evolved the ability to suppress not just PTI but ETI as well (will be discussed in section 1.4.1.2). ETI may induce genetic variations in a pathogen's effector repertoire through applying selective pressure on losing avirulent effectors or horizontal gene transfer for gaining ETI-suppressing effectors to enable pathogens to evade recognition. In response to this, host plant *R* genes have also diversified to recognize 'evolved effectors'. Maintaining R proteins may need many accessory proteins for their tight regulation as elevated defence responses reduce general fitness of plants. For plants, the appearance of alleles of *R* genes has to be balanced by the cost of maintaining them. This repeated process of detection and evasion between plants and pathogens, respectively, is termed an 'arms race' (Jones & Dangl, 2006).

1.3.2.1. Disease resistance proteins in plants

ETI is the second layer of the plant immune system that detects effectors and activates immunity. While there are various types of plant disease resistance (R) proteins activating ETI, the largest class comprises NLR-type R proteins. NLR proteins carry a central nucleotide-binding domain conserved in Apaf1, R proteins and CED4 (NB-ARC) domain and a C-terminal leucine rich repeat (LRR) domain. NLR proteins belong to the STAND (signal transduction ATPases with numerous domains) superfamily (Takken & Goverse, 2012; Jones et al., 2016).

NLR proteins contain two major groups based on their N-terminal structure. The TIR-NB-ARC-LRR (TNL) class contains a Toll/Interleukin-1 like receptor (TIR) homology region at its N-terminus. The other class, CC-NB-ARC-LRR (CNL), contains an N-terminal coiled–coil (CC) domain (Pan et al., 2000). So far, TNL proteins have been found only in dicot plants while CNL proteins are found in both dicot and monocot plants. Apart from the type of N-terminal domain in NLRs, the major difference between these two classes, CNL and TNL, is further delineated by sequence variation in their NB-ARC domains (Pan et al., 2000).

Interestingly, some NLR proteins carry one or more atypical domains (Meyer et al., 2003). For example, *A. thaliana* NLR protein RRS1 (resistance to

Ralstonia solanacearum 1) contains the C-terminal WRKY DNA-binding domain (Deslandes et al., 2003).

There are some atypical NB-ARC-LRR proteins that are classified as TNLs. For example, some of these does not possess an N-terminal TIR domains but their NB-ARC domain sequences are similar to TNLs. Some of them have TIR domain at their C-terminus instead of at the N-terminus (Meyers et al., 1999; Radwan et al., 2008). Additionally, R proteins lacking LRR domains or carrying more than one LRR domain are present in *A. thaliana, Populus* and *Oryza sativa* (Kohler et al., 2008; Meyers et al., 2003).

There are also small groups of NLR proteins that are not CNLs among non-TNL proteins. BEAF and DREF proteins with a zinc-finger DNA-binding domain, BED-NB-ARC -LRR, BED-NB-ARC, and the proteins lacking an additional N-terminal domain (NBS-(CC/BED)-LRR) are classified based on their NB-ARC domain sequences (Kohler et al., 2008; Meyers et al., 2003; T. Zhou et al., 2004). Furthermore, there is another type of R protein containing an RPW8-like N-terminal domain. This, so-called RNL (RPW8-NBS-LRR) is considered to belong to a subgroup of CNLs but the actual phylogenetic relationship with other members of CNLs remains unclear (Shao et al., 2014). Finally, TIR-CC-NB-LRR proteins that contain both TIR and CC domains have also been reported in *Arabidopsis* (Kohler et al., 2008; Meyers et al., 2003; T. Zhou et al., 2004).

The functions of each domain in NLR proteins have been studied extensively. LRR domains consist of tandem repeats of 20-30 amino acids with a consensus sequence of LxxLxLxxNxL (L-leucine; N-asparagine, threonine, serine or cysteine; and x for any amino acids). Several studies have shown that the LRR domain is important for recognition of effectors, providing a platform for protein-protein interaction (Bella et al., 2008). The NB-ARC domain is divided into several subdomains: NB, ARC1, ARC2 and ARC3. NB and ARC2 are found in the majority of plant NLR proteins (Kobe & Kajava, 2000). The NB domain shows ATPase activity and may be involved in conformational changes of NLR proteins (reviewed in (Głowacki et al., 2011)). In some cases, mutations in the ARC2 domain cause auto-activation of NLR proteins (Takken, Albrecht, & Tameling, 2006; Williams et al.,

2011). On the other hand, the ARC1 subdomain appears to be involved in binding to the LRR domain (Rairdan & Moffett, 2006).

The TIR domain in some NLR proteins seems to be involved in the recognition of avirulent (Avr) proteins. For example, in tobacco, association of TIR domain of a TNL protein N and p50 from tobacco mosaic virus is required for HR (Burch-Smith et al., 2007). The CC domain is also involved in intramolecular interactions associated with defence signaling. The EDIVD consensus motif that is found in many CNLs appears to function in intramolecular interactions between NB-ARC and LRR domains resulting in suppression of the autoimmune response (Rairdan et al., 2008). Furthermore, the CC domain of RPS5 interacts with PBS1, a well-characterized target of *P. syringae* effector AvrPphB (Ade et al. 2007). In addition to TIR/CC, NB and LRR domains, some NLR proteins carry additional domains that are required for their function. *Arabidopsis* RRS1, for example, uses its C-terminal WRKY DNA-binding domain to detect effectors and trigger an immune response (Deslandes et al., 2003). WRKY DNA-binding domains are found in WRKY transcription factors that were identified based on the conserved WRKYGQK motif in the DNA-binding domain. Many WRKY transcription factors seem to be important for regulation of defence gene expression (Eulgem & Somssich, 2007; Ulker & Somssich, 2004). The importance of the WRKY domain of RRS1 in effector recognition will be described in the next chapter.

1.3.2.2. Effector recognition mechanism by R proteins

1.3.2.2.1 Direct recognition

There are limited examples of direct recognition between pathogen effectors and NLR proteins. The rice NLR Pi-ta confers resistance to rice blast fungus, *Magnaporthe grisea,* by directly interacting with the fungal effector AVR-Pita (Jia et al., 2000). The *Arabidopsis* TNL protein RRS1-R interacts with *Ralstonia solanacearum* effector PopP2 and *Pseudomonas syringae* effector AvrRps4 (Deslandes et al., 2003). In flax (*Linum usitatissimum*), the flax rust fungus

Melampsora lini effector protein AvrL567 is directly recognised by flax NLRs L5, L6, and L7 (Ravensdale et al., 2012). In addition, paired rice NLR proteins RGA4 and RGA5 recognize AVR1-CO39 and AVR-Pia from *Magnaporthe oryzae* via direct physical interaction (Cesari et al., 2013). ATR1 alleles from *Hyaloperonospora arabidopsidis (Hpa)* are very diverse between strains and differentially recognized by the RPP1 receptor via direct interaction with its LRR domain (Steinbrenner et al., 2012). The LRR domain of RPP1 is sufficient for association with ATR1 but the ARC2 subdomain is required for the effective receptor activation (Steinbrenner et al., 2015)

Despite these examples showing that physical Avr-NLR interaction is required for activation of defence, the underlying mechanism is largely unknown.

1.3.2.2.2. Indirect recognition via guardee and decoys

Apart from a few examples of Avr-NLR direct interaction, the majority of NLR proteins recognize cognate Avr proteins indirectly. This indirect recognition of Avr proteins by corresponding NLR proteins can be explained by the 'guard' or 'decoy' strategy (Jones & Dangl, 2006; Van der Hoorn and Kamoun, 2008; Jones et al., 2016). Briefly, this theory suggests that NLR proteins are able to monitor the status of certain plant proteins referred to as 'guardees', which are modified by pathogen effectors. When pathogen effectors biochemically modify these guardee proteins, the NLR proteins can detect the 'modified-self' and activate defence signaling. For example, *Arabidopsis* RIN4 (RPM1 INTERACTING PROTEIN 4) is physically associated with at least two NLR proteins, RPS2 and RPM1 (Axtell and Staskawicz, 2003; Mackey et al., 2002). Modification of RIN4 by pathogen effectors can activate RPM1 and RPS2 differently. AvrRpm1 from *P. syringae* pv. *maculicola* and AvrB from *P. syringae* pv. *glycinea* induce RIN4 phosphorylation via the receptor-like cytoplasmic kinase RIPK, activating RPM1-mediated defences (Liu et al., 2011; Mackey et al., 2002). On the other hand, a cysteine protease, AvrRpt2 from *P. syringae* pv. *tomato*, cleaves RIN4, inducing RPS2-mediated defences (Axtell and Staskawicz, 2003; Kim et al., 2005).

While it is hard to draw a clear line between guardee and decoy, the latter specifically refers to an effector target-mimicking molecule without its own function in defence response but rather luring the effectors to modify them and thus trapping them for recognition by the NLR proteins (Van der Hoorn and Kamoun, 2008; Jones et al., 2016). In tomato, *P. syringae* effector AvrPto and AvrPtoB are recognized by a serine/threonine protein kinase Pto and a CNL protein Prf. AvrPto and AvrPtoB bind to the catalytic cleft of Pto, establishing contact with residues in the kinase P+1 loop (Dong et al., 2009; Wu et al., 2004; Xing et al., 2007). Interestingly, the same residues are required for maintaining Prf in an inactive state before effector recognition, indicating that there is a competition for Pto binding sites between Prf and effectors (Mucyn et al., 2009). Binding of AvrPto or AvrPtoB disrupts the P+1 loop of the Pto/Prf complex composed of heterodimerized Pto (a sensor Pto and an activator Pto) with a pair of coordinated Prf proteins, interfering with the negative regulation of the sensor Pto by Prf (Mucyn et al., 2009). This activates the second activator Pto in the Prf complex, leading to full activation of the complex and initiation of ETI (Ntoukakis et al., 2013). The C-terminal region of AvrPtoB possesses an E3 ligase activity that is required for suppressing HR. However, the Pto can bind to two different sites in AvrPtoB unlike Fen which cannot activate Prf-mediated defence activation and only can interact with one site near the AvrPtoB E3 ligase domain. Ability to bind the additional site in AvrPtoB distant from the E3 ligase domain seems to be required for Pto-Prf defence activation (Mathieu et al., 2014). AvrPphB from *P. syringae* pv. *phaseolicola* is recognized by a CNL protein, RPS5, via *Arabidopsis* serine-threonine kinase PBS1 (*AVRPPHB* SUSCEPTIBLE 1). AvrPphB has cysteine protease activity and is able to cleave itself, with this activity being required for the recognition by RPS5. PBS1 is directly cleaved by AvrPphB, thereby activating its guard, RPS5 (Qi et al., 2014). *P. syringae* effector HopZ1a is recognized by a CNL protein ZAR1 (HOPZ ACTIVATED RESISTANCE 1). For this ZAR1-mediated recognition, interaction of a pseudokinase ZED1 (HopZ-ETI deficient1), a putative decoy mimicking receptor-like protein kinases, with HopZ1a is required (Lewis et al., 2013).

Recently, the concept of an ‘integrated decoy’, a physically linked decoy within an NLR protein, has been introduced. The paired NLR proteins, RPS4 and

RRS1, are physically associated proteins recognising *Pseudomonas syringae* effector AvrRps4, *Ralstonia* effector PopP2 and an unknown effector of *Colletotrichum higginsianum* (Narusaka et al., 2009; Birker et al., 2009). As described earlier, RRS1 has an additional WRKY domain at the C-terminus after its canonical LRR domain. WRKY transcription factors are well known to be involved in plant immunity, and some pathogen effectors may target them to achieve virulence. Indeed, Sarris and colleagues showed that AvrRps4 and PopP2 can interact with other WRKY transcription factors, which strongly supports the theory that the WRKY domain in RRS1 is an integrated decoy (Sarris et al., 2015).

1.3.2.3. Genetic components required for effector-triggered immunity

The downstream components of ETI responses following Avr-NLR recognition are at least partially shared with PTI including MAP kinase cascades and defence-related transcription factors such as WRKYs. This induces the biosynthesis of phytohormones related to the defence response including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), cell wall thickening, lignification, transcriptional activation of pathogenesis-related (PR) genes and production of antimicrobial components in the endoplasmic reticulum (ER) that are secreted by vacuoles (reviewed in (Muthamilarasan & Prasad, 2013)). Accumulated SA in the infected area binds to NPR3 (NON-EXPRESSOR OF PR GENES 3) and mediates the degradation of NPR1 which is a cell death suppressor, eventually leading to an HR (Fu et al., 2012). The HR, a special form of programmed cell death (PCD), involves cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling, vacuolization and chloroplast disruption (Coll et al., 2011).

There are two partially independent signaling pathways that are activated by NLR proteins. One is the NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE1)-dependent and the other is the EDS1 (ENHANCED DISEASE SUSCEPTIBILITY1)-dependent pathway (Aarts et al., 1998). NDR1 is an integrin-like protein and plays a role in fluid loss and plasma membrane-cell wall adhesion (Knepper et al., 2011). Importantly, NDR1 is required for ETI mediated by many CNLs, such as RPM1 and

RPS2, both of which interact with RIN4 (Day et al., 2006). EDS1 is a homolog of eukaryotic lipases, which is required for ETI mediated by most TNLs (Aarts et al., 1998). However, a few NLR proteins including RPP7, RPP8 and RPP13 require both EDS1 and NDR1 for activation of defence (McDowell et al., 2000). . To date, the details of the NDR1-dependent defence activation are largely unknown.

EDS1 plays an important role in the plant response to both biotic and abiotic stress (Wiermer, Feys, & Parker, 2005). PAD4 (PHYTOALEXIN DEFICIENT 4) interacts strongly with EDS1 and both are required to activate immune responses (Feys, 2001). EDS1 and PAD4 function in transducing redox signals in response to certain biotic and abiotic stresses and are activators of SA accumulation. They were also shown to be regulators of JA and ET defence response pathways (Brodersen et al., 2006). In addition, SAG101 (SENESCENCE ASSOCIATED GENE 101), another homolog of EDS1, appears to form a complex with EDS1 and PAD4 in defence signaling (Zhu et al., 2011).

In *Arabidopsis,* SGT1 (SUPPRESSOR OF THE G2 ALLELE OF *SKP1*) acts as a co-chaperone of HSP90 (HEAT SHOCK PROTEIN 90) and, along with RAR1 (REQUIRED FOR MLA12 RESISTANCE 1), is required for ETI (Shen et al., 2003; Tor et al., 2002). In a recent model of the chaperone complex, RAR1 interacts via its CHORD domain (cysteine- and histidine-rich domain) with the CS (CHORD-containing protein and SGT1) of SGT1 and the ND domain (N-terminal ATPase domain). In addition, SGT1 interacts with the LRR domain of NLR proteins and possibly stabilize them (Yasuhiro et al., 2008; Yasuhiro et al., 2010).

Whereas most NLR proteins are localized in the cell cytosol and plasma membrane, the recent discovery of functional NLS (nuclear localization signal) motifs in several NLR proteins suggested the possible role of NLR proteins in the cell nucleus (Burch-Smith et al., 2007). It was reported that NLR proteins interact with WRKY transcription factors such as CaWRKY1 in *Capsicum* and WRKY11 and WRKY17 in *Arabidopsis,* which act as negative regulators of defence-related gene expression, implying the role of WRKY transcription factors in NLR-mediated defence gene expression (Journot-Cataliono et al., 2006; Oh et al., 2008). Recently, it was shown that NLR proteins can associate with ARGONAUTE4-like proteins

involved in small RNA-mediated transcriptional regulation in response to viral pathogens (Bhattacharjee et al., 2009).

1.3.2.4. ETI uncoupled from the hypersensitive response

The hypersensitive response (HR) is often accompanied by ETI and, therefore, has often been implicated as an indicator of ETI in many examples of known plant immune responses to biotrophic pathogens. Thus ETI-associated HR was explained as a physical barrier to restrict the growth of the pathogen. However, several lines of evidence suggest that the plant defence response and HR can be uncoupled and that HR may not be strictly required for defence activation (Coll et al., 2011). For example, there are several NLR proteins that trigger defence responses without cell death. The potato Rx protein recognizes potato virus X and triggers strong defence responses, and the barley *Rdg2a* gene can confer resistance to *Pyrenophora graminea*, both without macroscopic cell death (Bendahmane et al., 1999; Bulgarelli et al., 2010). Also, defence-related genes were upregulated by several pathogens in resistant bean plants without onset of cell death response (Jakobek and Lindgren, 1993).

Studies on several *Arabidopsis* mutants further support the hypothesis that HR is not always necessary for plant immunity. *defence no death (dnd)* mutants showed elevated enhanced SA accumulation and disease resistance to biotrophic pathogens but significantly reduced HR when compared to wild-type plants (Genger et al., 2008). Elevated resistance in *dnd* mutants was dependent on SA accumulation, indicating that SA is required for resistance but may not be directly associated with HR. In addition, the *Arabidopsis ndr1* mutant showed susceptibility to *Pto* DC3000 carrying AvrRpm1 and AvrB but developed an HR. This implies that *NDR1* is specifically required for AvrRpm1 and AvrB-triggered immunity but not cell death, again supporting the possibility of distinct pathways between defence responses and HR (Century et al., 1995).

1.4. The roles of effectors in pathogenicity

1.4.1 Effector delivery systems

Microbes have evolved several strategies to deliver their effectors into host cells. The type III secretion system (T3SS) is a direct effector delivery mechanism in many bacterial pathogens of plants and animals. The T3SS is important for the virulence of the pathogen since pathogens carrying an improperly assembled T3SS show significantly reduced virulence (He et al., 2004). The T3SS is encoded by *hrp (hypersensitive response and pathogenicity*) genes that are clustered in the bacterial chromosome. For consistency of the nomenclature, *hrp* genes that share high sequence similarities with *Yersinia* Ysc homologs were renamed as *hrc (hypersensitive response and pathogenicity conserved)* genes (Bogdanove et al., 1996). The *hrc/hrp* genes are relatively well conserved in several pathogenic bacteria including *Pseudomonas, Erwinia, Ralstonia and Xanthomonas*. *hrp/hrc* genes are clustered in so-called 'pathogenicity islands' that are distinguishable due to a specific G+C content ratio in their nucleotide sequences (Hacker et al., 1997). In the *hrp/ hrc* locus, there are three groups of genes: genes for transcriptional regulation of the T3SS, structural units of the T3SS and proteins that are secreted by the T3SS (Thomas et al., 2009). The mechanism by which the T3SS penetrates the plant cell is still unknown. However, evidence exists for at least some T3SS genes to have evolved from flagellum encoding genes with some T3SS components sharing high sequence similarity with flagellar assembly components (Van Gijsegem et al., 1995). In fact, the physical structure of the *Salmonella enterica* T3SS has been studied and its needle complex was found to closely resemble the flagellar assembly structure through visualization using electron microscopy (Diepold & Wagner, 2014).

The T3SS has been used for different purposes by bacteria. Intracellular pathogens like *Salmonella* use it for hijacking intracellular organelles during successful infections, while *Yersinia* uses it to resist phagocytosis. Most extracellular gram-negative plant pathogens make use of the T3SS to deliver virulence proteins to interfere with plant innate immunity. In many cases,

disruption of the T3SS by knocking out genes encoding basal structural components of the T3SS such as *hrcC* resulted in attenuated virulence (Wei et al., 2000). *hrp*/*hrc* genes can regulate T3SS genes. For example, alternative sigma factor HrpL is known to bind to a conserved *cis*-element *hrp* box in the promoters of T3SS-related genes to regulate their expression (Zwiesler-Vollick et al., 2002; Nissan et al., 2005).

Many plant pathogens utilize their T3SS to secrete proteins through the needle structure, penetrating the host cell membrane. There are two main classes of secreted proteins. The first class constitute so-called 'harpins' which are secreted into the extracellular space of the host and are possibly involved in the penetration of host cells (He, 1998). The second class are termed 'effectors' and secreted directly into the host. Translocation and folding of effectors are sometimes assisted by specific chaperones (He, 1998). Effectors can play major roles in the virulence of the pathogen by interacting directly with plant immune system. The diverse function of effectors will be discussed in the next section.

In eukaryotic parasites such as oomycete and fungal pathogens, a structure called a haustorium or invasive hypha is used to deliver effectors, and details of the mechanism are still under investigation (Petre and Kamoun, 2014). Eukaryotic effector translocations are known to be directed by signal peptides at the N-terminus (Petre and Kamoun, 2014). In some oomycete effectors such as ATR1 and ATR13 from *Hyaloperonospora arabidopsidis*, the RxLR motif is required for translocation of the effectors into host cells (Haldar et al., 2006). The RxLR motif is often found in many oomycete effectors and serves as an indicator of effectors with an orthologous RxLR-like motif represented in fungal effectors.

1.4.2 Roles of effectors in pathogenicity

Regardless of delivery systems or structures, the main role of proteins secreted into the host cells is to dampen the host immune system, facilitating successful infections. However, contributions of effectors in successful host colonization not directly related to the immune system, such as nutrient uptake

from the host cell, have also been reported (Macho, 2016). In this section, mechanistic details of bacterial effector functions will be introduced.

1.4.2.1 Suppression of PTI

PTI is the first layer of plant immunity that pathogens encounter and therefore PTI components are one of the early targets of pathogen effectors for suppression. PTI is initiated by PAMP recognition via PRRs and followed by downstream activation of RLCKs and MAPK cascades. Many effectors are reported to interfere with the recognition of PAMPs by PRR by directly interacting with PRRs or their associated proteins. *P. syringae* effector AvrPto can interact directly with RLKs including FLS2 and EFR in *Arabidopsis* to diminish PTI responses (J.-M. Zhou & Chai, 2008). AvrPtoB degrades FLS2 and CERK1 by its E3 ligase activity at the C-terminus and inhibits BAK1 kinase activity (Göhre et al., 2008; Shan et al., 2008; Gimenez-Ibanez et al., 2009). HopF2, also from *P. syringae,* has been reported to interact with BAK1 and interfere with BIK1 phosphorylation induced by flg22 (Zhou et al., 2014). Another *P. syringae* effector*,* HopAO1, has tyrosine phosphatase activity and interferes with tyrosine phosphorylation of EFR at an undefined residue(s) (Macho et al., 2014). *P. syringae* pv. *phaseolicola* effectors AvrPphB and AvrAC, a uridylyltransferase from *X. campestris* pv. *campestris*, both target BIK1. AvrPphB is a cysteine protease localized to the host cell plasma membrane and cleaves PBS1-like (PBL) kinases including BIK1 and PBL2 to interfere with PTI (Zhang et al., 2010). AvrAC adds uridine 5'-monophosphate to the activation loop of BIL1 and RIPK to inhibit phosphorylation of the activation loop and further block the downstream signaling (Feng et al., 2012).

PAMP recognition by PRRs often involves activation of MAPK cascades. Therefore, modification of the MAPK cascade is one of the strategies effectors employ to inhibit PTI. HopAI1 from *P. syringae* functions as a phosphothreonine lyase and directly inactivates MAP kinases MPK3 and MPK6 by removing the phosphate group from phosphothreonine in *Arabidopsis* (Zhang et al., 2007). In

addition, HopF2 interacts with and ADP-ribosylates MKK5 to suppress its phosphorylation activity on MPK6 and MPK4 (Wang et al., 2010).

Effectors also suppress PTI indirectly by interacting with proteins that are required for PTI. AvrB suppresses callose deposition after flg22 treatment by interaction with RAR1 which is a negative regulator of PTI (Shang et al., 2006). Some effectors can target *de novo* PRR biogenesis. *Pto* DC3000 effector HopU1 modifies RNA binding proteins such as GRP7 via its ADP-ribosyltransferase activity to delay RNA binding and impede PTI (Nicaise et al., 2013).

1.4.2.2. Suppression of ETI

The evolutionary arms race between pathogens and plants resulted in various strategies for pathogens to suppress not only PTI but ETI as well. Guo et al showed that the majority of the *Pto* DC3000 type III effectors can suppress either PTI, ETI or both (Guo et al., 2009). In their study, the majority of *Pto* DC3000 effectors were able to suppress HopA1-induced HR in *Arabidopsis*. Several examples of ETI suppression mechanisms by effectors have been reported. For example, *P. syringae* effector HopZ3 is an acetyltransferase and acetylates multiple members of the RPM1-mediated defence complex, therefore possibly inactivating it (Lee et al., 2015). Another *P. syringae* effector HopD1 specifically contributes to suppression of ETI but not PTI (Block et al., 2014). HopD1 interacts with *Arabidopsis* transcription factor NTL9, a positive regulator of ETI at the cell endoplasmic reticulum and interferes with the upregulation of NTL9-regulated ETI genes (Block et al., 2014).

1.4.2.3. Other roles of effectors in pathogenicity

Besides directly interacting with plant immune components, by modulating other aspects of host plant physiology, effectors can promote pathogenesis. Effector functions beyond immune suppression were originally highlighted in studies of some transcription activator-like effectors (TALEs) from different

Xanthomonas species. *Xanthomonas oryzae* TALEs PthXo1 and AvrXa7 induce expression of members of the SWEET sugar transporter family and promote sugar efflux from host cells (Chen et al., 2010). Changing sugar efflux affects osmotic pressure within the apoplastic spaces where these pathogens reside (Chen et al., 2010). Recently, the direct implement of a sugar transporter by a pathogen to pump sugar into the apoplast to be used by the pathogen has been reported (Yamada et al., 2016). In this study, sugar transporter 13 (STP13) was shown to associated with FLS2 in *Arabidopsis* and become phosphorylated by BAK1 to enhance the monosaccharide uptake activity upon flg22 treatment. Maintaining high humidity conditions at infection sites is critical for successful pathogenesis. Recently, *Pto* DC3000 effectors HopM1 and AvrE1 were reported to be required for establishment of an aqueous apoplast at the infection site, and artificial high-humidity conditions were sufficient to transform non-pathogenic *P. syringae* into virulent pathogens (Xin et al., 2016).

Bacterial effectors have also evolved to manipulate plant hormones as a virulence strategy. SA is an essential hormone for plant immunity against biotrophic pathogens. Some effectors can activate JA signaling to antagonize SA-dependent defence signaling (Kazan and Lyons, 2014). Other hormones that are known to be primarily involved in abiotic stress are also regulated by some effectors. Effectors from *P. syringae* that affect plant hormone levels include AvrPtoB, which modulates the abscisic acid (ABA) pathway (Truman, de Zabala, & Grant, 2006), and AvrRpt2, which promotes auxin accumulation in *Arabidopsis* (Chen et al., 2007). It has been hypothesized that abiotic stress-related hormone regulation by effectors would achieve beneficial conditions for the pathogen through negative crosstalk with pathways leading to SA biogenesis or by altering the physiological status of the host thereby indirectly affecting plant immune responses (Kazan and Lyons, 2014). However, emerging evidence also supports an independent role of auxin in plant immunity (Chen et al., 2007).

Plant secretion systems are also targeted by effectors. Cell cytoskeletal structures and vesicle trafficking systems are involved in secretion of defence compounds. HopZ1a interacts with tubulin and microtubules to interrupt the plant secretion system and cell wall-tethered defences (Lee et al., 2012). Futhermore,

HopW1 from *P. syringae* forms a complex with actin, disrupts the actin cytoskeleton and affects protein trafficking by vacuoles (Kang et al., 2014) .

Chloroplast and mitochondria are also targeted by effectors. *Pto* effector HopN1 localizes in chloroplasts and degrades tomato PsbQ, which is a member of the oxygen evolving complex of photosystem II (PSII), thus compromising ROS production and programmed cell death (Rodríguez-Herva et al., 2012). *P. syringae* effectors AvrRps4 (pv. *pisi)* and HopK1 (pv. *tomato)* are processed into a smaller form *in planta.* The N-terminus fragments share high amino acid sequence similarity and are required for respective virulence fuctions (Li et al., 2014). Both N-termini of AvrRps4 and HopK1 localize in the chloroplasts suggesting that the conserved N-terminal domain may carry a chloroplast-targeting peptide. *Pto* effector HopG1 targets mitochondria by its N-terminal sequences (Block et al., 2010). Indeed, HopG1 seems to target the mitochondria-localized kinesin motor protein, resulting in the disruption of actin cytoskeleton organization and promote the virulence (Shimono et al., 2016).

1.5. YopJ family effectors in plant

YopJ (*Yersinia* outer protein J) is a *Yersinia pestis* and *Yersinia pseudotuberculosis* effector that blocks the mammalian innate immune response by inhibiting the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NFκB) signaling pathways (Lewis et al., 2011). The YopJ superfamily of bacterial effectors is present in highly diverse groups of plant and mammalian pathogens. YopJ family effectors are found in various plant pathogens such as *Pseudomonas* (the HopZ family), *Xanthomonas* (AvrXv4, AvrRxv, AvrBsT and XopJ), *Erwinia* (ORFB), *Ralstonia* (PopP1 and PopP2) and a plant symbiont *Rhizobium* (Y4LO) (Ma et al., 2006).

Despite the great diversity in their sequence and host targets, YopJ family effectors have similar molecular functions, mainly as acetyltransferases with conserved functional residues. The catalytic triad in most of the family members consists of His/Glu/Cys and is identical to that of the C55 family of cysteine

proteases (Orth, 2000). A distinct feature of YopJ acetyltransferases from other acetyltransferases such as HATs (Histone acetyltransferases) and NATs (N-terminal acetyltransferases) is that they can acetylate multiple Ser/Thr/Lys residues in different positions within their target proteins (Ma and Ma, 2016). Several studies have proven that the conserved cysteine residue from the catalytic triad is required for enzymatic function among HopZ family effectors including HopZ1a, HopZ2 and HopZ4 (Lewis et al., 2012; Ma et al., 2006; Macho et al., 2010; Uestuen et al., 2014) as well as other YopJ family members. XopJ from *Xanthomonas* and PopP2 from *Ralstonia* also require this conserved cysteine residue for their enzymatic function, indicating the conserved biochemical function within the YopJ family (Bartetzko et al., 2009; Tasset et al., 2010). In particular, mutation of the conserved cysteine residue in PopP2 showed that it is required for acetyltransferase function as well as both trans- and auto-acetylation ability, both of which are required for avirulence (Tasset et al., 2010).

Additionally, several YopJ family effectors require the eukaryotic cofactor IP_6 (inositol hexakisphosphate) for activation of their enzymatic functions (Mittal et al., 2010). Recently, a study of the crystal structure of HopZ1a showed that the catalytic domain alone is insufficient for HopZ1a function. Furthermore, it was shown that a regulatory domain where IP_6 binds is required for HopZ1a avirulence activity (Zhang et al., 2016). In addition, some YopJ family effectors share the conserved myristoylation residue for plasma membrane localization. XopJ, HopZ2 and HopZ4 have a N-terminal conserved glycine residue for membrane localization, indicating that they may target membrane associated components (Lewis et al., 2008).

The HopZ family from *P. syringae* comprises 5 effectors so far: HopZ1, HopZ2, HopZ3, HopZ4 and the most recently identified HopZ5. HopZ1a triggers defence responses in many plant species including *A. thaliana*, soybean, sesame, rice and *N. benthamiana* (Lewis et al., 2008; Ma et al., 2006; Zhou et al., 2009). All HopZ family effectors are predicted to act as cofactor-activated acetyltransferases (Lewis et al., 2011).

1.5.1. Targets of YopJ family effectors in plants

Several examples of plant virulence targets of YopJ family effectors from plant pathogens have been identified. HopZ1a is an allele of HopZ1 from *P. syringae* pv. *syringae* and recognized in *Arabidopsis* accession Col-0 by a CNL protein ZAR1 (Lewis et al., 2010). There are allelic differences in HopZ1 among different *P. syringae* pathovars, HopZ1a, HopZ1b and HopZ1c but among them, only HopZ1a triggers ETI in *Arabidopsis* Col-0 (Lewis et al., 2008), which suggests that ZAR1 recognition of HopZ1 is highly specific. In *zar1* knock-out *Arabidopsis* plants, HopZ1a promotes growth of *Pto* DC3000. Using a yeast two-hybrid assay to identify the virulence targets of HopZ1a, JAZ (JASMONATE ZIM DOMAIN) proteins were found to associate with HopZ1a (Jiang et al., 2013). JAZ proteins are the transcriptional repressors of the JA signaling pathway, indicating a strategy that HopZ1a employs to promote virulence. JA, together with another phytohormone ethylene (ET), plays important roles in mediating defence responses against necrotrophic pathogens. JA/ET-mediated signaling acts antagonistically to SA signaling, which is required for defence against biotrophic pathogens (Bari and Jones, 2009). Therefore, promotion of JA/ET signaling by HopZ1a can help dampen plant innate immunity specifically against biotrophic pathogens like *P. syringae*. In addition, JA signaling is involved in stomatal closure thus having an effect on the access of pathogens to the plant apoplast (Bari and Jones, 2009). Indeed, HopZ1a suppresses stomata closure in *Arabidopsis* (Ma et al., 2015). Although the JAZ residues acetylated (if any) by HopZ1a are not yet defined, the HopZ1a-interacting domain of JAZ proteins was identified as its Jas domain (Jiang et al., 2013). The Jas domain of JAZ proteins is known to be required for interaction with COI1, an E3 ligase for proteasome-dependent degradation, as well as JAZ-mediated gene repression of JA-responsive transcription factors (Wager and Browse, 2012).

HopZ1a also targets tubulin to modify the microtubule network in host plants (Lee et al., 2012). Acetylation of tubulin by HopZ1a occurs on a lysine residue required for tubulin polymerization in animals, which may interfere with tubulin polymerization and the plant secretion system (Lee et al., 2012). This was

supported by the fact that expression of HopZ1a in both plant and animal cells disrupted the cellular microtubule network and provoked morphological changes, respectively (Lee et al., 2012). Also, in soybean, HopZ1a is known to target and degrade hydroxyisoflavanone dehydratase1 (GmHID1) which is involved in the production of antibacterial phytochemical precursor daidzein (Zhou et al., 2011).

The *Xanthomonas* effector AvrBsT also targets the microtubule network. AvrBsT acetylates tubulin-associated protein ACIP1 (acetylated interacting protein1) *in vitro* (Cheong et al., 2014). However, knocking out of ACIP1 did not attenuate the avirulence of AvrBsT in *Arabidopsis* but suppressing 7 ACIP1 family members in *Arabidopsis* by RNAi did suggesting that the ACIP1 family proteins function redundantly (Cheong et al., 2014). However, how acetylation of ACIP1 proteins by AvrBsT activates defence responses is largely unknown. This example is contrary to the observations for HopZ1a in which microtubule disruption promoted virulence. Targets of AvrBsT have also been studied in the natural host of *Xanthomonas*, pepper. SNF1-RELATED KINASE1 (snRK1) was identified as an interactor of AvrBsT using a yeast two-hybrid assay. Despite there being no direct evidence of acetylation, knocking down snRK1 transcript levels attenuated AvrBs1-triggered HR-suppression by AvrBsT, indicating that snRK1 is required for AvrBsT-mediated HR-suppression (Szczesny et al., 2010).

The *Pseudomonas* effector HopZ3 is known to acetylate multiple unrelated targets including RPM1-mediated defence components (Lee et al., 2015). HopZ3 promotes *P. syringae* growth in *Arabidopsis*, at least partly through acetylation of RIN4 and, interestingly, another effector AvrB3 that activates RPM1-mediated defence signalling (Lee et al., 2015). In addition, HopZ3 also targets PTI components such as PBL1 and BIK1 but it is unknown if they are regulated differentially following acetylation by HopZ3 (Lee et al., 2015).

The *Pseudomonas* effector HopZ2 is known to target MLO2, a susceptibility factor in barley (Lewis et al., 2012). Moreover, another *Pseudomonas* effector HopZ4 interacts with proteosomal subunit RPT6 in yeast and *in planta*, thus disrupting the proteasomal activity of plants to promote susceptibility (Üstün et al., 2014).

1.5.2. Examples of recognition of YopJ family effectors in plants

YopJ family effectors of plant pathogenic bacteria target multiple host components and the mechanisms by which NLRs recognize them are variable. For example, HopZ1a is recognized by the CNL protein ZAR1 and this recognition requires the catalytic cysteine and myristoylation of glycine residues of HopZ1a (Lewis et al., 2008). The HopZ1a recognition event involves a decoy protein. A pseudokinase ZED1 (HOPZ-ETI-DEFICIENT1) is required for HopZ1a recognition by ZAR1. ZED1 shares high sequence similarity with a class of receptor-like kinases but it has a mutation in the enzymatic domain (Lewis et al., 2013).

In contrast, *Ralstonia* effector PopP2 is recognized by a pair of *Arabidopsis* NLR proteins, RPS4 and RRS1, that also recognize another sequence-unrelated *Pseudomonas* effector AvrRps4 (Birker et al., 2009; Narusaka et al., 2009). As mentioned earlier, RRS1 is an atypical TNL protein with an additional WRKY domain at the C-terminus (Deslandes et al., 1998). PopP2 is believed to target WRKY transcription factors to promote pathogen virulence, a function of PopP2 utilized by RRS1 to trap PopP2. Consistently, PopP2 and RRS1 physically interact and PopP2 acetylates the RRS1 WRKY domain (Sarris et al., 2015). Acetylation of the RRS1 WRKY domain by PopP2 occurs on lysine residues in the WRKY heptad sequence and an acetylation mimic mutant of RRS1 (K1221Q) shows autoimmunity, supporting the role of acetylation by PopP2 in RRS1-dependent activation of immunity (Le Roux et al., 2015).

1.6. Suppressors of effector-triggered immunity in plants

Several suppressors of ETI have been identified through mutations in genes that showed enhanced resistance to pathogens. The first example is SRFR (suppressor of *rps4-RLD)* in *Arabidopsis*. Non-allelic *srfr* mutants *srfr1* and *srfr3* in the *Arabidopsis* RLD accession that is naturally susceptible to *Pto* DC3000 expressing AvrRps4 became resistant to *Pto* DC3000 expressing AvrRps4, while

their response to flagellin was unaltered (Kwon et al., 2004). SRFR1 was further characterized as a nucleus-localised protein with the tetratricopeptide repeats showing sequence similarity to transcriptional repressors in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Kwon et al., 2009). Following studies showed that SRFR1 acts as a general negative regulator of the defence responses activated by SNC1, RPS6 and RPS2 (Kim et al., 2009b; Kim et al., 2010; Li et al., 2010). In addition, SRFR1 interacts with TCP transcription factors and upregulates SA and JA/ET defence pathway genes (Kim et al., 2009a). SRFR1 is also involved in plant resistance responses against nematodes and chewing insects, suggesting a role of SRFR1 as a hub regulator of plant immunity (Nguyen et al., 2016).

A second example is SOBER1 (Suppressor of AvrBsT-elicited Resistance1) which suppresses AvrBsT-triggered immunity in *Arabidopsis* (Cunnac et al., 2007). SOBER1 specifically suppresses immunity triggered by the *Xanthomonas* YopJ acetyltransferase family effector, AvrBsT, in *Arabidopsis*. Transient co-expression of SOBER1 and AvrBsT suppressed AvrBsT-triggered cell death in *N. benthamiana* suggesting a conserved SOBER1-mediated suppression mechanism in different species. The carboxyl hydrolase family consists of a large number of proteins. According to the structural similarity, SOBER1 belongs to the group of phospholipases (Kirik and Mudgett, 2009). In *Arabidopsis*, phosphatidic acid (PA) accumulation seems to be important for AvrBsT-triggered immunity and SOBER1 is implied to negatively regulate AvrBsT-triggered immunity by inhibiting PA accumulation through its phospholipase A_2 (PLA_2) activity which downregulates the precursor of PA (Kirik and Mudgett, 2009).

1.7. Aims of the study

Plant pathogens are one of the main threats to agriculture, causing significant yield losses of economically important crops. *Psa,* the causal agent of bacterial canker disease in kiwifruits, has rapidly spread around the world, including New Zealand in 2010. Since the *Psa*-V outbreak in New Zealand in 2010,

over 90% of kiwifruit orchards in New Zealand have been contaminated with *Psa*-V. The gold variety Hort 16A is particularly susceptible to *Psa*-V when compared to the green variety Hayward. A new gold variety Sungold is known to be tolerant to *Psa*-V; however, the underlying mechanism is unclear. As there are different *Pseudomonas* strains in *Psa*-V-contaminated orchards, there is the possibility of genetic exchange between *Pseudomonas* strains to overcome the tolerance. A recent study shows that *Psa* exchanges copper resistance genes resulting in more copper-resistant *Psa.* Therefore, establishment of multiple layers of resistance is required.

Understanding the virulence strategy of this pathogen and the mechanism by which *Psa* effectors are recognized in non-host plants will be helpful to develop durable disease resistance in kiwifruit. Similar to other gram-negative plant pathogens, *Psa* utilizes a T3SS to deliver effectors in order to suppress plant innate immunity and successfully colonize host plants.

The first aim of this thesis was to profile the T3S effectors of *Psa* and investigate their functions, especially in activating plant immunity (Chapter 3). *Psa* triggers non-host resistance in *Nicotiana* spp.; therefore, basic *in planta* characteristics of *Psa* effectors, such as subcellular localization and HCD elicitation in *Nicotiana* spp., were examined. Each HCD triggering effector in *Nicotiana benthamiana* was further studied to identify the requirement of defence regulators in *Psa* effector-triggered immunity. Previously, *Psa* effectors were also screened in several *Arabidopsis* accessions to identify the avirulent effector(s) and understand the *Psa* effector-recognition mechanism (Jayaraman et al. 2017). In this screening, HopZ5, an acetyltransferase effector, was identified as an avirulence effector (Jayaraman et al. 2017). In chapters 4 and 5, this thesis focused on mapping an *Arabidopsis R* gene that confers recognition of HopZ5 in order to better understand the mechanism of the HopZ5 recognition system. Dissecting the recognition mechanism of HopZ5 would broaden our knowledge of how the plant immune system defends against bacterial pathogens and, ultimately, it would aid in the development of *Psa*-resistant kiwifruit varieties.

Chapter 2. Materials and Methods

2.1 Bacterial materials

2.1.1 List of bacteria strains

Pseudomonas syringae pv. *actinidiae* NZ V-13 and NZ LV-5 (McCann et al., 2013)

ICMP 18884 (V-13), ICMP 18803 (LV-5)

Pseudomonas syringae pv*. actinidiae* NZ V13 was used as a research object pathogen.

Pseudomonas syringae pv*. tomato* DC3000

NCPPB 4369

Pseudomonas syringae pv*. tomato* DC3000 was used for growth restriction assay and hypersensitive response assay

Pseudomonas fluorescens Pf0-1 (T3SS) (Thomas et al., 2009)

Pseudomonas fluorescens Pf0-1(T3SS) is *Pseudomonas fluorescens* Pf0-1 with introduced type III secretion system by Thomas et al. This was used for type III effectors expression.

Agrobacterium tumefaciens AGL1(Lazo de la Vega, 1991)

Agrobacterium tumefaciens AGL1 was used for *Agrobacterium*-mediated transient gene expression.

Escherichia coli DH5α

Escherichia coli DH5α was used as primary transformant for a new plasmid construct.

Escherichia coli HB101(pRK2013) (Figurski & Helinski, 1979)

Escherichia coli HB101 was used as a helper strain for triparental mating of *Pseudomonas* spp*.*

2.1.2 Media

For 1L content

L medium: 10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose (10g agar for L agar plate)

King's B medium: 20g bacteria peptone, 1.5g potassium hydrogen phosphate, 10ml glycerol (15g agar for king's B agar plate)

Murashige and Skoog medium plate: Murashige and Skoog medium (Sigma, Duchefa) 4.4g, sucrose (Sigma) 20g, pH5.8 adjusted with KOH, 8g Agar

2.1.3 Antibiotics

Antibiotics for bacteria were used in constant concentration throughout this study. 50μg/ml of kanamycin, 100μg /ml of ampicillin,100μg /ml of spectinomycin, 20μg/ml of gentamycin, 5μg /ml of tetracycline, 30μg/ml of chloramphenicol and 50μg/ml of rifampicin were used in bacterial media. Kanamycin, ampicillin and spectinomycin were dissolved in H_2O, rifampicin was in methanol, and tetracycline and chloramphenicol were in ethanol.

2.1.4 Triparental conjugation

Overnight grown liquid cultures of donor (*E. coli* DH5α), recipient (*Pseudomonas* spp.) and helper (*E. coli* HB101) at 37°C for *E. coli* and 28°C for *Pseudomonas* were mixed in a 1.5ml eppendorf tube in the following ratio: 0.6ml of recipient + 0.2ml of donor + 0.2ml at OD_{600}=1 of helper. The mixture was spun down for 3 minutes at 4000g, washed in 1ml of L broth and resuspended in 200μl

of L broth. 10 spots of 10μl of resuspended cells were spotted on an L agar plate for conjugation for 6-8 hours at 28°C and cells were restreaked on an L agar plate containing appropriate antibiotics for transconjugant selection.

2.1.5 Electroporation

E. coli DH5α, *A. tumefaciens* AGL1, *P. syringae* pv. *tomato* DC3000 and *P. fluorescens* Pf0-1 were transformed by electroporation in an ice-cold 0.1cm electroporation cuvette by MicroPulser ^{TM} electroporator (Bio-rad).

For *P. syringae* pv. *tomato* DC3000 and *P. fluorescens* Pf0-1 transformation, electrocompetent cells were prepared freshly every time. Overnight grown cultures with appropriate antibiotics (rifampicin for *P. syringae* pv. *tomato* DC3000 and chloramphenicol and tetracyclin for *P. fluorescens* Pf0-1) were spun down at 4000rpm for 3 minutes and washed with autoclaved 300mM sucrose 2 times. Cells were re-suspended in 80μl of 300mM sucrose for electroporation.

50-300ng of purified plasmid was transformed into 80μl of freshly made *Pseudomonas* electrocompetent cells, or 50μl of pre-frozen electrocompetent cells (Preparation of electrocompetent cells of *E. coli* and *A. tumefaciens* are in section 2.5.1.7.1), incubated for 1 and half hours for *Pseudomonas* strains, 1 hour at 37°C for *E. coli* and 2 hours at 28°C for *A. tumefaciens,* and plated on antibiotic selective plates.

2.2 Plant materials

2.2.1 List of plant materials

2.2.1.1 List of *Arabidopsis thaliana* materials

Table 2.2.1.1. List of *Arabidopsis thaliana* wild type accessions

Accession	Stock ID	Accession	Stock ID	Accession	Stock ID	Accession	Stock ID	Accession	Stock ID	Accession	Stock ID
Ag-0	KSPG 1	CS22491	KSPG 13	Hr-5	KSPG 28	Lp2-4	KSPG 40	Oy-0	KSPG 52	RLD	KSPG 62
An-1	KSPG 2	Cvi-0	KSPG 15	Kas-1	KSPG 29	Lp2-6	KSPG 41	Pna-10	KSPG 53	Rmx-A180	KSPG 63
Bla-1	KSPG 3	Edi-0	KSPG 17	Ken-0	KSPG 30	Lz-0	KSPG 42	Pna-17	KSPG 54	RRS-10	KSPG 64
Bor-1	KSPG 4	Ei-2	KSPG 18	Kin-0	KSPG 31	Mir-0	KSPG 43	Pro-0	KSPG 55	Se-0	KSPG 65
Bor-4	KSPG 5	Est-1	KSPG 19	Kondara	KSPG 32	Ml-0	KSPG 44	Prs-7	KSPG 56	Sf-2	KSPG 66
Br-0	KSPG 6	Fei-0	KSPG 20	Knox-10	KSPG 33	Mrk-0	KSPG 45	Pu2-7	KSPG 57	Shandara	KSPG 67
Bur-0	KSPG 7	Ga-0	KSPG 21	Knox-18	KSPG 34	Ms-0	KSPG 46	Ra-0	KSPG 58	Sorbo	KSPG 68
C24	KSPG 8	Got-1	KSPG 22	Kz-1	KSPG 35	Mz-0	KSPG 47	Rsch-4	KSPG 59	Sq-1	KSPG 69
Can-0	KSPG 9	Got-7	KSPG 23	Kz-9	KSPG 36	Nd-1	KSPG 49	Ren-1	KSPG 60	Sq-8	KSPG 70
CIBC-5	KSPG 10	Gu-0	KSPG 24	Kz-10	KSPG 37	Nfa-10	KSPG 50	Ren-11	KSPG 61	Tamm-27	KSPG 71
CIBC-17	KSPG 11	Hi-0	KSPG 26	LL-0	KSPG 39	Nok-3	KSPG 51	Pet-0	KSPG 514	Ts-1	KSPG 72
Uk-1	KSPG 75	Hp2-2	KSPG 27	Zdr-6	KSPG 86	HOG	KSPG 503	Pyl-1	KSPG 516	U112-3	KSPG 74
Uk-3	KSPG 76	Dr-0	KSPG 493	Col-0	KSPG 481	Hs-0	KSPG 504	Rld-2	KSPG 518	Van-0	KSPG 79
Uod-1	KSPG 77	Dra-0	KSPG 494	Akita	KSPG 483	Je-54	KSPG 505	RRS7	KSPG 520	Wc-2	KSPG 526
Uod-7	KSPG 78	El-0	KSPG 495	Ak-1	KSPG 484	Lan-0	KSPG 506	Rsch-0	KSPG 522	Wei-1	KSPG 527
Van-0	KSPG 79	Ep-0	KSPG 496	Bay-0	KSPG 485	Lip-0	KSPG 507	Sap-0	KSPG 524	Ws-3	KSPG 529
Var2-6	KSPG 80	Fei-0	KSPG 497	Bd-0	KSPG 486	Lm	KSPG 508	Shah	KSPG 526	Yo-0	KSPG 530
Wa-1	KSPG 81	Ge-2	KSPG 498	Be-0	KSPG 487	Mh-1	KSPG 509	St-0	KSPG 528		
Wei-0	KSPG 82	Gol-1	KSPG 499	Bla-11	KSPG 488	NFA-8	KSPG 510	Stw-0	KSPG 530		
Wil-2	KSPG 83	Gr	KSPG 500	Bu-2	KSPG 489	Nw-3	KSPG 511	Tamm-2	KSPG 532		
Ws-2	KSPG 84	Gre-0	KSPG 501	Cha-0	KSPG 490	Old-1	KSPG 512	Te-0	KSPG 534		
Wt-5	KSPG 85	HI-3	KSPG 502	Cl-0	KSPG 491	Ove-0	KSPG 513	Pi-0*	PKSPG 48		
Ct-1	KSPG 14	No-0	KSPG 536	Dijon-M	KSPG 492	Hi-0	KSPG 533	Ws-0	KSPG 621		

* Different numbering system

SAIL_113_H12 *sober1-3* Col-0 T-DNA knock out line for At4g22305 from NASC

Arabidopsis thaliana recombinant inbred lines Ct-1xCol-0 166 core-pop lines (the Jean-Pierre Bourgin Institute (IJPB) of the National Institute for Agricultural Research (INRA), Precise explanation in method section)

2.2.1.2 Other plant materials

Nicotiana benthamiana
Nicotiana tabacum Wisconsin 38
Nicotiana glutinosa
Nicotiana attenuata
Nicotiana benthamiana and *Nicotiana tabacum* Wisconsin 38 were obtained from Dr. Mattew Templeton from Plant and Food Research, New Zealand. *Nicotiana glutinosa* and *Nicotiana attenuata* seeds are obtained from Dr. Young Jin Kim from Korea University, Republic of Korea.

2.2.2 Growth condition of plants

2.2.2.1 *Arabidopsis thaliana*

A. thaliana was grown at 11-hour light/13 hour dark and at 21-23°C (Humidity uncontrolled, 40-60%). Artificial white light was used in the plant growth room. The age of plants used varied based on the purpose of the experiment. All seeds were stratified before germination for up to 1 week.

2.2.2.2 *Nicotiana* spp.

All *Nicotiana* spp. was grown at 11hour light/ 13hour dark and at 21-23°C (Humidity uncontrolled, 40-60%). Artificial white light was used in the plant growth room. The age of plants used also varied upon the purpose of the experiment. The precise age of the plants is stated in each experimental method.

2.3 Plasmid constructs

2.3.1 List of vectors used in this study

pUC19B (pICH41021)

pUC19B is *Bsa*I site silenced pUC19 vector (Yanischperron, Vieira, & Messing, 1985). pUC19B was used for establishing a module library for Golden Gate assembly. Each module of one gene was cloned into pUC19B by *Sma*I ligation and assembled together.

pICH86988

pICH86988 is a binary vector for Golden Gate assembly with 5' overhang AATG and 3' overhang TTCG. All modules of a gene in pUC19B were assembled into piCH86988 with a C-terminal epitope tag. The pICH86988 gene construct is expressed under a 35S promoter.

pICH86966

pICH86966 is a binary vector for Golden Gate assembly with 5' overhang GGAG and 3' overhang TTCG. A promoter module and a Nos terminator module are needed to express the gene.

pBBR 1MCS 5B : 128bp *avrRps4* promoter

pBBR 1MCS 5B is a *Bsa*I site silenced pBBR 1MCS 5 broad host vector. All modules of a gene in pUC19B were assembled into pBBR 1MCS 5B : *avrRps4* promoter with a C-terminal epitope tag.

pBBR 1MCS 5B 66

pBBR 1MCS 5B 66 is a derivative of pBBR 1MCS 5B with a Golden Gate assembly cassette with 5’ overhang GGAG and 3’ overhang TTCG.

pBBR 1MCS 5B 662

pBBR 1MCS 5B 662 is a derivative of pBBR 1MCS 5B with a Golden Gate assembly cassette with 5’ overhang GGAG and 3’ overhang GCTT.

pYL156

pYL156 is used for Virus-induced gene silencing.

2.3.2. Module constructs in pUC19B for Golden Gate assembly

Psa effector module constructs in pUC19B were designed and cloned as described in 2.5.1.2.2. Golden Gate assembly. Table 2.3.2.1.1. shows each Golden Gate module and overhangs and Table 2.3.2.1.2. shows primer sets used in module amplification.

SOBER1, other effectors, effector promoter and N-terminal region and genes module constructs are in Table 2.3.2.2.

Apart from *Psa* effector genes, all other genes in this study were cloned as a single module.

Table 2.3.2.1 *Psa* effector Golden Gate module primer sequences and overhangs

***Psa* Effector**	**Golden gate Overhang (5'/3')**	**Primers (F/R 5'-3')**
avrPto5	AATG / TTCG	GGTCTCGAATGGGAAACGTATGCGTTGGTG / GGTCTCACGAAGCCGTTGCGGGGAGCGAA
hopS2	AATG / TTCG	GGTCTCGAATGAAAAAGTCTGGCGCTGGAA / GGTCTCACGAAGCCAGATGAGCTCGCCAC
hopA1	AATG / TTCG	GGTCTCGAATGAACCCCATTCAGTCACGCT / GGTCTCACGAACCTTGAAAAACGCCGGTC

hopAZ1	AATG / TTCG	GGTCTCGAATGATCAATAGCATCCGTGGTGG / GGTCTCACGAACTTCCAGCGCGCCATGAAAA
hopH1	AATG / TTCG	GGTCTCGAATGATCACTCCATCTCGATATCCAGG / GGTCTCACGAATTGATGTGGCCTGTACTTCATG
hopAW1	AATG / TTCG	GGTCTCGAATGCGCGTGAGAGTATCAAACACT / GGTCTCACGAACGAGCGCACAGGCAGAATAT
avrRpm2	AATG / TTCG	GGTCTCGAATGGGTAATATATGTGGTACTTCTGGCTC / GGTCTCACGAAGAAATCGTCGTCAGAATCTGAC
avrRpm1	AATG / TTCG	GGTCTCGAATGGGCTGTGTATCGAGTACGTC / GGTCTCACGAATCCAGTAAAGCGGCTCATGAT
hopAY1 V-13	AATG / TTCG	GGTCTCGAATGAGACGGTACACTAGCATCGT / GGTCTCACGAACTTCACACGCTGAATAACCATG
hopAH1	AATG / TTCG	GGTCTCGAATGGTCGAGGACGTAAAAAATGCC / GGTCTCACGAAGGAGAATTGATCCCCTTTCGA
hopAI1 V-13	AATG / TTCG	GGTCTCGAATGCCCGTAAGCAAACCCATGCT / GGTCTCACGAAGCGATTCCAGGGCGGTG
hopAM1-1	AATG / TTCG	GGTCTCGAATGCACGCAAATCCTTTAAGCTCTT / GGTCTCACGAAGTCGCCTAGGAAATTATTTAGTTCC
hopBB1-1	AATG / TTCG	GGTCTCGAATGGGTAATATTTGTGGTACTTCCGG / GGTCTCACGAATTCATCAGACCGAGAATAGTTGTT
hopY1	AATG / TTCG	GGTCTCGAATGAACATAACGCCGCTCACGT / GGTCTCACGAACTGGTAGTTGATGCCCGTG
avrD1	AATG / TTCG	GGTCTCGAATGCAAGACCTTAGTTTTAGCAATATAGAAA / GGTCTCACGAAAAGGGGTAATAGATGCGCAACT
avrB4-1	AATG / TTCG	GGTCTCGAATGAAGGGGTAATAGATGCGCAACT / GGTCTCACGAAGCGATCAAAAAAGTTATTGTAGTGC
hopAO2	AATG / TTCG	GGTCTCGAATGCAGAATCATGTCATTACTTCCCGA / GGTCTCACGAAAAAGCGTTGTTGAGAGGGG
hopF2	AATG / TTCG	GGTCTCGAATGGGTAATGTTTGTGGTACTTC / GGTCTCACGAAGTCGTCCACTACCTGCGCT
hopAF1	AATG / TTCG	GGTCTCGAATGGTTTCTTTAGACGCTTATCAA / GGTCTCACGAAGCCAGTCACCAAATGTTTTATAA
hopZ5	AATG / TTCG	GGTCTCGAATGGGACTTTGTGCATCAAAA / GGTCTCACGAAGGATTCTATCGCTTTTCTTATTTTTCG
hopN1	AATG / TTCG	GGTCTCGAATGTATATCCAGCAATCTGGCGC / GGTCTCACGAATCGCAAGTGAAAGTCTGCT
hopX3	AATG / TTCG	GGTCTCGAATGGGTAATATTTTTGGTACTTCC /

			GGTCTCACGAAAAAGTTGGGCCGCCTGG
hopW1		AATG / TTCG	GGTCTCGAATGCCCAGTTGCGAGGTC / GGTCTCACGAAGTAAAGCTGATGGAGCGTAAA
hopZ3		AATG / TTCG	GGTCTCGAATGAATATCTCAGGTCTGAACAGA / GGTCTCACGAACGCCCTGACCCTGTTGG
hopQ1	Module 1	AATG / CAAC	GGTCTCGAATGCATCGTCCTATCACCG / GGTCTCTGTTGTAAGCGCGTGCAT
	Module 2	CAAC / TTCG	GGTCTCGCAACAATGCGACCGACAT / GGTCTCACGAAATCTGGGGCTACCGTCGA
avrE1	Module 1	AATG / ACGC	GGTCTCGAATGCAGTCATCATGGATCCAC / GGTCTCAGCGTCCAATTGAATGGTGCCTT
	Module 2	ACGC / ATTC	GGTCTCGACGCTAAAGGAAAGCCTGA / GGTCTCAGAATCAGCTTCAATTTCGGC
	Module 3	ATTC / GGCG	GGTCTCGATTCCCGAGCAGGCAAAC / GGTCTCACGCCTTCTAGTCCTGGAATG
	Module 4	GGCG / GAAG	GGTCTCGGGCGATATCAAAAGCCTGTCG / GGTCTCACTTCACTGGCATCACGACGTT
	Module 5	GAAG / CAAT	GGTCTCGGAAGATCATGGGTTGAGCAAAG / GGTCTCAATTGGGATCGGCATTCGGAT
	Module 6	CAAT / TTCG	GGTCTCGCAATAGCGATTCGTTTTCTGC / GGTCTCACGAAGCTCTTCAGTTCGAATCCCT
hopAE1	Module 1	AATG / GAAG	GGTCTCGAATGCCACCGCAGATAACACG / GGTCTCACTTCCTCTCGAGCAAACGACAG
	Module 2	GAAG / CACT	GGTCTCGGAAGATATAGAAGCTCGAGGCGG / GGTCTCAAGTGATTGACCACTGCACGGT
	Module 3	CACT / GCGC	GGTCTCGCACTTCAACGTCAGCGAGCAT / GGTCTCAGCGCGCTAGCTGCACCTCCTA
	Module 4	GCGC / TTCG	GGTCTCGGCGCTTTCAACCGCAATTC / GGTCTCACGAAAACCCGATCACTTCCGGAC
hopAS1	Module 1	AATG / ACGC	GGTCTCGAATGACCTTAAGAATCAATACTCGTT / GGTCTCAGCGTTTGACCAATAGCGGTGT
	Module 2	ACGC / CAGG	GGTCTCGACGCTTGCTACGCGTTCTCA / GGTCTCACCTGATCAACCTGCTTTCTG
	Module 3	CAGG / CTGT	GGTCTCGCAGGTTATTTCCGCGCGTAT / GGTCTCAACAGAGACTTCCGGGTTACG
	Module 4	CTGT / TTCG	GGTCTCGCTGTCAGCCAGATAGATGCG / GGTCTCACGAACGGTGGTGGAAATTGTGCC

hopAU1	Module 1	AATG / GCAG	GGTCTCGAATGCTCGACGTAAATAACAGC / GGTCTCACTGCACAGCATCATCGAAAGA
	Module 2	GCAG / CAGT	GGTCTCGGCAGGCTGCAATTACGTCGGT / GGTCTCAACTGAGCCTTAAGTTCACCCT
	Module 3	CAGT / TTCG	GGTCTCGCAGTGGACACAGAAAGTCTCC / GGTCTCACGAATTCCTGCTGTGTGCGAGCACTA
hopAV1	Module 1	AATG / GCAT	GGTCTCGAATGACAGCTGCTGGTAGACGC / GGTCTCAATGCTCGACAGTCAGATCCTTG
	Module 2	GCAT / GCCA	GGTCTCGGCATATTTCCGAGTCCAAGCT / GGTCTCATGGCTCGGGCAGAATCTGGTGT
	Module 3	GCCA / CCTG	GGTCTCGGCCATTGCCAAGCTCTACATCC / GGTCTCACAGGCTCAGTGTGTCTGCTTC
	Module 4	CCTG / TTCG	GGTCTCGCCTGAAAGATGAGAAGAAAGTC / GGTCTCACGAAATCCCAGTCAAACGGTGTTGG
hopD1	Module 1	AATG / ACGC	GGTCTCGAATGAATCCTCTACGATCTATTCAAC / GGTCTCAGCGTTTGGACCAAATGCTG
	Module 2	ACGC / CAAC	GGTCTCGACGCATTAATTGGACAGAA / GGTCTCAGTTGACACGTCCTCCAAGT
	Module 3	CAAC / TTCG	GGTCTCGCAACGTTCCAGGGCTATTTGA / GGTCTCACGAAAGGTGCAGGTTGTCGAGATGT
hopI1		AATG/TTCG	GGTCTCGAATGATCAACCTCACCCACATT / GGTCTCACGAACGCTCTAGCATCAGGTACCA
hopM1	Module 1	AATG / ACTG	GGTCTCGAATGAGCGACATGAGAATCAAT / GGTCTCACAGTCCGTCTTCAATCGCTTT
	Module 2	ACTG / CTGG	GGTCTCGACTGAATGAAAGTATTACGTCACTGG / GGTCTCACCAGTGGATGTGTGGACGATAC
	Module 3	CTGG / GTAC	GGTCTCGCTGGGTGAAATGATACCTTACG / GGTCTCAGTACGTGCCAGCATTGGT
	Module 4	GTAC / TTCG	GGTCTCGGTACTGAAACCGTGTTGAACA / GGTCTCACGAAGCGTCTTCGACCTTCTTC
hopR1	Module 1	AATG / CAGA	GGTCTCGAATGGTCAAGGTTACCTCTTCC / GGTCTCATCTGGTCTTGTAGCATCAGGT
	Module 2	CAGA / ACAT	GGTCTCGCAGAATGGACGACGTATGAG / GGTCTCAATGTGTAAGCCTGTTTCGCC
	Module 3	ACAT / CGAC	GGTCTCGACATCAGGGAAGATACTGTTTG / GGTCTCAGTCGCGAATCAATAACACGG
	Module 4	CGAC / GCCG	GGTCTCGCGACCGAGCAGCTCGATGAA /

			GGTCTCACGGCACTTTACGACGCAGTT
	Module 5	CGGC / AACA	GGTCTCGGCCGGGTCTTGATCAAGTTAT / GGTCTCATGTTAAGTTCAAGCCCTGTG
	Module 6	AACA / TTCG	GGTCTCGAACAGAGTCTCATCGCGAGTG / GGTCTCACGAACAGGTTATCGAGTTCACCCCA
hopAB3	Module 1	AATG / GGCA	GGTCTCGAATGGTGGGTATTAATAGAGCGGG / GGTCTCATGCCCGAGCATAAGCAGGCG
	Module 2	AATG / TTCG	GGTCTCGGGCACCGCAGCACGCAGGA / GGTCTCACGAAGGGGACTATTCTAAAAGCATACTTGG
hopAF1-2		AATG / TTCG	GGTCTCGAATGGGGCTATGTATTTCAAAAAACTC / GGTCTCACGAACTGTGCGACCAGATGTTTTATG
hopAG1	Module 1	AATG / GACA	GGTCTCGAATGAACCCTATAATACACAGCTTT / GGTCTCATGTCTTTATGATTCCCGTCTATCA
	Module 2	GACA / TTCG	GGTCTCGGACATCAAAGCGATAGAGGGA / GGTCTCACGAAGAAGGGGTTACGCCTGAAAGC
hopAI1 LV-5		AATG / TTCG	GGTCTCGAATGCCCATAAACAAACCCATGCTC / GGTCTCACGAAGCGATTCCAGGGCGGTGG
hopAY1 LV-5		AATG / TTCG	GGTCTCGAATGGTGGGTATCAACAGAGCA / GGTCTCACGAACTCCACGCGCTGAATAACCAT
hopE1		AATG / TTCG	GGTCTCGAATGTATAGAGTTTCCGGTAGCTCG / GGTCTCACGAAGTCAATCACATGCGCTTGGC
hopO1		AATG / TTCG	GGTCTCGAATGAATATCAGTCCTGTATCGGG / GGTCTCACGAACTCGTCTGAATTATCTGCTATCTCG
hopS1		AATG / TTCG	GGTCTCGAATGAAAATATCCGGCTCCACAT / GGTCTCACGAATTTTTGAGACAGTACACTAACGAC
hopT1		AATG / TTCG	GGTCTCGAATGATCAAAACAGTCAGCGATAAC / GGTCTCACGAAATGCTCGACTGGCTCGTCG
hopW1	Module 1	AATG / TGCC	GGTCTCGAATGAATCCAGCTCAGATCACG / GGTCTCAGGCAAGAGTCCAGTGCTACG
	Module 2	TGCC / TTCG	GGTCTCGTGCCCCGATCAGGAGAGGTA / GGTCTCACGAAACGGTCTTCGAAGGGCTTCC
hopA1		AATG / TTCG	GGTCTCGAATGCAACCGTTGGTATTTGAT / GGTCTCACGAATTTCGTGTTTCGAAGGGCCG
hopF1		AATG / TTCG	GGTCTCGAATGAAGAACTCGTTTGACCG / GGTCTCACGAAATCGAGGATATTGACCGGTAC
hopAR1		AATG / TTCG	GGTCTCGAATGAAGATAGGTACGCAGGCC / GGTCTCACGAACGAAACTCTGAACTCGTTTACGC

hopX1	AATG / TTCG	GGTCTCGAATGAAAATACATAACGCTGGCCC / GGTCTCACGAACGTATCTCGCTGAGGCGTGC
hopX2	AATG / TTCG	GGTCTCGAATGGGTTCGTGCGTATCAAAAGG / GGTCTCACGAACCAACTCAAGGGCGGGCG
hopAA1	AATG / TTCG	GGTCTCGAATGCACATCAACCGACCCGT / GGTCTCACGAACGAGCGCATAGGCCGAAAC

Table 2.3.2.2 Golden Gate module primer sequences overhangs for other genes

Promoter modules	**5' overhang**	**3' overhang**	**Primer sequences (F/R ,5'-3')**
SOBER1 native promoter (1-510bp)	GGAG	AATG	GGTCTCAGGAGTGGCAAGGGAGAAAACTTCAAC / GGTCTCACATTCGTGCTTGAGTTCCGTGAA
avrRps4 promoter (128bp) + N-terminal 136 amino acid (+2nt)	GGAG	AATG	GGTCTCAGGAGTTTCCCCGAAGATTAGGAACT / GGTCTCACATTCGTTTACCTCCACCCA
avrRpt2 promoter (171bp) + N-terminal 100 amino acid (+2nt)	GGAG	AATG	GGTCTCAGGAGATCGATTGATCTCTGGCTCA / GGTCTCACATTTCGGTGGAATCGGAAGCCA
YopJ effector modules			
avrBsT $_{11-350}$*	AATG	TTCG	GGTCTCAAATGGGTTCTAGCCGTTCTAGCCG / GGTCTCACGAATGATTCAATAGTTTTCCTAATTTTCCTCA
hopZ1a	AATG	TTCG	GGTCTCAAATGGGAAATGTATGCGTCGGCG / GGTCTCACGAAGCGCTGCTCTTCGGCAAG
SOBER1 variants			
SOBER1 Col	AATG	TTCG	GGTCTCAAATGGCTCGAACTTTCATCTTGT / GGTCTCACGAAGTGGAACATTTCTTTAAGACAATTAAGT
SOBER1 Ct-1	AATG	TTCG	GGTCTCAAATGGCTCGAACTTTCATCTTGT / GGTCTCACGAAGCTCCTTATTGCTGATCGAG
SOBER1 Br-0	AATG	TTCG	GGTCTCAAATGGCTCGAACTTTCATCTTGT / GGTCTCACGAAGCCTCTTCGGGAAACTGA

* Amino acid sequence 11-350

2.3.3. Constructs in binary vector

Psa effector module constructs were Golden Gate assembled into pICH86988 with a C-terminal YFP tag.

SOBER1 and *SOBER1* variants constructs were cloned into pICH86988 with a C-terminal 3X FLAG tag. *SOBER1*^{col} was also cloned into pICH86966 under native promoter (1510 bp upstream before ATG) and with a C-terminal 3X FLAG tag using Golden Gate assembly.

2.3.3 Constructs in broad host range vector

HopZ5 and HopZ3 were cloned into pBBR 1MCS 5B: 128bp *avrRps4* promoter + 136 N-terminal amino acids with C-terminal 6X HA epitope tag module. The HopZ1a module was cloned into pBBR 1MCS 5B 662 with 128bp *avrRps4* promoter + 136 N-terminal amino acid module with C-terminal 6X HA epitope tag module. AvrRpt2 and AvrBsT (11-350) were cloned under *avrRpt2* promoter + N100(+2nt) in pBBR 1MCS 5B-662 and pBBR 1MCS 5B 66, respectively.

2.4 Plant pathology

2.4.1 Bacterial infiltration in *Arabidopsis thaliana* and Hypersensitive Response assay

4-5-week-old *Arabidopsis* plants which were grown in 10 hours of light (artificial white light) at 25° C were used for infiltration. *Pseudomonas* strains were grown on King's B agar plates with selective antibiotics at 28 °C for 2 days and re-suspended in 10mM $MgCl_2$. The OD_{600} was adjusted accordingly for specific experiments. *Pto* DC3000 was infiltrated at OD_{600}=0.1 for scoring at 1DPI (Day Post Infiltration) or OD_{600}=0.6 for 12HPI (Hour Post Infiltration). *P. fluorescens* was infiltrated at

OD_{600}=0.2 and scored at 1DPI. 3-4 leaves per *Arabidopsis* plant were infiltrated with a blunt end syringe.

2.4.2 *Agrobacterium*-mediated transformation

2.4.2.1 *Agrobacterium*-mediated transient gene expression in *Nicotiana spp.*

5-6-week-old *N. benthamiana* and *N. tabacum* were mainly used for *Agrobacterium*-mediated gene expression (hereafter agroinfiltration). *A. tumefaciens* was cultured in L broth overnight for 2 days at 28°C and the overnight culture was spun down at 4000rpm for 5 minutes. The bacteria pellet was re-suspended in 10mM $MgCl_2$ 10mM MES solution and adjusted to the appropriate OD_{600}. *N. benthamiana* or *N. tabacum* leaves were infiltrated with a blunt end syringe.

2.4.2.2 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*

Stable transgenic *Arabidopsis* lines were generated by the floral dip method (J. Clough 1998). 1ml of overnight-liquid-cultured *Agrobacterium* harboring the construct to be transformed into *Arabidopsis* was diluted in 250ml of L broth and incubated for ~20 hours. The culture was cooled on ice for 30 minutes and then spun down at 5000rpm at 4°C for 15 minutes and resuspended in 5% sucrose. *Arabidopsis* siliques were removed before flower dipping. *Arabidopsis* flowers were dipped in ice-cold 400ml of OD=0.8 *Agrobacterium* solution in 5% sucrose for 2minutes. After dipping, the plants were covered with a plastic bag to increase humidity and the plastic bag was removed after 1 day. Transformed *Arabidopsis* seeds were selected on kanamycin MS plates.

2.4.3 Ion leakage assay

2.4.3.1 *Arabidopsis thaliana*

Pseudomonas strains were grown on King's B plate containing appropriate antibiotics for 1-2 days. *Pseudomonas* strains were scraped directly from the plate and re-suspended in 10mM $MgCl_2$ at OD_{600}=0.2. 3-5 leaves of 4-5-week-old *Arabidopsis* plants, which were grown in 10 hours of day light at 21-23 °C, were infiltrated with the bacterial inoculum with a blunt end syringe. 10 minutes after infiltration, 18 of $1cm^2$ leaf discs were harvested, soaked in 20ml of dH_2O and shaken for 30 minutes. 3 replicates of 4 leaf discs were taken carefully into 3ml dH_2O. The ion conductivity in a 60µl sample was measured with a Horiba Twin Conductivity Meter B-173 at specific time points. Measurements were recorded 1, 12, 24 and 48 hours after infiltration.

2.4.4 Bacterial growth assay in *Arabidopsis thaliana*

4-5-week old *Arabidopsis* plants which were grown in 10 hours of day light at 21-23°C were used for infiltration. *Pseudomonas syringae* pv. *tomato* DC3000 was grown on a King's B plate with appropriate antibiotics and infiltrated at OD_{600}=0.001 in 10mM $MgCl_2$. 3 leaf discs (total $0.76cm^2$, harvested with #3 cork borer) were ground in autoclaved 100µl of 10mM $MgCl_2$ and topped up to 500µl. 6 replicates per samples were harvested. 100µl of 500µl of sample was transferred to a 96-well plate and 100µl of autoclaved $MgCl_2$ was added, which was counted as X 1/50. 20µl of X1/50 sample was transferred to the next well, which contained 180µl of autoclaved $MgCl_2$, to make a 10 times serial dilution. Dilution went up to $1/5X10^6$. 20µl of each diluted sample was plated on a King's B antibiotic plate and grown for 2 days at 28°C for colony counting.

2.4.5 Virus-induced gene silencing (VIGS) in *Nicotiana benthamiana*

2.4.5.1 VIGS construct design

Fragments of 400 bp in the target genes, *SGT1, NDR1* and *EDS1* for VIGS were predicted by the Solgenomics VIGS tool (www.vigs.solgenomics.net). Using the primer sets below, the 400bp fragment from the target genes with flanking *EcoR*I-400bp-*Xho*I were amplified and *Sm*aI-ligated into pUC19B as done with Golden Gate modules (VIGS module). Equimolar pYL156 and VIGS modules were cut with *EcoR*I and *Xho*I and the backbone of pYL156 and the fragment of VIGS modules were gel purified from agarose gel. The same volume of gel eluted solution of pYL156 backbone and VIGS fragment were ligated as described in the ligation section.

Primers used for VIGS (5'-3'):
NbEDS1 vigs F GAATTCGTACAGTTGTAGCACTTCTTTTG
NbEDS1 vigs R CTCGAGCATCCAAAAGTTATACAGTATGGAC
NbNDR1 vigs F GAATTCATGTCAGACTATGGATCCAATAATAC
NbNDR1 vigs R CTCGAGACCTTGTACCTGACTCTAGTAGC
NbSGT1 vigs F GAATTCCTAAAGATGCTCAACCAACTGTC
NbSGT1 vigs R CTCGAGAGGGTTCAGCTTTTGCAAGG

2.4.5.2 VIGS

A. tumefaciens AGL1 harboring pTRV1 and pTRV2:target gene were incubated in L broth with antibiotics at 28°C overnight. Each culture was spun down at 4000rpm for 5 minutes and resuspended in 1mM MES 10mM $MgCl_2$ solution. *Agrobacterium* harboring pTRV1 and *Agrobacterium* harboring each pTRV2-gene were mixed together to OD_{600}=0.5 of both pTRV1 and pTRV2:target gene (total OD_{600}=1.0). This preparation was infiltrated gently into 1-2 cotyledons of 2 to 2 and half-week old *N. benthamiana* seedlings. The infected seedlings were grown for 4-5 weeks at 21-

23°C in 10-11 hours daylight until they were tested for agroinfiltration. Gene silencing was confirmed by amplifying the target gene from silenced *N. benthamiana* cDNA (Procedure is described in 2.5.2 RNA section).

The primers used in semi-quantitative polymerase chain reaction for each gene were:

EDS1 (5'- AACGAGGAAAAGATTGATGGTA - 3'/5'- TCCTTTCTTCCCTCAAAACTATC - 3')

SGT1 (5'- TAATGTGTCATCAGATGCCC - 3'/5'- ACTTCTTTCCAGTTTGTCGAC - 3')

NDR1 (5'- TAGTAAAGTGAAAGTGGATGGTTC - 3'/5' – GCAATCAACTGAGTCCAACAT – 3')

Actin(5'-GATGAAGATACTCACAGAAAGA-3'/5'-GTGGTTTCATGAATGCCAGCA-3')

2.5 Molecular Biology methods

2.5.1 DNA

2.5.1.1 Polymerase Chain reaction (PCR)

The basic PCR reagents for both Phusion DNA polymerase (Thermo) and Prime star GXL DNA polymerase (Takara) were 0.02U/µl polymerase, 1X Polymerase buffer (provided by the manufacturer), 0.25mM dNTP and 1mM each of forward and reverse primers in a 20µl reaction. The PCR cycle conditions are below. The number of cycles (n) varied upon genes between 28-40 cycles.

95°C – 1'

X n [95°C – 30''

55°C (Annealing temperature was optimized for each primer) – 30''

68°C – 1' per 1000 base pairs (kb)]

68°C – 3'

20°C hold, Lid temperature kept at 98°C

The PCR reagents for Taq polymerase (NEB and GenetBio) were 0.02U/µl Taq polymerase, 1X Taq buffer (provided by the manufacturer), 0.25mM dNTP and 1mM each of forward and reverse primers in 20µl reaction. The PCR cycle conditions are below. The number of cycles (n) varied upon genes between 28-40 cycles.

95°C – 1’

X n [95°C – 30’’
55°C (Annealing temperature was optimized for each primer) – 30’’
72°C – 1’ per 1000 base pairs (kb)]

72°C – 3’

20°C hold, Lid temperature kept at 98°C

2.5.1.2 Cloning

2.5.1.2.1. Blunt-end ligation

DNA fragments were amplified with Phusion DNA polymerase (Thermo) or Prime star GXL polymerase (Takara) and blunt-end-cloned into pUC19B with SmaI (NEB) and T4 ligase (NEB). PCR and ligation procedures are explained each section. 100-300ng of PCR product was ligated into 40ng of pUC19B vector with 0.25U/µl SmaI(NEB), 0.25U/µl T4 ligase (NEB) in 1X T4 buffer (NEB) total volume 15µl for 4 hours up to overnight at 25°C.

2.5.1.2.2. Golden Gate assembly

Golden Gate assembly module construct strategy

Golden Gate assembly is an easy and efficient cloning strategy for long genes and domain swapping. Genes were first divided into several modules to be assembled. The strategy for designing modules was: 1. No longer than 2kb, 2. No internal SmaI site, 3. Avoid internal BsaI site, but if not possible, mutagenesis is required afterward, 4. No more than 3 nucleotides overlap between overhangs, 5. To remove any SmaI or BsaI sites at the end of modules, synonymous nucleotide changes were introduced by primers. The 5' 4bp overhang of the first module of the gene was fixed to AATG and the 3' 4bp overhang of the last module before a C-terminal epitope tag was TTCG to keep in-frame and for convenience for different epitope tagging. C-terminal epitope tag constructs were originally from the Sainsbury Laboratory but were modified to have additional 2 nucleotides to be in frame.

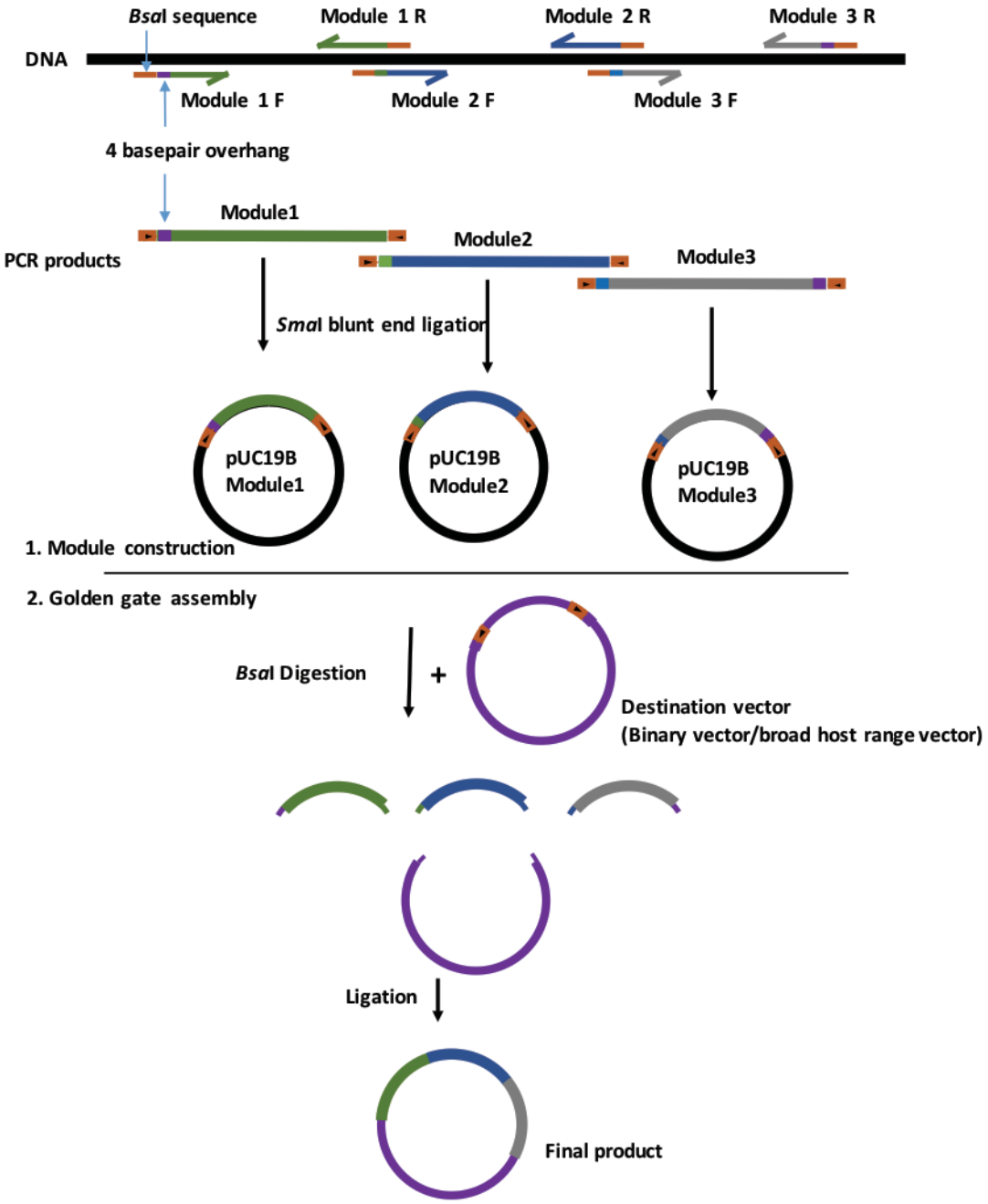


Figure 2.5.1.2.1. Cloning strategy using Golden Gate assembly in this study.
Genes were divided into ~1kb modules and each module was amplified with *Bsa*I flanking primers. Modules were blunt-end ligated with *Sma*I into the entry vector pUC19B. The *Bsa*I digesting direction is indicated with a black triangle. For Golden Gate assembly, all module constructs and the destination vector were mixed in one pot with *Bsa*I and ligase. *Bsa*I digests and produces directional sticky ends (4 bp overhangs) without leaving a *Bsa*I site. As there is no *Bsa*I left over, the final product is stable once these complementary overhangs have ligated.

Golden Gate assembly

~100ng of the modules in pUC19B were mixed with ~80ng of the destination vector with the equimolar epitope tag in 100ug BSA (Bovine Serum Albumin), 1X T4 ligase buffer (NEB), 0.5U/μl of T4 ligase (NEB) total volume 20μl.

The cloning cycle was;

x25 [37°C - 3’
      16°C - 4’

50°C - 5’

80°C - 5’

20°C - hold

The cloning product was sepharose purified (2.5.1.7.2. Sepharose purification) and electro transformed into *E. coli* DH5α (2.5.1.7.3. Electroporation). Positive colonies were determined by the disruption of the LacZ cassette in the vector by the insert. White colonies on X-gal (Sigma, Georgiachem)/IPTG (Sigma, Georgiachem) antibiotic L plates were selected for plasmid digestion confirmation (2.5.1.8. Plasmid DNA purification and 2.5.1.6. Restriction enzyme digestion).

2.5.1.3.2. TA cloning

LacZ cassette modules for modifying vectors into Golden Gate compatible or several gene modules with non-removable SmaI sites were cloned into commercial TA cloning vector pGEM-t easy (Promega). The insert was amplified with Taq polymerase (NEB) as described in 2.5.1.1 PCR section, and cloned into pGEM-t easy vector following the manufacturer’s instructions. The reagents 1X rapid reaction buffer, 50ng of pGEM-t easy vector, PCR product (amount varied), 1U/μl of T4 ligase in total volume 10μl were incubated for 1 hour at room temperature. The transforming procedure was identical to 2.5.1.2.1. Golden Gate assembly.

2.5.1.2.4. Subcloning

VIGS constructs were amplified with flanking 5'EcoRI-3'XhoI restriction sites by primers. These PCR fragments were blunt-end cloned into pUC19B first. Both pYL156 and VIGS constructs in pUC19B were EcoRI/XhoI treated using appropriate buffer as per the manufacturer's instructions and gel-purified after electrophoresis. Purified pYL156 and each VIGS construct fragment were mixed in approximately equimolar amounts and ligated. The final construct was selected by *E. coli* kanamycin resistance and confirmed by enzyme digestion.

2.5.1.3. Site-directed mutagenesis

Site-directed mutagenesis primers were designed according to the Stratagene protocol. The annealing temperature of the primers were calculated using this formula: Tm=81.5+0.41*(%GC-675)/(Number of basepairs of primer (25-45bp))-(Number of mutation)/(Number of base pairs of primer)*100 (Tm>79°C)

The site-directed mutagenesis PCR reagents by Phusion DNA polymerase (Thermo) were ~125µl of template plasmid, 1X Phusion high fidelity buffer, 0.2mM dNTP, 125ng of primers, 2U Phusion in total 50µl volume. PCR cycle for a point mutation was 30 seconds at 95°C, 12-16 cycles of (30 seconds at 95°C -1 minute at 55°C -(1mintute per kb of plasmid) at 68°C) and 5 minutes at 68°C.

25µl of PCR product was treated with 1µl of DpnI(NEB) for 1 hour at 37°C and transformed into *E. coli* DH5α after sepharose purification.

2.5.1.4. DNA gel purification

PCR product or restriction enzyme cut plasmid was run on 1% agarose gel by electrophoresis with ethidium bromide. The DNA bands were visualized under UV light and cut by blade. DNA was purified with DNA gel purification kit (Roche) as per the manufacturer's instructions.

2.5.1.5. Ligation

T4 ligase (NEB) was used in ligation in 1X T4 ligase buffer total volume 10-20μl.

2.5.1.6. Restriction enzyme

Restriction enzymes were used in the proper buffer that was recommended by the manufacturer.

2.5.1.7. Transformation of bacteria

2.5.1.7.1. Preparation of electrocompetent cells

E. coli

E. coli DH5α was grown overnight on an L plate without antibiotics and seed cultured in 30ml of L broth overnight. The seed culture of cells was diluted in 100 times the volume of autoclaved L broth and shaken at 28°C until OD_{600} reached 0.7. The culture was cooled down and centrifuged for 15 minutes at 4°C. The pellet was washed with 10% glycerol 4 times at 4°C. After the final washing, the pellet was resuspended in 1-2ml of 10% glycerol and aliquoted into 50μl in 0.5ml Eppendorf tubes.

***A. tumefaciens* AGL1**

1-2 days grown *A. tumefaciens* AGL1 on an ampicillin L plate was seed cultured in 30ml of L broth overnight. The seed culture of cells was diluted in 40-50 times volume of autoclaved L broth and shaken at 28°C until OD_{600} reached 0.7. The culture was cooled down and centrifuged for 15 minutes at 4°C. The pellet was washed with 10% glycerol 4 times at 4°C. After the final washing, the pellet was resuspended in 6-8ml of 10% glycerol and aliquoted into 50μl in 0.5ml eppendorf tubes

2.5.1.7.2. Electroporation

E. coli DH5α and *A. tumefaciens* AGL1 were transformed by electroporation in an ice-cold 0.1cm electroporation cuvette by a Micropulser tm electroporator (Bio-rad) with the provided setting (Ecl I for *E. coli* and Agr for *A. tumefaciens)*.

1µl of purified plasmid was transformed into 50µl of electrocompetent cells, incubated for 1 hour at 37°C for *E. coli* and 2 hours at 28°C for *A. tumefaciens,* and plated on an antibiotic selective plate.

2.5.1.8. Plasmid DNA purification

Plasmid DNA was purified based on alkaline lysis plasmid isolation either manually or by plasmid purification kit (Roche or GenetBio) as the manufacturer instructed in the protocol.

For manual plasmid isolation from bacteria, an overnight liquid culture of bacteria in L broth was spun down at 2500g for 5 minutes and lysed with 100µl of solution I by vortexing. 2 minutes after adding 200µl of solution II, 200µl of solution III was added.

For *Agrobacterium* plasmid purification, cells were incubated for 5 minutes after adding solution II. The mixture was spun down at 10000g for 10 minutes and then ~600µl of supernatant was mixed with 400µl of isopropanol, followed by spinning down for 1 minute at 13000rpm. The pellet was washed with 400µl of 70% ethanol twice and dried for 15 minutes until the pellet became transparent. The dried pellet was resuspended in 30-40µl of dH_20 with RNase A.

- solution I: 50 mM glucose/10 mM EDTA/ 25 mM Tris pH 8.0
- solution II: 0.2 N NaOH/1% SDS
- solution III: 60 ml 5M KOAc/11.5 ml 96% acetic acid/28.5 ml H2O

2.5.1.9. Colony PCR

One or two days old colonies grown on agar medium were resuspended in 100µl of dH_2O. 2µl of resuspended cells were used as template for polymerase chain reaction (PCR). PCR was run with Taq DNA polymerase for 30-35 cycles.

2.5.1.10. Electrophoresis

DNA samples were mixed with 10X DNA loading buffer (0.25% bromophenol blue 0.2M EDTA 50% glycerol) and loaded in a 1-2% (varied for purpose) TAE (40mM Tris acetate 1mM EDTA pH8.2-8.4) agarose gel. The gel was run at 120mV for various durations.

2.5.1.11. Genomic DNA purification

2.5.1.11.1. Genomic DNA purification kit (Thermo)

Genomic DNA from *A. thaliana* was purified using a genomic DNA purification kit provided by Thermo according to the manufacturer instructions.

Chelex DNA crude purification

1 cotyledon size (#1 cork borer) leaf disc was homogenized in 150µl of 5% chelex (Bio-rad) and incubated at 95°C for 5 minutes. After vortexing, the tissue was spun down at 13000rpm for 2 minutes and the supernatant was used as a DNA template (0.5-2µl per 20µl PCR reaction).

Boiling leaf tissue

1 cotyledon size (#1 cork borer) leaf disc was harvested and boiled at 96°C in 50µl lysis buffer (50mM EDTA pH8.0, 50mM Tris-HCl pH 7.5-8.0, 0.2% w/v SDS) for 15 minutes. After brief spinning down, 500µl of distilled water was added and mixed by vortexing. 2µl of this solution was used as a PCR template for 40 cycles.

CTAB (Cetrimonium bromide) DNA purification

3 #3 size leaf discs were ground in 100µl of Nuclear lysis buffer (0.2M Tris-HCl pH 7.5, 0.05M EDTA, 2M NaCl, 2% CTAB (Sigma)) in a 1.5ml eppendorf tube. After grinding, 150µl more of nuclear lysis buffer, 200µl of extraction buffer (0.1M Tris-HCl pH7.5, 0.005M EDTA, 0.35M sorbitol, 20mM Na-bisulfite (added just before use)) and 25µl of 5% sarkosyl (*N*-Laurylsarcosine) were added in the tube and incubated at 65°C for 1 hour. The samples were cooled down on ice for 15 minutes, 250µl of Phenol : Chloroform : Isoamyl alcohol (25 : 24 : 1) was added, incubated for 5 minutes after mixing by inverting and spun down at 13500rpm for 10 minutes. 250µl of clear supernatant was transferred to a new 1.5ml eppendorf tube, mixed with 250µl of isopropanol and incubated for 1 minute. The sample was spun down at 13500 rpm for 30 minutes. The pellet was washed once with 70 % ethanol and re-suspended in TE buffer with 0.5ug of RNase A.

Nuclei-enriched DNA purification

0.3-0.6g of *Arabidopsis* rosette leaves were harvested and ground finely in liquid nitrogen in a mortar and transferred to a 15ml falcon tube with 10ml of Nuclei Extraction buffer (10mM Tris-HCl pH9.5, 10mM EDTA, 100mM KCl, 500mM sucrose, 4mM spermidine, 1mM spermine, 1.2g/L $NaHSO_3$ (added just before use)). The thawed samples were filtered in 2 layers of miracloth into a 50ml ice-cold falcon tube. 2ml of Nuclei lysis buffer (10% Triton X-100 in Nuclei Extraction buffer) was added and gently mixed on ice for 2-5 minutes. The sample was spun down at 2000 rpm for 10 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in 500µl CTAB Extraction buffer (100mM Tris-HCl pH7.5, 0.7M NaCl, 10mM EDTA, 1% CTAB, autoclaved, 1.2g/L $NaHSO_3$ (added just before use without autoclaving)), incubated at 65°C for 30 minutes and chilled down at RT for 5 minutes. 350µl of Phenol: Chloroform : Isoamyl alcohol (25 : 24 : 1) was added, mixed by inverting and incubated for 5 minutes. After spinning down at 5000g for 10 minutes, 450µl of clear upper-phase liquid was transferred to a new 2ml eppendorf tube and mixed with 1/10X volume of 3M NaOAc pH5.2 and 2X volume of ice-cold 100% ethanol. The solution was mixed by inverting, incubated at -80°C for more than 10 minutes up to overnight and spun down at 10000g for 5 minutes.

The pellet was washed with 70% ethanol, air-dried for 3-5 minutes and resuspended in 100µl TE with 10ug/ml RNAseA. The final DNA sample was incubated at 65°C for 20 minutes to de-activate DNAse and kept at -80°C.

2.5.2. RNA

2.5.2.1. Trizol RNA purification

Leaf tissue was finely ground in liquid nitrogen. ~250µl of ground tissue powder was moved into an eppendorf tube and 1ml of Tri reagent was added. After vortexing and 5 minute-incubation at room temperature, 100µl of 1-bromo 3-chloropropane was added into the tube and mixed well. The mixture was centrifuged after 10 minute-incubation at room temperature at 12000g for 10minutes at 4°C. The supernatant was transferred into a new eppendorf tube and 250µl of isopropanol and 250µl of high salt precipitation solution (0.8M sodium citrate 1.2M NaCl 0.45uM filtered) was added. The mixture was centrifuged at 12000g for 15minutes at 4°C after 5-minute- incubation at room temperature. The pellet was washed with 70% ethanol at 8000g for 5 minutes at 4°C and air-dried at room temperature. The pellet was finally resuspended in 50µl of DEPC-treated H2O and 5µl of DNAse 10X reaction buffer and 5µl of DNAseI was added. After 15 minute-incubation at room temperature, 5µl of stop solution was added and incubated for 10 minutes at 70°C (DNAse 10X buffer, DNAseI, STOP solution from DNAseI AMPD1-1KT Sigma).

Extracted RNA was run on a 1.8-2% agarose gel by electrophoresis for integrity check and the concentration was measured with Nanodrop (Thermo) or Micro cuvette (Eppendorf).

2.5.2.2. cDNA synthesis (RT-PCR)

Up to 5µg of total RNA was used for first strand cDNA synthesis (Maxima kit FMTK1641, Thermo) as instructed by the manufacturer.

2.5.2.3. Semi-quantitative PCR

Genes were amplified from equal amounts of cDNA samples using gene-specific primers. EF1α was used for the cDNA quantity control for *Arabidopsis* and Actin was used for *Nicotiana benthamiana*.

2.5.3. Protein

2.5.3.1. Protein expression *in planta*

N. benthamiana was used for protein expression throughout the experiments. *A. tumefaciens* AGL1 harboring the gene construct was infiltrated in *N. benthamiana* at OD_{600}=0.1-0.4 with *Agrobacterium* harboring viral silencing suppressor P19 (Voinnet et al., 2003) at OD_{600}=0.1. 1-2 DPI; the leaf samples were harvested in liquid nitrogen and stored at -80°C.

2.5.3.2. Total plant protein extraction and immunoprecipitation

The harvested leaf tissue was ground in liquid nitrogen. A small amount (5-6 #3 cork borer size leaf discs) of ground powder was directly added to 5X protein loading buffer (250mM Tris-Cl 8% SDS 0.1% Bromophenol blue 40% glycerol 5mM Dithiothreitol (DTT) in H_2O, (Laemmli, 1970) and boiled at 95°C for 10 minutes followed by 1 minute centrifugation at 13000rpm. A large amount of ground tissue was thawed in a 0.5-1X volume of GTEN protein extraction buffer (10% Glycerol 25mM Tris 1mM EDTA 150mM NaCl pH7.5) with cOmplete protease inhibitor cocktail (Roche), detergent (IGEPAL, sigma), 5mM DTT and polyvinyl polypyrrolidone (PVPP). The sample was centrifuged at 5000rpm for 15 minutes at 4°C. For total protein detection, 200µl of supernatant was mixed with 50µl of 5X

protein loading buffer, boiled for 10 minutes at 95°C and briefly centrifuged at 13000rpm for 1 minute.

For immunoprecipitation, the supernatant was filtered with miracloth, spun down at 5000rpm for 15 minutes again. 15µl of slurry anti-FLAG affinity gel (A2220, Sigma) and GFP trap-A (gta-20, Chromotek) were pre-washed with GTEN buffer and used for immunoprecipitation. Affinity gel and 5-700µl of protein sample that was topped up with GTEN buffer up to 1.5ml in 1.5ml eppendorf tube was incubated in a rotator at 4°C for 2 hours. After washing 3 times, beads were boiled in 50µl of 5X protein loading buffer at 96°C for 10minutes and spun down at 13000 rpm for 1minute.

2.5.3.4. Western blot

The protein samples were loaded in an 8-10% acrylamide gel and separated by SDS-PAGE. The protein samples on the gel were transferred onto a PVDF membrane (Millipore) for 2 hours at 350mA. The membrane after protein transfer was blocked and antibody-treated. Horseradish peroxidase conjugated-anti-GFP-antibody (Santa-cruz, sc-9996 HRP), anti-flag antibody (F3165, Sigma), anti-mouse antibody (A99044, Sigma) and anti-acetylated lysine antibody (9681S, Cell signaling technology) were used to probe proteins in this study.

Super signal Pico and Femto (Thermo) were mixed in different ratios and applied to the membrane. Fuji medical X-ray film was exposed to the membrane to visualize, or LAS500 machine was used for visualization.

2.5.4. Genetic mapping

2.5.4.1. Crossing

Different *A. thaliana* accessions were crossed to generate F1 progenies and F1 individuals were self-fertilized to generate F2 populations. The petals of the

flower bud of *Arabidopsis* Ct-1 was removed and the remaining stigma was brushed with Col-0 stamen 10 times. F1 confirmation genotyping markers are provided in 2.5.4.4. List of Genotyping markers.

2.5.4.2. Recombinant inbred lines

Col-0 X Ct-1 F8 recombinant inbred lines were commercially available at the Jean-Pierre Bourgin Institute (IJPB) of the National Institute for Agricultural Research (INRA). Among 164 core RILs, 121 lines were infiltrated with pBBR 1MCS 5B : *avrRps4P*:*hopZ5*:HA construct harboring *P. fluorescens* Pf0-1 at OD_{600} =0.2 for a hypersensitive reaction (HR) assay. HR was scored 18-20 hours after infiltration. Genotype information for each RIL was provided by INRA (http://publiclines.versailles.inra.fr/). The lines that showed HR or no HR were compiled together and the genotypes compared to find the region that consistently originated from Ct-1 in HR showing lines or from Col-0 in no-HR showing lines.

The *A. thaliana* Col-0 genomic DNA sequence was provided by TAIR (www.arabidopsis.org). The Ct-1 genomic DNA sequence was available on http://mus.well.ox.ac.uk/19genomes/, which was precisely resequenced (Gan et al., 2011). Col-0 and Ct-1 genomic DNA sequences (fasta file) were pairwise aligned in Geneious 7.0 to find insertion-deletion regions or SNPs. INDEL markers were designed manually with sequence comparison and dCAPs markers were designed by dCAPs finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html).

2.5.4.3. Bulked segregant analysis

For Ga-0 x Ct-1 F2 bulk-segregation analysis, genomic DNA from F2 individuals was extracted by the Nuclei-enriched gDNA extraction method. Quantity was compared by running DNA on ethidium bromide containing gel for 5 minutes and approximately the same amount of F2 DNA samples was mixed in one tube. This mixture was used as the template DNA for genotyping with primers below.

2.5.4.4. List of Genotyping markers

Table 2.5.4.4.1. F1 genotyping markers

F1 parents	Marker name	Marker Type	Marker length for parent *Arabidopsis* accessions		Marker primer references or sequences (5'-3')
Ct-1 x Col-0	nga151[a]	SSLP	Col-0	150	(F) GTTTTGGGAAGTTTTGCTGG
			Ct-1	~100	(R) CAGTCTAAAAGCGAGAGTATGATG
Ct-1 x Ws-2	nga106[a]	SSLP	Ws-2	123	(F) TGCCCCATTTTGTTCTTCTC
			Ct-1	~100	(R) GTTATGGAGTTTCTAGGGCACG
Ga-0 x Ct-1	nga59[a]	SSLP	Ga-0	~110	(F) TTAATACATTAGCCCAGACCCG
			Ct-1	~130	(R) GCATCTGTGTTCACTCGCC
Ga-0 x sober1-3	nga6[a]	SSLP	Ga-0	~110	(F) TGGATTTCTTCCTCTCTTCAC
			sober1-3	~130	(R) ATGGAGAAGCTTACACTGATC
	sober1 T-DNA[c]	SSLP	Ga-0	~1200	(F) TTCTTATTAGGCGCCACAATG
			sober1-3	~700	(R) TCGTTTCGGACATAAAACGAC (LB2[b]) GCTTCCTATTATATCTTCCCAAATTACCAATACA

[a] (Bell and Ecker, 1994)
[b] (McElver et al., 2001)
[c] Designed with T-DNA express Primer design tool (http://signal.salk.edu/tdnaprimers.2.html)

Table 2.5.4.4.2. RIL genotyping markers

Marker name	Marker Type	Col-0	Ct-1	Primer sequence (F/R) 5'-3'
Marker59(c4_10609)	dCAPS (HindIII)	200bp	188bp	CCGTAGCTGTTGAGGAAGA / TTGTGGTATTTGAGCTTTGTCCTAAAGC
Marker60(c4_11878)	dCAPS (RsaI)	157bp	185bp	TCATTATTCACACTAACGATTATGTAG / TGATGAAATGATCTTTGGGAGG
Marker 200-2	SSLP	155bp	175bp	TATTTTCCCAACTCAAAGGG / GTACATGTCCAATGGAAACT
Marker 200-1	SSLP	264bp	227bp	CCTGATATTAACTGGATACTCA / ACAACCGCAGTCCCTAATTT
Marker200-3	SSLP	184bp	210bp	CCAAAATTTATTAGAACACTGC / GGGAAAGGAATCCAAAAAAAAT

Marker200-4	SSLP	207bp	245bp	GATCCAATGTACACCAAGA / TTACGACAGTGGTCTGTAA
Marker200-5	SSLP	173bp	150bp	CCTTTTTCTCAAGCATTGAAGA / AATCTTACCATCCTGTTAATATG
Marker200-6	SSLP	192bp	211bp	CAGCCACTTCTATATTAAGG / TTTAAAGGATAGAGAGCCTG
Marker200-8	SSLP	314bp	292bp	AGTACACCACATGCATACAA / CTGTGAGTGATTACATTTCAAA
Marker200-9	SSLP	192bp	226bp	GACCCCAAAATAAAAAAGTACA / CCATCTCCTTTTTATAGAAAG
Marker200-11	SSLP	128bp	141bp	CCATACGTAACACAAACCCT / GTACCATGATGTCAGTAAGC
Marker 200-6-2	SSLP	154bp	133bp	CTTGTGATTGATTTTTGCATGTT / TATTTGCAAAACCGCGGAATA
Marker 200-6-3	SSLP	216bp	194bp	CCTTTTGTCTAAAGGGGAAG / AGCTAGTGAGAAGAAAATTCTG
Marker 200-6-4	SSLP	100bp	113bp	ATATGATGTTTTGCATCAGGG / TTTGCGGAATTACCACTGCA
Marker 200-6-5	SSLP	310bp	229bp	GGGAAAAATGCATTGATTAGG / GGAGATTCTGAAAAATATAAGAG
Marker 200-6-6	SSLP	146bp	115bp	CAGATTCCGCTTCATTTGAAA / GAAAGCAAAAGCAAGAAATGG
Marker 200-6-7	SSLP	978bp	101bp	CCTAAGTCTTAGATATATGTAAG / GGATACTGGCCAAAATCATT
Marker 200-6-8	SSLP	267bp	307bp	GTAGTTAAAATGTTTTGCTAATTC / CCTTTGTTTTTCTTAGATAAATTTA
Marker 200-6-9	SSLP	102bp	90bp	CCACAAGACCCCATTTATTAT / TCATCATTCCCACAGTCGT
SOBER` genotyping	CAPS (BsrF1)	564bp	250/ 314bp	TTGTGGCGCCTAATAAGAATTTTC/TCAGTGG AACATTTCTTTAAGACAATT

Table 2.5.4.4.3. Bulk-segregation analysis markers

Marker name	Marker type	Location (Reference sequence Col-0 TAIR)	Approximate size		Primer sequences (F/R 5'-3')
			Ga-0	Ct-1	

Ch2A[a]	SSLP	Chromosome 2 1401kb	~190	~210	GTTTGGATCAGTCCCAGCTC / TGAAAAAGTGGTGGAACCAA
Ch3B[a]	SSLP	Chromosome 3 7140kb	~250	~200	CTCCAGCTCCACCACCAG / CCAAAAGACATTCTTCCACCA
Ch5B[a]	SSLP	Chromosome 5 4063kb	~250	~200	TACCAGTGCATGGTTTGCAT / ACGCAGGACATGTTTCCTCT
nga59[b]	SSLP	Chromosome 1 2768kb	~100	~150	TTAATACATTAGCCCAGACCCG /GCATCTGTGTTCACTCGCC
nga151[b]	SSLP	Chromosome 5 4848kb	~120	~100	GTTTTGGGAAGTTTTGCTGG / GTTTTGGGAAGTTTTGCTGG
nga106[b]	SSLP	Chromosome 5 5613kb	~120	~100	TGCCCCATTTTGTTCTTCTC / TGCCCCATTTTGTTCTTCTC
F13F21[b]	SSLP	Chromosome 1 17900b	~200	~250	GTTGAACGGTTTAGATTGAAGG / TACGGAGATGCTTAGCCGAGC
Chr1 2000K[c]	SSLP	Chromosome 1 ~2000K	~700	~650	GATTTGTTAATTCACTCCTCAAACA / GAGAACAAACAGTGCTAACAAAA
Chr2 18000K[c]	SSLP	Chromosome 2 ~18000K	~800	~400	CAGAGATGAGTTTTGGGATTTG / AACTAGAGTGGTACTAACACTCA
Chr4 9000K[c]	SSLP	Chromosome 4 ~9000K	~350	~400	GCTTGTTGGGATAGTGCATC / CATGAAATTAGGGTTCTGTAGAAGA

2.6. Confocal laser scanning microscopy for *Psa* effector subcellular localization assay

Psa effectors were transiently expressed in *N. benthamiana* leaf cells by agroinfiltration. 2 days after agroinfiltration, 2-3 8mm diameter leaf discs were sampled, mounted under water and viewed under a confocal microscope (Carl Zeiss LSM700). YFP fluorescence was excited at 488nm with a 20mW Argon laser and captured by the confocal channel in the emission range between 500 and 530nm.

Chapter 3. Multiple type III effectors from *Pseudomonas syringae* pv. *actinidiae* induce programmed cell death in *Nicotiana* species.

3.1. Objectives and contributions

3.1.1 Objectives

Bacterial effectors have diverse enzymatic functions and target various host components to dampen the plant immune system and promote successful proliferation of bacteria in host plants. Both PTI and ETI can be suppressed by various bacterial effectors. Numerous examples of effectors showing diverse enzymatic activities and affecting different host systems including immunity are described in Chapter 1.

Pseudomonas syringae pv. *actinidiae* (*Psa*) causes kiwifruit bacterial canker disease. Since the 2008 outbreak of highly virulent *Psa* (*Psa*-V) in Italy, *Psa*-V has spread worldwide, including Chile (2010) and New Zealand (2010). In addition, the low virulent strains (e.g. *Psa*-LV, biovar 4) were also reported in *Psa*-contaminated orchards in New Zealand. McCann et al. showed that *Psa*-J, *Psa*-K, *Psa*-LV and *Psa*-V belong to very distinctive phylogenetic clades (McCann et al., 2013). The authors further suggested that these distinctive clades are the result of various gene shifting processes between multiple source populations, suggesting the possibility of new virulence emergence in *Psa* populations. Considering the active transfer and conversion events of virulent effector genes in *Psa* source populations, it would be informative to study functions of effectors that are present in the *Psa* strains isolated in the same region such as *Psa*-LV and *Psa*-V.

In this research, one representative strain was selected from each clade for an effector characterization study, namely the highly virulent strain *Psa* NZ V-13 and the low virulent strain *Psa* NZ LV-5 (hereafter *Psa* V-13 and *Psa* LV-5), both isolated in Rangiuru, Bay of Plenty, New Zealand (Chapman et al., 2012).

In order to better understand the biological activities of *Psa* effectors and eventually, to establish a durable resistance to *Psa* in kiwifruit, characterization of

effectors and identification of *R* genes for multiple effectors is very important. In this chapter, the aim is to characterize *Psa* effectors predicted from McCann et al. (2013) for their localization *in planta* and avirulence activity in non-host plant *Nicotiana* spp. to find future candidates for *R* gene investigation.

3.1.2 Contributions

Several individuals have contributed in the work described in this chapter of the thesis.

These include:

Amandine Spiandore – for assistance with cloning of *Psa* V13 effector modules described in Chapter 3.

Jay Jayaraman – for cloning *Psa* LV5 effector modules in Chapter 3, *Psa* V13 effector GoldenGate cloning into broad host-range vector, and production of Figure 3.2.2.2 (confocal images of *Psa* effector localization *in planta*.

Without these significant contributions, this thesis would not have been able to achieve the results herein. All other work in this thesis is done by myself under supervision of Prof. Kee Hoon Sohn or Prof. Cecile Segonzac.

3.2 Results

3.2.1 Establishment of *Psa* effector library

In order to test avirulence activities of *Psa* effectors, two previously characterized *Psa* strains, V-13 and LV-5, were chosen for further analysis since

complete sets of effectors were reported (McCann et al., 2013). *Psa* V-13 and LV-5 carry 38 and 26 predicted effectors based on their genome sequences, respectively. Among these, 47 effectors were selected based on the following criteria, i) V-13 or LV-5 specific effectors ii) V-13 variants for the commonly present effectors with more than 90% amino acid identity iii) both V-13 and LV-5 variants for the commonly present effectors with less than 90% amino acid identity (Table 3.2.1.1). In addition, significantly truncated effectors (*hopA1*, *hopW1*, *hopAA1-1* and *hopAA1-2* from *Psa* V-13 were eliminated from the list, and only a single representative of the duplicated effectors (*hopBB1-1/hopBB1-2* (93.6 % identity) and *hopAM1-1/hopAM1-2* (100 % identity) from *Psa* V-13) were cloned. Among 10 effector homologues shared by V-13 and LV-5, *hopAI1* and *hopAY1* were cloned separately from both strains since shared peptide identity was less than 90% (*hopAI1*) or peptide sequence differed in length greater than 100 amino acids (*hopAY1*). Of the other 8 shared effectors, *avrE1*, *hopN1*, *hopR1*, *hopS2*, *hopAE1*, *hopAH1*, *hopAS1* and *hopAZ1* were representatively cloned from V-13 alone.

The brief screening scheme describing the characterization procedure of *Psa* effectors in *Nicotiana* spp. is shown in Fig. 3.2.1.1. In order to generate binary constructs for transient expression of the 47 selected *Psa* effectors *in planta*, the Golden Gate cloning method was used (Engler et al., 2008). Briefly, each *Psa* effector was divided into several modules of roughly 1 kb, polymerase chain reaction (PCR)-amplified with primers with flanking restriction enzyme *BsaI* sites and cloned into pICH41021 (modified pUC19 carrying mutated *BsaI* site). The number of modules for each effector and *BsaI* 4bp overhangs are listed in (Table 2.5.1.2.1.1.). Modules for each effector were assembled with a C-terminal YFP tag module into binary vector pICH86988 under a cauliflower mosaic virus (CaMV) 35S promoter and transformed into *A. tumefaciens* AGL1. The resulting *A. tumefaciens*-mediated transient expression library of *Psa* effectors was used throughout this study (Figure 3.2.1.1.).

Table 3.2.1.1. List of type III effectors from *Psa* V-13 and LV-5 used in this study

Psa effector	*Psa* V-13	*Psa* LV-5	Amino acid Identity (%)	Predicted Protein size (kDa)
AvrB4-1	o			36.0
AvrD1	o			34.6
AvrE1	o	o	97.1	195.3
AvrPto5	o			17.2
AvrRpm1	o			24.9
HopA1	o(ST)	o		42.2
HopD1	o			34.6
HopE1		o		24.1
HopF1		o		21.9
HopF2	o			22.0
HopH1	o			24.3
HopI1	o			49.3
HopM1	o			75.9
HopN1	o	o	99.6	38.7
HopO1		o		32.6
HopQ1	o			48.8
HopR1	o	o	99.0	210.3
HopS1		o		19.9
HopS2	o	o	94.9	18.7
HopT1		o		41.5
HopW1	o(ST)	o		82.9
HopX1		o		40.6
HopX2		o		38.0
HopF4b (HopX3)	o			40.9
HopY1	o	o		30.9
HopZ3	o			45.1
HopAA1	o(ST)	o		50.5
HopAB3		o		62.6
HopAE1	o	o	99.8	126.6
HopAF1	o			23.4
HopAF1-2	o			30.9
HopAG1		o		77.6
HopAH1	o	o	93.1	42.8
HopAI1^{+}	o	o	81.6	29.7
HopAM1-1	o			31.3
HopAO2	o			38.1
HopAR1		o		28.5
HopAS1	o	o	98.9	149.0
HopAU1	o			88.2
HopAV1	o			123.5
HopAW1	o			24.9
HopAY1 *	o	o	91.9	27.1(V-13)/35.4(LV-5)
HopAZ1	o	o	99.5	24.7
HopBB1-2	o			30.7

ST: Effectors which are highly truncated

*: *Psa* V-13 allele has shorter amino acid sequence than in *Psa* LV-5

+: Amino acid identity between alleles from *Psa* V-13 and LV-5 is less than 90%

O: Existence of the effector allele

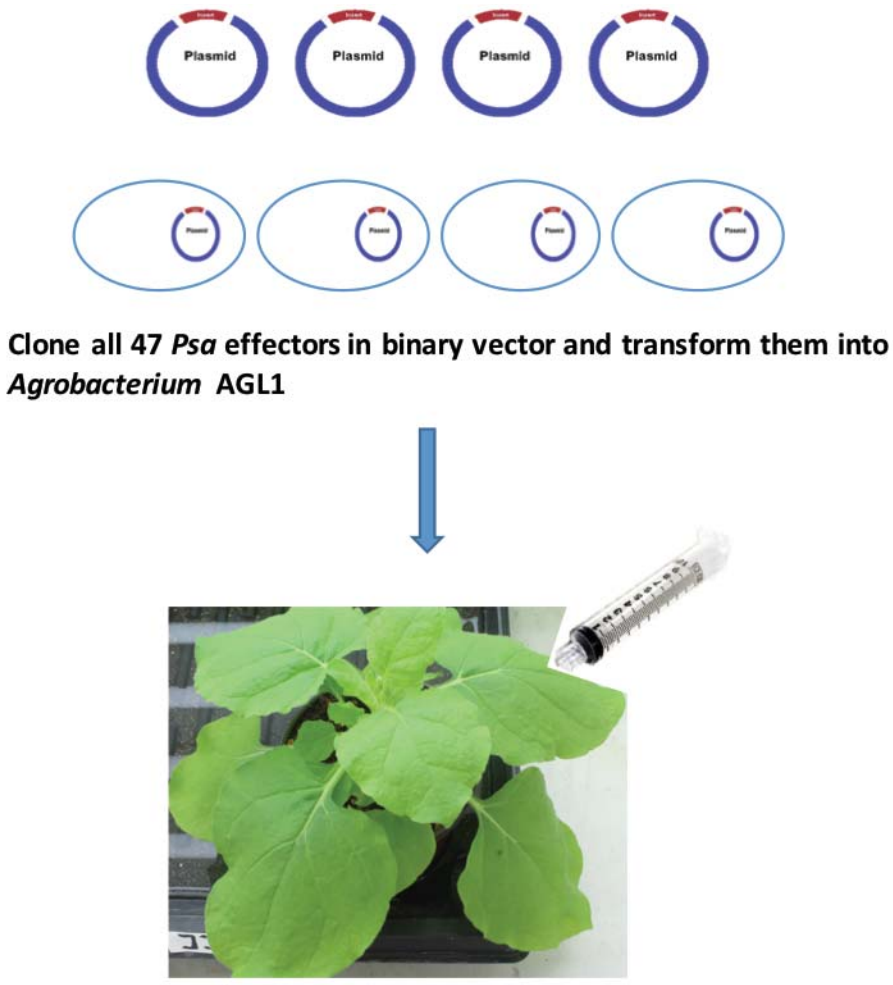


Figure 3.2.1.1. Schematic procedure of characterizing *Psa* effectors.
C-terminally YFP-tagged 47 *Psa* effectors were cloned into a binary vector pICH86988 (blue circles), mobilized into *Agrobacterium* AGL1 and transiently expressed in *N. benthamiana* or *N. tabacum* via *Agrobacterium*-mediated transient transformation for characterization.

3.2.2 Subcellular localization of *Psa* effectors in *N. benthamiana* leaf epidermal cells

As a step towards understanding *Psa* effector functions, *Psa* effector subcellular localization was examined. Each *Psa* effector was expressed in five-week old *N. benthamiana* leaf cells by *Agrobacterium*-mediated transient gene expression (hereafter, agroinfiltration) and subcellular localization of C-terminally YFP-tagged *Psa* effectors were analysed using confocal microscopy at 48 hours after infiltration (hpi) (Fig. 3.2.2.2). The summary of subcellular localization of *Psa* effectors is shown in Table 3.2.2.1. 23 effectors showed nucleocytoplasmic localization. Of these, 4 localized to the nucleolus while the remaining 19 were excluded from nucleolus (Fig. 3.2.2.2, Table 3.2.2.1). Only one effector, HopBN1, showed nuclear localization without any visible signal in the cytoplasm. In addition, 7 effectors localized in the cytoplasm but were excluded from the nucleus. HopM1 localized to chloroplasts, HopBB1-2 localized to endomembrane structures and in subnucleolar-foci and two effectors, HopAV1 and HopAZ1, localized to the cytoplasm but in strands resembling the cytoskeleton. Ten effectors localized to the cell periphery (largely absent from cytoplasmic strands), and two of them (HopT1 and AvrE1) showed punctate localization. As expected, these results showed that bacterial effectors can localize to different compartments of the host cell.

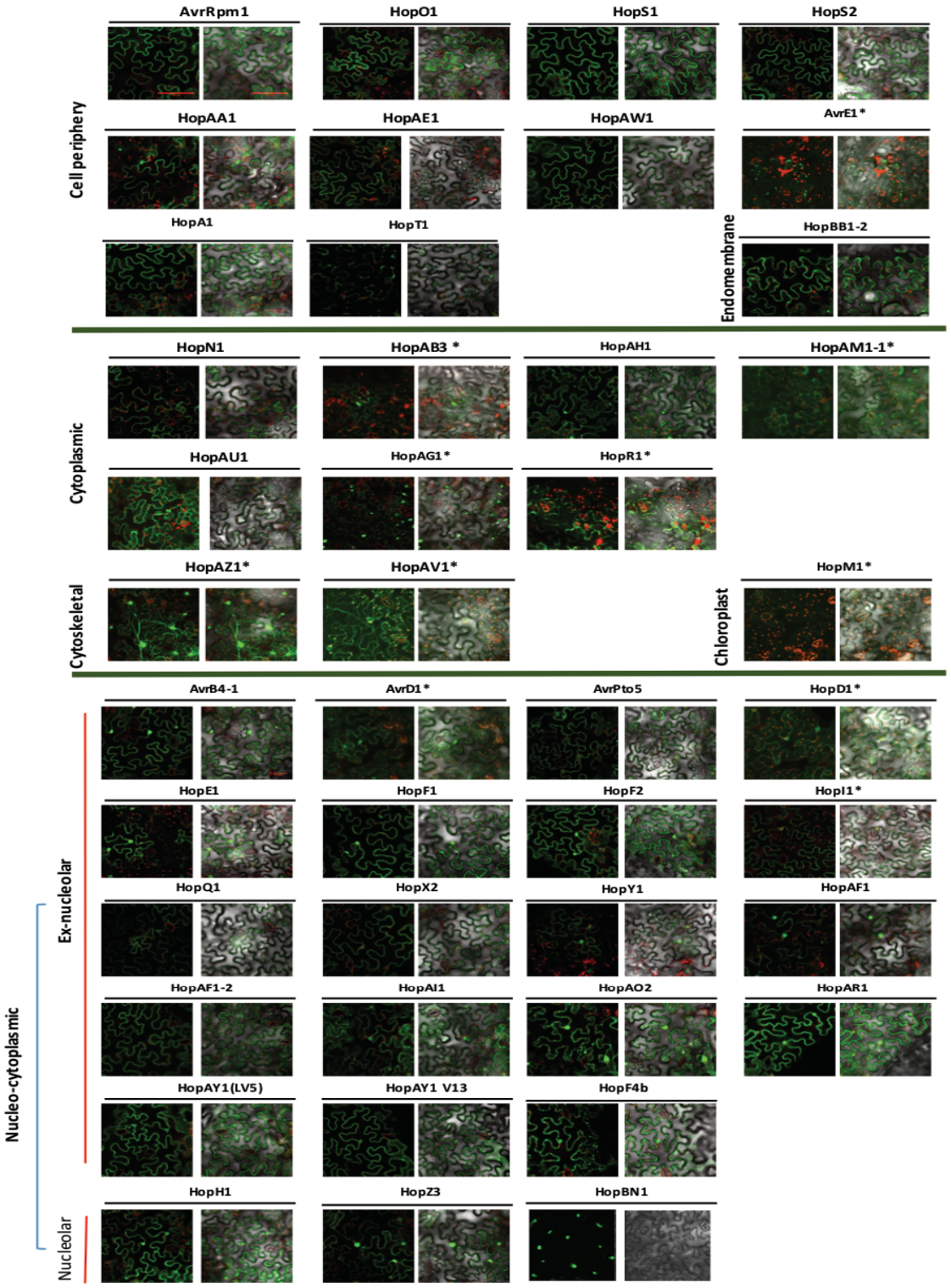


Figure 3.2.2.2. Subcellular localization of *Psa* effectors.

Psa effectors were transiently expressed in *N. benthamiana* leaf cells. 2 days after agro-infiltration, 2-3 8mm diameter leaf disc were sampled and viewed using confocal microscopy. The right panel shows the YFP signal and the left panel shows the merged image of YFP signal with the bright field. Scale bar indicates 50uM and is only present in AvrRpm1 as the same magnification was applied for all effector pictures. YFP fluorescence was excited at 488nm with a 20mW Argon laser and captured by the confocal channel in

the emission range between 500 and 530nm. An asterisk indicates effectors which are maximum projected. Chloroplast autofluorescence was excited at 543nm and detected at LP 560nm. This experiment has been conducted once.

Table 3.2.2.1. Summary of subcellular localization of *Psa* effectors in *N. benthaiana* leaf cells

Sub-cellular localization	*Psa* effectors
Cell periphery	AvrRpm1, HopAA1, HopAE1, HopAW1, HopO1, HopS1, HopS2, HopT1, AvrE1, HopA1
Cytoskeleton	HopAV1, HopAZ1
Endomembrane	HopBB1-2
Cytoplasm	HopAB3, HopAH1, HopAM1-1, HopAU1, HopN1, HopAG1, HopR1
Chloroplast	HopM1
Nucleus and cytoplasm (ex-nucleolus)	AvrB4-1, AvrD1, AvrPto5, HopAF1, HopAF1-2, HopAO2, HopAR1, HopAY1 (LV-5/V13) , HopD1, HopE1, HopF1, HopF2, HopI1 , HopQ1, HopX2, HopY1, HopF4b(HopX3), HopAI1(LV-5/V-13) HopH1, HopZ3
Nucleus	HopBN1

In order to confirm expression of *Psa* effector-YFP fusion proteins, effector-YFP proteins were transiently expressed in *N. benthamiana* leaf cells using the agroinfiltration method. Crude protein extracts were subjected to immunoblot analysis using anti-GFP antibodies. Out of 47 effectors tested, protein expression of 37 were confirmed (Fig. 3.2.2.3.) However, expression of 10 effectors (HopAM1-1, AvrE1, HopR1, HopAS1, HopI1, HopBN1, AvrD1, HopAV1, HopT1 and HopW1) could not be detected in our experimental conditions including HopX1, which was detected using confocal microscopy (Fig 3.2.2.2. and Fig 3.2.2.3.)

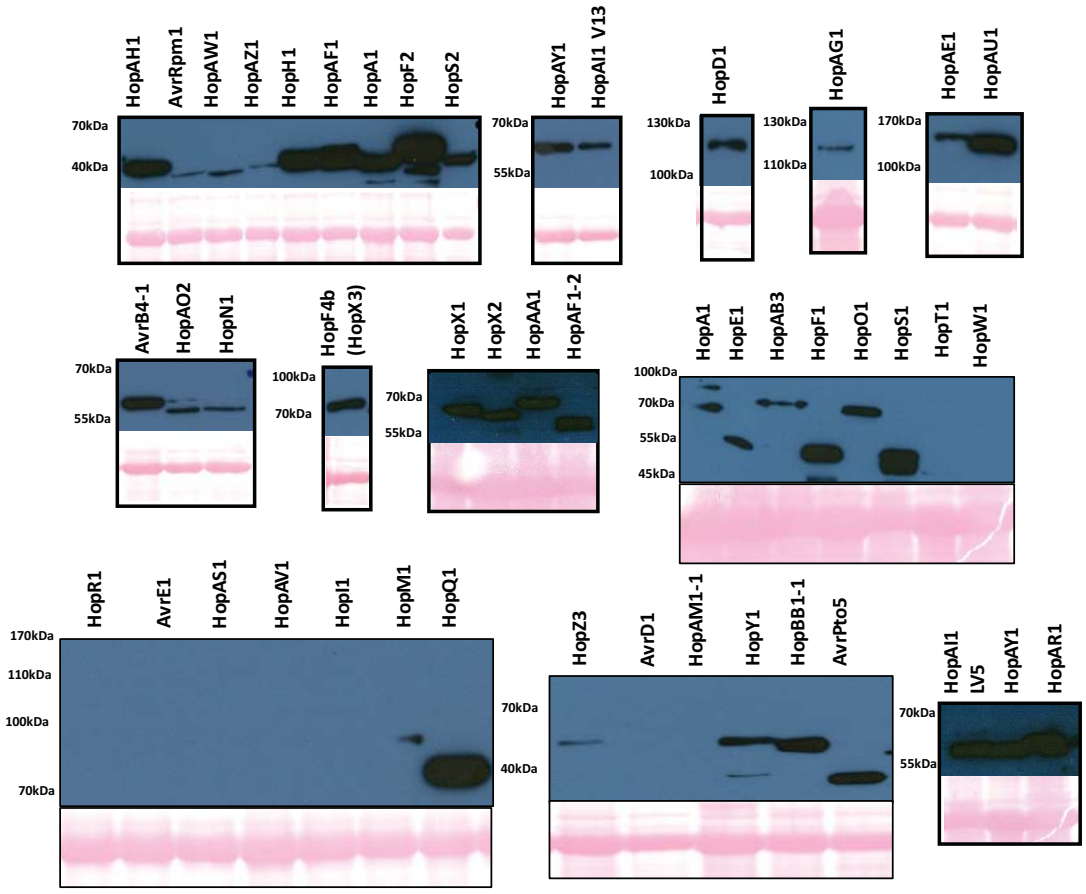


Figure 3.2.2.3. Immunoblot analysis of the C-terminally YFP-tagged *Psa* effectors in *N. benthamiana*

C-terminally YFP-tagged *Psa* effectors were transiently expressed in *N. benthamiana* leaves with P19. Total protein was separated by SDS-PAGE and probed with anti-GFP-HRP conjugated antibodies. The bottom panels show S-ponceau-red staining of Rubisco for loading control. This experiment has been conducted 2 times with similar results.

3.2.3 Multiple *Psa* effectors induce HR-like cell death in *Nicotiana* spp.

In order to identify *Psa* effectors that trigger HCD in *N. benthamiana* and *N. tabacum, Psa* effectors were transiently expressed by agroinfiltration in *N. benthamiana* and *N. tabacum* and HCD was scored.

Five-week-old *N. benthamiana* and *N. tabacum* leaves were used for agroinfiltration and HCD was scored at 2-4 days post infection (dpi). Agroinfiltration of each of the 17 *Psa* T3Es induced HCD in *N. tabacum.* Among these, 9 effectors also triggered HCD in *N. benthamiana* (Fig.3.2.3.1.). Interestingly, none of the tested *Psa* effectors triggered *N. benthamiana*-specific HCD. In *N. tabacum,* among the 17 T3Es that induced cell death, 16 triggered full collapse of the infected tissue (indicated as ++ in Fig.3.2.3.1) whereas one effector, AvrE1 triggered partial HCD (+ in Fig.3.2.3.1.) at 3- 4 dpi. In *N. benthamiana,* 7 effectors induced full collapse of the infected tissue and 3 induced partial patches of collapse at 4 dpi (Fig.3.2.3.1.). It is interesting to note that 7 effectors induced HCD only in *N. tabacum* but not in *N. benthamiana*. Also, HopAB3 and HopT1 triggered strong HCD in *N. tabacum* but a weaker response in *N. benthamiana.*

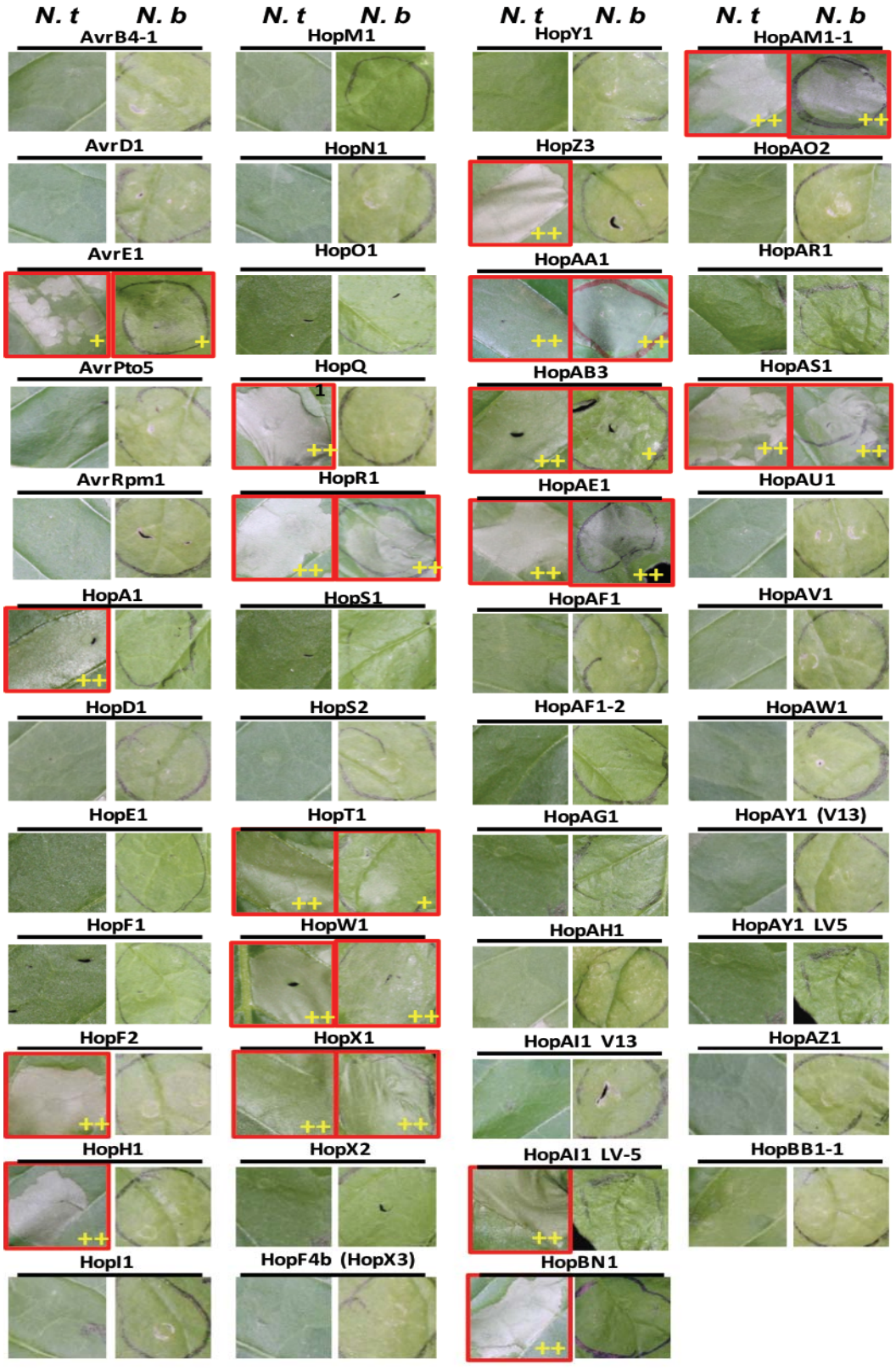


Figure 3.2.3.1. Multiple *Psa* effectors trigger HR-like cell death in *N. tabacum* and *N. benthamiana.*

C-terminally YFP tagged *Psa* effectors were transiently expressed in *N. benthamiana* and *N. tabacum* leaves. HCD was scored and photographed at 3 days after agro-infiltration. HCD triggering effectors are indicated with a red box. One plus (+) indicates punctate spots of HR and two pluses (++) indicate full collapse of

the infiltrated patch. This experiment has been conducted 7 times with similar results.

Different *Pseudomonas* strains share effector homologues. In order to see how these effector homologues are functionally conserved in triggering HCD in *Nicotiana* spp., HCD triggered by *Psa* effector homologues from other *Pseudomonas* strains were compared to that of *Psa* effectors based on previously published literature. Table 3.2.3.1. shows the HCD of *Psa* effectors in *N. benthamiana* and *N. tabacum* from this study and that of effector homologues from other *Pseudomonas* strains with their shared amino acid sequence identity (%). As *Pto* DC3000 is a well characterised model strain and shares 23 effector homologues with *Psa*, the cell death phenotype of many effector homologues from this strain was compared to *Psa* effectors. Out of 23 effector homologues from *Pto* DC3000, 19 effector homologues whose HR/HCD-inducing function was reported were included (Wei et al., 2007; Wroblewski et al., 2009).

Table 3.2.3.1. Cell death-inducing activities of published *Psa* effector homologues

Published effector homologues in other *Pseudomonas* strains	Strain	Cell death	Cell death phenotype of *Psa* effectors	AA identity (%) with *Psa* homologues	Reference
		N.b, / N.t	N.b, / N.t		
AvrB4-1	*Pph* 1448A	- / NA	- / -	49.2	1
AvrE1	*Pto* DC3000	+ / NA	+ / +	94.1	2
AvrRpm1	*Pma* M2	- / NA	- / -	59	1
HopA1	*Pto* DC3000	- / NA	- / +	93.9	2
HopD1	*Pto* DC3000	- / NA	- / -	98.6	2
HopE1	*Pto* DC3000	- / NA	- / -	99.1	2
HopF2	*Pto* DC3000	- / +	- / +	57.6	2,3
HopH1	*Pto* DC3000	- / NA	- / +	97.7	2
HopI1	*Pto* DC3000	- / NA	- / -	85.1	2
HopM1	*Pto* DC3000	+ / NA	- / -	66.9	2
HopN1	*Pto* DC3000	- / NA	- / -	97.7	2
HopO1-1[a]	*Pto* DC3000	- / NA	- / -	72.2	2
HopQ1-1[b]	*Pto* DC3000	+ / NA	- / +	99.1	2
HopR1	*Pto* DC3000	- / NA	+ / +	95.9	2
HopT1-1[c]	*Pto* DC3000	+ / NA	+ / +	70.4	2
HopW1-1[d]	*Pma* ES4326	+ / NA	+ / +	77.8	2
HopX1	*Pto* DC3000	+ / NA	+ / +	73.7	2
HopY1	*Pto* DC3000	- / NA	- / -	94.1	2
HopZ3	*Psy* B728A	- / NA	- / +	72.1	4
HopAA1-1[e]	*Pto* DC3000	+ / NA	+ / +	93.8	2
HopAB3	*Pto* T1	- / NA	+ / +	83.5	5
HopAF1	*Pto* DC3000	- / NA	- / -	82.2	2
HopAI1	*Pto* DC3000	- / NA	- / -	80.8	2
HopAM1*	*Pto* DC3000	- / NA	- / -	98.9	2

* Both *Psa* and *Pto* DC3000 have identical two copies of HopAM1 (HopAM1-1/HopAM1-2).

[a] *(Psa* HopO1 shares 95.6% of amino acid sequence identity with *Pto* DC3000 HopO1-2 and 53% with *Pto* DC3000 HopO1-3'*)*

[b] *(Pto* DC3000 HopQ1-2 is highly truncated (Buell et al., 2003*))*

[c] *(Psa* HopT1 shares 96.9% of amino acid sequence identity with *Pto* DC3000 *HopT1-2)*

[d] *(Psa* HopW1 shares 59.6% of amino acid sequence identity with *Pma* ES4326 *HopW1-2)*

[e] *(Psa* HopAA1 shares 77.2% of amino acid sequence identity with *Pto* DC3000 HopAA1-2 which does not trigger HR-like cell death in *N. benthamiana (Wei et al., 2007).*

References 1. (Wroblewski et al., 2009) 2. (Wei et al., 2007) 3. (Robert-Seilaniantz et al., 2006) 4. (Lee et al., 2015) 5. (Lin et al., 2006)

Abbreviations; Pto: Pseudomonas syrinagae pv. tomato, Pma: Pseudomonas maculicola, Pph: Pseudomonas phaseolicola, Psy: Pseudomonas syringae pv. syringae.

In *Pto* DC3000, there are two copies or more of HopO1 (-1, -2 and -3'), HopT1 (-1 and -2) and HopAA1 (-1 and -2). *Psa* HopO1 shared the highest amino acid sequence identity with *Pto* DC3000 HopO1-2 (95.6%) and *Psa* HopT1 with *Pto* DC3000 HopT1-2 (96.9%) (Table 3.2.3.1.). However, only HopO1 to HopO1-1 and HopT1 to HopT1-1 were compared because the HCD phenotype was only available for these homologues. *Psa* HopO1 and *Pto* DC3000 HopO1-1 (72.2%) did not induce cell death in *N. benthamiana,* and *Psa* HopT1 and *Pto* DC3000 HopT1-1 (70.4%) triggered cell death in *N. benthamiana* (Table 3.2.3.1.). *Psa* HopAA1 shares more amino acid sequence identity with *Pto* DC3000 HopAA1-1 (93.8%) than HopAA1-2 (77.2%), but HopAA1 failed to induce cell death in *N. benthamiana* much like HopAA1-2. (Wei et al 2007). In contrast, *Pto* DC3000 HopF2 and *Psa* HopF2 share relatively low amino acid sequence identity (57.6%), but both trigger cell death in *N. tabacum* and not in *N. benthamiana.*

In addition to *Pto* DC3000, effector homologues from different *P. syringae* strains were compared to *Psa* effectors (Lin et al., 2006; Wroblewski et al., 2009). *Pto* T1 HopAB3 (formally known as AvrPtoB) can suppress the Pto-mediated defence response in *N. benthamiana* (Lin et al., 2006) and shares high amino acid identity with *Psa* HopAB3 (84.5%). However, unlike HopAB3 from *Pto* T1, agroinfiltration of *Psa* HopAB3 induced HCD in *N. benthamiana* and *N. tabacum. Pto* DC3000 HopQ1-1 and HopR1 are nearly identical to *Psa* HopQ1 and HopR1 (99.1% and 95.9%), respectively, but only *Pto* DC3000 HopQ1-1 and *Psa* HopR1 trigger cell death in *N. benthamiana* (Table 3.2.3.1.). In contrast, *Pto* DC3000 HopF2 and *Psa* HopF2 share relatively low amino acid sequence identity (57.6%), but both trigger cell death in *N. tabacum.*

3.2.4 HR-like cell death induced by multiple *Psa* effectors partially requires *SGT1* in *Nicotiana benthamiana*

Significant reduction in gene expression of key components of plant immune response by virus-induced-gene silencing (VIGS) has been widely used to study effector-triggered defence signalling pathways (Liu et al., 2002; Peart et al.,

2002). EDS1*,* a eukaryotic lipase homologue, is known to regulate salicylic acid (SA) signalling in several TNL-mediated defence pathways by forming a complex with two other defence regulators, PAD4 and SAG101 (Zhu et al., 2011). NDR1 is a known component in several CNL-mediated immune signalling pathways independent of EDS1 (Aarts et al., 1998). SGT1 is a conserved regulator of various biological processes in eukaryotes together with HSP90 and is reported to be required for recognition by both CNLs and TNLs (Takahashi et al., 2003; Swiderski et al., 2009).

To test the requirement of these general immunity regulators for *Psa* effector-triggered HCD in *N. benthamiana*, VIGS was applied to generate the *N. benthamiana* plants silenced for *EDS1* (*TRV2:NbEDS1), NDR1 (TRV2:NbNDR1), SGT1 (TRV2:NbSGT1)* or empty vector control (*TRV2:EV)*. There were no abnormal morphological changes in *TRV2:EDS1* and *TRV2:NDR1 as* compared to *TRV2:EV.* However, *TRV2:SGT1* plants were severely stunted as shown previously (Peart et al., 2002). Semi-quantitative RT-PCR analysis confirmed that the level of *NbEDS1, NbSGT1,* and *NbNDR1* transcriptional expression was significantly reduced in the corresponding silenced plants as compared to control plants (Fig. 3.2.4.2.).

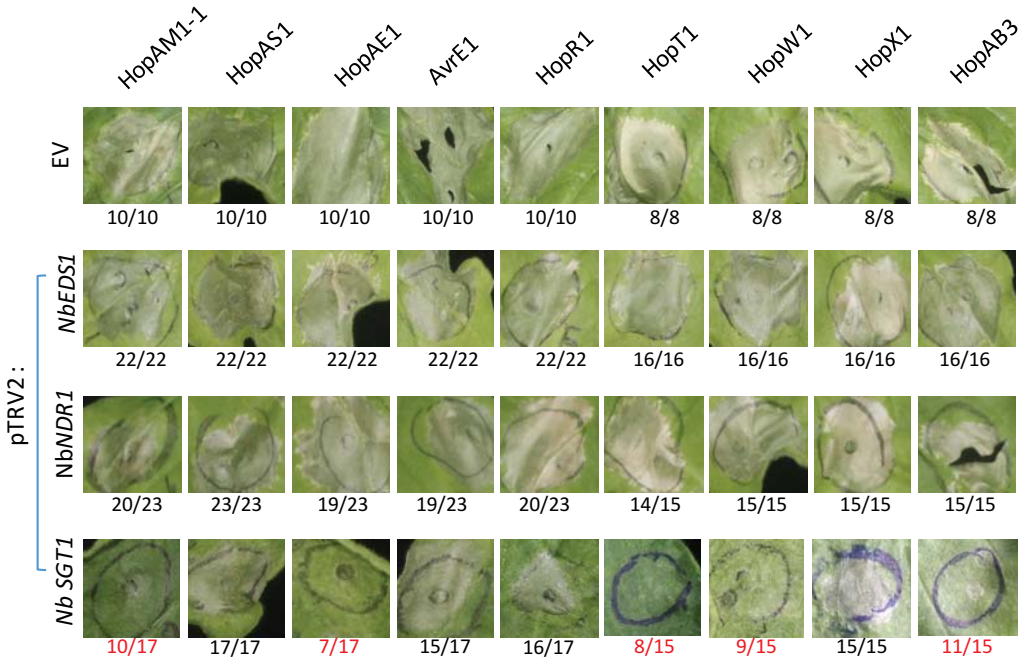


Figure 3.2.4.1. *NbSGT1* is partially required for HR triggered by multiple *Psa* effectors.

Psa effectors that trigger HCD in *N. benthamiana* were transiently expressed in *NbEDS1, NbNDR1* and *NbSGT1* virus-induced silenced *N. benthamiana.* HR was scored and photographed 4-5 days after agro-infiltration. The number of HCD showing patches out of total infiltrated patches are indicated under each picture. The number is colored red when effectors showed ~50% reduction in the number of HCD patches. This experiment has been conducted for 5 times with similar results.

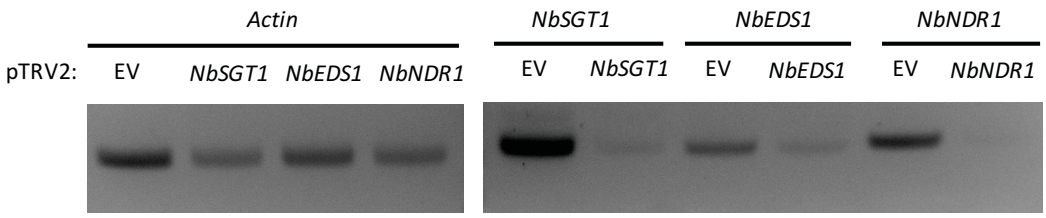


Figure 3.2.4.2. Confirmation of virus-induced gene silencing in *N. benthamiana*

Semi-quantitative PCR amplification of virus-induced gene silenced plants.
Total RNA was extracted by the Trizol RNA extraction method from 8mm diameter leaf discs. The *actin* gene was amplified to normalize the amount of total RNA and *NbEDS1, NbNDR1* and *NbSGT1* genes were amplified from EV and silenced plants with gene-specific PCR primers. This experiment has been conducted 3 times with similar results.

Nine effectors, HopAM1-1, HopAS1, HopAE1, AvrE1, HopR1, HopT1, HopW1, HopX1 and HopAB3 that triggered cell death in wild-type *N. benthamiana* were infiltrated into *NbEDS1-, NbNDR1*- and *NbSGT1*-silenced plants. None of the effector-triggered HCD responses were abolished in *NbEDS1*-silenced plants as compared to EV control plants (Fig. 3.2.4.1.). Interestingly, none of the effector-triggered HCD responses were significantly abolished in *NbEDS1*– or *NbNDR1*–silenced plants compared to EV-silenced plants (Fig.3.2.4.1.). On the other hand, in *NbSGT1*-silenced plants, 5 effectors, HopAM1-1 (7/17), HopAE1 (10/17), HopT1 (7/15), HopW1 (6/15) and HopAB3 (4/15) failed to trigger cell death. In contrast, HopAS1 (17/17), HopX1 (15/15) and HopR1 (16/17) triggered cell death consistently and reproducibly. This suggests that *SGT1* but not *NDR1* or *EDS1* could be a key component in HCD induced by several *Psa* effectors.

3.3 Discussion

Type III effectors are well known to be critical for successful pathogen infection in plants. It is not surprising that there is great enzymatic and functional diversity in effectors because of high evolutionary pressure from the plant immune system on pathogens. Non-host plants still stay healthy after pathogen infection and recent studies show effector recognition plays an important role in non-host resistance (Sohn et al., 2012; Wei et al., 2007). *Psa* causes kiwifruit canker disease in worldwide orchards and each clade seem to arise independently from geographically different source populations. Therefore, it is meaningful to study different *Psa* strains from the same source as they have a high chance of genetic interchange. Also, in this study, homologous *Psa* effectors and effectors from other *P. syringae* strains were compared and characterized.

An *Agrobacterium*-mediated transient gene expression assay was applied to establish a profile of *Psa* V-13 and LV-5 effectors' ability to induce HCD in non-host *Nicotiana* spp. together with subcellular localization *in planta* and requirement of known defence regulators. Agroinfiltration of each *Psa* effector-YFP protein was validated by an immunoblot assay. 10 effectors out of 47 effectors could not be

detected in our experimental conditions. Among these 10 effectors, agroinfiltration of each of six effectors (AvrE1, HopR1, HopT1, HopW1, HopAM1-1 or HopAS1) triggered HCD in *N. benthamiana.* Therefore, it is plausible that detection failure of these effectors might be linked to cell death-inducing activity (Fig. 3.2.2.3.). However, the remaining 4 effectors (HopI1, HopBN1, AvrD1 and HopAV1) whose expression could not be detected did not induce HCD. Although none of these 4 effector homologues have been reported to be cleaved *in planta,* it is possible that these effectors are cleaved or unstable in *N. benthamiana.* Interestingly, AvrE1, HopR1, HopBN1, HopI1, HopAM1-1, HopAV1 and HopT1 were detected by confocal microscopy but not in the protein expression assay. As those protein sizes are all above ~80 kDa, it is possible that our experimental procedure was not optimized for large proteins. HopW1 and HopAS1 could not be detected in either the protein expression assay or in the confocal assay possibly due to too low protein expression.

3.3.1. Diverse localization of effectors implicates their various targets in the host cell

Localization of an effector *in planta* implies the possible host target protein location. For example, membrane localized effectors such as AvrRpt2, AvrRpm1 and AvrB target membrane associated host immunity regulator RIN4 while nuclear localized effector PopP2 targets the corresponding NLR, RRS1, inside the nucleus (Axtell and Staskawicz, 2003; Eitas et al., 2008; Deslandes et al., 2003). Through the large-scale screening of sub-cellular localization of *Psa* effectors shown in this chapter, it is concluded that the nucleus and membrane are major target compartments for *Psa* effectors in host cell.

Several studies have shown that effectors can cause transcriptional re-programming of the host cell by directly interacting with DNA in the nucleus (Kay and Bonas, 2009; Nissan et al., 2006). It is therefore not surprising that nearly half of the *Psa* effectors (23 out of 47 effectors) are capable of localizing to the nucleus.

Since the majority of PRRs are localized at the cell surface, many effectors target the plasma membrane to interfere with host defence components accordingly (Zhou et al., 2014; Kim et al., 2005). Recently, an increasing amount of evidence is accumulating that supports the theory that an effector may target multiple host components. This could be the reason for the existence of a large number of nucleocytoplasmic effectors. *P. syringae* effector HopZ3 is known to target multiple host molecules in the nucleus and cytoplasm (Lee et al., 2015). As many effectors used in this study are larger than 50 kDa including the C-terminal YFP tag, which may not be permeable through the nuclear membrane without a nucleus localization signal (NLS), these nucleocytoplasmic effectors need a special signal to be imported and exported from the nucleus (Shimozono et al., 2009). HopZ3 from *P. syringae* pv. *syringae* B728a (approximately 76kDa including the YFP tag; 72% amino acid identity to *Psa* HopZ3) showed nucleo-cytoplasmic localization targets MPK3 and MPK6, while HopBB1 from *P. syringae* pv. *mori* 301020 (approximately 60kDa including the YFP tag; 93% amino acid identity to *Psa* HopBB1-2) localizes to the nucleus and targets TCP14 and JAZ3 for degradation. However, neither of these effectors possesses a well-defined NLS (Lewis et al., 2014; Yang et al., 2017). There is also a significant possibility that the YFP tag used for these effectors could cleave off the proteins *in planta* and be directed through diffusion into the nucleus (Seibel et al., 2007).

The plasma membrane houses complexes of cellular receptors involved in PTI, so effectors that can be expected to target membrane-associated host barriers to interfere with host cell defence would thus localize there accordingly (Kim et al., 2005; Zhou et al., 2014). Increasing evidence supports the theory that an effector may target more than one host molecule, which gives further credence to the large number of nucleocytoplasmic effectors. For example, the aforementioned *P. syringae* pv. *syringae* B728a effector HopZ3 targets host molecules at the plasma membrane (RIN4), cytoplasm (cytoplasmic kinases) and nucleus (Lee et al., 2015).

A future application of findings from this study will be to examine *Psa* effector function in relation to localization. HopZ1a and HopW1 are also known to target the cytoskeleton to disrupt PTI (Kang et al., 2014; Lee et al., 2012). Thus, it would be interesting to investigate functions of HopAZ1 or HopAV1 that both

appear to target the host cell cytoskeleton. A *hopAZ1* homolog from *P. savastanoi* pv. *savastanoi* NCPPB3335 was recently reported to suppress both ROS production and callose deposition, critical markers of PTI (Matas et al., 2014). *hopAZ1* is present in all sequenced strains of *Psa* and appears to be a 'core effector' (McCann et al., 2013). Interestingly, *hopAZ1* was reported to have been independently acquired three times into the effector repertoire of pathogens of hazelnut, *P. syringae* pv. *avellenae* that are from two different phylogroups of *P. syringae* strains, suggesting this effector is closely associated with a gain of virulence in this woody host (O'Brien et al., 2012). Taken together, our findings that HopAZ1 from *Psa* V13 localizes to the cell cytoskeleton suggests a host target that participates in PTI or other types of plant defence operated in this compartment. Identification of HopAZ1-interacting proteins in the cytoskeleton may help reveal the role of HopAZ1 in promoting host-specific pathogen virulence.

As many effectors localize to nucleus, cytoplasm or plasma membrane, this may imply that there are more host cellular machineries involved in plant defence. Also, as effectors localize to various host cellular compartments, it is possible for these effectors to have a synergistic effect on defeating the host cell defence systems. Although many research efforts have been highly concentrated on investigating the virulence function of effectors in plant defence, there is emerging evidence of other effector functions for successful pathogen proliferation. For example, *Xanthomonas* transcript-activator-like effectors (TALEs) PthXo1 and AvrXa7 manipulate gene expression of sugar transporters for nutrient acquisition (Chen et al., 2010). Also, plant hormone signalling is manipulated by various effectors, which can influence many aspects of plant cells not only directly to defence (reviewed in (Macho, 2016). For example, *P. syringae* effector AvrRpt2 is able to alter auxin physiology (Chen et al., 2007), which can loosen cell walls and indirectly change the flow of water and nutrient towards the intracellular space (Mutka et al., 2013). Therefore, diverse effector localization also implies various functions of effectors in successful pathogenicity.

3.3.2. From HCD-triggering effectors to developing resistance in kiwifruit

Not all HCD triggered by agroinfiltration of effectors may be due to immune responses (Vinatzer et al., 2006; Wroblewski et al., 2009). For instance, HopT1-1 triggered HCD but does not appear to be associated with significant growth restriction in *N. benthamiana* (Wei et al., 2007; Wroblewski et al., 2009). Conversely, *P. syringae* pv. *tomato* DC3000 lacking *hopQ1-1* can cause disease symptoms in the non-host *N. benthamiana* but agroinfiltration of HopQ1-1 does not result in significant cell death (Adlung and Bonas, 2017; Wei et al., 2007; Wroblewski et al., 2009). Utilization of VIGS to suppress T3E-triggered cell death can offer clues about the downstream mechanism of effector-triggered HCD. The requirement of known immunity-regulator genes would suggest that the effector-triggered HCD could be an immune response (Vinatzer et al., 2006). Wei and colleagues (2007), using VIGS, confirmed that *SGT1* was required for cell death triggered by *Pto*-delivered HopQ1-1. Similarly, the *Psy*B728a effector, HopAA1, partially contributes to growth restriction of this strain on *N. benthamiana* and requires *EDS1* for its HCD development (Vinatzer et al., 2006).

SGT1 is at least partially required for 5 *Psa* T3Es: HopAM1-1, HopAE1, HopT1, HopW1 and HopAB3. *hopAM1-1* is unique to *Psa* V13 while *hopT1*, *hopW1* and *hopAB3* are unique to *Psa* LV5. *hopAE1* alleles are present in both *Psa* strains. Interestingly, HopAM1$_{Pto\text{DC3000}}$ is associated with resistance in *Arabidopsis* accession Bur-0 (Iakovidis et al., 2016), while HopAB3, a homolog of AvrPtoB$_{Pto\text{DC3000}}$, is an avirulence effector in tomato (Lin et al., 2006). Unexpectedly, HopAS1-triggered cell death is not suppressed by *SGT1*-silencing despite it being noted as a avirulence determinant in *Arabidopsis* (Sohn et al., 2012). Interestingly, HopAS1 alleles (with 95.9% shared amino acid identity, Table 3) shared by *Psa* and *Pta* 11528, a virulent pathogen of *N. benthamiana*, suggest that it is not a likely candidate for contribution to NHR in *N. benthamiana* (Studholme et al., 2009). Cell death triggered by HopX1, AvrE1, and HopR1 were also largely unaffected by *SGT1*-silencing suggesting limited involvement in immune responses. Support for this

notion comes from previous observations suggesting that HopX1, AvrE1, and HopR1 trigger necrosis rather than HR (Badel et al., 2006; B. H. Kvitko et al., 2009; Lorang and Keen, 1995; Nimchuk et al., 2007). Further characterization of *Psa* T3Es that are responsible for NHR in *N. benthamiana* through gene knockouts in *Psa* would be useful for identification of avirulent effectors to map the *R* genes for *Psa.* According to our result, the strongest candidates would be HopAB3, HopAM1-1 and HopAE1.

Identification of *R*-genes involved in the recognition of effectors is challenging and laborious. Recently, however, two advances in the field have facilitated this process. Firstly, Brendolise and colleagues (2017) have developed a hairpin-RNAi library targeting NLRs in *N. benthamiana* that allows for rapid analysis of NLRs involved in the recognition of an effector. They have demonstrated that the hairpin library is not only efficient at identifying the sensor NLR involved (Prf in the case of AvrPto) but also helper NLRs as well. This system is highly amenable for our purposes and multiple NLRs may be identified for cloning and downstream analyses without first mapping the resistance loci involved.

The second technology is the development of RenSeq – a tool that capitalizes on the enrichment of specific NLR sequence(s) in a screened population of resistant plants (Jupe et al., 2013). This technology uses NLR enrichment and resequencing/reannotation to identify resistant alleles of NLRs even in crop plants that are still in the early stages of study. RenSeq and its derivative technologies have been used to identify candidate NLRs for multiple crop plants, including potato, tomato, and wheat (Jupe et al., 2013; Steuernagel et al., 2016; Witek et al., 2016). Application of RenSeq to identify tobacco and *N. benthamiana* NLRs for *Psa* effectors would necessitate identification of partners for these plants that lack recognition of the *Psa* effector in question. Then, following floral crosses and establishment of an F_2 segregating population, RenSeq may be deployed to identify the NLR that confers recognition of the *Psa* effector. This NLR may then be cloned for downstream deployment and analysis.

3.3.3. Comparative analysis of type III effectors from *Psa* and other *Pseudomonas strains*

Pseudomonas syringae strains share multiple effector homologues possibly by interchanging plasmids and homologous recombination (Guttman et al., 2014; Jackson et al., 2011). *Psa* shares 19 effector homologues with model strain *Pto* DC3000. Interestingly, among the 17 Psa effectors that trigger cell death in *N. benthamiana*, only three effectors, AvrE1, HopT1, and HopX1, are conserved in both strains. In contrast, while HopQ1-1 from *Pto* DC3000 triggered cell death in *N. benthamiana* and loss of *hopQ1-1* or *hopAD1* was sufficient to render this strain virulent (Wei et al., 2007, 2015), HopQ1 from *Psa* V-13 did not trigger HCD in our experiments. This suggests that the true host-range determinant effector(s) from *Psa* is likely a product of a complex dynamic between the *N. benthamiana R* gene repertoire and the pattern of expression for that effector *in vivo*.

In this study, a few *Psa* effectors localize differently from previously reported effector homologues. HopM1 from *Pto* DC3000 is known to target AtMIN7 (*Arabidopsis* HopM1 interactor 7), which is an ADP ribosylation factor-guanine nucleotide exchange factor (ARF-GEF) in *Arabidopsis* and destabilizes AtMIN7 in the trans-Golgi network and early endosome (Nomura et al., 2011). However, *Psa* HopM1 localizes to the chloroplasts in *N. benthamiana.* Possibly due to only 66.9% amino acid sequence identity between them, HopM1 homologues of *Psa* and *Pto* DC3000 seem to target different proteins. *Pto* DC3000 HopM1 is shown to be involved in proteasome signalling and interact with several E3 ligase domains (Nomura et al., 2011). However, previous findings have highlighted an alternative possibility since HopM1 was found to suppress SA-mediated immunity (DebRoy et al., 2004) as well as interact with 14-3-3 proteins to mediate pathogen virulence *in planta* (Lozano-Duran et al., 2014). This is particularly interesting since SA synthesis is primarily localized within the chloroplast and exported out by the multidrug and toxin extrusion-like transporter, EDS5, and 14-3-3 proteins have been associated with proteins targeted to the chloroplast (Sehnke et al., 2000; Serrano et al., 2013).

P. syringae effector HopD1 localizes to the endoplasmic reticulum with transcription factor NTL9 to compromise ETI in *Arabidopsis* (Block et al., 2014), but *Psa* HopD1 was distributed to the nucleus and cytoplasm despite high amino acid sequence similarity (98.6%). As the apparent expression level of *Psa* HopD1 under the confocal microscope was low, there is a possibility that the image with MAX projection misled *Psa* HopD1 localization. It is hard to distinguish endoplasmic reticulum localization and cytoplasmic localization without subcellular localization markers. Also, the cleavage of free YFP was not detected in an immunoblot assay; however, it is still possible that a small amount of free YFP was exaggerated by MAX projection in confocal microscopy. Also, *Pto* HopN1 and *Psa* HopN1 are 97.7% identical in amino acid sequence, but the former localizes in chloroplasts and interferes with photosynthesis (Rodríguez-Herva et al., 2012) while the latter localizes in the cytoplasm. HopAB3, the homologue of AvrPtoB, triggers cell death in *N. benthamiana* even though its closest homologue effector *Pto* T1 HopAB3, which is also cytoplasmic, is able to suppress Pto-mediated cell death in *N. benthamiana.* The amino acid identity is relatively high (83.5%), but those two effector homologues are differently recognized by the plant. Taken together, it appears that amino acid sequence identity alone cannot predict the cell death response by the plant, suggesting that different evolutionary pressures affect the dynamics of effector recognition by the plant against pathogens from different hosts. This may result from selective mutations of active residues in the effectors.

There are a large number of *Psa* effectors that are distributed to the nucleus and cytoplasm. In fact, there are examples of effectors targeting more than one cell compartment (Lee et al., 2015; Zhou et al., 2014; Lo et al., 2016), so there is a possibility that these effectors can also target multiple plant components.

Chapter 4 Identification of SOBER1 (Suppressor of AvrBsT-elicited Resistance 1) as the Suppressor of HopZ5-triggered Immunity in *Arabidopsis thaliana*

4.1 Objectives

Identification of *R* genes that confer resistance to pathogens is an important step to understand how pathogen effectors and plant immunity intersect. The more we understand plant immunity against pathogens, the greater our ability to engineer robust resistance by accumulating *R* genes. *Pseudomonas syringae* pv. *actinidiae* is the causal agent of kiwifruit canker disease and a recent outbreak of *Psa* V has contaminated 90% of New Zealand orchards (www.kvh.org.nz/maps_stats). In order to understand the effector-triggered resistance mechanisms against *Psa* V-13, the model plant *Arabidopsis thaliana* was utilized to accelerate the research of *R* gene(s). We previously cloned 34 predicted full-length effectors from *Psa* V-13 and delivered them independently via *Pseudomonas fluorescens* Pf0-1 with an introduced type III secretion system (T3SS) in order to identify an avirulent effector in a resistant *Arabidopsis* accession Ct-1. From this screen, a predicted member of the YopJ family of acetyltransferases, HopZ5, triggered HR and defence responses in *Arabidopsis* Ct-1.

The YopJ family consists of various effectors with an acetyltransferase activity from plant and animal pathogens. The significant feature of the family is a conserved catalytic triad, which is required for function as discussed in chapter 1. In our previous study, HopZ5 was characterized to be an acetyltransferase, the function of which is dependent on a conserved catalytic cysteine residue (Jayaraman et al., 2017).

In this chapter, the aim was to identify the genomic locus that is responsible for HopZ5-triggered immunity in *Arabidopsis* Ct-1. The phenotypic difference of HR between *Arabidopsis* accessions Col-0 (No HR) and Ct-1 (HR) upon HopZ5 delivery was genetically analysed in an F_2 population. In order to map the

HopZ5-triggered HR-conferring locus, 166 publicly available recombinant inbred lines (RILs) between Ct-1 and Col-0 were used. RILs enable faster mapping via associating the HR phenotype of each RIL with their genotypes directly.

4.2 Results

4.2.1 The *Psa* effector HopZ5, a YopJ family acetyltransferase effector, triggers hypersensitive response in *Arabidopsis* Ct-1

We previously identified *Arabidopsis* accession Ct-1 as being resistant to *Psa* V-13. In order to identify the avirulent effector from *Psa* V-13, we screened 34 *Psa* V-13 effectors in *Psa* V-13-resistant *Arabidopsis* accession Ct-1 (Jayaraman et al., 2017). The promoter region of a well-studied *Pseudomonas syringae* pv. *pisi* effector *avrRps4* was used for T3S-dependent expression of 34 *Psa* effectors and delivery by *Pf* Pf0-1 (T3SS). This screen revealed one effector that triggered HR in *Arabidopsis* accession Ct-1 but not in Col-0, namely HopZ5, a member of the YopJ acetyltransferase family (Fig. 4.2.1.1.).

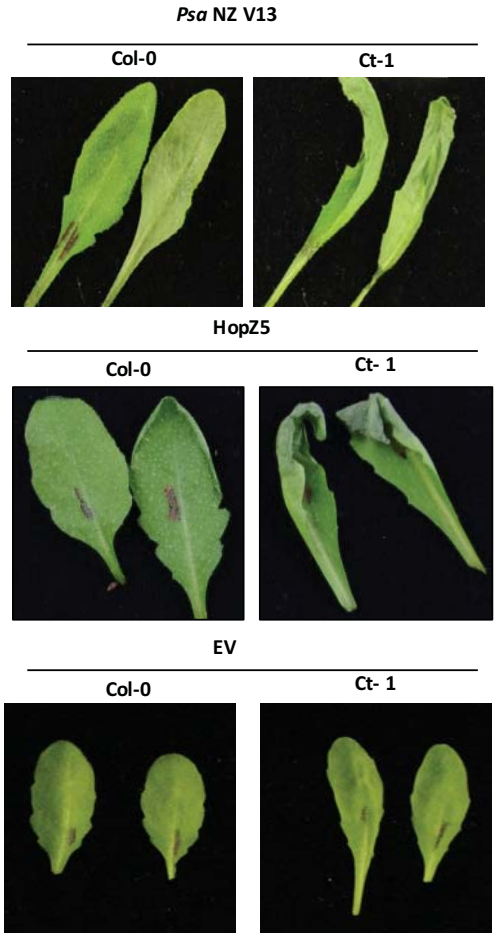


Figure 4.2.1.1. *Psa* NZ V-13 effector HopZ5, a member of acetyltransferase YopJ family, triggers hypersensitive response in *Arabidopsis* accession Ct-1 when delivered from the *Pseudomonas* type III secretion system

Psa V-13 triggers HR in *Arabidopsis* Ct-1 but not in Col-0. *Psa* V-13 was hand-infiltrated into fully developed leaves of five-week old *Arabidopsis* accession Col-0 and Ct-1 in 10mM $MgCl_2$ at OD600=0.2. HR was scored and photographed 1 day after infiltration. *Psa* V13 effector HopZ5, a member of acetyltransferase YopJ family triggers HR in *Arabidopsis* Ct-1. C-terminally HA-tagged HopZ5 was delivered into *Arabidopsis* leaves via *Pseudomonas fluorescens* Pf0-1 (T3SS). *P. fluorescens* was hand-infiltrated at OD_{600}=0.2 in 10mM $MgCl_2$. HR was scored and photographed 1 day after infiltration. This experiment has been conducted more than 3 times with similar results.

YopJ acetyltransferase family members share a conserved catalytic triad consists of Cys, His and Glu for their function (Ma and Ma, 2016). To test if HopZ5 also requires these conserved residues, site-directed mutagenesis of the conserved Cys residue was conducted. Cysteine 218, one of the catalytic triad residues, was mutated to alanine and this mutation completely abolished HopZ5 avirulence in *Arabidopsis* Ct-1 when delivered by the *Pf* Pf0-1 (T3SS) (Fig. 4.2.1.2.). In addition, mutation of lysine 278, the predicted autoacetylation residue, to arginine also attenuated HopZ5-triggered HR. Further analysis, including an ion leakage assay and an immunoprecipitation assay, supported the requirement of C218 and K278 in HopZ5 avirulence and autoacetylation (Jayaraman et al., 2017). Therefore, it is concluded that HopZ5 is an acetyltransferase YopJ family member and C218 and K278 were required for its catalytic activity and avirulence.

Figure 4.2.1.2. Conserved catalytic residue Cysteine218 and autoacetylation residue Lysine278 are required for avirulence activity of HopZ5 in *Arabidopsis* Ct-1

1 x 10^8 cfu/ml of *Pseudomonas fluorescens* Pf0-1 (T3SS) carrying pBBR-1MCS-5B:*avrRps4* promoter:*hopZ5 mutants (WT,C218A or K278R):HA,* pVSP61:*avrRpm1* and pBBR-1MCS-5B:*avrRps4* promoter (EV) were hand-infiltrated in 10mM $MgCl_2$. HR was scored and photographed 1 day after infiltration. This experiment has been conducted more than 3 times with similar results.

4.2.2 Genetic analysis of HopZ5-triggered HR

In order to analyse the genetic basis of HopZ5-triggered HR in *Arabidopsis*, accession Ct-1 (HR to HopZ5) and Col-0 (no HR to HopZ5), as well as Ct-1 and Ws-2 (no HR to HopZ5) were crossed to generate F_1 hybrids between these accessions. The HopZ5-triggered HR in F_1 hybrids and HR segregation in the F_2 populations were examined.

Both F_1 hybrids, Ct-1 x Col-0 F_1 and Ct-1 x Ws-2 F_1 did not show HR upon HopZ5 delivery via *Pf* Pf0-1 (T3SS) (Figure 4.2.2.1.). Those F_1 plants were allowed to self-pollinate for generation of F_2 population and HopZ5-triggered HR segregation analysis was carried out in the F_2 populations. In both F_2 population (Ct-1 x Col-0 and Ct-1 x Ws-2), HR segregated in a 1:3 = (HR):(No HR) ratio (19 HR to 55 No HR for Ct-1 x Col-0 and 30 HR to 88 No HR for Ct-1x Ws-2), which indicated that HopZ5-triggered HR was monogenic and recessive (Table 4.2.2.1.). The P value for both F_2 analysis chi-squared test results were greater than 0.05 (0.89 for Ct-1 x Col-0 (X^2 = 0.02) and 0.92 for Ct-1 x Ws-2 (X^2 = 0.01)), statistically supporting the 1:3 segregation ratio.

Table 4.2.2.1. Genetic analysis of HopZ5-triggered HR in F_2 hybrids

F_2	HR	No HR	n	X^2	P value
Ct-1 x Col-0	1(19)	3(55)	74	0.02	0.89
Ct-1 x Ws-2	1(30)	3(88)	118	0.01	0.92

n: Total number of tested population, X^2: Chi-squared test value

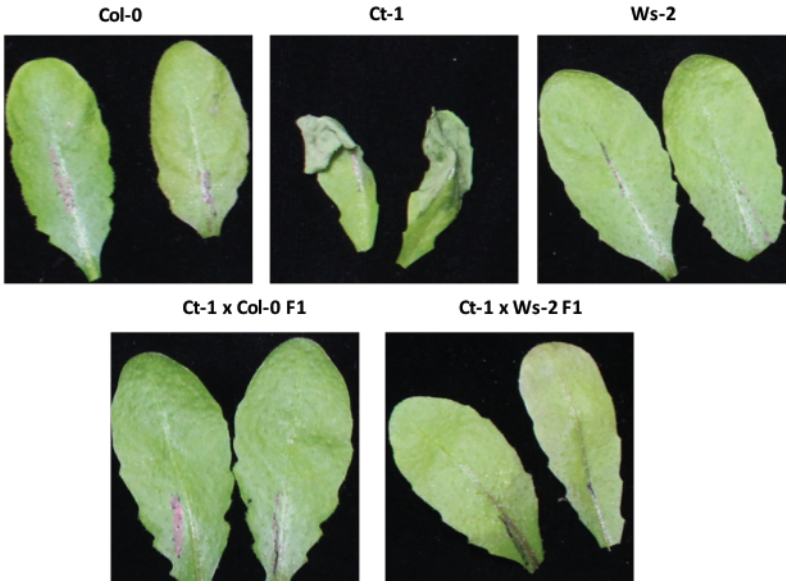


Figure 4.2.2.1. Phenotypic analysis of HopZ5-triggered HR in *Arabidopsis* wild-type accessions and F1 hybrids.
Leave of five-week old *Arabidopsis* F_1 hybrids between Ct-1 and Col-0 or Ct-1 and Ws-2 were infiltrated with 1 x 10^8 cfu/ml of *P. fluorecens* Pf0-1 (T3SS)s carrying pBBR-1MCS-5B:*avrRps4 promoter:hopZ5*:*HA*. HR was scored and photographed 1 day after infiltration. This experiment has been conducted more than 3 times with similar results.

4.2.3 The locus containing the suppressor of HopZ5-triggered HR harbours *SOBER1*.

Recombinant Inbred Lines (RILs) are powerful genetic tools for mapping genes. This system was employed, as RILs between Ct-1 and Col-0 were publicly available from the INRA *Arabidopsis* stock centre (France). Figure 4.2.3.1. introduces the construction of RILs between Ct-1 and Col-0 (Simon et al., 2008). Briefly, *Arabidopsis* accessions Ct-1 and Col-0 were crossed and the resulting F_1 plants were self-pollinated to generate an F_2 population. Around 500 F_2 plants were randomly selected and self-pollinated. A single seed (F_3) from each F_2 plant was randomly selected and processed to the next generation. 4 additional cycles were conducted to obtain individual F_7 populations and each line was genotyped. As a result, the phenotypes in the F_7 generation can be directly associated with genotyping data to obtain an approximate locus of the gene of interest.

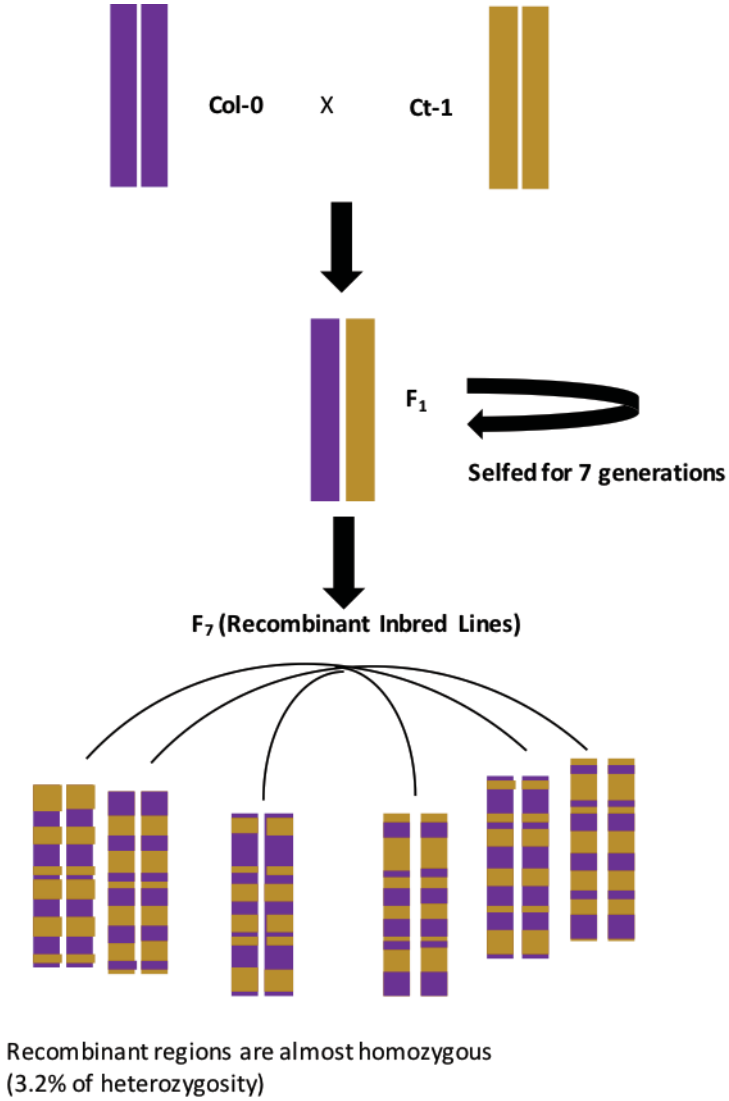


Figure 4.2.3.1. Schematic overview of construction of recombinant inbred lines used in this study.

Col-0 x Ct-1 F_1 was self-pollinated and generated F_2. Single seed was randomly selected from each ~500 Ct-1 x Col-0 F_2 plants and self-pollinated. Additional cycles of single-seed descent (SSD) were performed until F_7 seeds were generated (Simon et al., 2008). Purple indicates Col-0 DNA and brown indicates Ct-1 DNA. The F_7 graphic shows the homozygous recombinant regions in each F_7 individual.

In order to identify the recessive *R* gene that recognizes HopZ5 or the dominant suppressor of HopZ5-triggered HR, 119 Ct-1 x Col-0 RILs (F_8) were tested. 54 out of 119 RILs showed HR in response to Pf0-1(T3S)-delivered HopZ5. This is close to a 1:1 ratio (X^2 = 0.313, P = 0.42) corresponding to a single locus hypothesis from the F_2 analysis (Table 4.2.3.1.). Initial recombination analysis with genotyping data provided by INRA revealed that the target locus was on the lower arm of chromosome IV. No significant linkage was observed on other chromosomes. Additional chromosome IV genotyping primers were designed according to the SNP database from 19 MAGIC founder lines database (gbrowse.cbio.mskcc.org/gb/gbrowse/thaliana-19magic/; (Gan et al., 2011) for fine mapping. Briefly, single sequence length polymorphism (SSLP) or derived cleaved amplified polymorphic sequence (dCAPS) markers were designed at each interval and used to genotype RILs. The recombination point was narrowed down by markers in narrower intervals as shown in Table 4.2.3.2 and Fig.4.2.3.2. In depth recombination analysis narrowed the locus to the genomic region between marker 200-6-2 and 200-5, an approximately 400kb region, according to the Col-0 reference genome sequence (Table 4.2.3.2. and Fig. 4.2.3.2.). In this locus, one gene *SOBER1 (SUPPRESSOR OF AVRBST-ELICITED RESISTANCE 1)* became a strong candidate, as *SOBER1* is a known suppressor of *Xanthomonas* effector AvrBsT-triggered immunity, which is also a member of the YopJ acetyltransferase family (Cunnac et al., 2007). AvrBsT is one of the closest homologues of HopZ5, sharing 48.8% amino acid identity.

Table 4.2.3.1. Genetic analysis of HopZ5-triggered HR in recombinant inbred lines.

Recombinant Inbred Line	HR	No HR	n	X^2	P Value
Ct-1 x Col-0	1 (54)	1 (65)	119	0.313	0.42

n: Total number of tested population, X^2: Chi-squared test value

Table 4.2.3.2. Genotyping data of key recombinant inbred lines (RILs) in mapping the HR suppressor locus

Key RIL number	Phenotype	Marker									
		200-4	200-5	200-6	60	200-6-2	200-6-3	200-8	200-9	200-11	61
7RV42F8	HR	CT	CT	CT	CT						CT
7RV122F8	HR	CT	CT	CT	CT						CT
7RV182F8	No HR			COL	COL	COL	CT	CT	CT	CT	CT
7RV228F8	No HR	COL	COL	COL	COL						COL
7RV240F8	No HR			COL	COL	COL	COL	CT	CT	CT	CT
7RV276F8	No HR	COL	COL	COL	COL						COL
7RV316F8	No HR	CT	COL	COL	COL						COL
7RV326F8	HR				CT			CT	CT	CT	CT
7RV428F8	No HR			COL	COL	COL	CT				CT

RIL: Recombinant inbred line

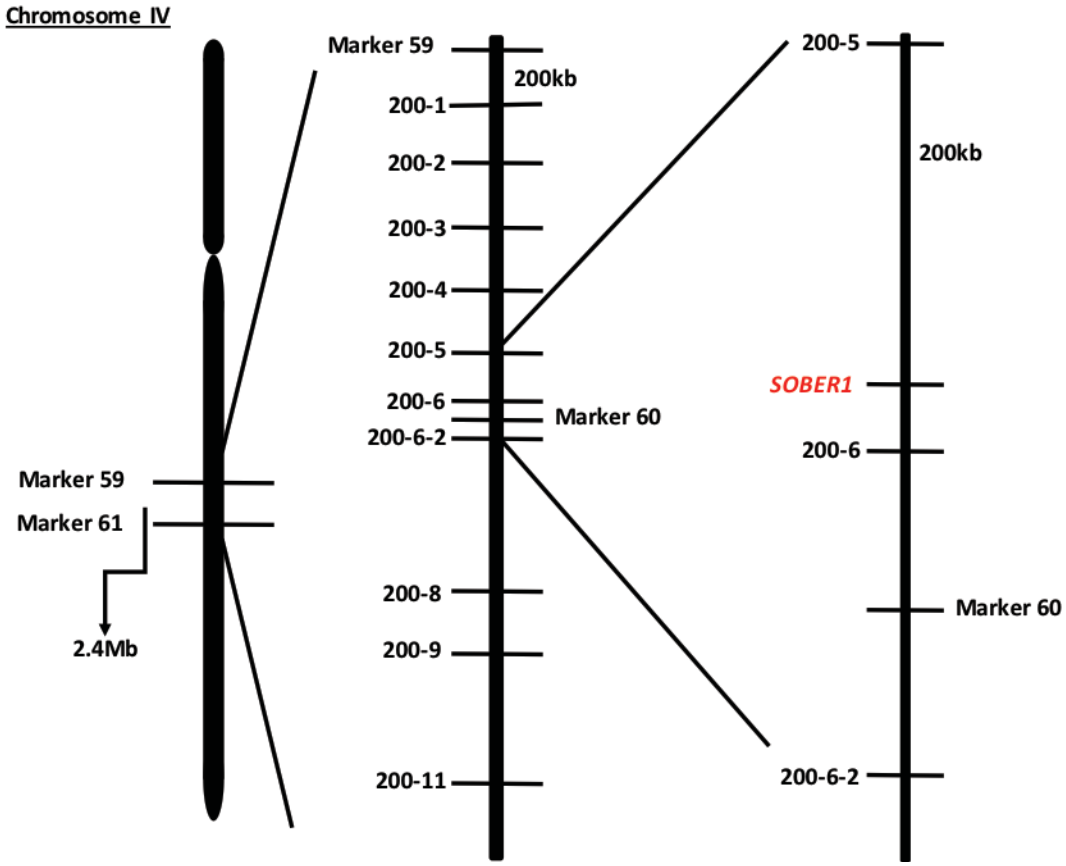


Figure 4.2.3.2. SOBER1 is present between markers 200-5 and 200-6 on chromosome 4.

Approximate genetic distance between markers are indicated based on Col-0 reference sequence. *SOBER1* is located between Marker200-5 and Marker 200-6-2, an approximately 400kb interval. Distance between markers represents the actual distance between markers. kb = 1000 base pairs

SOBER1 (*At4g22305*), a gene encoding a putative α/β serine hydrolase, consists of 6 exons spanning 1510 bp on the reverse strand of the lower arm of chromosome IV. The length of the open reading frame is 684 bp in Col-0 (Figure 4.2.3.3. A). The transcript of *SOBER1* in Col-0 is 1036 bp including 136 bp of 5' untranslated region (UTR) and 216 bp of 3' UTR. The exact promoter region is not defined precisely (Cunnac et al., 2007). The translated SOBER1 polypeptide is 228 amino acids long and 24.8 kDa. SOBER1 has highly conserved catalytic core residues among serine hydrolases containing Ser106, Asp160 and His192 (Figure 4.2.3.3. B).

Both genomic DNA (gDNA) and complementary DNA (cDNA) sequences of *SOBER1* in Ct-1 were amplified by PCR and the sequences were confirmed. *SOBER1* was successfully amplified from *Arabidopsis* Ct-1 cDNA, which meant truncated *SOBER1* was transcribed to mRNA in Ct-1. In Ct-1, a single nucleotide deletion in exon 5 caused a frameshift from 179 aa and an early stop codon at 198 aa (Fig. 4.2.3.3. A and B). Due to the frameshift from 179 aa, one of the catalytic core residues, His192, is absent in $SOBER1^{Ct\text{-}1}$, which likely results in a non-functional SOBER1 polypeptide.

In order to test if *SOBER1* was involved in the suppression of HopZ5-triggered HR in *Arabidopsis*, transgenic Ct-1 lines expressing $SOBER1^{Col\text{-}0}$ were generated. Transgenic Ct-1 lines carrying C-terminally FLAG tagged *SOBER1* was tested to determine if $SOBER1^{Col}$ could suppress HopZ5-triggered HR in Ct-1. *SOBER1* was transformed into Ct-1 under the control of two different promoters, 35S cauliflower mosaic virus (CaMV) promoter or native promoter (~1.5kb upstream). *Pf* Pf0-1 (T3SS)-delivered HopZ5 did not induce HR in either *SOBER1-FLAG* transgenic T_1 lines (Fig. 4.2.3.4). Different levels of SOBER1-FLAG protein expression, depending on the promoter, were detected using an immunoblot assay in T_2 plants (Figure 4.2.3.5.). This demonstrates that not only overexpression but also native expression of *SOBER1* successfully suppresses HopZ5-triggered HR when expressed in the Ct-1 background, indicating that *SOBER1* is the *bona fide* suppressor within the HopZ5-triggered HR locus.

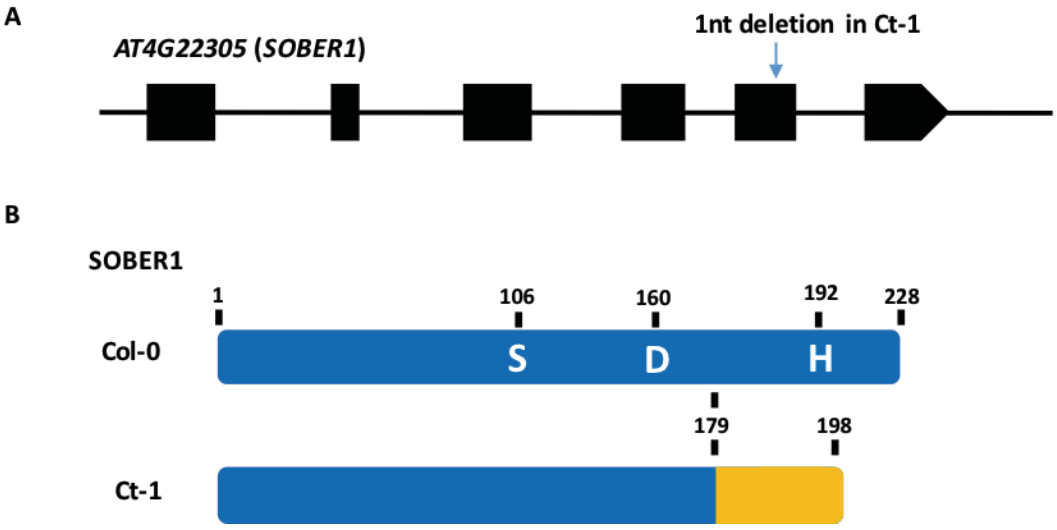


Figure 4.2.3.3. *SOBER1* is frameshifted and truncated due to a single nucleotide deletion in exon 5 in Ct-1.

A – Scheme of genomic sequence of *SOBER1 (AT4g22305).* Black boxes indicate exons and lines indicate introns. Mutation of *SOBER1* in Ct-1 occurs in exon 5.

B – Protein polymorphisms of SOBER1 between Col-0 and Ct-1. Three conserved catalytic residues serine 106 (S), aspartic acid 160 (D) and histidine 192 (H) are indicated. The amino acid sequence after the frameshift is colored yellow in Ct-1.

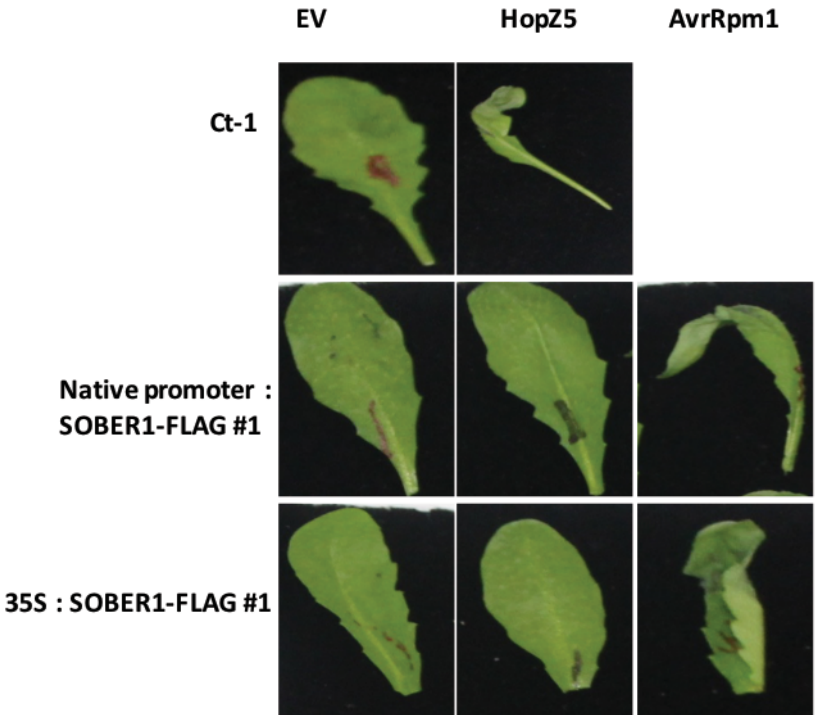


Figure 4.2.3.4. Transgenic expression of SOBER1 abolishes HopZ5-triggered HR in Ct-1.
1 x 10^8 cfu/ml of *P. fluorescens* Pf0-1 (T3SS) carrying pBBR 1MCS 5B:*avrRps4 promoter (*EV*),(hopZ5 : HA)* and pVSP61:*avrRpm1* were infiltrated to 35S promoter: *SOBER1-FLAG* and *native promoter : SOBER1-FLAG* transgenic T1 Ct-1. HR was scored and photographed 1 day after infiltration. This experiment has been conducted more than 3 times with similar results.

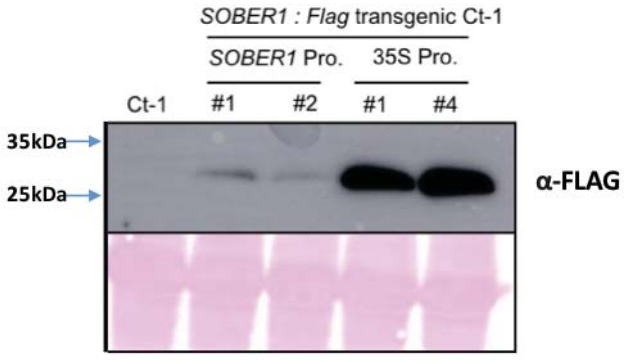


Figure 4.2.3.5. Immunoblot analysis of SOBER1-FLAG protein in stable *Arabidopsis* transgenic lines.
Total protein extract from Ct-1 and *SOBER1-FLAG* transgenic T_2 Ct-1 was separated by SDS-PAGE and detected with anti-FLAG antibodies. The bottom panel shows S-

ponceau red staining of rubisco for loading control. This experiment has been conducted 2 times with similar results.

4.2.4. SOBER1 suppresses HopZ5-triggered HR but not immunity in *Arabidopsis* accession Col-0.

In parallel, characterization of HopZ5 revealed that *Arabidopsis* accession Col-0 was resistant to HopZ5 despite the absence of HR (Jayaraman et al., 2017). Firstly, the growth of *Pto* DC3000 carrying *hopZ5* was restricted in Col-0 when compared to *Pto* DC3000 carrying empty vector, whereas it grew as much as empty vector strain in Ws-2. In addition, transgenic expression of HopZ5 in Col-0 resulted in a stunted autoimmune-like phenotype, elevated *PR1* gene expression and growth restriction of wild-type *Pto* DC3000, which suggested the existence of resistance against HopZ5 in Col-0.

If there is an immune receptor that recognises HopZ5 in Col-0, the HR phenotype is the only difference between Col-0 and Ct-1. Therefore, knocking out *SOBER1* would be expected to restore HR upon HopZ5 delivery. In order to test this hypothesis, a T-DNA knockout null mutant in the Col background, *sober1-3* (SAIL_113_H12) (Cunnac et al., 2007), was challenged with *Pf* Pf0-1 (T3SS) delivering HopZ5. As SAIL T-DNA knock out lines were generated in the Col-3 background, hereafter 'Col' will be used to refer to both Col-0 and Col-3 throughout this chapter and assume there is ignorable genetic differences between Col-0 and Col-3. *sober1-3* has a T-DNA insertion in the first intron and was confirmed as a null allele as no *hopZ5* transcript was detected by semi-quantitative RT-PCR (Cunnac et al., 2007). In *sober1-3* plants, HopZ5 delivered by *Pf* Pf0-1 (T3SS) triggered clear HR (Fig. 4.2.4.1.), which confirmed that *SOBER1* was the suppressor of HopZ5-triggered HR in Col.

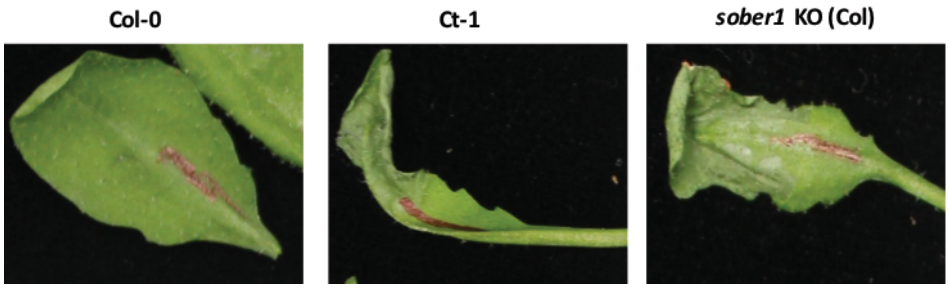


Figure 4.2.4.1. HopZ5-triggered HR is restored in Col-0 *sober1* knockout T-DNA mutant .
1 x 10^8 cfu/ml of pBBR-1MCS-5B: *avrRps4 promoter:hopZ5*:*HA* carrying *P. flourescens* Pf0-1 (T3SS) was infiltrated into leaves of five-week old *Arabidopsis* Col-0, Ct-1 and the *sober1* T-DNA knock-out line (SAIL_H12_113). HR was scored and photographed 1 day after infiltration. This experiment has been conducted more than 3 times with similar results.

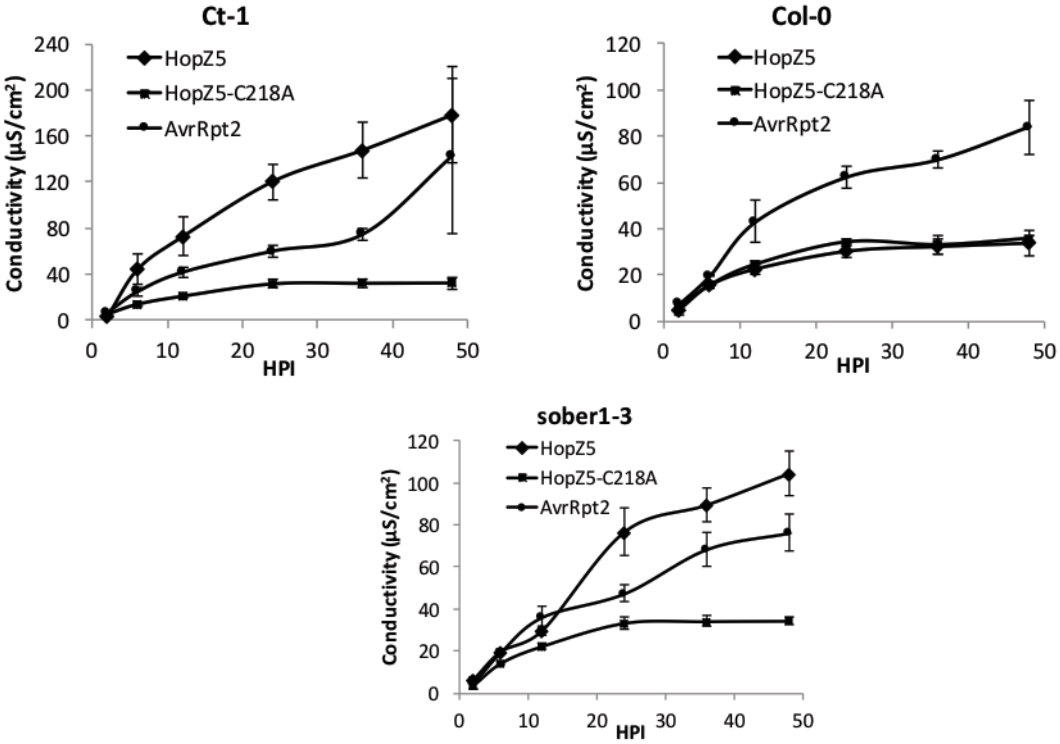


Figure 4.2.4.2. Electrolyte leakage in *sober1-3* increased when HopZ5 was delivered by *P. fluorescens* Pf0-1 (T3SS).
1 x 10^8 cfu/ml of *P. flouresens* Pf0-1 (T3SS) carrying pBBR-1MCS-5B:*avrRps4 promoter (EV), (hopZ5*:*HA) or* pVSP61:*avrRpt2* were infiltrated into leaves of five-week old *Arabidopsis* Col-0, Ct-1 and *sober1* T-DNA knock-out line (SAIL_H12_113). Leaf discs were harvested and floated on distilled water and electrolyte leakage was measured at indicated time points by a conductivity meter. Error bars indicate

standard error between 4 technical replications from one biological replication. This experiment has been conducted 3 times independently with similar results.

Electrolyte leakage from leaves infiltrated with *Pf* Pf0-1 (T3SS) carrying empty vector, *hopZ5* or *avrRpt2* was measured to quantify HopZ5-triggered HR in Col-0, Ct-1 and *sober1-3* (Fig. 4.2.4.2.). The maximum peak of conductivity for the negative control, HopZ5 C218A, and the positive control, AvrRpt2, were lower overall in Col-0 and *sober1-3* when compared to Ct-1, possibly due to differential HR activation in these genotypes. In Ct-1, the peak of HopZ5 delivery reached around 80% of that of AvrRpt2. In Col-0, delivery of HopZ5 or the catalytic null mutant, HopZ5 C218A, showed very low electrolyte conductivity; however, in *sober1-3,* HopZ5 triggered significantly higher electrolyte release, which was around 73% of the positive control, AvrRpt2. This result further supports the evidence that SOBER1 is a suppressor of HopZ5-triggered HR.

In order to test if HopZ5 triggers immunity, growth of *Pto* DC3000 carrying *hopZ5* was also examined in *sober1-3.* The growth of *Pto* DC3000 carrying *hopZ5* was restricted in *sober1-3*, as expected (Fig. 4.2.4.3.). Ct-1 and, interestingly, Col-0 also recognised HopZ5, as indicated by *Pto* DC3000 (*hopZ5*) growth restriction. However, whether *sober1-3* was more resistant to *Pto* DC3000 (*hopZ5*) than Col-0 was unclear.

In contrast, the growth of *Pto* DC3000 carrying *hopZ5* was not restricted in *Arabidopsis* accession Ws-2 (Jayaraman et al., 2017). Also, genetic analysis of HopZ5-triggered HR in the Ct-1 x Ws-2 F_2 population revealed that the HR segregates into 1:3=HR:noHR, indicating a single locus segregation (Table 4.2.2.1). To verify if this locus was also *SOBER1,* 148 F_2 individuals were tested for HopZ5-triggered HR and all 148 F_2 individuals were genotyped for *SOBER1* allelic differences. Clearly, 27 F_2 individuals that showed HR contained non-functional *SOBER1* polymorphisms while the rest of the F_2s with no HR phenotype had a full length *SOBER1* allele. This suggested two possibilities: 1. *SOBER1* could suppress the immune response, not only the HR, in Ws-2. 2. There are other components that contribute specifically to HopZ5-triggered immunity in Ws-2.

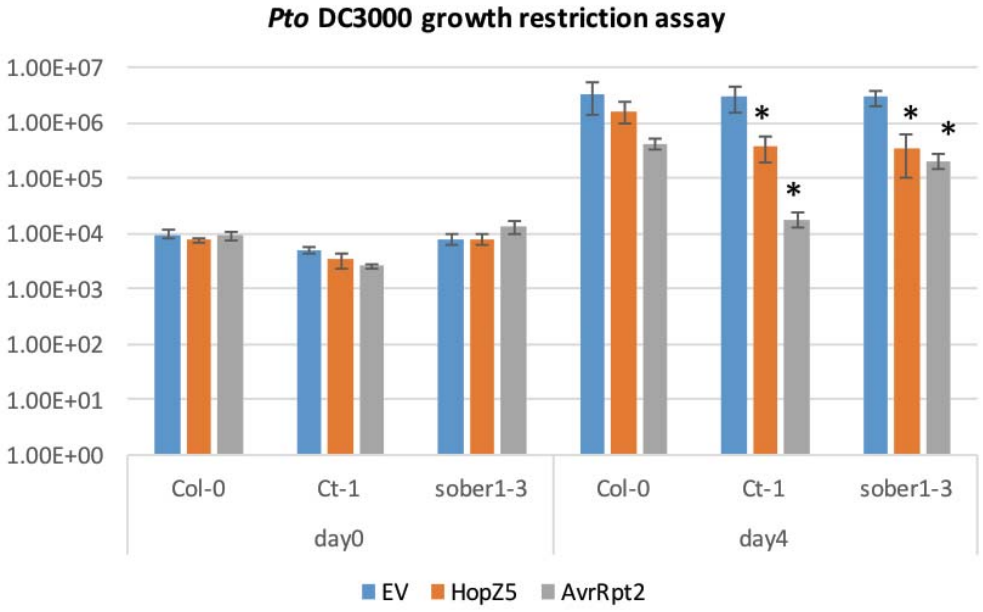


Figure 4.2.4.3. Growth of *Pto* DC3000 carrying HopZ5 is restricted in *sober1-3* T-DNA knockout Col.

5 x 10^5 cfu/ml of *Pto* DC3000 carrying pBBR 1MCS 5B:*avrRps4 promoter (*EV*),(hopZ5 : HA)* or pVSP61:*avrRpt2* were inoculated. The number of bacteria was counted at indicated time points. Error bars indicate standard error from 6 technical replecations from one biological replication. Asterisk indicates statistically significant differences from EV (student t-test, *p<0.05). This experiment has been conducted 2 times independently with similar results.

4.2.5 SOBER1, a putative phospholipase (or α/β carboxylase), requires conserved Ser106 and His192 to suppress HopZ5-triggered HR-like cell death in *Nicotiana.*

Phospholipases are primarily localized to membrane-associated cellular components such as the plasma membrane, microsome, endoplasmic reticulum (ER) or Golgi apparatus but some are nuclear, cytosolic or secreted (Du, 2003; Freyberg et al., 2001; Murelli et al., 2007). Therefore, SOBER1 was primarily expected to localize in membrane-associated cellular organelles.

To identify the subcellular localization of SOBER1, C-terminally YFP tagged SOBER1 protein was transiently expressed in *N. benthamiana* leaf cells using agroinfiltration (Fig. 4.2.5.1.). Interestingly, the SOBER1-YFP protein was localized in the nucleus and cytoplasm. This result raises a possibility of another function of SOBER1 *in vivo* other than hydrolysing PC in the membrane.

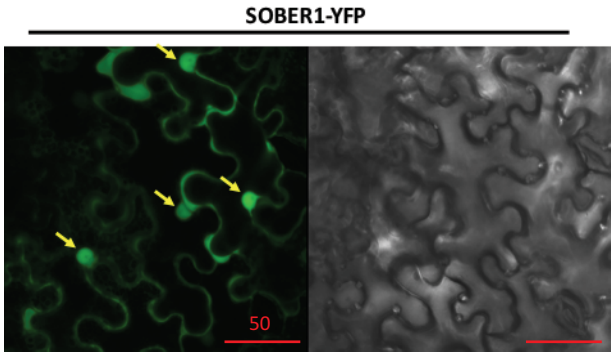


Figure 4.2.5.1. SOBER1-YFP localizes in nucleus and cytoplasm when transiently expressed in *N. benthamiana* leaf cells.
The SOBER1-YFP fusion protein was transiently expressed in 4-5-week-old *N. benthamiana* leaf cells and visualized using confocal microscopy. The right panel shows the YFP signal and the left panel shows the bright field. Pointers indicate nuclei. Scale bar (red bar) indicates 50uM. YFP fluorescence was excited at 488nm with a 20mW Argon laser and captured by the confocal channel in the emission range between 500 and 530nm. This experiment has been conducted once.

Since SOBER1 was identified as the suppressor of AvrBsT- or HopZ5-triggered immunity in *Arabidopsis*, it was tested if conserved catalytic residues of SOBER1 were required for HopZ5-triggered HCD in *Nicotiana* spp. (Fig. 4.2.5.2.). SOBER1 was shown to suppress AvrBsT-triggered HR in *N. benthamiana* (Cunnac et al., 2007). Mutation of catalytic residues Ser106 or His192 in SOBER1 disabled the suppression of AvrBsT-triggered HCD in *N. benthamiana*, indicating that enzymatic activity of AvrBsT is required for ETI suppression (Cunnac et al., 2007). Single amino acid mutations were introduced to generate mutant SOBER1 variants carrying S106A or H192A substitutions and each variant was tagged with a C-terminal FLAG tag. As expected, both SOBER1-FLAG variants carrying S106A or H192A did not suppress HopZ5-triggered HCD in *N. benthamiana* and *N. tabacum.* Therefore, SOBER1-mediated suppression of HopZ5-triggered HCD is dependent on SOBER1 catalytic residues in the same manner as suppression of AvrBsT-triggered HCD. Expression of HopZ5 variants was confirmed by an immunoblot assay (Fig. 4.3.5.3.). As HopZ5 triggers rapid cell death in *N. benthamiana,* a 2µM solution of $LaCl_3$ was applied every 24 hours to suppress cell death. $LaCl_3$ is known to suppress cell death but not defence signalling by interrupting Ca^{2+} channels (Rosales and Brown, 1992). C-terminally FLAG tagged SOBER1 variants and the negative control GFP were detected clearly on an anti-FLAG blot, so the lack of suppression phenotype was not due to a lower or a lack of SOBER1 expression.

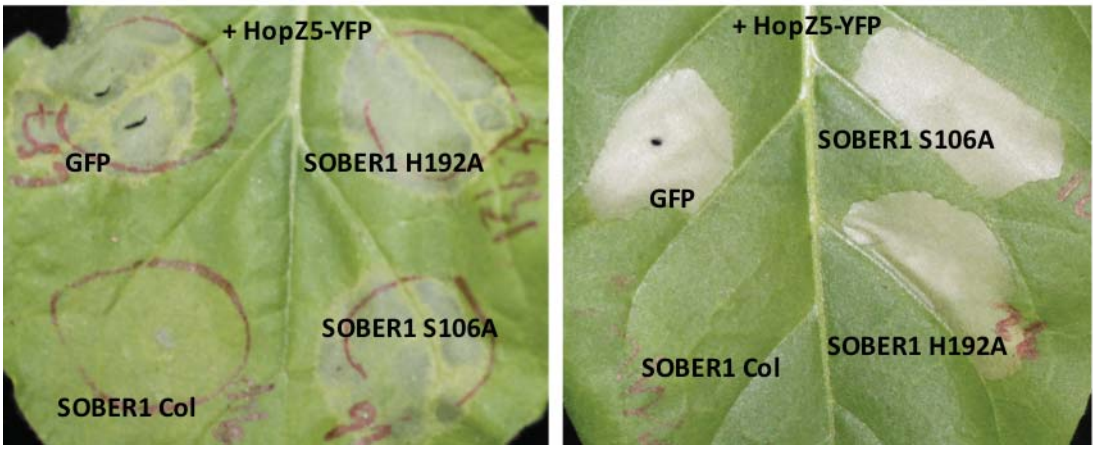


Figure 4.2.5.2. Conserved catalytic residues serine 106 and histidine 192 are required for SOBER1 to suppress HopZ5-triggered HR-like cell death in *Nicotiana*
HopZ5-YFP and SOBER1 (Col, H192A and S106A)-FLAG were transiently co-expressed in *N. benthamiana and N. tabacum*. The infiltration inoculum ratio was HopZ5: SOBER1 = 1:4 (OD_{600} 0.1 : 0.4). HR was scored and photographed 2 days after agro-infiltration. This experiment has been conducted 3 times with similar results.

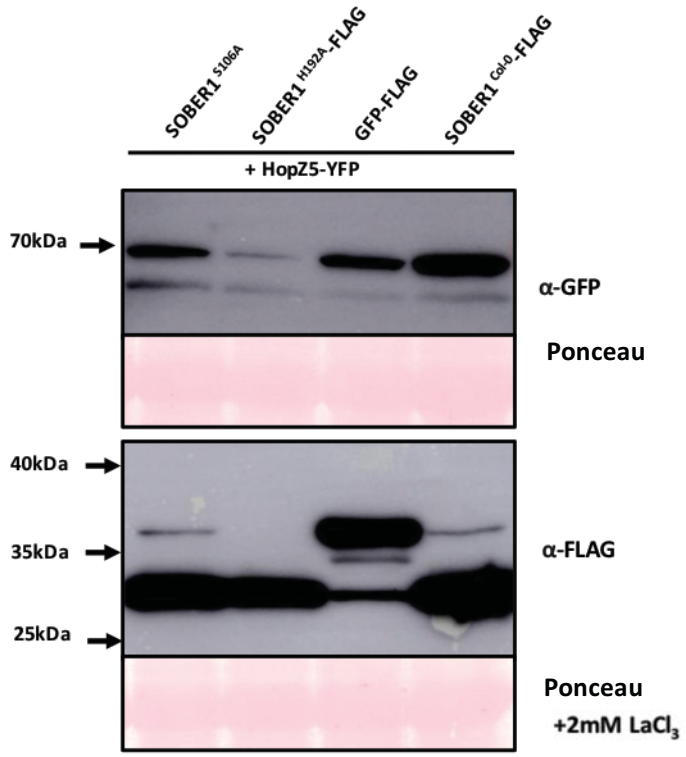


Figure 4.2.5.3 Immunoblot analysis of SOBER1-Flag and proteins transiently expressed in *N. benthamiana*.
HopZ5-YFP and SOBER1 (Col, H192A and S106A) were transiently co-expressed in *N. benthamiana* with 2mM of $LaCl_3$ to prevent cell death. The bottom panels show the S-ponceau red staining of rubisco for loading control. Total protein was separated by SDS-PAGE and probed with anti-GFP and anti-FLAG antibodies. This experiment has been conducted 2 times with similar results.

4.2.6 Natural variation of *SOBER1* in *Arabidopsis* accessions strongly correlates with HopZ5-triggered HR

In order to study the natural variation of HopZ5-triggered HR in *Arabidopsis*, 136 accessions were screened for HopZ5-triggered HR (Table 4.2.6.1.). Surprisingly, only 6 out of 136 accessions showed HR when HopZ5 was delivered by *P. fluorescens* Pf0-1 (T3SS). Among these 136 *Arabidopsis* accessions, for those which have genomic SNP data available, *SOBER1* sequence polymorphisms were compared using the GBrowse online tool from the 1001 *Arabidopsis* genome database website (http://signal.salk.edu/atg1001/3.0/gebrowser.php). Out of 6 accessions that showed HR to HopZ5, SNP data were available for 4 accessions (Ct-1, Pi-0, Ws-0 and Br-0). In this database, Ct-1 and Ws-0 had the same 1 nucleotide (nt) insertion in the *SOBER1* sequence resulting in an early stop codon. *SOBER1* polymorphisms in Br-0 and Pi-0 were not detected in this approach even though Pi-0 was reported to have the same mutation in *SOBER1* as in Ct-1 (Cunnac et al., 2007). To investigate *SOBER1* sequence polymorphisms, *SOBER1* was PCR-amplified from genomic DNA for all 6 accessions and resulting PCR fragments were sequenced. Ct-1, Dijion-M, Nw-3 and Pi-0 had the same 1 nucleotide deletion at exon 5 which leads to a frameshift from 179 aa and an early stop codon at 198 aa (hereafter, SOBER1^{Ct-1}) (Figure 4.2.5.1). Br-0 had a 1 nucleotide insertion in exon 4 which caused a frameshift from 117 aa resulting in a 165 aa-long truncated SOBER1 (SOBER1^{Br-0}). Both variations lack at least one of the predicted catalytic residues (His192) and, therefore, both are expected to be enzymatically inactive. These accessions showed a strong correlation between HopZ5-triggered HR and lack of *SOBER1.*

Table 4.2.6.1. Natural variation of HopZ5-triggered HR in 136 *Arabidopsis* accessions

Accession	Phenotype	Accession	Phenotype	Accession	Phenotype	Accession	Phenotype	Accession	Phenotype	Accession	Phenotype
Ag-0	No HR	CS22491	No HR	Hr-5	No HR	Lp2-4	No HR	Oy-0	No HR	RLD	No HR
An-1	No HR	Cvi-0	No HR	Kas-1	No HR	Lp2-6	No HR	Pna-10	No HR	Rmx-A180	No HR
Bla-1	No HR	Edi-0	No HR	Ken-0	No HR	Lz-0	No HR	Pna-17	No HR	RRS-10	No HR
Bor-1	No HR	Ei-2	No HR	Kin-0	No HR	Mir-0	No HR	Pro-0	No HR	Se-0	No HR
Bor-4	No HR	Est-1	No HR	Kondara	No HR	Ml-0	No HR	Prs-7	No HR	Sf-2	No HR
Br-0	HR	Fei-0	No HR	Knox-10	No HR	Mrk-0	No HR	Pu2-7	No HR	Shandara	No HR
Bur-0	No HR	Ga-0	No HR	Knox-18	No HR	Ms-0	No HR	Ra-0	No HR	Sorbo	No HR
C24	No HR	Got-1	No HR	Kz-1	No HR	Mz-0	No HR	Rsch-4	No HR	Sq-1	No HR
Can-0	No HR	Got-7	No HR	Kz-9	No HR	Nd-1	No HR	Ren-1	No HR	Sq-8	No HR
CIBC-5	No HR	Gu-0	No HR	Kz-10	No HR	Nfa-10	No HR	Ren-11	No HR	Tamm-27	No HR
CIBC-17	No HR	Hi-0	No HR	LL-0	No HR	Nok-3	No HR	Pet-0	No HR	Ts-1	No HR
Uk-1	No HR	Hp2-2	No HR	Zdr-6	No HR	HOG	No HR	Pyl-1	No HR	U112-3	No HR
Uk-3	No HR	Dr-0	No HR	Col-0	No HR	Hs-0	No HR	Rld-2	No HR	Van-0	No HR
Uod-1	No HR	Dra-0	No HR	Akita	No HR	Je-54	No HR	RRS7	No HR	Wc-2	No HR
Uod-7	No HR	El-0	No HR	Ak-1	No HR	Lan-0	No HR	Rsch-0	No HR	Wei-1	No HR
Van-0	No HR	Ep-0	No HR	Bay-0	No HR	Lip-0	No HR	Sap-0	No HR	Wil	No HR
Var2-6	No HR	Fei-0	No HR	Bd-0	No HR	Lm	No HR	Shah	No HR	Ws-3	No HR
Wa-1	No HR	Ge-2	No HR	Be-0	No HR	Mh-1	No HR	St-0	No HR	Yo-0	No HR
Wei-0	No HR	Gol-1	No HR	Bla-11	No HR	NFA-8	No HR	Stw-0	No HR	Epi-0	No HR
Wil-2	No HR	Gr	No HR	Bu-2	No HR	Nw-3	HR	Tamm-2	No HR	Pi-0	HR
Ws-2	No HR	Gre-0	No HR	Cha-0	No HR	Old-1	No HR	Te-0	No HR	Ws-0	HR
Wt-5	No HR	HI-3	No HR	Cl-0	No HR	Ove-0	No HR	Col-0	No HR		
Ct-1	HR	No-0	No HR	Dijon-M	HR	RCH4	No HR	Hi-0	No HR		

Orange color indicates the accessions showing HR to HopZ5

The sequences of *SOBER1* from other accessions that did not show HR to HopZ5 were also compared using the 1001 GBrowse database, but no significant polymorphisms were detected for all except Ga-0 (Fig. 4.2.6.1.), which will be discussed in Chapter 5. However, as the *Arabidopsis* SNP public database was unable to predict *SOBER1* polymorphisms for Br-0 and Pi-0, it is possible that there are more accessions harbouring a truncated SOBER1 variant among the 130 accessions that did not show HR to HopZ5. In these accessions, it is possible that

the corresponding *R* genes for HopZ5 are absent. Out of all accessible accessions analysed (>1000 in the database), only 12 more accessions (En-1, En-D, Er-0, ISF29, Haes-1.9791, Hof1, KBG2-13.9770, Kyoto, Zu-1, Nc-1, Ru-N2 and Tscha1) had insertion/deletion polymorphisms that caused a frameshift according to the public polymorphism database (http://signal.salk.edu/atg1001/3.0/gebrowser.php). Thus, *SOBER1* deletion allelic variation was extremely rare.

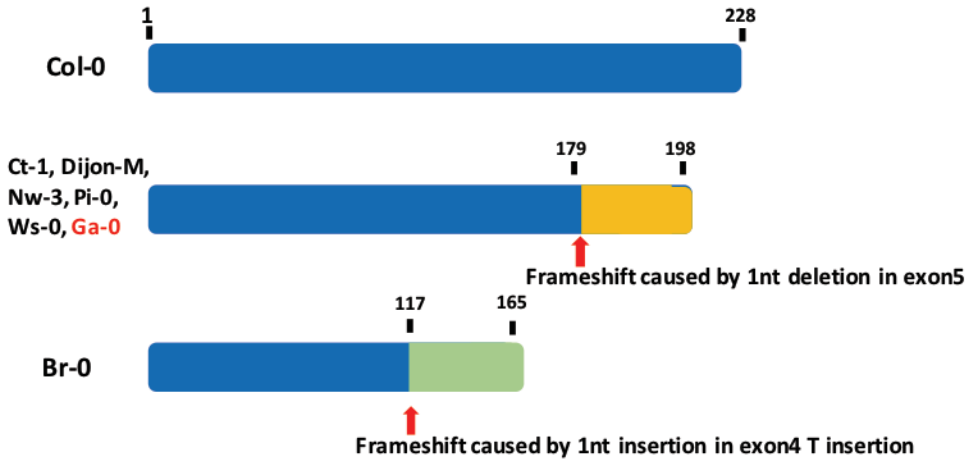


Figure 4.2.6.1. Representative natural sequence variations of SOBER1 in *Arabidopsis* accessions.
Different amino acid sequences after frameshift are colored yellow and green. Ga-0 is colored red due to the lack of HopZ5-triggered HR phenotype.

In order to investigate the naturally occurring sequence variations in SOBER1 that caused the loss of function, the SOBER1 natural variants were tested for their ability to suppress the HopZ5-triggered HCD phenotype in *Nicotiana* spp. The C-terminally FLAG-tagged SOBER1 natural variants with frameshift mutations were transiently expressed with HopZ5 in *N. benthamiana* (Figure 4.2.6.2.). As expected, SOBER1^{Ct-1} and SOBER1^{Br-0} did not suppress HopZ5-triggered HCD in *N. benthamiana* and *N. tabacum.* Expression of all constructs was validated by an immunoblot assay (Fig. 4.2.6.3.) with 2µM $LaCl_3$ to prevent cell death triggered by HopZ5. However, SOBER1^{Ct-1} –FLAG protein was barely detected similar to SOBER1^{Pi-0} as described in previous research (Cunnac et al., 2007).

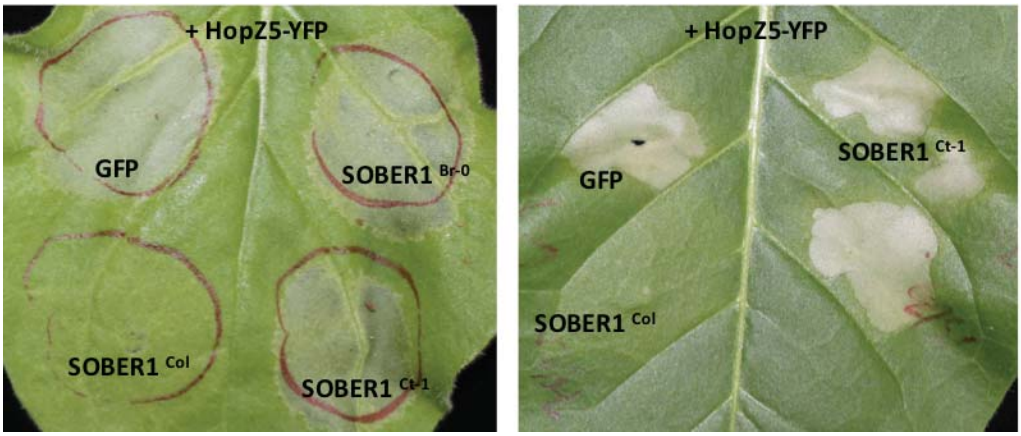


Figure 4.2.6.2. The C-terminally truncated natural variants of SOBER1 cannot suppress HopZ5-triggered HR-like cell death in *Nicotiana.*

HopZ5-YFP and SOBER1 natural variants (Col, Ct-1 and Br-0)-FLAG were transiently co-expressed in *N. benthamiana and N. tabacum*. The infiltration inoculum ratio was HopZ5 : SOBER1 = 1:4 (OD_{600} 0.1 : 0.4). HR was scored and photographed 2 days after agro-infiltration. This experiment has been conducted 3 times with similar results.

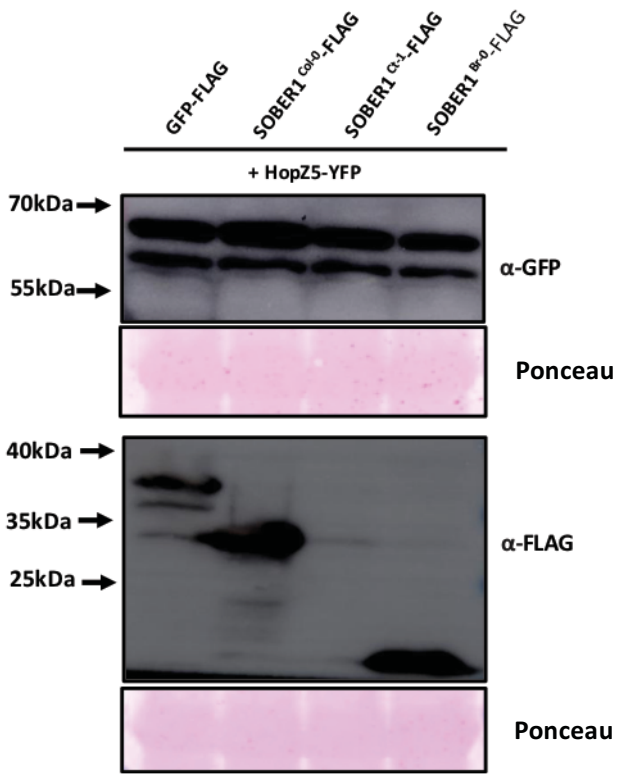


Figure 4.2.6.3. Immunoblot analysis of HopZ5-YFP and C-terminally truncated natural variants of SOBER1-Flag proteins expressed in *N. benthamiana* cells

HopZ5-YFP and SOBER1 (Col, Ct-1 and Br-0) were transiently co-expressed in *N. benthamiana* with 2mM of $LaCl_3$. Total protein was sampled 2 days after agro-infiltration and probed with anti-GFP and anti-FLAG antibodies. The bottom panels show the S-ponceau red staining of rubisco for loading control. This experiment has been conducted 2 times with similar results.

4.2.7 SOBER1 suppresses HCD induced by multiple YopJ family acetyltransferases in *Nicotiana*

As SOBER1 suppresses HR triggered by two YopJ acetyltransferase family effectors from different pathogens, HopZ5 and AvrBsT, a possibility that SOBER1 broadly suppresses activities of YopJ acetyltransferase effectors arose. In order to test the range of SOBER1 suppression of YopJ family effectors-induced HCD in *Nicotiana* spp.*,* two additional acetyltransferase effectors, HopZ3 from *Psa* V-13 and HopZ1a from *P. syringae* pv. *syringae,* were cloned with a C-terminal YFP tag. HopZ1a is an well characterised acetyltransferase effector which is recognised by the CNL ZAR1 in *Arabidopsis* (Lewis et al., 2013, 2010). HopZ3 from *Psa* V-13 is a homologue of HopZ3 (72% of amino acid identity) from *Pseudomonas syringae* pv. *syringae* which is known to acetylate multiple targets to promote virulence in *Arabidopsis* (Lee et al., 2015). The $AvrBsT_{11\text{-}350}$ amino acid sequence was used for assays in this study including agroinfiltration, as HCD in *Nicotiana benthamiana* by $AvrBsT_{11\text{-}350}$ was validated in Cunnac et al. 2007. SOBER1-FLAG and GFP-FLAG were transiently co-expressed with different YopJ family effectors in *N. tabacum.* Interestingly, SOBER1 suppressed HCD induced by multiple acetyltransferase effectors, HopZ5, HopZ3 and AvrBsT but not HopZ1a (Fig. 4.2.7.1). Transient expression of each construct was confirmed by an immunoblot assay with 2µM $LaCl_3$ in *N. benthamiana* due to the cell death from HopZ5 expression (Fig. 4.2.7.2.). In general, effector protein accumulation appeared higher when co-expressed with SOBER1-FLAG.

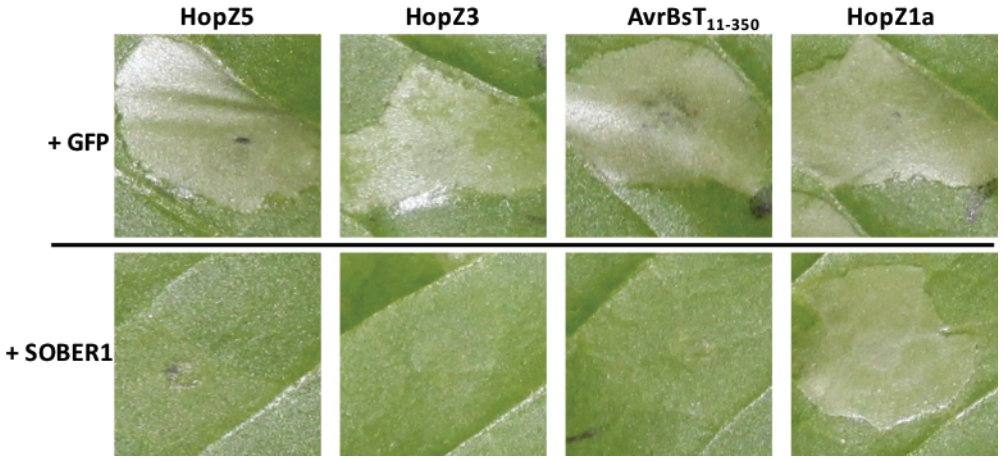


Figure 4.2.7.1. SOBER1 suppresses HR-like cell death triggered by multiple acetyltransferase effectors in *N. tabacum*.
Acetyltransferase effectors HopZ5-YFP, HopZ3-YFP, AvrBsT$_{11-350}$-YFP and HopZ1a-YFP were transiently co-expressed with SOBER1 –FLAG or GFP-FLAG in *N. tabacum*. The infiltration inoculum ratio was effector : SOBER1 or GFP-FLAG = 1:4 (OD_{600} 0.1 : 0.4). HR was scored and photographed 2 days after agro-infiltration. This experiment has been conducted more than 3 times with similar results.

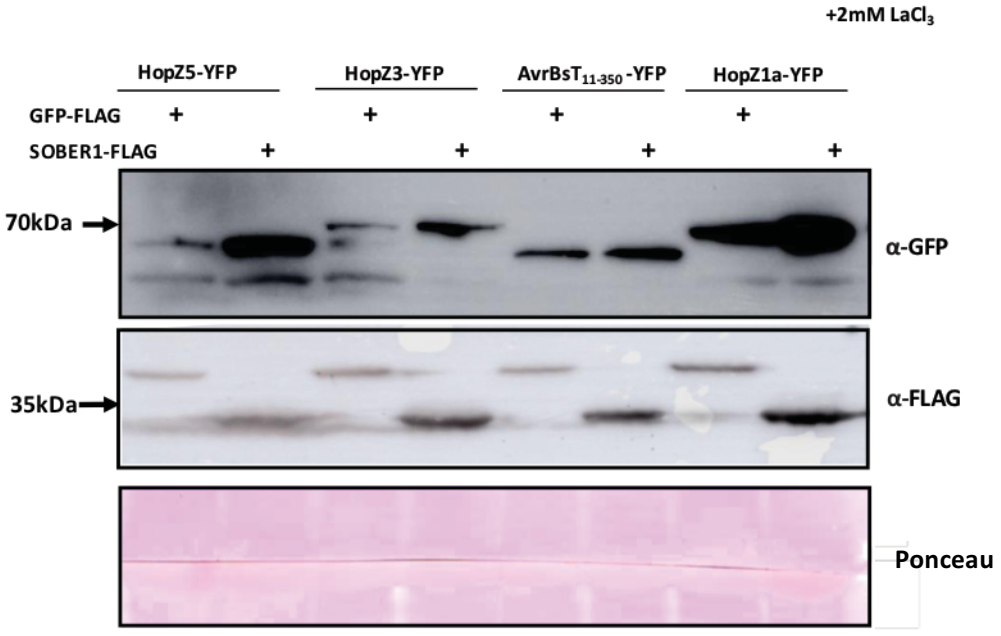


Figure 4.2.7.2. Immunoblot analysis of several acetyltransferase effectors and SOBER1.
Acetyltransferase effectors HopZ5-YFP, HopZ3-YFP, AvrBsT$_{11-350}$-YFP and HopZ1a-YFP were transiently co-expressed with SOBER1 –FLAG or GFP-FLAG in *N.*

benthamiana with 2mM $LaCl_3$. The infiltration inoculum ratio was effector : SOBER1 or GFP-FLAG = 1:4 (OD_{600} 0.1 : 0.4). Total protein was sampled 2 days after agro-infiltration and probed with anti-GFP and anti-FLAG antibodies. The bottom panel shows the S-ponceau red staining of rubisco for loading control. This experiment has been conducted 2 times with similar results.

In order to test if SOBER1-mediated immune suppression is restricted to the YopJ acetyltransferase family, 7 *Psa* effectors that triggered HR in *N. tabacum* but are not predicted as acetyltransferases were co-expressed with SOBER1. In this experiment, no other effectors apart from HopZ3 were affected by SOBER1 (Figure 4.2.7.3.).

There is a possibility that HopZ5, HopZ3 and AvrBsT are recognized by the same system and that SOBER1 can suppress cell death triggered by these effectors. While HopZ1a recognition by *Arabidopsis* ZAR1 in accession Col-0 has been characterized (Lewis et al., 2010), immune receptors for the other effectors have not been defined as yet. As Col-0 has a functional SOBER1, HopZ1a-triggered HR appears unaffected by *SOBER1* in *Arabidopsis*. HopZ5, HopZ3, AvrBsT and HopZ1a were cloned and delivered by *Pto* DC3000 into several *Arabidopsis* accessions lacking a functional *SOBER1* allele (Table 4.2.7.1). For delivery of *Xanthomonas* effector AvrBsT through the *Pseudomonas* type III secretion system, $AvrBsT_{11\text{-}350}$ was cloned under the *avrRpt2* promoter and the N-terminal 100 amino acid sequence of *avrRpt2 (*$AvrRpt2_{100}AvrBsT_{11\text{-}350}$) as conducted in Cunnac et al. (2007). Interestingly, when delivered from *Pto* DC3000, HopZ5 and AvrBsT triggered an HR in all accessions lacking *SOBER1*, however, HopZ3 did not trigger HR in any of accessions tested.

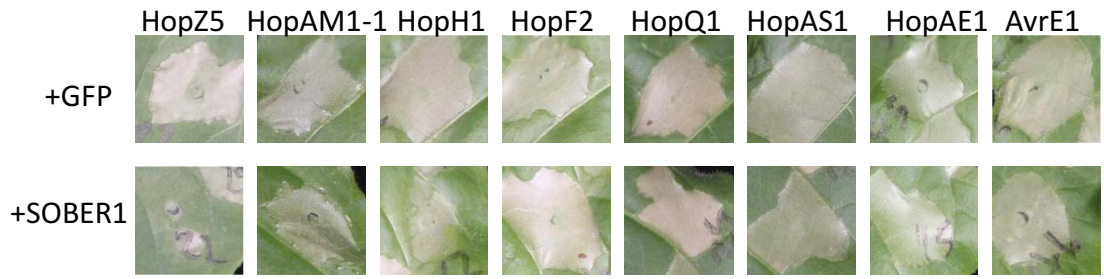


Figure 4.2.7.3. SOBER1 cannot suppress HR-like cell death triggered by non-acetyltransferase *Psa* effectors in *N. tabacum*.
Psa effectors HopZ5-YFP, HopAM1-1-YFP, HopH1-YFP HopF2-YFP, HopQ1-YFP, HopAS1-YFP, HopAE1-YFP and AvrE1-YFP were transiently co-expressed with SOBER1 –FLAG or GFP-FLAG in *N. tabacum*. The infiltration inoculum ratio was effector : SOBER1 or GFP-FLAG = 1:4 (OD_{600} 0.1 : 0.4). HR was scored and photographed 2 days after agro-infiltration. This experiment has been conducted 2 times with similar results.

Table 4.2.7.1 YopJ family acetyltransferase effector-triggered HR when delivered by *Pto* DC3000 in *Arabidopsis* accessions without functional SOBER1

Accession	SOBER1 polymorphism	YopJ family effectors			
		HopZ5	$AvrRpt2_{100}AvrBsT_{11-350}$	HopZ3	HopZ1a
Col-0	Full length	no HR	no HR	no HR	HR
Ct-1	Truncated	HR	HR	no HR	HR
Pi-0	Truncated	HR	HR	no HR	N.T*
Nw-3	Truncated	HR	HR	no HR	N.T
Br-0	Truncated	HR	HR	no HR	N.T
Dijon-M	Truncated	HR	HR	no HR	N.T
Ws-0	Truncated	HR	HR	no HR	N.T
Ws-2	Full length	no HR	no HR	no HR	N.T
sober1-3	Not transcribed	No HR	No HR	N.T	N.T

*Not tested

4.2.8 Autoacetylation of HopZ5 is stabilized by SOBER1 in *N. benthamiana*

Autoacetylation is one of the significant features of the YopJ acetyltransferase effector family. Mutation of the residues that are autoacetylated or required for autoacetylation often abolishes functions of acetyltransferase effectors (Cheong et al., 2014; Lee et al., 2015).

In order to test *in vivo* autoacetylation of the predicted lysine residue of 4 acetyltransferase effectors and the effect of SOBER1 on autoacetylation, an immunoprecipitation immunoblot assay was conducted. Each YFP-tagged effector was expressed with SOBER1-FLAG or the negative control, GFP-FLAG, in *N. benthamiana*. Immunoprecipitation using a GFP-binding protein was conducted prior to immunoblot analysis to avoid detection of random acetylated lysines in the plant extract. *Psa* HopZ3 (sharing 72% amino acid identity with *P. syringae* pv. *syringae* HopZ3) and AvrBsT could not be detected by the anti-AcK antibody, as expected (Fig. 4.2.8.1). Interestingly, HopZ1a, which was not predicted to autoacetylate a lysine residue, was detected by anti-AcK antibodies. Which lysine residue(s) was acetylated in HopZ1a is unclear. The HopZ5 catalytic mutant, C218A, a non-functional mutant, was added as a negative control.

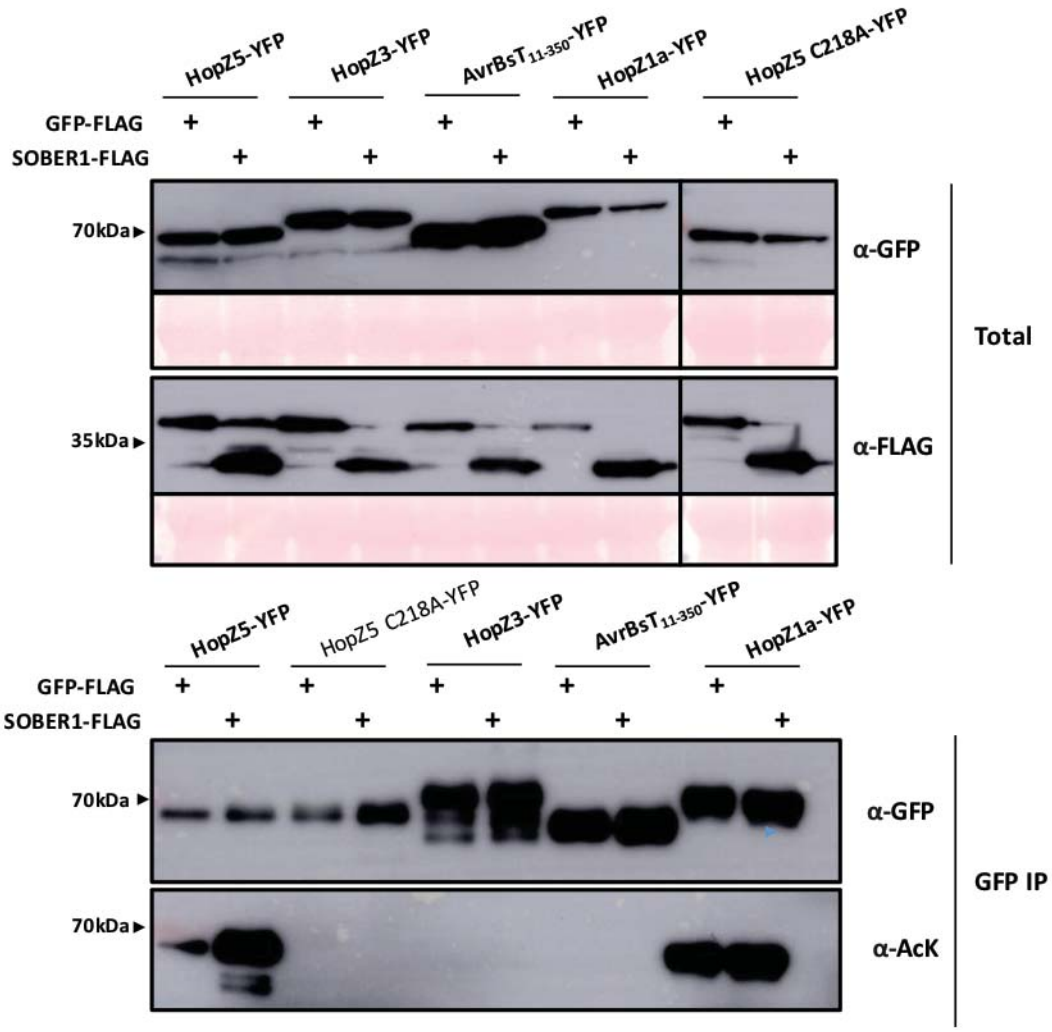


Figure 4.2.8.1. Autoacetylation of HopZ5 is stabilized by co-expression of SOBER1 *in planta*.

Acetyltransferase effectors HopZ5-YFP, HopZ3-YFP, AvrBsT$_{11-350}$-YFP and HopZ1a-YFP were transiently co-expressed with SOBER1–FLAG or GFP-FLAG in *N. benthamiana* with 2mM $LaCl_3$. Total protein extracts were anti-GFP immunoprecipitated and probed with anti-GFP and anti-AcK antibodies. The bottom panel for the total extract shows the S-ponceau red staining of rubisco for loading control. This experiment was conducted for 2 times with similar results.

Strikingly, HopZ5 Lys autoacetylation was stabilized in the presence of SOBER1. While the HopZ1a anti-AcK band intensity was similar between negative control and SOBER1, HopZ5 intensity was much higher in the presence of SOBER1. SOBER1 did not affect the amount of immunoprecipitated HopZ5 protein.

4.2.9 *Pto* DC3000 delivery of HopZ5 does not trigger HR in *sober1-3* Col

As shown above, HopZ5 triggered clear HR in *sober1-3* (Col T-DNA knockout) when delivered by *Pf* Pf0-1 (T3SS) (Fig. 4.2.4.1.). In contrast, it was reported that $AvrBsT_{11\text{-}350}$ did not trigger HR in *sober1-3* when delivered by *Pto* DC3000. This suggests that there may be a secondary player of AvrBsT immunity in Col apart from *SOBER1* which interferes with AvrBsT-triggered HR in *sober1-3* (Cunnac et al., 2007). In order to test if *Pto* DC3000 delivery of HopZ5 also triggers HR in Ct-1, HopZ5 was delivered into *sober1-3* plants by *Pto* DC3000 delivery. Intriguingly, HopZ5 failed to trigger HR in *sober1-3* when delivered by *Pto* DC3000 while *Pf* Pf0-1 (T3SS) delivery clearly triggered HR (Fig. 4.2.9.1). This result is particularly interesting because the growth of *Pto* DC3000 carrying HopZ5 was successfully restricted in *sober1-3* (Fig. 4.2.3.5.). This result again supports the distinct signalling pathway between HopZ5-triggered HR and immunity.

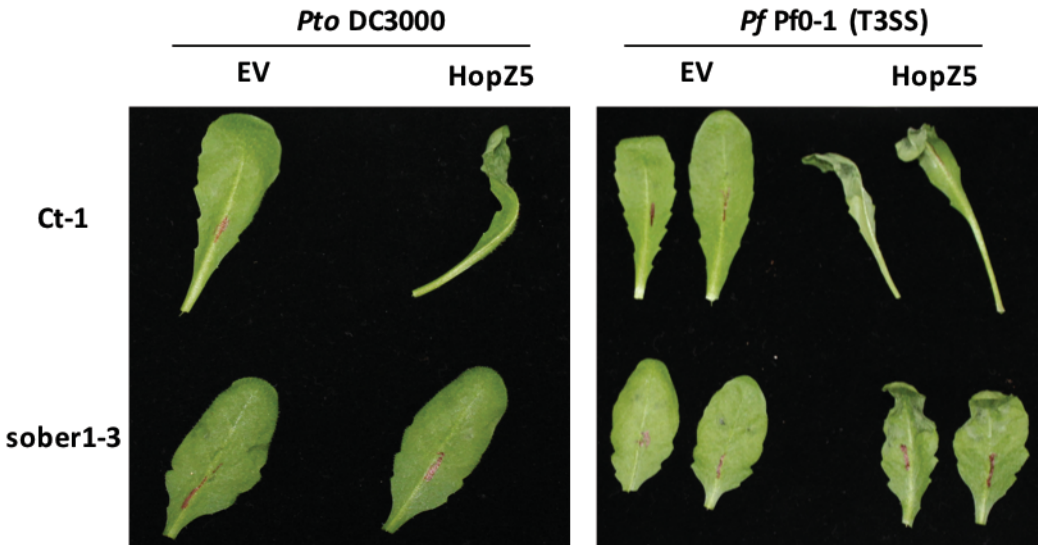


Figure 4.2.9.1. HopZ5 does not trigger HR in *sober1-3* (Col) via *Pto* DC3000 delivery. 3 x 10^8 cfu/ml of *Pto* DC3000 and 1 x 10^8 cfu/ml of *P. fluorescens* Pf0-1 (T3SS) carrying *pBBR-1MCS-5B:avrRps4 promoter (EV). (hopZ5:HA)* were infiltrated into *Arabidopsis* Ct-1 and *sober1-3.* HR was scored and photographed 13 hours (*Pto* DC3000) and 1 day (*P. fluorescens* Pf0-1) after infiltration. This experiment was conducted more than 3 times with similar results.

4.3 Discussion

According to the genetic analysis between *Arabidopsis* Col-0 and Ct-1, HopZ5-triggered HR segregated in a 3:1 ratio (No HR:HR). This result suggested two hypotheses: 1. A single recessive *R* gene. 2. A dominant suppressor of HopZ5-triggered HR. Although there are very few examples of recessive *R* genes (Deslandes et al., 1998; Sama et al., 2014), the F_2 segregation result implied that the gene in Col-0 required for HopZ5-triggered HR was recessive. By fine mapping utilizing RILs between Col-0 and Ct-1, SOBER1, a reported suppressor of AvrBsT-elicited HR both in *Arabidopsis* and *N. benthamiana*, was mapped as the suppressor of HopZ5-triggered HR in *Arabidopsis.* Interestingly, AvrBsT also belongs to the acetyltransferase YopJ family and shared significant amino acid sequence homology with HopZ5.

As very little is known about SOBER1 or SOBER1 homologues/orthologues functions, a possible hypothesis for the SOBER1-mediated suppression mechanism and different aspects of HopZ5-triggered immunity will be discussed in this section based on what is known about general plant defence.

4.3.1. Natural variation in SOBER1-mediated suppression of HR and immunity.

According to genetic analysis in the F_2 populations between Col-0 and Ct-1, HopZ5-triggered HR phenotype was due to a single recessive gene. The lack of HopZ5-triggered HR is governed by the presence of functional *SOBER1* in both Col-0 and Ws-2. In Col-0, there appears to be separate nodes of signalling between HR and immune response when HopZ5 is delivered (Jayaraman et al., 2017). In contrast, HopZ5 does not trigger HR or *Pto* DC3000 growth restriction in Ws-2. In this case, SOBER1 seems to suppress both HopZ5-triggered immunity and HR in Ws-2.

Although HR is often accompanied by resistance, several examples of resistance without HR have been reported (Bendahmane et al., 1999; Coll et al., 2011). Recent studies have revealed a few HR-specific regulators in plants. The *Arabidopsis dnd1 (defence no death 1)* mutant lacking a cyclic nucleotide-gated ion channel protein showed elevated disease resistance to *Pto, Hyaloperonospora parasitica* and *Botrytis cinerea* with a greatly reduced HR that was dependent on *NDR1*-regulated SA signalling (Genger et al., 2008). Interestingly, Zurbriggen and colleagues showed that reduction of plastid-generated ROS in tobacco by expressing flavodoxin (Fld), an electron shuttle present in prokaryotes and algae, dramatically reduced cell death when challenged by non-host pathogen *Xanthomonas campestris* pv. *vesicatoria* without affecting defence gene expression (Zurbriggen et al., 2009). There are also opposite examples implying that HR is not an obligatory defence response. In the Avr4/Cf-4 recognition event in *N. benthamiana,* virus-induced gene silencing of phospholipase C6 (PLC6) abolished defence responses but not HR (Vossen et al., 2010). The *ndr1-1* mutation in *Arabidopsis* can increase susceptibility to a bacterial pathogen carrying AvrB or AvrRpm1 without affecting the HR development (Century et al., 1995). However, how these regulators can selectively suppress immunity or HR is poorly understood.

In addition to qualitative disease resistance, quantitative resistance (QR), a product of quantitative trait loci (QTLs) is an emerging and important parameter in the field of plant disease resistance research. Qualitative resistance is often conferred by one or two major alleles that follow Mendelian phenotypic segregation (Kushalappa et al., 2016). For example, resistant to *Puccinia hordei* in barley cultivar Cebada Capa is conferred by a single gene, *Rph7g*, while cv Vada shows a high level of partial resistance to *P. hordei* due to the presence of several quantitative disease resistance genes (Niks et al., 2015). Quantitative resistance is governed by a combination of QTLs in which each additively contribute to the overall resistance phenotype at quantifiable levels (Yeo et al., 2014). In addition, in an example of sheath blight resistance in rice, 6 identified QTLs have a positively additive effect (Liu et al., 2014). The additive effect of QR genes may imply that there are several nodes of defence pathways, which can be separately activated or work independently. The combinatorial effect of QTLs may vary between plant

accessions (Niks et al., 2015). Results in this study support this idea, namely, the different levels of electrolyte leakage and *Pto* DC3000 growth restriction observed in Ct-1 and Col (WT and *sober1-3*). Both HopZ5 and AvrRpt2 showed reduced electrolyte leakage in the Col background compared to that in Ct-1. *Pto* DC3000 growth restriction was also greater in Ct-1 as compared to Col-0. These results suggest different levels of defence responses exist in Ct-1 and Col, possibly conferred by different QTL sets influencing quantitative resistance by the relevant R protein(s).

Pathogen recognition by the plant immune system essentially involves induction of ROS, which often upregulates the level of SA. A major source of ROS production in plant immune responses are the chloroplasts (Zurbriggen et al., 2009). Notably, pathogen-induced ROS and SA production show a biphasic pattern. It is hypothesized that the first phase possibly potentiates the second phase forming a tight, self-amplifying feedback circuit. While the first phase is involved in both PTI and ETI, the second phase participated primarily in ETI or artificially exaggerated PTI (high dose of PAMP treatment in suspension-cultured plant cells), which implies a possibility of quantitative regulation of the signalling circuit. Despite a lack of direct evidence, HR and plant defence responses could be affected by this kind of quantitative regulation. Different intrinsic secondary inputs (ROS/SA) can be generated from the same primary input (pathogen sensing) as determined by the genome/epigenome of the plant. As a result, different accessions can exhibit a unique intrinsic phenotype from the same input. SOBER1 suppresses HopZ5-triggered immunity/HR starting from an unknown SOBER1-mediated biochemical reaction, but has a different result for the different genetic pools in Col-0 and Ws-2.

4.3.2. Mechanisms by which SOBER1 suppresses plant immunity

SOBER1 is classified as a CEH (carboxylic ester hydrolase) 28 and belongs to the tertiary structural clan C whose polypeptide sequence is predicted to have an

α/β carboxylase fold of the first β-strand antiparallel to the other 7 strands on the same β sheet (Chen et al., 2016). Most CEH 28 group members possess carboxylesterase, serine esterase and phospholipase activities associated with conserved catalytic residues, such as Ser113/Asp166/His197 for *Pseudomonas aeruginosa* carboxylesterase. Cunnac and colleagues aligned the SOBER1 amino acid sequence with other CEH28 members, human acyl protein thioesterase (HsAPT1) and *Pseudomonas fluorescens* esterase EstB. As a result, the conserved catalytic residues of SOBER1 were predicted to be Ser106, Asp158 and His192 (Cunnac et al., 2007) (Fig. 4.2.3.3.).

Previously, SOBER1 was reported to interfere with the accumulation of phosphatidic acid (PA) associated with AvrBsT-elicited defence in *Arabidopsis*. It was suggested that SOBER1 may have phospholipase A2 (PLA_2) activity to hydrolyse phosphatidylcholine (PC), a precursor of PA, which is dependent on Ser106 and His192 (Kirik and Mudgett, 2009). PA is a simple phospholipid involved in the regulation of various stresses and its importance in regulating plant defence responses is currently emerging (Canonne et al., 2011; Li et al., 2009; Testerink and Munnik, 2011). There are two major pathways of PA generation via different types of phospholipases. One is from breaking membrane-associated phosphatidylcholine (PC) from its head group by phospholipase D (PLD). The other is via two steps: first, phosphatidylinositol-(4,5)- biphosphate (PIP_2) is converted to diacylglycerol (DAG) by phospholipase C (PLC), and second, DAG phosphorylation by diacylglycerol kinase (DGK) generating PA (Testerink and Munnik, 2005). PA upregulation by both pathways is known to be involved in plant defence responses (Kirik and Mudgett, 2009; de Jong et al., 2004; Andersson et al., 2006).

It was proposed that PA accumulation through the PLD-mediated pathway was involved in AvrBsT-triggered immunity in *Arabidopsis* and that the phospholipase A_2 activity of SOBER1 interrupted the PA upregulation by downregulating its precursor, PC, effectively antagonizing the AvrBsT-triggered defence response (Kirik and Mudgett, 2009). However, multiple studies indicated that PLD-mediated PA accumulation is a general response involved in plant immunity which does not correlate with our discovery that SOBER1 selectively suppresses ETI (Testerink and Munnik, 2005). For example, AvrRpm1 or AvrRpt2-

triggered electrolyte leakage in *Arabidopsis* was significantly reduced upon PLD-specific inhibitor n-butanol treatment (Andersson et al., 2006). Moreover, there are multiple lines of evidence for a role of SA as a stimulus for PA, which is a broad plant immune regulator (Zhang and Xiao, 2015). As SOBER1-mediated HR suppression is specific to several acetyltransferases, it is unlikely that a major function of SOBER1 is to down-regulate PA level. It is likely that PA is a downstream component of ETI rather than a specific regulator of particular ETI signalling.

In this chapter, SOBER1 was shown to suppress HCD triggered by multiple acetyltransferase effectors but not all YopJ family effectors in *N. tabacum*, for example HopZ1a. This implies that SOBER1 suppresses HCD triggered by multiple but specific acetyltransferase effectors in *N. tabacum,* indicating that the suppression mechanism is positioned at a shared pathway of certain acetyltransferase effectors. Alternatively, SOBER1 may target multiple host components that are selectively required for immunity triggered by each effector.

An interesting fact about SOBER1-mediated suppression of effector-triggered HR in *N. tabacum* is the different localisation of effectors. HopZ5 is localized at the plasma membrane by myristoylation while HopZ3 and AvrBsT are localised to the nucleus and cytoplasm (Jayaraman et al., 2017; Chapter 3 herein; Cheong et al., 2014) as is SOBER1. This result implies that SOBER1 possibly suppresses downstream of the effector recognition event by the cognate immune receptor. A proposed mechanism for SOBER1-mediated HR suppression is illustrated in Figure 4.3.2.1. HopZ5, AvrBsT and HopZ3 may be recognised by the same or different R proteins that trigger common defence signals. If SOBER1 suppresses this pathway upstream of PA regulation, immunity triggered by other effectors that is dependent on PA regulation, such as AvrRpm1, would be interfered by SOBER1. However, it is well known that AvrRpm1 triggers RPM1-dependent immunity in Col-0 where SOBER1 is fully functional in suppressing HopZ5-triggered HR. Thus, it is unlikely that SOBER1 directly down-regulates the PA level. However, involvement of PA accumulation in HopZ5 or HopZ3-triggered HR is not yet defined. An interesting alternative hypothesis is that SOBER1 suppression may occur at the level of effector recognition. If HopZ5 and HopZ3 share a guardee

or decoy molecule to be recognised by their corresponding R proteins, it is plausible that SOBER1 targets and perturbs this molecule (Fig. 4.3.2.1.).

Autoacetylation of YopJ acetyltransferase effectors from both plant and animal pathogens has been broadly reported. HopZ3 from *P. syringae* autoacetylates Cys300, HopZ1a autoacetylates mainly on Thr346 and AvrBsT autoacetylates an unknown residue(s) *in vitro* (Lee et al., 2015; Cunnac et al., 2007). HopZ5 autoacetylates lysine residue(s) *in vivo* (Jayaraman et al., 2017). Autoacetylation-defective mutants of these effectors are attenuated in their avirulence or virulence function (Lee et al., 2015; Ma et al., 2015). Even though acetylation of the putative HopZ1a autoacetylated Lys289 was not validated, HopZ1a K289R mutant partially is attenuated in virulence and avirulence (Rufián et al., 2015). AvrBsT lysine 282 is a predicted autoacetylated lysine residue and mutation of this residue did not affect autoacetylation *in vitro* yet K282R was impaired in avirulence in *Arabidopsis* (Cheong et al., 2014). In this study, *in vivo* lysine autoacetylation was shown in HopZ5 and HopZ1a albeit the residue is not specified. Strikingly, lysine autoacetylation of HopZ5 was stabilised when co-expressed with SOBER1 while HopZ1a lysine autoacetylation was not. This result is very interesting as in the previous study (Jayaraman et al., 2017) Lys278 mutation abolished lysine acetylation and significantly attenuated HopZ5-triggered HCD in *Nicotiana*. If the lysine acetylation of HopZ5 is related to its avirulence in *Nicotiana,* it seems more likely to show less lysine acetylation when SOBER1 is present and suppresses its HCD. A mechanistic linkage between SOBER1-mediated HCD seems to be a novel phenomenon in YopJ family effector recognition.

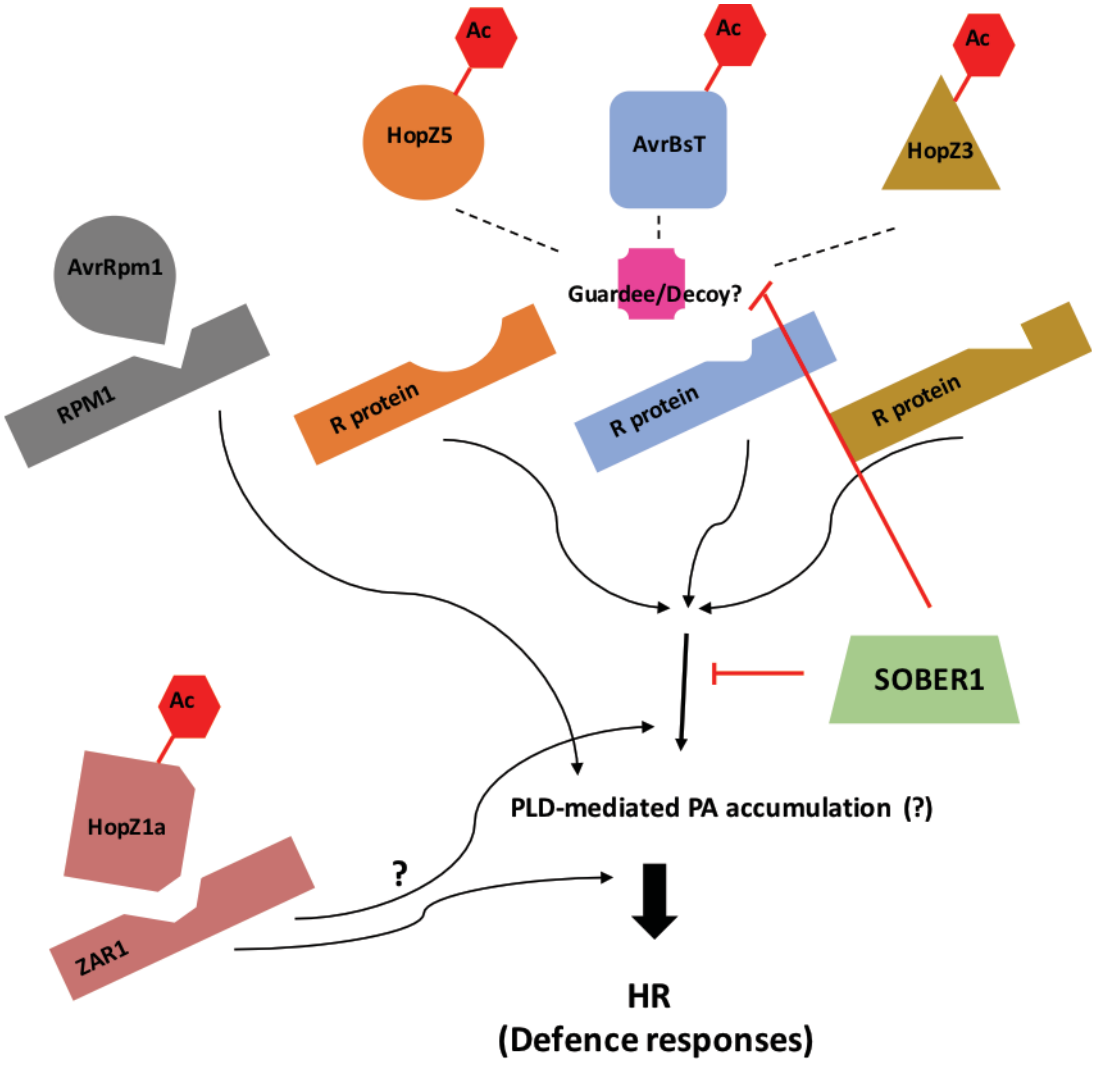


Figure 4.3.2.1. Schematic model of SOBER1-mediated acetyltransferase effector-triggered HR.

HopZ5, AvrBsT and HopZ3 may be recognised by the same or different R proteins that trigger common defence signals. SOBER1 may suppress this pathway upstream of PA regulation as SOBER1-independent immunity triggered by other effectors that is dependent on PA regulation, such as AvrRpm1. Thus, it is unlikely that SOBER1 directly down-regulates PA level. Alternatively, SOBER1 suppression may occur at the level of effector recognition as all these effectors share acetyltransferase activity. If HopZ5 and HopZ3 share a guardee or decoy molecule that is recognised by their corresponding R proteins, it is plausible that SOBER1 targets and perturbs this molecule. However, this is specific to HopZ5, HopZ3 and AvrBsT as immunity triggered by another acetyltransferase effector HopZ1a upon ZAR1 recognition is not suppressed by SOBER1. Ac: Acetylation

4.3.3. Interplay between effectors from pathogens

An interesting feature about HopZ5-triggered HR was the different HR phenotypes in *sober1-3* depending on the delivery system. The main difference between *Pto* DC3000 and *Pf* Pf0-1 (T3SS) is the presence of an effector repertoire of its own in the former. The fact that HopZ5 can trigger HR in *sober1-3* when it is delivered alone from *Pf* Pf0-1 but not by *Pto* DC3000 together with an additional 28 effectors implies that there would be one or more effectors in *Pto* DC3000 which may interfere with HopZ5-triggered HR but not immunity in the Col background. Also, as the growth of *Pto* DC3000 carrying *hopZ5* was still restricted, this *Pto* DC3000 effector-mediated suppression effect is again limited to HR, not defence responses. Suppression of ETI has been considered as one of the major roles of pathogen effectors (Macho et al., 2010; Block et al., 2014; Abramovitch et al., 2006; Guo et al., 2009). However, HR-specific suppression by an effector has not been reported. This result again supports the distinct pattern of HopZ5-triggered immunity in *Arabidopsis* with a level of separation between cell death signalling and defence responses. Transgenic expression of a prokaryote gene, Fld, in tobacco plastids suppressed HR specifically without affecting immunity (Zurbriggen et al., 2009). Therefore, it is possible that a *Pto* DC3000 effector suppresses an HR-specific pathway, maybe in shared signalling with the SOBER1-mediated HR suppression. Another possibility is the actual interplay between effectors attenuating or changing the function of the effector, as implied in the case of *Psy* HopZ3 interacting with AvrRpm1 and AvrB3 and attenuating RPM1-dependent resistance (Lee et al., 2015). Notably, *PR1* gene expression is upregulated in Col-0 only when HopZ5 is delivered by *Pto* DC3000 but not by *Pf* Pf0-1 (Jayaraman et al., 2017). These lines of evidence again support the separation of HR and immunity in Col and the complex interplay of pathogen effectors.

Chapter 5 Identification of the genomic locus conferring HopZ5-triggered immunity in *Arabidopsis thaliana*

5.1. Objectives and contributions

5.1.1. Objectives

Interaction between pathogen effectors and the host defence system, especially the mechanism of effector detection by R proteins, has been extensively studied over the past few decades. Understanding various effector recognition systems is important not only for tracing evolutionary footsteps, but also for practical agricultural applications. To understand the mechanism by which HopZ5 is recognised in *Arabidopsis*, the *R* gene that confers HopZ5 recognition in a resistant *Arabidopsis* accession Ct-1 was attempted to be mapped. However, *SOBER1* was identified as the dominant suppressor of HopZ5-triggered HR.

By combining this large-scale screening of HopZ5-triggered HR in *Arabidopsis* accessions with the public database of 1001 *Arabidopsis* genomes sequence browser, one accession called Gabelstein-0 (Ga-0), which lacks *SOBER1* but shows no HR to HopZ5, was identified; therefore, it can be used to map the resistance gene for HopZ5 as a susceptible parent.

In this chapter, firstly, the genetic requirements of known immune regulators in HopZ5-triggered HR were investigated to better understand HopZ5-triggered immunity in *Arabidopsis*. Secondly, the initial bulk-segregation analysis for mapping of the *hopZ5* resistance gene in the F2 population between Ct-1 and Ga-0 is reported.

5.1.2. Contributions

Jay Jayaraman has contributed in the work described in this chapter of the thesis, specifically, he conducted one independent biological replication out of three for Figure 5.2.1.2.

Without these significant contributions, this thesis would not have been able to achieve the results herein. All other work in this thesis is done by myself under supervision of Prof. Kee Hoon Sohn or Prof. Cecile Segonzac.

5.2 Results

5.2.1. Different genetic components are required for HopZ5-triggered HR and growth restriction in *Arabidopsis*.

In order to test the requirement of known immune-regulating components in HopZ5-triggered immunity in *Arabidopsis*, several mutations, such as *sid2-1* (Wildermuth et al., 2001)*, eds1-2* (Falk et al., 1999)*, ndr1-1* (Century et al., 1995) and *pad4-1* (Jirage et al., 1999) were introduced into the Ct-1 background (*sober1*$^{Ct\text{-}1}$) by crossing. Double mutant $F_{3\text{-}4}$ plants were selected for testing HopZ5-triggered HR. Many *R* gene-mediated defence responses require *EDS1/PAD4* or *NDR1* depending on the N-terminal domain structure of the resulting protein (Aarts et al., 1998). The EDS1/PAD4 complex is known to be involved in downstream signalling of most TIR-NBS-LRRs while NDR1 is required for downstream signalling of multiple CC-NBS-LRRs.

Interestingly, HopZ5-triggered HR was independent of *EDS1, PAD4* and *NDR1* (Fig. 5.2.1.1). AvrRps4 did not trigger HR in *eds1-2* Ct-1 and AvrRpt2 did not trigger HR in *ndr1-1* Ct-1 plants as expected (Century et al., 1995a). *PAD4* was not required for both AvrRps4 and AvrRpt2-triggered HR. The plant hormone, salicylic acid (SA), is known to play an important role in defence signalling activated by many *R* genes. Therefore, the *sid2-1* mutant which impairs SA accumulation upon pathogen invasion was tested (Wildermuth et al., 2001). HopZ5 still was able to trigger HR in *sid2-1* Ct-1 plants.

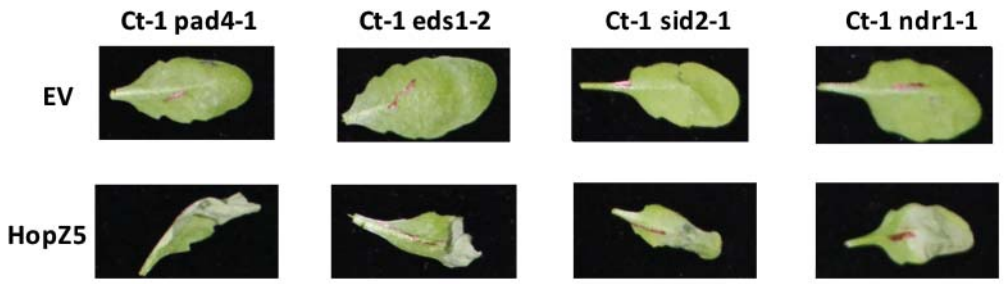


Figure 5.2.1.1. HopZ5-triggered HR does not require known regulators of plant immunity, *EDS1, PAD4, SID2* or *NDR1*.
Known *Arabidopsis* mutants *pad4-1, eds1-2, sid2-1* and *ndr1-1* were integrated into Ct-1 background with truncated *SOBER1* by crossing. pBBR 1MCS 5B:*avrRps4 promoter (EV),* (*hopZ5:HA)* carrying *P. fluorescens* Pf0-1 was infiltrated into fully expanded five-week old *Arabidopsis* leaves. HR was scored and photographed 1 day after infiltration. This experiment was conducted 3 times with similar results.

HopZ5-triggered immunity was separated from HR in Col-0 indicating that HR and immunity may have different signalling pathways in the case of response to HopZ5. In order to test if these immune signalling components affect pathogen growth restriction, *Pto* DC3000 carrying HopZ5 growth was tested in those mutants in the Ct-1 background. Interestingly, in contrast to the HR result, growth restriction of *Pto* DC3000 carrying HopZ5 was abolished and this strain grew to the same level as EV in *eds1-2* and *pad4-1,* or grew more than EV in *sid2-1* (Fig. 5.2.1.2.). *Pto* DC3000 carrying HopZ5 still grew less in *ndr1-1,* but this was not statistically significantly different from EV. This fact may imply the partial involvement of *NDR1* in HopZ5 recognition. *Pto* DC3000 carrying AvrRpt2 or AvrRps4 was used as the controls for *ndr1-1* or *eds1-2,* respectively. This is an unexpected result, as AvrBsT,

first reported as an acetyltransferase effector which was suppressed by SOBER1, required *NDR1* and only partially *EDS1*, *PAD4* and *SID2* for *Pto* DC3000 growth restriction (Cunnac et al., 2007). This indicates that SOBER1 may suppress two different effector recognition systems, in addition implying that HopZ5 and AvrBsT are recognized by different immune signalling systems.

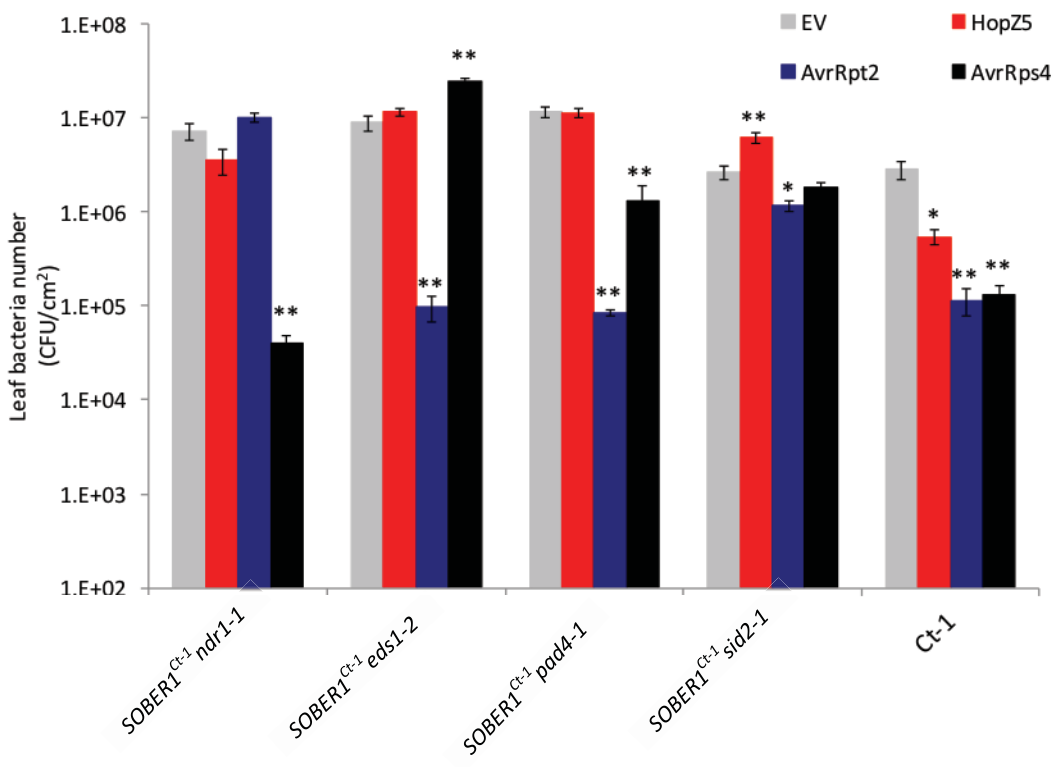


Figure 5.2.1.2. HopZ5-triggered immunity does require known regulators of plant immunity, *EDS1, PAD4, SID2* but not *NDR1*.
Known *Arabidopsis* mutants *pad4-1, eds1-2, sid2-1* and *ndr1-1* were integrated into the Ct-1 background with Ct-1 version of truncated *SOBER1* allele (*SOBER1*$^{Ct-1}$*)* by crossing. pBBR 1MCS 5B:*avtRps4 promoter (EV*), (*hopZ5:HA)* carrying *Pto* DC3000 (5 x 10^5 cfu/ml) was syringe-infiltrated in 4-5-week Arabidopsis leaves. Error bars indicate standard error from 6 technical replications from one biological replication. The number of bacteria on the leaves was counted at 4dpi. This experiment was conducted 3 times independently with similar results.

5.2.2 *Arabidopsis* accession Ga-0 carries a truncated *SOBER1* variant and does not trigger HR in response to HopZ5

In chapter 4, 136 *Arabidopsis* accessions were screened for HopZ5-triggered HR and correlation between HopZ5-triggered HR and absence of full-length *SOBER1* was observed. SNPs in *SOBER1* sequences from accessions that did not show HR to HopZ5 were compared to the Col-0 reference sequence from the public genomic sequence browser (http://signal.salk.edu/atg1001/3.0/gebrowser.php).

Among the accessions with SNP data available, one accession, Ga-0 had the same nucleotide deletion in exon 5 (cytosine 86 deletion) as in Ct-1. This deletion

leads to a frameshift after glycine 179 introducing an early stop codon (Fig. 5.2.1.1). The Ga-0 *SOBER1* deletion polymorphism was confirmed by *SOBER1* PCR product sequencing. To test if the lack of HR phenotype in Ga-0 is specific to HopZ5, EV, AvrRpt2 and HopZ5 were delivered by *Pto* DC3000. Ga-0 clearly showed HR to AvrRpt2 but not to HopZ5 (Fig. 5.2.1.1.) while Ct-1 showed HR to both AvrRpt2 and HopZ5. Therefore, Ga-0 was chosen for further genetic analysis of *SOBER1*-independent absence of HopZ5-triggered HR, which possibly involves *R* genes. Hereafter, the *R* genes will be referred to as *SOBER1*-independent HopZ5-HR-mediating genes in Ct-1.

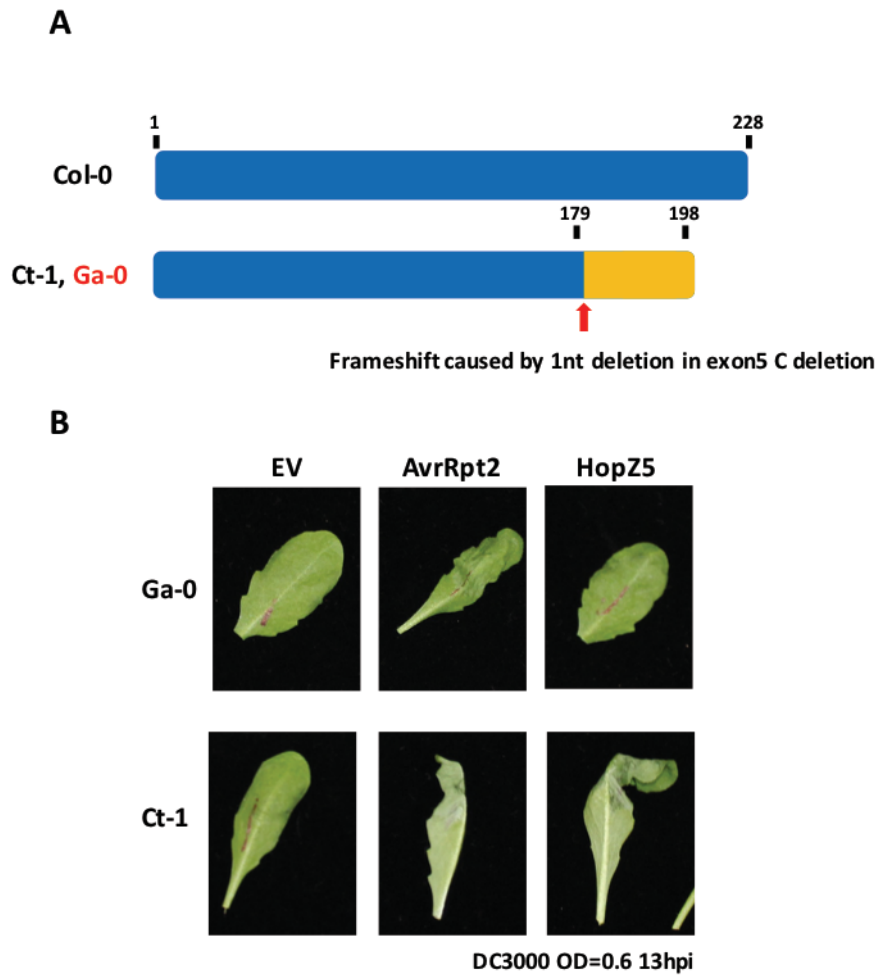


Figure 5.2.1.1 Ga-0 does not show HR to HopZ5 despite the truncated SOBER1.
A – Ga-0 contains the same one nucleotide deletion at exon 5 that causes a frameshift from 179G and an early stop codon in Ct-1 (indicated with yellow color). B – Ga-0 does not show HR to HopZ5 delivery by *Pto* DC3000. 3 x 10^8 cfu/ml of *Pto* DC3000 carrying pBBR-1MCS-5B:avrRps4 promoter (EV), (*hopZ5:HA)* and pBBR-1MCS-5B 662 :*avrRpt2* were infiltrated into *Arabidopsis* Ct-1 and Ga-0 leaves. HR

was scored and photographed at 13hpi. This experiment was conducted 3 times with similar results.

5.2.3 Two different genes may be involved in HopZ5-triggered immunity independent of *SOBER1*

For genetic analysis of *SOBER1*-independent HopZ5-triggered HR in *Arabidopsis*, Ct-1 or *sober1-3* (Col) were crossed to Ga-0. Interestingly, F_1 progenies between Ct-1 x Ga-0 and *sober1-3* x Ga-0 showed different phenotypes upon HopZ5 delivery via Pf0-1 (T3SS). In the Ga-0 x Ct-1 F_1 hybrid, HopZ5-triggered clear HR was not evident for the Ga-0 x *sober1-3* F_1. This result implied that there might be different HopZ5 *R* genes in Ct-1 and Col-0 (Fig. 5.2.3.1.).

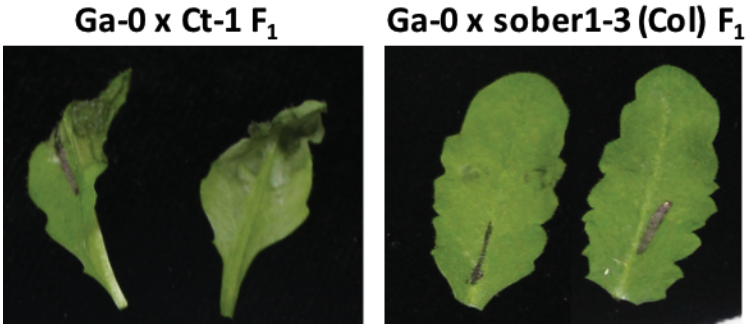


Figure 5.2.3.1. Different phenotypes of HopZ5-triggered HR in Ga-0 x Ct-1 F_1 and Ga-0 x sober1-3 (Col) F_1 hybrids.
1 x 10^8 cfu/ml of pBBR 1MCS 5B:*avtRps4 promoter (hopZ5:HA)* carrying *P. fluorescens* Pf0-1 was infiltrated into *Arabidopsis* Ga-0 x Ct-1 and Ga-0 x *sober1-3* F_1 hybrids leaves. Photographs were taken 1 day after infiltration. This experiment was conducted 2 times with similar results.

Segregation of HopZ5-triggered HR in the F_2 population between Ga-0 and Ct-1 was further analysed. Out of a total of 401 F_2 individuals, the expected number of F_2 individuals not showing HR was 100 (E=100.25, ¼, Table 5.2.3.1.), assuming there is a single dominant locus involved in HopZ5-triggered HR. Surprisingly, 83 individuals did not show HR, which was significantly different from expectations according to the chi-squared test (X^2=3.95, *P*=0.047<0.05). However, if there are two genes involved redundantly, one dominant and the other recessive, the

expected number of F_2 individuals showing no HR would be around 75 (E=75.18, 3/16, Table 5.2.3.2.) and this is not statistically different from 83 (X^2=0.9985, *P*=0.318>0.05).

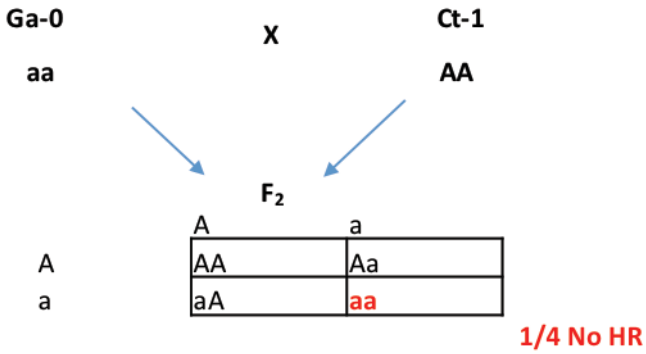
Table 5.2.3.1. Genetic analysis of HopZ5-triggered HR in F_2 hybrids

F_2	HR	No HR	n	X^2	P value
Ga-0 x Ct-1	13 (318)	3 (83)	401	0.9985	0.318
Ga-0 x sober1-3	1(13)	3 (35)	48	0.125	0.724

n: Total number of tested population, X^2: Chi-squared test value

Interestingly, a preliminary HR segregation test of the Ga-0 x *sober1-3* F_2 population revealed 13 HR-showing individuals out of 48 (1/4, X^2=0.125 *P*=0.724>0.05) in total, which suggests that each parent harbours a single recessive allele. It is possible that the recessive gene in Ct-1 is the same in Col-0.

Hypothesis 1. Single dominant allele (A) involved in HopZ5-triggered HR



Hypothesis 2. Two unlinked loci independently conferring HopZ5-triggered HR.

A dominant allele (A), homozygous recessive alleles (bb)

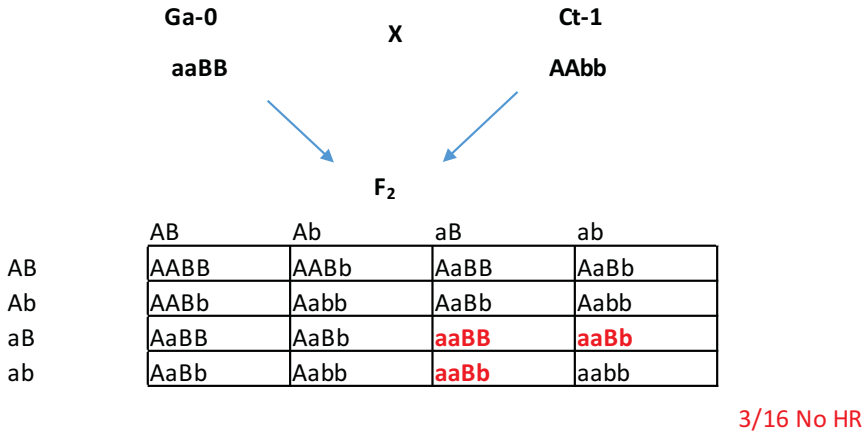


Figure 5.2.3.2. Two independent unlinked loci are possibly involved in HopZ5-triggered HR between Ct-1 and Col.
Punnett square analysis for two different hypotheses of genetic segregation of HopZ5-triggered HR. Red genotypes are hypothesized not to show HR to HopZ5.

5.2.4 The dominant locus that confers HopZ5 recognition is located on the upper arm of chromosome 3

To map the dominant allele in the Ga-0 x Ct-1 F_2 population, bulked segregant analysis (Michelmore et al., 1991) was conducted with F_2 individuals that did not show HopZ5-triggered HR. Genomic DNA from F_2 individuals which did not show HR to HopZ5 was extracted by a nuclei-enriched genomic DNA extraction method and quantified by agarose gel electrophoresis. An approximately equal amount of genomic DNA from each F_2 individual was pooled into a tube to avoid dosage-correlated preferential amplification of genotyping markers.

Due to the incomplete and imprecise Ga-0 sequence in public databases, only a few genotyping primers could be used in this analysis, which affected the resolution of the mapping. Among 50 primers, including previously published genotyping primers for different accessions of *Arabidopsis* and newly designed ones according to the public sequence database in this study, only 10 primers could be used: two markers, each in chromosome 1 and 2, one marker each for chromosome 3 and 4, and three markers for chromosome 5 (Table 2.5.3.3.3.).

As a result, one marker on the upper arm of chromosome 3 (Chr3B) amplified Ga-0 preferentially in the F_2 genomic DNA pool (Figure 5.2.3.1. A). Although this marker tended to amplify the Ct-1 DNA fragment when Ga-0 and Ct-1 genomic DNA was mixed in a one to one ratio, it clearly only amplified the Ga-0 DNA fragment in the F_2 genomic DNA pool. This means F_2 individuals that did not show HR to HopZ5 only contain the Ga-0 allele at this locus.

As NB-LRRs are a major group of R proteins, any NB-LRRs near this locus are strong candidates for HopZ5 recognition. Figure 5.2.3.1. B shows the map of predicted full length NB-LRRs on chromosome 3 of the Col-0 reference genome with respective locations of the markers. This map was drawn based on (Meyers et al., 2003) and the numbers indicate the name of the locus according to TAIR references (www.arabidopsis.org). There are six NB-LRR genes on the upper arm of chromosome 3; three are CC-NB-LRRs and another three are TIR-NB-LRRs. As HopZ5-triggered immunity seems to involve a mainly *EDS1*-dependent SA signalling

pathway, the 3 TNLs are stronger candidates. None of these genes are reported as *R* genes for other pathogens.

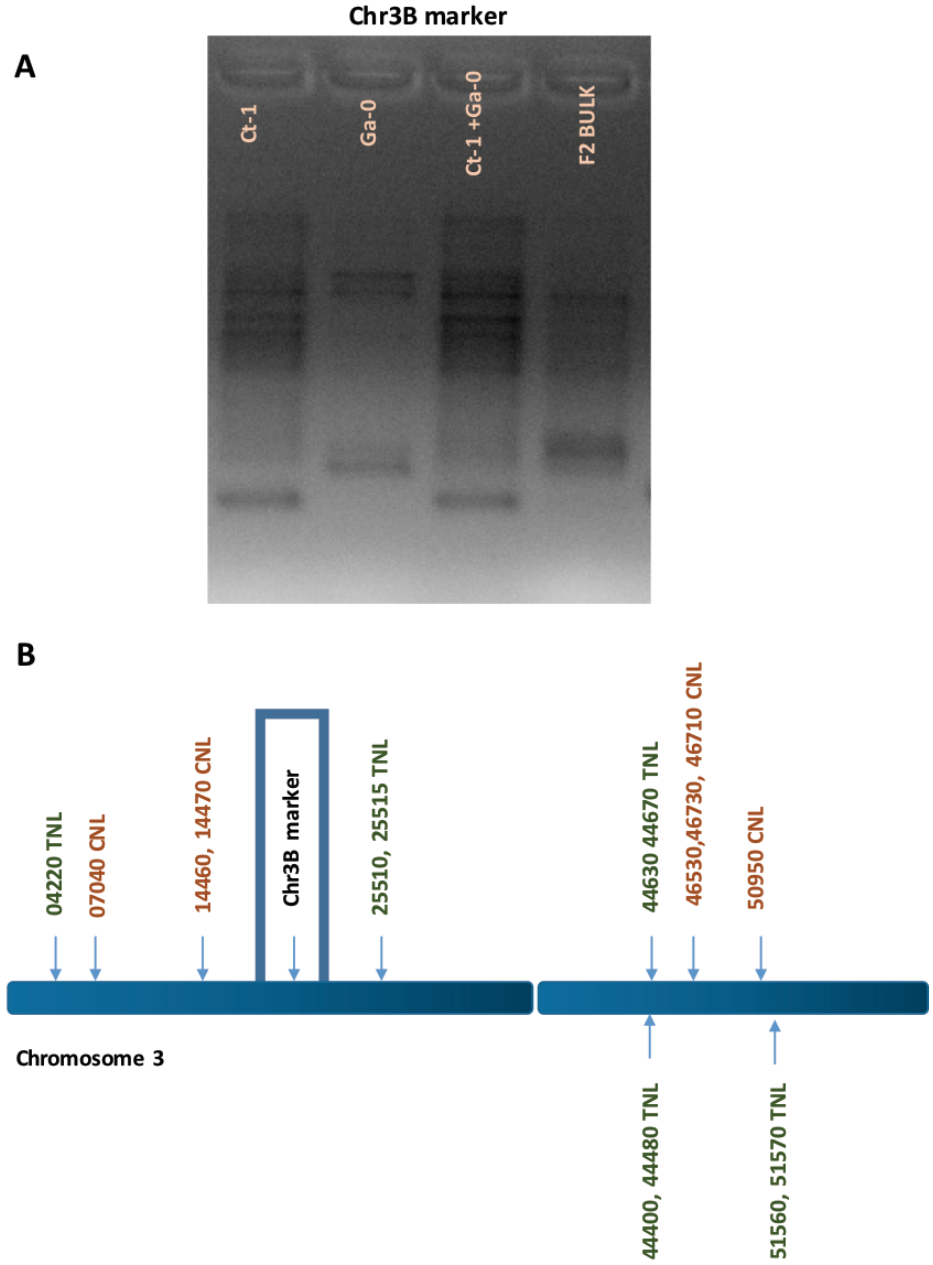


Figure 5.2.3.1. The dominant allele involved in HopZ5-triggered HR is located on chromosome 3.
A – Bulked segregant analysis shows Ga-0 specific amplification of Chr3B marker.
B – Relative location of full-length NB-LRR genes on chromosome 3 to Chr3B marker. Full length means that genes are predicted to have full TIR/CC domain, NB domain and LRR domain in Meyers et al. (2002) for the Col-0 reference sequence. Numbers indicate the locus name after chromosome number according to the TAIR system (for example, AT3G04220 is written as 04220). TIR-NB-LRRs and CC-NB-LRRs are indicated as TNL (green color) and CNL (orange color), respectively.

5.3 Discussion

5.3.1. HopZ5-triggered immunity but not HR may involve EDS1/PAD4-dependent SA regulatory signalling.

Many NB-LRR genes require several nodes of signalling depending on their N-terminal domain (Aarts et al., 1998). Most thus far characterized TNLs require the *EDS1/PAD4*-dependent SA signalling pathway and most CNLs require *NDR1.* In this study, HopZ5-triggered HR is independent of any genetic components tested (*EDS1, PAD4, NDR1* and *SID2).* However, *Pto* DC3000 carrying HopZ5-mediated growth restriction required *EDS1, PAD4* and *SID2,* which implies that HopZ5-triggered immunity is regulated by *EDS1/PAD4*-dependent SA signalling possibly through TNL *R* genes. Corresponding to HopZ5-triggered immunity in Col-0, HopZ5-triggered HR seems to follow a separate signalling pathway from HopZ5-triggered immunity in Ct-1 as well. The idea of HR not being necessary for immunity is emerging with accumulating examples of HR disconnected from immunity. Immunity triggered by the *P. syringae* effectors AvrRpm1 and AvrB mediated by RPS2 requires *NDR1* but HR is not affected in *Arabidopsis* (Century et al., 1995). TMV recognition by the CNL HRT unusually requires *EDS1/PAD4* for immunity but not for HR activation in *Arabidopsis* (Chandra-Shekara et al., 2004). In addition, the potato *Rx* gene mediates PVX (Potato virus X) resistance without HR (Bendahmane et al., 1999).

Why HR is occurring in many resistant plants is still under debate. If HR is not always contributing to plant immunity, it could be a consequence of plant-microbe interaction by sharing signalling patterns between apoptosis and defence whether by chance or not. From an evolutionary point of view, many known *R* gene systems which induce systemic cell death have fitness costs (Tian et al., 2003) partially because they need to be successfully suppressed until pathogen invasion. Furthermore, HR associated with R protein activity can be hijacked by necrotrophic pathogens (Hammond-Kosack and Rudd, 2008). In this perspective, having *SOBER1*

as a suppressor of HR but not resistance would be an evolutionarily more advanced strategy. However, different suppression effects of *SOBER1* in different accessions still need to be explained.

As HopZ5-triggered immunity follows an *EDS1/PAD4*-dependent SA regulatory node, the first possibility for the cognate immune receptor is a TNL. However, there are also examples of non-TNL type immune receptors requiring *EDS1/PAD4*-dependent signalling. As mentioned above, the CNL *HRT* and the atypical *R* gene *RPW8*, which is not an NB-LRR, require *EDS1* for defence against TMV and powdery mildew, respectively (Chandra-Shekara et al., 2004; Xiao et al., 2001).

Of note are the 6 *Arabidopsis* accessions lacking *SOBER1*, which showed HR to HopZ5 delivery in Chapter 4, which also showed HR to AvrBsT. However, AvrBsT-triggered immunity and HR in Pi-0 required *NDR1* (Cunnac et al., 2007) unlike HopZ5. Possibly, it can be a coincidence to have two *R* genes recognizing two closely related homologous effectors, but also the same receptor via a different mechanism (possibly by a different decoy or guardee). If the latter is true, the HopZ5/AvrBsT *R* gene would be expected to be non TNLs as so far there are more examples of non TNLs requiring *EDS1* than TNLs requiring *NDR1*. On the other hand, in the case of AvrB recognition by a pair of genes *RPM1* and *TAO1* in *Arabidopsis*, two genes can recognize the same (or similar) effector(s) and at least one of these genes is broadly spread among *Arabidopsis* accessions (Eitas et al., 2008). Similarly, the tobacco TNL *N* and CNL *NRG1* mediate TMV resistance but require *EDS1* and *SGT1* respectively (Peart et al., 2005), which could also be the case for HopZ5 and AvrBsT recognition.

5.3.2. Two *R* genes in Ct-1, one *R* gene in Col-0: do Ct-1 and Col-0 share the same recessive gene?

Figure 5.3.2 shows a summarized hypothesis about HopZ5 *R* genes between *Arabidopsis* Ct-1, Ga-0 and Col-0. It seems that there are two redundant components for HopZ5-triggered HR in Ct-1. A similar case has been reported by

Chandara and colleagues, which is the presence of the redundant recessive immune component *RRT* along with dominant *HRT* for TMV resistance in *Arabidopsis* (Chandra-Shekara et al., 2004) even though the precise mechanism is still elusive. Also, *TAO1*, which is considered to be required for full AvrB recognition together with RPM1, is also recessive while *RPM1* is dominant (Eitas et al., 2008). As the 13:3 segregation implies the full redundancy of two genes, it is less likely for those two genes to be in a hierarchical position of the signalling pathway. Furthermore, considering that both alleles are effectively suppressed by SOBER1, they would be either downstream of SOBER1-suppression or upstream, but share the common downstream signalling pathway suppressed by SOBER1. Whether these two genes are additively supportive in HopZ5-triggered immunity, like the RPM1/TAO1 pair in recognition, remains to be tested after those genes are mapped.

As one of two in Ct-1 and the one in Col-0 is expected to segregate recessively, the question arises if those recessive genes are the same. In order to address this question, crosses between Ct-1 x Ga-0 F_1 and Ct-1 x *sober1* (Col) F_1 would be useful tool to analyse the segregation of the hybrid F_2 population. If the recessive *R* genes in Ct-1 and Col-0 are the same, it is expected that HopZ5-triggered HR will segregate in a 5:3 ratio, HR to no HR.

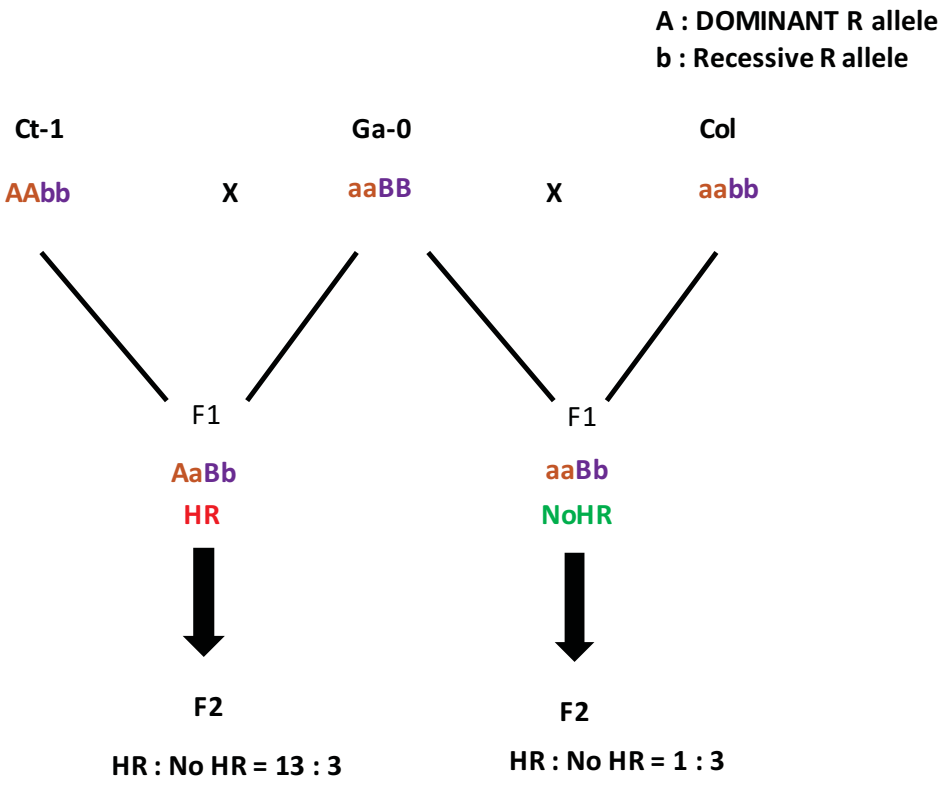


Figure 5.3.2. Summary hypothesis of *hopZ5* resistance gene genetics between *Arabidopsis* accessions Ct-1, Ga-0 and Col

Chapter 6. General discussion and future directions

6.1 Understanding the diversity of *Psa* effectors; dynamic cellular environmental changes by effector proteins

Throughout this study, multiple results supported various roles of type III effectors in plant-microbe interactions. In chapter 1, the diversity of subcellular localization and avirulence activities following distinct signalling nodes for multiple effectors was shown. Pathogens effectors have been studied for decades and can now be described as 'multifunctional, redundant and cooperative' (Cunnac et al., 2011). Redundancy and hierarchy of effector sets in pathogen virulence are well known from studies including the research focused on a minimal virulence set of effectors in *Pto* DC3000 (Cunnac et al., 2011). Effectors are mainly studied for their avirulence or virulence activities. However, evidence of other functions for effectors that indirectly affect pathogen propagation *in planta* is accumulating. Recently, HopM1 and AvrE1 have been reported to be involved in high humidity maintenance around the infection site to increase pathogen virulence (Xin et al., 2016). Also, effectors that take part in sugar transport towards the *in planta* site of infection and changing the physiological flow of sugar that is normally allocated for plant development. These activities are also known to benefit the pathogen (Chen et al., 2010). Profiling effector characteristics could give broad suggestions about their roles *in planta.* Studying each effector function in either host immunity activation/suppression, or in contribution to virulence indirectly through non-immune functions would help to understand effector-mediated host cell physiological changes. Promising future work would involve investigation of how each of the effectors in *Psa* contributes to avirulence in non-host *Nicotiana* spp. by serial step-by-step deletion of effectors. The first candidates would be effectors triggering HCD in *Nicotiana* spp.

The fact that HopZ5-triggered HR is abolished in *sober1-3* plants when delivered by *Pto* DC3000 strongly suggests the presence of effector-mediated

inhibition of HopZ5-triggered HR in the Col background. This underlines the interplay between effectors when a non-native effector is introduced into a pathogen as determining this new effector's contribution towards virulence/avirulence. Future work to reveal the mechanism underlying this phenotype would involve testing HopZ5 delivery by a series of effector deletion mutants of *Pto* DC3000 to identify effectors contributing to the suppression of HopZ5-triggered HR. *Pto* DC3000 effector deletion mutants, with several effector clusters inactivated, have been used for studies of effector redundancy and hierarchy studies (Kvitko et al., 2009; Cunnac et al., 2011). This suppression of HR could be instigated by one or multiple effectors subtly changing host cellular dynamics in favour of HR attenuation. Once the suppressing effector is identified, the plant suppression target could also be mapped using *sober1-3* x Ct-1 or RIL individuals from the Col-0 x Ct-1 cross that carry only the Ct-1 allele of *SOBER1.*

Testing *Psa* V-13 in *sober1-3* would also be an interesting direction in future work. *Psa* V-13 and *Pto* DC3000 share effector homologues. Comparing if homologous effectors in different effector pools can differentially affect HopZ5-triggered HR for *Psa* versus *Pto* could facilitate research into the complex nature of interconnection between effectors.

6.2 Different signalling pathways are required for HR and immune responses with shared regulators

HopZ5-triggered immunity in *Arabidopsis* has a distinctive phenotype different from canonical immune responses, and involving the selective HR suppressor SOBER1. HopZ5-triggered HR and immune responses seem to follow different pathways that can be suppressed specifically by SOBER1. In addition, SOBER1 suppresses cell death triggered by several YopJ acetyltransferase effectors in *N. benthamiana,* which implies that SOBER1 negatively regulates the common mechanism of triggering cell death shared by acetyltransferase effectors. Understanding how SOBER1 suppresses HopZ5-triggered HR as well as cell death

triggered by other acetyltransferases could significantly broaden our understanding of HR and immune signalling pathways.

SOBER1 suppresses HopZ5-triggered HR specifically in *Arabidopsis* Col-0. Considering results in chapter 4 and 5 together, HopZ5-triggered HR suppression by SOBER1 can be hypothesized as in Figure 6.2.1. HopZ5 is recognised by its corresponding R protein directly or indirectly involving a guardee/decoy.

Perception of HopZ5 by an immune receptor in Col-0 signals through downstream components. Each circle represents a set of signalling pathway nodes involved in defence gene regulations, physiological regulations or cell death triggering responses. These signalling events involve various regulation of signalling molecules, which can be shared in different pathways by their multifunctional activities possibly in a quantitative manner. The fine tune regulations of each signalling pathways would create different patterns of crosstalk between them and causes different outcomes, which is shown as overlaps of circles in the figure. The location of each circle does not mean the epistasis of each pattern, but EDS1/PAD4-mediated SA regulation seems to be only involved in HopZ5-triggered defence responses not in HR, suggesting the involvement of EDS1/PAD4 after the HR-immunity connection. If SOBER1 suppressed this connecting reaction between an HR-provoking pattern and another defence signalling pattern, this connection between them would no longer exist, resulting in resistance without HR (red arrows). If SOBER1 alters the recognition step of HopZ5, the altered signal may go directly through to the immune response signalling, not through the HR connecting pattern (purple arrows). The main point of this model is that HR and defence responses are not completely separated; they are branching from one to another, but interconnected in many reactions that can be diversely regulated.

Understanding atypical resistance could broaden the concept of plant immunity. It would be particularly interesting to study the mechanism underlying HopZ5-triggered immunity in *Arabidopsis*. In order to understand more about HopZ5-triggered immunity, identification of the cognate R protein and the HopZ5 target *in planta* would be necessary. Mapping two possible redundant *R* genes suggested from the Ga-0 x Ct-1 F_2 analysis should be prioritised. If these two genes

work as an additive pair, this would support the quantitative threshold regulation hypothesis in plant immune responses.

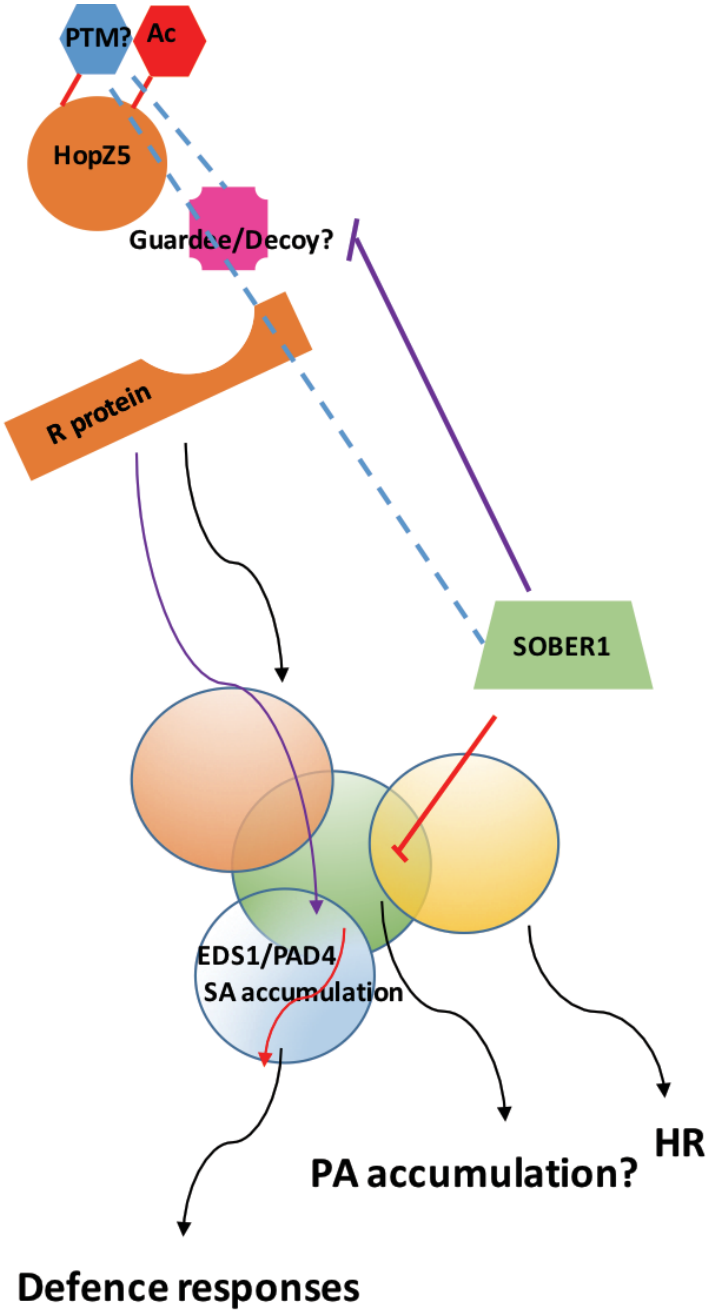


Figure 6.2.1. Summary hypothesis of HopZ5–triggered immunity and SOBER1-mediated suppression

Perception of HopZ5 by an immune receptor in Col-0 signals through downstream reactions. Each circle represents a set of signalling pathway nodes involved in defence gene regulations, physiological regulations or cell death triggering responses. Signalling can be shared in different pathways possibly in a quantitative

manner, which is shown as overlaps of circles. The location of each circle does not mean the epistasis of each pattern, but EDS1/PAD4-mediated SA regulation seems to be only involved in HopZ5-triggered defence responses not in HR, suggesting the involvement of EDS1/PAD4 after the HR-immunity connection. If SOBER1 suppressed this connecting reaction between an HR-provoking pattern and another defence signalling pattern, this connection between them would no longer exist, resulting in resistance without HR (red arrows). If SOBER1 alters the recognition step of HopZ5, the altered signal may go directly through to the immune response signalling, not through the HR connecting pattern (purple arrows).
PTM: Post-translational modification
Ac: Acetylation
PA: Phosphatidic acid
SA: Salicylic acid

Apart from HopZ5, SOBER1 also suppresses cell death triggered by multiple acetyltransferase effectors in *Nicotiana*. This suggests the presence of a shared mechanism of these acetyltransferase effectors although they appear to be recognized by different immune receptors and signalling pathways (Cunnac et al., 2007). Investigation of which step SOBER1 suppresses in cell death triggered by several YopJ acetyltransferase effectors would reveal a shared pathway by different immune receptors. It would be interesting to test if the same plant immunity molecular components such as EDS1 or NDR1 are required in this context. Investigation of the involvement of PA accumulation in immune responses triggered by different YopJ family effectors would be of interest in future research.

6.3. Possible roles of SOBER1 and SOBER1-like proteins in plant immunity

SOBER1 belongs to the α/β hydrolase superfamily. α/β hydrolases have a conserved folding structure with highly diverse amino acid sequences and enzymatic functions. EDS1 and PAD4 also have an α/β hydrolase structure, but their enzymatic activities are yet to be determined (Falk et al., 1999; Jirage et al., 1999). Although SOBER1 falls in a subgroup of phospholipases and cysteine carboxylesterases, there is a possibility that its actual enzymatic activity is different or that it is multifunctional. α/β hydrolases represent one of the largest protein groups with conserved structures with or without catalytic activity. Indeed, there are already examples of multifunctional α/β hydrolases (Hiraiwa, 1999; Vincent et al., 2003). There are many SOBER1-like homologues and orthologues across plant species, but at least some of them are non-functional (Cunnac et al., 2007). Also, truncated SOBER1 is very rare in *Arabidopsis*. This suggests a possible involvement of SOBER1 or SOBER1-like proteins in general physiological pathway(s) in plants, not only specific to immune suppression. If so, unravelling other physiological activities of SOBER1 could be helpful to understand the crosstalk between different signalling pathways.

Although *in vitro* phospholipase activity of SOBER1 has been reported, associated with PA accumulation in AvrBsT-triggered immunity (Kirik and Mudgett, 2009), there is no direct *in vivo* evidence. Studying if the phospholipase function of SOBER1 is generally involved in immunity triggered by other effectors would be critical to confirm SOBER1 enzymatic activity. Screening *Arabidopsis* accessions to find accessions that recognise HopZ3 would be useful for investigation of a general role of PA, as HopZ3 is not recognised by any HopZ5/AvrBsT-recognising *Arabidopsis* accessions. If PA is not a direct substrate of SOBER1, identifying the physical interactor of SOBER1 by immunoprecipitation or yeast-2-hybrid would enlighten how SOBER1 suppresses HopZ5-triggered HR.

References

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., Parker, J.E., 1998. Different requirements for EDS1 and NDR1 by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. Proc. Natl. Acad. Sci. U. S. A. 95, 10306–10311.

Abramovitch, R.B., Janjusevic, R., Stebbins, C.E., Martin, G.B., 2006. Type III effector AvrPtoB requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity. Proc. Natl. Acad. Sci. U. S. A. 103, 2851–2856. https://doi.org/10.1073/pnas.0507892103

Ade, J., DeYoung, B.J., Golstein, C., Innes, R.W., 2007. Indirect activation of a plant nucleotide binding site-leucine-rich repeat protein by a bacterial protease. Proc. Natl. Acad. Sci. U. S. A. 104, 2531–2536. https://doi.org/10.1073/pnas.0608779104

Adlung, N., Bonas, U., 2017. Dissecting virulence function from recognition - cell death suppression in *Nicotiana benthamiana* by XopQ/HopQ1-family effectors relies on EDS1-dependent immunity. Plant J. https://doi.org/10.1111/tpj.13578

Andersson, M.X., Kourtchenko, O., Dangl, J.L., Mackey, D., Ellerström, M., 2006. Phospholipase-dependent signalling during the AvrRpm1- and AvrRpt2-induced disease resistance responses in *Arabidopsis thaliana*. Plant J. 47, 947–959. https://doi.org/10.1111/j.1365-313X.2006.02844.x

Axtell, M.J., Staskawicz, B.J., 2003. Initiation of RPS2-specified disease resistance in Arabidopsis is coupled to the AvrRpt2-directed elimination of RIN4. Cell 112, 369–377.

Badel, J.L., Shimizu, R., Oh, H.-S., Collmer, A., 2006. A *Pseudomonas syringae* pv. *tomato avrE1/hopM1* mutant is severely reduced in growth and lesion formation in tomato. Mol. Plant. Microbe Interact. 19, 99–111. https://doi.org/10.1094/MPMI-19-0099

Bari, R., Jones, J.D.G., 2009. Role of plant hormones in plant defence responses. Plant Mol. Biol. 69, 473–488. https://doi.org/10.1007/s11103-008-9435-0

Bartetzko, V., Sonnewald, S., Vogel, F., Hartner, K., Stadler, R., Hammes, U.Z., Börnke, F., 2009. The *Xanthomonas campestris* pv. *vesicatoria* type III effector protein XopJ inhibits protein secretion: evidence for interference with cell wall-associated defense responses. Mol. Plant-Microbe Interact. MPMI 22, 655–664. https://doi.org/10.1094/MPMI-22-6-0655

Bell, C.J., Ecker, J.R., 1994. Assignment of 30 Microsatellite Loci to the Linkage Map of Arabidopsis. Genomics 19, 137–144. https://doi.org/10.1006/geno.1994.1023

Bendahmane, A., Kanyuka, K., Baulcombe, D.C., 1999. The *Rx* gene from potato controls separate virus resistance and cell death responses. Plant Cell 11, 781–792.

Birker, D., Heidrich, K., Takahara, H., Narusaka, M., Deslandes, L., Narusaka, Y., Reymond, M., Parker, J.E., O'Connell, R., 2009. A locus conferring resistance to *Colletotrichum higginsianum* is shared by four geographically distinct

Arabidopsis accessions: *Penetration resistance of Arabidopsis to Colletotrichum*. Plant J. 60, 602–613. https://doi.org/10.1111/j.1365-313X.2009.03984.x

Block, A., Guo, M., Li, G., Elowsky, C., Clemente, T.E., Alfano, J.R., 2010. The *Pseudomonas syringae* type III effector HopG1 targets mitochondria, alters plant development and suppresses plant innate immunity. Cell. Microbiol. 12, 318–330. https://doi.org/10.1111/j.1462-5822.2009.01396.x

Block, A., Toruño, T.Y., Elowsky, C.G., Zhang, C., Steinbrenner, J., Beynon, J., Alfano, J.R., 2014. The *Pseudomonas syringae t*ype III effector HopD1 suppresses effector-triggered immunity, localizes to the endoplasmic reticulum, and targets the *Arabidopsis* transcription factor NTL9. New Phytol. 201, 1358–1370. https://doi.org/10.1111/nph.12626

Bogdanove, A.J., Beer, S. v., Bonas, U., Boucher, C.A., Collmer, A., Coplin, D.L., Cornelis, G.R., Huang, H.C., Hutcheson, S.W., Panopoulos, N.J., Van Gijsegem, F., 1996. Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. Mol. Microbiol. 20, 681–683.

Boller, T., 1995. Chemoperception of Microbial Signals in Plant Cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 189–214. https://doi.org/10.1146/annurev.pp.46.060195.001201

Boller, T., Felix, G., 2009. A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. Annu. Rev. Plant Biol. 60, 379–406. https://doi.org/10.1146/annurev.arplant.57.032905.105346

Brendolise, C., Montefiori, M., Dinis, R., Peeters, N., Storey, R.D., Rikkerink, E.H., 2017. A novel hairpin library-based approach to identify NBS-LRR genes required for effector-triggered hypersensitive response in *Nicotiana benthamiana.* Plant Methods 13, 32. https://doi.org/10.1186/s13007-017-0181-7

Brodersen, P., Petersen, M., Bjørn Nielsen, H., Zhu, S., Newman, M.-A., Shokat, K.M., Rietz, S., Parker, J., Mundy, J., 2006. *Arabidopsis* MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. Plant J. Cell Mol. Biol. 47, 532–546. https://doi.org/10.1111/j.1365-313X.2006.02806.x

Canonne, J., Froidure-Nicolas, S., Rivas, S., 2011. Phospholipases in action during plant defense signaling. Plant Signal. Behav. 6, 13–18. https://doi.org/10.4161/psb.6.1.14037

Century, K.S., Holub, E.B., Staskawicz, B.J., 1995. NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. Proc. Natl. Acad. Sci. U. S. A. 92, 6597–6601.

Chandra-Shekara, A.C., Navarre, D., Kachroo, A., Kang, H.-G., Klessig, D., Kachroo, P., 2004. Signaling requirements and role of salicylic acid in HRT- and rrt-mediated resistance to turnip crinkle virus in Arabidopsis. Plant J. Cell Mol. Biol. 40, 647–659. https://doi.org/10.1111/j.1365-313X.2004.02241.x

Chapman, J.R., Taylor, R.K., Weir, B.S., Romberg, M.K., Vanneste, J.L., Luck, J., Alexander, B.J.R., 2012. Phylogenetic relationships among global populations of *Pseudomonas syringae* pv. *actinidiae*. Phytopathology 102, 1034–1044. https://doi.org/10.1094/PHYTO-03-12-0064-R

Chen, L.-Q., Hou, B.-H., Lalonde, S., Takanaga, H., Hartung, M.L., Qu, X.-Q., Guo, W.-J., Kim, J.-G., Underwood, W., Chaudhuri, B., Chermak, D., Antony, G., White, F.F., Somerville, S.C., Mudgett, M.B., Frommer, W.B., 2010. Sugar transporters for intercellular exchange and nutrition of pathogens. Nature 468, 527–532. https://doi.org/10.1038/nature09606

Chen, Y., Black, D.S., Reilly, P.J., 2016. Carboxylic ester hydrolases: Classification and database derived from their primary, secondary, and tertiary structures: Classification and Database of CEHs. Protein Sci. 25, 1942–1953. https://doi.org/10.1002/pro.3016

Chen, Z., Agnew, J.L., Cohen, J.D., He, P., Shan, L., Sheen, J., Kunkel, B.N., 2007. *Pseudomonas syringae* type III effector AvrRpt2 alters *Arabidopsis thaliana* auxin physiology. Proc. Natl. Acad. Sci. U. S. A. 104, 20131–20136. https://doi.org/10.1073/pnas.0704901104

Cheong, M.S., Kirik, A., Kim, J.-G., Frame, K., Kirik, V., Mudgett, M.B., 2014. AvrBsT acetylates Arabidopsis ACIP1, a protein that associates with microtubules and is required for immunity. PLoS Pathog. 10, e1003952. https://doi.org/10.1371/journal.ppat.1003952

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J.D.G., Felix, G., Boller, T., 2007. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature 448, 497–500. https://doi.org/10.1038/nature05999

Clarke, C.R., Chinchilla, D., Hind, S.R., Taguchi, F., Miki, R., Ichinose, Y., Martin, G.B., Leman, S., Felix, G., Vinatzer, B.A., 2013. Allelic variation in two distinct *Pseudomonas syringae* flagellin epitopes modulates the strength of plant immune responses but not bacterial motility. New Phytol. 200, 847–860. https://doi.org/10.1111/nph.12408

Coll, N.S., Epple, P., Dangl, J.L., 2011. Programmed cell death in the plant immune system. Cell Death Differ. 18, 1247–1256. https://doi.org/10.1038/cdd.2011.37

Cui, H., Tsuda, K., Parker, J.E., 2015. Effector-triggered immunity: from pathogen perception to robust defense. Annu. Rev. Plant Biol. 66, 487–511. https://doi.org/10.1146/annurev-arplant-050213-040012

Cunnac, S., Chakravarthy, S., Kvitko, B.H., Russell, A.B., Martin, G.B., Collmer, A., 2011. Genetic disassembly and combinatorial reassembly identify a minimal functional repertoire of type III effectors in *Pseudomonas syringae*. Proc. Natl. Acad. Sci. U. S. A. 108, 2975–2980. https://doi.org/10.1073/pnas.1013031108

Cunnac, S., Wilson, A., Nuwer, J., Kirik, A., Baranage, G., Mudgett, M.B., 2007. A Conserved Carboxylesterase Is a SUPPRESSOR OF AVRBST-ELICITED RESISTANCE in Arabidopsis. PLANT CELL ONLINE 19, 688–705. https://doi.org/10.1105/tpc.106.048710

Dangl, J.L., Jones, J.D., 2001. Plant pathogens and integrated defence responses to infection. Nature 411, 826–833. https://doi.org/10.1038/35081161

Day, B., Dahlbeck, D., Staskawicz, B.J., 2006. NDR1 interaction with RIN4 mediates the differential activation of multiple disease resistance pathways in Arabidopsis. Plant Cell 18, 2782–2791. https://doi.org/10.1105/tpc.106.044693

de Jong, C.F., Laxalt, A.M., Bargmann, B.O.R., de Wit, P.J.G.M., Joosten, M.H.A.J., Munnik, T., 2004. Phosphatidic acid accumulation is an early response in the Cf-4/Avr4 interaction. Plant J. Cell Mol. Biol. 39, 1–12. https://doi.org/10.1111/j.1365-313X.2004.02110.x

DebRoy, S., Thilmony, R., Kwack, Y.B., Nomura, K., He, S.Y., 2004. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. Proc. Natl. Acad. Sci. U. S. A. 101, 9927–9932. https://doi.org/10.1073/pnas.0401601101

Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S., Marco, Y., 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proc. Natl. Acad. Sci. U. S. A. 100, 8024–8029. https://doi.org/10.1073/pnas.1230660100

Deslandes, L., Pileur, F., Liaubet, L., Camut, S., Can, C., Williams, K., Holub, E., Beynon, J., Arlat, M., Marco, Y., 1998. Genetic characterization of *RRS1*, a recessive locus in *Arabidopsis thaliana* that confers resistance to the bacterial soilborne pathogen *Ralstonia solanacearum*. Mol. Plant-Microbe Interact. MPMI 11, 659–667. https://doi.org/10.1094/MPMI.1998.11.7.659

Du, G., 2003. Phospholipase D2 Localizes to the Plasma Membrane and Regulates Angiotensin II Receptor Endocytosis. Mol. Biol. Cell 15, 1024–1030. https://doi.org/10.1091/mbc.E03-09-0673

Eitas, T.K., Nimchuk, Z.L., Dangl, J.L., 2008. *Arabidopsis* TAO1 is a TIR-NB-LRR protein that contributes to disease resistance induced by the *Pseudomonas syringae* effector AvrB. Proc. Natl. Acad. Sci. U. S. A. 105, 6475–6480. https://doi.org/10.1073/pnas.0802157105

Engler, C., Kandzia, R., Marillonnet, S., 2008. A One Pot, One Step, Precision Cloning Method with High Throughput Capability. PLoS ONE 3, e3647. https://doi.org/10.1371/journal.pone.0003647

Falk, A., Feys, B.J., Frost, L.N., Jones, J.D., Daniels, M.J., Parker, J.E., 1999. EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proc. Natl. Acad. Sci. U. S. A. 96, 3292–3297.

Felix, G., Duran, J.D., Volko, S., Boller, T., 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. Cell Mol. Biol. 18, 265–276.

Feng, F., Yang, F., Rong, W., Wu, X., Zhang, J., Chen, S., He, C., Zhou, J.-M., 2012. A *Xanthomonas* uridine 5'-monophosphate transferase inhibits plant immune kinases. Nature 485, 114–118. https://doi.org/10.1038/nature10962

Feys, B.J., 2001. Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO J. 20, 5400–5411. https://doi.org/10.1093/emboj/20.19.5400

Freyberg, Z., Sweeney, D., Siddhanta, A., Bourgoin, S., Frohman, M., Shields, D., 2001. Intracellular localization of phospholipase D1 in mammalian cells. Mol. Biol. Cell 12, 943–955.

Furukawa, T., Inagaki, H., Takai, R., Hirai, H., Che, F.-S., 2014. Two Distinct EF-Tu Epitopes Induce Immune Responses in Rice and *Arabidopsis*. Mol. Plant.

Microbe Interact. 27, 113–124. https://doi.org/10.1094/MPMI-10-13-0304-R

Gan, X., Stegle, O., Behr, J., Steffen, J.G., Drewe, P., Hildebrand, K.L., Lyngsoe, R., Schultheiss, S.J., Osborne, E.J., Sreedharan, V.T., Kahles, A., Bohnert, R., Jean, G., Derwent, P., Kersey, P., Belfield, E.J., Harberd, N.P., Kemen, E., Toomajian, C., Kover, P.X., Clark, R.M., Rätsch, G., Mott, R., 2011. Multiple reference genomes and transcriptomes for *Arabidopsis thaliana*. Nature 477, 419–423. https://doi.org/10.1038/nature10414

Genger, R.K., Jurkowski, G.I., McDowell, J.M., Lu, H., Jung, H.W., Greenberg, J.T., Bent, A.F., 2008. Signaling Pathways That Regulate the Enhanced Disease Resistance of *Arabidopsis* "*Defense, No Death*" Mutants. Mol. Plant. Microbe Interact. 21, 1285–1296. https://doi.org/10.1094/MPMI-21-10-1285

Gimenez-Ibanez, S., Hann, D.R., Ntoukakis, V., Petutschnig, E., Lipka, V., Rathjen, J.P., 2009. AvrPtoB Targets the LysM Receptor Kinase CERK1 to Promote Bacterial Virulence on Plants. Curr. Biol. 19, 423–429. https://doi.org/10.1016/j.cub.2009.01.054

Głowacki, S., Macioszek, V., Kononowicz, A., 2011. R proteins as fundamentals of plant innate immunity. Cell. Mol. Biol. Lett. 16, 1–24. https://doi.org/10.2478/s11658-010-0024-2

Göhre, V., Spallek, T., Häweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J.W., Robatzek, S., 2008. Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. Curr. Biol. CB 18, 1824–1832. https://doi.org/10.1016/j.cub.2008.10.063

Guo, M., Tian, F., Wamboldt, Y., Alfano, J.R., 2009. The majority of the type III effector inventory of *Pseudomonas syringae* pv. *tomato* DC3000 can suppress plant immunity. Mol. Plant-Microbe Interact. MPMI 22, 1069–1080. https://doi.org/10.1094/MPMI-22-9-1069

Gust, A.A., Biswas, R., Lenz, H.D., Rauhut, T., Ranf, S., Kemmerling, B., Götz, F., Glawischnig, E., Lee, J., Felix, G., Nürnberger, T., 2007. Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in *Arabidopsis*. J. Biol. Chem. 282, 32338–32348. https://doi.org/10.1074/jbc.M704886200

Guttman, D.S., McHardy, A.C., Schulze-Lefert, P., 2014. Microbial genome-enabled insights into plant–microorganism interactions. Nat. Rev. Genet. 15, 797–813. https://doi.org/10.1038/nrg3748

Hacker, J., Blum-Oehler, G., Mühldorfer, I., Tschäpe, H., 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. 23, 1089–1097.

Hammond-Kosack, K.E., Rudd, J.J., 2008. Plant resistance signalling hijacked by a necrotrophic fungal pathogen. Plant Signal. Behav. 3, 993–995.

He, S.Y., 1998. TYPE III PROTEIN SECRETION SYSTEMS IN PLANT AND ANIMAL PATHOGENIC BACTERIA. Annu. Rev. Phytopathol. 36, 363–392. https://doi.org/10.1146/annurev.phyto.36.1.363

He, S.Y., Nomura, K., Whittam, T.S., 2004. Type III protein secretion mechanism in mammalian and plant pathogens. Biochim. Biophys. Acta BBA - Mol. Cell Res. 1694, 181–206. https://doi.org/10.1016/j.bbamcr.2004.03.011

Hind, S.R., Strickler, S.R., Boyle, P.C., Dunham, D.M., Bao, Z., O'Doherty, I.M., Baccile, J.A., Hoki, J.S., Viox, E.G., Clarke, C.R., Vinatzer, B.A., Schroeder, F.C., Martin, G.B., 2016. Tomato receptor FLAGELLIN-SENSING 3 binds flgII-28 and activates the plant immune system. Nat. Plants 2, 16128. https://doi.org/10.1038/nplants.2016.128

Hiraiwa, M., 1999. Cathepsin A/protective protein: an unusual lysosomal multifunctional protein. Cell. Mol. Life Sci. CMLS 56, 894–907. https://doi.org/10.1007/s000180050482

Iakovidis, M., Teixeira, P.J.P.L., Exposito-Alonso, M., Cowper, M.G., Law, T.F., Liu, Q., Vu, M.C., Dang, T.M., Corwin, J.A., Weigel, D., Dangl, J.L., Grant, S.R., 2016. Effector-Triggered Immune Response in *Arabidopsis thaliana* Is a Quantitative Trait. Genetics 204, 337–353. https://doi.org/10.1534/genetics.116.190678

Jackson, R.W., Vinatzer, B., Arnold, D.L., Dorus, S., Murillo, J., 2011. The influence of the accessory genome on bacterial pathogen evolution. Mob. Genet. Elem. 1, 55–65. https://doi.org/10.4161/mge.1.1.16432

Jakobek, J.L., Lindgren, P.B., 1993. Generalized Induction of Defense Responses in Bean Is Not Correlated with the Induction of the Hypersensitive Reaction. Plant Cell 5, 49–56. https://doi.org/10.1105/tpc.5.1.49

Jayaraman, J., Choi, S., Prokchorchik, M., Choi, D.S., Spiandore, A., Templeton, M.D., Rikkerink, E.H.A., Segonzac, C., Sohn, K.H., 2017. A bacterial acetyltransferase triggers immunity in *Arabidopsis thaliana* independent of hypersensitive response. Sci. Rep.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., Valent, B., 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J. 19, 4004–4014. https://doi.org/10.1093/emboj/19.15.4004

Jiang, S., Yao, J., Ma, K.-W., Zhou, H., Song, J., He, S.Y., Ma, W., 2013. Bacterial Effector Activates Jasmonate Signaling by Directly Targeting JAZ Transcriptional Repressors. PLoS Pathog. 9, e1003715. https://doi.org/10.1371/journal.ppat.1003715

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M., Glazebrook, J., 1999. *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. Proc. Natl. Acad. Sci. U. S. A. 96, 13583–13588.

Jones, J.D.G., Dangl, J.L., 2006. The plant immune system. Nature 444, 323–329. https://doi.org/10.1038/nature05286

Jones, J.D.G., Vance, R.E., Dangl, J.L., 2016. Intracellular innate immune surveillance devices in plants and animals. Science 354, aaf6395-aaf6395. https://doi.org/10.1126/science.aaf6395

Jupe, F., Witek, K., Verweij, W., Sliwka, J., Pritchard, L., Etherington, G.J., Maclean, D., Cock, P.J., Leggett, R.M., Bryan, G.J., Cardle, L., Hein, I., Jones, J.D.G., 2013. Resistance gene enrichment sequencing (RenSeq) enables reannotation of the NB-LRR gene family from sequenced plant genomes and rapid mapping of resistance loci in segregating populations. Plant J. 76, 530–544. https://doi.org/10.1111/tpj.12307

Kang, Y., Jelenska, J., Cecchini, N.M., Li, Y., Lee, M.W., Kovar, D.R., Greenberg, J.T., 2014. HopW1 from *Pseudomonas syringae* disrupts the actin cytoskeleton to promote virulence in *Arabidopsis*. PLoS Pathog. 10, e1004232. https://doi.org/10.1371/journal.ppat.1004232

Kay, S., Bonas, U., 2009. How *Xanthomonas* type III effectors manipulate the host plant. Curr. Opin. Microbiol. 12, 37–43. https://doi.org/10.1016/j.mib.2008.12.006

Kazan, K., Lyons, R., 2014. Intervention of Phytohormone Pathways by Pathogen Effectors. Plant Cell 26, 2285–2309. https://doi.org/10.1105/tpc.114.125419

Kim, M.G., da Cunha, L., McFall, A.J., Belkhadir, Y., DebRoy, S., Dangl, J.L., Mackey, D., 2005. Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in *Arabidopsis.* Cell 121, 749–759. https://doi.org/10.1016/j.cell.2005.03.025

Kim, S.H., Gao, F., Bhattacharjee, S., Adiasor, J.A., Nam, J.C., Gassmann, W., 2010. The *Arabidopsis* resistance-like gene SNC1 is activated by mutations in SRFR1 and contributes to resistance to the bacterial effector AvrRps4. PLoS Pathog. 6, e1001172. https://doi.org/10.1371/journal.ppat.1001172

Kim, S.H., Kwon, S.I., Bhattacharjee, S., Gassmann, W., 2009a. Regulation of defense gene expression by *Arabidopsis* SRFR1. Plant Signal. Behav. 4, 149–150.

Kim, S.H., Kwon, S.I., Saha, D., Anyanwu, N.C., Gassmann, W., 2009b. Resistance to the *Pseudomonas syringae* effector HopA1 is governed by the TIR-NBS-LRR protein RPS6 and is enhanced by mutations in SRFR1. Plant Physiol. 150, 1723–1732. https://doi.org/10.1104/pp.109.139238

Kirik, A., Mudgett, M.B., 2009. SOBER1 phospholipase activity suppresses phosphatidic acid accumulation and plant immunity in response to bacterial effector AvrBsT. Proc. Natl. Acad. Sci. U. S. A. 106, 20532–20537. https://doi.org/10.1073/pnas.0903859106

Knepper, C., Savory, E.A., Day, B., 2011. The role of NDR1 in pathogen perception and plant defense signaling. Plant Signal. Behav. 6, 1114–1116. https://doi.org/10.4161/psb.6.8.15843

Kushalappa, A.C., Yogendra, K.N., Karre, S., 2016. Plant Innate Immune Response: Qualitative and Quantitative Resistance. Crit. Rev. Plant Sci. 35, 38–55. https://doi.org/10.1080/07352689.2016.1148980

Kvitko, B.H., Park, D.H., Velasquez, A.C., Wei, C.F., Russell, A.B., Martin, G.B., Schneider, D.J., Collmer, A., 2009. Deletions in the Repertoire of *Pseudomonas syringae* pv. *tomato* DC3000 Type III Secretion Effector Genes Reveal Functional Overlap among Effectors. Plos Pathog. 5. https://doi.org/10.1371/journal.ppat.1000388

Kvitko, B.H., Park, D.H., Velásquez, A.C., Wei, C.-F., Russell, A.B., Martin, G.B., Schneider, D.J., Collmer, A., 2009. Deletions in the Repertoire of *Pseudomonas syringae* pv. *tomato* DC3000 Type III Secretion Effector Genes Reveal Functional Overlap among Effectors. PLoS Pathog. 5, e1000388. https://doi.org/10.1371/journal.ppat.1000388

Kwon, S.I., Kim, S.H., Bhattacharjee, S., Noh, J.-J., Gassmann, W., 2009. SRFR1, a suppressor of effector-triggered immunity, encodes a conserved

tetratricopeptide repeat protein with similarity to transcriptional repressors. Plant J. Cell Mol. Biol. 57, 109–119. https://doi.org/10.1111/j.1365-313X.2008.03669.x

Kwon, S.I., Koczan, J.M., Gassmann, W., 2004. Two *Arabidopsis srfr* (*suppressor of rps4-RLD)* mutants exhibit avrRps4-specific disease resistance independent of RPS4. Plant J. Cell Mol. Biol. 40, 366–375. https://doi.org/10.1111/j.1365-313X.2004.02213.x

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lawrence, G.J., Finnegan, E.J., Ayliffe, M.A., Ellis, J.G., 1995. The *L6* gene for flax rust resistance is related to the *Arabidopsis* bacterial resistance gene *RPS2* and the tobacco viral resistance gene N. Plant Cell 7, 1195–1206. https://doi.org/10.1105/tpc.7.8.1195

Le Roux, C., Huet, G., Jauneau, A., Camborde, L., Trémousaygue, D., Kraut, A., Zhou, B., Levaillant, M., Adachi, H., Yoshioka, H., Raffaele, S., Berthomé, R., Couté, Y., Parker, J.E., Deslandes, L., 2015. A receptor pair with an integrated decoy converts pathogen disabling of transcription factors to immunity. Cell 161, 1074–1088. https://doi.org/10.1016/j.cell.2015.04.025

Lee, A.H.-Y., Hurley, B., Felsensteiner, C., Yea, C., Ckurshumova, W., Bartetzko, V., Wang, P.W., Quach, V., Lewis, J.D., Liu, Y.C., Börnke, F., Angers, S., Wilde, A., Guttman, D.S., Desveaux, D., 2012. A bacterial acetyltransferase destroys plant microtubule networks and blocks secretion. PLoS Pathog. 8, e1002523. https://doi.org/10.1371/journal.ppat.1002523

Lee, J., Manning, A.J., Wolfgeher, D., Jelenska, J., Cavanaugh, K.A., Xu, H., Fernandez, S.M., Michelmore, R.W., Kron, S.J., Greenberg, J.T., 2015. Acetylation of an NB-LRR Plant Immune-Effector Complex Suppresses Immunity. Cell Rep. 13, 1670–1682. https://doi.org/10.1016/j.celrep.2015.10.029

Lewis, J.D., Abada, W., Ma, W., Guttman, D.S., Desveaux, D., 2008. The HopZ family of *Pseudomonas syringae* type III effectors require myristoylation for virulence and avirulence functions in *Arabidopsis thaliana*. J. Bacteriol. 190, 2880–2891. https://doi.org/10.1128/JB.01702-07

Lewis, J.D., Lee, A.H.-Y., Hassan, J.A., Wan, J., Hurley, B., Jhingree, J.R., Wang, P.W., Lo, T., Youn, J.-Y., Guttman, D.S., Desveaux, D., 2013. The *Arabidopsis* ZED1 pseudokinase is required for ZAR1-mediated immunity induced by the *Pseudomonas syringae* type III effector HopZ1a. Proc. Natl. Acad. Sci. U. S. A. 110, 18722–18727. https://doi.org/10.1073/pnas.1315520110

Lewis, J.D., Wan, J., Ford, R., Gong, Y., Fung, P., Nahal, H., Wang, P.W., Desveaux, D., Guttman, D.S., 2012. Quantitative Interactor Screening with next-generation Sequencing (QIS-Seq) identifies *Arabidopsis thaliana* MLO2 as a target of the *Pseudomonas syringae* type III effector HopZ2. BMC Genomics 13, 8. https://doi.org/10.1186/1471-2164-13-8

Lewis, J.D., Wilton, M., Mott, G.. A., Lu, W., Hassan, J.A., Guttman, D.S., Desveaux, D., 2014. Immunomodulation by the *Pseudomonas syringae* HopZ Type III Effector Family in *Arabidopsis*. PLoS ONE 9, e116152. https://doi.org/10.1371/journal.pone.0116152

Lewis, J.D., Wu, R., Guttman, D.S., Desveaux, D., 2010. Allele-Specific Virulence Attenuation of the Pseudomonas syringae HopZ1a Type III Effector via the *Arabidopsis* ZAR1 Resistance Protein. PLoS Genet. 6, e1000894. https://doi.org/10.1371/journal.pgen.1000894

Li, G., Froehlich, J.E., Elowsky, C., Msanne, J., Ostosh, A.C., Zhang, C., Awada, T., Alfano, J.R., 2014. Distinct *Pseudomonas* type-III effectors use a cleavable transit peptide to target chloroplasts. Plant J. Cell Mol. Biol. 77, 310–321. https://doi.org/10.1111/tpj.12396

Li, M., Hong, Y., Wang, X., 2009. Phospholipase D- and phosphatidic acid-mediated signaling in plants. Biochim. Biophys. Acta 1791, 927–935. https://doi.org/10.1016/j.bbalip.2009.02.017

Li, Y., Li, S., Bi, D., Cheng, Y.T., Li, X., Zhang, Y., 2010. SRFR1 negatively regulates plant NB-LRR resistance protein accumulation to prevent autoimmunity. PLoS Pathog. 6, e1001111. https://doi.org/10.1371/journal.ppat.1001111

Lin, N.-C., Abramovitch, R.B., Kim, Y.J., Martin, G.B., 2006. Diverse AvrPtoB Homologs from Several *Pseudomonas syringae* Pathovars Elicit Pto-Dependent Resistance and Have Similar Virulence Activities. Appl. Environ. Microbiol. 72, 702–712. https://doi.org/10.1128/AEM.72.1.702-712.2006

Liu, J., Elmore, J.M., Lin, Z.-J.D., Coaker, G., 2011. A receptor-like cytoplasmic kinase phosphorylates the host target RIN4, leading to the activation of a plant innate immune receptor. Cell Host Microbe 9, 137–146. https://doi.org/10.1016/j.chom.2011.01.010

Liu, Y., Chen, L., Fu, D., Lou, Q., Mei, H., Xiong, L., Li, M., Xu, X., Mei, X., Luo, L., 2014. Dissection of additive, epistatic effect and QTL × environment interaction of quantitative trait loci for sheath blight resistance in rice. Hereditas 151, 28–37. https://doi.org/10.1111/hrd2.00026

Liu, Y., Schiff, M., Marathe, R., Dinesh-Kumar, S.P., 2002. Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J. Cell Mol. Biol. 30, 415–429.

Lo, T., Koulena, N., Seto, D., Guttman, D.S., Desveaux, D., 2016. The HopF family of *Pseudomonas syringae* type III secreted effectors. Mol. Plant Pathol. https://doi.org/10.1111/mpp.12412

Lorang, J.M., Keen, N.T., 1995. Characterization of avrE from *Pseudomonas syringae* pv. *tomato*: a *hrp*-linked avirulence locus consisting of at least two transcriptional units. Mol. Plant-Microbe Interact. MPMI 8, 49–57.

Lozano-Duran, R., Bourdais, G., He, S.Y., Robatzek, S., 2014. The bacterial effector HopM1 suppresses PAMP-triggered oxidative burst and stomatal immunity. New Phytol. 202, 259–269. https://doi.org/10.1111/nph.12651

Ma, K.-W., Jiang, S., Hawara, E., Lee, D., Pan, S., Coaker, G., Song, J., Ma, W., 2015. Two serine residues in *Pseudomonas syringae* effector HopZ1a are required for acetyltransferase activity and association with the host co-factor. New Phytol. 208, 1157–1168. https://doi.org/10.1111/nph.13528

Ma, K.-W., Ma, W., 2016. YopJ Family Effectors Promote Bacterial Infection through a Unique Acetyltransferase Activity. Microbiol. Mol. Biol. Rev. MMBR 80, 1011–1027. https://doi.org/10.1128/MMBR.00032-16

Macho, A.P., 2016. Subversion of plant cellular functions by bacterial type-III effectors: beyond suppression of immunity. New Phytol. 210, 51–57. https://doi.org/10.1111/nph.13605

Macho, A.P., Guevara, C.M., Tornero, P., Ruiz-Albert, J., Beuzón, C.R., 2010. The *Pseudomonas syringae* effector protein HopZ1a suppresses effector-triggered immunity. New Phytol. 187, 1018–1033. https://doi.org/10.1111/j.1469-8137.2010.03381.x

Macho, A.P., Schwessinger, B., Ntoukakis, V., Brutus, A., Segonzac, C., Roy, S., Kadota, Y., Oh, M.-H., Sklenar, J., Derbyshire, P., Lozano-Duran, R., Malinovsky, F.G., Monaghan, J., Menke, F.L., Huber, S.C., He, S.Y., Zipfel, C., 2014. A Bacterial Tyrosine Phosphatase Inhibits Plant Pattern Recognition Receptor Activation. Science 343, 1509–1512. https://doi.org/10.1126/science.1248849

Mackey, D., Holt, B.F., Wiig, A., Dangl, J.L., 2002. RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell 108, 743–754.

Matas, I.M., Castañeda-Ojeda, M.P., Aragón, I.M., Antúnez-Lamas, M., Murillo, J., Rodríguez-Palenzuela, P., López-Solanilla, E., Ramos, C., 2014. Translocation and functional analysis of *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 type III secretion system effectors reveals two novel effector families of the *Pseudomonas syringae* complex. Mol. Plant-Microbe Interact. MPMI 27, 424–436. https://doi.org/10.1094/MPMI-07-13-0206-R

McCann, H.C., Rikkerink, E.H.A., Bertels, F., Fiers, M., Lu, A., Rees-George, J., Andersen, M.T., Gleave, A.P., Haubold, B., Wohlers, M.W., Guttman, D.S., Wang, P.W., Straub, C., Vanneste, J., Rainey, P.B., Templeton, M.D., 2013. Genomic Analysis of the Kiwifruit Pathogen *Pseudomonas syringae* pv. *actinidiae* Provides Insight into the Origins of an Emergent Plant Disease. PLoS Pathog. 9, e1003503. https://doi.org/10.1371/journal.ppat.1003503

McDowell, J.M., Cuzick, A., Can, C., Beynon, J., Dangl, J.L., Holub, E.B., 2000. Downy mildew (Peronospora parasitica) resistance genes in *Arabidopsis* vary in functional requirements for NDR1, EDS1, NPR1 and salicylic acid accumulation. Plant J. Cell Mol. Biol. 22, 523–529.

McElver, J., Tzafrir, I., Aux, G., Rogers, R., Ashby, C., Smith, K., Thomas, C., Schetter, A., Zhou, Q., Cushman, M.A., Tossberg, J., Nickle, T., Levin, J.Z., Law, M., Meinke, D., Patton, D., 2001. Insertional mutagenesis of genes required for seed development in *Arabidopsis thaliana*. Genetics 159, 1751–1763.

Meyers, B.C., Kozik, A., Griego, A., Kuang, H., Michelmore, R.W., 2003. Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. Plant Cell 15, 809–834.

Michelmore, R.W., Paran, I., Kesseli, R.V., 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. U. S. A. 88, 9828–9832.

Mittal, R., Peak-Chew, S.Y., Sade, R.S., Vallis, Y., McMahon, H.T., 2010. The acetyltransferase activity of the bacterial toxin YopJ of *Yersinia* is activated by eukaryotic host cell inositol hexakisphosphate. J. Biol. Chem. 285, 19927–19934. https://doi.org/10.1074/jbc.M110.126581

Murelli, R.P., Cheung, A.K., Snapper, M.L., 2007. Conformationally restricted (+)-cacospongionolide B analogues. Influence on secretory phospholipase A2 inhibition. J. Org. Chem. 72, 1545–1552. https://doi.org/10.1021/jo061407a

Mutka, A.M., Fawley, S., Tsao, T., Kunkel, B.N., 2013. Auxin promotes susceptibility to *Pseudomonas syringae* via a mechanism independent of suppression of salicylic acid-mediated defenses. Plant J. Cell Mol. Biol. 74, 746–754. https://doi.org/10.1111/tpj.12157

Narusaka, M., Kubo, Y., Shiraishi, T., Iwabuchi, M., Narusaka, Y., 2009. A dual resistance gene system prevents infection by three distinct pathogens. Plant Signal. Behav. 4, 954–955.

Nguyen, P.D.T., Pike, S., Wang, J., Nepal Poudel, A., Heinz, R., Schultz, J.C., Koo, A.J., Mitchum, M.G., Appel, H.M., Gassmann, W., 2016. The *Arabidopsis* immune regulator SRFR1 dampens defences against herbivory by *Spodoptera exigua* and parasitism by *Heterodera schachtii*. Mol. Plant Pathol. 17, 588–600. https://doi.org/10.1111/mpp.12304

Nicaise, V., Joe, A., Jeong, B., Korneli, C., Boutrot, F., Westedt, I., Staiger, D., Alfano, J.R., Zipfel, C., 2013. *Pseudomonas* HopU1 modulates plant immune receptor levels by blocking the interaction of their mRNAs with GRP7. EMBO J. 32, 701–712. https://doi.org/10.1038/emboj.2013.15

Niks, R.E., Qi, X., Marcel, T.C., 2015. Quantitative Resistance to Biotrophic Filamentous Plant Pathogens: Concepts, Misconceptions, and Mechanisms. Annu. Rev. Phytopathol. 53, 445–470. https://doi.org/10.1146/annurev-phyto-080614-115928

Nimchuk, Z.L., Fisher, E.J., Desveaux, D., Chang, J.H., Dangl, J.L., 2007. The HopX (AvrPphE) family of *Pseudomonas syringae* type III effectors require a catalytic triad and a novel N-terminal domain for function. Mol. Plant-Microbe Interact. MPMI 20, 346–357. https://doi.org/10.1094/MPMI-20-4-0346

Nissan, G., Manulis, S., Weinthal, D.M., Sessa, G., Barash, I., 2005. Analysis of Promoters Recognized by HrpL, an Alternative σ-Factor Protein from *Pantoea agglomerans* pv. *gypsophilae*. Mol. Plant. Microbe Interact. 18, 634–643. https://doi.org/10.1094/MPMI-18-0634

Nissan, G., Manulis-Sasson, S., Weinthal, D., Mor, H., Sessa, G., Barash, I., 2006. The type III effectors HsvG and HsvB of gall-forming *Pantoea agglomerans* determine host specificity and function as transcriptional activators. Mol. Microbiol. 61, 1118–1131. https://doi.org/10.1111/j.1365-2958.2006.05301.x

Nomura, K., Mecey, C., Lee, Y.-N., Imboden, L.A., Chang, J.H., He, S.Y., 2011. Effector-triggered immunity blocks pathogen degradation of an immunity-associated vesicle traffic regulator in *Arabidopsis*. Proc. Natl. Acad. Sci. U. S. A. 108, 10774–10779. https://doi.org/10.1073/pnas.1103338108

O'Brien, H.E., Thakur, S., Gong, Y., Fung, P., Zhang, J., Yuan, L., Wang, P.W., Yong, C., Scortichini, M., Guttman, D.S., 2012. Extensive remodeling of the *Pseudomonas syringae* pv. *avellanae* type III secretome associated with two independent host shifts onto hazelnut. BMC Microbiol. 12, 141. https://doi.org/10.1186/1471-2180-12-141

Orth, K., 2000. Disruption of Signaling by *Yersinia* Effector YopJ, a Ubiquitin-Like Protein Protease. Science 290, 1594–1597. https://doi.org/10.1126/science.290.5496.1594

Pan, Q., Wendel, J., Fluhr, R., 2000. Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. J. Mol. Evol. 50, 203–213.

Peart, J.R., Lu, R., Sadanandom, A., Malcuit, I., Moffett, P., Brice, D.C., Schauser, L., Jaggard, D.A.W., Xiao, S., Coleman, M.J., Dow, M., Jones, J.D.G., Shirasu, K., Baulcombe, D.C., 2002. Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. Proc. Natl. Acad. Sci. U. S. A. 99, 10865–10869. https://doi.org/10.1073/pnas.152330599

Peart, J.R., Mestre, P., Lu, R., Malcuit, I., Baulcombe, D.C., 2005. NRG1, a CC-NB-LRR Protein, together with N, a TIR-NB-LRR Protein, Mediates Resistance against Tobacco Mosaic Virus. Curr. Biol. 15, 968–973. https://doi.org/10.1016/j.cub.2005.04.053

Petre, B., Kamoun, S., 2014. How Do Filamentous Pathogens Deliver Effector Proteins into Plant Cells? PLoS Biol. 12, e1001801. https://doi.org/10.1371/journal.pbio.1001801

Ravensdale, M., Bernoux, M., Ve, T., Kobe, B., Thrall, P.H., Ellis, J.G., Dodds, P.N., 2012. Intramolecular Interaction Influences Binding of the Flax L5 and L6 Resistance Proteins to their AvrL567 Ligands. PLoS Pathog. 8, e1003004. https://doi.org/10.1371/journal.ppat.1003004

Robert-Seilaniantz, A., Shan, L., Zhou, J.-M., Tang, X., 2006. The *Pseudomonas syringae* pv. *tomato* DC3000 Type III Effector HopF2 Has a Putative Myristoylation Site Required for Its Avirulence and Virulence Functions. Mol. Plant. Microbe Interact. 19, 130–138. https://doi.org/10.1094/MPMI-19-0130

Rodríguez-Herva, J.J., González-Melendi, P., Cuartas-Lanza, R., Antúnez-Lamas, M., Río-Alvarez, I., Li, Z., López-Torrejón, G., Díaz, I., Del Pozo, J.C., Chakravarthy, S., Collmer, A., Rodríguez-Palenzuela, P., López-Solanilla, E., 2012. A bacterial cysteine protease effector protein interferes with photosynthesis to suppress plant innate immune responses. Cell. Microbiol. 14, 669–681. https://doi.org/10.1111/j.1462-5822.2012.01749.x

Rosales, C., Brown, E.J., 1992. Calcium channel blockers nifedipine and diltiazem inhibit Ca2+ release from intracellular stores in neutrophils. J. Biol. Chem. 267, 1443–1448.

Rufián, J.S., Lucía, A., Macho, A.P., Orozco-Navarrete, B., Arroyo-Mateos, M., Bejarano, E.R., Beuzón, C.R., Ruiz-Albert, J., 2015. Auto-acetylation on K289 is not essential for HopZ1a-mediated plant defense suppression. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.00684

Sama, v. S. a. K., Rawat, N., Sundaram, R.M., Himabindu, K., Naik, B.S., Viraktamath, B.C., Bentur, J.S., 2014. A putative candidate for the recessive gall midge resistance gene *gm3* in rice identified and validated. TAG Theor. Appl. Genet. Theor. Angew. Genet. 127, 113–124. https://doi.org/10.1007/s00122-013-2205-7

Sarris, P.F., Duxbury, Z., Huh, S.U., Ma, Y., Segonzac, C., Sklenar, J., Derbyshire, P., Cevik, V., Rallapalli, G., Saucet, S.B., Wirthmueller, L., Menke, F.L.H., Sohn, K.H., Jones, J.D.G., 2015. A Plant Immune Receptor Detects Pathogen

Effectors that Target WRKY Transcription Factors. Cell 161, 1089–1100. https://doi.org/10.1016/j.cell.2015.04.024

Sehnke, P.C., Henry, R., Cline, K., Ferl, R.J., 2000. Interaction of a plant 14-3-3 protein with the signal peptide of a thylakoid-targeted chloroplast precursor protein and the presence of 14-3-3 isoforms in the chloroplast stroma. Plant Physiol. 122, 235–242.

Seibel, N.M., Eljouni, J., Nalaskowski, M.M., Hampe, W., 2007. Nuclear localization of enhanced green fluorescent protein homomultimers. Anal. Biochem. 368, 95–99. https://doi.org/10.1016/j.ab.2007.05.025

Serizawa, S., Ichikawa, T., Takikawa, Y., Tsuyumu, S., Goto, M., 1989. Occurrence of bacterial canker of kiwifruit in Japan: Description of symptoms, isolation of the pathogen and screening of bactericides. Jpn. J. Phytopathol. 55, 427–436. https://doi.org/10.3186/jjphytopath.55.427

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., Geisler, M., Mauch, F., Nawrath, C., Métraux, J.-P., 2013. Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. Plant Physiol. 162, 1815–1821. https://doi.org/10.1104/pp.113.218156

Shan, L., He, P., Li, J., Heese, A., Peck, S.C., Nürnberger, T., Martin, G.B., Sheen, J., 2008. Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. Cell Host Microbe 4, 17–27. https://doi.org/10.1016/j.chom.2008.05.017

Shang, Y., Li, X., Cui, H., He, P., Thilmony, R., Chintamanani, S., Zwiesler-Vollick, J., Gopalan, S., Tang, X., Zhou, J.-M., 2006. RAR1, a central player in plant immunity, is targeted by Pseudomonas syringae effector AvrB. Proc. Natl. Acad. Sci. 103, 19200–19205. https://doi.org/10.1073/pnas.0607279103

Shao, Z.-Q., Zhang, Y.-M., Hang, Y.-Y., Xue, J.-Y., Zhou, G.-C., Wu, P., Wu, X.-Y., Wu, X.-Z., Wang, Q., Wang, B., Chen, J.-Q., 2014. Long-term evolution of nucleotide-binding site-leucine-rich repeat genes: understanding gained from and beyond the legume family. Plant Physiol. 166, 217–234. https://doi.org/10.1104/pp.114.243626

Shimono, M., Lu, Y.-J., Porter, K., Kvitko, B.H., Henty-Ridilla, J.L., Creason, A., He, S.Y., Chang, J.H., Staiger, C.J., Day, B., 2016. The *Pseudomonas syringae* type-III effector HopG1 induces actin remodeling to promote symptom development and susceptibility during infection. Plant Physiol. https://doi.org/10.1104/pp.16.01593

Shimozono, S., Tsutsui, H., Miyawaki, A., 2009. Diffusion of Large Molecules into Assembling Nuclei Revealed Using an Optical Highlighting Technique. Biophys. J. 97, 1288–1294. https://doi.org/10.1016/j.bpj.2009.06.024

Simon, M., Loudet, O., Durand, S., Berard, A., Brunel, D., Sennesal, F.-X., Durand-Tardif, M., Pelletier, G., Camilleri, C., 2008. Quantitative Trait Loci Mapping in Five New Large Recombinant Inbred Line Populations of *Arabidopsis thaliana* Genotyped With Consensus Single-Nucleotide Polymorphism Markers. Genetics 178, 2253–2264. https://doi.org/10.1534/genetics.107.083899

Sohn, K.H., Saucet, S.B., Clarke, C.R., Vinatzer, B.A., O'Brien, H.E., Guttman, D.S., Jones, J.D.G., 2012. HopAS1 recognition significantly contributes to

Arabidopsis nonhost resistance to *Pseudomonas syringae* pathogens. New Phytol. 193, 58–66. https://doi.org/10.1111/j.1469-8137.2011.03950.x

Steinbrenner, A.D., Goritschnig, S., Krasileva, K.V., Schreiber, K.J., Staskawicz, B.J., 2012. Effector Recognition and Activation of the *Arabidopsis thaliana* NLR Innate Immune Receptors. Cold Spring Harb. Symp. Quant. Biol. 77, 249–257. https://doi.org/10.1101/sqb.2012.77.014860

Steinbrenner, A.D., Goritschnig, S., Staskawicz, B.J., 2015. Recognition and activation domains contribute to allele-specific responses of an *Arabidopsis* NLR receptor to an oomycete effector protein. PLoS Pathog. 11, e1004665. https://doi.org/10.1371/journal.ppat.1004665

Steuernagel, B., Periyannan, S.K., Hernández-Pinzón, I., Witek, K., Rouse, M.N., Yu, G., Hatta, A., Ayliffe, M., Bariana, H., Jones, J.D.G., Lagudah, E.S., Wulff, B.B.H., 2016. Rapid cloning of disease-resistance genes in plants using mutagenesis and sequence capture. Nat. Biotechnol. https://doi.org/10.1038/nbt.3543

Studholme, D.J., Ibanez, S.G., MacLean, D., Dangl, J.L., Chang, J.H., Rathjen, J.P., 2009. A draft genome sequence and functional screen reveals the repertoire of type III secreted proteins of *Pseudomonas syringae* pathovar *tabaci* 11528. BMC Genomics 10, 395. https://doi.org/10.1186/1471-2164-10-395

Swiderski, M.R., Birker, D., Jones, J.D.G., 2009. The TIR domain of TIR-NB-LRR resistance proteins is a signaling domain involved in cell death induction. Mol. Plant-Microbe Interact. MPMI 22, 157–165. https://doi.org/10.1094/MPMI-22-2-0157

Szczesny, R., Büttner, D., Escolar, L., Schulze, S., Seiferth, A., Bonas, U., 2010. Suppression of the AvrBs1-specific hypersensitive response by the YopJ effector homolog AvrBsT from *Xanthomonas* depends on a SNF1-related kinase. New Phytol. 187, 1058–1074. https://doi.org/10.1111/j.1469-8137.2010.03346.x

Takahashi, A., Casais, C., Ichimura, K., Shirasu, K., 2003. HSP90 interacts with RAR1 and SGT1 and is essential for RPS2-mediated disease resistance in *Arabidopsis.* Proc. Natl. Acad. Sci. U. S. A. 100, 11777–11782. https://doi.org/10.1073/pnas.2033934100

Tasset, C., Bernoux, M., Jauneau, A., Pouzet, C., Brière, C., Kieffer-Jacquinod, S., Rivas, S., Marco, Y., Deslandes, L., 2010. Autoacetylation of the *Ralstonia solanacearum* effector PopP2 targets a lysine residue essential for RRS1-R-mediated immunity in *Arabidopsis*. PLoS Pathog. 6, e1001202. https://doi.org/10.1371/journal.ppat.1001202

Testerink, C., Munnik, T., 2011. Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. J. Exp. Bot. 62, 2349–2361. https://doi.org/10.1093/jxb/err079

Testerink, C., Munnik, T., 2005. Phosphatidic acid: a multifunctional stress signaling lipid in plants. Trends Plant Sci. 10, 368–375. https://doi.org/10.1016/j.tplants.2005.06.002

Thomas, W.J., Thireault, C.A., Kimbrel, J.A., Chang, J.H., 2009. Recombineering and stable integration of the *Pseudomonas syringae* pv. *syringae* 61 hrp/hrc cluster into the genome of the soil bacterium *Pseudomonas fluorescens* Pf0-

1. Plant J. Cell Mol. Biol. 60, 919–928. https://doi.org/10.1111/j.1365-313X.2009.03998.x

Tian, D., Traw, M.B., Chen, J.Q., Kreitman, M., Bergelson, J., 2003. Fitness costs of *R*-gene-mediated resistance in *Arabidopsis thaliana*. Nature 423, 74–77. https://doi.org/10.1038/nature01588

Üstün, S., König, P., Guttman, D.S., Börnke, F., 2014. HopZ4 from *Pseudomonas syringae*, a member of the HopZ type III effector family from the YopJ superfamily, inhibits the proteasome in plants. Mol. Plant-Microbe Interact. MPMI 27, 611–623. https://doi.org/10.1094/MPMI-12-13-0363-R

van der Hoorn, R.A.L., Kamoun, S., 2008. From Guard to Decoy: a new model for perception of plant pathogen effectors. Plant Cell 20, 2009–2017. https://doi.org/10.1105/tpc.108.060194

Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., Boucher, C., 1995. The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. Mol. Microbiol. 15, 1095–1114.

Vanneste, J.L., Yu, J., Cornish, D.A., Tanner, D.J., Windner, R., Chapman, J.R., Taylor, R.K., Mackay, J.F., Dowlut, S., 2013. Identification, Virulence, and Distribution of Two Biovars of *Pseudomonas syringae* pv. *actinidiae* in New Zealand. Plant Dis. 97, 708–719. https://doi.org/10.1094/PDIS-07-12-0700-RE

Vatansever, F., de Melo, W.C.M.A., Avci, P., Vecchio, D., Sadasivam, M., Gupta, A., Chandran, R., Karimi, M., Parizotto, N.A., Yin, R., Tegos, G.P., Hamblin, M.R., 2013. Antimicrobial strategies centered around reactive oxygen species – bactericidal antibiotics, photodynamic therapy, and beyond. FEMS Microbiol. Rev. 37, 955–989. https://doi.org/10.1111/1574-6976.12026

Vinatzer, B.A., Teitzel, G.M., Lee, M.-W., Jelenska, J., Hotton, S., Fairfax, K., Jenrette, J., Greenberg, J.T., 2006. The type III effector repertoire of *Pseudomonas syringae* pv. *syringae* B728a and its role in survival and disease on host and non-host plants. Mol. Microbiol. 62, 26–44. https://doi.org/10.1111/j.1365-2958.2006.05350.x

Vincent, F., Charnock, S.J., Verschueren, K.H.G., Turkenburg, J.P., Scott, D.J., Offen, W.A., Roberts, S., Pell, G., Gilbert, H.J., Davies, G.J., Brannigan, J.A., 2003. Multifunctional xylooligosaccharide/cephalosporin C deacetylase revealed by the hexameric structure of the Bacillus subtilis enzyme at 1.9A resolution. J. Mol. Biol. 330, 593–606.

Voinnet, O., Rivas, S., Mestre, P., Baulcombe, D., 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. Cell Mol. Biol. 33, 949–956.

Vossen, J.H., Abd-El-Haliem, A., Fradin, E.F., van den Berg, G.C.M., Ekengren, S.K., Meijer, H.J.G., Seifi, A., Bai, Y., ten Have, A., Munnik, T., Thomma, B.P.H.J., Joosten, M.H.A.J., 2010. Identification of tomato phosphatidylinositol-specific phospholipase-C (PI-PLC) family members and the role of PLC4 and PLC6 in HR and disease resistance. Plant J. Cell Mol. Biol. 62, 224–239. https://doi.org/10.1111/j.1365-313X.2010.04136.x

Wager, A., Browse, J., 2012. Social Network: JAZ Protein Interactions Expand Our Knowledge of Jasmonate Signaling. Front. Plant Sci. 3. https://doi.org/10.3389/fpls.2012.00041

Wang, Y., Li, J., Hou, S., Wang, X., Li, Y., Ren, D., Chen, S., Tang, X., Zhou, J.-M., 2010. A Pseudomonas syringae ADP-ribosyltransferase inhibits *Arabidopsis* mitogen-activated protein kinase kinases. Plant Cell 22, 2033–2044. https://doi.org/10.1105/tpc.110.075697

Wei, C.-F., Kvitko, B.H., Shimizu, R., Crabill, E., Alfano, J.R., Lin, N.-C., Martin, G.B., Huang, H.-C., Collmer, A., 2007. A *Pseudomonas syringae* pv. *tomato* DC3000 mutant lacking the type III effector HopQ1-1 is able to cause disease in the model plant *Nicotiana benthamiana*. Plant J. Cell Mol. Biol. 51, 32–46. https://doi.org/10.1111/j.1365-313X.2007.03126.x

Wei, H.-L., Chakravarthy, S., Mathieu, J., Helmann, T.C., Stodghill, P., Swingle, B., Martin, G.B., Collmer, A., 2015. *Pseudomonas syringae* pv. *tomato* DC3000 Type III Secretion Effector Polymutants Reveal an Interplay between HopAD1 and AvrPtoB. Cell Host Microbe 17, 752–762. https://doi.org/10.1016/j.chom.2015.05.007

Wei, W., Plovanich-Jones, A., Deng, W.-L., Jin, Q.-L., Collmer, A., Huang, H.-C., He, S.Y., 2000. The gene coding for the Hrp pilus structural protein is required for type III secretion of Hrp and Avr proteins in Pseudomonas syringae pv. tomato. Proc. Natl. Acad. Sci. 97, 2247–2252. https://doi.org/10.1073/pnas.040570097

Wildermuth, M.C., Dewdney, J., Wu, G., Ausubel, F.M., 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 414, 562–565. https://doi.org/10.1038/35107108

Williams, S.J., Sornaraj, P., deCourcy-Ireland, E., Menz, R.I., Kobe, B., Ellis, J.G., Dodds, P.N., Anderson, P.A., 2011. An Autoactive Mutant of the M Flax Rust Resistance Protein Has a Preference for Binding ATP, Whereas Wild-Type M Protein Binds ADP. Mol. Plant. Microbe Interact. 24, 897–906. https://doi.org/10.1094/MPMI-03-11-0052

Willmann, R., Lajunen, H.M., Erbs, G., Newman, M.-A., Kolb, D., Tsuda, K., Katagiri, F., Fliegmann, J., Bono, J.-J., Cullimore, J.V., Jehle, A.K., Götz, F., Kulik, A., Molinaro, A., Lipka, V., Gust, A.A., Nürnberger, T., 2011. *Arabidopsis* lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. Proc. Natl. Acad. Sci. U. S. A. 108, 19824–19829. https://doi.org/10.1073/pnas.1112862108

Witek, K., Jupe, F., Witek, A.I., Baker, D., Clark, M.D., Jones, J.D.G., 2016. Accelerated cloning of a potato late blight–resistance gene using RenSeq and SMRT sequencing. Nat. Biotechnol. https://doi.org/10.1038/nbt.3540

Wroblewski, T., Caldwell, K.S., Piskurewicz, U., Cavanaugh, K.A., Xu, H., Kozik, A., Ochoa, O., McHale, L.K., Lahre, K., Jelenska, J., Castillo, J.A., Blumenthal, D., Vinatzer, B.A., Greenberg, J.T., Michelmore, R.W., 2009. Comparative large-scale analysis of interactions between several crop species and the effector repertoires from multiple pathovars of Pseudomonas and Ralstonia. Plant Physiol. 150, 1733–1749. https://doi.org/10.1104/pp.109.140251

Xiao, S., Ellwood, S., Calis, O., Patrick, E., Li, T., Coleman, M., Turner, J.G., 2001. Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by

RPW8. Science 291, 118–120. https://doi.org/10.1126/science.291.5501.118

Xin, X.-F., Nomura, K., Aung, K., Velásquez, A.C., Yao, J., Boutrot, F., Chang, J.H., Zipfel, C., He, S.Y., 2016a. Bacteria establish an aqueous living space in plants crucial for virulence. Nature 539, 524–529. https://doi.org/10.1038/nature20166

Yamada, K., Saijo, Y., Nakagami, H., Takano, Y., 2016. Regulation of sugar transporter activity for antibacterial defense in *Arabidopsis*. Science 354, 1427–1430. https://doi.org/10.1126/science.aah5692

Yang, L., Teixeira, P.J.P.L., Biswas, S., Finkel, O.M., He, Y., Salas-Gonzalez, I., English, M.E., Epple, P., Mieczkowski, P., Dangl, J.L., 2017. *Pseudomonas syringae* Type III Effector HopBB1 Promotes Host Transcriptional Repressor Degradation to Regulate Phytohormone Responses and Virulence. Cell Host Microbe 21, 156–168. https://doi.org/10.1016/j.chom.2017.01.003

Yeo, F.K.S., Hensel, G., Vozábová, T., Martin-Sanz, A., Marcel, T.C., Kumlehn, J., Niks, R.E., 2014. Golden SusPtrit: a genetically well transformable barley line for studies on the resistance to rust fungi. TAG Theor. Appl. Genet. Theor. Angew. Genet. 127, 325–337. https://doi.org/10.1007/s00122-013-2221-7

Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S., Mengiste, T., Zhang, Y., Zhou, J.-M., 2010. Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. Cell Host Microbe 7, 290–301. https://doi.org/10.1016/j.chom.2010.03.007

Zhang, Q., Xiao, S., 2015. Lipids in salicylic acid-mediated defense in plants: focusing on the roles of phosphatidic acid and phosphatidylinositol 4-phosphate. Front. Plant Sci. 6, 387. https://doi.org/10.3389/fpls.2015.00387

Zhang, Z.-M., Ma, K.-W., Yuan, S., Luo, Y., Jiang, S., Hawara, E., Pan, S., Ma, W., Song, J., 2016. Structure of a pathogen effector reveals the enzymatic mechanism of a novel acetyltransferase family. Nat. Struct. Mol. Biol. 23, 847–852. https://doi.org/10.1038/nsmb.3279

Zhou, H., Lin, J., Johnson, A., Morgan, R.L., Zhong, W., Ma, W., 2011. *Pseudomonas syringae* Type III Effector HopZ1 Targets a Host Enzyme to Suppress Isoflavone Biosynthesis and Promote Infection in Soybean. Cell Host Microbe 9, 177–186. https://doi.org/10.1016/j.chom.2011.02.007

Zhou, J., Wu, S., Chen, X., Liu, C., Sheen, J., Shan, L., He, P., 2014. The *Pseudomonas syringae* effector HopF2 suppresses *Arabidopsis* immunity by targeting BAK1. Plant J. Cell Mol. Biol. 77, 235–245. https://doi.org/10.1111/tpj.12381

Zhu, S., Jeong, R.-D., Venugopal, S.C., Lapchyk, L., Navarre, D., Kachroo, A., Kachroo, P., 2011. SAG101 forms a ternary complex with EDS1 and PAD4 and is required for resistance signaling against turnip crinkle virus. PLoS Pathog. 7, e1002318. https://doi.org/10.1371/journal.ppat.1002318

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D.G., Boller, T., Felix, G., 2006. Perception of the Bacterial PAMP EF-Tu by the Receptor EFR Restricts *Agrobacterium*-Mediated Transformation. Cell 125, 749–760. https://doi.org/10.1016/j.cell.2006.03.037

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D.G., Felix, G., Boller, T., 2004. Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature 428, 764–767. https://doi.org/10.1038/nature02485

Zurbriggen, M.D., Carrillo, N., Tognetti, V.B., Melzer, M., Peisker, M., Hause, B., Hajirezaei, M.-R., 2009. Chloroplast-generated reactive oxygen species play a major role in localized cell death during the non-host interaction between tobacco and *Xanthomonas campestris* pv. *vesicatoria*: Chloroplast ROS in a non-host interaction. Plant J. 60, 962–973. https://doi.org/10.1111/j.1365-313X.2009.04010.x

Zwiesler-Vollick, J., Plovanich-Jones, A.E., Nomura, K., Bandyopadhyay, S., Joardar, V., Kunkel, B.N., He, S.Y., 2002. Identification of novel *hrp*-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv. *tomato* DC3000 genome. Mol. Microbiol. 45, 1207–1218.