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«Investigating putative subcellular targets of *Ralstonia solanacearum* type III effectors»

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"You can learn all that there is to know about their ways in a month, and yet after a hundred years they can still surprise you at a pinch."

— J.R.R. Tolkien, The Fellowship of the Ring

To my family

Τίτλος μεταπτυχιακής εργασίας:

«Αναζήτηση των πιθανών υποκυτταρικών στόχων παραγόντων παθογένειας (effectors) του παθογόνου *Ralstonia solanacearum*»

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Contents

Abstract

In a world with an ever-growing population and an ever-changing climate, sustainable agriculture and the pathogens that threaten it are crucial for food security. *Ralstonia solanacearum* is an aggressive Gram-negative bacterium with a wide host range, which includes plant species of both monocots and dicots. Its pathogenicity mostly depends on the Type III Secretion System (T3SS) delivery machine. The effector proteins secreted from the T3SS into the host cell can interfere with various eukaryotic cell processes, including immunity responses. NLRs (Nucleotide-binding Leucine-rich repeats Receptors) are plant intracellular immunity receptors, that activate plant defense, upon effector recognition. The Integrated Decoy or ID model describes NLR domains serving as baits, that seem to have derived from the original subcellular target of the effector. In this study, we used yeast to hybrid screening in order to identify the interactions between pathogen effectors and Decoy domains. A screening for interactions between pathogen effectors and a list of frequently appearing IDs could uncover the effectors' putative original targets. We tested 6 effectors from the soil-borne bacterial pathogen *Ralstonia solanacearum* against 23 Integrated Decoys deriving from plant NLRs and we identified 11 putative interactions. Understanding such interplay on a protein level can give insight to virulence mechanisms of bacterial wilt disease and provide novel targets for genome editing approaches, that introduce resistance.

Περίληψη

Η ανθρώπινη πληθυσμιακη έκρηξη και η αδιαμφισβήτητη κλιματική αλλαγή καθιστούν την βιώσιμη αγροτική παραγωγή αναπόσπαστο κομμάτι της διασφάλισης τροφής. Παθογόνοι οργανισμοί που προσβάλλουν τα φυτά αποτελούν μια από τις κύριες πηγές κινδύνου ενάντια στην απόδοση της παγκόσμιας παραγωγής τροφής και μεταξύ αυτών το φυτοπαθογόνο βακτήριο *Ralstonia solanacearum* θεωρείται από τα πιο επικίνδυνα. Η παθογένειά του βασίζεται κυρίως στο εκκριτικό σύστημα τύπου ΙΙΙ (Type III Secretion System, T3SS) και στις πρωτεΐνες τελεστές (effectors), που μέσω αυτού εγχέει στο κύτταρο του ξενιστή. Οι effectors στοχεύουν στην διατάραξη ευκαρυωτικών κυτταρικών διεργασιών προς όφελος του βακτηρίου, όπως την αναστολή της ενεργοποίησης της άμυνας. Ενδοκυτταρικοί υποδοχείς της φυτικής άμυνας, που ανήκουν στην οικογένεια των NLR (Nucleotide-binding Leucine-rich-repeats Receptors), έχουν εξελιχθεί να αναγνωρίζουν κάποιους effectors και να σηματοδοτούν την ενεργοποίηση της άμυνας. Ένα από τα μοντέλα αναγνώρισης NLR-effector είναι αυτό του ενσωματωμένου δολώματος (Integrated Decoy ή ID). Σύμφωνα με το ID μοντέλο, οι πραγματικοί στόχοι των πρωτεϊνών αυτών έχουν ενσωματωθεί ως μη συντηρημένες επικράτειες σε υποδοχείς NLR, και αποτελούν «δόλωμα» (decoy) για την αναγνώριση των effectors. Έτσι, καθίσταται δυνατή η αναζήτηση των υποκυτταρικών στόχων μιας βακτηριακής πρωτεΐνης effector, μέσω της διερεύνησης τυχόν αλληλεπιδράσεών της με ID επικράτειες των NLR υποδοχέων. Στην παρούσα εργασία εξετάστηκε η αλληλεπίδραση μεταξύ έξι πρωτεϊνών παθογένειας (effectors) του βακτηρίου *Ralstonia solanacearum* και είκοσι τριών ID επικρατειών που απαντούν συντηγμένες σε NLR υποδοχείς διαφορετικών ειδών φυτών και αποκαλύφθηκαν 11 αλληλεπιδράσεις. Η κατανόηση τέτοιων σχέσεων σε πρωτεϊνικό επίπεδο μπορεί ξεδιαλύνει τους μηχανισμούς παθογένειας και να υποδείξει υποψήφιους στόχους για την τεχνητή επίτευξη της ανθεκτικότητας έναντι του φυτοπαθογόνου *Ralstonia solanacearum*.

Introduction

The world's population is estimated to reach 9.1 billion people in the next 30 years (FAO 2009). The continuous population growth along with the undeniable climate change provoke important challenges to provision of food supplies and the so-called food security (Ericksen 2008). Increasing and sustaining food production may not be the only way to approach this global concern, but it certainly is one of the largest obstacles to be overcome (Prosekov and Ivanova 2018). A standard 10-16% of the global agricultural yield is annually lost due to plant pathogens and in extreme cases a disease has the capacity to even destroy half of the total harvest (Chakraborty and Newton 2011). Better understanding and efficient prevention of crops diseases can be achieved through uncovering the exact molecular mechanisms of pathogenicity and the molecular interactions between the host and the invading microbe (P. P. Singh et al. 2018).

Plant-Microbe Interactions

Plants, lacking cell and somatic mobility, are equipped with a cell-autonomous innate immunity system to recognize and cope with the various microorganisms constantly found in their habitats (P. P. Singh et al. 2018). Perception of invading pathogens occurs extracellularly or intracellularly through the corresponding immunity receptors (van der Burgh and Joosten 2019). In their long history of coevolution, microbes and plants have developed sophisticated mechanisms to achieve successful colonization and resistance, respectively (Sherif et al. 2015). These mechanisms can be concisely illustrated in a four-layered comprehensive model, shown in Figure 1 (Jones and Dangl 2006; Sherif et al. 2015).

In the first part (Figure 1A), a plant gains resistance through the extracellular Pattern-Recognition Receptors (PRRs) that can recognize conserved patterns contained in structural and chemical microbial components (Boller and Felix 2009). These patterns are collectively known as Pathogen-Associated Molecular Patterns (PAMPs) or Microbe-Associated Molecular Patterns (MAMPs) and their recognition by PRRs result in activation of a series of defense responses called **Pattern-Triggered Immunity (PTI)** (Jones and Dangl 2006). A well-studied example of a molecular motif of this kind is flg22, a highly conserved peptide of the bacterial flagellin, which is known to be sensed by a receptor kinase in *Arabidopsis*, eliciting PTI (Boller and Felix 2009). PRRs are cell-surface receptors, that typically are Receptor-like Proteins (RLPs) or Receptor-like Kinases (RLKs) and can activate MAP kinases signaling cascades leading to PTI responses, which contribute to restriction of the pathogen's further distribution (Jones and Dangl 2006; Kourelis and Hoorn 2018). PTI responses include callose deposition and antimicrobial compounds aggregation at the infection site, increased production of reactive oxygen species (ROS) and transcriptional activation of numerous defense-related genes (Sherif et al. 2015). In models that have been proposed to describe the plant innate immunity system, PTI is considered to be a first

non-specific layer of the plant's defense against different microbial invaders (Boller and Felix 2009; Jones and Dangl 2006).

The second level (Figure 1B) refers to cases of successful pathogens that secrete virulence factors with the ability to suppress PTI responses (Sherif et al. 2015). In general, invaders and parasites utilize proteins and other small molecules to manipulate host structure and physiology and promote successful colonization (P. P. Singh et al. 2018). These molecules, conjointly called **effectors**, introduce a state known as **Effector-Triggered Susceptibility (ETS)** and may derive from miscellaneous organisms, such as bacteria, viruses, fungi, oomycetes, nematodes and insects (Zipfel et al. 2012). The effectors' functions can usually be determined through their outcome regarding the host's physiology (Alfano and Collmer 2004; Boller and Felix 2009). These virulence factors are known to predominantly disturb signaling pathways involved in the host's immunity responses, but they often appear to have multiple functions (Dean 2011). The PTI responses can be manipulated by effectors in several different levels (Gimenez-Ibanez et al. 2018). Effectors target host molecules that seem to play critical roles in pathogen perception, signal transduction, defense-related genes expression, protein translocation and vesicle transportation (Sherif et al. 2015). Distinct other effector functions may even include manipulation of the host's cellular structure, such as HopZ1a from *Pseudomonas syringae*, which interferes with microtubule networks and disrupts cell-wall-dependent defense (Zipfel et al. 2012). Different organisms have developed separate delivery mechanisms for effectors injection (Hogenhout et al. 2009). Bacteria attempting to colonize a plant host use complex machineries to translocate effector proteins across their outer membrane, the so-called secretion systems (Cornelis 2000; Galán et al. 2014). Until today, nine secretion systems of various architectures and functions have been characterized (N. Singh and Wagner 2019). Among them, the **Type III Secretion System (T3SS)** of Gram-negative bacteria has been studied more thoroughly and is considered to be the most sophisticated protein secretion system (Kusmierek et al. 2019). T3SS is a needle-like apparatus that is formed upon contact with the eukaryotic cell and facilitates injection of bacterial **type III effectors (T3Es)** (Alfano and Collmer 2004; Cornelis 2000). T3SS has been closely linked to virulence of many animal and plant pathogens, for instance the Gram-negative bacteria *Salmonella* spp. and *Ralstonia solanacearum,* respectively (Buttner and Bonas 2002; Stéphane Genin and Denny 2012).

Injected effectors inside the plant cell and the modifications they cause on the targeted host proteins are intracellular signals indicating invasion (Boller and Felix 2009; Cook, Mesarich, and Thomma 2015). Such alerts are sensed by eukaryotic intracellular receptors, that have the ability to stimulate a more severe defensive mechanism, described in Figure 1C, known as **Effector-Triggered Immunity (ETI)** (Jones and Dangl 2006; Sherif et al. 2015)**.** Numerous studies and the several models that have been proposed to describe plant innate immunity, agree on the fact that most of the intracellular sensors of invasion belong to the **Nucleotide-binding Leucine-rich Receptors (NLRs)** (Boller and Felix 2009; van der Burgh and Joosten 2019; Cook, Mesarich, and Thomma 2015; Duxbury et al. 2016; Jones and Dangl 2006; Kourelis and Hoorn 2018). NLRs are found in both plants and animals with similar function and

structure and have probably occurred through convergent evolution (Duxbury et al. 2016). Although a significant structural NLR diversity appears among species, a typical structure can be defined (X. Li, Kapos, and Zhang 2015). The term NLR derives from the conserved domains of these receptors, since NLRs often carry a nucleotide binding domain (NB-ARC), a C-terminal leucine-rich repeat domain (LRR) and a variable N-terminal domain that mediates signal transduction (X. Li, Kapos, and Zhang 2015). In plants, the N-terminal signaling domain can either be a Toll/Interleukin-1 Receptor (TIR) domain, a Coiled-coil (CC) domain or an RPW8 domain, sorting plant NLRs in the three corresponding subclasses (Cesari 2018; Eitas and Dangl 2010). The presence of the NB-ARC domain suggests that NLRs belong to the Signal Transduction ATPase with Numerous Domains (STAND) protein family and this domain plays a role in self-regulation of the receptor through nucleotide binding and hydrolysis (Duxbury et al. 2016; Eitas and Dangl 2010; Lukasik and Takken 2009). The LRR domain is usually located in the C-terminus serving as a stable surface for interaction with other proteins (Lukasik and Takken 2009). Evolutionary pressure has granted a wide variation in NLRs operating models (Cesari 2018). These models are of significant interest for this study and are assiduously described in the chapter "NLR ligand recognition model". Effector recognition by NLRs typically stimulates ETI responses, which often result in a **Hypersensitive Response (HR)** (Jones and Dangl 2006). In this third layer (Figure 1C) of the complex molecular interactions between plants and microorganisms, the plant blocks pathogen dispersal by triggering HR, a spatially limited programmed cell death of the cells surrounding the invasion site (Jones and Dangl 2006; Sherif et al. 2015). ETI also provides resistance to the host through the salicylic acid hormonal pathway that is responsible for **Systemic Acquired Resistance (SAR)** (Tsuda and Somssich 2015). Apart from NLRs, PRRs are an additional part of ETI, since they sense effectors that are secreted by the pathogen in the apoplast as extracellular danger signals and can also trigger HR/SAR defense responses (Alfano and Collmer 2004; Boller and Felix 2009; van der Burgh and Joosten 2019).

The fourth and final part of the illustrated model (Figure 1D) determines the outcome of the interaction, that can eventually be **susceptibility** or **resistance** (Duxbury et al. 2016; Jones and Dangl 2006). Successful pathogens tend to discard effectors or evolve new ones to avoid perception or even suppress ETI responses (Khan et al. 2018). In addition, in many reported cases, pathogens acquire effectors that can target and suppress ETI, through interference with signaling and implementation of cell death or by manipulating hormonal pathways (Sherif et al. 2015). On the other hand, resistant plants have evolved new receptors to perceive effectors and have developed several sophisticated mechanisms through which these receptors, mostly NLRs, recognize their ligands and activate the appropriate signaling pathways (Adachi, Derevnina, and Kamoun 2019; Tsuda and Somssich 2015).

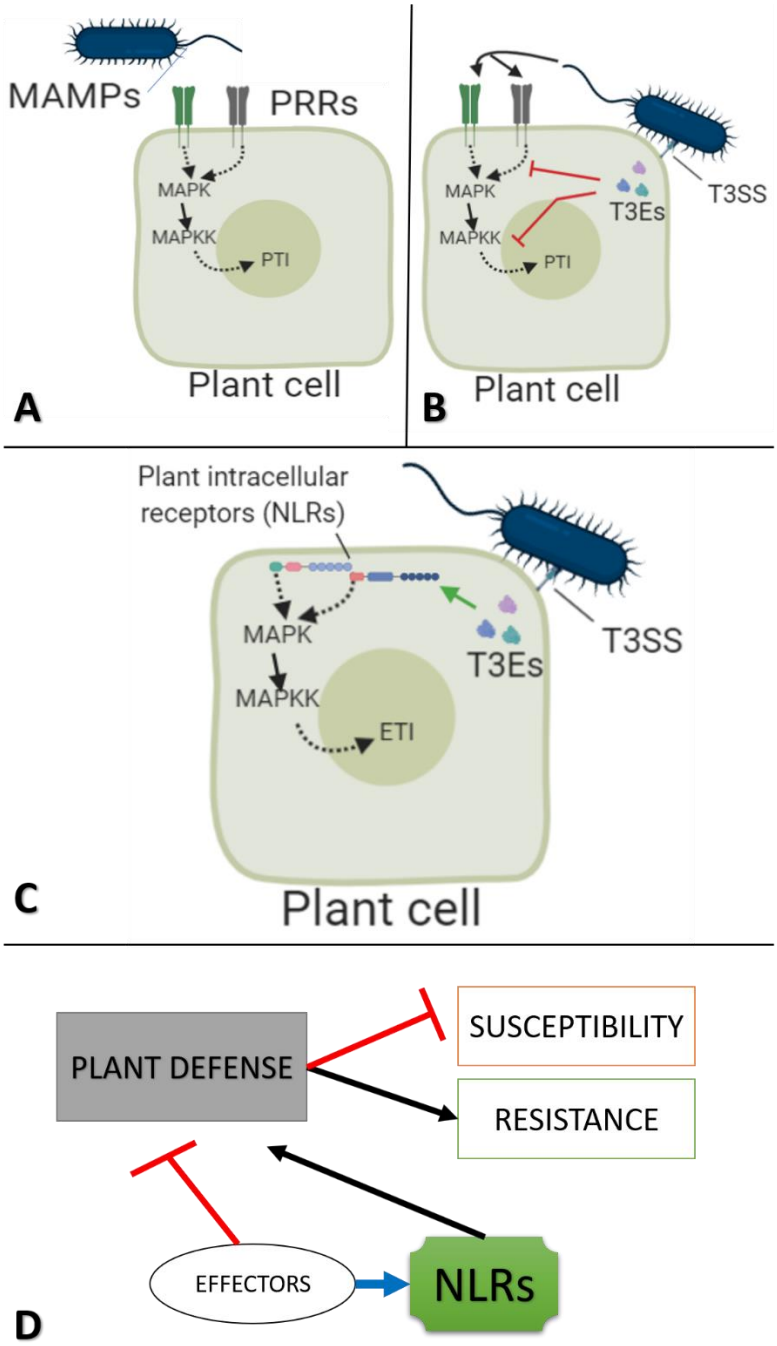

Figure 1: The complex molecular interactions between an invading pathogen and a plant cell, that have been developed through an evolutionary race. **A)** Extracellular Pattern Recognition Receptors (PRRs) sense conserved structural and biochemical molecular motifs among pathogens and signal the initiation of defense responses known as Pattern-triggered Immunity (PTI). **B)** Successful pathogens secrete effector proteins inside the host cell and block host PTI responses. **C)** Intracellular Nucleotide-binding Leucine-rich Receptors (NLRs) have evolved to recognize effectors deriving from pathogens and trigger a second more drastic layer of immunity called Effector-triggered Immunity (ETI). **D)** The blue arrow symbolizes the part where the outcome of the interaction is defined. If effectors escape recognition by NLRs, the host is susceptible, while resistance is achieved through successful perception of all effectors that can block activation of defense. Evolutionary pressure grands pathogen fitness in virtue of losing recognized effectors or acquiring new ones that can suppress ETI. The same forces result in evolution of novel plant NLRs or recognition mechanisms, to sense these new effectors and grand resistance. This figure was inspired by Jones and Dangl 2006; Sherif et al. 2015 and was created with bioRender www.biorender.com

Nucleotide-binding Leucine-rich repeats Receptors (NLRs)

Plant resistance to pathogens was not studied on a genetic basis until the late 19th century (Kapos, Devendrakumar, and Li 2019). The first attempt to give insight to the host-pathogen relationship between plant pathogens and crops was made by Flor, when he described **resistance (R)** plant genes each of which couples with a single **avirulence (Avr)** gene of a pathogen (Flor 1971). According to Flor, only when both genes were present in the host and the pathogen respectively, resistance would occur (Flor 1971). Avr genes are clearly correlated to pathogen effector proteins, just like R genes are to plant NLRs and this correlation is now widely accepted, since it matches the early biochemical receptor-ligand interpretation that followed Flor's model (Duxbury et al. 2016; Kapos, Devendrakumar, and Li 2019; X. Li, Kapos, and Zhang 2015). NLRs have become popular tools when it comes to introducing resistance to the few plant species that make up the fragile global food production (Cesari 2018; P. P. Singh et al. 2018). After decades of research, several sophisticated mechanisms underlying NLR function have been uncovered (Baggs, Dagdas, and Krasileva 2017; Tsuda and Somssich 2015).

NLR singletons and complexes

Operation of NLRs seem to vary, concerning the number of molecules that take part in a response (Adachi, Derevnina, and Kamoun 2019). Several NLRs can function as single units (Figure 2A), possessing the domains required both for recognition of effector molecules and for signaling activation of defense (Cesari 2018). This type of behavior matches Flor's model, where one receptor senses one virulence factor, but it seems to occur only in limited circumstances (Baggs, Dagdas, and Krasileva 2017; Flor 1971). As described earlier, typical NLR structure include a nucleotide binding domain (NB-ARC), a C-terminal leucine-rich repeat (LRR) responsible for recognition and a variable N-terminal domain that resolves signal transduction (X. Li, Kapos, and Zhang 2015). For example, RESISTANCE TO PSEUDOMONAS SYRINGAE 5 (RPS5) has been shown to sense AVRPphB activity and stimulate downstream ETI signaling in a way that depends on its N-terminal Coiled-Coil (CC) domain (Eitas and Dangl 2010; Sherif et al. 2015).

A larger amount of studies report NLRs that depend on other NLR proteins to operate, functioning as a pair, where the one partner mediates recognition and the other is responsible for signaling (Sarris et al. 2015; Williams et al. 2014). The two proteins are often genetically linked and are referred to as the sensor and the helper NLR (Figure 2B) (Adachi, Derevnina, and Kamoun 2019; Baggs, Dagdas, and Krasileva 2017). The RESISTANCE to *Pseudomonas syringae* 4 (RPS4) and RESISTANCE to *Ralstonia solanacearum* 1 (RRS1) are a well-studied interacting NLR pair in *Arabidopsis* (Le Roux et al. 2015). The non-typical C-terminal WKRY domain of RRS1 interacts with effectors deriving from both pathogens, causing rearrangements of the RPS4/RRS1 heterocomplex (Sarris et al. 2015). The two NLRs interaction through their TIR domains seems to be crucial for activation of defense (Williams et al. 2014).

Recently, it was shown that NLRs can recruit several other partners, forming a resistosome that mediates immunity responses as a complex (Jing Wang, Chern, and Chen 2019; Jizong Wang et al. 2019). These studies provided evidence that plant NLRs function through oligomerization forming complexes that resemble NLR inflammasomes, already found in animals (Jizong Wang et al. 2019). The structure of the ZAR1-RKS1 (HOPZ-ACTIVATED RESISTANCE 1 – RESISTANCE-RELATED KINASE 1) was resolved through cryo-EM studies and shed light to the biochemical mechanisms underlying NLR function (Jing Wang, Chern, and Chen 2019). The two proteins directly interact through the ZAR1 LRR domain, forming a heterodimer (Feng and Tang 2019). Upon recognition of a bacterial effector or its modified target and subsequent ATP-dependent activation, the complex oligomerizes into a pentamer (Fugure 2C) and is translocated to the plasma membrane forming a pore that initiates cell-death processes (Dangl and Jones 2019).

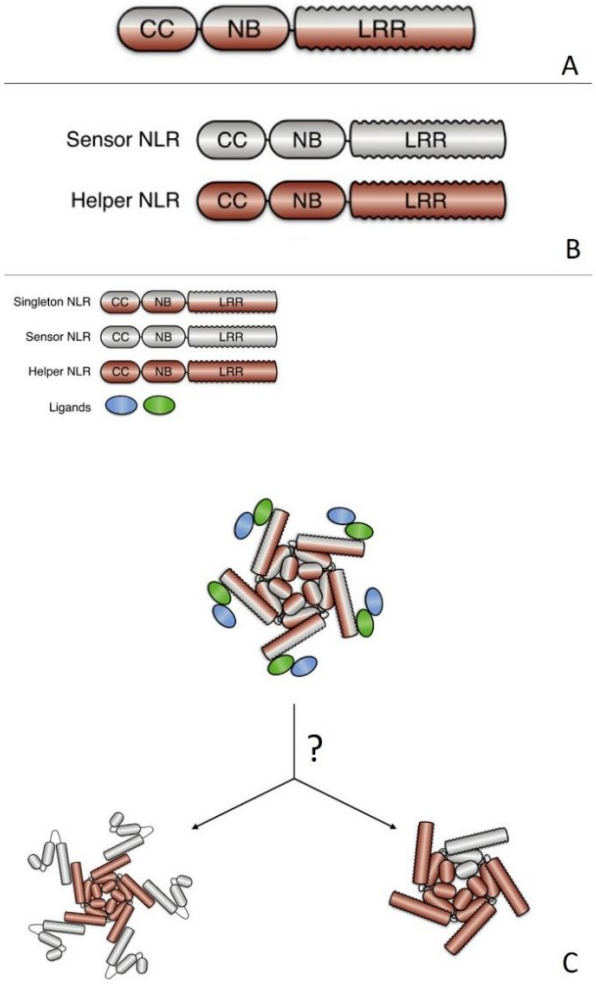

Figure 2: Different operation models of NLRs, concerning the number of molecules that take part in a response. A) NLRs that operate as single units carry the domains required both for effector recognition and for signaling. B) NLR commonly operate in pairs, with one sensor NLR responsible for effector recognition and one helper NLR that mediates signaling. C) The first resolved structure of a plant resistosome showed that the CC-NLR ZAR1 forms a pentameric complex. ZAR1 carries both recognition- and signaling-mediating domains. The sensor/helper NLR heterocomplexes could either resemble this structure (left arrow) or assemble in a way that mimics the mammalian inflammasome (right arrow). Figure found in Adachi, Derevnina, and Kamoun 2019, Current Opinion in Plant Biology.

NLR ligand recognition models

Further divergence emerges upon consideration of the different mechanistic models through which NLRs sense their ligands (Baggs, Dagdas, and Krasileva 2017; Cesari 2018). As mentioned above, Flor's model or the **"gene-for-gene" model** refers to the few cases, where an NLR senses an effector through direct recognition (Figure 3A) (Flor 1971; X. Li, Kapos, and Zhang 2015). For example, the fungal *Melampsora lini* effector AvrL567 is directly sensed by the TIR-NLR L6, resulting in *Linum* resistance (Duxbury et al. 2016). Furthermore, the oomycete effector ARABIDOPSIS THALIANA RECOGNIZED 1 (ATR1) was found to directly interact with the LRR domain of RECOGNITION OF PERONOSPORA PARASITICA 1 (RPP1), resulting in HR triggering through the TIR RPP1 domain (Krasileva, Dahlbeck, and Staskawicz 2010). The small numbers of such cases is consistent with the evolutionary challenges that plants face while coping with rapidly evolving, due to their shorter lifespans, pathogens (Adachi, Derevnina, and Kamoun 2019). NLR-encoding genes are certainly diversifying at a slower pace in comparison with pathogen effector genes and a surveillance system mainly depending on direct recognition would create an evolutionary disadvantage for hosts (Baggs, Dagdas, and Krasileva 2017).

Indirect recognition is the case for most of the NLR-effector reported interactions and it refers to two different mechanisms (Kourelis and Hoorn 2018). According to the **"guardee" model** (Figure 3B), the NLR monitors modifications on an additional functional plant protein, caused by an effector (Baggs, Dagdas, and Krasileva 2017). The second host protein is called the guardee and it is originally targeted by the effector to disrupt and manipulate various pathways, aiming to promote virulence (Cesari 2018). For instance, RPM1-Interacting Protein 4 (RIN4) is an Arabidopsis protein that is known to be targeted by four *Pseudomonas syringae* T3Es, causing different modifications (Cesari 2018). *Arabidopsis* possesses at least two NLR proteins, RPM1 and RPS2, that guard RIN4 and elicit defense responses and homologs of both the guardee and the corresponding R proteins have apparently arisen in several plant species (Afzal, Kim, and Mackey 2013). Guardees are proteins that mediate various essential cellular responses and they seem to be targeted by multiple pathogen effectors (Khan et al. 2018; Sarris et al. 2015). Similarly, in some cases evolution has granted more than one plant R proteins that are able to sense this targeting and initiate defense mechanisms (Duxbury et al. 2016; Tsuda and Somssich 2015).

The **"decoy" model** (Figure 3C) correspondingly describes situations where the guarded protein mimics the structure of the effector target, lacking its original function (van der Hoorn and Kamoun 2008). The recognition and signaling mechanisms parallel that of the guardee model (X. Li, Kapos, and Zhang 2015). For example, the *Arabidopsis* protein PBS1 is a decoy protein and its cleavage by the *Pseudomonas syringae* protease effector AvrPphB is downstream sensed by NLRs that trigger HR (Shao et al. 2003). The decoy's role is to serve as a bait for effectors and to mediate NLR recognition of effectors through the modifications they cause on it (Cesari 2018). Decoys anew illustrate how indirect recognition broadens the list of different effectors that a relatively small set of NLR proteins can perceive (van der Burgh and Joosten 2019). It is possible that decoys not only serve the surveillance system, but also compete with

the original targets for effector binding, hence protecting functional host proteins (van der Hoorn and Kamoun 2008).

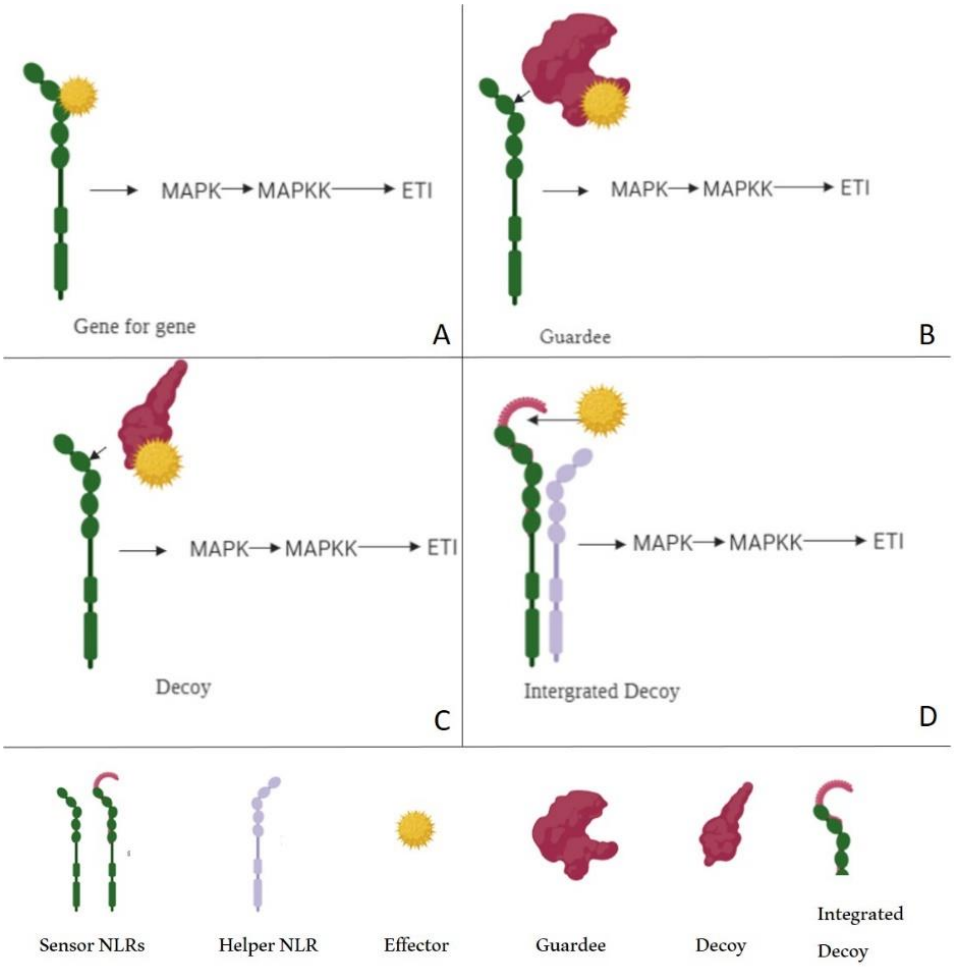

Figure 3: The mechanistic models that describe the recognition of effectors by NLRs and subsequent signaling events to activate defense. A) The gene-for-gene model: an NLR directly recognizes an effector and triggers ETI. B) The guardee model: an NLR senses modifications caused by effectors on targeted host proteins (guardees). It subsequently triggers ETI directly or by pairing with other NLR proteins. C) The decoy model: NLRs sense modifications caused by effectors on decoy proteins that mimic functional effector targets. ETI is triggered by the same NLR or by pairs of sensor-helper NLRs. D) The Integrated Decoy (ID) model: only observed in pairs of sensor-helper NLRs. The sensing partner carries a decoy fused on the molecule that interacts with effectors to mediate pathogen perception. This figure was inspired by Baggs, Dagdas, and Krasileva 2017; Cesari 2018; Jones and Dangl 2006 and was created with bioRender www.biorender.com

The latest advance in describing NLR-effectors interaction is the **"Integrated Decoy (ID)" model** (Cesari et al. 2014). All examined NLR pairs so far seem to carry additional non-canonical domains fused to the typical domains of the sensing partner (Figure 3D) (Kroj et al. 2016). A well-studied case is that of the WRKY domain found fused to the *Arabidopsis* RRS1, the sensor NLR of the RRS1-RPR1 pair (Le Roux et al. 2015; Sarris et al. 2015). This domain was shown to interact with at least two effectors and trigger RPR1-mediated signaling events that result in defense activation (Sarris et al. 2015). Such NLR-integrated domains are called **Integrated decoys (IDs)** and derive from functional domains of various eukaryotic proteins with crucial roles in cellular

processes (Baggs, Dagdas, and Krasileva 2017; Sarris et al. 2016). Hence, it is believed that this model is an expansion of the "decoy" model, where the decoy, or more precisely its effector-interacting region, has been fused to the NLR after duplication (Wu et al. 2015). It is argued whether these domains have lost their original function and only serve as baits, or if they maintain some activity (Baggs, Dagdas, and Krasileva 2017; Wu et al. 2015). An *in silico* analysis showed that NLR-IDs are probably a conserved mechanism between mosses and plant species and across all lineages of angiosperms (Sarris et al. 2016). They also investigated similarities between known effector targets and the IDs and provided a list of plant protein functional domains that appear more regularly fused to NLRs (Figure 4) (Baggs, Dagdas, and Krasileva 2017; Sarris et al. 2016). To synopsize, the ID model describes NLR pairs, whose interacting partner contains a decoy or guardee region, fused to its usual domains, to more efficiently sense the effector and activate defense (Cesari 2018). Therefore, IDs may provide the means for uncovering the subcellular host molecules or motifs that are originally targeted by pathogen effectors (Cesari 2018; Sarris et al. 2016; P. P. Singh et al. 2018).

Figure 4: Word cloud analysis that revealed plant proteins or protein domains most commonly found fused to NLRs as Integrated Decoys (IDs). The world cloud was constructed on the family level and font size indicates frequency. Many of these domains were found to overlap with known effector subcellular targets. This figure was found in Sarris et al. 2016, BMC Biology.

The plant pathogen *Ralstonia solanacearum*

Ralstonia solanacearum is a Gram-negative Beta-Proteobacterium that causes bacterial wilt disease to an expanded list of plants, including economically important crops (Álvarez, Biosca, and López 2010). Bacterial wilt disease is considered to be one of the most economically important plant diseases caused by pathogens of our time (Mansfield et al. 2012). Concerning agriculture, *Ralstonia solanacearum* is known for crop yield loss of many solanaceous species, such as *Solanum melongena* (eggplant), *Solanum tuberosum* (potato), *Nicotiana tabacum* (tobacco), and *Lycopersicon esculentum* (tomato) (Álvarez, Biosca, and López 2010). However, strains that reside in this diverse group not only infect members of the Solanaceae family, but they can collectively cause disease to more than 250 different plant species of monocots and dicots, along with the model organism *Arabidopsis thaliana* (Peeters et al. 2013). *Ralstonia solanacearum* strains also show a broad geographic distribution and are found in nearly every sector between the Tropics of Cancer and Capricorn (Gnanamanickan 2006). Despite this soil-borne bacterium's success in warm and moist climates, there are subsets of highly aggressive strains that can colonize plants and cause disease in comparably lower temperatures (Clarke et al. 2015). The pathogen is considered to be highly destructive and has a quarantine status in several zones, including the European Union (Álvarez, Biosca, and López 2010).

Genetic studies have brought to light an impressive intra-species diversity and *Ralstonia solanacearum* is now considered a species complex (Gnanamanickan 2006). Originally the strains of this diverse group were classified into five races according to their host range and into six biovars considering their different biochemical properties (Fegan and Prior 2005). Molecular analysis of an extended list of strains introduced the most recent system of classification, that of the four phylotypes and the sequevars they are further divided into (Peeters et al. 2013). The DNA-based classification system of phylotypes approximately represents the strains' geographic origin (Stéphane Genin and Denny 2012). Phylotype I strains have probably originated in Asia, phylotype II strains in America, phylotype III in Africa and Phylotype IV strains in Indonesia (Ailloud et al. 2015). Although many studies utilize older classification systems to study different subgroups of the *Ralstonia solanacearum* species complex, the phylotype system is the most accurate, since the same relationships are observed regardless the gene used for classification (Stéphane Genin and Denny 2012).

Despite being treated as a plant pathogen, *Ralstonia solanacearum* can adopt different survival strategies and can lastingly endure various habitats, e.g. aquatic or soil environments or even metal-polluted soils (Stéphane Genin and Boucher 2004). When found near a host, the bacterium senses molecules secreted by its roots and uses swim motility to move towards that location through chemotaxis (Lowe-Power, Khokhani, and Allen 2018). After root invasion via wounds and natural openings, wilting symptoms appear on the plant due to voluminous colonization of its xylem vessels by *Ralstonia solanacearum* (Álvarez, Biosca, and López 2010; Stéphane Genin and Boucher 2004). Apparently, the bacterium can manipulate the host's metabolism in a way that results in a more nutrient-rich xylem sap to extendedly settle in the

vascular system (Lowe-Power et al. 2018). During colonization, two forms of *Ralstonia solanacearum* can be isolated (Lowe-Power et al. 2018). The two versions differ biologically and the one is an immobile form adapted to consume various nutrients, while the other is a planktonic form with adaptations that favor virulence and further colonization of host tissues (Lowe-Power, Khokhani, and Allen 2018).

Considering virulence factors, *Ralstonia solanacearum* is a highly persistent pathogen equipped with several intrusive mechanisms (Meng 2013). The flagellum and the type IV pili seem to be essential for the bacterium's twitching and swimming motility, biofilm formation and attachment to the root or other surfaces (Peeters et al. 2013). The wilting symptoms are predominately caused due to xylem vessel occlusion by a heterogenous polymer produced by Ralstonia solanacearum, termed the Extracellular Polysaccharide I (EPSI) (Álvarez, Biosca, and López 2010). EPSI is also presumed to form a protective layer around the bacteria, that secure the cells from potential antimicrobial substances secreted by the host (Meng 2013). *Ralstonia solanacearum* additionally uses a type-II secretion system that secretes virulence-promoting molecules, mostly Cell-Wall-Degrading Enzymes (CWDEs) and other enzymes that detoxify reactive oxygen species (ROS) (Stéphane Genin and Denny 2012; Peeters et al. 2013). Despite the numerous disease reinforcements, a Type III Secretion System (T3SS) and the type III effector (T3Es) molecules it delivers are known to be the main virulence determinants of *Ralstonia solanacearum*, as it is the case for most Gram-negative bacterial pathogens (Coll and Valls 2013; Meng 2013).

Ralstonia solanacearum type III secretion system & type III effectors

Ralstonia solanacearum assembles its T3SS by generating a threadlike surface device, known as the Hrp pilus, through a mechanism that resembles the formation of the flagellum (Cornelis 2000). Assembly of the T3SS and protein secretion through the pilus are regulated processes and, as mentioned above, they are the most essential virulence determinants of this pathogen (Coll and Valls 2013). Genes related to T3SS assembly and secretion are transcriptionally controlled by the regulatory protein HrpB, comprising the HrpB regulon of *Ralstonia solanacearum* (Coll and Valls 2013; Cunnac et al. 2004). The HrpB regulon consists of a large number of genes dispersed across the chromosome and the megaplasmid, the two circular molecules that make up the bacterium's total genome, and is activated consequently to host cell contact and attachment (Cunnac et al. 2004; Salanoubat et al. 2002). Activation of T3SS initiates when PhrA, an outer membrane receptor, senses cell wall components of the host (Peeters et al. 2013). Through a transcriptional regulatory cascade, this sensing results in downstream activation of HrpG, which is a crucial regulator of the whole process and responsible for activation of HrpB (Stéphane Genin and Denny 2012). T3SS genes related to T3SS assembly and secretion, as well as the T3E genes, are all directly transcriptionally controlled by HrpB (Coll and Valls 2013).

Remarkably, *Ralstonia solanacearum* is able to secrete more than 100 proteins in the extracellular medium, when cultured (Poueymiro and Genin 2009). However, this bacterium mostly stands out for its extended T3E repertoire, since injection of more than 70 effectors inside the plant cell, using a T3SS-dependent translocation, has been

demonstrated (Deslandes and Genin 2014; Mukaihara, Tamura, and Iwabuchi 2010). Despite the great intraspecies diversity, more than 30 T3E genes seem to be conserved among all sequenced strains, comprising the core effectorome of the species complex (Deslandes and Genin 2014). Genomic data signify that *Ralstonia solanacearum* T3E repertoire is larger in comparison with other plant pathogenic bacteria (Peeters et al. 2013). A great T3E repertoire heterogeneity is nevertheless observed within the species (Coll and Valls 2013). Another striking feature is the presence of multigenic families that have probably occurred through gene duplication and appear to be shared between several *Ralstonia solanacearum* strains, but are also found in other plant pathogens (Stéphane Genin and Denny 2012). For example, The Transcription Activators Like (TAL) effectors are a multigenic effector family conserved among both *Ralstonia solanacearum* and *Xanthomonas* spp. strains (Ailloud et al. 2015).

Effectors are often associated with host specificity, since adequate effector perception by a plant immunity receptor molecule can lead to defense responses linked to resistance (Alfano and Collmer 2004). This may be true for most plant pathogens, but very limited cases of *Ralstonia solanacearum* T3Es have been reported that are specifically connected to pathogenicity on a plant host (Coll and Valls 2013; Zheng et al. 2019). One of the few examples is RipG7, a protein secreted by *Ralstonia solanacearum* in a T3SS-dependent manner that is essential for successfully infecting of *Medicago truncatula* (K. Wang et al. 2016). *Ralstonia solanacearum* T3Es are generally considered multifunctional and of probable overlapping functions, on the grounds that multiple effectors mutations are required to considerably modify pathogenicity on susceptible hosts (Deslandes and Genin 2014). Little is known about the exact molecular mechanisms of disease improvement that is carried out by *Ralstonia solanacearum* T3Es (Peeters et al. 2013). RipAK has been shown to localize in the plant cell peroxisomes, where its interaction with host catalases results in HR suppression (Sun et al. 2017). RipAY is known to associate with plant host thioredoxins, in the process of suppressing immunity responses triggered by salicylic acid (Sang et al. 2018). The precise nature of the host targets are yet to be uncovered, but through years of research it is possible to determine the pathways and host processes that effectors aim to interfere with (Hogenhout et al. 2009). While several well-studied plant pathogens utilize T3Es to mostly interfere with host signaling pathways and transcription regulatory mechanisms mostly involved in defense, *Ralstonia solanacearum* T3Es functions mainly result in host metabolism alteration (Khan et al. 2018). Since this pathogen successfully colonizes the xylem, it is possible that T3Es facilitate host physiology adjustment in a way that enriches the xylem in nutrients (Lowe-Power et al. 2018; Lowe-Power, Khokhani, and Allen 2018). The few but notable examples and the systemic analysis that illustrate *Ralstonia solanacearum* T3Es function suggest sophisticated pathogenicity mechanisms for this bacterium, that are T3E-dependent (Lowe-Power, Khokhani, and Allen 2018; Peeters et al. 2013).

Perspective

Unlike other Gram-negative bacteria that utilize T3SS, very limited cases of *Ralstonia solanacearum* T3Es have been associated with distinct host molecules or cellular pathways (Coll and Valls 2013; Mansfield et al. 2012). However, this destructive plant pathogen is apparently supplied with a considerable amount of multifunctional effectors, serving to manipulate the host physiology in favor of the pathogen, to escape the host's surveillance system and to suppress the responses of plant innate immunity (Poueymiro and Genin 2009). Therefore, sufficient awareness of bacterial wilt disease could be accomplished through uncovering the subcellular targets of type III effectors secreted by *Ralstonia solanacearum* (Lowe-Power, Khokhani, and Allen 2018; Meng 2013; P. P. Singh et al. 2018). Considering pathogen-host interactions, bacterial effectors and plant NLRs appear to be in an evolutionary contest aiming to avoid recognition and to ensure resistance, respectively (Jones and Dangl 2006). Throughout this evolutionary race, the original targets of effectors, or decoys that mimic them, were fused to NLRs as Integrated Decoys (IDs) to facilitate recognition (Kourelis and Hoorn 2018; Sarris et al. 2015, 2016). In this study, we hope to bring to light putative subcellular targets of *Ralstonia solanacearum* important effectors, through a screening for their possible interaction with various ID domains fused to NLRs from different plant species.

Proteins of interest

Five effectors belonging to the core effectorome of both the *Ralstonia solanacearum* species complex and the highly aggressive temperate climate-adapted subgroup R3B2 and additionally the putative acetyltransferase PopP1 were selected for studying (Clarke et al. 2015). The six in total effectors of interest, PopP1, RipE1, RipW, RipAR, RipAB and RipF1 are listed in Table 1. These effectors were further tested for interaction against selected IDs, through a yeast two-hybrid screening.

PopP1 or RipP1 (RSc0826) is a member of the YopJ-like family of cysteine proteases (Deslandes and Genin 2014). YopJ proteins of *Yersinia enterocolitica* were the first representative proteins of this diverse family that includes effectors deriving from plant symbionts and both animal and plant pathogens (Bastedo et al. 2019). YopJ family members are mostly acetyltransferases with a conserved cysteine at the active site and are able to target serine, threonine or lysine residues of their various substrates (Bastedo et al. 2019). PopP1 is closely related to PopP2 , another *Ralstonia solanacearum* type III effector of the same family that is thoroughly studied and has been proved to target WRKY transcription factors and to show acetyltransferase activity (Sarris et al. 2015). PopP1 also possesses a domain with putative acetyltransferase activity (L. Chen et al. 2018). We hypothesize that PopP1 can act in a similar manner as PopP2 and therefore possibly target WRKY transcription factors.

RipE1 (Rsc3369) is a conserved type III effector amongst all *Ralstonia solanacearum* strains that have been sequenced so far (Stephane Genin et al. 2013). This protein is a member of the AvrPphE family of effectors and shares homology with the *Pseudomonas syringae* HopX type III effectors (Stephane Genin et al. 2013; Nimchuk et

al. 2007). RipE1 has yet to be linked to a defined molecular function, but it has been associated with avirulence to bacterial wilt resistant eggplant cultivars (Pensec et al. 2015). Here, we intend to uncover putative subcellular targets of this conserved pathogenicity factor.

RipW or formerly PopW (RSc2775) is a two-domain harpin that is secreted via *Ralstonia solanacearum* T3SS and is believed to target unidentified cell wall components of the host (J. G. Li et al. 2010). Harpins are glycine-rich and heat-stable proteins that are secreted by plant pathogenic bacteria to promote disease (T. Wei et al. 2015). The exact molecular mechanism through which PopW benefits the pathogen has not been elucidated and the subcellular localization of the protein is unclear (H. Liu et al. 2016). However, this effector seems to be of great biotechnological interest, since it has been used as a biocontrol agent against other plant pathogens and has also been linked to abiotic stress tolerance (J.-G. Li et al. 2011; H. Liu et al. 2016). We aim to expose subcellular targets of RipW to help establish its use for biotechnological applications and to give insight to its role in bacterial wilt disease.

RipAR (RSp1236) is a putative ubiquitin ligase injected by *Ralstonia solanacearum* inside the plant cell (Deslandes and Genin 2014). This type III effector protein shares homology with the members of the IpaH family of effectors, which carry a novel E3 ubiquitin ligase (NEL) domain (Nakano, Oda, and Mukaihara 2017). RipAR demonstrates a poly-ubiquitination in vitro activity, despite it lacking the IpaH typical LRR domain (Nakano, Oda, and Mukaihara 2017). RipAR has the previously described putative enzymatic activity and is relatively conserved among different *Ralstonia solanacearum* strains, but its molecular target is unknown (Bastedo et al. 2019). Therefore, this effector makes an interesting point for our study.

RipAB or formerly PopB (RSp0876) is encoded through the *Ralstonia solanacearum* popABC operon and has been shown to be secreted through the pathogen's T3SS (Stéphane Genin and Boucher 2004; Mukaihara, Tamura, and Iwabuchi 2010). This effector carries a functional nuclear localization signal and it is believed that is especially localizes in the nuclei (Guéneron et al. 2000). Recently, a study showed that RipAB is able to manipulate the Ca^{2+} signaling pathway at the transcriptional level in potato (Zheng et al. 2019). We intend to investigate whether RipAB interacts with conserved domains found in various families of transcription factors.

RipF1 or formerly PopF1 (RSp1555) is one of the effectors that are conserved in the *Ralstonia solanacearum* species complex (Clarke et al. 2015; Stephane Genin et al. 2013). No molecular host target has been assigned to this protein, yet it is considered a translocator and a member of the HrpF/NopX family of effectors (Meyer et al. 2006). This T3SS transporter was included in our screening, since multifunctionality of Ralstonia solanacearum T3Es is often underlined.

Table 1: A list of the *Ralstonia solanacearum* type III effectors that were chosen to be investigated in this study. These proteins were all demonstrated to be secreted in a T3SS-dependent manner by Mukaihara, Tamura, and Iwabuchi 2010.

NAME	GENE CODE	REFERENCES
PopP1	RSc0826	(L. Chen et al. 2018; Clarke et al. 2015; Mukaihara, Tamura, and Iwabuchi 2010)
RipE1	RSc3369	(Clarke et al. 2015; Mukaihara, Tamura, and Iwabuchi 2010; Pensec et al. 2015)
RipW	RSc2775	(Clarke et al. 2015; J. G. Li et al. 2010; Mukaihara, Tamura, and Iwabuchi 2010; T. Wei et al. 2015)
RipAR	RSp1236	(Clarke et al. 2015; Mukaihara, Tamura, and Iwabuchi 2010; Nakano, Oda, and Mukaihara 2017)
RipAB	RSp0876	(Clarke et al. 2015; Guéneron et al. 2000; Mukaihara, Tamura, and Iwabuchi 2010)
RipF1	RSp1555	(Clarke et al. 2015; Meyer et al. 2006; Mukaihara, Tamura, and Iwabuchi 2010)

The selected effectors were tested for interaction, through yeast-two-hybrid screening assays, against the twenty-three cloned IDs from monocots and *Brassica* plants that are listed in Table 2. These domains were selected, as they derive from functional plant proteins, but also often appear integrated as decoy domains in plant NLRs (Sarris et al. 2016). Any interactions with the Calm-binding domain (ID number 23) were not to be taken under consideration, since later analysis showed that the construct's sequence was not correct.

Table 2: The list of Integrated Decoys obtained from NLR genes from various plant species. The IDs were cloned in yeast expression vectors to serve as a yeast-two-hybrid screening library for uncovering putative subcellular targets of effector proteins.

#	ID NAME	ORIGIN	YEAST EXPRESSION VECTOR CONSTUCTION
1	WRKY-1	*Arabidopsis thaliana*	Vasiliki Michalopoulou
2	WRKY-2	*Oryza sativa*	Vasiliki Michalopoulou
3	WRKY-3	*Hordeum vulgare*	Vasiliki Michalopoulou
4	Thior-1	*Vitis vinifera*	Paraskevi Kallemi
5	Thior-2	*Gossypium raimondii*	Paraskevi Kallemi
6	Kinase-1	*Hordeum vulgare*	Vasiliki Michalopoulou
7	Kinase-2	*Hordeum vulgare*	Vasiliki Michalopoulou
8	Kinase-3	*Hordeum vulgare*	Vasiliki Michalopoulou
9	HMA	*Brassica*	Synthesis: SynBio Tech©, design: Panagiotis Sarris, cloning: Vasiliki Michalopoulou
10	TCP	*Brassica*	Synthesis: SynBio Tech©, design: Panagiotis Sarris, cloning: Vasiliki Michalopoulou
11	LIM	*Brassica*	Synthesis: SynBio Tech©, design: Panagiotis Sarris, cloning: Vasiliki Michalopoulou
12	Ubox	*Brassica*	Synthesis: SynBio Tech©, design: Panagiotis Sarris, cloning: Vasiliki Michalopoulou
13	TFSII N	*Brassica*	Synthesis: SynBio Tech©, design: Panagiotis Sarris, cloning: Vasiliki Michalopoulou
14	TIR	*Brassica*	Synthesis: SynBio Tech©, design: Panagiotis Sarris, cloning: Vasiliki Michalopoulou
15	B3	*Hordeum vulgare*	Vasiliki Michalopoulou
16	CG	*Hordeum vulgare*	Vasiliki Michalopoulou
17	Exo70	*Hordeum vulgare*	Vasiliki Michalopoulou
18	HSF	*Hordeum vulgare*	Vasiliki Michalopoulou
19	PP2C	*Hordeum vulgare*	Vasiliki Michalopoulou
20	TRX	*Hordeum vulgare*	Vasiliki Michalopoulou
21	ZFBED-1	*Hordeum vulgare*	Vasiliki Michalopoulou
22	ZFBED-2	*Hordeum vulgare*	Vasiliki Michalopoulou
23	Calm-binding	*Hordeum vulgare*	Vasiliki Michalopoulou

Materials & Methods

Screening for protein-protein interactions – The yeast-two hybrid system

The yeast-two hybrid system is a molecular technique that utilizes the transcription mechanism of *Saccharomyces cerevisiae* (yeast) cells to investigate interactions between macromolecules (Brückner et al. 2009). The method has been expanded over the years and converted into a very effective tool to uncover protein-protein interactions (Brückner et al. 2009; J. O. Liu 2002; Stanley Fields 1993). To apply this technique, the construction of two hybrid proteins is required; the two different domains of a transcriptional activator are fused with two proteins of interest "X" and "Y", respectively (Lin and Lai 2017). An interaction between the proteins "X" (prey) and "Y" (bait) in the *Saccharomyces cerevisiae* nucleus would bring the DNA-binding domain and the transcriptional activation domain together, causing reconstruction of the transcriptional factor and thus, the expression of a reporter gene (Stanley Fields 1993). In this study, we used the yeast strain PJ69 (James, Halladay, and Craig 1996), that is appropriate for a GAL4p-based yeast two hybrid screening.

Yeast strain and vectors

In yeast, the protein GAL4 is a positive regulator that controls, along with the negative regulator GAL80 and the exogenous carbon source, the expression of the genes related to galactose metabolism (Evans 2009). GAL4 is a transcriptional activator and is often used to engineer Gal4-based yeast strains appropriate for yeast two-hybrid system application, like the strain PJ69 4A that was used in this study (James, Halladay, and Craig 1996). This strain contains Gal4-responsive promoter elements (TATA box and Upstream Activating Sequence, UAS) that control the reporter genes HIS3, ADE2 and LacZ (Evans 2009; Shaffer et al. 2012). Here, the genetic selection marker ADE2 was chosen as the most reliable of the three, as it gave no false positives (Sarris Lab unpublished data) and is considered to have high induced expression levels while not being leaky (Evans 2009). The complete genotype of the *S. cerevisiae* strain PJ69 4A is presented in Table 3.

Table 3: Genotype of the *Saccharomyces cerevisiae* strain PJ69 4A, that was used in this study (James, Halladay, and Craig 1996).

Strain	Genotype	Source
PJ69 4A	MATα	James *et al*. (1996)
	gal4Δ, gal80Δ	
	trp1-901 leu2-3 ura3-52 his3-200	
	LYS2::GAL1-HIS3 *GAL2-ADE2* *met2::GAL7-lacZ*	

The genes encoding for the selected *Ralstonia solanacearum* effector proteins were cloned in fusion with the Activation Domain (AD) of the GAL4 protein, in the yeast

expression vector pGADT7. Similarly, the Integrated Decoys (IDs) derived from various NLRs and plant families were cloned in fusion with the Binding Domain (BD) of the GAL4 protein, in the yeast expression vector pGBKT7. After double transformation with the two plasmids, interaction between the effector (prey) and the ID (bait) would reconstruct the regulatory protein Gal4, activating expression of the reporter genes and allowing the transformant to grow on appropriate selective medium (Brückner et al. 2009). Both plasmids' basic features appear in in Table 4.

Table 4: The yeast expression vectors pGADT7 and pGBKT7 that were used in this study (Laboratories 2007).

Vector	Antibiotic resistance (*E. coli* selection)	Nutritional marker (*S. cerevisiae* selection)	N-terminal fusion (MCS, designed for expression)
pGADT7	Ampicillin	*LEU2*	GAL4 activation domain (AD)
pGBKT7	Kanamycin	*TRP1*	GAL4 DNA binding domain (BD)

Yeast competent cells preparation & transformation

Competent *Saccharomyces cerevisiae* PJ69 4A were prepared *de novo* right before each transformation event, since competent yeast cells cannot be stored for long periods of time (Evans 2009). Competent yeast cells were prepared and transformed using a modified version of the LiAc/ssDNA/PEG protocol, described below. (Gietz and Schiestl 2008).

A single *Saccharomyces cerevisiae* PJ69 4A colony was cultured overnight in 5-10ml YPDA medium (Table S1) at 30°C. This culture was used to prepare a fresh one, with OD_{600}=0.2 and a final volume of n*10ml, where n the number of transformants needed. The new culture was incubated at 30°C for 3.5-4h, until its OD_{600} reached ~0.8-1. The culture was then divided in Falcon 50ml tubes and the cells were pelleted by a 4-minutes centrifugation at 3000rpm. YPDA was discarded and equal volume of sterilized dH_2O was used to dilute the cells. After a second centrifugation step, the cell pellet was diluted in a total volume of 1mL LiAc 0.1M and transferred in sterile Eppendorf 1.5ml tubes. The cells were centrifuged 11000g for 1 minute and, after disposal of the supernatant, the pellets were diluted with a total volume of n*100μl LiAc 0.1M, where n the number of transformants needed. The solution was distributed equally in n sterile Eppendorf 1.5ml tubes. After a quick spin (10sec), the supernatant was removed and 240μl of PEG 50% were added on the side of each tube, to protect the cells from any osmotic pressure until transformation.

For double transformation, a mixture described in Table 5 was added in each tube correspondingly, along with LiAc 0.1M. After intensively mixing (vortex ~1 minute), the cells were left to rest at 30°C for 25 minutes and then heat-shocked by an incubation at 42°C for 25 minutes. Finally, the cells were again pelleted, diluted in 200μl of selective SDC-Leu-Trp medium (Table S1) and plated on the same solid medium.

Table 5: The mixture containing pDNA, that was used for double transformation of competent yeast PJ69 4A cells in this study.

Component	Quantity
PREY pGADT7 containing *Ralstonia solanacearum* effectors fused to GAL4 Activation Domain	~ 1µg
BAIT pGBKT7 containing plant ID domains fused to GAL4 DNA-binding domain	~ 1µg
Carrier DNA UltraPure™ Salmon Sperm DNA Solution (Invitrogen) 2mg/ml (boiled for 5 minutes and chilled on ice for 5-10 minutes)	10µl
dH_2O (sterile)	Up to 75µl

Selection of transformants

Cells not carrying both plasmids, that contain LEU2 and TRP1 functional genes respectively, were prevented from growing on the selective SDC-Leu-Trp (Table S1) agar plates. SDC-Leu-Trp selective medium allows growth of yeast cells that only have received both plasmids, since the genes *LEU2* and *TRP1* are mutated in the yeast strain's genome. After incubation at 30°C for 2-3 days, colonies that had grown were streaked on new SDC-Leu-Trp plates and incubated for another 2-3 days to seperate distinctive single colonies of approximately 2mm were visible. Those colonies were then chosen as appropriate for protein-protein interaction testing, using the yeast-two hybrid assay.

Protein-protein interactions testing

Single *S. cerevisiae* colonies PJ69 4A carrying both corresponding plasmids (bait and prey) were selected as described above. Each colony was diluted in 10µl of sterilized dH2O. Half of the diluted cells (5µl) were cultured at on a selective SDC-Leu-Trp plate as a droplet left to dry in sterile conditions. Similarly, the remaining 5µl were cultured on a selective SDC-Leu-Trp-Ade plate (Table S). After an incubation of 2-3 days at 30°C, colonies that grew in the lack of adenine were considered to carry plasmids with bait and prey proteins interacting with each other. Such an interaction would result in the reconstruction of the GAL4 protein and the transcriptional activation of the ADE2 gene. The SDC-Leu-Trp plate served as a control to show that colonies chosen for the assay did indeed carry both plasmids, even if their bait and prey proteins did not interact. For each transformation event, four controls were used during the two-hybrid assay. The negative control (ctrl-) was a colony carrying the empty vectors pGADT7 and pGBKT7 and should not grow lacking adenine. The positive control (ctrl+) was a colony carrying plasmids containing bait and prey that are known to interact with each other and should grow lacking adenine. For this study, the *Pseudomonas syringae* effector AvrRPS4 (cloned in pGADT7) and the *Arabidopsis thaliana* ecotype WS2 WKRY ID from NLR RRS1 (cloned in pGBKT7) were chosen (Le Roux et al. 2015; Sarris et al. 2015). In addition, transformants carrying each pGADT7::RsEffector plasmid and the empty pGBKT7 vector and transformants carrying each pGBKT7::plantID and the empty pGADT7 vector were also cultured on a selective plate lacking adenine, to rule out any autoactivation.

Gene Construction in yeast expression vectors – The Golden Gate Cloning method

A list of Integrated Decoy (ID) domains found in NLRs from different plant species (Table 2) had been cloned in the yeast expression vector pGBKT7 fused to the DNA-binding domain of the GAL4 protein and serve as baits for various yeast-two hybrid screenings in the lab (Vasiliki Michalopoulou, PhD candidate, Sarris Lab). Therefore, for this study, the gene construction referred to the genes encoding for the selected *Ralstonia solanacearum* effectors. These genes were cloned in the final yeast expression vector pGADT7, in fusion to the Activation Domain of GAL4, via the Golden Gate Cloning method (Engler, Kandzia, and Marillonnet 2008). The primers used to obtain the genes through amplification with Polymerase Chain Reaction (PCR) are listed in Table S2 (Supplementary) and were designed to meet the standard Golden Gate Cloning requirements (Engler, Kandzia, and Marillonnet 2008):

- Insertion of BsaI flanking sites to the genes of interest.
- Deletion of endogenous BsaI sites (BsaI domestication) of the genes of interest via silent in frame mutations. To fill this requirement, genes that had endogenous BsaI sites were partially amplified.

Amplifying the genes of interest

The genes of interest, or the parts constituting them, were amplified via PCR, using the Phusion High- Fidelity Polymerase (New England Biolab, NEB) and primers constructed by Macrogen Incorporation. The primers are listed in Table S4. The amplicons were then recovered using the DNA extraction from agarose gel protocol, coming with the MACHEREY-NAGEL GmbH & Co's *NucleoSpin® Gel and PCR Clean-up* kit.

TA cloning in pBluescript vector

The cleaned PCR products were A-tailed, using 0.05 units per reaction ul of TaqPolymerase (Minotech) and 1x of its corresponding buffer, along with 0.2mM dNTAs for a 30-minute incubation at 72°C. The A-tailed products were cleaned using the PCR Clean-up protocol, coming with the MACHEREY-NAGEL GmbH & Co's *NucleoSpin® Gel and PCR Clean-up* kit. The A-tailed products were cloned in the BsaI domesticated and T-tailed vector pBluescript SK(-) produced in the lab by Dr. Glykeria Mermigka, Sarris Lab via the TA cloning method (Zhou, Gomez-Sanchez, and Montgomery 2000). An amount of ~0.33 units per reaction ul of T4 DNA Ligase (New England Biolab, NEB), along with 1x of its corresponding buffer was used for ligation, via a 2-hour incubation in room temperature.

Golden Gate Cloning assembly

The genes of interest were cloned in a final yeast expression vector via the Golden Gate Cloning method. (Engler, Kandzia, and Marillonnet 2008) This method is based on typeIIS restriction endonucleases, that recognize and cleave the DNA outside

their recognition site. In this study, BsaI-HF® (NEB)was used for this purpose, whose recognition and cleavage pattern are shown in Figure 5.

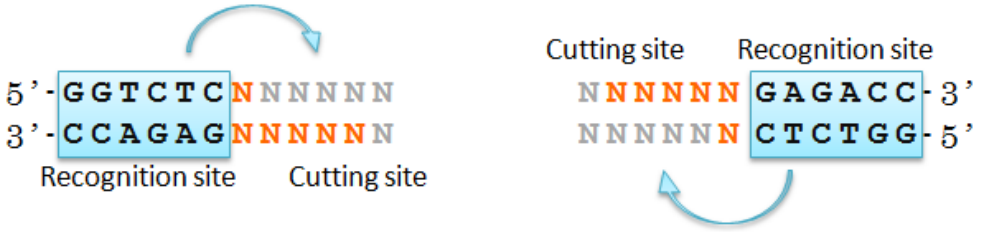

Figure 5: Recognition and cleavage patterns of the typeIIS restriction endonuclease BsaI. This type of restriction enzyme is used in the Golden Gate Cloning method. Figure posted online by the iGEM Hokkaido U Team: http://2011.igem.org/Team:HokkaidoU_Japan

Chemically competent *E. coli* cells preparation and transformation

E. coli DH10B or STELLAR cells were grown to an OD600 of ~0.6 and were centrifuged for 10 minutes at 2500rpm. The pellet was washed with 0.1 volumes of freshly prepared TFBI solution (Table S3). After a 30-minute rest and a second centrifugation, the pellet was resuspended in 1/25 volume of freshly prepared TFBII solution (Table S3). Finally, 100µL aliquots of cell suspension were frozen in liquid nitrogen and stored at -80°C for future use. For transformation, cells aliquots were thawed and appropriate quantity of plasmid DNA (pDNA) extract or ligation product was added. After a resting time of 30 minutes at 4°C, the cells were heatshocked (1 minute at 42°C & 1 minute at 4°C). LB medium (Table S1) was added up to 1mL and the cells were incubated at optimum conditions (37°C, rotating). After 1 hour, the cells were plated for corresponding antibiotic selection on LB solid medium.

Nucleic acids extraction

Genomic DNA extraction from *Ralstonia solanacearum* GMI1000 was performed via the CTAB extraction method (William, Feil, and Copeland 2004). All plasmids, that were constructed or used in this study, were isolated using the Alkaline Lysis method (Fritsch and Maniatis 2003)

Plasmid sequencing

All plasmids were sequenced by the Custom DNA Sequencing Services of Macrogen Incorporation or Eurofins Genomics, using the universal primers T3 and T7promoter or additional endogenous primers (Table S4) that were designed to read of inserts larger than 1400bp.

Cultures

Escherichia coli DH10B or STELLAR cells were cultured in LB medium (Table S1) at 37°C. *Ralstonia solanacearum* GMI1000 cells were cultured in NA medium (Table S1) at 28°C. *Saccharomyces serevisiae* PJ69 4A cells were cultured in YPDA medium or in appropriate dropout medium (Table S1) at 30°C.

Results

Gene Construction Validation

The results that are presented in this section provide evidence for sequence verification of the yeast expression vectors carrying the genes of interest that were used in our study. For the purpose of BsaI domestication, genes with endogenous BsaI sites were partially amplified, as it is shown in Table 6.

Table 6: The genes of interest were partially amplified, according to the number of endogenous BsaI sites they contained. BsaI domestication was achieved through silent mutation at those sites.

Gene name	Number of endogenous BsaI sites	Obtained in gene parts
popP1	0	popP1 (whole)
ripE1	0	ripE1 (whole)
ripW	0	ripW (whole)
ripAR	1	ripAR part 1, ripAR part 2
ripAB	2	ripAB part 1, ripAB part 2, ripAB part 3
ripF1	0	ripF1 (whole)

Gene amplification (PCR)

The genes and the parts of genes of interest were successfully obtained via PCR from genomic DNA of *Ralstonia solanacearum* strain GMI1000 and subsequent extraction from the corresponding agarose gel. Expected amplicon size in base pairs verified the success of the reaction. The electrophoresis agarose gels from which the genes and gene parts were recovered are demonstrated in Figures 6-8.

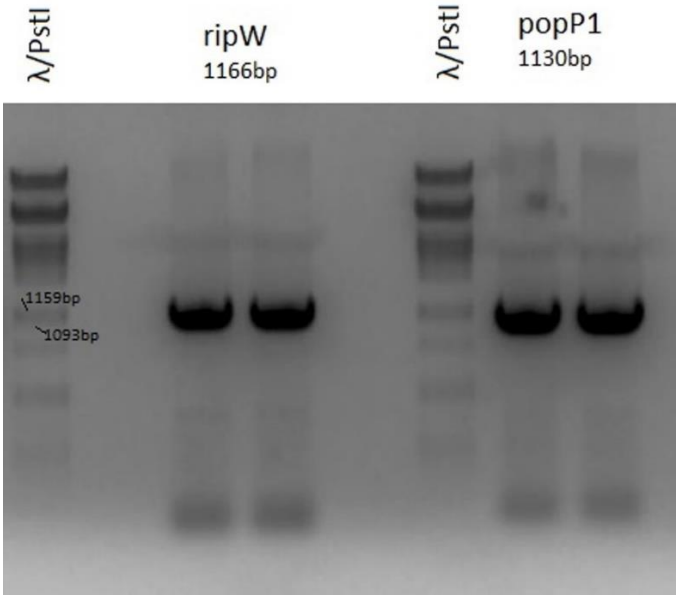

Figure 6: Electrophoresis agarose 1.2% gel of the PCR reactions for ripW and popP1 genes amplification. The primers that were used are listed in Table S4. ripW was amplified, along with the appropriate BsaI

flanking sites, using the primers ripW-FW and ripW-REV and the expected amplicon was 1166bp in length. popP1 was amplified, along with the appropriate BsaI flanking sites, using the primers popP1-FW and popP1-REV and the expected amplicon length was 1130bp. Genomic DNA extracted from *Ralstonia solanacearum* GMI1000 was the template for these PCR reactions.

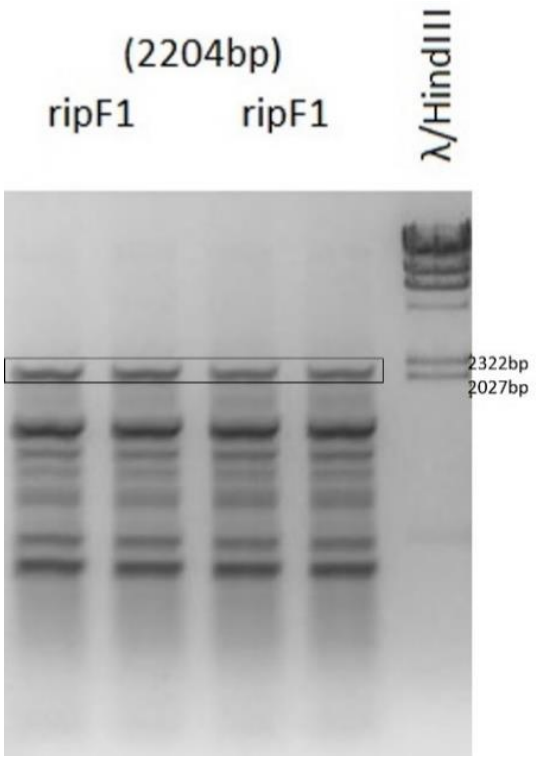

Figure 7: Electrophoresis agarose 1.2% gel of the PCR reaction for ripF1 gene amplification. The primers that were used are listed in Table S4. ripF1 was amplified, along with the appropriate BsaI flanking sites, using the primers ripF1-FW and ripF1-REV and the expected amplicon was 2204bp in length. Genomic DNA extracted from *Ralstonia solanacearum* GMI1000 was the template for this PCR reaction.

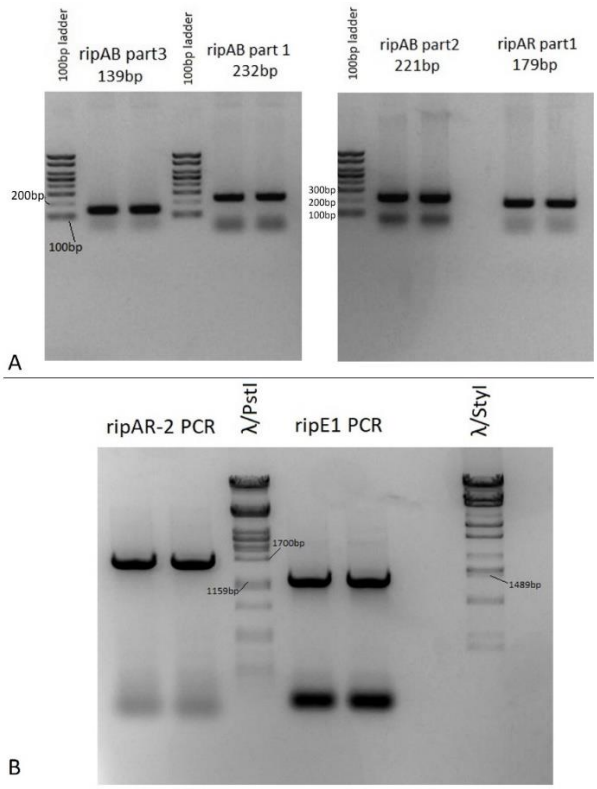

Figure 8: Electrophoresis agarose 1.2% gels of the PCR reaction for ripE1, ripAB and ripAR genes amplification. The primers that were used are listed in Table S4. ripE1 was amplified (**Figure 8B**), along with the appropriate BsaI flanking sites, using the primers ripE1-FW and ripE1-REV and the expected amplicon was 1301bp in length. ripAB had to be partially amplified to ensure elimination of endogenous BsaI sites with the appropriate silent mutations. ripAB part 1 was amplified, along with the appropriate BsaI flanking sites and single nucleotide substitution, using the primers ripAB-FW1 and ripAB-REV1 and the expected amplicon was 232bp in length **(Figure 8A, left)**. ripAB part 2 was amplified, along with the appropriate BsaI flanking sites, using the primers ripAB-FW3 and ripAB-REV3 and the expected amplicon was 221bp in length **(Figure 8A, right)**. ripAB part 3 was amplified, along with the appropriate BsaI flanking sites and single nucleotide substitution, using the primers ripAB-FW3 and ripAB-REV3 and the expected amplicon was 139bp in length **(Figure 8A, left)**. ripAB parts were designed to assemble a ripAB gene that would carry the silent mutation C207T (Arg). ripAR had to also be be partially amplified to ensure elimination of endogenous BsaI sites with the appropriate silent mutations. ripAR part 1 was amplified, along with the appropriate BsaI flanking sites and single nucleotide substitution, using the primers ripAR-FW1 and ripAR-REV1 and the expected amplicon was 179bp in length **(Figure 8A, right)**. ripAR part 2 was amplified, along with the appropriate BsaI flanking sites and single nucleotide substitution, using the primers ripAR-FW3 and ripAR-REV3 and the expected amplicon was 1684bp in length **(Figure 8B)**. ripAR parts were designed to assemble a ripAR gene that would carry the silent mutation G150A (Ser). Genomic DNA extracted from *Ralstonia solanacearum* GMI1000 was the template for these PCR reactions.

TA cloning

All amplicons that were successfully obtained were subsequently cloned in pBluescript II SK (-) vector in a TA cloning manner. Their sequence was initially verified through restriction digestions (Figures 9-12) and subsequently via DNA sequencing (data not shown).

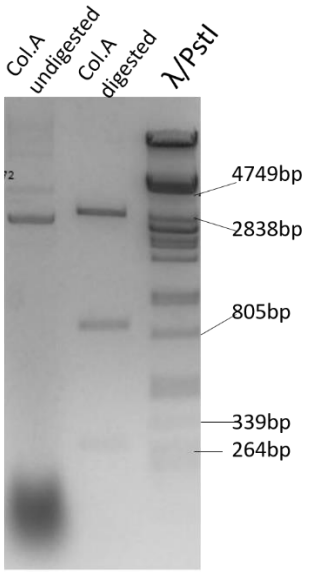

Figure 9: Agarose gel 1.2% that demonstrates the electrophoresis of diagnostic digestion with restriction enzymes. The plasmid pBSK::popP1 was digested with BamHI and HindIII restriction enzymes (Minotech). The expected pattern was 2931-890-271bp (or 2931-873-288bp if the cloning had happened reversed) and colony A seemed to carry the correct plasmid. This plasmid's sequence was further confirmed through DNA sequencing (data not shown) and it was used for the next assembly.

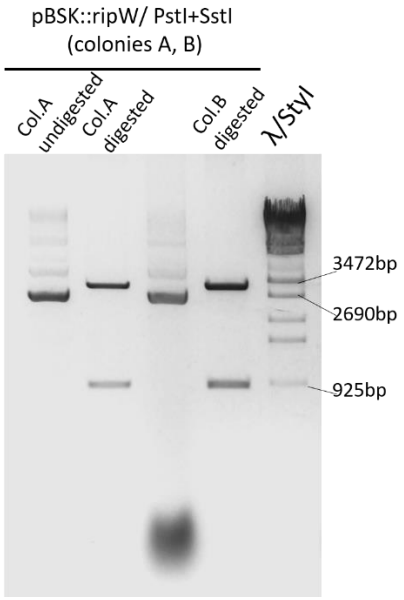

Figure 10: Agarose gel 1.2% that demonstrates the electrophoresis of diagnostic digestion with restriction enzymes. The plasmid pBSK::ripW was digested with PstI and SstI (SacI isomer) restriction enzymes (Minotech). The expected pattern was 3794-286-48bp (or 3176-904-48bp if the cloning had happened reversed) and colony A seemed to carry the correct plasmid. This plasmid's sequence was further confirmed through DNA sequencing (data not shown) and it was used for the next assembly.

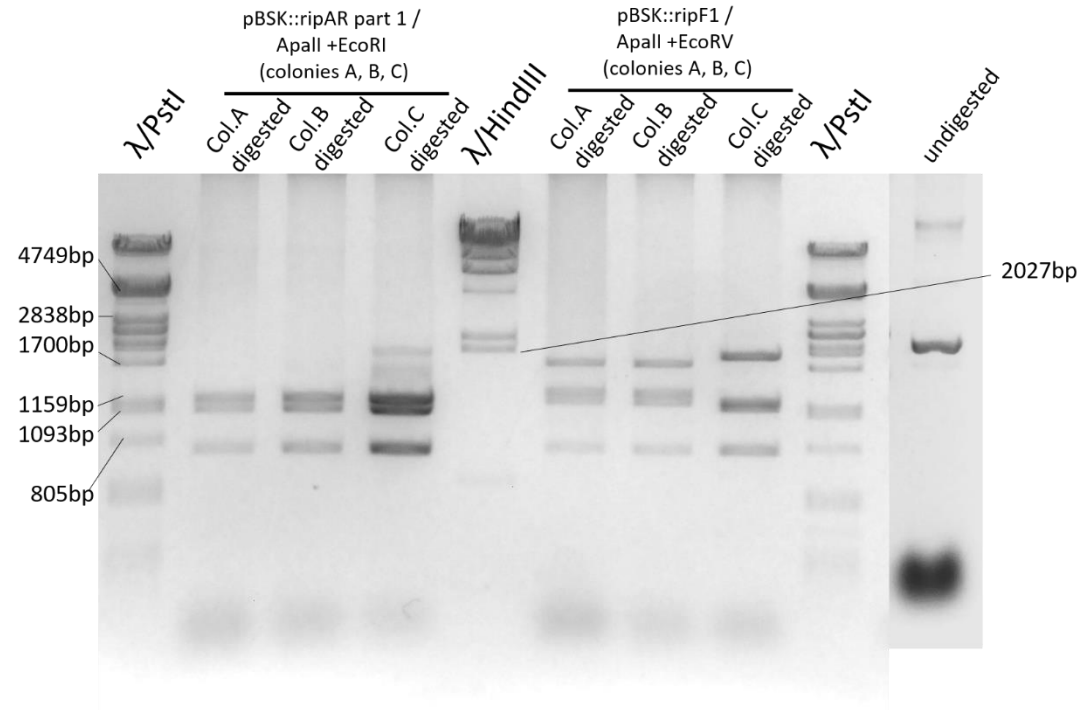

Figure 11: Agarose gel 1.2% that demonstrates the electrophoresis of diagnostic digestions with restriction enzymes. **Left:** The plasmid pBSK::ripAR part 1 was digested with ApaII and EcoRI restriction enzymes (Minotech). The expected pattern was 1246-1129-766bp and colony A seemed to carry the correct plasmid. **Right:** The plasmid pBSK::ripF1 was digested with ApaII and EcoRV restriction enzymes (Minotech). The expected pattern was 1781-1349-1246-790bp (or 1953-1246-1173-794bp if the cloning had happened reversed) and colony A seemed to carry the correct plasmid. The selected plasmids' sequence was further confirmed through DNA sequencing (data not shown) and they were used for the next assembly.

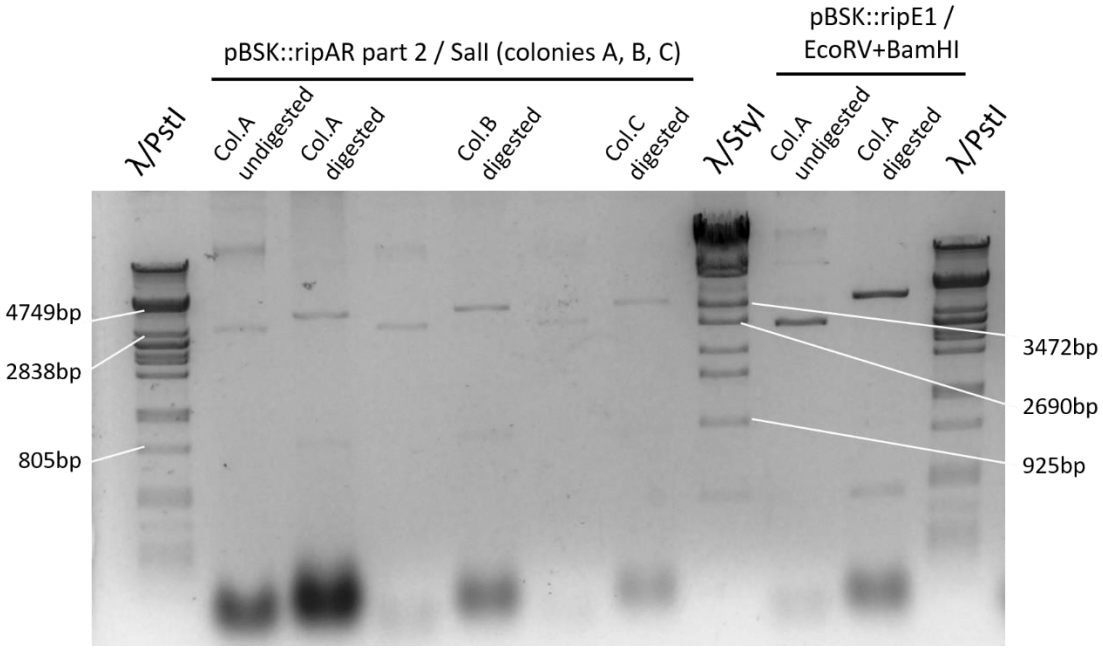

Figure 12: Agarose gel 1.2% that demonstrates the electrophoresis of diagnostic digestions with restriction enzymes. **Left:** The plasmid pBSK::ripAR part 2 was digested with SalI restriction enzyme (Minotech). The expected pattern was 3793-853bp (or 3771-875bp if the cloning had happened reversed) and colony A seemed to carry the correct plasmid. **Right:** The plasmid pBSK::ripE1 was digested with BamHI and EcoRV restriction enzymes (Minotech). The expected pattern was 3334-929bp (or 3847-416bp if the cloning

had happened reversed) and colony A seemed to carry the correct plasmid. The selected plasmids' sequence was further confirmed through DNA sequencing (data not shown) and they were used for the next assembly.

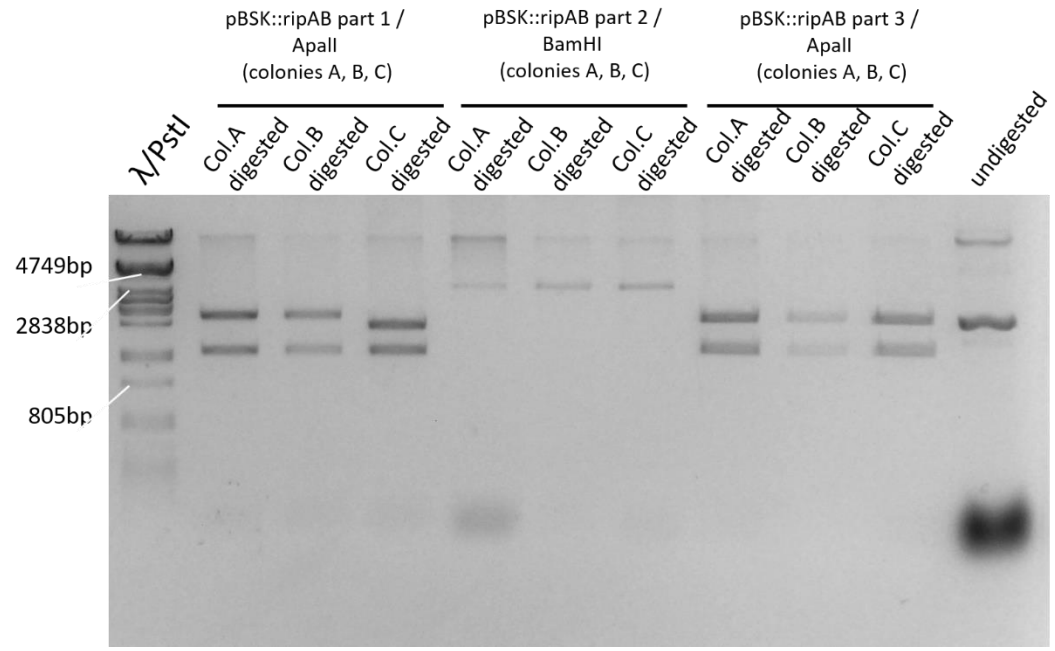

Figure 13: Agarose gel 1.2% that demonstrates the electrophoresis of diagnostic digestions with restriction enzymes. **Left:** The plasmid pBSK::ripAB part 1 was digested with ApaII restriction enzyme (Minotech). The expected pattern was 1948-1246bp and colony A seemed to carry the correct plasmid. **Center:** The plasmid pBSK::ripAB part 2 was digested with BamHI restriction enzyme (Minotech). The expected pattern was 3143-40bp (or 2954-229bp if the cloning had happened reversed) and colony A seemed to carry the correct plasmid. **Right:** The plasmid pBSK::ripAB part 3 was digested with ApaII restriction enzyme (Minotech). The expected pattern was 1855-2246bp and colony A seemed to carry the correct plasmid. The selected plasmids' sequence was further confirmed through DNA sequencing (data not shown) and they were used for the next assembly.

Golden Gate cloning

Sequenced pBluescript II SK (-) plasmids, containing the genes and gene parts of interest were used for the Golden Gate cloning assembly of the functional *Ralstonia solanacearum* T3E genes in the yeast expression vector pGADT7. Golden Gate cloning products were digested with the appropriate restriction enzymes to confirm the success of the cloning reaction. The electrophoresis agarose gels of the restrictive digestion reactions are demonstrated in Figures 14-16.

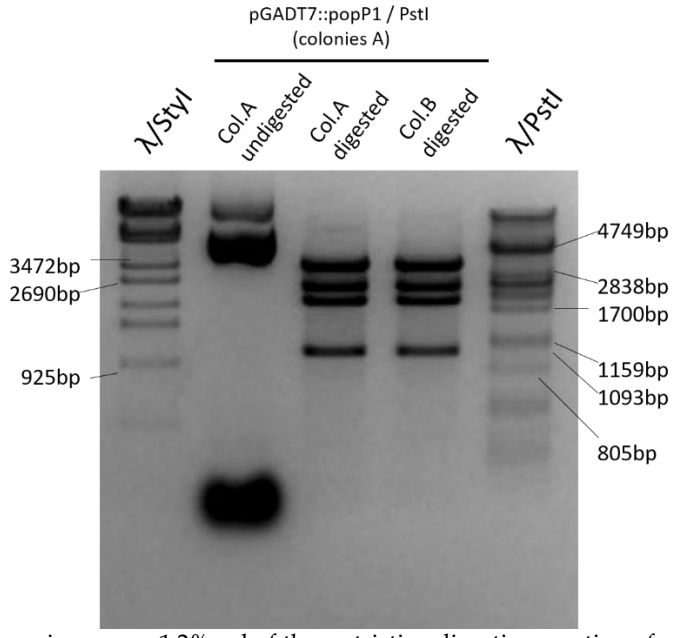

Figure 14: Electrophoresis agarose 1.2% gel of the restrictive digestion reaction of pGADT7::popP1. The plasmid was constructed via the Golden Gate Cloning method and it was digested with the restriction enzyme PstI. The expected pattern after this digestion was four DNA bands of length 3490, 2470, 2003 and 1041 base pairs, respectively. Colonies A and B are carrying the correct plasmid, which was later used for the yeast two-hybrid screening assays.

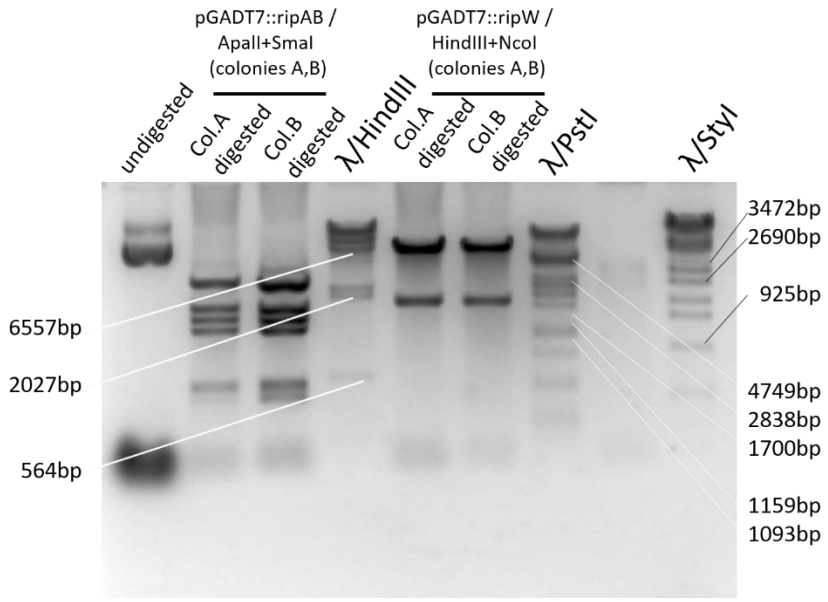

Figure 15: Electrophoresis agarose 1.2% gel of the restrictive digestion reactions of pGADT7::ripAB and pGADT7::ripW. The plasmids were constructed via the Golden Gate Cloning method. pGADT7::ripW

was digested with the restriction enzymes HindIII and NcoI. The expected pattern after this digestion was two DNA bands of length 7187 and 1853 base pairs, respectively. Colonies A2 and B2 are carrying the correct plasmid, which was later used for the yeast two-hybrid screening assays. pGADT7::ripAB was digested with the restriction enzymes ApaII and SmaI. The expected pattern after this digestion was six DNA bands of length 2976, 1799, 1503, 1246, 497 and 421 base pairs, respectively. Colonies A2 and B2 are carrying the correct plasmid, which was later used for the yeast two-hybrid screening assays.

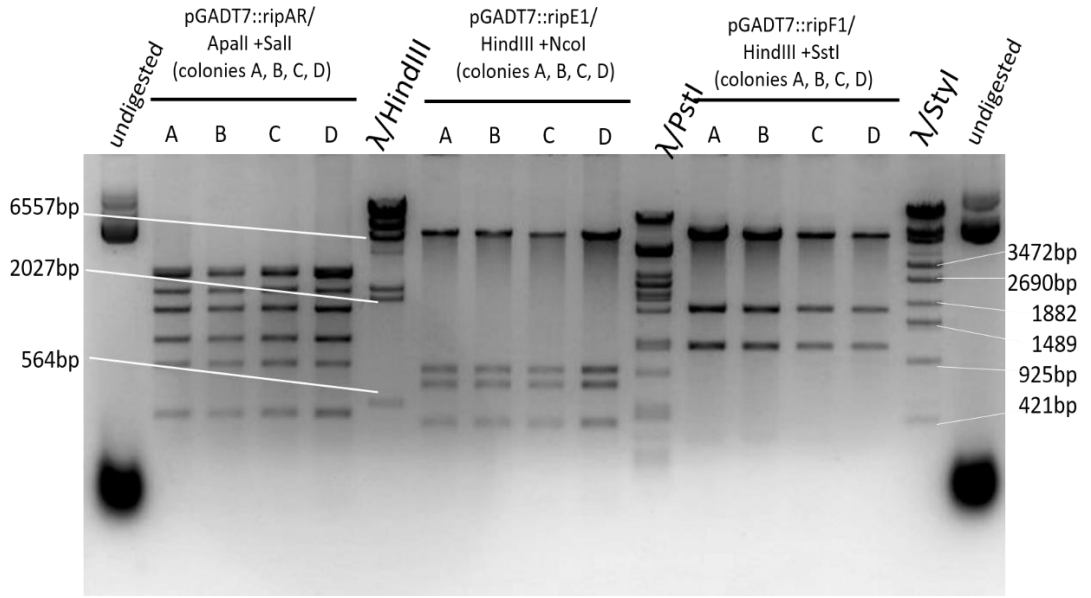

Figure 16: Electrophoresis agarose 1.2% gel of the restrictive digestion reactions of pGADT7::ripAR, pGADT7::ripE1 and pGADT7::ripF1. The plasmids were constructed via the Golden Gate Cloning method. pGADT7::ripAR was digested with the restriction enzymes ApaII and SalI. The expected pattern after this digestion was six DNA bands of length 2976, 2288, 1799, 1246, 909 and 497 base pairs, respectively. Colonies A and B are carrying the correct plasmid, which was later used for the yeast two-hybrid screening assays. pGADT7::ripE1 was digested with the restriction enzymes HindIII and NcoI. The expected pattern after this digestion was four DNA bands of length 7187, 852, 707 and 429 base pairs, respectively. Colonies A and B are carrying the correct plasmid, which was later used for the yeast two-hybrid screening assays. pGADT7::ripF1 was digested with the restriction enzymes HindIII and SstI (SacI isomer). The expected pattern after this digestion was four DNA bands of length 7187, 1759 and 1132 base pairs, respectively. Colonies C and D are carrying the correct plasmid, which was later used for the yeast two-hybrid screening assays.

Protein-Protein Interaction Results

A total of 138 possible interactions between Ralstonia solanacearum type III effectors (Table 1) and plant NLR ID domains (Table 2) were tested through yeast two-hybrid assays. The positive results that are presented in Figures 12-20 have been repeated three times for validation.

Our results indicate possible interaction between the effector PopP1 and a WRKY domain found integrated as decoy (Figure 17). PopP1 interacts with WRKY-3, which is found in a *Hordeum vulgare* NLR, but not with the WRKY IDs from other species, that were included in this screening.

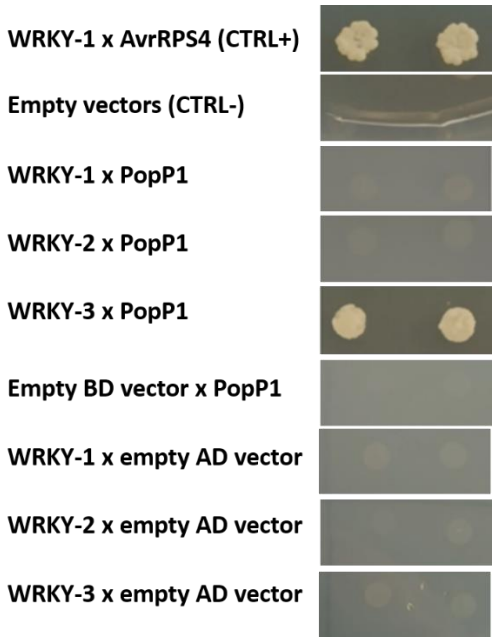

Figure 17: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. PopP1 was found to interact with a WRKY ID domain from *Hordeum vulgare* (barley). In this experiment additional controls were used to rule out any false positives caused by auto-binding activity of PopP1 or auto-activation capabilities of the WRKY domains. pGADT7::PopP1 was used along with pGBKT7_RFP in the "Empty BD vector x PopP1" transformant. Similarly, pGBKT7::WRKY was used along with pGADT7_RFP in the "WRKY x empty AD vector" transformants. PopP1 did not interact with the other WRKY IDs included in this screening.

The effector RipE1 seems to interact with the same WRKY ID domain as PopP1 (Figure 18). In fact, RipE1 exhibited associations with multiple plant NLR ID domains. Apart from the *Hordeum vulgare* WRKY ID, it was also found to interact with an N-terminal TFSII ID domain from *Brassica napus* (Figure 18), a CG ID domain (Figure 19), an Exo70 ID domain (Figure 19) and a C-terminal TRX domain (Figure 20) found fused in NLRs of monocot plants. Another, yet weaker, interaction was demonstrated between RipE1 and an HSF (Figure 20) domain from barley.

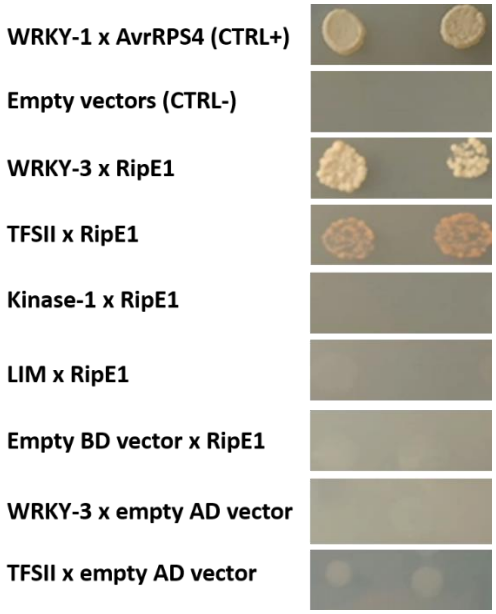

Figure 18: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. In this experiment additional controls were used to rule out any false positives caused by auto-binding activity of RipE1 or auto-activation capabilities of the ID domains. pGADT7::RipE1 was used along with pGBKT7_RFP in the "Empty BD vector x RipE1" transformant. Similarly, pGBKT7::ID was used along with pGADT7_RFP in the "ID x empty AD vector" transformants. RipE1 was found to interact with the WRKY-3 and the TFSII ID domains.

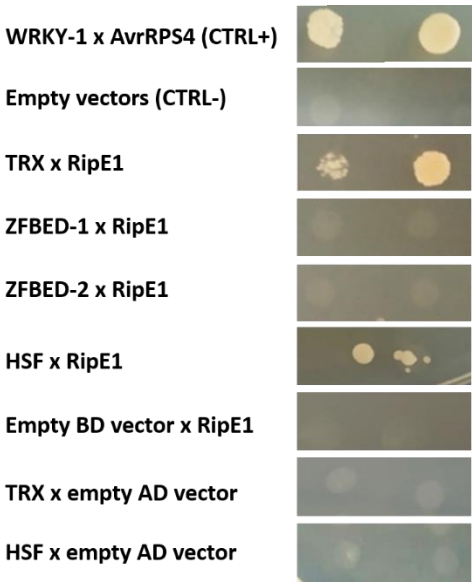

Figure 19: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. In this experiment additional controls were used to rule out any false positives caused by auto-binding activity of RipE1 or auto-activation capabilities of the ID domains. pGADT7::RipE1 was used along with pGBKT7_RFP in the "Empty BD vector x RipE1" transformant. Similarly, pGBKT7::ID was used along with pGADT7_RFP in the "ID x empty AD vector" transformants. RipE1 was found to interact with the TRX ID domain and to weakly interact with the HSF ID domain.

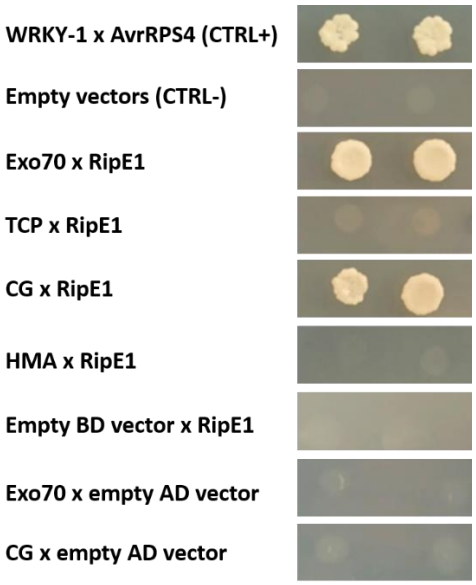

Figure 20 *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. In this experiment additional controls were used to rule out any false positives caused by auto-binding activity of RipE1 or auto-activation capabilities of the ID domains. pGADT7::RipE1 was used along with pGBKT7_RFP in the "Empty BD vector x RipE1" transformant. Similarly, pGBKT7::ID was used along with pGADT7_RFP in the "ID x empty AD vector" transformants. RipE1 was found to interact with the Exo70 and the CG ID domains.

Surprisingly, the effector RipAR appeared to associate with plant IDs, in a similar manner as RipE1. Interactions were discovered between RipAR and the ID domains WRKY-3 and TFSII (Figure 21), CG and Exo70 (Figure 22), but not with the TRX ID (Figure 22).

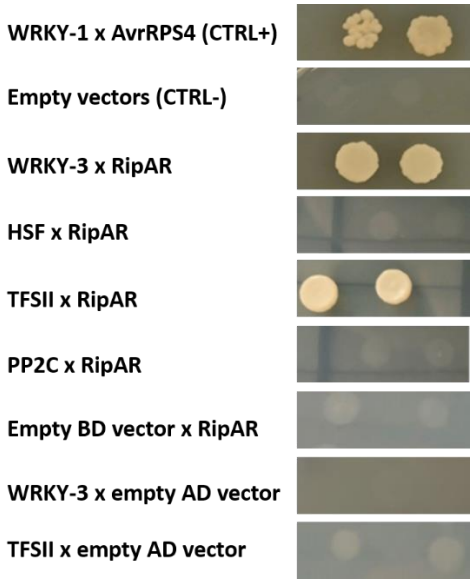

Figure 21: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of

leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. In this experiment additional controls were used to rule out any false positives caused by auto-binding activity of RipAR or auto-activation capabilities of the ID domains. pGADT7::RipAR was used along with pGBKT7_RFP in the "Empty BD vector x RipE1" transformant. Similarly, pGBKT7::ID was used along with pGADT7_RFP in the "ID x empty AD vector" transformants. RipAR was found to interact with the WRKY-3 and the TFSII ID domains.

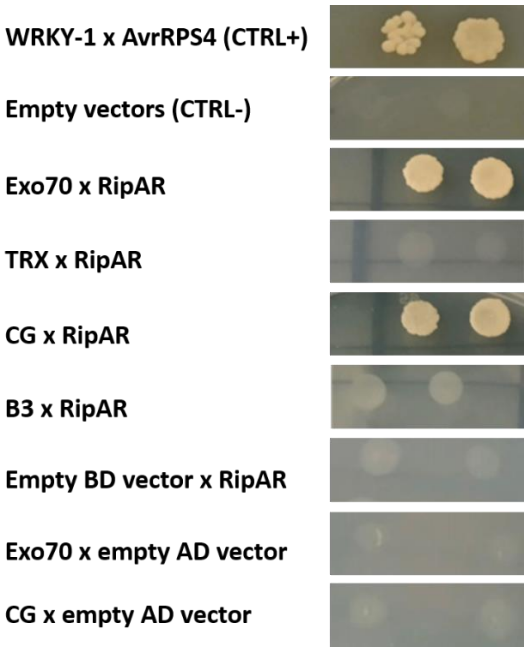

Figure 22: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. In this experiment additional controls were used to rule out any false positives caused by auto-binding activity of RipAR or auto-activation capabilities of the ID domains. pGADT7::RipAR was used along with pGBKT7_RFP in the "Empty BD vector x RipE1" transformant. Similarly, pGBKT7::ID was used along with pGADT7_RFP in the "ID x empty AD vector" transformants. RipAR was found to interact with the Exo70 and the CG ID domains, but not with TRX.

Finally, a TCP domain fused in a *Brassica napus* NLR conceivably interacts with the effector RipW (Figure 23).

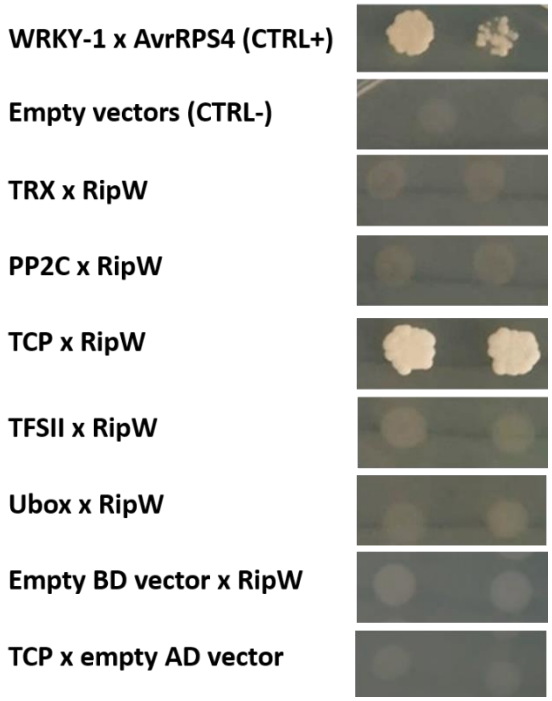

Figure 23: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. In this experiment additional controls were used to rule out any false positives caused by auto-binding activity of RipW or auto-activation capabilities of the ID domains. pGADT7::RipW was used along with pGBKT7_RFP in the "Empty BD vector x RipW" transformant. Similarly, pGBKT7::ID was used along with pGADT7_RFP in the "ID x empty AD vector" transformants. RipW was found to interact with the TCP ID domain.

The same list of IDs (Table 2) was tested for interactions against the effectors RipF1 and RipAB, yet no such interactions were brought to light (Supplementary Figures S10-S13).

The interactions indicated by our results, between the effectors PopP1, RipE1, RipAR, RipW and the various ID domains, are comparatively displayed in Figure 24, were yeast cells carrying the corresponding plasmids were cultured in the lack of adenine in subsequently diluted concentrations. Finally, all interactions detected in this screening and the appropriate checks for any false positives are collectively demonstrated in Figure 25 and in Table 7.

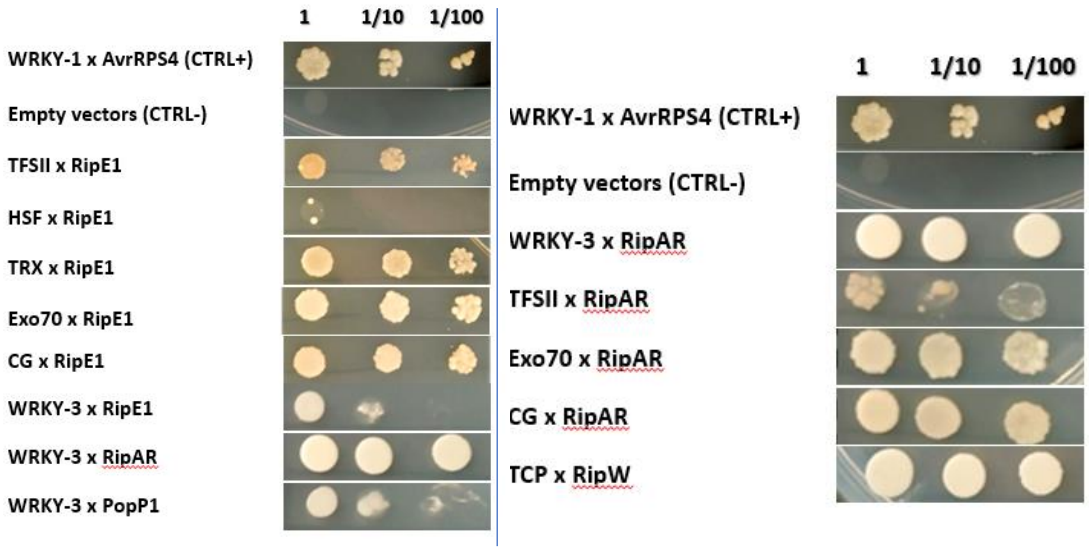

Figure 24: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. Cells were cultured in different concentrations, prepared by subsequent dilutions (1, 1/10 and 1/100), to validate the interactions and compare their effectiveness. This Figure depicts all the interactions that were uncovered, including the weak interaction between RipE1 and the HSF ID.

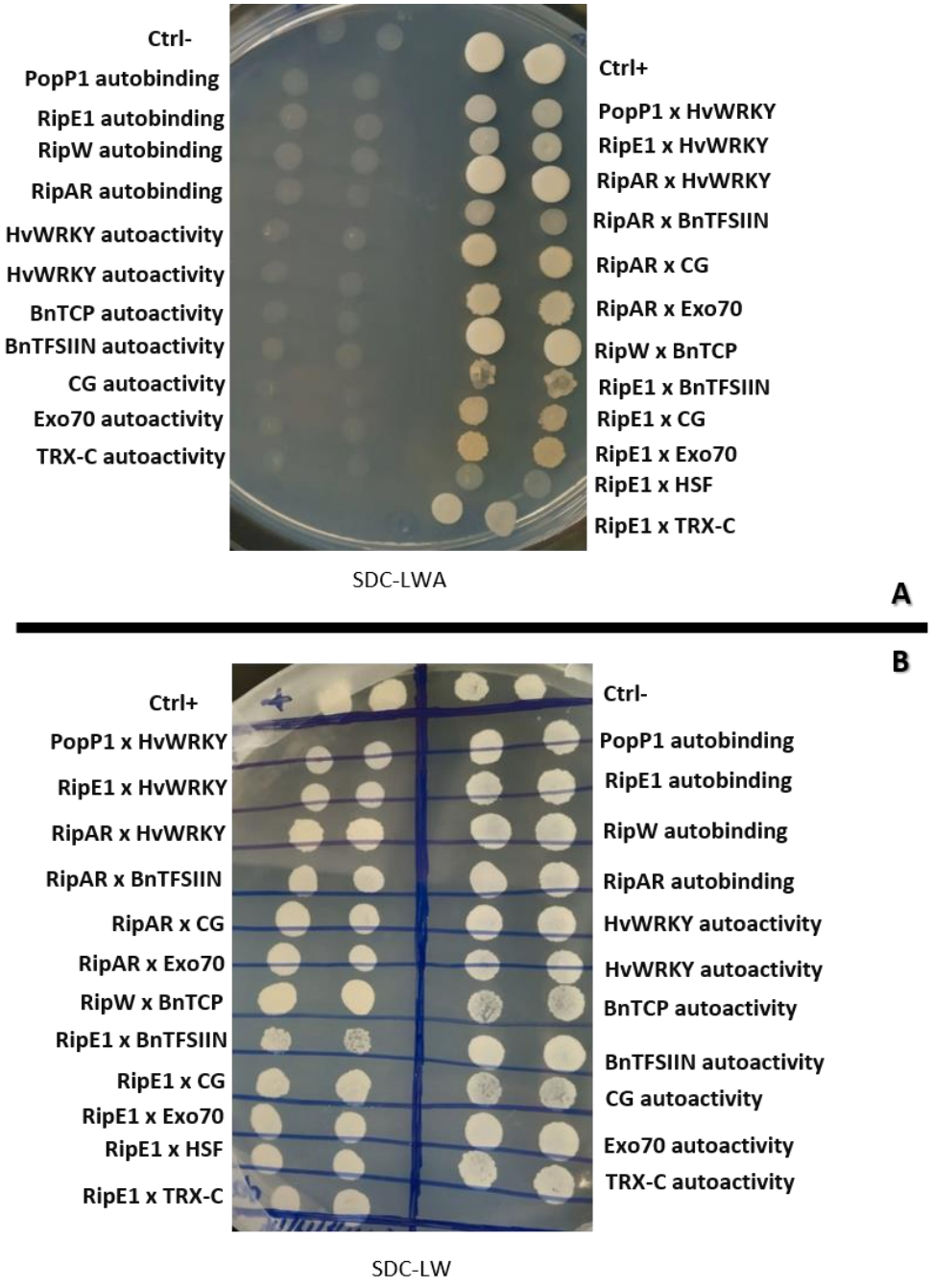

Figure 25: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. The appropriate auto-binding effector capacity and ID auto-activity was checked using transformants that carry pGADT7::RsEFFECTOR, pGBKT7_RFP or pGADT7_RFP, pGBKT7::plantID, respectively. In Figure B it is demonstrated that the yeast colonies chosen for the screening, grow in the lack of leucine and tryptophan. This proves that they carry indeed the correct plasmids, which are being expressed.

Table 7: All the interactions uncovered between *Ralstonia solanacearum* T3Es (horizontal) and plant NLR IDs (vertical) in this study. The ● symbolizes interactions that were confirmed by repeating the experiment at least three times and running all the appropriate controls. The ○ symbolizes interactions that were not observed after the experiment was repeated or they appear to be weak, since the corresponding yeast transformant demonstrated a relatively low growth rate.

	PopP1	RipE1	RipW	RipAR	RipAB	RipF1
AtWRKY						
OsWRKY						
HvWRKY	●	●		●		
VvThiorN						
GrThiorN						
Kinase C						
Kinase N C010						
Kinase N C135						
BnHMA						
BnTCP			●			
BnLIM						
BnUbox						
BnTFSII N		●		●		
BnTIR						
B3						
CG		●		●		
Exo70		●		●		
HSF		○				
PP2C-C						
TRX-C		●				
ZFBED_N_C045						
ZFBED_N_C350						
Calm-binding						

Discussion

When the Integrated Decoy model was first proposed, it predicted that non-canonical sensor NLR domains are original effector targets duplicated and fused to NLRs (Cesari et al. 2014). Accumulating data suggest that this prediction is accurate and could provide the means to uncover effector targets through large-scale screenings (Baggs, Dagdas, and Krasileva 2017; Kapos, Devendrakumar, and Li 2019; Sarris et al. 2015, 2016; Wu et al. 2015). The results of this study, that reveal interactions between *Ralstonia solanacearum* effectors and plant IDs, could provide clues and direct research for ultimately uncovering the targets of these effectors. It should be noted that, negative results do not necessarily mean no interaction, since the expression of constructs has not been validated through western blot assays. Thus, we discuss here the possible biological meaning of the positive results that were detected.

Three different *Ralstonia solanacearum* effectors interact with the same WRKY ID plant domain

WRKY domains appear to be frequently fused to plant NLR-ID proteins that are present in many monocot and dicot plant species (Sarris et al. 2016). These highly conserved amino acid sequences are the most characteristic feature of the WRKY superfamily of transcription factors (X. Chen et al. 2019). WRKY transcription factors are unique to plants and it is suggested that the numerous members of the family are involved in multiple physiological responses (Eulgem et al. 2000). Members of the WRKY superfamily can be transcriptional activators or suppressors of responses to biotic and abiotic stress, developmental pathways and other important processes (Rushton et al. 2010). Considering the crucial role of WRKYs in the two basic aspects of plant innate immunity, PTI and ETI, it is not surprising that they are a common target between pathogen effectors (Kalde et al. 2003; Rushton et al. 2010). Accordingly, WRKY domains as Integrated Decoys can serve for recognition of multiple effectors deriving from different pathogens (Baggs, Dagdas, and Krasileva 2017). Such cases have been reported before, with the prominent example being the *Arabidopsis* RRS1-WRKY domain, which recognizes both the *Pseudomonas syringae* AvrRps4 and the *Ralstonia solanacearum* PopP2 effector (Sarris et al. 2015). Our results report three different *Ralstonia solanacearum* effectors interacting with a WRKY domain found fused in a *Hordeum vulgare* NLR protein. This ID contains two WRKY domains, resembling several functional WRKY proteins (Eulgem et al. 2000). Both WRKY domains are most closely related to *Arabidopsis* WRKY Group III proteins, according to BLAST searches. Proteins of the Arabidopsis WRKY Group III transcription factors are known to be involved in different signaling pathways of plant defense (Kalde et al. 2003). Thus, PopP1, RipE1 and RipAR could be utilized by the pathogen to promote bacterial wilt disease by targeting WRKY members of such pathways and suppressing different defense mechanisms.

Two *Ralstonia solanacearum* effectors interact with multiple plant ID domains

Many studies indicate that effector proteins are no longer considered specific molecules and they frequently appear to target or associate with multiple host proteins (Bastedo et al. 2019; Hogenhout et al. 2009). Among the genes involved in plant-microbe interactions, effector genes are the most rapidly evolving and effector function is often an example of remarkable novelty (Zipfel et al. 2012). This study uncovered two *Ralstonia solanacearum* core effectors, that seem to associate with multiple plant IDs deriving from host proteins with distinct function and subcellular localization.

As mentioned above, RipE1 and RipAR interact with the same WRKY ID and most likely interact with WRKY transcription factors. Host factors involved in controlling the epigenetic and transcriptional profile of eukaryotes are commonly targeted by adapted pathogens through virulence mechanisms, including secreted effectors (Khan et al. 2018; Silmon De Monerri and Kim 2014). Our results indicate that both these effectors could target additional groups of plant transcription factors. One of these groups is the Transcription elongation factor (TFIIS) family, since RipE1 and RipAR can interact with the TFIIS N ID. TFIIS N ID is a domain fused to the *Brassica napus* NLR encoded by *Bn_LOC106419748*. BLAST search and SMART annotation revealed that TFSII N domain is found in members of the Transcription elongation factor (TFIIS) family. TFIIS members are conserved eukaryotic proteins, which mediate enhancing of mRNA cleavage by RNA polymerase II (Kettenberger, Armache, and Cramer 2003). RipE1 and RipAR are also associated with the CG ID domain. CG is a domain contained in calmodulin-binding transcription activators (CAMTA) (Bouché et al. 2002; da Costa e Silva 1994). CAMTA proteins mostly localize in the nucleus and they are calcium-responsive activators (Bouché et al. 2002). Calcium signaling is a vital process for plant immunity and the components of the corresponding pathways are likely to be targeted by pathogen effectors (van der Burgh and Joosten 2019; Feng and Tang 2019).

RipE1 and RipAR effectors seem to interact with the Exo70 ID domain and it is possible that they originally target the Exo70B1 protein of Arabidopsis and other hosts (BLAST search). Exo70 is a subunit of the exocyst complex, which is required for the exocytosis complex of eukaryotes (TerBush et al. 1996). Exo70 is known to interact with proteins responsible for vesicle transportation during exocytosis (Robinson et al. 1999). Effectors secreted by pathogens can target such proteins to promote disease by interfering with the exocytosis of antimicrobial compounds during PTI or to suppress ETI processes that are related to cell-death (Sherif et al. 2015). TRX is an ID domain fused to NLRs and is usually detected in thioredoxins (SMART database). Thioredoxins are proteins with crucial engagement in regulation of reactive oxygen species (ROS) during plant stress responses (Zhang et al. 2011). ROS production is a major procedure to achieve PTI, since they have significant antimicrobial activity (van der Burgh and Joosten 2019). Thioredoxin domains are frequently fused to NLRs as IDs (Sarris et al. 2016) and are commonly targeted by pathogens to disrupt immunity (Khan et al. 2018; Y. Wei, Sang, and Macho 2017) . Here we report a *Ralstonia*

solaanacearum effector, RipE1, that potentially targets thioredoxins, among its other functions.

A harpin binds to ID characteristic of Transcription Factor family

RipW has been characterized as a harpin targeting plant cell wall components and triggering immunity responses extracellularly (H. Liu et al. 2016). However, we observed a strong interaction between RipW and a TCP ID of the NLR encoded by *Bn_LOC106305604* from *Brassica napus*. TCP is a molecular motif mostly found in proteins comprising the TCP family of transcription factors (Lauter et al. 2014). TCP proteins are key regulators of plant growth and development and members of this family can control cell division (Gutie et al. 2005; Lauter et al. 2014). The balance between plant growth and plant immunity responses is governed by several hormonal cross-talking pathways, but the molecular mechanisms are mostly unknown (Denancé et al. 2013). Pathogens often target crucial components of these complex processes and manipulate plant hormonal pathways to suppress immunity responses (Han and Kahmann 2019). Although RipW's ability to reach the host nucleus is questionable, it would seemingly give this effector the capacity to manipulate fundamental regulatory pathways of plant immunity.

Conclusions

We conclude that *Ralstonia solanacearum* elemental type III effectors display multifunctionality and could target several different proteins in the host nucleus and the cytosol. Effectors can be localized to different host cell compartments (Gimenez-Ibanez et al. 2014) and the study of PopP1, RipE1, RipAR and RipW subcellular localization would shed light to our findings. In addition, we hypothesize that PopP1 can act in a similar manner as PopP2, which belongs to the same family of effectors and targets WRKY transcription factors (Le Roux et al. 2015; Sarris et al. 2015). Our findings did not uncovered IDs that interact with the effectors RipAB and RipF1. An ID that derives from the original target of RipAB may not be included in the list of IDs tested. In the case of RipF1, its suggested function as a translocator could be singular and there is a possibility that this effector can interact with other pathogen effectors, rather than with plant proteins.

Future Work

In order to evaluate our findings and exclude artifacts, it is urgent to determine the subcellular localization of the effectors of interest. The suggested approach is the transient expression of the proteins fused with fluorescent tags in host leaves and the subsequent confocal microscopy observations (Choi et al. 2017). Interactions with possible biological meaning should be then confirmed via other protein-protein interaction testing methods, such as co-immunoprecipitation (co-IP) and Biomolecular Fluorescence Complementation (BioFC). Yeast two-hybrid, co-IP and BioFC could be also used to examine interactions between the effectors of interest and their real

molecular targets, from which the corresponding IDs most likely derived. However, more sophisticated methods could be considered if available. Understanding such interplay on a protein level can give insight to virulence mechanisms of bacterial wilt disease. If an effector's target is determined, any putative enzymatic activity could be proven through the appropriate assays. Our pipeline could be used, along with an extended list of effectors and IDs, in order to better understand bacterial wilt disease or other microbial plant diseases. Finally, we propose that the real molecular targets of effectors could provide novel targets for genome editing approaches, that introduce resistance to susceptible crops.

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Supplementary

Table S 1: Culture media used to grow microorganisms in this study. For solid media a final concentration of agar 15g/L was added before autoclaving.

Culture media	Components	Final concentration
LB	NaCl	10 g/L
	Yeast extract	5 g/L
	Tryptone	10 g/L
NA (nutrient agar)	NaCl	5 g/L
	Yeast extract	3 g/L
	Peptone	5 g/L
YPDA (rich medium)	Bacto-Yeast extract	1%
	Bacto-peptone	2%
	Glucose	2%
	Adenine	30mg/L
SDC (minimum medium)	CSM (-His-Leu-Trp-Ade)	0.06%
	YNB	0.67%
	Glucose	2%
	Amino Acids (100x) *(100x concentrated Histidine is 2mg/mL in dH_2O, 100x concentrated Adenine is 4mg/ml in HCl 0.5M)*	1x

Table S 2: The concentrations of antibiotics and components of the blue-white selection, that were used in this study.

Antibiotics	Final concentration
Ampicillin	100µg/ml
Kanamycin	50µg/ml
Blue-white screening components	**Final concentration**
x-GAL	80µg/ml
IPTG	0.5mM

Table S 3: Solutions that were used for competent *E. coli* preparation in this study.

Protocol	Solution name & pH	Components	Component concentration
Competent *E. coli* cells preparation	TFBI *pH=5.8*	CH_3COOK RbCl $MnCl_2$▪$4H_2O$ $CaCl_2$▪$2H_2O$ Glycerol	30 mM 100 mM 50 mM 10 mM 15%
	TFBII *pH=6.5*	MOPS CaCl2 RbCl Glycerol	10 mM 10 mM 10 mM 15%

Table S 4: List of all the primers that were used in this study.

Name	Length (bp)	Sequence (5' --> 3')	Tm (°C)	Purpose
ripAB-FW1	35	aaGGTCTCa**AATG**AGCCACAGCAAAATCAAGGCTG	**70**	Insert BsaI 5' flanking site to RipAB effector of *R.solanacearum*
ripAB-REV1	30	aaGGTCTCa**GAGC**GA**a**ACCATCGTGCTGCC	**72**	Eliminate internal BsaI site from RipAB effector of *R.solanacearum*
ripAB-FW2	29	aaGGTCTCa**GCTC**AAGTCGCGCTCGTCGT	**73**	Eliminate internal BsaI site from RipAB effector of *R.solanacearum*
ripAB-REV2	27	aaGGTCTCa**CTGA**GGATCCGGCCGAGC	**71**	Eliminate internal BsaI site from RipAB effector of *R.solanacearum*
ripAB-FW3	34	aaGGTCTCa**TCAGG***t*TCTCCGGAAGACGCGCTGA	**76**	Eliminate internal BsaI site from RipAB effector of *R.solanacearum*
ripAB-REV3	41	aaGGTCTCa**AAGC**TCAGTCGTCGTCGTCCTTGATCTTCATC	**71**	Insert BsaI 5' flanking site to RipAB effector of *R.solanacearum*
ripAR-FW1	28	aaGGTCTCa**AATG**CCGCCTCCCATCCGG	**74**	Insert BsaI 5' flanking site to RipAR effector of *R.solanacearum*
ripAR-REV1	27	aaGGTCTCa**GCCT**CGGTCT*t*GACGGCA	**71**	Eliminate internal BsaI site from RipAR effector of *R.solanacearum*
ripAR-FW2	26	aaGGTCTCa**AGGC**TGCAACCGCCTCA	**71**	Eliminate internal BsaI site from RipAR effector of *R.solanacearum*
ripAR-REV2	31	aaGGTCTCa**AAGC**TCAGCCGTGGTCCGTCGG	**74**	Insert BsaI 5' flanking site to RipAR effector of *R.solanacearum*
ripF1-FW	36	aaGGTCTCa**AATG**AGTACCAACATCTCTAGCGCAGC	**70**	Insert BsaI 5' flanking site to RipF1 effector of *R.solanacearum*
ripF1-REV	27	taGGTCTCa**AAGC**TCAGGCCGCCGCCG	**74**	Insert BsaI 5' flanking site to RipF1 effector of *R.solanacearum*

ripE1-FW	27	aaGGTCTCa**AATG**CCGCCCGTCCTGCC	**76**	Insert BsaI 5' flanking site to RipE1 effector of *R.solanacearum*
ripE1-REV	31	aaGGTCTCa**AAGC**TCAGCTTTCCGTGGCGGG	**71**	Insert BsaI 5' flanking site to RipE1 effector of *R.solanacearum*
ripW-FW	31	aaGGTCTCc**AATG**TCCATCCAGATTGATCGC	**65**	Insert BsaI 5' flanking site to RipW effector of *R.solanacearum*
ripW-REV	34	aaGGTCTCa**AAGC**TCAGCCCGAGTAGGCCTTGTA	**70**	Insert BsaI 5' flanking site to RipW effector of *R.solanacearum*
popP1-FW	38	aaGGTCTCa**AATG**AAAAGACTATTCAGAGCATTGGGCG	**69**	Insert BsaI 5' flanking site to PopP1 effector of *R.solanacearum*
popP1-REV	37	aaGGTCTCa**AAGC**TCACGACTCCAGGGCATGTCGAAT	**73**	Insert BsaI 5' flanking site to PopP1 effector of *R.solanacearum*
ripF1-endog	18	TGTTCTTCGCCATCGGCT	**67**	Endogenous primer for pBLSK::ripF1 sequencing
Universal primer T3	17	ATTAACCCTCACTAAAG		Macrogen Incorporation and Eurofin Genomics Sequencing services
Universal primer T7promoter	20	TAATACGACTCACTATAGGG		Macrogen Incorporation and Eurofin Genomics Sequencing services

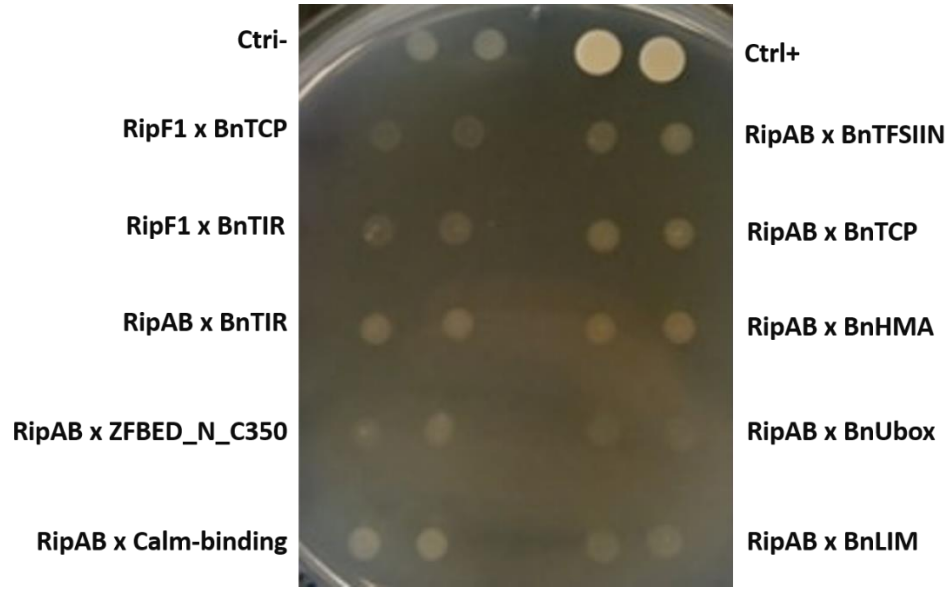

Figure S 1: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. No interactions were detected between IDs tested and the effectors RipAB, RipF1.

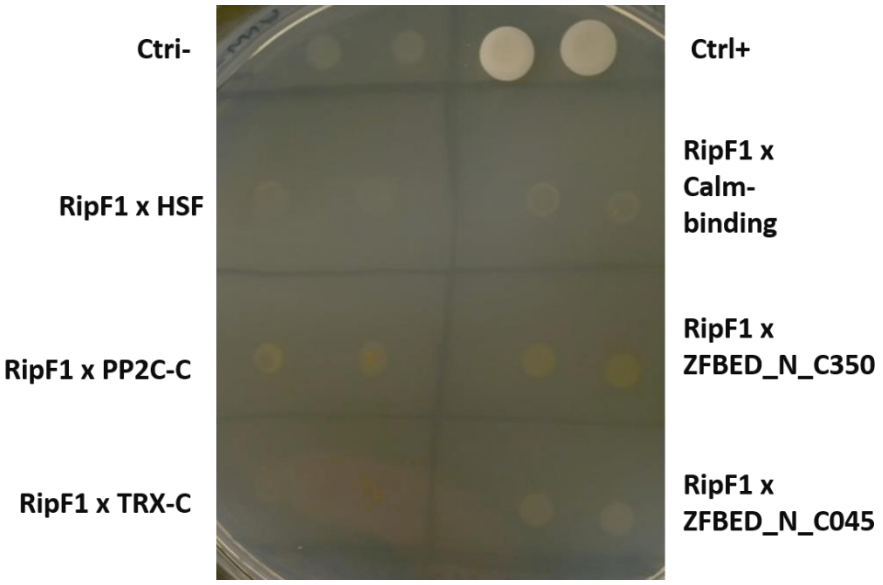

Figure S 2: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. No interactions were detected between IDs tested and the effector RipF1.

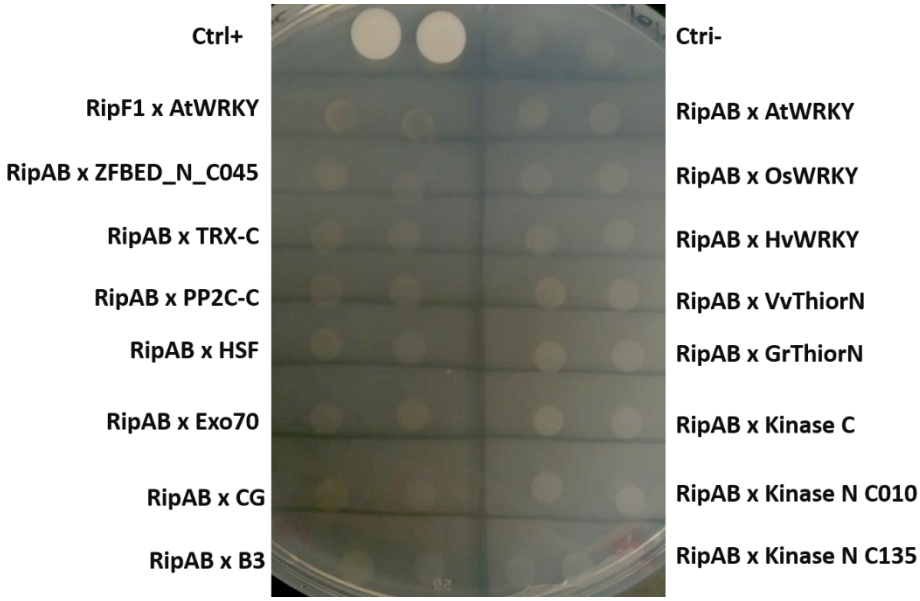

Figure S 3: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. No interactions were detected between IDs tested and the effectors RipAB, RipF1.

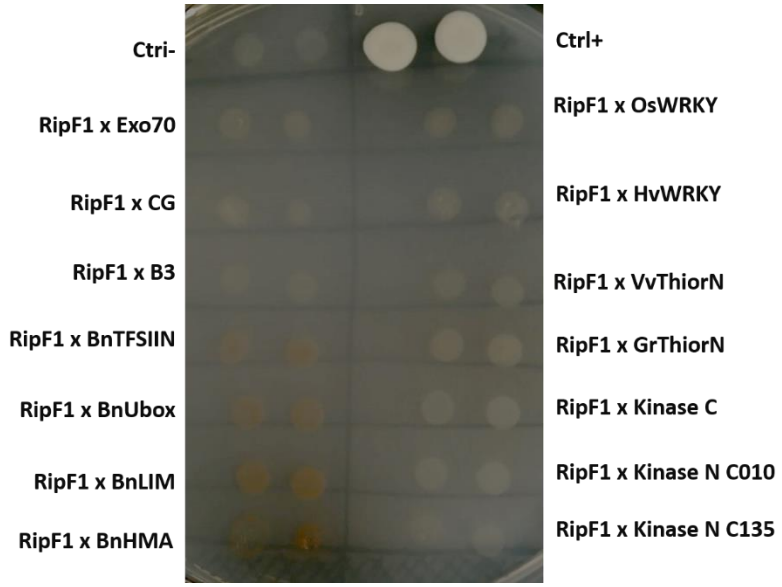

Figure S 4: *Saccharomyces cerevisiae* PJ69 4A cells were transformed for each interaction tested with both plasmids that are appropriately described in Table 5 and were grown in culture media in the lack of leucine, tryptophan and adenine. Cells transformed with the plasmids not containing effector or ID, respectively, were used as the negative control Ctrl-. Cells transformed with the plasmids containing the effector AvrRPS4 and the *Arabidopsis* WRKY ID, respectively, were used as the positive control Ctrl+. No interactions were detected between IDs tested and the effector RipF1.