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"Investigation for the detection of putative intracellular targets of *Shigella*."

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HERAKLION

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To my family

“Investigation for the detection of putative intracellular targets of *Shigella*.”
Master Thesis

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Contents

ABSTRACT

Shigella is a dangerous primate-restricted pathogen strongly linked with worldwide diarrhea-associated mortality. Its growing development of antibiotic resistance and the lack of an effective vaccine constitutes a health issue, especially for developing countries. *Shigella* infects its hosts by injecting a suite of effector proteins into the human colonic epithelial cells, causing the shigellosis disease. A better understanding of the *Shigella* pathogenicity will aid the fight against shigellosis. Despite *Shigella* being a human pathogen, it recently became evident that it can infect and proliferate in *Arabidopsis thaliana,* employing effectors used for pathogenesis in humans. That indicates that *Shigella* interacts with both a human and a plant host. In this thesis, we sought to elucidate and highlight the presence of common strategies and principles governing the host-pathogen interactions between plants and animals. In a cross-kingdom approach, we attempted to check whether the integrated decoys of the cytoplasmic nucleotide-binding leucine-rich repeat (NLR) receptors of the plant innate immune system can recognize and interact with the effector proteins of the highly specialized human pathogen *Shigella*. We employed a yeast two-hybrid system in which twelve *Shigella* effector proteins were individually coexpressed with each of twenty-three plant NLR integrated decoys (IDs) in yeast cells (*Saccharomyces cerevisiae*). With the yeast two-hybrid screenings, we tried to detect interactions between these effector proteins and IDs. The transformation of yeast cells with the *Shigella* effector proteins showed that OspF, VirA, IpgD, IpaJ, and OspC1 are toxic upon expression in yeast leading to growth inhibition. A mild autoactivation ability was observed during the yeast two-hybrid screenings for the EXO70 ID and a stronger one for the WRKY_Barley of the *Hordeum vulgare*. Interestingly, a putative interaction was identified between the effector protein IpaJ and the WRKY_Barley ID. The study of the interplay of plant proteins with the proteins from animal pathogens could improve the understanding of the virulence strategies that animal pathogens employ. Furthermore, this framework can highlight novel targets for the prevention and treatment of the diseases that human pathogens cause. In future experiments, we will further study the IpaJ – WRKY_Barley pair to validate the presence of such an interaction and analyze the putative underlying molecular mechanism. Furthermore, we will focus on *Shigella* effector proteins

that are homologous to effector proteins from plant pathogens, which have known plant protein targets. Finally, we aim to investigate whether such plant proteins can interact with the *Shigella* effectors.

ΠΕΡΙΛΗΨΗ

Η *Shigella* είναι ένα παθογόνο βακτήριο το οποίο προσβάλλει πρωτεύοντα και ευθύνεται παγκοσμίως για μεγάλο ποσοστό θανάτων που σχετίζονται με διάρροια. Τα τελευταία χρόνια, το βακτήριο έχει αναπτύξει ανθεκτικότητα σε πολλά ευρέως χρησιμοποιούμενα αντιβιοτικά, ενώ δεν υπάρχει μέχρι στιγμής κάποιο αποτελεσματικό εμβόλιο για την πρόληψη της νόσου. Αυτό δημιουργεί σοβαρά προβλήματα στην παγκόσμια υγεία, και ιδιαίτερα στις αναπτυσσόμενες χώρες. Για να μπορέσει να μολύνει τα επιθηλιακά κύτταρα του εντέρου και να πολλαπλασιαστεί, η *Shigella* αξιοποιεί ένα σύνολο πρωτεϊνών τελεστών (effector proteins) τις οποίες εγχέει στο κυτταρόπλασμα των κυττάρων μέσω του τύπου ΙΙΙ εκκριτικού συστήματός της. Η καλύτερη κατανόηση των μηχανισμών παθογένειας της *Shigella* είναι απαραίτητη για την ανάπτυξη αποτελεσματικότερων στρατηγικών για την καταπολέμησή της. Παρόλο που η *Shigella* θεωρείται ένα εξειδικευμένο ανθρωποπαθογόνο, πρόσφατα παρατηρήθηκε ότι έχει την ικανότητα να μολύνει και να πολλαπλασιάζεται στο φυτό *Arabidopsis thaliana*, αξιοποιώντας τις ίδιες πρωτεΐνες τελεστές που χρησιμοποιεί για να μολύνει και τα κύτταρα του ανθρώπου. Έτσι, φαίνεται ότι ο τρόπος με τον οποίο δρουν οι πρωτεΐνες τελεστές της *Shigella* για να τροποποιήσουν τις κυτταρικές λειτουργίες προς όφελος του παθογόνου είναι κοινές ανεξάρτητα από τον ξενιστή στον οποίο βρίσκεται. Μέσω αυτής της εργασίας επιδιώκουμε να αναδείξουμε την ύπαρξη βασικών αρχών που διέπουν την αλληλεπίδραση μεταξύ παθογόνου και ξενιστή οι οποίες είναι κοινές μεταξύ φυτών και ζώων. Σε αυτά τα πλαίσια επιχειρήσαμε να ελέγξουμε εάν οι επικράτειες φυτικών κυτταροπλασματικών υποδοχέων NLRs (nucleotide-binding leucine-rich repeat) που χρησιμεύουν ως δολώματα για την ανίχνευση πρωτεϊνών τελεστών φυτικών παθογόνων (επικράτειες integrated decoys, IDs) μπορούν να αλληλεπιδράσουν με πρωτεΐνες τελεστές του ανθρωποπαθογόνου βακτηρίου *Shigella*. Για αυτό το σκοπό αξιοποιήσαμε δοκιμασίες δύο υβριδίων σε κύτταρα ζύμης (*Saccharomyces cerevisiae*) στα οποία συνεκφράστηκαν δώδεκα πρωτεΐνες τελεστές του βακτηρίου *Shigella* και είκοσι τρεις επικράτειες ID που εντοπίζονται σε υποδοχείς NLRs από διαφορετικά είδη φυτών. Αρχικά, παρατηρήθηκε ότι οι πρωτεΐνες τελεστές OspF, VirA, IpgD, IpaJ, και OspC1 της *Shigella* είναι τοξικοί όταν εκφράζονται στη ζύμη και προκαλούν ήπια ή έντονη αναστολή της αύξησης. Επιπλέον,

παρατηρήθηκε ότι η επικράτεια EXO70 ID από το φυτό *Hordeum vulgare* (κριθάρι) μπορούσε σε ορισμένες περιπτώσεις να προκαλεί ήπια αυτοενεργοποίηση της έκφρασης των γονιδίων μαρτύρων, ενώ η επικράτεια WRKY_Barley ID, από το ίδιο φυτό, προκαλούσε πιο έντονη αυτοενεργοποίηση. Μία πιθανή αλληλεπίδραση εντοπίστηκε μεταξύ της επικράτειας WRKY_Barley ID και της πρωτεΐνης τελεστή IpaJ. Η αξιοποίηση των φυτών ως μοντέλα για τη μελέτη της αλληλεπίδρασης μεταξύ πρωτεϊνών ζωικών παθογόνων και φυτικών πρωτεϊνών μπορεί να βελτιώσει την αντίληψή μας για τους μηχανισμούς εμφάνισης και αντιμετώπισης της παθογένειας που προκαλείται από τα ζωικά παθογόνα και να παρέχει νέους στόχους για την πρόληψη και αντιμετώπιση των ασθενειών που προκαλούνται από αυτά. Σε μελλοντικά πειράματα θα διερευνήσουμε περεταίρω την ύπαρξη αλληλεπίδρασης μεταξύ της επικράτειας WRKY_Barley ID και της πρωτεΐνης τελεστή IpaJ. Επιπλέον, θα εστιάσουμε σε πρωτεΐνες τελεστές του βακτηρίου *Shigella* που εμφανίζουν ομολογία με τελεστές φυτοπαθογόνων οι οποίοι στοχεύουν γνωστές φυτικές πρωτεΐνες. Είναι πιθανό αυτές οι φυτικές πρωτεΐνες να μπορούν να αλληλεπιδράσουν και με τους τελεστές τις *Shigella* αναδεικνύοντας την ομοιότητα στον τρόπο αλληλεπίδρασης μεταξύ παθογόνου και ξενιστή.

1) INTRODUCTION

1.1) The innate immune response in plants and animals

The ability of an organism to distinguish itself from no-self is of crucial importance for its survival. This trait emerged early in the evolution of eukaryotes and later gave rise to the immune system of plants and animals (Ausubel, 2005). Although plants do not have circulated cells (e.g., macrophages, lymphocytes) and lymph, like the adaptive immune system of animals, there are many similarities between their innate immune systems (Ausubel, 2005).

The first stage of pathogen recognition involves transmembrane receptors called PRRs (Pathogen Recognition Receptors). These proteins recognize specific microbe or pathogen-associated molecular patterns (MAMPs or PAMPs) found on microbial components (Dangl et al., 2013; Su et al., 2018). Well-studied PAMPs are present on the lipopolysaccharide (LPS), bacterial flagellin, and peptidoglycan (Dangl et al., 2013; Su et al., 2018). The most extensively studied PRRs in animals are the Toll-like receptors (TLRs), which are conserved between mammals and insects (Ausubel, 2005). The activation of TLRs enhances their dimerization and enables signaling pathways involving many proteins, such as adaptor proteins (e.g., MyD88), kinases, and ubiquitin ligases (Ausubel, 2005). These induce the nuclear translocation of transcription factors (e.g. NF-κB) leading to upregulation of genes encoding for antimicrobial peptides and cytokines, and inflammation development (Ausubel, 2005; Puhar and Sansonetti, 2014).

The PRRs of plants work similarly. The binding of the appropriate ligand on the PRR, e.g., the binding of the Flg22 peptide on the FLS2 receptor, induces alterations in their conformation, leading to their activation and heterodimerization with co-receptors (Ausubel, 2005; Dangl et al., 2013; Su et al., 2018). In this form, the intracellular kinase domain of the PRRs phosphorylates intercellular proteins like the receptor-like cytoplasmic kinases (RLCKs) and activates downstream signaling cascades, which culminate in the activation of transcription factors (e.g., WRKY family), changes in gene expression and the activation of the cellular response known as PTI (PAMP-triggered immunity) (**Figure**

1) (Ausubel, 2005; Dangl et al., 2013; Su et al., 2018). Typical means of PTI involves changes in the physicochemical properties of the cell wall (e.g., callose deposition), the induction of antimicrobial substances and phytohormones, and the production of reactive oxygen species (ROS) (Bacete et al., 2018; Su et al., 2018).

The second stage of recognition requires cytoplasmic receptors of the host cells (Dangl et al., 2013). In animals, pathogens engulfed into the host cells can be dissolved into lysosomes or autophagosomes (Puhar and Sansonetti, 2014). Additional signals are secreted by the cell to induce the adaptive immune system and attract macrophages (**Figure 2A**) (Puhar and Sansonetti, 2014). Macrophages can phagocytose pathogens (Puhar and Sansonetti, 2014). In their cytoplasm, receptors called NLRs (Nucleotide-binding leucine-rich repeat proteins) (e.g., NLRC4) can detect conserved damage- or pathogen-related components (Maekawa et al., 2011). NLRs have a tripartite organization involving a variable N-terminal domain, a nucleotide-binding domain (NBD) and a C-terminal domain with leucine-rich repeats (LRRs) (Ausubel, 2005; Mermigka et al., 2020; Saur et al., 2020). When activated, the NLRs cooperate and create an elaborate ring-shaped complex termed inflammasome (Puhar and Sansonetti, 2014; Mermigka et al., 2020; Saur et al., 2020). The recruitment of caspase-1 by the inflammasome causes cell death known as pyroptosis (Puhar and Sansonetti, 2014; Mermigka et al., 2020; Saur et al., 2020). Pyroptosis includes the activation of the gasdermins that open pores on the plasma membrane and the secretion of IL-1 and IL-18, inducing the activation, migration, and proliferation of immune cells (**Figure 2A**) (Puhar and Sansonetti, 2014; Mermigka et al., 2020; Saur et al., 2020).

During the hosts and pathogens coevolution, pathogens evolved effector proteins that surpass and inhibit the aforementioned defense mechanisms (Dangl et al., 2013; Su et al., 2018). Effectors allow pathogens to invade, survive, proliferate, and transmit within the host and between different hosts (**Figure 2B**) (Dangl et al., 2013; Su et al., 2018). In plants, biotrophic pathogens remain in the apoplast for their nutrition and proliferation, and they do not internalize and proliferate into the cytoplasm (Su et al., 2018). However, they inject effector proteins through their secretion systems that manipulate PTI by targeting its pathways, leading to effector-triggered susceptibility (ETS) (Su et al., 2018). To prevent ETS, plants have evolved an additional defense level called effector-triggered immunity (ETI) (**Figure 1**) (Dangl et al., 2013; Su et al., 2018). ETI is supported by specialized

cytoplasmic NLRs, which strongly resemble those of animals. The NLRs of plants contain an N-terminal signaling Coiled-Coil (CC) or Toll-Interleukin 1 (TIR) domain, a nucleotide-binding (NB) domain, and a C-terminal leucine-rich repeat (LRR) domain (Cesari et al., 2014). NLRs can recognize the injected effectors directly through their CC and TIR domains or indirectly by a decoy (Dangl et al., 2013; Cesari et al., 2014; Su et al., 2018). The decoy is a cellular protein mimicking the original host target of the effector. A decoy can be either guarded (guardee) after being modified by a pathogen-associated alteration during the infection or permanently integrated on the NLR protein sequence (integrated decoy – ID) (Cesari et al., 2014; Sarris et al., 2015; Saur et al., 2020). Integrated decoys can interact with both bacterial and fungal effectors (Saur et al., 2020). IDs mimic various host proteins targeted by effectors, especially kinases and transcription factors (e.g., WRKY) (Sarris et al., 2016). An analysis of 40 angiosperm genomes revealed 720 putative NLR IDs, 265 of which were unique in specific plant genomes (Sarris et al., 2016). The detection of the effector proteins of the pathogens by the NLRs triggers their activation (Cesari et al., 2014; Sarris et al., 2015; Mermigka et al., 2020; Saur et al., 2020). The activated NLRs interact with each other and form the resistosome, a supercomplex analogous to the inflammasome (Mermigka et al., 2020; Saur et al., 2020). The resistosome has signaling properties and induces a localized and programmed cell death known as hypersensitivity response (HR), which limits the transmission of the pathogen between cells (Mermigka et al., 2020; Saur et al., 2020).

An aspect of the convergent evolution of plants and animals is the similarity of their cell-death-mediating supercomplexes. The resistosomes of plants and the inflammasomes and apoptosomes of animals are all ring-shaped complexes, with variable oligomerization states that consist of three main layers (e.g., LRR, NOD, and TIR domains for the TIR NLRs) (**Figure 3**) (Mermigka et al., 2020; Saur et al., 2020).

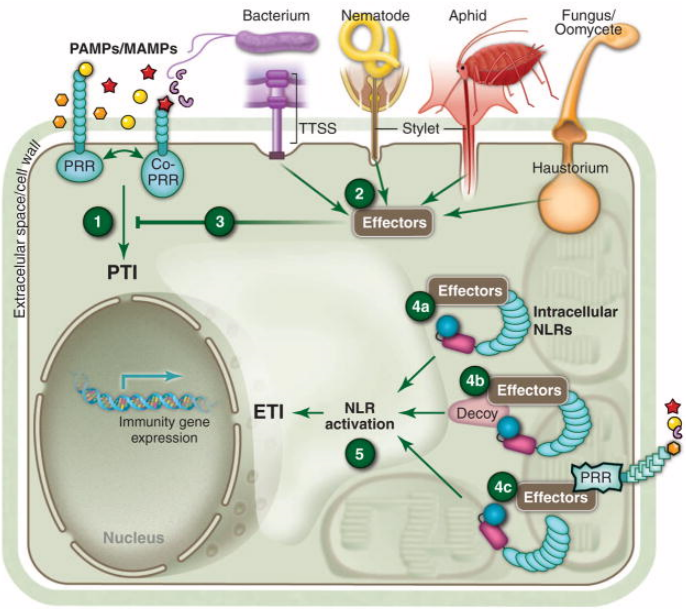

Figure 1. Immune response of plant cells against pathogens (Dangl et al., 2013). The components of plant pathogens contain microbe or pathogen-associated molecular patterns (MAMPs or PAMPs). When recognized by membrane receptors of the plant cells, they can induce an immune response called PAMP-triggered immunity (PTI) (1). The pathogens translocate effector proteins (2) that manipulate the cellular functions and inhibit PTI development (3). Resistant plants encode for intracellular nucleotide-binding leucine-rich repeat receptors (NLRs) that can interact with the effector proteins in three ways: directly with their N-terminal domain (4a), indirectly by guarded or integrated decoy proteins mimicking the real targets of the effector-proteins (4b), and by sensing effector-mediated alteration in host cell components, like the intercellular domain of the pathogen recognition receptors (PRRs) (4c). The interaction between NLRs and effector proteins triggers a response known as effector-triggered immunity (ETI) (5), resulting in a localized and programmed cell death known as hypersensitivity response (HR).

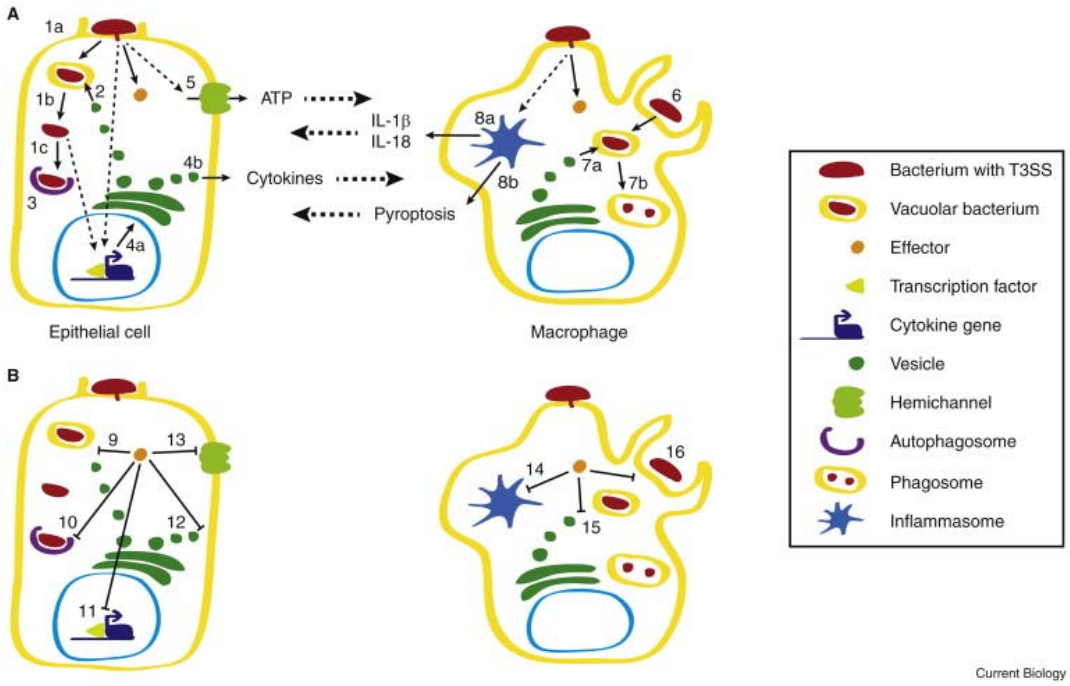

Figure 2. The immune response of mammalian epithelial cells and macrophages against pathogens (Puhar and Sansonetti, 2014). A) The animal pathogens are frequently invading epithelial cells (1a). After their engulfment, they can reside inside the vacuole (1b) or burst out of it (1c). The residing pathogens are degraded after the fusion of vacuoles with lysosomes (2), while the cytoplasmic pathogens are eliminated inside autophagosomes (3). PAMPs and the bacterial T3SS activity can induce signaling cascades (thin dashed arrows), culminating in the nuclear translocation of transcription factors and the expressional upregulation of immune response-associated genes (4a). The response is enhanced by the production and secretion of cytokines (4b), endogenous ATP (5) and other substances with host cell-attracting and alerting properties (thick dashed arrows). Macrophages can phagocytose (6) and digest pathogens (7a,b). The detection of conserved components (e.g., T3SS) (thin dashed arrows) in the cytoplasm of macrophages induces the inflammasome formation, which leads to IL-1b and IL-18 secretion (8a) and pyroptotic cell death (8b), which signals the neighboring epithelial and immune cells (thick dashed arrows). B) The pathogen effector proteins surpass and inhibit the defense mechanisms described above (9-16) and allow the invasion, survival, proliferation, and transmission of the pathogens into the host.

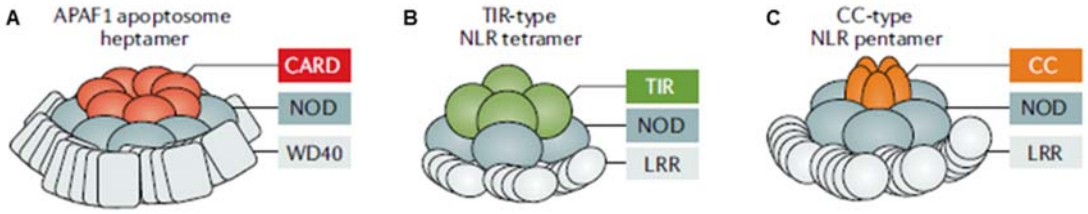

Figure 3. The similarity of the cell-death-mediating supercomplexes of plants and animals. The activated NLRs of animals and plants form ring-shaped complexes, with variable oligomerization states that consist of three main layers. **A**) the apoptosome in animals, **B**) the plant resistosome formed by TIR-type NLRs and **C**) the plant resistosome formed by CC-type NLRs**.** Figure adapted by (Saur et al., 2020).

1.2) The type III secretion system (T3SS)

The type III secretion system (T3SS) is a macromolecular machine of Gram negative bacteria which surpasses the bacterial and host cell membranes and directly delivers effector proteins from bacterium to the host cell (**Figure 4A**) (Puhar and Sansonetti, 2014). Roland Rosqvist and colleagues firstly elucidated its activity in 1994 by studying the translocation of the effector protein YopE (Yersinia outer protein E) by the T3SS of the pathogens *Yersinia pestis*, which causes the plague disease, and *Yersinia pseudotuberculosis*, causing the Far East scarlet-like fever (Rosqvist et al., 1994). T3SS is homologous to the bacterial flagellum and thus, also termed non-flagellar T3SS (NF-T3SS) (Abby and Rocha, 2012). The two complexes diversified when an ancestral motility-dedicated structure lost core elements associated with motility and recruited secretion-associated proteins (e.g., secretins) (Abby and Rocha, 2012). This system is present in a plethora of pathogenic gram-negative animal- and plant-pathogens such as *Shigella*, *Salmonella*, *Escherichia coli*, *Yersinia*, *Bordetella*, *Pseudomonas aeruginosa*, *Chlamydia*, *Burkholderia, Pseudomonas syringae*, *Xanthomonas*, and *Ralstonia* (Coburn et al., 2007; Abby and Rocha, 2012). T3SS targets specific cells, in contrast to toxins which are spread systemically and affect multiple cells irreversibly (Puhar and Sansonetti, 2014). The effectors injected by T3SS have a complicated activity due to synergistic and antagonistic effects. While T3SS is a universal interface that allows cross-kingdom communication

between hosts and bacteria (both pathogenic and beneficial), most studies focus on the harmful interactions (Puhar and Sansonetti, 2014).

T3SS consists of approximately 20 different proteins organized in distinct oligomers (**Figure 4B**) (Burkinshaw and Strynadka, 2014; Diepold and Wagner, 2014). Stimuli from the host environment induce its complete assembly (Burkinshaw and Strynadka, 2014). The complex can be dissected in four essential parts: the basal body, the export apparatus, the needle, and its tip (Burkinshaw and Strynadka, 2014) (**Figure 4**). The basal body comprises the inner membrane ring, the periplasmic domain, and the outer membrane ring (Burkinshaw and Strynadka, 2014). The export apparatus, which is essential for secretion, is localized to the inner membrane (Burkinshaw and Strynadka, 2014; Diepold and Wagner, 2014). It contains five membrane proteins and an ATPase complex (Burkinshaw and Strynadka, 2014; Diepold and Wagner, 2014). The basal body and the export apparatus are initially assembled before the extension of the complex to the extracellular space (Barison et al., 2013; Burkinshaw and Strynadka, 2014; Diepold and Wagner, 2014). The needle creates the passage between bacterium and the host cell (Cornelis, 2006; Burkinshaw and Strynadka, 2014; Diepold and Wagner, 2014). It is longer and thinner in plants to transverse their thick cell wall, and it is called a pilus (Cornelis, 2006). The opening of the tip, known as the translocation pore, is regulated by both internal and external cues, and it controls the delivery of the effector proteins (Wagner et al., 2018). Apart from the structural proteins, the T3SS features non-structural proteins such as translocators and chaperons (Burkinshaw and Strynadka, 2014; Diepold and Wagner, 2014; Wagner et al., 2018). Translocators regulate the transfer of the effector proteins between the parts of the T3SS and eventually into the host-cell cytoplasm (Wagner et al., 2018). Chaperons bound to many of the effectors by recognizing specific signal peptides and accompany them through the passage while retaining them to the unfolded state until entering the host cell (Wagner et al., 2018).

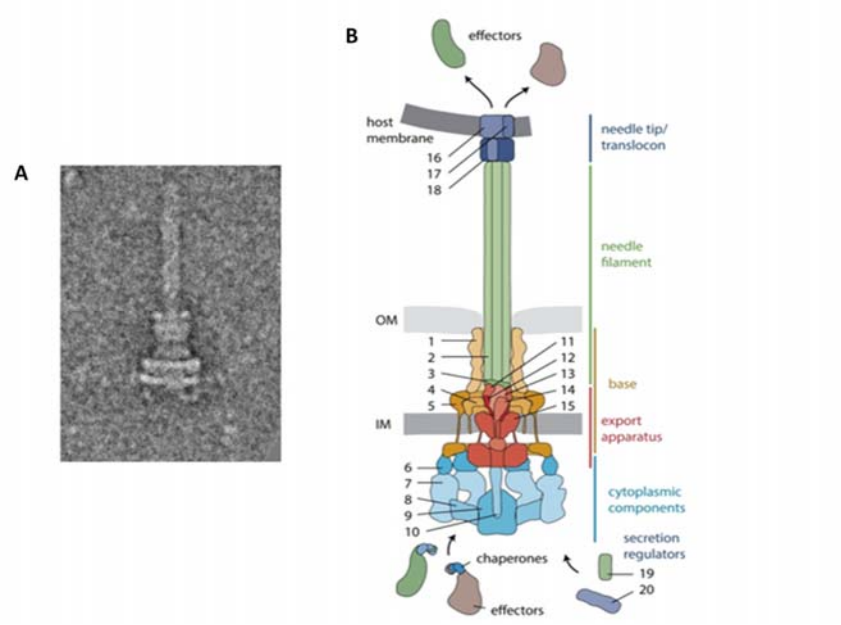

Figure 4. The structure of the Type III Secretion System (T3SS). A) Electron micrograph of *Salmonella* Pathogenicity Island 1 (SPI1) T3SS (Puhar and Sansonetti, 2014). B) The basic structural units and components of the T3SS(Wagner et al., 2018).

1.3) Pathogenicity and virulence of *Shigella*

Shigella is a genus of Gram-negative, facultatively anaerobic, non-motile, non-spore-forming, and rod-shaped bacilli (Hale and Keusch, 1996). *Shigella* is a member of the *Enterobacteriaceae* family and closely related to *Escherichia coli* (Hale and Keusch, 1996; Fukushima et al., 2002; Anderson et al., 2016; Shears, 2017). The genus got its name from Kiyoshi Shiga, a Japanese physician that first isolated *Shigella dysenteriae* from patient feces (Lampel et al., 2018). Dr. Shiga cultured *S. dysenteriae* on an alkaline agar medium and observed its agglutination in the serum of infected individuals during a dysentery outbreak in 1897 (Lampel et al., 2018). In the following years, between 1897 and 1931, three more *Shigellae* species were described: *Shigella flexneri* by Simon Flexner in 1900, *Shigella sonnei* by Carl Sonne in 1915, and *Shigella boydii* by J.S.K. Boyd in 1931 (Anderson et al., 2016; Lampel et al., 2018). Each species, except *Shigella sonnei,* is divided into different serotypes according to their LPS antigenic diversity (Anderson et al., 2016). *S. flexneri* is among the major causes of bacterial dysentery in developing countries

due to the lack of proper water sanitation (Anderson et al., 2016). At the same time, *S. sonnei* is more common in the industrialized world (Anderson et al., 2016). *S. boydii* is geographically restricted in South-East Asia and Bangladesh, and *S. dysenteriae* is responsible for occasional outbreaks (Anderson et al., 2016).

Shigella is a primate-restricted pathogen and mainly infects humans (Lampel et al., 2018). According to recent estimates, *Shigella* is the second leading cause of diarrhea-associated mortality after the rotavirus, with about 200,000 deaths annually (Troeger et al., 2018). Mortality is higher in developing countries, children younger than five, and adults older than 70 years old (Troeger et al., 2018). Even an infectious dose of 10-100 bacteria is sufficient for the infection (Shears, 2017). The subsequent disease is called Shigellosis or bacillary dysentery and is characterized by acute colonic inflammation and death of the intestinal epithelial cells (Shears, 2017; Baker and Chung, 2018). That results in aggressive watery diarrhea with mucus and blood, leading to dehydration and malnutrition (Shears, 2017; Baker and Chung, 2018). A significant complication provoked mainly by the Shiga toxin of *S. dysenteriae* is the hemolytic uremic syndrome (HUS) (Shears, 2017). Other rarer cases are bacteremia and encephalopathy (Shears, 2017; Baker and Chung, 2018).

Shigella infects the colon by invading colonic epithelial cells (**Figure 5**) (Ashida et al., 2009; Anderson et al., 2016; Mattock and Blocker, 2017; Baker and Chung, 2018; Liu et al., 2019). Before the invasion, the bacteria need to surpass two barriers: the microbiota residing on the gut surface and the thick mucus layer coating the gastrointestinal tract (Anderson et al., 2016). *Shigella* genes encode for strain-specific antimicrobial substances called colicins suggested to compete and restrict specific host microbe-populations and mucolytic molecules, enabling its invasion in the lower layer where the epithelial cells are located (Anderson et al., 2016). However, *Shigella* cannot enter into the colonic epithelial cells directly through the apical surface. *Shigella* bacterial cells are initially engulfed by the microfold cells (M-cells) of the colon and then transferred by transcytosis and taken up by the resident macrophages of the gut lymphoid tissue (Ashida et al., 2009; Mattock and Blocker, 2017; Baker and Chung, 2018; Liu et al., 2019). Macrophages internalize the bacteria in phagosomes. *Shigella* overcomes the destruction by disrupting the phagosome. Then, it manipulates the intrinsic cell signaling and induces the death of the macrophage through pyroptosis (Ashida et al., 2009; Mattock and Blocker, 2017; Baker and Chung,

2018; Liu et al., 2019). Effectors of the bacterium can interact with caspase-1 and NLRs (e.g., NLRC4), inducing the formation of the inflammasome (Hermansson et al., 2016).

Additionally, macrophages produce and release proinflammatory cytokines like IL-1β and IL-18 (Ashida et al., 2009; Mattock and Blocker, 2017). Cytokines lead to intense inflammation and enhanced migration of polymorphonuclear (PMN) cells (Ashida et al., 2009; Mattock and Blocker, 2017; Liu et al., 2019). After bursting out of the macrophages, the pathogens invade the epithelial cells through the basolateral surface (Ashida et al., 2009; Mattock and Blocker, 2017; Liu et al., 2019). The penetration of the PMN cells between epithelial cells facilitates the invasion and transmission of the pathogen between cells (Ashida et al., 2009; Mattock and Blocker, 2017; Liu et al., 2019). The IL-8 produced by infected epithelial cells also has PMN cell-attracting abilities (Ashida et al., 2009; Mattock and Blocker, 2017). Actin polymerization control is necessary for directing the engulfment of the pathogen into the epithelial cells (Ashida et al., 2009; Mattock and Blocker, 2017). As in the macrophage, *Shigella* bursts out of the epithelial engulfment (Ashida et al., 2009; Mattock and Blocker, 2017). However, in this case, after avoiding autophagy and lysosomal destruction, it inhibits apoptosis (Ashida et al., 2009; Mattock and Blocker, 2017). Then it starts proliferating in the cytoplasm and disseminating into the neighboring epithelial cells (Ashida et al., 2009; Mattock and Blocker, 2017). Although *Shigella* is non-motile, it can exploit and control the actin network of the host cell for moving inside the cell and between different cells (Ashida et al., 2009; Mattock and Blocker, 2017). During the last stages of its infection cycle, *Shigella* attempts to dampen the host immune response (Ashida et al., 2009; Mattock and Blocker, 2017). However, the already overly-induced inflammation and attraction of PMN cells eventually eliminate the *Shigella* infection in most patients despite aiding its transmission in the first stages (Ashida et al., 2009; Mattock and Blocker, 2017).

The pathogenicity toolbox of *Shigella* is expressed by a 220 Kb virulence plasmid (Ashida et al., 2009; Mattock and Blocker, 2017; Baker and Chung, 2018; Liu et al., 2019). The virulence plasmid possesses approximately 50-60 virulence genes encoding for the T3SS components, effector proteins, chaperons, and regulators (Ashida et al., 2009; Mattock and Blocker, 2017; Baker and Chung, 2018). The circular bacterial chromosome also carries some infection-related genes, encoding for siderophores, toxins, receptors, and

surface antigens, grouped mainly in transferable elements called pathogenicity islands (PAI) (Mattock and Blocker, 2017). Virulence, invasion, T3SS assembly, and effector translocation are induced at 37°C (the human host temperature) and inhibited at a lower temperature (extrahost temperature) (Maurelli et al., 1984). The organic compound Congo red also has an inducing effect on the virulence of the pathogen (Parsot et al., 1995). Attachment to the host cell membrane is further required for the infection *in vivo*. Upon contact with host colonic epithelial cells, the invasion plasmid antigen B (IpaB) protein of the T3SS interacts with cholesterol-rich domains on the membrane. Henceforth secretion of bacterial effector proteins is induced (Enninga et al., 2005). As *Shigella flexneri* invades the cell, the transcriptional regulator virulence factor F (VirF) is expressed and activates the expression of virulence factor B (VirB), which is responsible for the transcriptional upregulation of virulence-associated genes (T3SS machinery and some secreted proteins) (Mavris et al., 2002a; Torres, 2004; Gall et al., 2005). The AraC family transcriptional activator MxiE [membrane expression of Ipa (Invasion plasmid antigen) E] interacts with its coactivator invasion plasmid gene C (IpgC). It is proposed that MxiE binds to a specific conserved motif (MxiE box) on the promoters of some effector-coding genes such as *virA*, *ospC1,* and *ipaH9.8*, inducing their expression (Mavris et al., 2002b; Gall et al., 2005; Bongrand et al., 2012). The effector proteins produced by these genes are then translocated via the T3SS into the host cell cytoplasm.

Understanding the mechanisms controlling the pathogenicity and virulence of *Shigella* had a significant contribution in eliminating its influence on global health (Lampel et al., 2018). The improvements in hygiene and sanitation and the development of antibiotics between the 20th and 21st centuries significantly reduced dysentery incidences (Lampel et al., 2018). However, the selection pressure under various antibiotics such as ciprofloxacin and the last-resort compound azithromycin combined with horizontal resistance-gene transfer between bacteria has led to a global spread of multiresistant *Shigella* strains (Nüesch-inderbinen et al., 2016; Lampel et al., 2018; Ahmad et al., 2021). The development of broad-acting and highly protective vaccines against immunogenic proteins conserved among serotypes (e.g., Ipa proteins, VirG) is a priority in the fight against infection (Camacho et al., 2013).

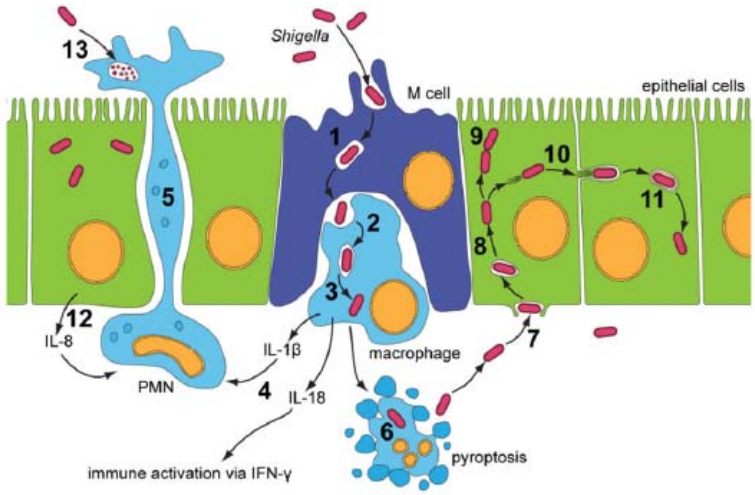

Figure 5. The infectious cycle of *Shigella* (Mattock and Blocker, 2017)**.** *Shigella* cells are initially engulfed by the epithelial M-cells through membrane ruffling (1), transferred to the M-cell pocket at the basal epithelial surface, and endocytosed by the resident colonic macrophages (2). After the phagosome disruption (3), the bacterial cells manipulate the host-cell signaling pathways inducing pyroptotic cell death (6) and release of the pro-inflammatory cytokines IL-1β and IL-18 (4). The cytokines attract PMN cells that traverse the epithelium and facilitate the pathogen's basolateral invasion (5). *Shigella* manipulates the host-cell actin network and guides the formation of membrane protrusions, enabling its engulfment by the epithelial cells (7). Then it disrupts the engulfment (8), hijacks the physiological cellular processes, and proliferates (9). Furthermore, it directs the polymerization of actin, enabling intracellular transport and intercellular transmission (10). Again, after the engulfment into the neighboring cells, the vacuole disruption is induced (11). The infected cells emit the PMN-attracting cytokine IL-8 (12). Eventually, PMN cells eliminate the infection of the colonic epithelium (13).

1.4) The virulence strategies of pathogens can be universal and host-independent

Pathogens can infect a wide range of different hosts (generalists) or a single host species (specialists) (Bäumler and Fang, 2013). The ability of a pathogen to adhere, invade and proliferate in the host cells and get efficiently transmitted to new hosts determine the specificity of a pathogen and restricts it to a single or a number of hosts (Bäumler and Fang, 2013). However, new data indicate the conservation of the mechanisms leading to pathogenesis and the ability of otherwise specialized pathogens to invade and proliferate

in a broader range of hosts (Schikora et al., 2008; Üstün et al., 2012; Bhavsar et al., 2013; Neumann et al., 2014; Jo et al., 2019).

Recently, *Shigella* was found to infect a plant host (Jo et al., 2019). *Shigella* could enter and colonize *Arabidopsis thaliana* leaves and roots, leading to symptom development in a strain-dependent manner (Jo et al., 2019). The colonization of the plant tissues and the proliferation of the bacterial cells require T3SS and virulence genes involved in human pathogenicity (Jo et al., 2019). The translocation of effectors into plant cells inactivated mitogen-activated protein kinases (MAPKs) and suppressed the plant immune response (Jo et al., 2019). Interestingly, the effectors OspF (outer *Shigella* protein F) and OspG (outer *Shigella* protein G) adopt a subcellular localization similar to the one in animal cells (Jo et al., 2019). OspF has a nuclear localization and reduces the phosphorylation of MAPKs, while OspG has nuclear and cytosolic localization, and its target in plant cells is not identified yet (Jo et al., 2019).

This cross-kingdom infection ability was also observed in other bacterial species apart from *Shigella*. *Pseudomonas aeruginosa* is a species of gram-negative, opportunistic, and biofilm-forming pathogenic bacillus (Alhazmi, 2015; Azam and Khan, 2019). It affects mainly immune-compromised patients and patients with cystic fibrosis (CF), and its multi-resistance against many antibiotics has caused a growing global health problem (Alhazmi, 2015; Azam and Khan, 2019). The *P. aeruginosa* strain PA14 was found to infect both *Arabidopsis thaliana* and *Mus musculus* (mouse) using the same arsenal of virulence genes (Rahme et al., 1995, 2000; Baldini et al., 2014). Later studies elucidated a more extensive list of distant evolutionary hosts, including *Lactuca sativa* (lettuce) (Rahme et al., 2000; Baldini et al., 2002), *Drosophila melanogaster* (Baldini et al., 2002), *Galleria mellonella* (greater wax moth) (Baldini et al., 2002), *Caenorhabditis elegans* (Rahme et al., 2000), *Danio rerio* (zebrafish) (Llamas and van der Sar, 2014), and *Dictyostelium discoideum* (slime mold) (Filion and Charette, 2014). However, the genes identified as essential for the pathogenesis in both plants and mammals have been associated with fundamental virulence processes such as transcriptional and post-transcriptional regulation, and secretion of exotoxins (Rahme et al., 1995, 2000). The effector proteins, which are injected by the bacterial secretion systems directly into the cytoplasm of host cells and possess a more

specialized activity, constitute a very promising course for the investigation of common or conserved virulence strategies.

Contrary to the mainstream idea that *Salmonella* pathogenic potential is limited between its main hosts, a study revealed that *Salmonella typhimurium* was able to infect and proliferate in *Arabidopsis thaliana* tissues and even get internalized in plant cells (Schikora et al., 2008). *Salmonella* is a genus of gram-negative, facultatively anaerobic, flagellated, and motile bacilli of the family Enterobacteriaceae (Giannella, 1996). *Salmonella enterica* subspecies infect and colonize an extensive list of hosts in Amniotes (reptiles, birds, and mammals) (Lamas et al., 2018). *Salmonella enterica* subsp. *enterica* is responsible for gastrointestinal infections in mammalian hosts, such as humans, livestock and other warm-blooded hosts (Lamas et al., 2018). *Salmonella enterica* subsp. *enterica* uses a set of effector proteins translocated by its T3SS into the host-cells, in order to hijack physiological processes of the host and allow for colonization, cytoplasmic internalization into vacuoles, intake of nutrients, growth, replication, and dissemination (Figueira and Holden, 2012; Ramos-Morales, 2012; Jennings et al., 2017). The *Salmonella* infection of *Arabidopsis thaliana* led to the development of symptoms, induction of jasmonic acid and immune response pathways, and transcriptional upregulation of pathogenesis-related genes (Schikora et al., 2008). The immune response pathways induced were associated with the mitogen-activated protein kinase kinase 3 (MKK3) and mitogen-activated protein kinase 6 (MPK6) (MKK3/MPK6 pathways) (Schikora et al., 2008). Further studies showed that the phosphothreonine lyase SpvC (*Salmonella* plasmid virulence C), a *Salmonella* effector protein, can attenuate the PTI mechanism via dephosphorylation and inactivation of the MPK6 protein of *Arabidopsis thaliana* (Neumann et al., 2014). SpvC is even essential for infection and proliferation of the pathogen in plants (Neumann et al., 2014). Another study proved that the E3 ubiquitin ligase effector protein SspH2 (*Salmonella* secreted protein H2) interacts with the NLR co-chaperone SGT1 (Suppressor of G2 allele of SKP1) and enhances innate immune response in both human cells and *Arabidopsis thaliana* (Bhavsar et al., 2013). The *Salmonella enterica* effector SseF (Secretion system effector F) can also interact with the plant immune system, triggering SGT1-mediated HR-like symptoms when expressed or translocated in *Arabidopsis thaliana* leaves (Üstün et al., 2012).

The conservation of the virulence strategies of pathogens and the innate immune response of the host enables cross-kingdom approaches in the study of host-pathogen interactions. The genetic tractability of plants, their fast generation time, ease of handling, low maintenance cost, and fewer ethical concerns render them an attractive model for such studies (Baldini et al., 2002).

1.5) Perspective and research hypothesis

The newly described ability of *Shigella* to infect the plant model *Arabidopsis thaliana* by using its T3SS to inject and recruit specific effector proteins manipulating the plant cells (Jo et al., 2019) allows the study of common cross-kingdom principles that govern the host-pathogen interactions. Furthermore, the effector proteins injected by the T3SS of phytopathogens within plant cells result in the suppression of the host plant innate immune response and facilitate the proliferation and spread of the pathogens (Dangl et al., 2013). As a response to the effector proteins of the phytopathogens, plants have evolved an additional level of defense: plant protein domains that mimic the targets of specific pathogen effector proteins are fused with some of the plant cytoplasmic NLR receptors (Dangl et al., 2013; Sarris et al., 2015, 2016). These integrated domains (IDs) serve as decoys for detecting the effectors and activating localized cell death, eliminating the proliferation and spread of the pathogens (Dangl et al., 2013).

In this study, using the above paradigms, we sought to highlight the presence of similar strategies in perception and interaction with pathogens between plants and animals by employing a yeast two-hybrid screening assay. We selected to clone twelve effector proteins of *Shigella sonnei* (**Table 1**) to be expressed in yeast cells and tested for interaction, through yeast two-hybrid screenings, with a list of twenty-three cloned IDs from angiosperm plant species (**Table 2**). *Shigella sonnei* has been demonstrated to proliferate more intensely in *A. thaliana* in contrast to other *Shigella* species and strains (Jo et al., 2019). The selection of these twelve effector proteins was based on their similarities to effector proteins from phytopathogens (e.g., IpaH1.4, OspF), functions of interest (e.g., targeting kinases and exocytosis), and limited knowledge regarding their targets in humans. The selected IDs resemble domains of functional plant proteins. They are present in plant

NLRs and serve as decoys for the detection of effector proteins of plant pathogens (Sarris et al., 2016). Apart from the detection of any interaction and the elucidation of the common strategies of the innate immunity, this project seeks to show that plants are plausible alternative models for providing new insights into the function of effector proteins of animal pathogens like *Shigella*, and can even bring to light new putative targets in animals based on their interaction with proteins from plants.

Table 1. The twelve *Shigella sonnei* effector proteins we selected for our experiments, their enzyme activity, and their target and function. The table is based on Mattock and Blocker (2017).

Effectors	**Enzyme Activity**	**Target and Function**
IpaH1.4	E3 ubiquitin ligase	Ubiquitination of HOIP (HOIL-1-interacting protein) (de Jong et al., 2016; Bastedo et al., 2019)
OspF	Phosphothreonine lyase	Dephosphorylation of MAPKs
OspG	Kinase	Inhibition of $SCF^{\beta-TrCP}$
VirA	GTPase-activating protein (GAP)	Inactivation of Rab proteins
IpaJ	Cysteine protease	Cleavage of ARF1-GTP
IpgB1	Guanine nucleotide exchange factor (GEF)	Activation of Rac1 and Cdc42
IpgD	Inositol 4-phosphatase	Conversion of PtdIns(4,5)P_2 to PtdIns(5)P
IpaH7.8	E3 ubiquitin ligase	Ubiquitination of glomulin
OspB	unspecified	Phosphorylation of ERK and p38 MAPK
OspC1	unspecified	Phosphorylation of ERK
OspZ	unspecified	Blocking of p65 nuclear translocation
IpaH3	E3 ubiquitin ligase (Zhu et al., 2008)	unspecified target

Table 2. The conserved plant NLR IDs used, the plant species from which they are originated and the function attributed to the domains they resemble.

Integrated Decoys	Origin	Functional resemblance
1. B3	*Hordeum vulgare*	A highly conserved DNA binding domain found exclusively in transcription factors of plants (Yamasaki et al., 2004).
2. CG	*Hordeum vulgare*	CG-1 domains are DNA binding domains found in the CAMTA (Calmodulin -binding Transcription Activator) transcription factors (Bouché et al., 2002).
3. EXO70	*Hordeum vulgare*	The EXO70 is one subunit of the vesicle-tethering complex exocyst, required to target the complex to the membrane (Sekereš et al., 2017).
4. HSF	*Hordeum vulgare*	The heat shock factor (HSF) is a transcriptional activator of heat shock genes (Clos et al., 1990).
5. Kinase C 6. Kinase N_C010 7. Kinase N_C153	*Hordeum vulgare*	Protein kinases catalyze the covalent attachment of phosphate groups on target proteins (Newton, 1995). PKC (Protein Kinase C) is a family of serine/threonine protein kinases (Newton, 1995). Thus, it controls other proteins' function through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on them (Newton, 1995).
8. PP2C	*Hordeum vulgare*	Protein phosphatase 2C (PP2C) is a class of serine/threonine protein phosphatases (Wenk et al., 1992). The removal of phosphate groups from various proteins

		alters many cellular signaling pathways (Wenk et al., 1992).
9. TRX 10. VvThiorN 11. GrThiorN	*Hordeum vulgare* *Vitis vinifera* *Gossypium raimondii*	Thioredoxins (TRX) are ubiquitous small disulfide-containing disulfide oxidoreductases (Holmgren, 1985).
12. WRKY_Barley 13. WRKY-RPR1-O.sativa 14. WS2 WRKY 1	*Hordeum vulgare* *Oryza sativa* *Arabidopsis thaliana*	The WRKY domain is the DNA binding domain of the WRKY superfamily of transcription factors found exclusively in plants (Eulgem et al., 2000).
15. ZFBED_N_C045 16. ZFBED_N_350	*Hordeum vulgare*	BED (BEAF and DREF)-type zinc finger (ZF) domain is a small zinc-finger forming DNA binding domain found in regulatory factors and transposases of animals, plants, and fungi (Aravind, 2000).
17. HMA	*Brassica napus*	HMA (Heavy-metal-associated) domain is a conserved heavy metal transport or detoxification-associated protein domain (Bull and Cox, 1994; Fukuoka et al., 2009; Maqbool et al., 2015). Such domains can also serve as effector protein targets and decoys (Bull and Cox, 1994; Fukuoka et al., 2009; Maqbool et al., 2015).
18. TCP	*Brassica napus*	TCP (TB1, CYC, and PCFs) domain serves in DNA binding and protein-protein interaction, and it is found in plants (Cubas et al., 1999).

19. LIM	*Brassica napus*	LIM (LIN-11, Isl-1, and MEC-3) domains mediate protein-protein interactions necessary for cellular processes like cytoskeletal organization and plant cell development (Kadrmas and Beckerle, 2004).
20. Ubox	*Brassica napus*	The U-box domain consists of ~70 amino acids (Hatakeyama et al., 2001). It is conserved from yeast to humans and catalyzes the ubiquitination of itself and heterologous substrates (Hatakeyama et al., 2001).
21. TFSIIN	*Brassica napus*	Transcription factor S-II domain of the N-terminal region of transcription elongation factor S-II (TFSIIN) interacts with the RNA polymerase II and ensures efficient transcription elongation (Kim et al., 2007).
22. ZnFC3H1	*Brassica napus*	ZnFC3H1 domain is a zinc finger (ZnF) domain (Klug, 1999). Such domains are relatively small, bind zinc or other metals, and endow proteins with DNA binding specificities (Klug, 1999).
23. TIR	*Brassica napus*	TIR (Toll - interleukin 1 – resistance) domain is an intracellular signaling domain found in plants and animals (Burch-Smith and Dinesh-Kumar, 2007; O'Neill and Bowie, 2007). It mediates protein-protein interactions, and it is associated with host defense and resistance to disease (Burch-Smith and Dinesh-Kumar, 2007; O'Neill and Bowie, 2007).

2) MATERIALS AND METHODS

2.1) Culture of bacterial cells

Escherichia coli DH10B or Stellar™ cells were cultured in Lysogeny Broth (LB) (10 g tryptone, 5 g yeast extract, 10 g NaCl). Ampicillin (100 μg/mL) was added for selection of the vectors pBluescript II SK(-) phagemid and pGADT7-RFP, and kanamycin (50 μg/μL) was added for selection of the pGBKT7-RFP vector after autoclaving. Solid media were formed by adding a final concentration of agar 15 g/L before autoclaving. The empty pBluescript II SK (-) phagemid vector carries the coding sequence of the *lacZ* gene encoding for the enzyme β-galactosidase leading to the appearance of the transformed *E. coli* colonies with blue color in the presence of the organic compound X-gal. On the other hand, when an insert is integrated into this vector, it disrupts the coding sequence of the *lacZ* gene and the expression of the β-galactosidase, leading to the appearance of white colonies and enabling the picking of colonies carrying the desired construct by blue-white selection on solid media. For the blue-white selection, 80 μg/mL X-gal and 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) were added in LB with agar after autoclaving and before the solidification of the medium.

2.2) Preparation of chemically competent *E. coli* cells

E. coli DH10B cells were grown on LB plates from a competent cell stock and incubated overnight at 37°C. A single colony was selected, inoculated in liquid LB, and incubated overnight at 37°C, 200 rpm. A volume from the fresh liquid culture was inoculated in a hundred times greater volume of LB without antibiotics and incubated at 37°C, 200 rpm. A photometer (Biochrom WPA CO7000 Medical Colorimeter, Cambridge, United Kingdom) measured the optical density of the culture. When it reached an OD_{590} (optical density at 590 nm) of 0.5-0.6, it divided into equal aliquots. Each aliquot was centrifuged at 2500 rpm, 4°C for 10 min. After discarding the supernatant, the pellet was washed with transformation buffer I (CH_3COOK 30 mM, RbCl 100 mM, $MnCl_2$ 50 mM, $CaCl_2$ 10 mM,

and glycerol 15%, v/v) in a volume equal to 0.3 times the volume of the aliquot (0.3 volume), and kept at 4°C for 30 min. Then, cells were pelleted by centrifugation at 2500 rpm, 4°C for 10 min, and the supernatant was discarded. The pellet got resuspended in 1/25 volume of transformation buffer II (MOPS 10 mM, $CaCl_2$ 75 mM, RbCl 10 mM, and glycerol 15%, v/v). All the above steps were conducted under sterile conditions and at 4°C. The samples were split into aliquots of 100 µL and frozen immediately in liquid nitrogen. Then they were stored at -80°C.

2.3) Plasmid DNA isolation with alkaline lysis

Cultures of *E. coli* in LB were incubated overnight at 37°C, 200 rpm until saturation (concentration more than 10^9 cells/mL). The saturated cultures were centrifuged at 11000 × g, 25°C, for 30 s. The supernatant was discarded, and the pellet was resuspended by vortexing in 100 µL alkaline lysis solution I, pH 8.0 (glucose 50 mM, Tris-HCl 25 mM, EDTA 10 mM, 4°C) per 3 mL of culture. Then, 200 µL of alkaline lysis solution II (NaOH 0.2 N, SDS 1% w/v, 25°C) and 150 µL of alkaline lysis solution III (60 mL potassium acetate 5 M, 11.5 mL glacial acetic acid, 28.5 mL H_2O, 4°C) were added per 3 mL of culture. Each sample was mildly mixed after the addition of solutions II and III. After incubation at 4°C for 3 min, it was centrifuged at 13100 × g for 5 min at 4°C. The supernatant was transferred to a clean tube, and the DNA was precipitated with two volumes of absolute ethanol by shaking, incubating at 25°C for 2 min, and centrifuging at 13100 × g for 15 min. The supernatant was discarded, and the pellet washed with the addition of 500 µL 70% v/v ethanol and centrifuged at 13100 × g for 5 min. Finally, the ethanol was removed, and the pellet was dried and resuspended in 30 µL sterile distilled water (dH_2O).

2.4) Isolation of purified plasmid DNA

Purified plasmid DNA was used for the T tailing of the pBluescript II SK (-) phagemid vector, for sequencing the pBluescript:effector protein-coding sequence constructs and the

Golden Gate cloning procedure. The isolation of purified plasmid DNA was performed with the NucleoSpin® plasmid DNA purification kit (Macherey-Nagel™, Düren, Germany) according to the manufacturer's instructions. The DNA was doubly eluted in 50 µL sterile dH_2O.

2.5) Obtain and dilution of *Shigella sonnei* DNA

Total DNA from the bacterial species *Shigella sonnei* was extracted and sent in dried form by the laboratory of professor Jeong Mee Park (jmpark@kribb.re.kr, Daejeon, South Korea). Before use, it was resuspended in 70 µL sterile dH_2O and mixed well. Incubation at 30°C enhanced the resuspension.

2.6) Primer designs

Gene-specific "Golden Gate" compatible primers were computationally designed (**Table 3**) to be used for amplification of twelve *Shigella sonnei* effector-coding genes. The coding sequences of the effector proteins were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/). The primers were designed to insert 5' BsaI flanking sites on both sides of each effector coding sequence enabling their use for the Golden Gate procedure. The selection of the ideal primer lengths and melting temperatures (Tm) were based on calculations using the New England BioLabs® Tm calculator (https://tmcalculator.neb.com/). The primers were constructed by Macrogen Incorporation (Amsterdam, the Netherlands). The primers were resuspended in sterile dH_2O to a final concentration of 100 µM, according to the manufacturer's instructions.

Table 3. Gene-specific "Golden Gate" compatible primers designed for the amplification of twelve *Shigella sonnei* effector-coding genes.

Effectors	Forward Primer	Length (bp)	Reverse Primer	Length (bp)	Tm (°C)	Product length (bp)
IpaH1.4	TTGGTCTCTAATGATTA AATCAACCAATA	29	TTGGTCTCTAAGCTTAT GCGATATGATTTG	30	49	1751
IpaH7.8	TTGGTCTCTAATGTTCT CTGTAAATAATACAC	32	TTGGTCTCTAAGCTTAT GAATGGTGCAGTC	30	55	1721
OspC1	TTGGTCTCTAATGAATA TATCAGAAACAC	29	TTGGTCTCTAAGCTTAA ATATATTTATTGTCAGAT	35	50	1436
OspF	TTGGTCTCTAATGCCCA TAAAAAAGCC	27	TTGGTCTCTAAGCCTAC TCTATCATCAAACG	31	52	743
OspG	TTGGTCTCTAATGAAAA TAACATCTACCAT	30	TTGGTCTCTAAGCCTAT AAATATTTCCTGTTTAA	34	50	614
OspZ	TTGGTCTCTAATGATTA GTCCCATCAA	27	TTGGTCTCTAAGCTTAA GTAACAGGCATTC	30	51	716
IpaH3	TTGGTCTCTAATGTTAC CGATAAATAATAAC	31	TTGGTCTCTAAGCTTAT GAATGGTGCAGTT	30	52	1730
IpaJ	TTGGTCTCTAATGTCGG AACAACGGAA	27	AAAGCCTCATTAGTTAT AAC	37	58	803
IpgB1	TTGGTCTCTAATGCAAA TTCTAAACAAAAT	30	TTGGTCTCTAAGCTTAA TTTGTATTGCTTTGAC	33	53	650
IpgD	TTGGTCTCTAATGCACA TAACTAATTTGG	29	TTGGTCTCTAAGCTTAT ACAAATGACGAATAC	32	51	1640
OspB	TTGGTCTCTAATGAATT TAGATGGTGT	27	TTGGTCTCTAAGCCTAA TCCAGTTCTTTATT	31	49	890
VirA	TTGGTCTCTAATGCAGA CATCAAACAT	27	TTGGTCTCTAAGCTTAA ACATCAGGAGATATG	32	52	1226

2.7) Amplification of the *Shigella* effector protein-coding genes

For the amplification of the *Shigella* effector protein-coding genes, 30 ng of total DNA from *Shigella sonnei* was mixed with 1× Phusion HF Buffer, 0.5 µM forward primer, 0.5 µM reverse primer, 0.2 mM dNTPs, 0.4 U Phusion™ High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA), and sterile dH_2O up to a total volume of 20 µL. PCR cycling conditions consisted of an initial denaturation at 98°C for 30 s followed by 34 cycles of denaturation at 98°C for 10 s, annealing for 30 s at the melting temperature (Tm) of each primer pair (**Table 3**), and extension at 72°C for 30 s to 1 min, depending on the expected product length (**Table 3**). In detail, for OspF, OspG, OspZ, and IpaJ, the extension time was 20 s. For IpgB1, and OspB the extension time was 30 s, and for IpaH1.4, IpaH7.8, IpaH3, OspC1, IpgD, and VirA, it was 60 s. The extension time is determined as 15-30

s/kb by the manufacturer, using plasmid DNA as a template. Finally, a final extension step was performed at 72°C for 5 min.

2.8) Purification of the PCR products

The total volume of the PCR products was loaded on a 1.4% w/v agarose gel and run for 20 min under 100 V. Gel extraction and purification of the PCR products was performed with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel™, Düren, Germany) according to the manufacturer's instruction. The DNA was eluted twice in 25 μL sterile dH_2O.

2.9) T-tailing of the "Golden Gate" compatible pBluescript II SK(-) phagemid vector

The T-tailed and BsaI compatible pBluescript II SK(-) phagemid vector was prepared based on the T-vector preparation protocol of Zhou and Gomez-Sanchez (Zhou and Gomez-Sanchez, 2000). Initially, for the vector digestion with a blunt-end restriction enzyme, 10 μg of plasmid DNA were mixed with 1× CutSmart® Buffer (NEB, Ipswich, MA, USA), 50 U EcoRV (NEB, Ipswich, MA, USA), and sterile dH_2O up to 50 μL. The digestion reaction was incubated at 37°C for 2 h. The digested vector was purified by the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel™, Düren, Germany) according to the manufacturer's instructions, except for the final elution. For the final elution, the DNA was doubly eluted with sterile dH_2O in a total volume of 86 μL instead of a total volume of 50 μL. For the addition of T-overhangs to the blunt-ended pBluescript II vector, the 86 μL of the pBluescript II vector were mixed with 1× Taq Pol Buffer (Minotech, Heraklion, Greece), 2.5 mM $MgCl_2$, 1 mM dTTP, and 5 U Taq DNA Pol (Minotech, Heraklion, Greece), and incubated at 72°C for 2 h. Purification of the T-tailed pBluescript II vector was performed as previously described, except for the final elution. The T-tailed vector was doubly eluted with sterile dH_2O in a total volume of 44 μL. For the ligation of vectors lacking the T-overhangs, 44 μL of T-tailed pBluescript II vector were mixed with

1× T4 ligase buffer and 400 U T4 DNA ligase (NEB, Ipswich, MA, USA), and incubated overnight at 16°C. The DNA was purified by gel extraction and doubly eluted with sterile dH_2O in a final volume of 30 μL.

2.10) A-tailing of the amplified *Shigella* effector genes

Each amplified *Shigella* effector gene underwent an A-tailing procedure to be integrated into an already T-tailed "Golden Gate" compatible (without BsaI sites) pBluescript II SK(-) phagemid vector before its integration to the Golden Gate compatible pGADT7 vector. The pBluescript vector is used as an intermediate vector carrying the genes of the effector-proteins with the BsaI sites before their integration in the final pGADT7 yeast expression vector.

For each A-tailing reaction, the purified effector-protein genes (25 μL) were mixed with 1× Taq Pol Buffer (Minotech, Heraklion, Greece), 25 nM dATPs, 1 U Taq DNA Pol, and sterile dH_2O up to a total volume of 50 μL and incubated at 72°C for 30 min.

2.11) Ligation of the A-tailed *Shigella* effector genes into T-tailed pBluescript vectors

The mass of insert used for ligation is calculated by the formula below:

$$m_{insert}[ng] = \frac{m_{vector}\,[ng] \times size\ of\ insert\,[bp]}{size\ of\ vector\,[bp]} \times ratio_{\left(\frac{vector}{insert}\right)}$$

For the ligation reactions involving the pBluescript vector, 50 ng of the vector was used. A vector/insert molecular ratio of 1:3 was mixed with 1× T4 DNA Ligase Buffer, 400 U T4 DNA Ligase (NEB, Ipswich, MA, USA), and sterile dH_2O up to a total volume of 20 μL. Then, the samples were incubated at 25°C for 2 h.

2.12) Transformation of chemo-competent *E. coli* cells

A total of 10 μL of the ligation reaction was transformed into chemically competent DH10B cells. After incubation at 4°C for 30 min, the cells were heat-shocked at 42°C for 1 min and returned immediately to 4°C for 1 min. Then, LB was added up to a total volume of 1 mL. After incubation at 37°C with shaking at 200 rpm for 1 h, the cells were centrifuged at 2100 × *g* for 2 min, the supernatant was discarded, and the pellet was resuspended in the remaining solution, plated on solid LB with ampicillin (100 μg/mL) and incubated at 37°C overnight.

2.13) Diagnostic digestions with restriction enzymes

The transformation of the *E. coli* cells and the successful integration of the effector-coding sequences into the pBluescript and pGADT7 vectors were validated with diagnostic digestions. For each digestion, 5 μL of plasmid DNA isolated with alkaline lysis were mixed with 1× K Buffer (Minotech, Heraklion, Greece), 0.1 mg/mL RNase A, 10 U of each enzyme (determined by the plasmid and its insert), and sterile dH_2O up to a total volume of 20 μL. Then, the reaction was incubated at 37°C for 1 h.

2.14) Verification of the pBluescript::*Shigella* effector gene constructs with sequencing

The pBluescript::*Shigella* effector gene constructs were verified with Sanger sequencing. Primer T7 (5'-TAATACGACTCACTATAGGG-3') was used as a forward primer and T3 (5'- ATTAACCCTCACTAAAG-3') as a reverse primer. Sequencing was performed in Macrogen (Amsterdam, the Netherlands).

2.15) Golden Gate cloning

The effector protein-coding sequences were passed from the intermediate pBluescript vector to the final yeast expression vector pGADT7 via the Golden Gate cloning method (Engler et al., 2008; Engler and Marillonnet, 2014). For each Golden Gate cloning reaction, 100 ng of the yeast expression vector pGADT7 and an equimolar amount of each effector protein-coding sequence insert integrated into the pBluescript vector were mixed with 1× T4 ligase buffer, 0.1 mg/mL bovine serum albumin (BSA), 20 U BsaI-HF®v2 (NEB, Ipswich, MA, USA), and 400 U T4 DNA ligase (NEB, Ipswich, MA, USA). The first step of the reaction involved digestion with the BsaI restriction enzyme at 37°C for 3 min followed by ligation of the entry constructs to the recipient vector with the T4 DNA ligase at 16°C for 4 min, both repeated 25 times. The second step required final digestion with BsaI at 37°C for 10 min to redigest and eliminate any empty vector that still carried the BsaI recognition sites. The third was an enzyme inactivation step at 80°C for 5 min. The mass of the insert used in each reaction was calculated based on the formula below:

$$m_{insert}[ng] = \frac{m_{vector}\ [ng]}{size\ of\ vector\ [bp]} \times size\ of\ insert\ [bp]$$

2.16) Yeast strain and vectors

We amplified and integrated the coding sequence of the twelve core effector proteins of *Shigella sonnei* into the pGADT7-RFP yeast expression vector. The effector-coding sequences were conjugated with the GAL4 transcriptional activation domain (AD) coding sequence. The twenty-three conserved plant NLR integrated decoy (ID)-coding sequences were already cloned into pGBKT7-RFP vectors, conjugated with the GAL4 DNA binding domain (BD) coding sequence (Dr. Vasiliki Michalopoulou, Sarris Lab). The chimeric effector and ID proteins expressed into yeast cells functioned as preys and baits, respectively, for the Yeast Two-Hybrid (Y2H) assays. Strain AH109, a derivative of the strain PJ69-2, was used (**Table 4**) (James et al., 1996; Clontech Laboratories, 2007). The AH109 strain has a lesion in URA3, LEU2, HIS3, TRP1, and ADE2 genes and carries three

reporter genes with upstream activating sequences (UAS) and TATA boxes; ADE2, HIS3, and *lacZ* (Clontech Laboratories, 2007). The pGADT7-RFP and pGBKT7-RFP vectors carry the LEU2 and TRP1 genes, respectively. That enables auxotrophic complementation in a minimum growth medium lacking leucine and tryptophan (SDC –LW, transformation-selective medium). The interaction between an effector protein and an ID and the subsequent physical association of the GAL4 AD and BD induce the expression of the reporter genes, like the ADE2. Thus, the screening for putative interactions was based on nutritional selection into a minimum growth medium lacking leucine, tryptophan, and adenine (SDC –LWA, interaction-selective medium).

Table 4. Genotype of the *Saccharomyces cerevisiae* strain AH109 used in this study (Clontech Laboratories, 2007).

Strain	Genotype
AH109	MATa trp1-901, leu2-3, 112, ura3-52, his3-200 gal4Δ, gal80Δ LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3 GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2 URA3::MEL1$_{UAS}$-MEL1 $_{TATA}$-lacZ

2.17) Competent yeast cells preparation and transformation

AH109 yeast cells were grown on solid YPDA medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose, 30 mg/L adenine, 2% w/v agar, pH 6.0-6.5) and incubated at 28°C for 3 days. A single white colony was picked and cultured overnight in 15 mL liquid YPDA medium (without agar) at 28°C, 200 rpm for every series of transformations. This culture was used to prepare a fresh one with OD_{590}=0.2, and a final volume of n x 10 mL, where n the number of the desired transformants. The fresh culture was incubated at 28°C, 200 rpm until the OD_{590} measured by a photometer (Biochrom WPA CO7000 Medical Colorimeter, Cambridge, United Kingdom) reached a range between 0.5 and 0.8. After separating into aliquots, the cells were pelleted with centrifugation at 3000 rpm, 4 min, 4°C. The supernatant was discarded, and the pellet resuspended in an equal volume of

sterilized dH_2O. A second centrifugation was carried out, the supernatant was discarded, and the pellet resuspended in 1 mL LiAc 0.1 M and transferred in sterile 1.5 mL Eppendorf tubes. Cells were then pelleted by centrifugation at 11000 × *g* for 1 min, the supernatant was discarded, and the pellet was resuspended in n x 100 µL LiAc 0.1 M, where n the number of the desired transformants. Then, the mixture was aliquoted in n × 1.5 mL tubes. After a 10-sec spindown, the supernatant was discarded, and 240 µL PEG 50% w/v was added to each one. Subsequently, 36 µL LiAc 1 M and a mixture containing 500 ng of plasmid vectors carrying the bait (pGBKT7 vector containing plant ID domains fused to the GAL4 DNA-binding domain) and prey (pGADT7 vector containing *Shigella sonnei* effectors fused to the GAL4 transcription activation domain), isolated by alkaline lysis, and sterile dH_2O up to 75 µL were added. After mixing well, the samples were incubated at 28°C for 25 min for recovery. The mixture was heatshocked at 42°C for 25 min. The cells were centrifuged at 11000 × *g* for 1 min, the supernatant discarded, and the pellet resuspended in 200 µL sterile dH_2O and plated on solid SDC minimum medium without leucine and tryptophan (SDS –LW) (0.055% w/v CSM (-His –Leu –Trp –Ade –Met –Ura), 0.67% w/v YNB, 2% w/v glucose, 20 mg/L His, 20 mg/L Met, 20 mg/L Ura, 20 mg/L Ade, 2% w/v agar, pH 5.6) (transformation-selective or control medium). The plates were incubated at 28°C for 3 to 10 days, depending on the presence and intensity of growth inhibition due to the *Shigella* effectors. Before proceeding to the yeast two-hybrid assay, the yeast cultures were inoculated on new SDC –LW plates and incubated at 28°C for three days to obtain single colonies.

2.18) Yeast two-hybrid assay (Y2H)

Plates with SDC minimum medium without leucine, tryptophan, and adenine (SDC –LWA) (0.055% CSM (-His –Leu –Trp –Ade –Met –Ura), 0.67% YNB, 2% glucose, 20 mg/L His, 20 mg/L Met, 20 mg/L Ura, 2% agar, pH 5.6) were used as interaction-selective plates, and SDC –LW plates as non-selective control plates. A single transformed colony was picked for each bait-prey combination and diluted in 10 µL or 7 µL sterile dH_2O. A 5 or 3.5 µL drop, was respectively put on both the interaction-selective and the control plate. The process was repeated for another colony for each transformation as a technical

repetition. The plates were incubated at 28°C for 3-10 days and occasionally checked for interaction-induced growth. Yeast cells transformed with the empty pGADT7 and pGBKT7 vectors that should not grow in the absence of adenine were used as negative controls (-Ctrl). Yeast cells transformed with plasmids encoding for bait and prey known to interact, enabling growth in the absence of adenine, was used as positive controls (+Ctrl). In this study, the *Pseudomonas syringae* effector AvrRPS4 encoded by the pGADT7 vector was used as bait, and the *Hordeum vulgare* WKRY ID (WRKY_Barley ID) encoded by the pGBKT7 vector was used as bait. When the growth of yeast cells transformed with plasmids encoding for a specific pair of Shigella effector protein (prey) and plant NLR ID (bait) was observed in the lack of adenine, two additional controls were used. One of the additional controls was yeast cells transformed with the pGADT7::*Shigella* effector protein construct and the empty pGBKT7 vector. The other was yeast cells transformed with the corresponding pGBKT7::ID construct and the empty pGADT7 vector. The additional controls served to rule out any autoactivation incidence.

3) RESULTS

A total of 276 pairs of *Shigella* effector proteins and plant NLR ID domains were screened through yeast two-hybrid experiments for identifying putative interactions between twelve *Shigella* effector proteins (**Table 1**) and 23 plant NLR IDs (**Table 2**).

3.1) Some *Shigella* effector proteins are toxic upon expression in yeast

After yeast transformation, growth inhibition of yeast cells encoding for specific effector proteins was observed, while the yeast cells were grown into minimum growth medium lacking leucine and tryptophan (SDC –LW, transformation-selective or control medium). Specifically, the effector proteins IpaJ, IpgD and VirA caused intense inhibition, while OspF, OspC1, and IpgB1, in some cases, led to intermediate growth implications (image not shown). Interestingly, the delay of the transformants growth that was caused by each effector protein depended upon its coexpression with the different IDs used as baits. For example, OspF coexpressed with either the Kinase N_C153 of the *Hordeum vulgare* or the WS2 WRKY 1 ID of the *Arabidopsis thaliana* elicited a more intense growth inhibition of the transformed yeast cells compared to other IDs coexpressed with the same effector. Similarly, an intense growth inhibition was observed for IpaJ with WRKY_Barley and Kinase N_C153 IDs of the *Hordeum vulgare*. In the latter's case, the growth was highly delayed as yeast colonies appeared ten days after the transformation, instead of three, and were notably smaller than the controls. When these colonies were picked for the yeast two-hybrid assay, they grew neither on the control nor on the interaction-selective plate.

3.2) Screening for interactions between *Shigella* effector protein IpaH1.4 and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector IpaH1.4 with the EXO70 ID of the *Hordeum vulgare* gave signs of growth as tiny dots into minimum growth medium lacking leucine, tryptophan and adenine (SDC –LWA, interaction-selective medium) eight days after the Y2H assay. Additionally, transformants coexpressing IpaH1.4 with the WRKY_Barley ID of the *Hordeum vulgare* showed growth comparable to that of the positive control into the SDC –LWA medium five days after the assay. No significant growth was observed for the transformants carrying the other IpaH1.4-ID combinations (**Figure 6**).

3.3) Evaluation of the autoactivation capability of the EXO70 and WRKY_Barley IDs

To evaluate whether EXO70 and WRKY_Barley IDs were capable of autoactivation and the growth induced by an interaction between the IpaH1.4 effector protein and the IDs, we transformed competent yeast cells with the following four bait-prey combinations for each ID: pGADT7:IpaH1.4 and pGBKT7:ID (EXO70 or WRKY_Barley), pGADT7:IpaH1.4 and empty pGBKT7 vector, empty pGADT7 vector and pGBKT7:ID (EXO70 or WRKY_Barley), and empty pGADT7 and pGBKT7 vectors. After eight days, yeast strains carrying pGBKT7:EXO70 ID with pGADT7:IpaH1.4 and pGBKT7:EXO70 ID with the empty pGADT7 vector appeared to grow as dots on the interaction-selective plate. During the following two days, yeast colonies grew more prominent, and subsequently merged, and got a red hue (**Figure 7**). The red color, apparent in many of the transformed colonies, occurs due to the ADE2 mutation that blocks adenine biosynthesis leading to the accumulation of a red pigment (Ugolini and Bruschi, 1996). Upon transcriptional activation of the reporter ADE2 gene, the metabolic pathway is appropriately conducted, and the yeast patches appear white as usual. After four days, WRKY_Barley ID alone could also permit visible growth that increased by the following

four days (**Figure 8**). The color, in this case, was white, indicating less stress may be due to more intense transcriptional activation of the ADE2 reporter gene. By the above experimental procedures, we concluded that EXO70 has a very mild autoactivation ability that can be visible eight days after the Y2H assay, and WRKY_Barley ID has a stronger autoactivation ability observed four to five days after the assay. The autoactivation of the reporter genes by these IDs can lead to an increased presence of false-positive results.

3.4) Screening for interactions between *Shigella* effector protein IpaJ and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector IpaJ with the Kinase N_C153 ID of the *Hordeum vulgare* showed extreme growth inhibition into the SDC –LW medium after transformation and before the yeast two-hybrid assay. These transformants showed visible growth ten days after the transformation, and the colonies formed were abnormally small compared to the controls. The colonies carrying IpaJ with the Kinase N_C153 ID did not grow either on the control SDC –LW or the interaction-selective SDC-LWA medium during the Y2H assay (**Figure 9**). Interestingly, yeast cells coexpressing the *Shigella* effector IpaJ with the WRKY_Barley ID of the *Hordeum vulgare* had growth comparable to that of the positive control after just two days, unlike the pairs of other effectors, e.g., IpaH1.4, with this ID, where the yeast growth was observed in dots about four days after the assay (**Figure 9**, **Figure 10**). That could indicate a putative interaction between IpaJ and the WRKY_Barley ID. No significant growth was observed for the other IpaJ-ID combinations (**Figure 9**).

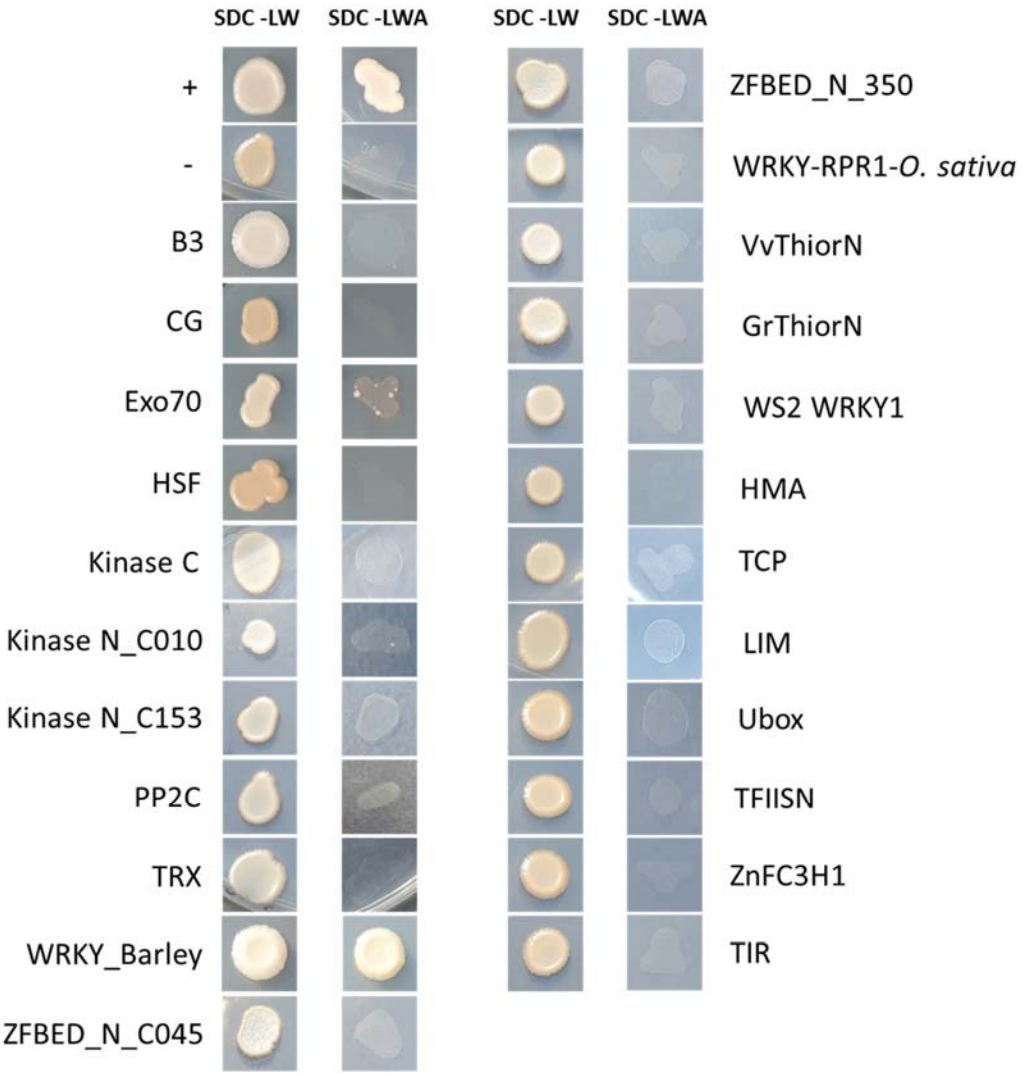

Figure 6: Screening for interactions with the *Shigella* effector protein IpaH1.4. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-IpaH1.4. The transformed colonies were washed in water, plated on transformation-selective SDC (-Leu-Trp) (SDC –LW) medium, as control, and on interaction-selective SDC (-Leu-Trp-Ade) (SDC –LWA) medium and incubated at 28°C for eight days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Signs of growth in tiny dots are visible for transformants with the Gal4 BD-EXO70 - Gal4 AD-IpaH1.4 pair on the SDC-LWA medium. A growth comparable to that on the control plate was observed for transformants with the Gal4 BD-WRKY_Barley - Gal4 AD-IpaH1.4 pair. No significant growth was observed for the other bait-pray combinations.

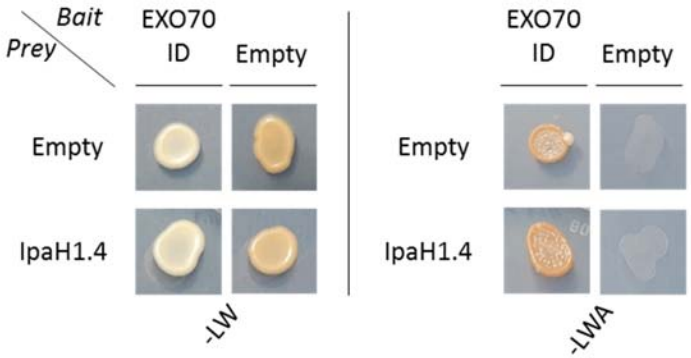

Figure 7: Evaluation of the autoactivation capability of the EXO70 ID. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for ten days. The EXO70 ID alone can autoactivate the expression of the reporter genes, enabling the growth of the transformed yeast cells independently of the presence of the effector protein.

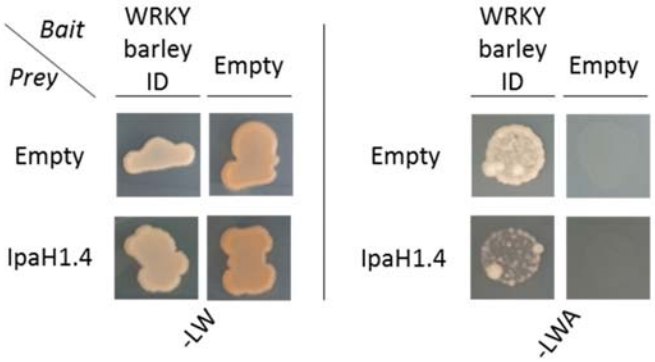

Figure 8: Evaluation of the autoactivation capability of the WRKY_Barley ID. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight days. The WRKY_Barley ID alone can autoactivate the expression of the reporter genes, enabling the growth of the transformed yeast cells independently of the presence of the effector protein.

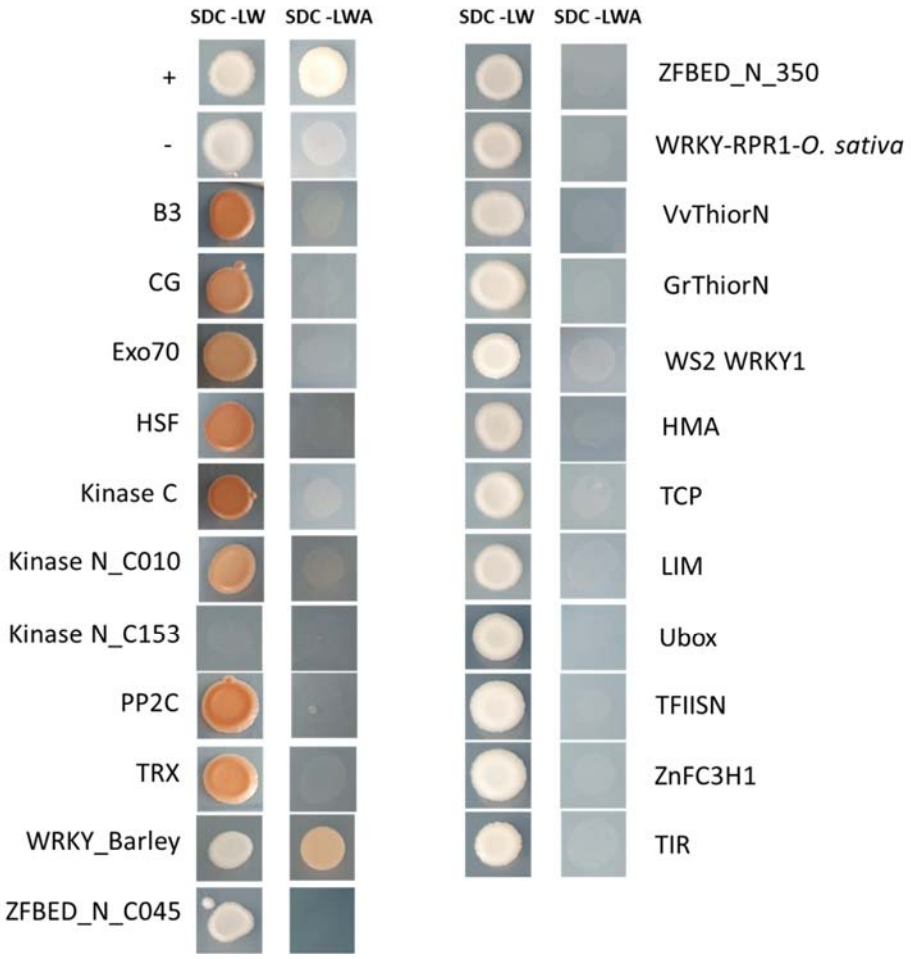

Figure 9: Screening for interactions with the *Shigella* effector protein IpaJ. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-IpaJ. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for three (WRKY_Barley-IpaJ pair) or ten days (the rest of the combinations). Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Despite those transformants carrying Gal4 BD-Kinase N_C153 - Gal4 AD-IpaJ pair had been grown on SDC-LW plates after transformation, albeit extremely delayed, their growth was inhibited completely on both the control and the interaction-selective medium of the assay. Interestingly, the growth of yeast strains transformed with the Gal4 BD-WRKY_Barley - Gal4 AD-IpaJ pair was profound after just two days post the assay, while growing of transformants carrying WRKY_Barley ID with the other *Shigella* effector proteins needed four days to appear in the form of tiny dots. That suggests a putative interaction. No significant growth was observed for the other bait-pray combinations.

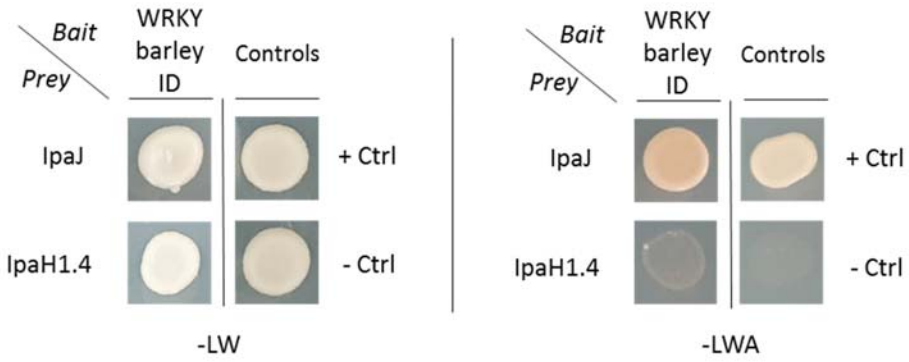

Figure 10: Growth of yeast cells coexpressing the *Shigella* effector protein IpaJ and the WRKY_Barley ID appears earlier than the autoactivation. The transformed colonies were washed in water, plated on non-selective SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C. The growth of the transformants encoding for the Gal4 BD WRKY_Barley-Gal4 AD IpaJ bait-prey pair appeared much earlier and more intense than those expressing Gal4 BD WRKY_Barley and Gal4 AD IpaH1.4. It was visible from just two days after the assay and comparable to that of the positive control. In comparison, in the other case, it was supported by the autoactivation of the WRKY_Barley ID, and it started being visible in tiny dots after four to five days.

3.5) Screening for interactions between *Shigella* effector protein OspF and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector OspF with the EXO70 ID of the *Hordeum vulgare* gave signs of growth as tiny dots into minimum growth medium SDC –LWA eight days after the Y2H assay. A more intense growth was observed for transformants expressing OspF with the WRKY_Barley ID of the *Hordeum vulgare* five days after the assay. No significant growth was observed for the transformants carrying the other OspF-ID combinations (**Figure 11**).

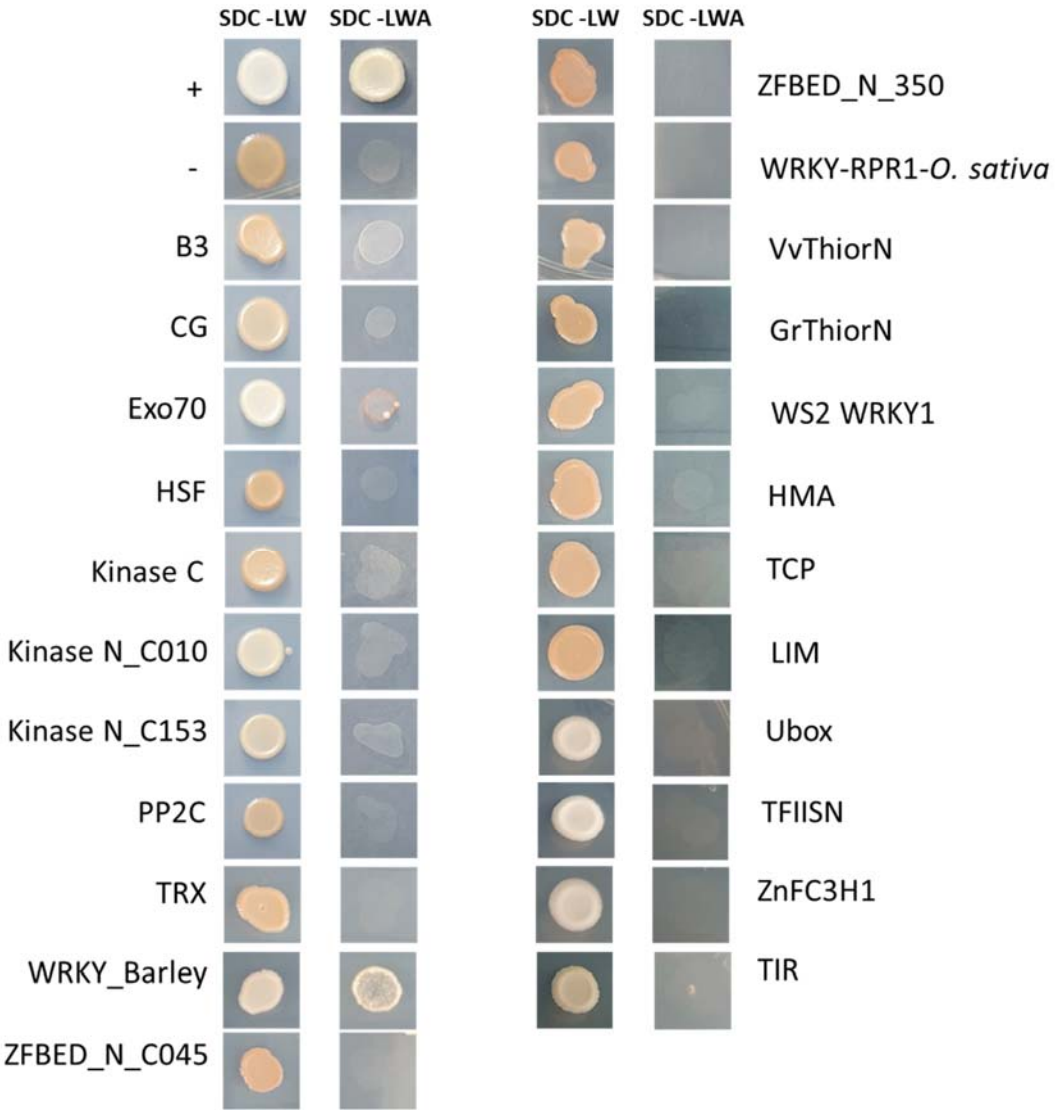

Figure 11: Screening for interactions with the *Shigella* effector protein OspF. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-OspF. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Signs of growth in tiny dots are visible for the transformants with Gal4 BD-EXO70 - Gal4 AD-OspF pair on the SDC-LWA medium. A more intense growth was observed for the transformants with Gal4 BD-WRKY_Barley - Gal4 AD-OspF pair. No significant growth was observed for the other bait-pray combinations.

3.6) Screening for interactions between *Shigella* effector protein OspG and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector OspG with the WRKY_Barley ID of the *Hordeum vulgare* grew after five days into minimum growth medium SDC –LWA. No significant growth was observed for the transformants carrying the other OspG-ID combinations (**Figure 12**).

3.7) Screening for interactions between *Shigella* effector protein VirA and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector VirA with the WRKY_Barley ID of the *Hordeum vulgare* grew after five days into minimum growth medium SDC –LWA. No significant growth was observed for the transformants carrying the other VirA-ID combinations (**Figure 13**).

3.8) Screening for interactions between *Shigella* effector protein IpgB1 and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector IpgB1 with the WRKY_Barley ID of the *Hordeum vulgare* grew in large dots after five days into minimum growth medium SDC –LWA. No significant growth was observed for the transformants carrying the other IpgB1-ID combinations (**Figure 14**).

3.9) Screening for interactions between *Shigella* effector protein IpgD and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector IpgD with the WRKY_Barley ID of the *Hordeum vulgare* grew after five days into minimum growth medium SDC –LWA. No

significant growth was observed for the transformants carrying the other IpgD-ID combinations (**Figure 15**).

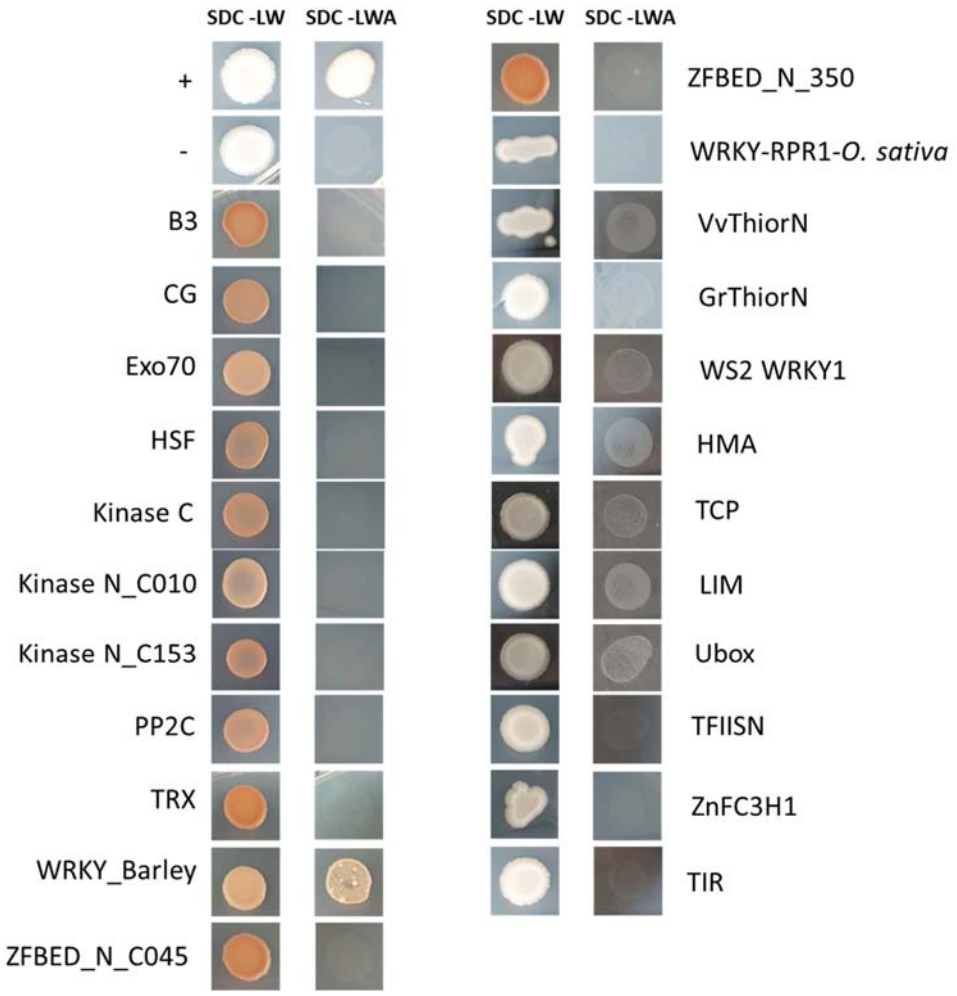

Figure 12: Screening for interactions with the *Shigella* effector protein OspG. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-OspG. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight to ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Growth was observed for the transformants with the Gal4 BD-WRKY_Barley - Gal4 AD-OspG pair on the SDC-LWA medium. No significant growth was observed for the other bait-pray combinations.

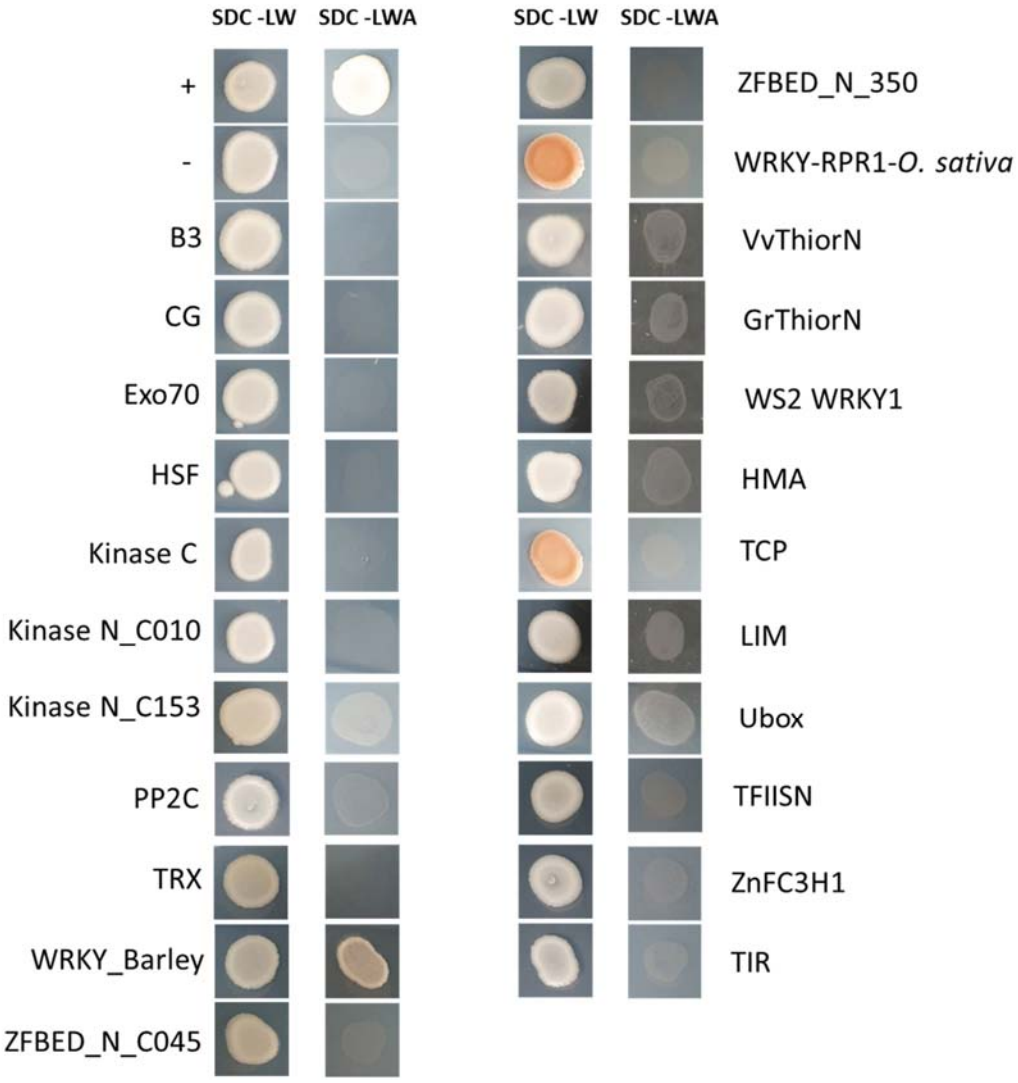

Figure 13: Screening for interactions with the *Shigella* effector protein VirA. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-VirA. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight to ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Growth was observed for the transformants carrying the Gal4 BD-WRKY_Barley - Gal4 AD-VirA pair on the SDC-LWA medium. No significant growth was observed for the other bait-pray combinations.

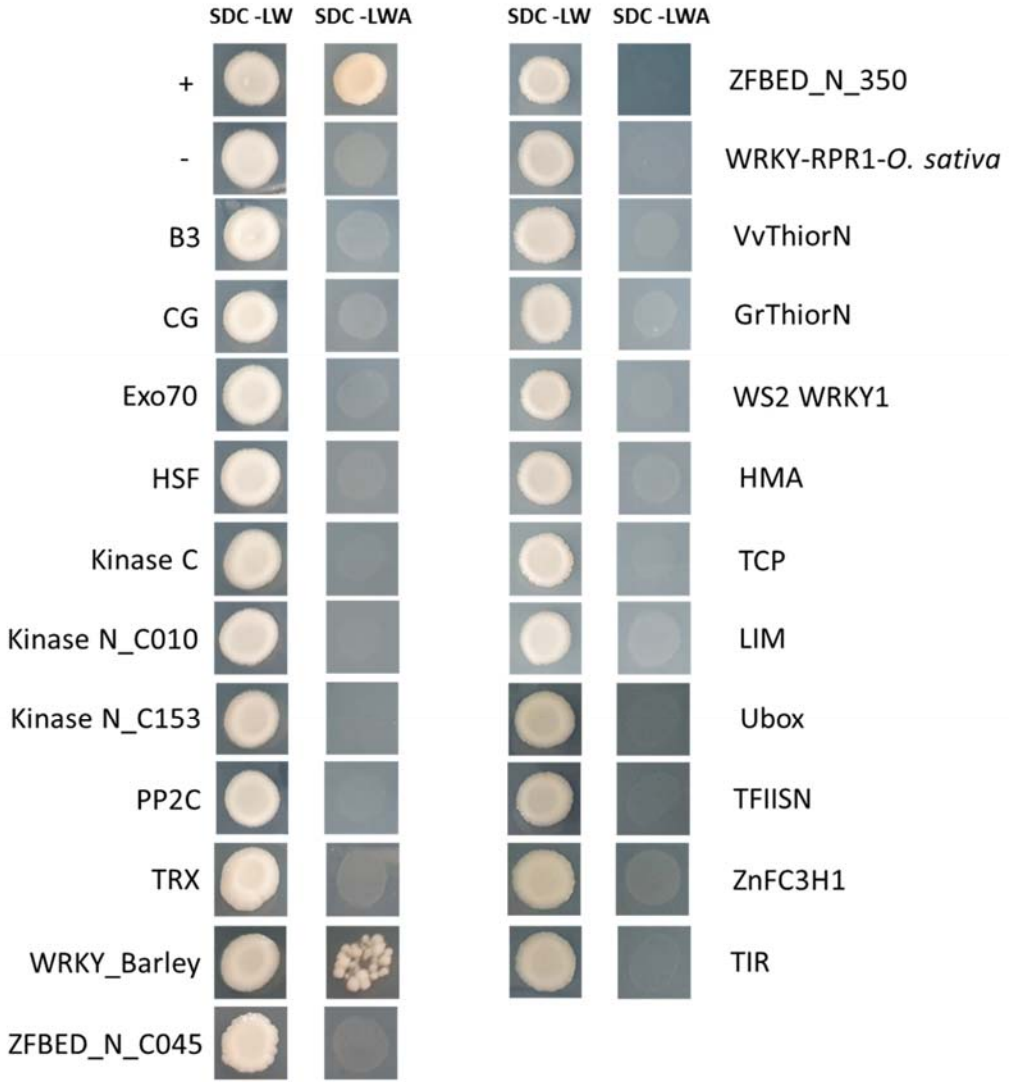

Figure 14: Screening for interactions with the *Shigella* effector protein IpgB1. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-IpgB1. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight to ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Growth in large dots was observed for the transformants carrying the Gal4 BD-WRKY_Barley - Gal4 AD-IpgB1 pair on the SDC-LWA medium. No significant growth was observed for the other bait-pray combinations.

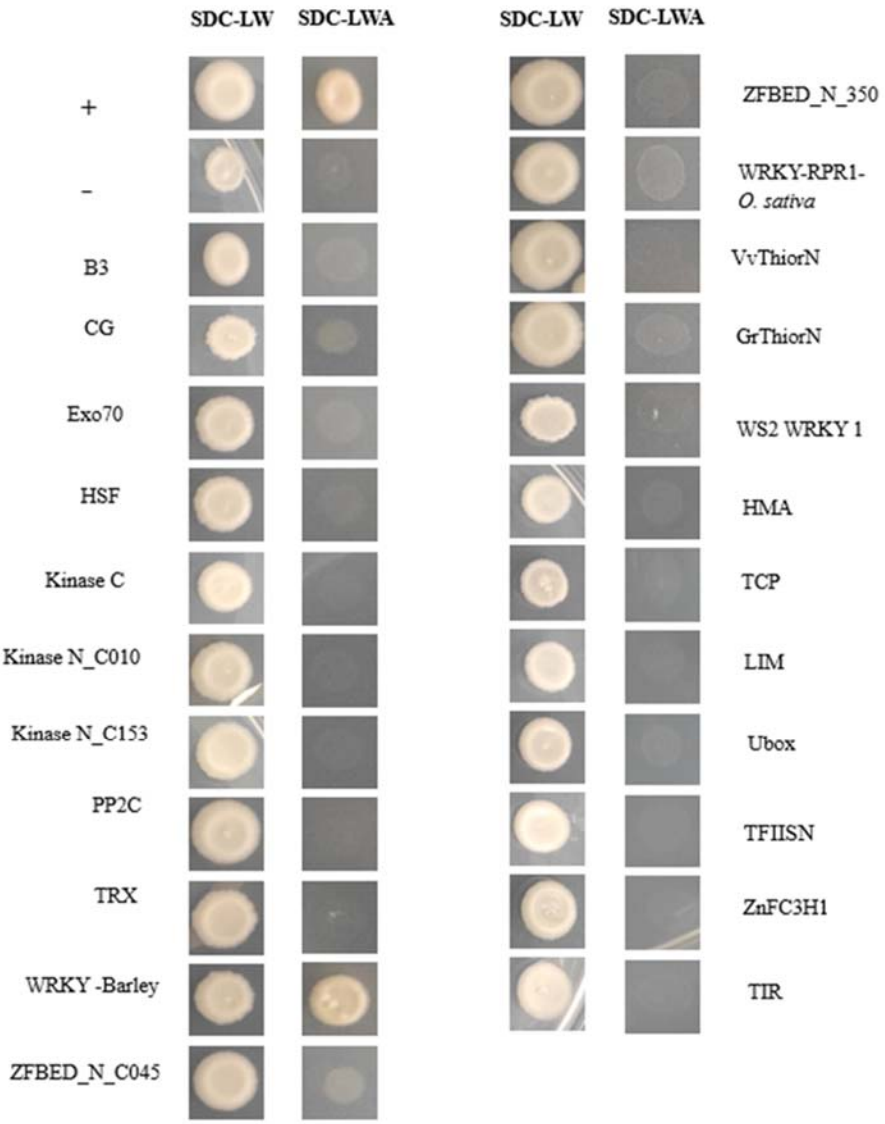

Figure 15: Screening for interactions with the *Shigella* effector protein IpgD. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-IpgD. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight to ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Growth was observed for the transformants carrying the Gal4 BD-WRKY_Barley - Gal4 AD-IpgD pair on the SDC-LWA medium. No significant growth was observed for the other bait-pray combinations.

3.10) Screening for interactions between *Shigella* effector protein IpaH7.8 and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector IpaH7.8 with the WRKY_Barley ID of the *Hordeum vulgare* grew after five days into minimum growth medium SDC –LWA. No significant growth was observed for the transformants carrying the other IpgD-ID combinations (**Figure 16**).

3.11) Screening for interactions between *Shigella* effector protein OspB and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector OspB with the WRKY_Barley ID of the *Hordeum vulgare* grew after five days into minimum growth medium SDC –LWA. No significant growth was observed for the transformants carrying the other IpgD-ID combinations (**Figure 17**).

3.12) Screening for interactions between *Shigella* effector protein OspC1 and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector OspC1 with the WRKY_Barley ID of the *Hordeum vulgare* grew in large dots after five days into minimum growth medium SDC –LWA. No significant growth was observed for the transformants carrying the other IpgD-ID combinations (**Figure 18**).

3.13) Screening for interactions between *Shigella* effector protein OspZ and plant NLR ID domains

Yeast cells coexpressing the *Shigella* effector OspZ with the WRKY_Barley ID of the *Hordeum vulgare* grew after five days into minimum growth medium SDC –LWA. No

significant growth was observed for the transformants carrying the other IpgD-ID combinations (**Figure 19**).

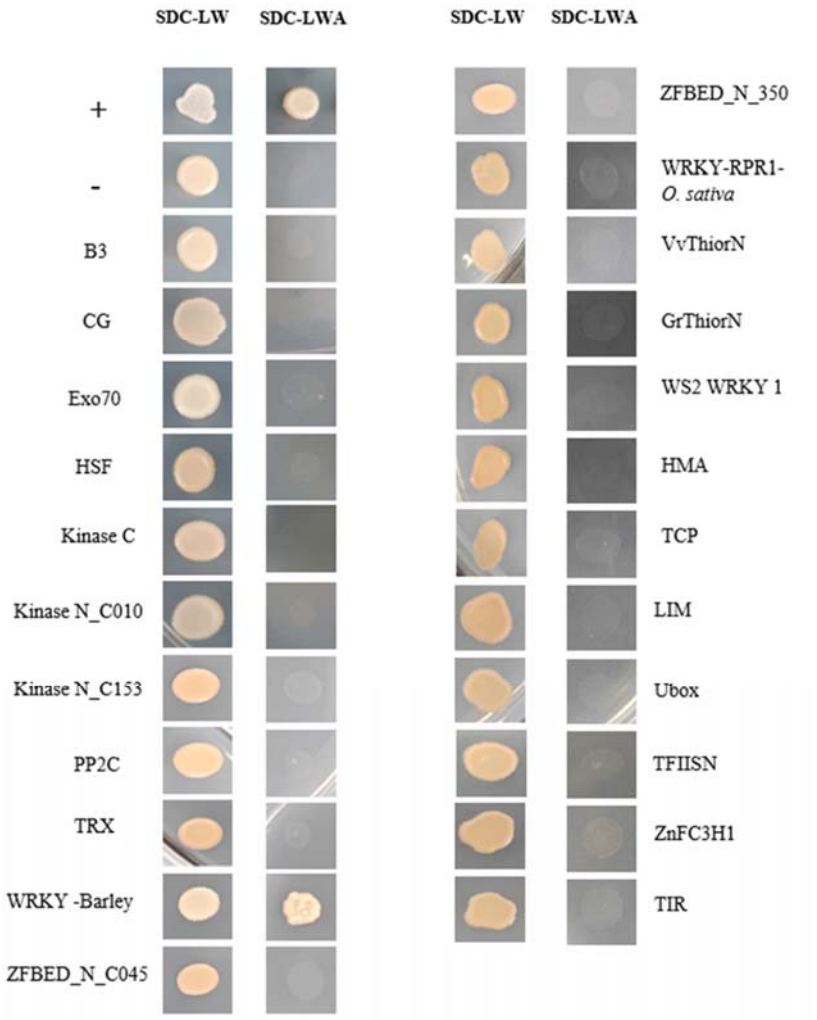

Figure 16: Screening for interactions with the Shigella effector protein IpaH7.8. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-IpaH7.8. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight to ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Growth was observed for the transformants carrying the Gal4 BD-WRKY_Barley - Gal4 AD-IpaH7.8 pair on the SDC-LWA medium. No significant growth was observed for the other bait-pray combinations.

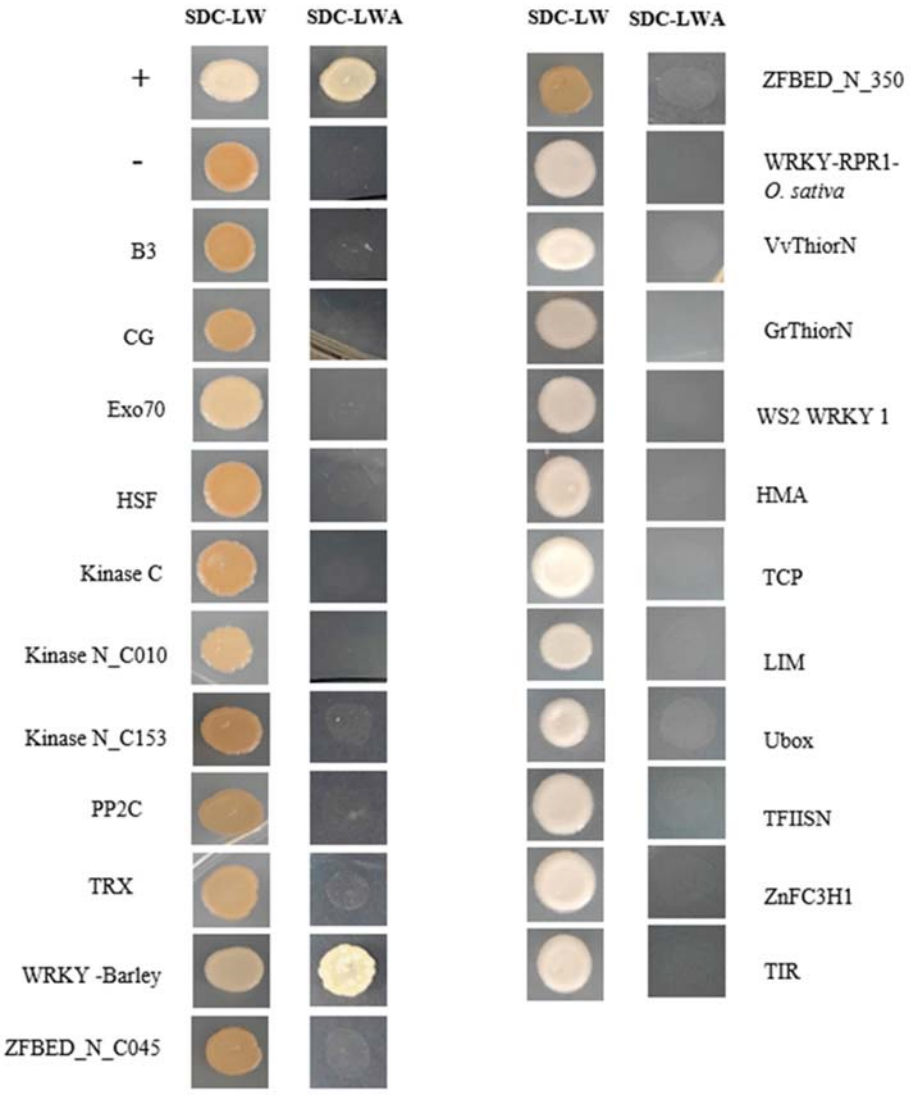

Figure 17: Screening for interactions with the *Shigella* effector protein OspB. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-OspB. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight to ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Growth was observed for the transformants carrying the Gal4 BD-WRKY_Barley - Gal4 AD-OspB pair on the SDC-LWA medium. No significant growth was observed for the other bait-pray combinations.

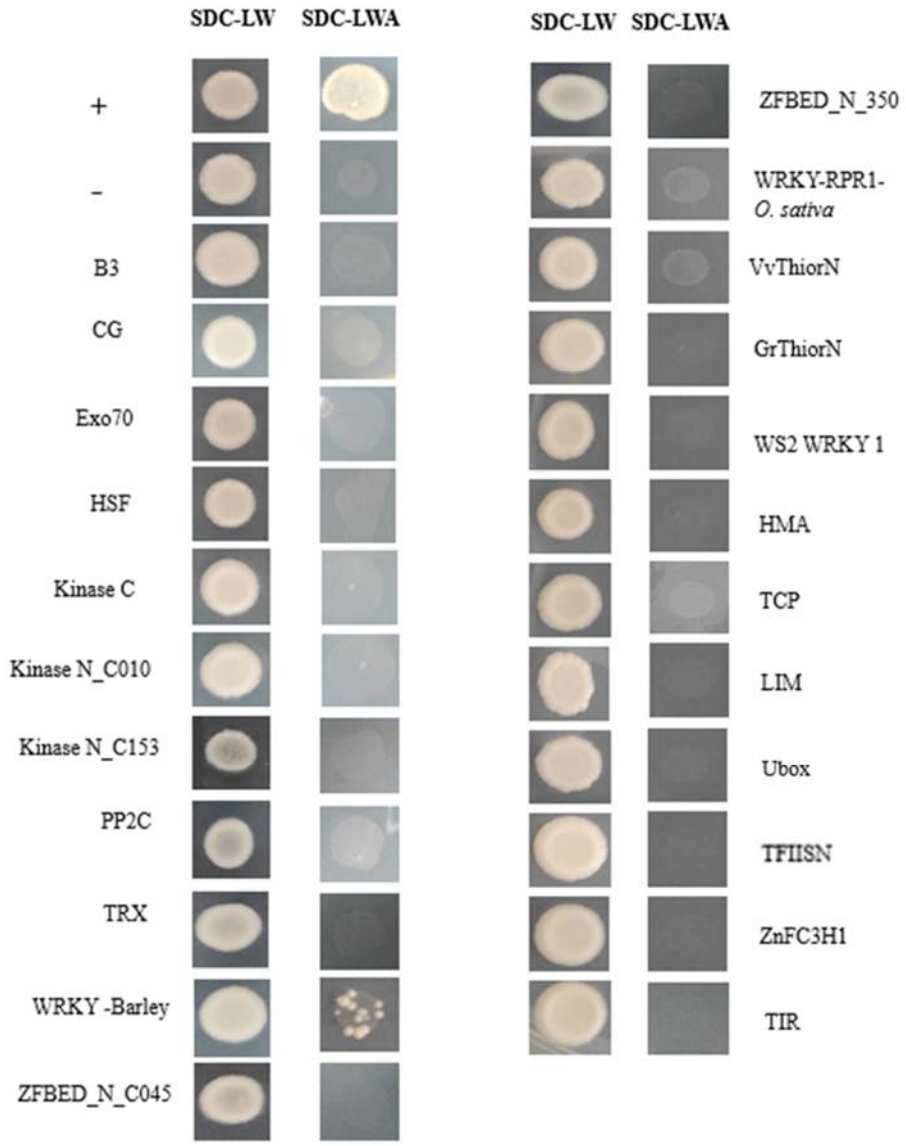

Figure 18: Screening for interactions with the *Shigella* effector-protein OspC1. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-OspC1. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight to ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Growth in large dots was observed for the transformants with the Gal4 BD-WRKY_Barley - Gal4 AD-OspC1 pair on the SDC-LWA medium. No significant growth was observed for the other bait-pray combinations.

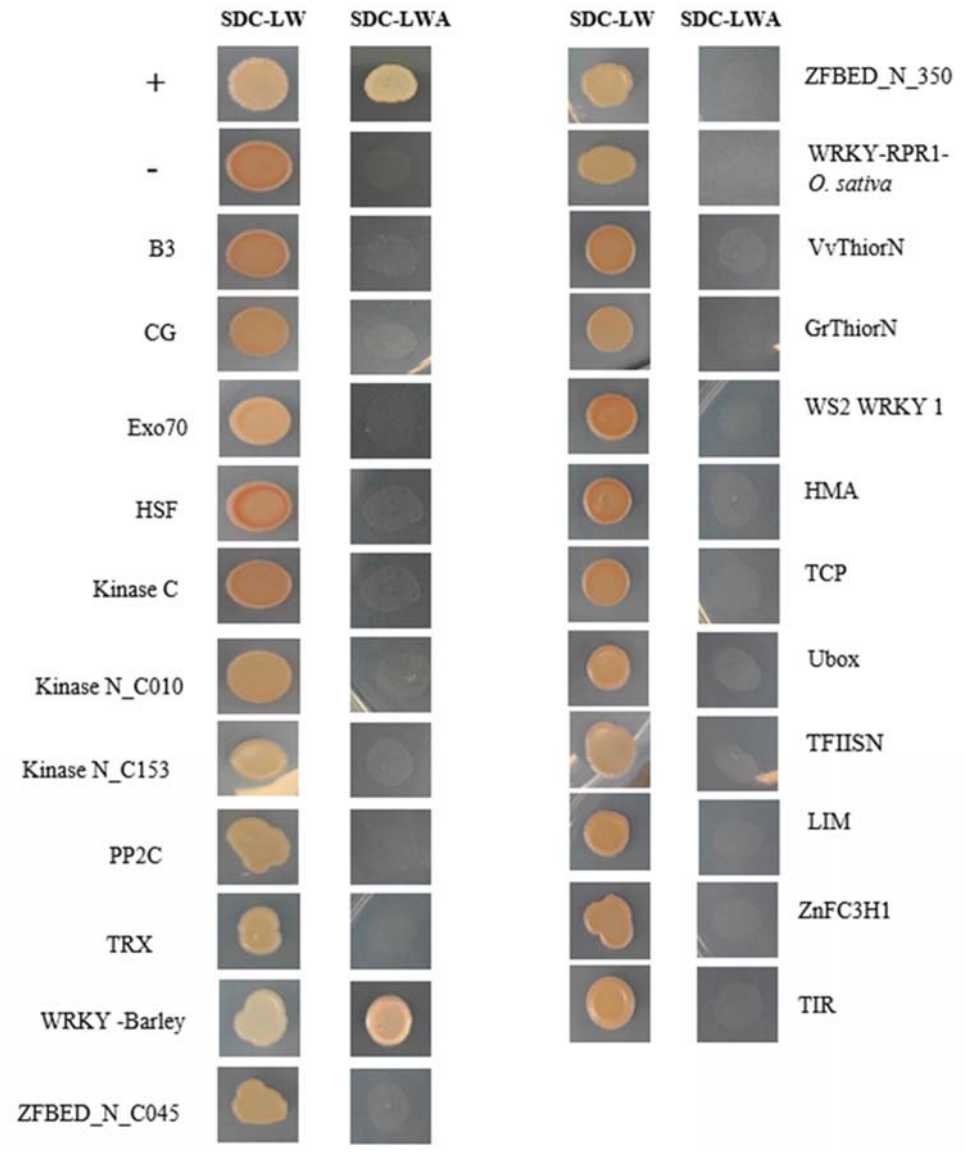

Figure 19: Screening for interactions with the *Shigella* effector-protein OspZ. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-OspZ. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight to ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). Growth was observed for the transformants carrying the Gal4 BD-WRKY_Barley - Gal4 AD-OspZ pair on the SDC-LWA medium. No significant growth was observed for the other bait-pray combinations.

3.14) Screening for interactions between *Shigella* effector protein IpaH3 and plant NLR ID domains

No significant growth was observed for the transformants carrying any of the IpaH3-ID combinations (**Figure 20**).

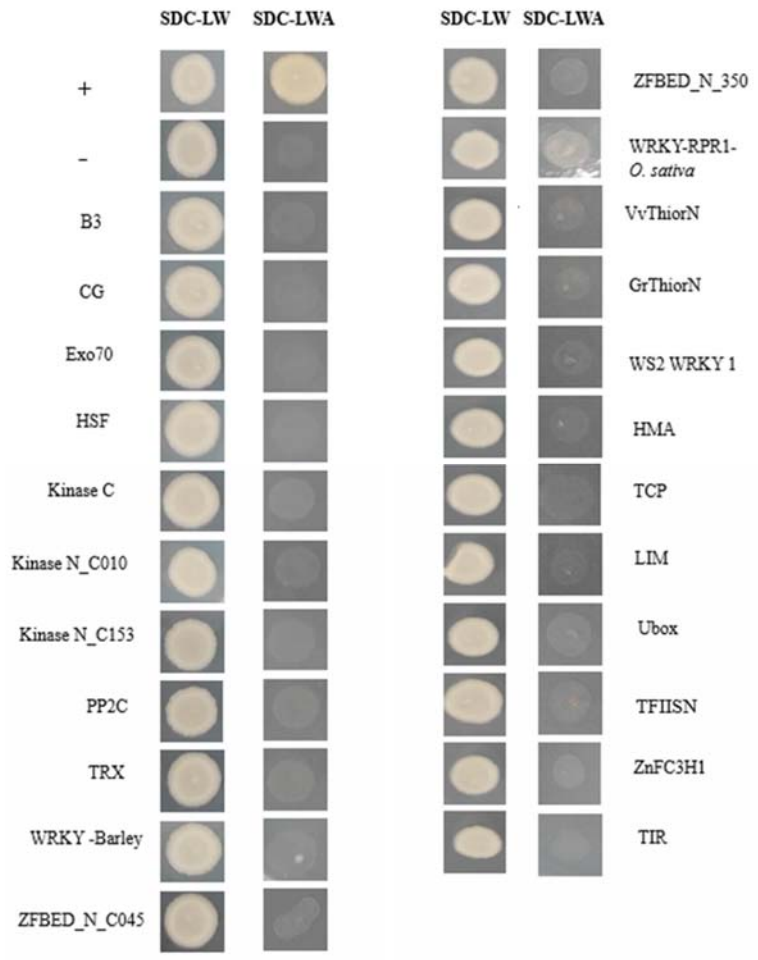

Figure 20: Screening for interactions with the *Shigella* effector protein IpaH3. Y2H assay of AH109 yeast cells containing plasmids encoding Gal4 BD fused with 23 conserved plant IDs and Gal4 AD-IpaH3. The transformed colonies were washed in water, plated on control SDC –LW and interaction-selective SDC –LWA plates, and incubated at 28°C for eight to ten days. Yeast transformants encoding Gal4 BD-WRKY_Barley ID and Gal4 AD-AvrRps4 were used as a positive control (+), while transformants carrying empty pGADT7:RFP and pGBKT7:RFP vectors were used as a negative control (-). No significant growth was observed for any of the 23 bait-pray combinations.

4) DISCUSSION

Shigella is a genus of primate-restricted pathogenic bacilli (Lampel et al., 2018). Its transmission is usually associated with insufficient or nonexistent water sanitation, and it follows the fecal-oral route. It mainly affects the developing countries, but it also leads to occasional outbreaks in the developed world (Anderson et al., 2016). Many strains have become multiresistant against most commercially available antibiotics, and a highly efficient vaccine is not available yet (Camacho et al., 2013; Nüesch-inderbinen et al., 2016; Lampel et al., 2018; Ahmad et al., 2021). Therefore, a further understanding of the pathogen's means of pathogenesis is required. *Shigella* manipulates the host cellular functions by injecting an arsenal of effector proteins capable of interacting with specific targets within host cells, enabling its proliferation, dissemination, and pathogenesis (Ashida et al., 2009; Mattock and Blocker, 2017). Thus, investigating the activity of *Shigella* effector proteins, their host targets, and their interaction is crucial for developing novel approaches for eliminating the disease.

Recent publications have illustrated that otherwise, animal-restricted pathogens could decently infect plant hosts utilizing the same means of pathogenesis (Schikora et al., 2008; Jo et al., 2019). The innate immune system in plants and animals features similarities regarding the recognition of pathogens and their components, and the orchestration of their elimination (Ausubel, 2005; Puhar and Sansonetti, 2014; Mermigka et al., 2020; Saur et al., 2020). These shared traits partially result from similar evolutionary pressure within the host-pathogen interactions and coevolution (Ausubel, 2005). In a broader sense, the fundamental non-self recognition principles of innate immunity are evolutionarily conserved and descended from the first eukaryotes.

We attempted to follow a cross-kingdom approach to highlight the similarity of the principles governing the host-pathogen interactions between plants and animals. By employing a yeast two-hybrid system, we coexpressed twelve *Shigella* effector proteins with twenty-three conserved plant NLR integrated decoys into the yeast strain AH109. The interaction detection laid on the ability of the yeast transformants to survive and grow in a

minimum medium lacking leucine, tryptophan, and adenine. A total of 276 combinations were screened.

Yeast two-hybrid (Y2H) assay is a powerful tool for detecting protein-protein interactions in a eukaryotic system *in vivo*, and it is plausible for comprehensive high-throughput screening procedures (Van Criekinge and Beyaert, 1999; Brückner et al., 2009). Y2H exploits transcription factors like GAL4 to activate the expression of reporter genes when two chimeric proteins carrying their transcriptional activation domain (AD) and DNA binding domain (BD), respectively, interact with each other (Van Criekinge and Beyaert, 1999; Brückner et al., 2009). The physical association of the domains of the transcription factors is necessary and sufficient for their function, a property known as modularity (Van Criekinge and Beyaert, 1999; Brückner et al., 2009). The expression of the proteins in the heterologous yeast system is an additional advantage given of the Y2H assay regarding the study of *Shigella;* an extremely dangerous human pathogen that requires increased culture and handling attention and safety procedures (Slagowski et al., 2008).

A potential issue of the heterologous expression of foreign proteins in yeast is the occurrence of toxicity. Some proteins can exert toxic effects when expressed and targeted to the yeast nucleus by binding or proteolyze endogenous proteins or causing wider unspecified disturbances, leading to lethality and growth defects (Van Criekinge and Beyaert, 1999; Slagowski et al., 2008). The strategies followed in such cases involve low copy plasmids and inducible promoters (Van Criekinge and Beyaert, 1999; Mehla et al., 2017). Indeed, some of the *Shigella* effector proteins, translocated into the host cell by the T3SS during infection, have been shown to cause growth inhibition and toxicity upon expression in yeast cells. Specifically, VirA, IpgD, and IpaJ had been shown to completely inhibit yeast growth, while OspC1, OspF, and IpgB1 caused intermediate growth inhibition (Slagowski et al., 2008). Effector proteins of *Shigella* target and interact with specific intracellular proteins of the human colonic epithelial cells leading to alteration of the cellular physiological processes (Ashida et al., 2009; Mattock and Blocker, 2017). Such functions are evolutionarily conserved even between distant organisms like humans and yeast. That renders the effectors capable of interacting with intrinsic yeast proteins, causing

disturbance of the cellular homeostasis and leading to lethality and growth inhibition (Slagowski et al., 2008; Burnaevskiy et al., 2013).

Indeed, we observed growth retardation of yeast cells carrying specific effector coding sequences integrated into pGADT7 vectors while growing into minimum growth medium lacking leucine and tryptophan (transformation-selective medium) before picking colonies for the Y2H assay. As described in the studies of Slagowski et al. (Slagowski et al., 2008), effectors causing intense inhibition were IpaJ, IpgD, and VirA, while OspF and OspC1 led to intermediate growth implications. However, excluding the yeast cells carrying the kinase N_C153-IpaJ bait-pray combination, we did not observe complete growth inhibition for our VirA, IpgD, and IpaJ transformants, as suggested by the Slagowski et al., (2008) study. A possible explanation for this discrepancy could be that Slagowski et al. (2008) used the effector proteins as fusions with GFP. The fluorescence protein tends to increase the stability and toxicity of the expressed effector proteins (Slagowski et al., 2008). Additionally, Slagowski et al. (2008) screened the growth of the different transformants by measuring the optical density of liquid cultures in parallel. In contrast, we examined the growth on the solid medium of the transformation-selective plates where even single colonies could be observed and distinguished. Furthermore, another reason for the discrepancy of the growth rates could be the different strains used by Slagowski et al., (2008) and our study; here, we used the AH109 strain, whereas Slagowski et al., (2008) used the wild-type yeast strain S288C (BY4741 MATa). Strain S288C (BY4741 MATa) could be more sensitive to the toxic effects of the effectors than the AH109 strain that we used.

Another possible issue that can be encountered during Y2H experiments is the autoactivation effect. Some proteins can initiate transcription of the reporter genes by themselves when fused to the DNA binding domain, independently from the interaction with the protein fused to the transcription activation domain, leading to false-positive results (Van Criekinge and Beyaert, 1999; Shivhare et al., 2021). We observed a very weak autoactivation ability for the EXO70 ID (**Figure 7**) and a stronger one for the WRKY_Barley ID (**Figure 8**). Yeast cells transformed with pGADT7:IpaH1.4 and pGBKT7:EXO70 ID showed signs of growth in the form of tiny dots into the interaction-selective medium about eight days after the assay (**Figure 6**). The autoactivation of the

EXO70 ID was barely visible at cotransformation with OspF (**Figure 11**), while it did not get observed with other effectors. The WRKY_Barley ID had a more intense autoactivating capability, as the growth of yeast cells encoding for this ID was clearly visible at 4 to 5 days in coexpression with all the effectors except IpaH3.

Interestingly, the yeast strains encoding for Gal4 AD-IpaJ and Gal4 BD-WRKY_Barley ID showed early growth comparable to that of the positive control (**Figure 9**, **Figure 10**). That could imply the presence of an interaction between the *Shigella* effector protein IpaJ and the WRKY_Barley ID of the *Hordeum vulgare*. IpaJ is a cysteine protease proven to cleave the Golgi-associated N-myristoylated protein ARF1 leading to Golgi fragmentation and disruption of the vesicular trafficking (Burnaevskiy et al., 2013). It can also mediate the de-myristoylation of other substrates, like the phosphatidylinositol kinase VPS15, altering various signaling pathways in parallel (Burnaevskiy et al., 2013). The residues C90, H230, and D242 are necessary for its protease activity (Burnaevskiy et al., 2013; Li et al., 2019). Myristoylation is the covalent, post-translational attachment of myristic acid (tetradecanoic acid) to the a-amino group of a glycine residue (Podell and Gribskov, 2004). That group can get exposed and prone to such a modification after the first methionine removal or caspase-mediated cleavage (Podell and Gribskov, 2004). The WRKY_Barley ID has a glycine residue after the first methionine but is predicted as non-myristoylated by the ExPASy myristoylation prediction tool based on its amino acid sequence. In addition, the amino-terminal domain of the ID is directly integrated into both the Gal4 BD in the expression vector and into the NLR in the plant cells. Therefore, it is not accessible for N-myristoylation and thus protein-protein interaction based on such a modification. That indicates that a myristoylation-independent mode of interaction is necessary.

A recent publication elucidated that the IpaJ effector protein of *Salmonella enterica* serovar Pullorum can hamper the ubiquitination of the NF-κB inhibitor IκBα upon TNFα-stimulation, leading to the inhibition of its TNFα-induced degradation (Li et al., 2019). Thus the NF-κB nuclear translocation and the expression of downstream response genes are reduced (Li et al., 2019). Despite *Shigella* IpaJ shares 49% homology with that of *Salmonella* serovar Pullorum, the catalytic residues are conserved. Interestingly enough, the *Shigella* effector protein could also inhibit the activation of the TNFα induced NF-κB signaling (Li et al., 2019). IκBα is not a myristoylation target. Thus, IpaJ effector proteins

could indirectly interfere with the IκBα ubiquitination by targeting and cleaving other myristoylated targets, e.g., E3 ubiquitin ligases, or directly interact with it by a yet unknown activity. The latter could explain a mode of interaction between *Shigella* IpaJ and WRKY_Barley ID.

Although we did not detect any other sign of interaction, that does not invalidate our hypothesis about the similarities regarding the modes of effector-receptor interactions between plants and animals. The NLR-decoy fusions are widespread among the flowering plants as they possess an agent of resistance against the pathogens, acting as baits to entrap and recognize their effectors and induce the response of the host (Cesari et al., 2014; Sarris et al., 2016). The integrated domains resemble protein activities with high enrichment in kinases, transcription factors (e.g., WRKY), and proteins involved in redox reactions (Sarris et al., 2016). Such targets are common for effector proteins of both plant and animal pathogens. Hence, the presence of plant receptors capable of recognizing and interacting with effector proteins from animal pathogens is rational.

In this project, we coexpressed in yeast cells twelve selected *Shigella* effectors with all the integrated decoys we had available. Thus, we examined many conditions (e.g., bait-prey pairs) in parallel. We chose this method to initially address our hypothesis as it is quite novel without any similar work in the current bibliography. Another approach would initially involve focusing on specific animal pathogen effector proteins that share similarities with phytopathogen effectors. Then the targets of the plant pathogen effectors into the plant cells should be assessed based on databases, experimental knowledge, and published studies. Eventually, a targeted investigation of the interaction between these selected animal pathogen effectors and plant proteins might be more productive. For example, OspF of *Shigella*, together with SpvC of *Salmonella*, and HopAI1 of *Pseudomonas syringae*, represent a family of T3SS effectors conserved in both plant and animal bacterial pathogens (Li et al., 2007; Zhu et al., 2007). OspF harbors a phosphothreonine lyase activity and hinders the action of MAPKs such as the extracellular signal-regulated kinases 1 and 2 (Erk1/2), p38, and c-Jun N-terminal kinase (JNK) (Li et al., 2007; Zhu et al., 2007). This way, it can hijack some of the main cellular signaling pathways, leading to fine-tuned immune suppression (Li et al., 2007; Zhu et al., 2007). Upon injection into plant cells, both OspF and SpvC can reduce the levels of active MAPKs

of *Arabidopsis thaliana,* interfering with the PTI response (Schikora et al., 2008; Jo et al., 2019). Other *Shigella* effectors targeting the activation of MAPKs are OspC1 and OspB (Mattock and Blocker, 2017). In future experiments, we plan to investigate whether these effectors can interfere with the activation of MAPKs in plants.

Such cross-kingdom approaches are important, since they show the conservation of the basal defense mechanisms and the host manipulation strategies of the pathogens. These approaches enable the utilization of plants and their components as putative models for studying the means of pathogenesis. Plant models provide genetic tractability, short generation time, low cost, easy handling, low maintenance expenses, and limited ethical consideration (Baldini et al., 2002). Therefore, they could comprise plausible alternatives for efficient study of the activity of effector proteins with known targets as for the identification of putative targets for effectors that have not profound interactors yet. Additionally, putative human pathogens that survive and proliferate endophytically are a newly described and emphasized phenomenon that possesses a considerable health hazard (Schikora et al., 2008; Jo et al., 2019). By unraveling the mechanisms of such unconventional interactions, we could develop new targeted plant disinfection and cleansing procedures before delivery and consumption to reduce health risks.

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