Study of *N*-acyl-homoserine lactones for plant protection against plant and human pathogens and their counteraction

by

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To my family,

“When you want something, all the universe conspires in helping you to achieve it”

(P. Coelho)

To my supervisors and colleagues,

“Really great people make you feel that you too can become great”

(Mark Twain)

Eternal gratitude to all of you

SUMMARY

Nowadays, efficient disease management encompasses diverse concepts, including the activation and reinforcement of the plant immune system. Therefore, biocontrol agents integrate priming of crop plants for stronger defense and systemic responses. To ensure maximum crop yield even upon pathogen pressure, it is fundamental to understand the mode of action of new biocontrol agents. Due to the ability to modulate plant-microbe interactions, bacterial quorum sensing molecules might be a good alternative for modern plant protection strategies. Quorum sensing (QS) refers to the communication system by which bacteria regulate (in a density-dependent manner) genes involved in diverse behaviors, such as biofilm formation, antibiotic resistance or virulence. In Gram-negative bacteria, the role of QS molecules is often played by *N*-acyl homoserine lactones (AHLs). In plants, AHLs can induce priming for a faster and more efficient immune response against pathogens. The objective of this thesis was to study the immune response and the physiological alteration upon AHL-priming in the model plant *Arabidopsis* and to translate those results into important crop plants, like barley, wheat, tomato and alfalfa. Based on previous data, I chose to work with the long-chain oxo-C14-HSL and the oxo-C14-HSL-producing rhizobacterium *Sinorhizobium meliloti*.

We could demonstrate that oxo-C14-HSL primed *Arabidopsis* plant for a broad-spectrum resistance based on a salicylic acid/oxylipin-dependent systemic signal. In addition, oxo-C14-HSL caused enhanced production of reactive oxygen species and transcriptional activation of defense-related genes in crop plants. The outcomes of the oxo-C14-HSL-induced priming were reinforcement of plant cell wall and stomata defense response, which helped to avoid pathogen entry and proliferation. Furthermore, oxo-C14-HSL could arrest the proliferation of the human pathogen *Salmonella enterica* serovar Thyphimurium in *Arabidopsis* plants even though this resistance effect might be limited when AHL-producing *S. meliloti* forms a symbiotic relationship with its native plant host *Medicago sativa.* On the other side, *Salmonella* inject effector proteins into the host cells in order to manipulate the immune system. We could show that the effector protein SpvC deactivates *At*MPK6 and *At*MPK3.

Taking together, quorum-sensing molecules have a positive impact on plants and AHL-induced resistance could be a model for plant priming and open new strategies for crop plant protection.

ZUSAMMENFASSUNG

Heutzutage umfasst effizienter Pflanzenschutz unterschiedliche Konzepte, die auch die Aktivierung und Stärkung des pflanzlichen Immunsystems beinhalten. Eine Alternative des biologischen Pflanzenschutzes ist das Konzept der „Sensibilisierung“ (*Priming*) von Kulturpflanzen, welches eine Stärkung der pflanzlichen Abwehr bewirkt. Um einen maximalen Ernteertrag auch bei Pathogendruck zu gewährleisten, ist es von grundlegender Bedeutung, die molekularen Wirkungsweisen von Pflanzenschutzmitteln zu verstehen. Bakterielle Quorum Sensing Moleküle haben die Eigenschaft, Pflanzen-Mikroben-Interaktionen zu modulieren und könnten deshalb eine gute Alternative im modernen Pflanzenschutz sein. Quorum sensing (QS) ist ein Kommunikationssystem, mit dem Bakterien ihr Verhalten in einer Population steuern können und Gene regulieren, die beispielsweise für Biofilmformation, Antibiotikaresistenz oder Virulenz wichtig sind. QS Moleküle von Gram-negativen Bakterien werden meistens in Form von *N*-Acyl-Homoserin-Lactone (AHLs) produziert. Diese AHLs können ebenfalls von Pflanzen erkannt werden und lösen eine „Sensibilisierung“ (*Priming*) zur schnelleren und effizienteren Immunantwort aus. Das Ziel dieser Arbeit war es, die Immunantwort und die physiologische Veränderung der Modellpflanze *Arabidopsis* nach einer AHL-Sensibilisierung zu studieren und diese Ergebnisse in wichtigen Kulturpflanzen Gerste, Weizen, Tomaten und Luzerne, zu übertragen. Aufgrund existierender Daten, wurde das langkettige AHL Molekül oxo-C14-HSL und das oxo-C14-HSL-produzierende Rhizobakterium *Sinorhizobium meliloti* zur Behandlung von Modell- und Kulturpflanzen verwendet. Wir konnten eine oxo-C14-HSL-induzierte Resistenz von *Arabidopsis* Pflanzen demonstrieren und konnten zeigen, dass diese Resistenzinduktion auf der Grundlage eines Salicylsäure/Oxylipin abhängigen systemischen Signals liegt. Darüber hinaus bewirkt eine oxo-C14-HSL Behandlung bei Gerste und Weizen eine verstärkte Produktion von reaktiven Sauerstoffspezies und eine transkriptionelle Regulation von verteidigungsrelevanten Genen. Das oxo-C14-HSL-induzierte *Priming* bewirkt eine Verstärkung der Pflanzenzellwand und eine Stomata Abwehrreaktion und verhindert somit das Eindringen des Krankheitserregers und deren Proliferation in der Pflanze. Darüber hinaus konnte oxo-C14-HSL die Vermehrung des

Humanpathogens *Salmonella enterica* serovar Thyphimurium in *Arabidopsis* Pflanzen verhindern. Dieser Resistenzeffekt ist jedoch limitiert, wenn AHL-produzierende *S. meliloti* eine symbiotische Beziehung mit ihrem nativen Pflanzenwirt *Medicago sativa* eingehen. Auf der anderen Seite kann *Salmonella* als Pflanzenpathogen, Effektorproteine in die Wirtszellen injizieren, um das Immunsystem zu manipulieren. Wir konnten zeigen, dass das Effektorprotein SpvC die pflanzlichen Abwehrproteine AtMPK6 und AtMPK3 deaktiviert.

Zusammenfassend haben Quorum Sensing Moleküle eine positive Wirkung auf Pflanzen. Außerdem ist die AHL-induzierte Resistenz ein Modell zur pflanzlichen „Sensibilisierung“ (*Priming*) für schnellere und stärkere Reaktion auf kommende Stresssituationen und eröffnet somit neue Strategien im modernen Pflanzenschutz.

LIST OF PUBLICATIONS

1. Neumann C, Fraiture M, **Hernández-Reyes C**, Akum FN, Virlogeux-Payant I, Chen Y, Pateyron S, Colcombet J, Kogel K-H, Hirt H, Brunner F and Schikora A (2014) The Salmonella effector protein SpvC, a phosphothreonine lyase is functional in plant cells. *Frontiers in Microbiology*, 5:548
2. **Hernández-Reyes C**, Schenk ST, Neumann C, Kogel KH and Schikora A (2014). N-acyl-homoserine lactones-producing bacteria protect plants against plant and human pathogens. *Microbial biotechnology*, 7 (6): 580-588.
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4. **Hernández-Reyes** C and Schikora A (2013). *Salmonella,* a cross-kingdom pathogen infecting humans and plants. FEMS Microbiology Letters, 343 (1): 1-7

ABBREVIATIONS

ABA: Abscisic acid
ACC: 1-aminocyclopropane 1-carboxylic acid
AGPs: Arabinogalactan proteins
AHL: N-acyl homoserine lactone
BABA: β-aminobutyric acid
BAK1: BR1-Associate Kinase 1
BSA: Bovine serum albumin
BSMT1: benzoic acid/SA carboxyl methyltransferase 1
BTH: Benzo (1,2,3,) thiadiazole-7-carbothioic acid *S*-methyl ester
ºC: degree Celsius
$CaCl_2$: Calcium chloride
CaM: Calcium-modulating protein
$Ca(NO_3)_2 \bullet 4H_2O$ Calcium Nitrate tetrahydrate
$Ca(SO_4)_2$: Calcium sulfate
CDPKs: Calcium-dependent protein kinases
CERK1: Chitin Elicitor Receptor Kinase
CH: Calponin homology domain
cis-OPDA: cis-12-oxo-phytodienoic acid
COI-1: Coronatine Insensitive 1
Co-IP: co-immunoprecipitation
COR: Coronatine
CSPs: Cold shock proteins
$CuSO_4$: Copper (II) sulfate
$CoCl_2 \bullet 7H_2O$: Cobalt (II) chloride heptahydrate
cv.: Cultivar
CWAs: Cell wall appositions
DAB: 3'3-Diaminobenzidine tetrachloride
dai: days after infection
dd: double distilled
DFR: Dihydroflavonol 4-reductase
DIR1: Defective in induced resistance 1
DMSO: Dimethyl sulfoxide
DTT: Dithiothreitol
EDTA: Ethylenediaminetetraacetic acid
EGTA: Ethylene glycol tetraacetic acid
ET: Ethylene
ESP: Exopolysaccharide
ext.: extension
Fe: Iron
FLS2: Flagellin-sensitive 2
FRK1: flg22-induced receptor-like kinase 1
f. sp.: Forma specialis
FWD: Forward
g: gram
GA: Gibberellic acid

GlcNAc: N-acetylglucosamine
GSH: Glutathione
GST: Glutathione S-transferase
h: hour
hai: hours after inoculation
hat: hours after treatment
HBO_3: Hydrogen borate
HR: hypersensitive response
HRGPs: Hydroxyproline-rich glycoproteins
HSP70: Heat shock protein 70
HSL: Homoserine lactone
IQGAP1: IQ-motif- containing GTPase-activating protein
IS: Insertion sequence
ISR: Induced systemic resistance
IT: Infection thread
JA: Jasmonic acid
JA-Ile: Jasmonoyl isoleucine
JAR1: Jasmonic acid amido-synthethase 1
JAZ: Jasmonate ZIM-domain
KCl: Potassium chloride
KH_2PO_4: Potassium dihydrogen orthophosphate
K_2HPO_4: Dipotassium phosphate
KNO_3: Potassium nitrate
KOH: Potassium hydroxide
L: Liter
LB: Lysogeny broth
LCOs: (lipochitooligosaccharides)
LOX: Lipoxygenase
LPS: Lipopolysaccharide
LRR: Leucine-rich repeat
LYKs: Lysine motif (LysM)-containing receptor-like kinases
M4: biotin-labeled oxo-C14-HSL
MAMP: Microbe-associated molecular pattern
MAPK: Mitogen-activated protein kinase
MED4-1: MeSA esterase 4
MES: 2-(*N*-morpholino)ethanesulfonic acid
MeSA: Methyl salicylate
$MgCl_2$: Magnesium chloride
$MgSO_4$ □ $7H_2O$: Magnesium phosphate heptahydrate
µL: microliter
min: minute
mL: milliliter
mmol: millimol
$MnSO_4$: Manganese (II) sulfate
MS: Murashige-Skoog
MSMO: Murashige-Skoog minimal organics
MTI: MAMP-triggered immunity

MVBs: Multivesicular bodies
MYB72: MYB domain protein 72
NAA: 1-Naphthaleneacetic acid
NaCl: Sodium chloride
NASC: Nottingham Arabidopsis Stock Centre
Na_2HPO_4 ⍰ $2H_2O$: Di-sodium hydrogen phosphate dihydrate
NaH_2PO_4: Monosodium phosphate
nm: nanometers
$Na_2MoO_4 \bullet H_2O$: Sodium molybdate
Na_2SO_4: Sodium sulfate
NDR1: Non-race-specific disease resistance 1
NH_4NO_3: Ammonium nitrate
Ni-NTA: Nickel- Nitrilotriacetic acid
NLR: Nucleotide-binding leucine-rich repeat receptor
NPR1: Non-expresser of PR gene 1
OD_{600}: Optical density at a wavelength of 600 nm.
OPDA: 12-oxo-phytodienoic acid
ORF: Open Reading Frame
PA: Phosphatidic acid
PAL1: Phenylalanine ammonia-lyase 1
PAMP: Pathogen-associated molecular patterns
PCR: Polymerase chain reaction
PDK: Phosphoinositide-dependent kinase
PEG: Polyethylene glycol
PGPF: Plant growth-promoting fungi
PGPR: Plant growth-promoting rhizobacteria
PI4K: Phosphatidylinositol-4- kinases
PIOX: Pathogen-induced oxygenase
PIPES: 1,4-Piperazinediethanesulfonic acid
PLC: Phospholipase C
PLD: Phospholipase D
PMBs: Paramural bodies
PNM: Plant Nutrient Medium
PR1: Pathogenesis-related protein 1
PRPs: Proline-rich proteins
PRRs: Pathogen recognition receptors
PRX7: Peroxidase 7
Pst: *Pseudomonas syringae* pv. *tomato*
PTI: Pattern-triggered Immunity
Pto: *Pseudomonas syringae pv tomato*
PUFAs: Polyunsaturated fatty acids
pv.: Pathovar
QS: Quorum sensing
RBOH: Respiratory burst oxidase homologs
REV: Reverse
RIN4: RPM1-interacting protein 4
RLKs: Receptor-like kinases

RNIs: Reactive nitrogen intermediates
ROP: Rho-like protein
ROS: Reactive oxygen species
RPM1:
RPS2:
RT: Room Temperature
s: second
SA: salicylic acid
SAR: Sytemic acquired resistance
SDS: Sodium dodecyl sulfate
SCF^{COI1}: Skp1/Cullin1/F-box protein COI1
SGT1: Suppressor of G2 allele of Skip1
SOC: Super Optimal Broth
SPI-1: *Salmonella* Pathogenicity Island-1
Spv: Salmonella virulence plasmid
str.: strain
STS: Silver thiosulfate solution
T3Es: Type 3 effectors
T3SS: Type 3 secretion system
TE: Tris-EDTA
TEMED: Tetramethylethylenediamine
TIR-NBS-LRR: Toll/interleukin-1 receptor nucleotide-binding leucine-rich repeat
TLP5: Tubby-like protein 5
Tris: tris(hydroxymethyl)aminomethane
TY: Tryptone-Yeast
Xoo: *Xanthomonas oryzae* pv *oryzae*
$ZnCl_2$: Zinc chlroride
$ZnSO_4 \bullet 7H_2O$: Zinc Sulfate Heptahydrate

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1 INTRODUCTION

1.1 Plant immunity

1.1.1 MAMP-triggered immunity (MTI)

Unlike animals, plants do not have an adaptive defense system nor somatic mobile cells specialized for defense, therefore they depend on the immune system of singular cells and the systemic signals to fight off pathogens [1]. The first basal barriers e.g., cell wall and waxy cuticular layers, are defensive structures that should prevent the entry of pathogens. However, this structural protection might be ineffective against certain pathogens; for example, pathogenic bacteria which enter via pores or wounds, or fungi that directly enter into plant epidermal cells. Therefore, important steps for the establishment of resistance against pathogens are signaling cascades and inducible defense mechanisms.

The Inducible plant immune system is usually divided into two branches according to the pathogen recognition sites in plant cells: MAMP-Triggered Immunity (MTI) and Effector-Triggered Immunity (ETI). MTI involves transmembrane pattern recognition receptors (PRRs) that respond to highly conserved and slowly evolving Microbial/Pathogen-Associated Molecular Patterns (M/PAMPs). Among the most prominent MAMPs is the 22-amino acid sequence of bacterial flagellin, which is sensed by the Leucine-Rich Repeat Receptor Kinase (LRR-RK) Flagellin-sensitive 2 (FLS2). This PRR with its co-receptor BR1-Associate Kinase 1 (BAK1) mediate an extracellular recognition event and activate an intracellular signaling cascade [2, 3]. Another example of bacterial MAMP is the PRR protein Xa21 that recognizes the sulfated 17-amino acid peptide of Ax21, a type 1 secreted protein derived from *Xanthomonas oryzae* pv *oryzae* (*Xoo*). Lysine Motif Receptor Kinases (LysM-RKs), such as the Chitin Elicitor Receptor Kinase 1 (CERK1), have a critical role in perception of the fungal cell wall component and prominent MAMP chitin, [4].

One of the early events attributed to MTI are the elevation of cytoplasmic Ca^{2+} concentration and accumulation of Ca^{2+} in the thylakoid lumen of chloroplasts. The changes in $[Ca^{2+}]_{cyt}$ are sensed by calcium-binding proteins e.g., calmodulin and

Calcium-Dependent Protein Kinases (CDPKs) [5, 6]. Additionally, Ca^{2+} elevation leads to other plant immune responses that include production of reactive oxygen species (ROS), Salicylic Acid (SA) and stomata closure [4]. The rapid release of Reactive Nitrogen Intermediates (RNIs) and ROS, also known as oxidative burst, trigger the cross-linking of phenolic compounds and glycoproteins for cell wall reinforcement, synthesis of SA and activation of Mitogen-Activated Protein Kinase (MAPK) cascades [4, 7-10]. For instance, FLS2 receptor protein is acquainted to induce MTI-related responses such as oxidative burst and callose deposition [2, 3].

MAPK cascades are downstream of the surface-located PRRs, and the cascade intrinsically consists of MAP kinase kinase kinase (MAP3K) that phosphorylates two amino acids of the downstream MAP kinase kinase (MAP2K) activation loop [11]. MAP2Ks in turn again double phosphorylate the downstream MAP kinases with the purpose of targeting various enzymes, other kinases or transcription factors [12-14]. The activation of MAPKs alters a wide array of responses, including changes in gene expression, protein trafficking and hormone signaling [14]. One MAPK signaling cascade acting downstream of the FLS2-BAK1 receptor complex is the MEKK1 – MKK4/MKK5 – MPK3/MPK6 cascade, which allows the prompt flg22-induced expression of members of a large gene family of transcription factor, known as WRKY [12, 15, 16]. These transcription factors, such as WRKY22 and WRKY29, have a DNA binding domain that interact with the W-box (TTGAXX/T) motif of the promoters present in defense-associated genes [4].

In addition to flg22 or chitin, it was recently demonstrated that bacterial nucleic acids induce MTI and might be also considered as MAMPs. Intact RNA from *Pseudomonas syringae* pv tomato DC3000 (*Pst* DC3000) elicited superoxide production, callose deposition and induction of the MPK3 and MPK6 as defense responses in *Arabidopsis* leaves [17].

The next step in MTI is the expression of *Pathogenesis-Related* (PR) genes. Their products, PR-proteins, are defense proteins with antimicrobial activity, and they are present in many organisms, including plants, animals and nematodes. PR-proteins are generally absent or present at a basal level in healthy tissues, but they are increasingly synthesized under stress conditions or pathogen attack. Many PR-proteins (at least 17) have been identified in various plants, and their expression is

part of the local-induced defense mechanism that is initiated by transcription factors or signaling compounds, as well as a response of the systemic induced defense that is described below [18].

MTI can also be activated by human pathogens like *Salmonella enterica* or *Escherichia coli* O157:H7. Plants can sense multiple *Salmonella* MAMPs like flg22, LPS (carrying the *O*-antigen) and conserved regions of cold shock proteins (CSPs); however, the divergence of flg22 sequence in certain *Salmonella* strains provide evidence of MAMPs evolving in order to avoid the activation of plant immune system [19]. After 10 min of *Salmonella* recognition by FLS2, the stress-induced MPK3/MPK6 and MPK4 are activated, as well as the plant defense-associated genes *PDF1.2*, *PR1* and *PR2* [20, 21]. MTI-induced callose depositions can be observed 24 hours after perception. The defense hormones SA, Jasmonic Acid (JA) and Ethylene (ET) are involved in the interaction between *S. enterica* and plants. JA and ET-responsive genes are strongly induced after 2 hours, while SA accumulation can be observed 24 hours after triggering MTI with *Salmonella enterica* Typhimurium or *Salmonella*-derived flg22 [21]. The inhibitory role of ethylene against *Salmonella* colonization was demonstrated in *Medicago sativa* plants grown on medium supplemented with the ethylene precursor ACC (1-aminocyclopropane 1-carboxylic acid), where colonization rate was significantly lower, in comparison to control [22]. Accordingly, three genes involved in ethylene synthesis/degradation were up-regulated in *Medicago truncatula* plants infected with *S. enterica* Typhimurium.

1.1.2 Effector-triggered immunity (ETI)

The second branch of the immune system usually operates when successful pathogens have evolved mechanisms to suppress MTI. Effector proteins that are secreted into the host cytoplasm by Type 3 Secretion Systems (T3SS) exploit a great range of biochemical activities to modify host cell proteins, and contribute to pathogen's virulence.

To counteract effector proteins activity, plants evolved another branch of plant immunity, the so-called Effector-Triggered Immunity (ETI). Plant resistance proteins (R-proteins), which are intracellular receptor proteins of the Nucleotide-Binding

Leucine-Rich Repeat (NB-LRR) protein family, can directly or indirectly interact with avirulence gene products of pathogens. The guard hypothesis refers to the indirect recognition of pathogen effector proteins. R proteins guard a specific plant protein target and detect its changes following interaction with the effector protein. One example demonstrating this mechanism is the function of RPM1 (Resistance to *P. syringae* pv *maculicola* 1) and RPS2 (Resistance to *P. syringae* 2), which are two NB-LRR proteins that guard the effector target RIN4 (RPM1-interacting protein 4). Two unrelated *P. syringae* effectors, AvrB and AvrRpm1, can bind to RIN4 and promote its phosphorylation, thus activating RPM1-mediated immune responses. RPS2-derived defense responses arise from the perception of the cleavage and degradation of RIN4, which is caused by the cysteine protease activity of the effector protein AvrRpt2 [23]. NB-LRR proteins can recognize pathogen effectors from different kingdoms and induced resistance in a similar manner, however NB-LRR-mediated disease resistance is only effective against pathogens growing on living tissue, or in other words against obligated biotrophic and hemi-biotrophic pathogens [1]. For example, the effector protein SseF from *Salmonella* is recognized by a R-protein and elicits HR-like symptoms in *Nicotiana bentamiana*. A loss of symptoms was observed upon silencing of the suppressor *SGT1* (Suppressor of G2 allele of Skip1) and the plasma membrane NDR1 (non-race-specific disease resistance 1) [19, 24].

Responses downstream of ETI mostly converge with MTI signaling. Defenses responses such as the expression of ROS production, activation of MAPK signaling cascades, induction of WRKY transcription factors, PR-proteins and callose deposition are regulated by complex mechanisms that involved hormones crosstalk in both MTI and ETI, which suggests that the two immune systems are evolutionary interrelated and mechanistically interconnected [25].

1.1.3 Systemically Induced resistance

In general, induced defense is defined as an enhancement of effective basal resistance via specific biotic or abiotic stimuli experienced by the plant before the subsequent challenge with a pathogen [26]. Two additional forms of induced resistance are Systemic Acquired Resistance (SAR) and Induced Systemic Resistance

(ISR). SAR is assigned to the response in distal parts of the plant to fortify uninfected tissues against pathogen invasion [27]. SAR requires the accumulation of endogenous SA at the infection site. In this manner, SA is subsequently converted by SA methyltransferase BSMT1 into the mobile molecule methyl salicylate (MeSA) for long distance signaling via the plasmodesmata or phloem. In systemic leaves, MeSA is perceived and de-methylated into SA by MeSA esterase, and SA binds to the high-affinity SA receptor NPR4 (NPR1-like protein 4) to avoid degradation of NPR1 (Non-expresser of PR-1 gene). The expression of immune-related genes like TGA factors and Pathogenesis-related (PR) proteins, are activated by NPR1 to facilitate cell survival [27-29]. Likewise, lipid-based molecules e.g., azelaic acid can act as mobile signals in SAR, where is also important the presence of the protein DIR1 (Defective In Induced Resistance 1) [28].

Another type of induced resistance is Induced Systemic Resistance (ISR), which is mediated by Plant growth-promoting rhizobacteria (PGPR), such as *Pseudomonas fluorescens* and *Bacillus* spp. ISR, unlike SAR, does not relay in the accumulation of PR-proteins or SA, but instead depends on JA and ET signaling pathways. Both hormones act synergistically for the activation of defense responses, where JA and derivatives boost the expression of defense-related genes like thionins [30, 31]. The root-specific transcription factor MYB72 (MYB domain protein 72) is a fundamental convergence point in ISR that is activated by beneficial microorganisms. MYB72 is suggested to require other signaling elements because its overexpression does not enhance resistance towards foliar pathogens [32, 33]. In addition, NPR1 is needed for JA/ET-dependent ISR triggered by many PGPR, such as *P. fluorescens* WCS417r and some plant growth-promoting fungi (PGPF). While NPR1 is connected to a nuclear function in SA signaling, NPR1 might function in the cytosol in JA/ET signaling and ISR. Simultaneous activation of SAR and ISR leads to an enhanced immune system, however the molecular mechanism of NPR1 in ISR needs to be further elucidated [34]. Moreover, despite the strong relationship between beneficial microorganism that induced ISR independently from SA, it has been demonstrated that some bacterial strains like *Pseudomonas aeruginosa* 7NSK2 may confer enhanced resistance in bean and tomato plants but not in a non-accumulating SA tomato mutant (*NahG*), suggesting that SA plays a role in induced resistance by this

bacterium. *Paenibacillus alvei* K165 and *P. fluorescens* SS101 can also induce SA-dependent SAR [33].

1.1.4 Priming

ISR is based on enhanced sensitivity to JA and ET rather than on an increased production of these hormones. Priming is defined as a sensitization process for enhanced resistance in the whole plant. Primed plants show relatively mild transcriptional changes in comparison to the transcriptional reprogramming following a pathogen attack. The primed state is almost not detectable unless plants are challenge with a pathogen to reveal the faster and stronger activation of cellular defense responses, which are typical characteristics of priming [35]. An example is the fungus *Trichoderma asperellum* that colonizes cucumber roots. This commercially biocontrol microbe induces defense priming and ISR against *Pseudomonas syringae* pv. *lachrymans*. The *Trichoderma*-induced priming is associated with the systemic gene expression of the orthologous of *AtMPK3*, the *Trichoderma*-induced protein kinase (*TIPK*) [36].

Priming is also associated to SAR, where plants present accumulation of MPK3/6 mRNA transcript and inactive proteins after treatment with benzothiadiazole (BTH), a SA analog and SAR activator. Furthermore, MPK3 might have a greater role than MPK6 in priming and SAR, however both enzymes are needed for the full priming and SAR effect. The enhanced expression of *PR1* and *PAL1* (*Phenylalanine ammonia-lyase 1*), a marker gene for SAR, is related to the increased of both MPK3 and MPK6 activities [37].

Molecular modifications of histones might participate as a memory effect after the primary infection that is intensified after a second stress stimulus. A proposed theory that could explain the rapid and stronger defense genes responses includes chromatin modifications, which are important for gene expression regulation. Acetylation of histones H3 and H4, as well as trimethylation of H3K4 on promoters and coding sequences are correlated to gene activation. In *Arabidopsis* plants, priming of *WRKY29* promoter is associated with the trimethylation and dimethylation of H3K4, and the acetylation of H3K9, H4K5, H4K8 and H4K12;

however, these modifications do not induce *WRKY29* transcription. Interestingly, other *WRKY* gene promoters have increased histone modifications following a pathogen challenge or BTH treatment [38]. The exposure to the pathogen induces systemic signals corresponding to SAR that are stored in distal leaves as histone alterations, and that could serve as a quick preparation in transcription before a stressful situation [38]. Moreover, priming appears to be a transgenerational phenomenon with an epigenetic basis. Progeny from *Pst*-inoculated *Arabidopsis* plants can be primed to induce SA defense genes against biotrophic pathogens. *NPR1* expression is required for transgenerational SAR and can be observed even after one stress-free generation [39, 40].

1.2 Quorum-sensing (QS) molecules

In agriculture, alternatives including integrated farming practices, resistant hybrids, transgenic crops and biological control are required for sustainable disease management. Induced resistance via natural molecules or biologicals is an interesting strategy resulting in a broad-spectrum and possibly long-lasting alternative, even though effectiveness might vary from 20 to 85% depending on the agent [41, 42]. Chemical priming is currently used in practice with the advantage of obtaining lower fitness costs than direct defense activation [43]. BABA (β-aminobutyric acid) is an example of a priming-inducing chemical that acts synergistically with diverse fungicides, and thus important for integrated plant disease management [44]. Another example is the compound BTH, which induces SA-defense responses that lead to SAR and offers protection against bacterial, fungal and viral diseases in monocot and dicot plants [42]. On the other hand, plants can activate defense responses after detection of secondary metabolites from beneficial bacteria. The metabolites from microbial inoculants might be responsible for the biocontrol activity, which is characterized by the inhibition of pathogens and enhanced systemic resistance [45]. The naturally occurring and plant-root colonizing *Bacillus amyloliquefaciens* FZB42 is able to stimulate plant growth and inhibit stem canker- and black scurf-causing pathogens in potato plants due to its capacity to synthesize antibiotic and antifungal metabolites, including polyketides and

lipoproteins [31]. Surfactin, for example, is a cyclic lipopeptide from *Bacillus* spp. that activates early defense responses and ISR in tobacco and tomato plants against the necrotrophic pathogen *Botrytis cinerea* [46].

Bacterial quorum sensing (QS) compounds are also important modulators of microbe-plant interactions. Cell-to-cell communication molecules are released into the cellular environment to sense the density and distribution of a bacterial population. This bacterial sensing mechanism optimizes the fitness of the population by regulating gene expression according the environmental conditions [45]. QS-controlled behaviors such as bioluminescence, virulence and biofilm formation are unproductive when they are initiated by an individual bacterium, but can become very effective with increasing bacterial density [47]. QS can occur between and among species; for example, using a mouse chronic lung infection model, *Burkholderia cepacia* was shown to perceived the AHLs produced by *Pseudomonas aeruginosa in vitro* and *in vivo* but the effect was not observed vice versa [48]. Gram-positive bacteria usually produce oligopeptides and furanosyl borate diesters as autoinducers (the former name for QS molecules). Gram-negative bacteria produce *N*-acyl homoserine lactones (AHLs), which are small lipid molecules containing a homoserine lactone residue coupled to an acyl side chain. The divergence of these signaling molecules is related to the acyl side-chain length (4 to 18 Carbon atoms), a substitution at the C3 position and the saturation level within the side chain [49].

The *Vibrio fischeri* LuxRI system is used as a model to describe AHL-based quorum sensing. AHLs are synthesized by the cytoplasmic LuxI synthase, and accumulate intra- and extracellularly since these molecules can diffuse passively through the bacterial membrane. At a stimulatory concentration, AHL molecules form a complex with the AHL-receptor protein LuxR and promote the expression of QS-regulated genes [47].

Many pathogenic bacteria rely on the production of QS molecules to induce the virulent phenotype. The broad-host-range pectobacteria are the best-characterized group of phytopathogens that depend on AHLs to regulate the production of plant cell wall-degrading enzymes. Furthermore, *Pseudomonas syringae* pv. *tabaci* produces 3-oxo-C6-HSL and C6-HSL to promote virulence and acquire iron [50]. On the other hand, not all QS-derived molecules have a negative impact on plants, and

plant responses might vary according to the length of the acyl chain of AHLs. The short-chain C6-HSL increases transpiration and stomatal conductance in *Phaseolus* vulgaris, and plant growth in *Hordeum vulgare*. *Arabidopsis* plants have enhanced lateral root formation in the presence of C10-HSL. Moreover, AHLs from *Serratia liquefaciens* (C6-HSL), *Serratia plymuthica* (oxo-C6-HSL), *P. aeruginosa* (oxo-C12-HSL) and *Sinorhizobium meliloti* (oxo-C14-HSL, oxo-C16-HSL) can trigger defense responses [51-54].

1.3 Aims of the thesis

The objective of this work was to elucidate the mechanism by which oxo-C14-HSL enhances resistance in different crop plants against a broad pathogen spectrum, including the human pathogen *Salmonella enterica* serovar Typhimurium. Understanding the mode of action of the AHL molecule is a fundamental step for further agricultural applications. Therefore, this work describes the priming responses in *Arabidopsis*, barley, wheat, tomato and alfalfa plants after treatment with the oxo-C14-HSL-producing *S. meliloti* bacterium and a secondary challenge. The results of this thesis demonstrated that the long AHL molecule has a positive effect on plant defense, and the mechanism is based on a salicylic acid/oxylipin-dependent systemic signal. AHL-induced resistance mainly triggers ROS production, activation of defenses-related genes, cell wall reinforcement and stomata responses. Likewise, the presence of *S. meliloti* producing oxo-C14-HSL lowers the proliferation of the human pathogen *Salmonella enterica* serovar Typhimurium in *Arabidopsis* plants. Conversely, *Salmonella* proliferation was not affected when the AHL-producing rhizobacteria forms a symbiotic relationship with its native host *Medicago sativa*, and similar results to the control were observed.

Moreover, one strategy used by *Salmonella* for a successful infection is the injection of effector proteins that suppress the plant immune system. For this reason, an additional objective of this work was to study the translocation of effector proteins into *Arabidopsis* plant cells, and to elucidate the involvement of several *S.* Typhimurium effector proteins, especially the phosphothreonine lyase SpvC. For this purpose, three effector proteins, SpvC, AvrA and SptP, were cloned to prove their

translocation into plant cells (the experiments are still in process). We could show that SpvC reduced activation of plant immune responses by deactivating active *At*MPK3 and *At*MPK6, and that this effector protein is required for full virulence of *Salmonella* in plants.

2 Materials and Methods

2.1 Cloning

Plant and bacterial genes were amplified from cDNA in a two-step PCR reaction. The first PCR was performed using ORF-specific primers and the second PCR using primers containing the attB recombination sites for the Gateway system.

First PCR reaction setup

cDNA	2 µL	
Phusion HF Buffer (5x)	10 µL	(New England Biolabs)
dNTPs [2mM]	2 µL	(Thermo Scientific, Germany)
Gene FWD [10mM]	2.5 µL	(*see* Supplmental Table 1)
Gene REV [10 mM]	2.5 µL	
Milli-Q water	30.5 µL	
Phusion DNA polymerase	0.5 µL	(New England Biolabs)
Total reaction volume	50 µL	

Second PCR reaction setup

First PCR reaction product	10 µL	
Phusion HF Buffer (5x)	10 µL	(New England Biolabs)
dNTPs [2mM]	2 µL	
U3 primer [10mM]	2.5 µL	(*see* Supplmental Table 1)
U5 primer [10 mM]	2.5 µL	
Milli-Q water	22.5 µL	
Phusion DNA polymerase	0.5 µL	(New England Biolabs)
Total reaction volume	50 µL	

Thermocycler setup (35 cycles)

	Initial denat.	**Denat.**	**Annealing**	**Extension**	**Final ext.**	**Cooling**
Temperature	98 °C	98 °C	50 °C	72 °C	72 °C	4 °C
Time	5 min	15 sec	30 sec	--- min	5 min	∞

A-tailing reaction setup

PCR product	8 µL	
10x BD Buffer	1 µL	(Promega, Germany)
10 mM dATPs	1 µL	(Promega, Germany)
2 mM $MgCl_2$	0.5 µL	
Taq (DCS polymerase)	0.5 µL	

The reaction was incubated for 15 min at 70 °C

pGEM-T Ligation reaction setup

PCR amplicon	4.5 µL

Ligation Buffer (2x)	10 µL	(Promega, Germany)
pGEM-T easy vector	1 µL	(Promega, Germany)
Milli-Q water	3.5 µL	
T4 DNA ligase	1 µL	

The reaction was incubated overnight at 16 °C

2.1.1 Gateway cloning

The amplified genes containing the attB sites were transfer into multiple types of vectors. The first vector used for this system is the entry vector, which requires a BP reaction to generate entry clones.

BP reaction setup

Second PCR amplicon	--- µL	
TE-Buffer	--- µL	
Gateway Entry vector PDONR207 (Gentamycin resistance)	1 µL	(Invitrogen)
BP Clonase	1 µL	(Invitrogen)
Total volume	5 µL	

Incubated overnight at room temperature

Proteinase K	1µL	(Invitrogen)

Incubated overnight at 37 °C for 10 min

BamHI digestion

Colony PCR amplicon	5 µL	
BamHI buffer	1 µL	(Fermentas, Germany)
BamHI enzyme	1 µL	
Milli-Q water	3 µL	
Total Volume	10 µL	

Incubated at 37 °C for 2 hours

The PCR amplicon is combined with PEG/$MgCl_2$ buffer and TE-buffer (pH 8)

TE buffer	**(For 50mL)**	
1M Tris (pH 8.0)	5 mL	(Carl Roth, Germany)
0.5M EDTA (pH 8.0)	1 mL	(Carl Roth, Germany)
dd H_20	44 mL	

The gene of interest previously cloned into the Gateway entry vector was transferred into Gateway destination vectors or to Gateway-compatible binary vectors via LR reaction from Invitrogen.

LR reaction setup

TE-Buffer	--- µL	
Gateway Entry vector PDONR207 (Gentamycin resistance)	--- µL	(Invitrogen)
Gateway Destination vector*	--- µL	
LR Clonase	1 µL	(Invitrogen)
Total volume	5 µL	

Incubated overnight at room temperature

Proteinase K	1 µL	(Invitrogen)

Incubated overnight at 37 °C for 10 min

Colony PCR reaction setup

Salmonella enterica str. 14028s genomic DNA	pEC75 1 µL	
10x BD Buffer	2.5 µL	(DNA cloning Service, Germany)
2mM dNTPs	2.5 µL	(Fermentas, Germany)
FWD specific primer	1.5 µL	(see Supplmental Table)
REV specific primer	1.5 µL	
Milli-Q water	14 µL	
2 mM $MgCl_2$	2 µL	(DNA cloning Service, Germany)
Taq (DCS polymerase)	0.13 µL	(DNA cloning Service, Germany)
Total reaction volume	25 µL	

Thermocycler setup (35 cycles)

	Initial denat.	**Denat.**	**Annealing**	**Extension**	**Final ext.**	**Cooling**
Temperature	95 °C	94 °C	(see Table)	72 °C	72 °C	4 °C
Time	5 min	30 sec	30 sec	--- min	7 min	∞

2.2 Cell transformation

2.2.1 DH5α competent cells

From a 20 mL single-colony DH5α cell preculture, three 100 mL SOC medium bacterial cultures were prepared (start OD_{600}≈0.05) and were grown at 19ºC until an OD_{600}≈0.5. The cultures were transferred to falcon tubes to pellet the bacteria at 4000 rpm, and then incubated on ice for 10 min. The bacteria were washed in 20 mL ice cold TB buffer per 100 mL culture and incubated on ice for 10 min. Centrifugation step was repeated and bacteria were resuspended in 10 mL TB buffer. DMSO buffer was prepared by mixing 1.4 mL DMSO in 8.6 mL TB buffer, and combined with the

bacterial solution. After 10 min incubation on ice, 200 µL aliquots were prepared and directly frozen with liquid Nitrogen.

SOB medium	**For 1L**	
Tryptone	20 g	(Carl Roth, Germany)
Yeast extract	5 g	(Carl Roth, Germany)
10 mM NaCl	0.6 g	(Carl Roth, Germany)
2.5 mM KCl	10 g	(Carl Roth, Germany)
10 mM $MgCl_2$	2 g	
10 mM $MgSO_4$	2.5 g	
In ddH_2O		
Adjust pH to 7.0 with NaOH		

SOC medium	**For 1L**	
Sterile SOB medium		
20 mM Glucose	7.2 g	(Carl Roth, Germany)
In ddH_2O		
Adjust pH to 7.0 with NaOH		

2.2.1.1 Bacterial transformation (Heat-shock)

The BP or LR reaction mixes were combined with 75 µL of DH5α competent cells, and incubated on ice for 15 min The cells were heat-shocked for 45 sec at 42 °C, and shook for 2 hours at 37 °C after adding 950 µL of SOC medium. The transformed cells were centrifuged for 3 min at 5000 rpm, resuspended in 200 µL SOC medium and plated on selective-LB plates. After O/N incubation at 37 ºC, colony PCR and agarose gel electrophoresis were performed to verify the presence of the plasmid in bacteria using as forward primer part of the promoter sequence of the vector, and as reverse primer the end sequence of the gene of interest (see Supplemental Table).

A second bacterial transformation using BL21 cells was required for the protein expression vectors, pDEST17 and pDEST15.

LB medium agar	**For 1L**	
Tryptone	5 g	(Carl Roth, Germany)
Yeast extract	10 g	(Carl Roth, Germany)
NaCl	0.5 g	(Carl Roth, Germany)
Agar	10 g	(Carl Roth, Germany)
In ddH_2O		
Adjust pH to 7.0		

2.2.1.2 Bacterial Transformation (electroporation)

A bacterial cell was selected as a starter for a liquid preculture, which is grown at 37ºC overnight. Subsequently, the starter is used to inoculate 100 mL SOC liquid media (start OD_{600}≈0.05) until an OD_{600}≈0.5 is reached. The liquid culture is then centrifuged at 4000 rpm and washed 1x with 20 mL ice-cold H_20 and 1x with 4 mL ice-cold 10% glycerol. Finally, the pellet is resuspended in 2 mL 10% glycerol and the 100µL aliquots are frozen immediately with liquid Nitrogen.

2.2.2 Arabidopsis thaliana protoplasts

An *Arabidopsis thaliana* green cell culture was obtained from the Max Planck Institute for Plant Breeding Research (Cologne, Germany). Cell cultures were invigorated every week by taking 5 mL of plant cell culture into 45 mL fresh Arabidopsis-cell growing medium. For protoplast isolation, five-day-old cell cultures were centrifuged at 1500 rpm for 2 min. The cells pellet (around 15 mL) were then resuspended in 50 mL enzyme solution and incubated in round petri dishes with slow shaking under dark conditions until >95% of the cells formed individual round shaped protoplast (1 ½ -2 hours). Protoplasts were transfer to 50 mL falcon tubes and centrifuged for 15 min at 1000 rpm; protoplasts in optimal conditions floated to the top after centrifugation. Subsequently, protoplasts were transfer to 15 mL falcon tubes using a cut-pipette tip and washed twice with protoplast culture medium. Centrifugation step was repeated but for 7 min. The floating protoplasts were resuspended in MMG solution to obtain ~ 2-5 x 10^6 protoplasts/ mL, and incubated on ice for ½ hour before transformation [55, 56].

***Arabidopsis*-cell growing medium**	**For 1L**	
MSMO	4.4 g	(Sigma-Aldrich, Germany)
Adjust pH to 5.7. Autoclave		
Sucrose	30 g	(Carl-Roth, Germany))
Myo-inositol	100 mg	(AppliChem GmbH, Germany)
Thiamine	0.4 mg	(Sigma Aldrich, Germany)

Filter sterilize and add to autoclaved medium

Kinetin (stock: 1 mg/mL in DMSO)	100 µL	(Duchefa Biochemie, The Netherlands)
NAA (stock: 1 mg/mL in ethanol)	500 µL	(Duchefa Biochemie, The Netherlands)

Protoplast culture medium	**For 200 mL**
MSMO	0.86 g
0.4 M Sucrose (13.7%)	27.38 g
MES	100 mg
$CaCl_2 \bullet 2H_2O$	150 mg
NH_4NO_3	50 mg
Adjust pH to 5.7	

Enzyme solution	**For 50 mL**	
Cellulase Onozuka R10 (1%)	0.5 g	(Duchefa Biochemie, The Netherlands)
Pectolyase Y-23	0.1 g	(Duchefa Biochemie, The Netherlands)

Dissolve in protoplast culture medium overnight at 4 ºC with slow agitation

MMG solution	**For 200 mL**
4 mM MES	156 mg
0.4 M Mannitol	7.8 mg
15 mM $MgCl_2$	285.6 mg
Adjust pH to 5.7	

2.2.2.1 Protoplast transformation

In a 2mL eppendorf tube, 100 µL protoplasts (2 $x10^5$ protoplasts/mL) were mixed with 10 µL (7 µg) of pAUL17, which is an HA-tagged plant protein expressing vector [57], or with pAUL17::*AvrA*. For transformation, 150 µL PEG solution was added and mixed with the protoplasts by ticking against the tube. After 15 min at room temperature and in dark, protoplasts were washed with 1.24 mL 0.275 M $Ca(NO_3)_2 \bullet 4H_2O$ and mixed by inverting the tube a couple of times. The transformed protoplasts were spin-down for 5 min at 800 rpm. The supernatant was removed and protoplasts were incubated overnight under dark conditions with 500 µL of after-transformation solution; the eppendorf tube needed to be inclined for incubation.

PEG solution	**For 10 mL**
PEG 6000	3 g
Mannitol	0.82 g
$Ca(NO_3)_2 \bullet 4H_2O$	0.24 g

Adjust pH to 9.0 with KOH

After transformation solution	**For 200 mL**
4 mM MES	156 mg
0.5 M Mannitol	18.22 mg
20 mM KCl	298.24 mg
Adjust pH to 5.7	

2.3 Protein biochemistry studies

2.3.1 Protein expression

Proteins were expressed in BL21 *E. coli* cells containing the Gateway pDEST17 or pDEST15 protein expression vectors. A small bacterial culture of each plasmid was incubated overnight at 37 °C in LB-ampicillin medium and used for a 100 mL culture (start OD_{600}≈0.05) that was induced with IPTG (1µL/mL culture) when the OD_{600} approached 0.6. The culture was incubated overnight at room temperature, and subsequently the cells were collected by centrifugation at 4000 rpm and 4 °C for 10 min. To verify the expression of the protein, the pellet (50 mL cell culture) is resuspended in 1.5 mL lysis buffer containing lysozyme (GST-tag) or 5 mL of lysis buffer (His-tag). Later, β-mercaptoethanol and sarkosyl (also lysozyme, RNase A and protease inhibitor for His-tag) are added to the lysate before rotating overnight at 4 °C. Sonication was performed 3x for 5 min with 5 min on ice in between. Finally, the lysate is centrifuge for 15 min at 4 ºC and 4000 rpm. Silver or Coomasie staining confirmed the expression of the proteins.

Lysis buffer (GST-tag)		
50 mM Tris-HCl (pH 7.5)		(Carl Roth, Germany)
5 mM $ZnCl_2$		
300 mM NaCl		
Lysosyme (1 mg/mL)		
+ β-mercaptoethanol		
(working concentration: 10 mM)		
+ Sarkosyl (stock: 400 mg/mL)	0.5 mL	

Lysis buffer (His-tag)	**For 100 mL**	
20 mM Sodium phosphate	284 mg	(Carl-Roth, Germany)
2 mM $MgCl_2$	40.66 mg	

100 mM NaCl	584 mg
20 mM imidazole	36.16 mg
Adjust pH to 7.4	
	For 20 mL
+ β-mercaptoethanol	200 µL
+ Protease inhibitor	1 tablet
+ Lysosyme (1mg/mL)	
RNase A	200 µL

SDS Gel 12%

Resolving gel

Tris-HCl 1.5 M (pH 8.8)	2.5 mL	
ddH_2O	3.4 mL	
Acrylamide solution (30%)	4.0 mL	(Carl Roth, Germany)
SDS (0.1%)	100 µL	(Carl Roth, Germany)
TEMED	10 µL	(Sigma-Aldrich, USA)
Ammonium persulphate (10%)	100 µL	(Carl Roth, Germany)

Stacking gel

Tris-HCl 0.5 M (pH 6.8)	600 µL
ddH_2O	3.6 mL
Acrylamide solution (30%)	1 mL
SDS (0.1%)	50 µL
TEMED	10 µL
Ammonium persulphate (10%)	50 µL

Running buffer (pH 8.3)

Tris	25 mM
Glycine	192 mM
SDS	0.1% w/v
In ddH_2O	

Silver staining

Solution A (Fixing)

Ethanol	40%
Acetic acid	10%

Solution B (Adjusting)

Sodium thiosulphate	200 mg/L

Solution C (Staining)

Silver nitrate 2g/L	
Formaldehyde	750 µL/L

Solution D (Developing)

Sodium carbonate	30 g/L
Formaldehyde	500 µL/L
Sodium thiosulphate	4 mg/L

Solution E (Conserving)

Ethanol	25%	
Glycerol	3%	(Carl Roth, Germany)
Solution wash/stop		
Acetic acid	5%	

The SDS gels were fixed in solution A for 2 hours or overnight. Then the gels were washed 1x with 30% ethanol and 1x with water for 20 min each. Incubation with solution B for 1 min was performed before washing again 3x times with water. The gels were developed in solution D until bands were visible. The reaction was stopped with stop solution for 2 min, and finally the gels were conserved in solution E overnight.

Coomasie fast staining

Solution A	
Coomasie R250	0.05%
Isopropanol	25%
Acetic acid	10%
Solution B	
Coomasie R250	0.05%
Isopropanol	10%
Solution C	
Coomasie R250	0.002%
Acetic acid	10%
Solution D	
Acetic acid	10%

The SDS gels were submerged in Solution A and simmered for around 2 min using the microwave. Gels were cooled down at RT and washed with water. The same steps were repeated for solutions B, C and D.

2.3.2 Protein purification

The induced pellet from the pDEST15 vector was purified following the GST-tagged protein purification form Macherey-Nagel, which consists in binding the GST-tagged protein in a column containing glutathione agarose, which is equilibrated with PBS bufferbefore adding the lysate. The column is washed twice with PBS, followed by two elution steps.

PBS buffer (GST-tag)	**For 500 mL**	
10 mM Na_2HPO_4	0.89 g	(Carl Roth, Germany)
1.8 mM KH_2PO_4	0.122 g	(Carl Roth, Germany)
2.7 mM KCl	0.10 g	(Carl Roth, Germany)
140 mM NaCl	4.091 g	
Sarkosyl (2%)	10 g	
Titron X-100 (4%)	20 g	
In ddH_2O		
Adjust pH to 7.3		

Elution buffer (GST-tag)	**For 200 mL**	
50 mM Tris base	1.21 g	(Carl Roth, Germany)
10 mM Glutathione	0.614 g	(Serva, Germany)
Sarkosyl (2%)	4 g	
Titron X-100 (4%)	8 g	
In ddH_2O		
Adjust pH to 8.0		

For the His-tagged proteins, the induced pellet from the pDEST17 plasmid was purified with equilibrated columns enclosing Ni-NTA resin (QIAGEN) under native conditions. For equilibration, 750 µL of the resin was centrifuged at 500 rpm for 5 min, and then washed with 5 mL binding buffer before another step centrifugation. The lysate was incubated together with the equilibrated beads for 1 hour at 4 ºC with continuous rotation. Subsequently, columns were equilibrated with 5 mL binding buffer, and the lysate-bead mix was added. The columns were washed 1x time with 4 mL washing buffer and eluted 4x with 0.5 mL elution buffer.

Binding buffer (His-tag)	**For 100 mL**	
20 mM Sodium phosphate	284 mg	(Carl-Roth, Germany)
500 mM NaCl	2.92 g	
20 mM imidazole	36.16 mg	
Sarkosyl (2%)	2 g	
Adjust pH to 7.4		

Washing buffer (His-tag)	**For 500 mL**	
50 mM NaH_2PO_4	3.45 g	(Carl Roth, Germany)
300 mM NaCl	8.77 g	(Carl Roth, Germany)
20 mM imidazole	0.68 g	(Carl Roth, Germany)
Sarkosyl 2%	10 g	
Triton X-100 (4%)	20 g	
In ddH_2O		
Adjust pH to 8.0		

Elution buffer (His-tag)	**For 500 mL**	
50 mM NaH_2PO_4	3.45 g	(Carl Roth, Germany)
300 mM NaCl	8.77 g	(Carl Roth, Germany)
250 mM imidazole	8.5 g	(Carl Roth, Germany)
Sarkosyl 2%	10 g	
Triton X-100 (4%)	20 g	
In ddH_2O		
Adjust pH to 8.0		

After confirming with a 12% SDS gel that the eluate contains the desire protein, GST- and His-tagged proteins were dialyzed overnight.

Dialysis buffer	**For 2L**
50 mM NaH_2PO_4	13.8 g
20 mM NaCl	1.2 g
In ddH_2O	
Adjust pH to 8.0	
Dialyse at 4 ºC with slow agitation overnight	

2.3.3 Western blot analysis

Frozen leaves samples treated with flg22, chitin or bacteria were shattered for 20 sec in a tissue lyser (QIAGEN). Total proteins were extracted by vigorously vortexing with LACUS buffer and centrifuged at 14,000 rpm at 4 °C for 20 min; protein concentration of the supernatant was measured by Bradford (BioRad, protein assay). An equal amount of proteins of each sample was loaded and separated on a 12% SDS-polyacrylamide gel (BioRad mini format 1-D electrophoresis system), and a BioRad semi-dry blotting system was used for protein transfer on a polyvinylidene fluoride (PVDF) membrane. Western blot analyses were done using specific antibodies (see Table 1). Immunodetection was done using Super signal® west Pico chemiluminescent substrate.

LACUS buffer		
Tris HCl (pH 7.8)	25mM	
$MgCl_2$	10mM	
EGTA	15mM	(Carl Roth, Germany)
NaCl	75mM	
DTT	1mM	
+ Protease inhibitor	1 tablet	(Roche, Germany)

+ Phosphatase inhibitor 1 tablet (Roche, Germany)

Towbin buffer

Tris base	25mM	
Glycine	192mM	
Methanol	20%	(Carl Roth, Germany)

PBS buffer

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Adjust pH to 7.4	

PBS-Tween buffer (For 1L)

PBS buffer	1L
Tween-20	0.5 mL

TBS buffer (10x)

Tris base	50mM
NaCl	150mM
Adjust pH to 7.4	

TBS-Tween buffer (1x) (For 1L)

TBS buffer (10x)	100mL
Tween 20	1mL

Table 1. Antibodies used for Western blot

Rabbit anti-MPK6	1:5000	(Sigma-Aldrich, Deisenhofen, Germany)
Rabbit anti-phospho-p44/42	1:1000	(Cell Signaling, Danvers, USA)
Goat anti-Rabbit (HRP)	1:10000	(Pierce Thermo Scientific, USA)
Mouse anti-His	1:1000	(Dianova, Germany)
Rabbit anti-GST	1:1000	(Dianova, Germany)
Mouse anti-cyaA	1:1000	(Santa Cruz Biotechnology Inc., USA)
Rabbit anti-mouse (HRP)	1:1000	(Antibodies-online Inc., USA)
Rat anti-HA	1:1000	(Roche, Germany)
Goat anti-rat (HRP)	1:1000	(Pierce Thermo Scientific, USA)

2.4 Protein interactions studies

2.4.1 Pull-down (Co-IP) assay

Putative AHL-interacting plant proteins were purified and immunoprecipitated together with a biotin-labeled oxo-C14-HSL (M4) to prove their interaction. First, the M4 molecules were immobilized with streptavidin beads (sepharose conjugate) and BSA was added to minimize protein unspecific binding; biotin was used as negative control. Then, the modified molecules were incubated with the GST-/His-tagged plant protein, and the interaction was detected via western blot using GST or His specific antibodies (see Table 1).

Washing (beads)

Lacus buffer	400 µL	
Streptavidin beads	20 µL	(Cell Signaling Technology, Germany)
BSA (10 mg/mL)	15 µL	

Rotate for 30 min. Centrifuge for 2 min at 1000 rpm and at 4 ºC.
Discard supernatant

Coating

Lacus buffer	250 µL
6 mM M4 or Biotin	30 µL
BSA (10 mg/mL)	15 µL

Rotate for 30 min. Centrifuge for 2 min at 1000 rpm and at 4 ºC.
Discard supernatant

Protein incubation

Lacus buffer	250 µL
1 µg GST-/His-tagged Plant protein	30 µL
BSA (10 mg/mL)	15 µL

Rotate overnight at 4 ºC. Centrifuge for 2 min at 1000 rpm and at 4 ºC.
Wash 4x with Lacus buffer.

2.4.2 Split-YFP assay

For the bimolecular fluorescence complementation assay (BiFC analysis), AvrA effector protein, AtMKK1 and AtMKK4 were cloned into the pBiFC2 vector (N-terminal and C-terminal half of YFP). Empty vectors expressing split YFP domains were used as controls. The subcellular localization of the interaction was visualized

using a confocal laser-scanning microscope (Leica SP8) after transiently expression of the proteins via particle bombardment.

2.4.1.1 Particle bombardment

Arabidopsis thaliana leaf epidermal cells were transiently transformed with 1.5 µg GFP-fusion constructs (p2GF7.0 vector) under control of the *35S* promoter *via* particle bombardment; the mCherry expression vector (0.5 µg) was used as transformation marker. Tungsten particles (25 mg/mL) were coated with the purified expression vectors, in the presence of 1 mM $CaNO_3$. The coated particle mix was incubated at room temperature for 10 min and centrifuged briefly. Subsequently, 20 µL per shot of the supernatant was removed before sonication for 10 sec. The amount used of coated particles per shot was 5.9 µL.

The particle bombardment of *A. thaliana* leaves (placed on 1% agarose Petri-dishes) was performed in a vacuum chamber at -0.8 bar with a helium-driven particle accelerator operated at a pressure of 10 bar. After bombardment, the leaves were kept under low-light conditions at room temperature for 24 hours. The mCherry and GFP presences were observed in a fluorescence (Axioplan 2, Zeiss, Germany) and confocal microscope (Leica SP8).

2.5 Plant growth conditions

2.5.1 Arabidopsis thaliana

A. thaliana Col-0 seeds (NASC ID: N70000) were surface sterilized (3 min 50% ethanol with 0.5% Tritron X-100, briefly rinsed with 95% ethanol) and directly placed for germination on Petri dishes with ½ Murashige and Skoog medium (Sigma Aldrich, Germany), including vitamins and 0.4% gelrite; the plants were grown at 22 °C with 150 mmol/m^2 light in a photoperiod of 8 h light and 16 h dark (PERCIVAL SCIENTIFIC CLF).

½ MS medium For 1L

MS with vitamins	2.2 g	(Duchefa Biochemie, Netherlands)
Sucrose	10 g	(Sigma-Aldrich, Germany)
MES hydrate	0.5 g	(Sigma-Aldrich, USA)
NaCl [140 mM]	8.182 g	

In ddH_2O
Adjust pH with KOH to 5.4

2.5.2 Hordeum vulgare and Triticum aestivum

Barley (*Hordeum vulgare* cv. Golden Promise) and wheat (*Triticum aestivum* cv. Bobwhite) seeds were dipped shortly in sterile water and subsequently in 70% ethanol. Then, the seeds were immersed for 90 min in 6% sodium hypochlorite with continuous stirring. Seeds were rinsed two times with sterile water pH 3.0 and several times with sterile water pH 7.0 until no trace of sodium hypochlorite was detected.

Barley and wheat plants were grown on soil for pathogenesis assays or under sterile conditions for transcriptional analyses at 19 ºC and long day photoperiod. For the sterile system, plants were grown in one-liter glass jars containing partially solidified 1/10 strength plant nutrient medium (PNM).

PNM medium (1/10)	**For 1L**	
1M KNO_3	0.5 mL	(Carl Roth, Germany)
1M KH_2PO_4	0.05 g	(Carl Roth, Germany)
1M K_2HPO_4	0.025 g	(Carl Roth, Germany)
1M $MgSO_4 \bullet 7H_2O$	2 mL	
1M $Ca(NO_3)_2$	0.2 mL	
1M Fe-EDTA *	2.5 mL	
1M NaCl	0.025 g	(Carl Roth, Germany)
Gelrite	4 g	

In ddH_2O
Adjust pH with KOH to 5.6

*** Fe-EDTA**

$FeSO_4 \bullet 7H_2O$	2.2 g	(Roth, Germany)
dd H_2O	10 g	
Na_2EDTA	0.5 g	(Carl Roth, Germany)

Bring to boil. Stir t=30 min while cooling.
Bring to final volume if 450 mL

2.5.3 Solanum lycopersicum

Tomato (*Solanum lycopersicum* cv. Moneymaker) seeds were surface sterilized and germinated as described for *Arabidopsis* seeds. Seedlings were transfer to soil and grown at 25°C, 80% humidity and long photoperiod.

2.5.4 Medicago sativa

Alfalfa or lucerne (*Medicago sativa*) seeds were surface sterilized with 70% ethanol for 35 min, following 3x washing with sterile H_2O. Additionally, seeds were treated with sodium hypochlorite (6%) and washed again 8x with sterile H_2O. After sterilization, seeds were vernalized on 1% gelrite plates (supplemented with 1µg/mL GA) for 3 days at 4 ºC under dark conditions. For germination, seeds were transfer to BNM-gelrite plates for one day under in dark and lastly transferred to soil.

BNM medium	**For 1L**	
1 M $Ca(SO_4)_2$	2 mL	(Carl Roth, Germany)
1 M KH_2PO_4	0.5 mL	(Carl Roth, Germany)
0.1 M $ZnSO_4 \bullet 7H_2O$	160 µL	(Carl Roth, Germany)
1 M $MgSO_4 \bullet 7H_2O$	0.5 mL	
1 M HBO_3	5 µL	
1 M Fe-EDTA	2.5 mL	
1 M $MnSO_4$	5 µL	(Carl Roth, Germany)
0.5 M $Na_2MoO_4 \bullet 2H_2O$	2 µL	
0.1 M $CuSO_4$	10 µL	
0.1 M $CoCl_2 \bullet 7H_2O$	10 µL	
2 mM MES	390.5 mg	
Gelrite	4 g	
In ddH_2O. Adjust pH to 6.5 and autoclave		
+ 10 µM **STS**	0.5 mL (stock: 20 mM)	
0.1 M Sodium thiosulfate		
0.1 M Silver nitrate		
For experiments use 20 mM 1:4 (Silver:thiosulfate)		
Filter sterilize and keep in dark conditions		

Fahraeus medium	**For 1L**	
$CaCl_2$	0.132 g	(Carl Roth, Germany)
KH_2PO_4	0.1 g	(Carl Roth, Germany)
$ZnCl_2 \bullet HBO_3$	0.07 mg	(Carl Roth, Germany)
$MgSO_4 \bullet 7H_2O$	0.12 g	

Fe-citrate	5 mg	
$MnCl_2$	0.07 mg	(Carl Roth, Germany)
$Na_2MoO_4 \bullet 2H_2O$	0.07 mg	
$CuSO_4 \bullet 5H_2O$	0.07 mg	
$Na_2HPO_4 \bullet 2H_2O$	0.075 g	

In ddH_2O. Adjust pH to 7.0 and autoclave

TB medium	**For 1L**	
10 mM PIPES	3 g	(Sigma-Aldrich, Germany)
15 mM $CaCl_2$	2.2 g	(Carl Roth, Germany)
250 mM KCl	18.6 g	(Carl Roth, Germany)
In ddH_2O		
Adjust pH to 6.7 with KOH 5N		
+ 55 mM $MnCl_2$	10.9 g	(Carl Roth, Germany)
Sterilize by filtration		

2.6 AHL-priming

2.6.1 oxo-C14-HSL treatment

Ten-day-old barley or wheat plants cultivated on 1/10 PNM agar and under sterile conditions were treated with oxo-C14-HSL at final concentration of 6 µM. The oxo-C14-HSL stock solution was prepared by dissolving the molecule from Sigma-Aldrich in acetone. All experiments were performed with the solvent control acetone.

2.6.2 Sinorhizobium meliloti inoculation

Sinorhizobium meliloti (*Ensifer meliloti*) Rm2011 *expR+* containing the pWBexpR plasmid (M. McIntosh) and *S. meliloti* (pBBR2-attM) carrying the lactonase gene *attM* from *Agrobacterium tumefaciens* were grown in medium agar. The rhizosphere was inoculated with *S. meliloti expR+* and *S. meliloti attM* culture solution (both OD_{600} = 0.2), or watered with 10 mM $MgSO_4$ as control.

TY medium agar	**For 1L**	
Tryptone	16 g	(Carl Roth, Germany)
Yeast extract	10 g	(Carl Roth, Germany)
NaCl	5 g	(Carl Roth, Germany)
Agar	10 g	(Carl Roth, Germany)

In ddH_2O
Adjust pH to 7.0

2.6.2.1 AHL extraction

AHLs from *S. meliloti* liquid cultures were extracted by vortexing with $CHCl_3$ and discarding the aqueous phase after centrifugation. The $CHCl_3$ phase was then evaporated using an ultra-speed vacuum centrifuge for 20 min. The remaining residue was dissolved in 20 µL acetone. Detection of oxo-C14-HSL was carried by dropping 10 µl of the extracted AHLs on a LB plate containing the reporter bacteria *Escherichia coli* strain MT102 carrying the pJBA89 plasmid (Ap^r; pUC18Not-*luxR*-P_{luxI}-RBSII-*gfp* (ASV)-T_0-T_1) [58]. After 2 hours the fluorescence was observed using a fluorescence stereomicroscope (Leica M205 C) with an excitation of 480/40 nm and emission of 510 nm filters. Other alternative was to drop the 10 µL of the extracted AHLs on 100 µL of the reporter bacteria (OD_{600}≈0.4). The luminescence was measured 6 hours later using the Tecan infinite m200 Pro device.

2.7 Pathogen challenge

2.7.1 Blumeria graminis f. sp. hordei treatment

Ten-day-old barley leaves (cv. Golden Promise) previously pretreated with oxo-C14-HSL molecule or *S. meliloti* strains were inoculated with *Blumeria graminis f. sp. hordei* by blowing spores from infected barley leaves (~100conidia/cm^2). The inoculated leaves were kept on 1% Agar-H_2O plates at room temperature under low-light condition for two days; thereafter, leaves were DAB stained to see the production of reactive oxygen species [59], conidial germination and development of elongated secondary hyphae using an Axioplan 2 fluorescence microscope (Zeiss, Germany).

2.7.2 Xanthomonas campestris pv. translucens treatment

Xanthomonas campestris pv. *translucens* bacteria were grown in Wilbrink's medium until an OD_{600}≈0.5 was reached. Subsequently, the bacteria was centrifuged at 4000 rpm, washed and resuspended in a solution of 10 mM $MgSO_4$? $7H_2O$ and Silwet L-77 (0.2 µL/mL). Finally, ten-day-old wheat leaves (cv. Bobwhite) previously pretreated with oxo-C14-HSL molecule were sprayed with *Xanthomonas* solution (OD_{600}≈0.2).

Wilbrink's medium agar	**For 1L**	
Bactopeptone	5 g	(Becton, Dickinson and Company, Germany)
Sucrose	10 g	(Carl Roth, Germany)
K_2HPO_4	0.5 g	(Carl Roth, Germany)
$MgSO_4 \bullet 7H_2O$	0.25 mL	(Carl Roth, Germany)
Na_2SO_4 (anhydrous)	0.05 mL	(Carl Roth, Germany)
Agar	10 g	(Carl Roth, Germany)
In ddH_2O		

2.7.3 Puccinia graminis treatment

Ten-day-old wheat plants (cv. Bobwhite) previously pretreated with oxo-C14-HSL or control (acetone) were sprayed with *Puccinia graminis* f. sp. *tritici* urediniospores, which were collected from infected plants (density of ~ 10^6 spores / ml). The inoculated plants were placed for 12 h in dark and 100% relative air humidity. Subsequently, inoculated plants were exposed to normal light condition and kept for 11 days in a growth chamber with an average of 19°C and 90% relative air humidity.

2.7.4 Phytophthora infestans treatment

A *Phytophthora infestans* isolate was obtained from infected potato foliage, and was invigorated by monthly passage through potato tubers. The oomycete fungus cultures (16- to 22-day-old) were maintained on solid V8 juice agar in the dark at 15°C. To obtain *P. infestans* spore solution, the culture was soaked with sterile distilled water; the spore density was counted using a Fuchs-Rosenthal counting chamber. The sporangial suspension was placed at 5˚C for 3 h to improve the zoospore release, and the final solution was adjusted to a density of about 80,000 spores/ml. For the treatment, plants were drenched with the inoculation solution

using a pneumatic spray gun and kept at 16°C in the dark with 100% relative air humidity. After 48 h, plants were exposed to a 16/8 (dark/light) regime and 65% relative air humidity.

2.7.5 *Salmonella* Typhimurium treatment

Soil-grown 4-week-old *Arabidopsis thaliana Col*-0 plants or one-day-germinated *Medicago sativa* seedlings were pretreated with *S. meliloti* strains before assessing the proliferation rate in plants of Salmonella *enterica* serovar Typhimurium strain 14028s, which carries the pEC75 plasmid that confers resistance to ampicillin. Salmonella was syringe-infiltrated in *Arabidopsis* leaves, while alfalfa seedling were submerge in a *Salmonella* bacterial solution. First, bacteria were grown until OD_{600} = 0.8 in LB medium, washed and resuspended in 10 mM $MgCl_2$ for *Arabidopsis* leaves and in Fahraeus medium for *Medicago* seedlings. Infiltration solution was adjusted to OD_{600} = 0.1 (1.5 x 10^7 CFU/ml) and bacterial population was monitored during 6 days post infiltration using selective LB medium containing ampicillin as described in [20]. Alfalfa seedling were treated with 20 mL bacterial solution (1.4 x 10^5 CFU/ml) and bacterial growth was evaluated 3 dai.

XLD medium agar	**For 1L**	
Yeast extract	3 g	(Carl Roth, Germany)
L-lysine	5 g	(Carl Roth, Germany)
Xylose	3.75 g	(Carl Roth, Germany)
Lactose	7.5 g	(Carl Roth, Germany)
Sucrose	7.5 g	(Carl Roth, Germany)
Sodium deoxycholate	1 g	(Carl Roth, Germany)
Sodium chloride	5 g	
Sodium thiosulfate	6.8 g	
Ferric ammonium citrate	0.8 g	
Phenol red	0.08 g	(Sigma-Aldrich, Germany)
Agar	10 g	

In ddH_2O. Heat for 15 min in pot with boiling H_2O, shaking every couple of min.
Do not autoclave.

MES buffer

MES	50mM

Adjust pH to 5.6 with KOH

2.7.5.1 *Salmonella* Typhimurium growth under SPI-1 inducing conditions

First, a small culture (5mL) of Salmonella Typhimurium was grown at 37 ºC with continuous shaking for approximately 12 hours. Subsequently, bacterial culture was adjusted to OD_{600} = 0.05 in a new flask containing LB 0.3 NaCl. The bacteria were grown at 37 ºC without shaking [60].

2.7.6 Chitin treatment

Barley plants previously treated with acetone or oxo-C14-HSL were treated with chitin from crab shell. For the preparation of chitin, 100 mg/mL of chitin from shrimp shell (Sigma- Aldrich, Germany) is ground using a mortar and then shortly microwaved. The shells are centrifuge and the supernatant is used for the treatment.

2.7.7 Flagellin treatment

To investigate whether AvrA localization is modify by the plant defense elicitor flagellin, transiently transformed *Arabidopsis* leaves were treated for 30, 60 and 120 min with 100nM flg22. Cells were observed by fluorescence microscopy to detect the GFP and mCherry (control) expression.

2.8 Cytological staining techniques

2.8.1 DAB staining

A fresh DAB solution was prepared by dissolving DAB tetrachloride (Sigma Aldrich, Germany) in H_2O at a concentration of 1 mg/mL, and the pH adjusted with NaOH to 3.8-4-0. Subsequently, leaf base and apex were cut under H_2O and the leaf base place into the DAB solution for 4 hours. For distaining, the leaves transferred into ethanol:chlorophorm:trichloroacetic acid (4:1:0.15%) solution for 2 days and preserved in 50% glycerol before cytological observations.

2.8.2 Calcofluor staining

This staining solution was used to see fungal structures under the light microscope

Calcofluor staining solution	**For 50 mL**
50 mM Tris	0.35 g
Calcofluor	0.15 g

Microwave 20 sec and store at 4 ºC

2.9 Aequorin assay

Eleven-day-old *Arabidopsis* transgenic pMAQ2 seedlings, which express the Ca^{2+}sensor apoaequorin, were transferred to white 96-well plates (Greiner, Germany). Each seedling was treated for 6 hours with 100 µL of 2.36 mM Coelenterazine (stock solution: 1mg/mL ethanol) to have a working concentration of 10 µM. Plants were treated afterwards with 50 µL of 60 mM oxo-C14-HSL or acetone, and immediately the luminescence was measured in a Tecan infinite m200 Pro device (integration time: 2000 ms; Kinetic cycles 25). Arabidopsis wild type (Col-0) seedlings were used for background control and 50 µL of 1 M $CaCl_2$ was added at the last three cycles as positive control.

2.10 Transcriptional analyses

Barley cv. Golden Promise leaves pretreated with oxo-C14-HSL or acetone, and subsequently inoculated with *Blumeria graminis* f. sp. *hordei* were harvested at 0, 24 and 48 hours after inoculation (hai). Plant material was homogenized and the total RNA was extracted using Trizol. cDNA synthesis was perform using 2 µg of total RNA according to qScript cDNA Synthesis Kit (Quanta BioScience Inc.) Primers used for quantitative RT-PCR are found in the Supplemental Table 1. All expression values were normalized to expression of *HvUBQ60*.

3 RESULTS

3.1 oxo-C14-HSL primes *Arabidopsis* plants for induced resistance

3.1.1 Transcriptional reprograming in AHL-primed plants

The molecular basis of priming in plants is still poorly understood even though some signs of the "primed" state have been elucidated, e.g. the accumulation of inactive MPK3 and MPK6 after a challenge [37]. In addition, plants treated with benzo (1,2,3,) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) present a stronger activation of the MAP kinases that is followed by the up regulation of the defense genes *PAL1* and *PR-1* [61]. Noticeably, a similar effect was described in *Arabidopsis* roots treated with the long chain AHL oxo-C14-HSL. Here, this molecule positively modulated the activation of MPK3 and MPK6, and the expression of the transcription factors *WRKY22* and *WRKY29*, in conjunction with the *PR-1* gene [62]. For this reason, microarray-based transcriptional profiling of *Arabidopsis thaliana* was performed in order to reveal the AHL-priming effect in plants. Considering that plants react differently to *N*-acyl homoserine lactones, differences in gene expression of plants treated with three different AHLs were evaluated using a binary logarithm of 1 and *p* value of 0.05 for the *Arabidopsis* (4x44k) Gene Expression Microarrays (Agilent Technology). In Schenk *et al*. treatment with *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-3-oxo-decanoyl-L-homoserine lactone (oxo-C10-HSL) or *N*-3-oxo-tetradecanoyl-L-homoserine lactone (oxo-C14-HSL) resulted in 954 genes differentially regulated within which 55 genes reacted to all AHL treatments, and more specifically 45% of these genes belong to stress response and signaling mechanisms [63]. Regarding the 177 genes that only reacted to oxo-C14-HSL, 25% were related to the biotic category i.e., genes encoded signaling kinases (but not MAP kinases), receptor kinases and proteins involved in Ca^{2+} signaling. Furthermore, when MAMP-triggered immunity (MTI) was triggered with 100µM flg22, the transcriptional reprogramming in oxo-C14-HSL-primed plants showed also biotic stress as its most outstanding category.

Concerning the biotic stress group, genes related to the common defense hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) were not differentially expressed; however, there was an enrichment of genes related to signaling, cell wall composition and secondary metabolism. Accordingly, receptor kinases, calcium-modulated proteins (or Calmodulin-binding proteins) and G-proteins were differentially expressed in primed plants.

3.1.2 Role of Ca-signaling in AHL-priming

The concentration of cytosolic free calcium ($[Ca^{2+}]_{cyt}$) is important to direct certain physiological responses [64]. In order to test the possible involvement of Ca-signaling in AHL-priming, we made use of transgenic *Arabidopsis* seedlings expressing the Ca-responding aequorin gene from *Aequorea victoria.* Those plants were primed with oxo-C14-HSL and incubated with coelenterazine to measure the cytosolic free calcium after triggering the immune responses with flg22. The enzymatic activity of aequorin, which oxidizes coelenterazine into coelenteramide upon Ca^{2+} binding, is proportional to the Ca^{2+} concentration in cell cytoplasm, and it can be measured by detecting the emission of light produced by the active aequorin enzyme [64]. Figure 1 represents three biological repetitions and shows the luminescence from oxo-C14-HSL-treated and control-treated seedlings after 100µM flg22, in addition of 1M $CaCl_2$ to test the highest possible enzymatic activity in the system. No significant differences were identified between AHL-primed and control seedlings, nor in leaf samples (data not shown) suggesting that oxo-C14-HSL does not enhance cytosolic free Ca^{2+} influx.

3.1.3 Modification of cell wall

Microarray data also revealed higher expression of genes related to secondary metabolites (phytoalexins and flavonoids) in oxo-C14-HSL primed plants, as well as genes related to the most abundant structural cell wall proteins, the hydroxyproline-rich glycoproteins (HRGPs). Deposition of mechanical barriers like the cross-linking of HRGPs and phenolic compounds, which is driven by reactive oxygen species (ROS) in

the apoplast, is essential for limiting pathogen expansion.

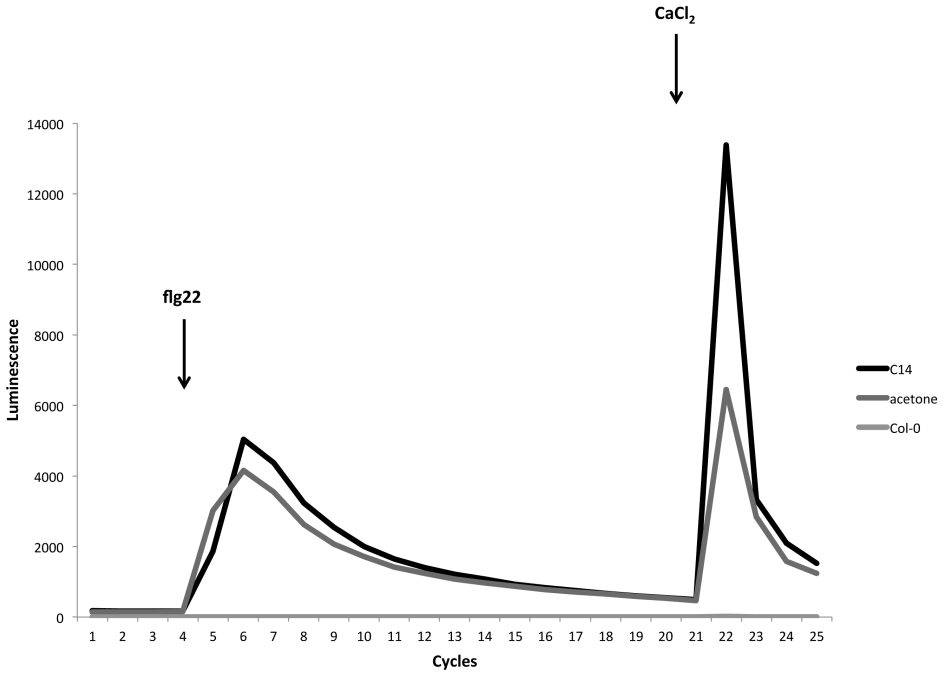

Figure 1. oxo-C14-HSL treatment does not lead to an increase of cytosolic free Ca^{2+} in transgenic *Arabidopsis* seedlings. The transgenic *Arabidopsis* line expressing the jellyfish aequorin gene, which oxidizes coelenterazine into coelenteramide after Ca^{2+} binding, were pretreated with oxo-C14-HSL or acetone (as control treatment) for 3 days before incubation with coelenterazine for 6 hours. The luminescence of the seedlings was assessed as a correlation to the concentration of cytosolic free Ca^{2+} before and after addition of the MAMP flg22 (100µM); $CaCl_2$ was added during the last 5 cycles to detect the maximal enzymatic activity of aequorin. In comparison to wild type (Col-0) *Arabidopsis* plants, the transgenic lines emitted light after flg22 treatment, however no differences were detected between control and oxo-C14-HSL seedlings implying that the cytosolic free Ca^{2+} influx is not augmented in oxo-C14-HSL primed plants.

In consequence, soluble phenolics, cell wall-bound phenolics and lignins were measured in seedlings (for local response) and leaves (for systemic response) treated with the oxo-C14-HSL molecule after flg22 challenge. The amount in milligrams of tannic acid equivalent (TAE) and lignin per gram of dried weight was significantly higher 24 hat in oxo-C14-HSL plants than in control treatments, indicating that remodeling of the cell wall in AHL-induced resistance is related to the increase and cross-linking of soluble phenolics, cell wall-bound phenolics and lignins.

Because callose depositions are found along with cells where cross-linkage of cell wall compounds occurred, this defense response was also estimated in control and oxo-C14-HSL pretreated plants [65]. Callose depositions were classified in four categories according to the amount of spots found in the observed area (4.9 mm^2). In both local and systemic leaf tissue, control plants displayed significantly more the category I (0-5 spots) and category II (5-20 spots), while oxo-C14-HSL-treated plants

exhibited significantly more the category III (20-100 spots) and category IV (>100 spots) 24 hat. These results in connection with the extensive phenolic cross-linkage in oxo-C14-HSL plants indicate that AHL priming of *Arabidopsis thaliana* plants reinforces the structure of the cell wall as a defense measurement against a pathogenic attack [63].

3.1.4 AHL-derived systemic signal

The systemic enhancement of basal resistance before a subsequent pathogen challenge is probably based on hormone(s) signaling. The systemic effect of AHL-induced resistance was evaluated according to the SA-dependent Systemic Acquired Resistance (SAR) and the JA/ ET-dependent Induced Systemic Resistance (ISR) theorem. Analysis of mutants revealed that the Non-Expresser of PR-1 gene (NPR1) is necessary for AHL induced resistance. The proliferation of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) was not attenuated 96 hai in *npr1* mutant plants pretreated with oxo-C14-HSL as seen in wild type plants [63]. In contrast, the AHL induced resistance effect was still evident in the *coi1-16* and *jar1-1* mutants, which suggests that the induced resistance caused by AHLs is independent of jasmonic acid. Complementary experiments were performed to confirm the involvement of SA in AHL priming and independence of JA. Accumulation of these hormones was measured in distal leaves from plants treated in the rhizosphere with oxo-C14-HSL for 3 days, and additionally inoculated with *Pst* in order to trigger the immune responses. SA together with *cis*-OPDA was significantly accumulated 24h after *Pst* inoculation, but not JA or its active derivative JA-Ile [63].

The accumulation of *cis*-12-oxo-phytodienoic acid (*cis*-OPDA), which is a precursor of jasmonic acid but however it possesses signaling functions distinct from Ja-Ile [66], proposed the participation of oxylipins in AHL induced resistance; therefore, the expression of two *cis*-OPDA-dependent and JA-independent genes, glutathione S-transferase 6 (*GST6*) and heat shock protein 70 (*HSP70*), was quantified in AHL-primed plants after challenge with *Pst.* Results demonstrated enhanced expression of both genes in oxo-C14-HSL-treated plants, which confirms the involvement of oxylipins in AHL-priming. Likewise, the expression of *GST6* and *HSP70* was analyzed

in two mutants in systemic acquired resistance [67], *bsmt1* and *med4-1*, and in a mutant line compromised in induced systemic resistance (ISR), *myb72*. In comparison to wild type plants, the expression of oxylipin-related genes in AHL-treated plants did not changed in *bsmt1* and *med4-1*; however, changes were observed in *myb72* mutant, proving that the conversion of SA into MeSA (important for SAR) is a prerequisite for AHL induced resistance. These results were verified as well by the *Pst* proliferation assay in all three mutants [63].

In Montillet *et al*., SA and *cis*-OPDA signaling pathways were suggested to coordinate flg22-triggered stomatal closure; for this reason, closed stomata were counted in AHL-treated plants 2 and 24 hours after infection with *Pseudomonas* (*Pst*) [68]. Notably, 60 to 70% of the stomata were closed in oxo-C14-HSL-treated plants at both time points, whereas in control plants only 50% of the stomata were closed. In conclusion, oxo-C14-HSL systemic resistance in *Arabidopsis* plants inhibits pathogen entry as another immune response by regulating stomata closure [63].

3.2 AHL-induced resistance in crop plants

3.2.1 Resistance in barley plants was induced by the AHL-producing *Sinorhizobium meliloti* strain

In addition to *Arabidopsis*, resistance against *Blumeria graminis* f. sp. *hordei* in *Hordeum vulgare* (barley) cultivar Golden Promise was enhanced in plants pretreated with oxo-C14-HSL [62]. To expand our observations and support the idea that oxo-C14-HSL produced by rhizobacteria can also enhance the resistance in crop plants, we analyzed the interaction between barley and AHL-producing *Sinorhizobium meliloti* (*Ensifer meliloti*) Rm2011 strain (expR^{+}). *S.meliloti* Rm2011 is a wild type strain disrupted in one *ExpR* copy due to an insertion sequence (IS) within the *ExpR* ORF. The ISRm2011-1 insertion is a member of the ISRm1 family of IS elements, and generates 5-bp target site duplications upon insertion [69]. In addition to the necessity of a functional copy of the ExpR gene for the production of symbiotically active EPS II, its absence causes a debilitated quorum sensing response i.e., the synthesis of oxo-C14-HSL is diminished [70]. For this reason, the wild type *S.*

meliloti strain was transformed with pWBexpR vector (from M. McIntosh) for the production of oxo-C14-HSL and used as an expR$^+$ strain. Conversely, S*. meliloti* Rm2011 expressing the lactonase *attM* gene from *Agrobacterium tumefaciens* (pBBR2-attM) was employ as an AHL negative (AHL$^-$) control strain [71].

To this end, ten-day-old barley plants previously pretreated in the rhizosphere three times with $MgSO_4$ (control), *S. meliloti attM* (negative control) or the *S. meliloti* strain *expR*$^+$ were inoculated with the biotrophic fungus that causes powdery mildew, *B. graminis*. After two days, leaves were stained with 3'3-diaminobenzidine tetrachloride (DAB), and fungal structures were stained with a calcofluor solution in order to examine the formation of papillae or hypersensitive response, which indicates a resistance reaction of the plant, or the development of elongated secondary hyphae, indicating a susceptible phenotype (Figure 2A). The results showed that oxo-C14-HSL produced by *S. meliloti expR*$^+$ induced the resistance, as revealed by the decreased amount of formed elongated secondary hyphae, and increase of the hypersensitive response in expR$^+$-treated plants in comparison to plants treated with $MgSO_4$ or with the lactonase expressing strain *S. meliloti attM*; (Figure 2B). Simultaneously, disease symptoms were monitored in barley plants five days after inoculation with *B. graminis*. The control and *S. meliloti attM* treated plants clearly exhibited more mycelia development than plants treated with *S. meliloti expR*$^+$ (Figure 2C). The experiments were repeated at least three times, and the production of AHL molecules by *S. meliloti* bacteria was controlled using the biosensor bacterium *E. coli* MT102 (Figure 2D) [58]. Notwithstanding that there was no significantly increase of papillae formation in *S. meliloti expR*$^+$, oxo-C14-HSL-producing *S. meliloti* strain can induced resistance in barley plants in the same manner as the pure AHL molecule, wherein the resistance is reflected mainly in the increase of hypersensitive response [72].

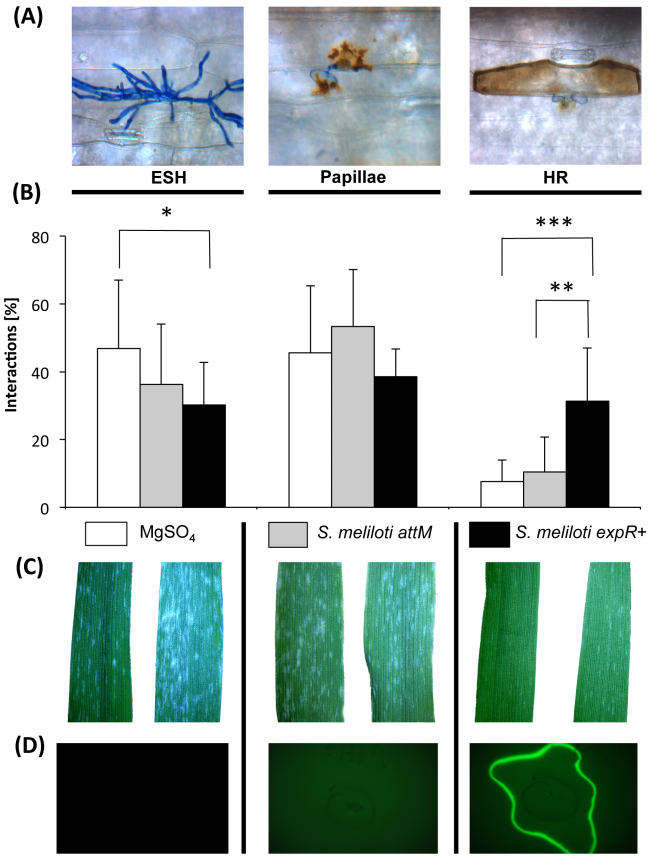

Figure 2. oxo-C14-HSL produced by *S. meliloti* induces resistance against *B. graminis* in barley. Before spreading powdery mildew (~100 conidia/cm^2) in barley plants cv. Golden Promise, a pretreatment was performed three times in the rhizosphere of these plants with $MgSO_4$ (control), the lactonase-expressing strain *S. meliloti attM,* or the *S. meliloti expR+* strain to see the impact of AHL producing bacteria in plant resistance. **(A)** The formation of elongated secondary hyphae (ESH), which exemplifies susceptibility against the pathogen, and the formation of papillae or hypersensitive response (HR), both indicating resistance, was assessed 2 dai by DAB staining infected leaves. **(B)** The percentage of fungi interaction sites in three independent experiments was evaluated. Control treatment samples significantly contained more elongated secondary hyphae, while *S. meliloti expR+* treatment significantly triggered more hypersensitive response than other treatments (*P ≤ 0.05; **P ≤ 0.005; ***P ≤ 0.0005 in Student's *t*-test) **(C)** Accordingly, leaves in control or *S. meliloti attM* treatments displayed a higher amount of mycelia development 5dai than AHL-producing *S. meliloti expR+,* indicating that this strain can induced resistance against *Blumeria graminis* f. sp. *hordei.* **(D)** *Escherichia coli* strains MT102 was used as biosensor for the detection of oxo-C14-HSL in all *Sinorhizobium meliloti* treatments.

(Published data, see reference [72])

3.2.2 AHL-induced resistance in barley is associated with enhanced expression of *HvPRX7* and *HvPR1*

One of barley's defense responses against *Blumeria graminis* is the formation of papillae, which are cell wall appositions formed by the cross-linking of phenolic compounds. The enzymes responsible for the cross-linkage using hydrogen peroxide as a substrate are peroxidases [73]. Considering our previous results, which revealed that in oxo-C14-HSL treated plants the formation of papillae after inoculation with pathogen was significantly higher than in control treatment, as well as a significantly increase of hypersensitive response (HR) in both oxo-C14-HSL- and *S. meliloti expR*$^+$-treated barley plants, we looked at the expression of related genes [62]. For this

reason, the expression of *HvPRX7*, a key enzyme involved in ROS production, was evaluated at 0, 24 and 48 hours after infection with *Blumeria graminis* in oxo-C14-HSL-treated or control sterile plants. Both treatments appeared to have papillae formation when germinated spores are interacting with epidermal cells, however cell wall appositions in oxo-C14-HSL-treated plants presented more accumulation of hydrogen peroxide, which was detected via DAB staining (Figure 3A). Furthermore, the transcript level of was up regulated 24 and 48 hai in plants treated with oxo-C14-HSL, however significant difference to control was observed only at 24 hai (Figure 3B).

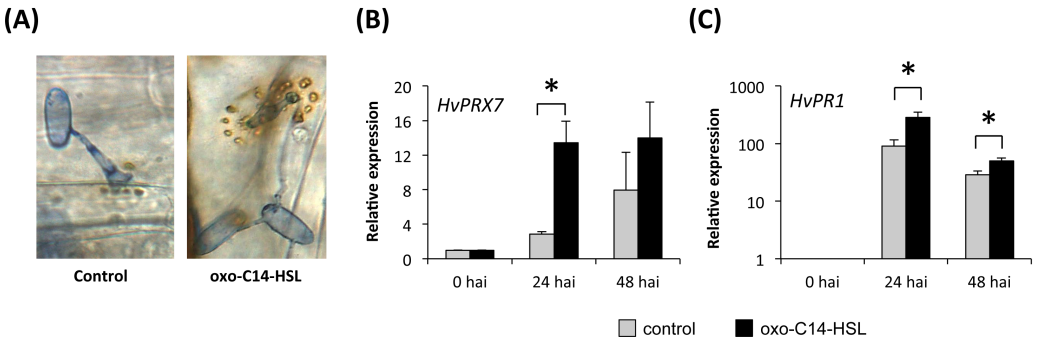

Figure 3. Barley AHL-induced resistance is associated with enhanced up regulation of *HvPRX7* and *HvPR-1b*. Golden promise (barley) plants were pretreated with oxo-C14-HSL or acetone (control) for ten days, and subsequently infected with *Blumeria graminis* f. sp. *hordei* by blowing infected plants (~100 conidia/cm^2). Leaves were DAB stained 2dai and fungal structures were observed under the microscope with a calcofluor staining solution. **(A)** Both treatments revealed the formation of papillae that were accompanied with the accumulation of hydrogen peroxide in fungal interacting sites. **(B)** The gene expression of *HvPRX7* was evaluated 24 and 48 hai. In comparison to the control, the significant gene up regulation of this enzyme in oxo-C14-HSL treatment is an indication that the penetration defense is enhanced by the formation of cell wall appositions using H_2O_2 as a substrate. **(C)** Moreover, leaves of AHL-treated plants showed that the barley Pathogenesis-related protein 1b (PR-1b) gene expression is significantly enhanced 24 and 48 hai, suggesting that this enzyme also participated in AHL-induced resistance for papillae formation and HR, and hence cell wall reinforcement (*P ≤ 0.05 in Student's *t*-test). Results were calculated from at least three biological repetitions.

(Published data, see reference [72])

The expression of *Pathogenesis-Related Protein 1 (PR-1)* is induced by pathogen attack or salicylic acid. In barley *pr-1b* mutant, *Blumeria graminis* was reported to overcome cell wall defense more frequently than in barley wild type plants, indicating the role of PR-1b in penetration resistance. Moreover, the expression of *PR-1b* is correlated with the accumulation of H_2O_2 during papillae formation and HR [74]. Consequently, the expression of *PR-1b* was measured in plants treated for three days with oxo-C14-HSL and subsequently inoculated with *B. graminis* for one and two days. In comparison to control, treatment with oxo-C14-HSL significantly induced the expression of *PR-1b* at both time points (Figure 3C); therefore, it is suggested that priming with oxo-C14-HSL might enhance resistance via the accumulation of H_2O_2, formation of papillae and HR reactions as a result of

significantly enhanced expression of *HvPRX7* and *HvPR-1b* [72].

3.2.3 AHL-producing *S. meliloti* strain *expR*$^+$ does not induce resistance against *Xanthomonas campestris* pv. *translucens*

To test the AHL-induced resistance in wheat, *Triticum aestivum* cultivar Bobwhite seeds were sterilized, sowed on soil and their rhizosphere was inoculated three times with $MgSO_4$ (control), *S. meliloti attM* (negative control) or the AHL-producing *S. meliloti expR*$^+$ strain. Ten-day-old plants were then inoculated with the hemibiotrophic bacteria *Xanthomonas campestris* pv. *translucens,* which causes the black chaff disease in wheat. The bacterial solution (OD_{600}≈0.2) was sprayed on detached leaves, and the symptoms were evaluated during five days after inoculation. At the very early stage of disease development, all treatments presented small oblong aqueous, translucent, green light spots (Figure 4A). In later stages of the disease (Figure 4B), all treatments presented brown-yellowish spots with a sticky slime. It is assumable from the results that *S. meliloti expR*$^+$-treated leaves showed no differences compared to control in early, as well as in later stages of disease.

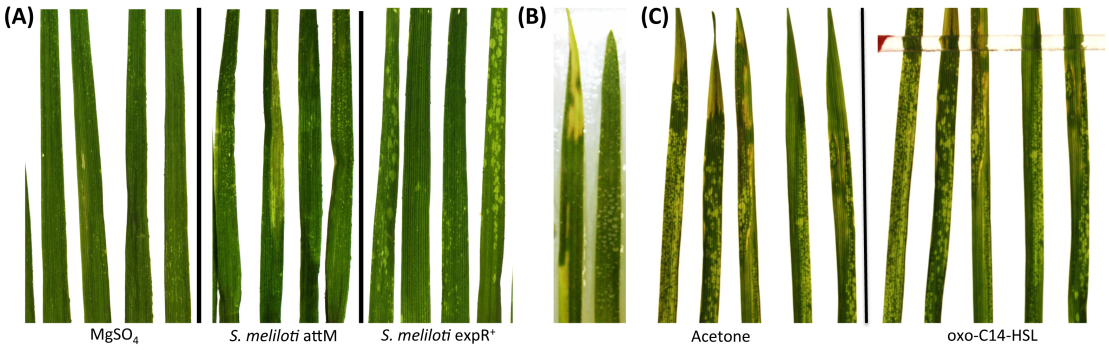

Figure 4. Resistance against *Xanthomonas campestris* pv. *translucens* is not induced in wheat plants treated with S. meliloti expR+ or oxo-C14-HSL. Wheat plants (cv. Bobwhite) were inoculated three times with $MgSO_4$ (control), *S. meliloti attM* or *S. meliloti expR+* before spraying on 10-day-old leaves with the pathogenic bacteria *Xanthomonas campestris* pv. *translucens* (OD_{600}≈0.2). Representative images of black chaff symptoms were taken 5 dai. **(A)** In all treatments, leaves present oblong aqueous translucent spots of light green color, which are characteristic for the first stage of disease development. **(B)** Representation of later stages of black chaff disease; leaves present yellow spots an a sticky slime (exudate). **(C)** Similarly, no differences between control (acetone) and oxo-C14-HSL treatment were found in wheat plants infected with *Xanthomonas*.

A similar experiment was performed with wheat plants treated with the molecule oxo-C14-HSL or acetone (control), where no differences were found between both

treatments (Figure 4C) suggesting that treatments with AHL-producing bacteria or oxo-C14-HSL do not induced resistance in wheat plants against *Xanthomonas campestris* pv*. translucens*.

3.2.4 Stem rust disease development is restrained by accumulation of H_2O_2 in guard cells of plants treated with oxo-C14-HSL.

In Schenk *et al*., we postulated that in *Arabidopsis thaliana* AHL-induced resistance is involved in stomatal closure as a defense response restricting one of the entry routes of plant pathogens [63]. Therefore, to ascertain if the purified AHL molecule or the AHL-producing *S. meliloti expR*+ strain have a similar impact in crop plants, *Puccinia graminis* f. sp. *tritici* was selected to test AHL-induced resistance in wheat due to the ability of this pathogen to enter mesophyll tissues via stomata.

Wheat (cv. Bobwhite) was grown in glass jars containing semi-solid PNM media (for sterile conditions) or on soil for 10 days. Roots were treated during this period three times with oxo-C14-HSL or acetone (in sterile conditions), or $MgSO_4$, *S. meliloti attM* or *S. meliloti expR+* on soil*.* After this pretreatment, plants were inoculated with *Puccinia graminis* f. sp*. tritici.* urediniospores (collected from infected wheat plants) were suspended in sterile H_2O (1 x 10^5 spores/mL), and pump-sprayed on the pretreated wheat leaves. Plants were kept in dark and 100 % relative humidity for 16 hours for optimal spore germination, and afterwards exposed to light for development of fungal structures [75]. Leaves were stained 2dai with 3'3-diaminobenzidine tetrachloride (DAB) and fungal structure with calcofluor in order to examine the fungal development and production of hydrogen peroxide (H_2O_2).

Our observation revealed that after germination of the *P. graminis* urediniospores (arrow), the hyphae followed a directional growth towards the stomatal openings (arrowhead) in all plants (Figure 5A). However, the difference between plants occurred in the accumulation of H_2O_2 in guard cells of oxo-C14-HSL-treated plants; the percentage of guard cells with increased H_2O_2 accumulation was significantly higher in oxo-C14-HSL treatment i.e. 78% of the guard cells accumulated H_2O_2 while in control plants only 15% guard cells accumulated H_2O_2 (Figure 5B). Interestingly, these results correspond to the enhanced expression of barley *Peroxidase 7*

(*HvPRX7*), which product cross-links the phenolic compounds during papillae formation using hydrogen peroxide. Moreover, eleven days after inoculation the enhanced presence of pustules on control plants indicated a stronger fungal development if compared to in AHL-treated plants (sterile conditions) (Figure 5C).

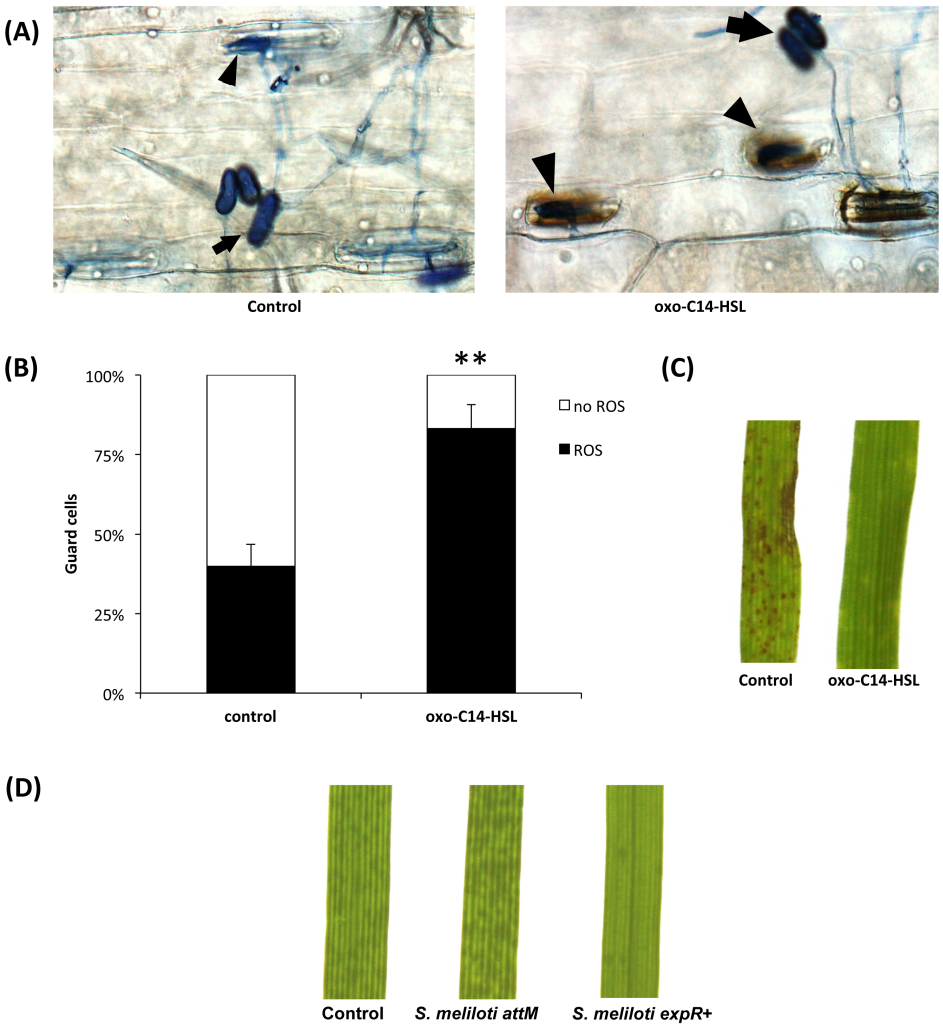

Figure 5. oxo-C14-HSL-induced resistance in wheat plants includes H_2O_2 production against *Puccinia graminis* f. sp. *tritici*. Sterile wheat plants (cv. Bobwhite) were inoculated three times with oxo-C14-HSL (acetone as control treatment), and soil-grown wheat plants were inoculated with $MgSO_4$ (control), *S. meliloti attM* or *S. meliloti expR+*. *Puccinia graminis* f. sp. *tritici* urediniospores solution (1 x10^5 spores/mL) was then sprayed on 10-day-old wheat plants. **(A)** After two days infection, leaf DAB staining showed the urediniospore (arrow) germination with directional growth of hyphae towards stomata (arrowhead). **(B)** In comparison to control, oxo-C14-HSL treated plants significantly triggered the accumulation of hydrogen peroxide (H_2O_2) in guard cells interacting with the pathogen (**P ≤ 0.005 in Student's t-test). **(C)** Eleven days after infection, control (acetone) plants presented higher pustule development than plants treated with oxo-C14-HSL. **(D)** Accordingly, control- and *S. meliloti attM*-treated plants had a stronger development of stem rust disease 5dai by the formation of pustules.

(Published data, see reference [72])

Similarly, pustule emergence in control or *S. meliloti attM*-treated plants was higher five days after inoculation, in comparison to *S. meliloti expR*$^+$-treated plants (Figure 5D), which indicates that proliferation of the stem rust-causing fungus is arrested if plants are treated with oxo-C14-HSL pure molecule or with the bacterial strain

producing this homoserine lactone [72].

3.2.5 *S. meliloti expR*$^+$ induced resistance against *Phytophthora infestans* pv. *tomato* in tomato plants

To further test the effectiveness to induced resistance using *S. meliloti expR*$^+$ strain, we use an additional model system. For this purpose, *Lycopersicum esculentum* (tomato) cultivar Moneymaker seeds were sown on soil, and plants grew for 4 weeks. During this time, *Sinorhizobium meliloti expR*+ and *Sinorhizobium meliloti* attM bacterial solutions (OD_{600}≈0.3) were inoculated every week in the rhizosphere before pathogen challenge; $MgSO_4$ was used as control. First, *Pseudomonas syringae* pv. *tomato* was selected to trigger the defense response in tomato plants, but these bacteria did not proliferate on this tomato variety. Therefore, another pathogen was chosen. *Phytophthora infestans* pv. *tomato* is one of the most common pathogens of tomato nowadays. The spore suspension (8 x 10^4 spores/mL) was sprayed on plants using a pneumatic spray gun until plants were soak with the solution. After one week, plants were examined for disease progress, and the infested leaf area of each plant was rated as a percentage of total leaf area (from 0 to 100). The mean of 5 replicates per treatment was used to obtain the rate of treatment efficiency by using Abbott's formula, where [76]:

$$EF = \left(\frac{Mtr - Mte}{100 - Mte}\right)$$

EF: percentage of treatment efficiency

Mtr: percentage of treatment severity

Mte: percentage of control severity

The disease progress was seen separately in the three different treatments. The first set of true leaves of plants treated with *S. meliloti expR*$^+$ presented only first stage symptoms of late blight disease, which is characterized by water-soaked spots that turned into brownish-black lesions (Fig. 6). In the second stage symptoms, as

observed in plants treated with *S. meliloti attM*, the foliage turns yellow and then black, until the final stage (e.g., control-treated plants) when foliage curls, shrivels and dies (Figure 6A). We conclude that the symptoms represented on the images coincide with the effectiveness of the treatments. In comparison to control, the treatment with AHL-producing *S. meliloti expR*$^{+}$ was 25% more effective for controlling the damage of late blight disease, while the non-AHL-producing *S. meliloti attM* had only 5% of treatment effectiveness (Figure 6B). In summary, the induction of resistance by oxo-C14-HSL-producing *S. meliloti* strain in tomato plants significantly hampered *Phytophthora infestans* infection [72]. The results described above are based on three biological repetitions.

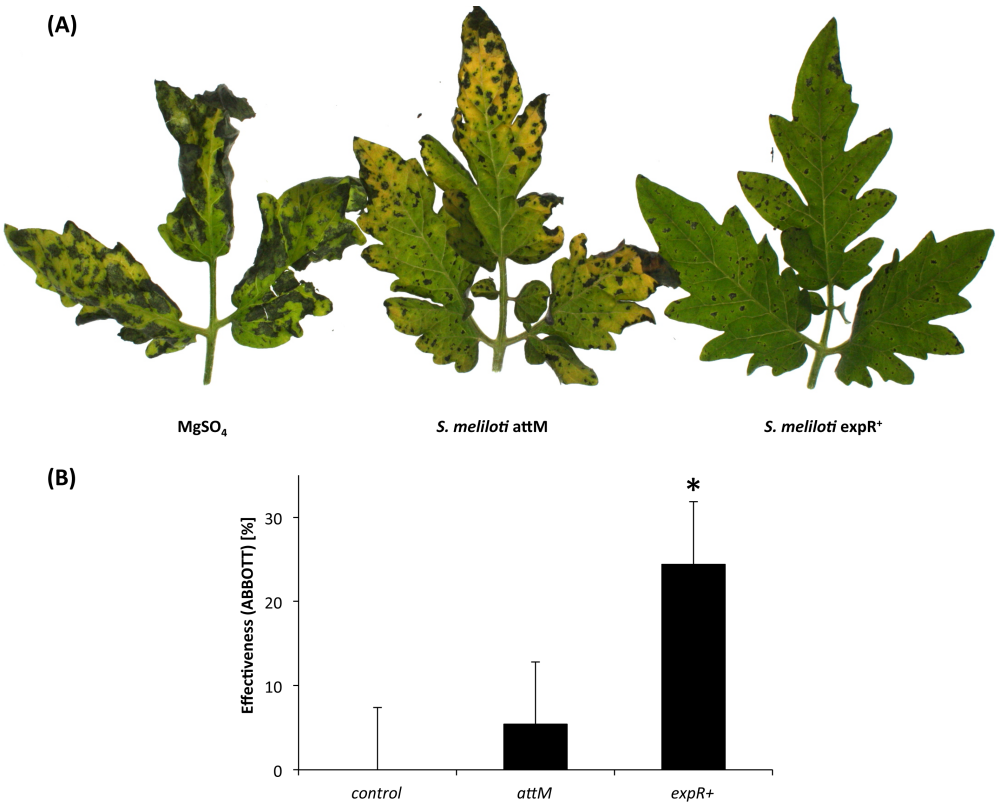

Figure 6. The resistance in tomato plants is increased with oxo-C14-HSL producing *S. meliloti* strain (expR+) against *Phytophthora infestans*. After inoculating four times tomato (cv. Moneymaker) rhizosphere with $MgSO_4$ (control), *S. meliloti attM* or *S. meliloti expR+* (OD_{600}≈0.3), spore suspension (8 x10^4 spores/mL) of *Phytophthora infestans* pv. tomato was sprayed on four-week-old tomato plants. **(A)** Late blight symptoms were documented 7dai and **(B)** the percentage of treatment efficiency was determined using Abbott's formula (*P ≤ 0.05 in ANOVA); data represents mean from 3 independent repetitions. The treatment with *S. meliloti expR+* strain significantly reduced disease development in tomato plants against the oomycete. It is noteworthy the induced resistance caused by AHL-producing *S. meliloti* strain expR+ in comparison to *S. meliloti* attM and control treatment. Production of AHLs in each treatment was controlled using the biosensor bacterium *Escherichia coli* strain MT102

(Published data, see reference [72])

Taking into account all the results, the oxo-C14-HSL priming effects counteract different biotrophic and hemibiotrophic pathogens in diverse plant species (Fig. 7).

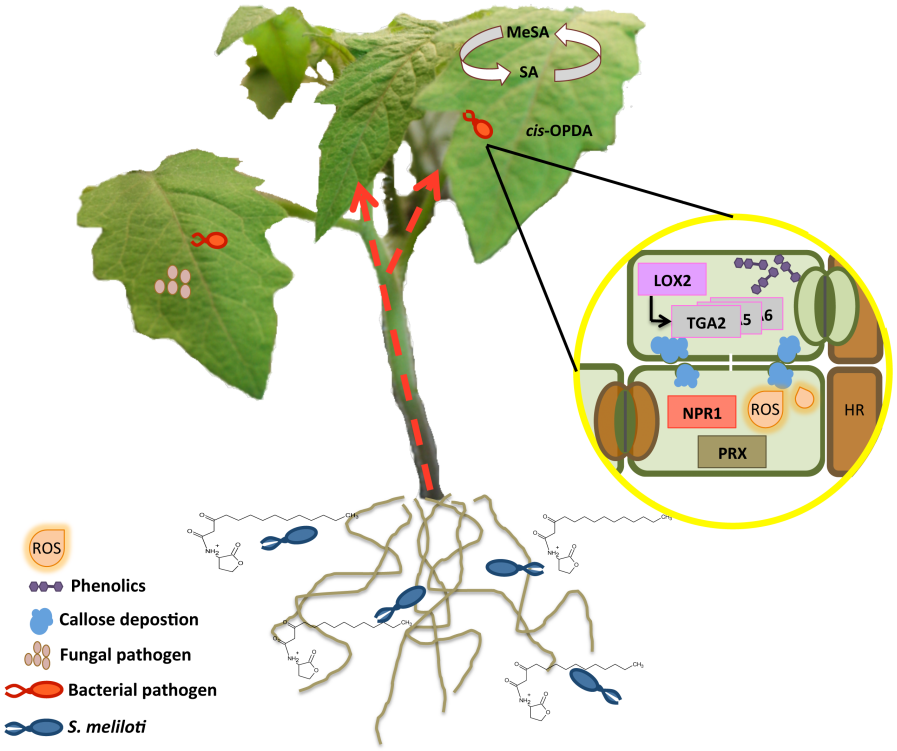

Figure 7. Plant defense responses in oxo-C14-HSL-induced priming. Treatment with oxo-C14-HSL (pure molecule or produced by *Sinorhizobium meliloti*) induces a systemic signaling that includes salicylic acid and oxylipins pathway for the up regulation of defense genes and stomata closure. Other defense responses are the enhancement of callose depositions and cross-linking of phenolic compounds to reinforce cell wall structure. ROS production and HR inhibit pathogen colonization in infected and nearby cell, as well as in guard cells.

The resulting immune responses are achieve through a systemic signal from roots, and salicylic acid and oxylipins pathways are required for the activation of relevant defense genes. As a result, enhanced ROS production in infected and nearby cells inhibits the proliferation of pathogens by causing hypersensitive response [58] and strengthening the plant cell wall structure i.e. phenolic compounds and lignin concentrations are augmented as well as the formation of papillae and callose depositions. Finally, stomata pores close to obstruct the main entry of pathogens, where additional ROS are produced in guard cells.

3.3 Perception of AHLs in *Arabidopsis* plants

3.3.1 *At2g47500* product may be involved in oxo-C14-HSL perception in *Arabidopsis* plants.

In Schikora *et al*. was mentioned that when *Arabidopsis* roots are treated with the long oxo-C14-HSL, the molecule is not transported systematically along the plant as it occurs with the short chain C6-HSL; therefore, it was suggested that plant proteins in or adjacent to the cell membrane may interact with the long AHLs molecules and transmit the signal [62, 77]. One candidate protein is *At2g47500* product, an IQ-motif-containing GTPase-activating protein (IQGAP1) homologue in *Arabidopsis thaliana*. IQGAP1 was demonstrated to interact with the *Pseudomonas aeruginosa* N-3-oxo-dodecanoyl- L-homoserine lactone (3-oxo-C12-HSL) in human intestinal epithelial CaCo-2 cells [78]. Consequently, *At2g47500* gene was amplified from cDNA clone RAFL15-38-117 (Riken Bioresource Center, Japan) with the purpose of transferring the candidate gene into Gateway protein expression vectors; however, the full ORF was not successfully cloned into neither bacterial nor plant protein expression vectors. As a result, three protein domains of *At2g47500* were cloned separately into the pDEST17 vector carrying a 6x-His tag: the calponin homology domain (1-318 AA), the kinesin coiled-coil domain (319-758 AA) and the C-terminus (759-983 AA) (Figure 8A).

Subsequently, the three domains were purified with Ni-NTA agarose purification kit (QIAGEN) with some modifications. Unfortunately, the recombinant protein parts were packaged into inclusion bodies, which made the purification challenging. In the Materials and Methods section of this work, optimized lysis and purification buffers are presented for challenging GST- or 6x-His tagged proteins using the ionic surfactant sarkosyl and the nonionic surfactant Triton X-100 (based on the results from Tao *et al*. [79]). In Figure 8B, western blots show the successful detection of the purified *At2g47500* domains using an antibody raised against the 6x-His-tag. Nevertheless, the interaction between oxo-C14-HSL and *At2g47500* domains, or oxo-C14-HSL and other plant proteins still need to be determined.

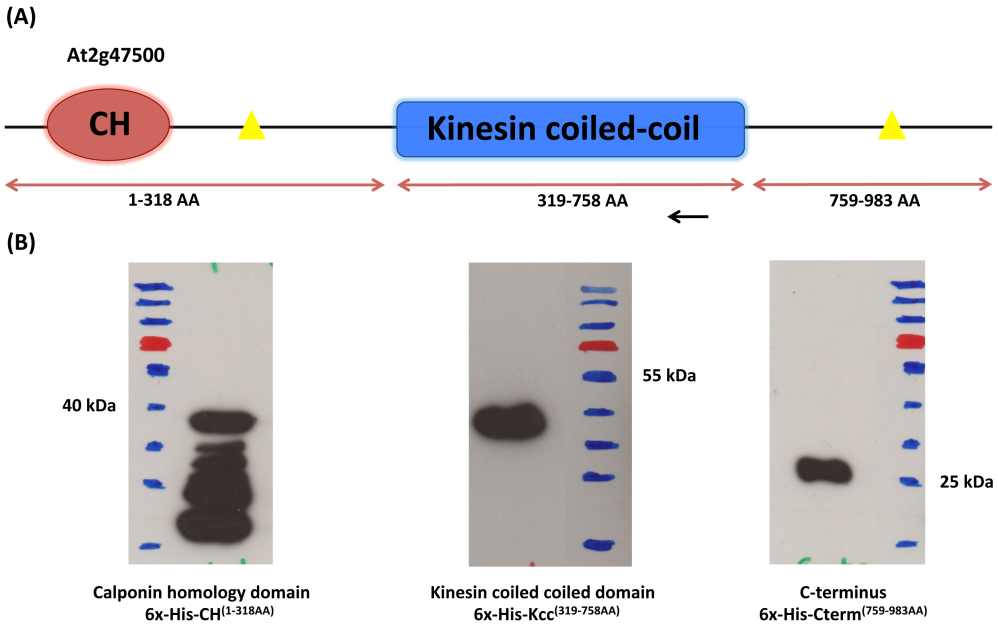

Figure 8. At2g47500 domains were successfully purified for AHL interaction studies. The three domains of the *HsIQGAP1* homologue in Arabidopsis thaliana, *At2g47500*, were cloned into the Gateway pDEST17 proteins expression vector: **(A)** Calponin homology domain (1-318 AA), kinesin coiled-coil domain (319-758 AA) and the C-terminus (759-983 AA). **(B)** Purification was carried out using two surfactants to avoid encapsulation of the proteins in inclusion bodies, and protocol was modified for Ni-NTA agarose purfication kit from QIAGEN. The 6x-His-$CH^{(1-318AA)}$ domain, 6x-His-$Kcc^{(319-758AA)}$ and 6x-His-$Cterm^{(759-983AA)}$ have an atomic mass of 34.9 kDa, 48.7 Kda and 25.4 KDa, respectively. Western blots were perform to test the detection of all domains using an α–His-tag antibody.

3.4 The possible use of AHL-priming for plant protection against human pathogens

3.4.1 *Salmonella* Typhimurium proliferation is inhibited by *S. meliloti expR*$^+$ in *Arabidopsis* leaves

Activation of Mitogen-Activated Protein Kinases (MAPKs), enhanced expression of PR proteins and increase production of ROS are some of the plant immune responses activated by plant pathogens and also the human pathogen *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) [80]. According to the above, and because *Salmonella* bacterium is an important pathogen worldwide that is able to colonize a great variety of host plants, new methods are sought for its biocontrol. An alternative strategy of how to address this issue was suggested in this PhD work. I suggest using the AHL-producing *Sinorhizobium meliloti expR*$^+$ strain due to its capacity to boost the expression of PR proteins and the production of ROS, and therefore enhance the plant immune system. It was assessed whether this strain could be used as an alternative strategy to restrict *Salmonella* growth in plant hosts.

For this reason, the rhizosphere of four-week-old *Arabidopsis* plants was irrigated every week with $MgSO_4$, *S. meliloti attM* or *S. meliloti expR*$^+$. Three days after the last pretreatment, *Salmonella* Typhimurium solution (OD_{600}≈0.1) was infiltrated into the abaxial leaf surfaces of leaves, and the proliferation of *Salmonella* was accessed 2 hours and 6 days after infiltration. The first time point is an indication that in all treatments, leaves were inoculated with similar amount of bacteria. At 6 dai significant differences in the proliferation of the pathogen were seen between plants treated with *S. meliloti attM* and *S. meliloti expR*$^+$. In plants treated with the AHL-producing *expR*$^+$ strain, the growth of *Salmonella* was inhibited significantly resulting in ten times less *Salmonella* CFU in a leaf disc (Figure 9A).

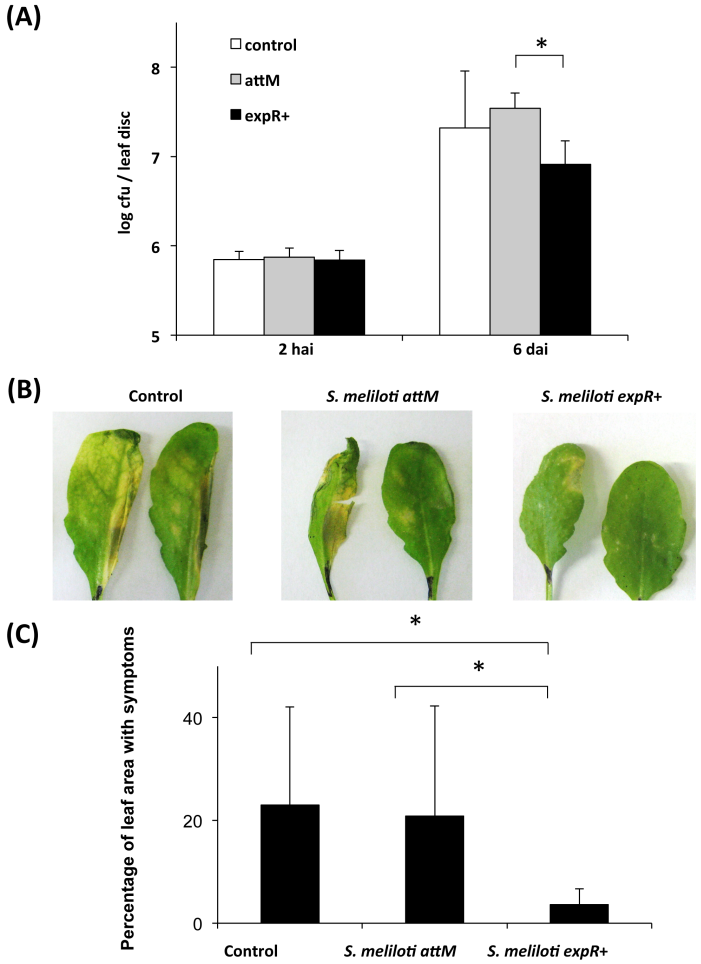

Figure 9. *Salmonella* growth is restricted in *Arabidopsis* plants through pretreatments with AHL-producing *Sinorhizobium meliloti*. Thale cress (*Arabidopsis thaliana*) plants were pretreated with $MgSO_4$ (control), *S. meliloti attM* or *S. meliloti expR+* (OD_{600}≈0.3) for 4 weeks before syringe-infiltration with *Salmonella enterica* serovar Typhimurium solution (OD_{600}≈0.1). The proliferation of the pathogen was determined 2 hai and 6 dai by cutting leaf discs from infected leaves and plating the plant-pathogen homogenate in selective medium. **(A)** Results showed that *S. meliloti expR+* inhibited significantly the proliferation of *Salmonella* in comparison to treatment with *S. meliloti attM.* **(B)** Accordingly, leaves from *S. meliloti expR+*- treated plants presented less disease symptoms than other treatments; leaves from control plants presented extensive chlorosis **(C)** Infection leaf symptoms were quantified using a specific algorithm [81]. Plants pretreated with rhizobacteria producing AHLs had a significant lower percentage of symptoms in comparison to the control or *the* rhizobacteria expressing the lactonase (*attM*) (*P ≤ 0.05 in Student's *t*-test).

(Published data, see reference [72])

Together with these results, a variation in infection symptoms was observed among the treatments, where the control treatment presented the highest chlorosis and plants treated with *S. meliloti expR*$^{+}$ showed the lowest disease symptoms (Figure 9B). These results were confirmed by quantifying the symptoms of each leaf using an algorithm described by Schikora *et al*. [81] (Figure 9C). Accordingly, it is proposed that induction of resistance by the *S. meliloti* strain producing oxo-C14-HLS could be an alternative approach to restrain the *Salmonella* proliferation in plants [72].

3.4.2 oxo-C14-HSL have an impact on the *in vitro* growth of *S.* Typhimurium

To further investigate the ability of AHL molecules to suppress the colonization of plants with *Salmonella*, *in vitro* studies were performed. *Salmonella enterica* serovar Typhimurium bacteria were grown in LB medium and the liquid cultures were supplied with 6 µM oxo-C14-HSL (Sigma Aldrich, Germany). The absorbance of each culture was measured in order to assess the growth rate (Figure 10).

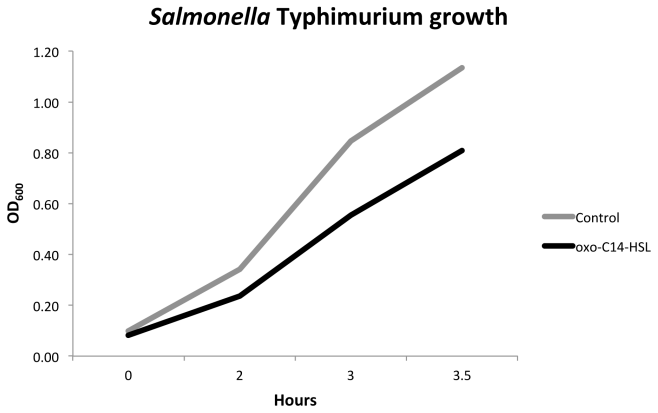

Figure 10. *Salmonella* Typhimurium *in vitro* growth is delayed by the presence of oxo-C14-HSL. Salmonella liquid cultures were grown with or without the presence of 6 µM oxo-C14-HSL. The bacterial optical density was monitored until OD_{600} ≈1.0 . After two hours, the bacterial solutions containing the AHL molecule exhibited a slower growth in comparison to control bacterial solutions.

The observations revealed that the bacterial growth decreased in the presence of AHL molecules. The differences in growth were clearly seen already three hours after the addition of AHL. From this stage on, the control cultures showed faster growth rate, in comparison to cultures containing the oxo-C14-HSL. The outcome of this experiment indicates that AHL molecules can affect *Salmonella* growth and that it

may influence its colonization capacity in the presence of AHL-producing rhizobacteria.

3.4.3 *S. meliloti expR*$^{+}_{(ch)}$ produced more AHLs at early culture stages

Rhizobacteria producing the long-chain AHL oxo-C14-HSL can have a positive influence on the plant immune system. As a consequence, the production and the amount of AHLs produced by *S. meliloti* bacteria needs to be controlled for efficient plant protection approaches. Henceforth, two different strains were tested to study the resistance effect of AHLs in symbiotic relationships, both from M. McIntosh (Loewe-Zentrum für Synthetische Mikrobiology). For this reason, the concentration of AHLs was evaluated first in the AHL-producing *S. meliloti* strains before inoculation of *Medicago sativa* (alfalfa or lucerne) plants with those bacteria. The quorum sensing molecules were extracted at different optical densities from a *S. meliloti* strain having a mutation in the AHL synthase *sinI* gene, and from the *S. meliloti expR*$^{+}_{(ch)}$, which is the *S. meliloti Rm2011* strain that has a chromosomal "repaired" in the copy of the *expR* gene. The production of AHLs was assessed using a luminescence assay. The luminescence was measured using the reporter bacteria *E.coli LuxR*$^{+}$*LuxI::LuxCDABE* (OD_{600}≈0.4). *S. meliloti ΔsinI* emitted the same background luminescence as the reporter bacteria, proving that there is no accumulation of AHLs in this strain (Fig. 11).

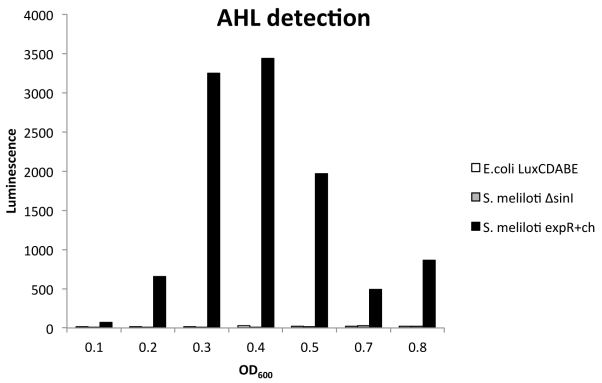

Figure 11. Production of AHLs in *S. meliloti expR+*$_{(ch)}$ strain is higher when optical density (OD_{600}) reaches 0.3 and 0.4 *S. meliloti ΔsinI* and *S. meliloti expR+*$_{(ch)}$ strains were grown in TY medium from OD_{600}≈0.05 to 0.8 to determine the production of AHLs, which were extracted from the rhizobacteria with chloroform. The *E.coli LuxR* $^{+}$*LuxI::LuxCDABE* strain was then used as reporter bacteria to measure the luminescence of each sample. The *Sinorhizobium meliloti expR+ch* showed higher concentration of AHLs when the OD_{600} reached 0.3 and 0.4; the background luminescence from the reporter bacteria and the *S. meliloti ΔsinI* was in parallel measured.

In contrast, *S. meliloti expR*$^{+}_{(ch)}$ showed high concentration of AHLs, especially at early stages of the culture (OD_{600} between 0.3 and 0.4). For this reason, *Medicago sativa* rhizosphere was inoculated with *S. meliloti expR*$^{+}_{(ch)}$ and *S. meliloti ΔsinI* at OD_{600}≈0.3.

3.4.4 AHL-producing *S. meliloti* strain does not enhance resistance against *S.* Typhimurium in alfalfa sprouts

As previously mentioned, AHLs produced by *Sinorhizobium meliloti* can induce resistance in various crop plants, however it has not yet been demonstrated if the same effect exists when this bacterium form a symbiotic relationship with its native host. In order to access the possible impact of AHL-producing S. meliloti on its host plant, we evaluated the resistance against *Salmonella* bacteria in *Medicago* plants. *Medicago sativa* seeds were germinated and pretreated overnight with $MgSO_4$, *S. meliloti ΔsinI* or *S. meliloti expR*$^{+}_{(ch)}$ under continuous shaking at 28 °C; non-attached bacteria were removed by washing. After three days, alfalfa sprouts were soaked overnight in *Salmonella* solution (1.4 x10^5 CFU/mL) and subsequently washed before evaluating the proliferation of the pathogen at 1 and 5 days after inoculation (Figure 12A). Because samples contained two types of bacteria, plants and bacterial-plant homogenates were plated on XLT-medium selective plates. *Salmonella* colonies appeared black due to their ability to reduce thiosulphate, while *S. meliloti* grew as yellow colonies because they are not able to reduce thiosulphate but still capable to ferment the sugar present in the medium (Figure 12B). The experiment was performed at least four times without obtaining significant differences in the proliferation of *Salmonella* Typhimurium on plants after the different *S. meliloti* strains treatments (Figure 12C), indicating that oxo-C14-HSL from *S. meliloti expR*$^{+}_{(ch)}$ did not enhanced the resistance against *Salmonella* bacteria in *Medicago sativa* seedlings. It its worth to mention that AHL production was not tested during *Medicago-S.meliloti* interactions, and genes involved in AHL production are downregulated after successful colonization of the host plant (*see* Discussions) [82].

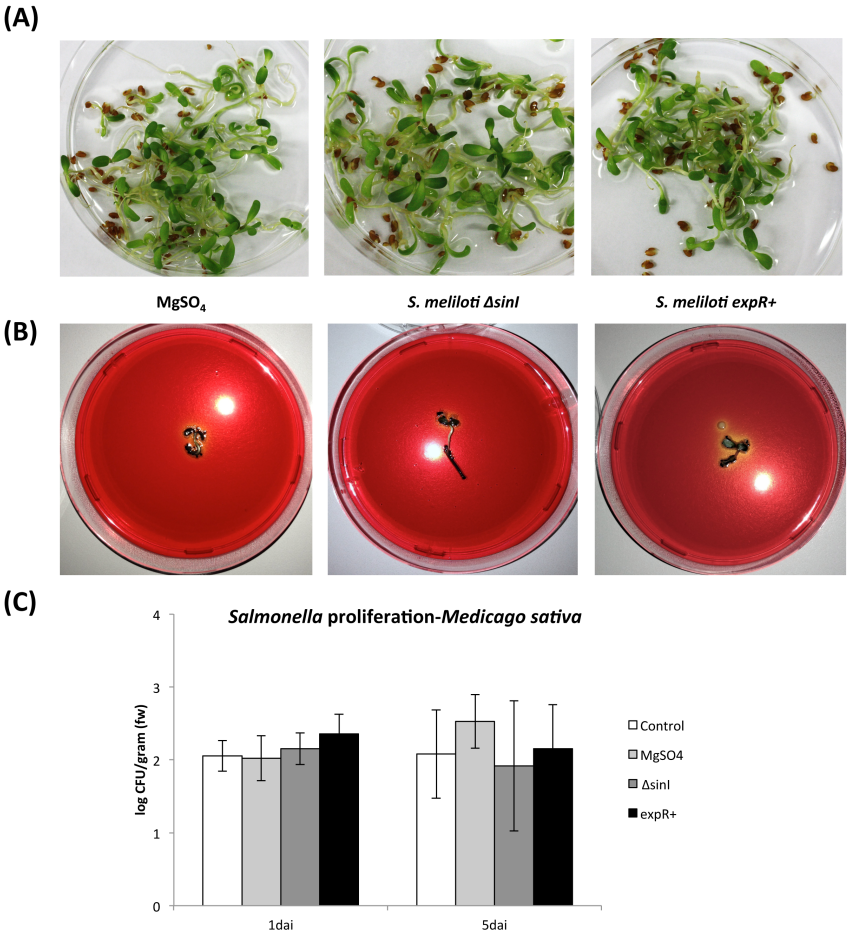

Figure 12. ***S. meliloti expR+*** $_{(ch)}$ **do not enhaced AHL-induced resistance in *Medicago* seedlings against *Salmonella*.** Alfalfa (*Medicago sativa*) seedlings were pretreated overnight with $MgSO_4$, *S. meliloti ΔsinI* or *S. meliloti expR+* $_{(ch)}$. After three days, seedlings were dipped overnight into *Salmonella* Typhimurium solution (≈1.4 x10^5) and washed before assessing the pathogen growth. **(A)** Images showed that alfalfa sprouts in all treatments did not presented disease symptoms 5 days after infection with *Salmonella*. **(B)** However, *Salmonella* infection was successful since black colonies developed along the roots and leaves; additionally, yellow colonies from *S. meliloti expR+* $_{(ch)}$ were also detected. **(C)** No significant differences in the growth of Salmonella 1dai and 5dai were observed, implying that AHLs produced by *S. meliloti expR+* $_{(ch)}$ did not increase resistance against the human pathogen.

3.5 *Salmonella* effector proteins

3.5.1 *Salmonella* effector protein SpvC suppresses the plant immune system

Salmonella enterica is recognized today as a cross-kingdom pathogen colonizing animals and plants [83]. Moreover, the bacterial strategies to suppress the different defense mechanisms in animals and plants are probably, at least partially, conserved. Therefore, to ascertain the infection mode of this bacterium in plants, the function of effector proteins of *Salmonella* Typhimurium were studied using the model plant *Arabidopsis thaliana*. The first candidate of this study was *SpvC*, whose

gene is located on the *Salmonella* plasmid virulence (*Spv*) locus, and its product is required for the virulence phenotype in animals [84].

In Neumann *et al.*, we demonstrated that when the effector protein SpvC is co-transformed with *pFRK1-Luciferase* (*pFRK1-Luc)* in *Arabidopsis* protoplasts, the PAMP-triggered activation of the luciferase activity is significantly suppressed six hours after flg22 treatment in comparison to the negative GFP negative control and the expression of a known suppressor of *pFRK1* activity, the AvrPto effector from *Psudomonas syringae* [85]. Moreover, to confirm these results, the native transcription levels of the flg22-induced receptor-like kinase 1 (*FRK1*), the expression levels of the transcription factor *WRK17,* and the protein-transport protein *Sec61* were analyzed. The expression of all genes is usually up-regulated after treatment with flg22; however, the expression induction was inhibited in SpvC-expressing protoplasts [85]. These results are comparable to those observed with the effector AvrPto from *Pseudomonas syringae*, suggesting that SpvC can intermit with the early MAMP-triggered signaling.

Due to the fact that SpvC is a member of the OspF effector protein family (phosphothreonine lyases), SpvC was expected to interact with plant MAPKs, similarly to its function in animal cells. To test this assumption, pull-down assays were performed by combining the total protein extract from *Arabidopsis thaliana* leaves and the 6x-His- or GST-tagged SpvC protein. The His- or GST- tagged proteins were captured by Ni-NTA or GTH affinity binding purification, respectively. The effector protein-kinase interaction was probed via protein immunoblots using monoclonal antibodies against the MAPKs: AtMPK6, AtMPK4 or AtMPK3. Western blots revealed that AtMPK6 could be detected in the complex with 6xHis-SpvC-protein but no signals were detectable for AtMPK4 or AtMPK3, suggesting that SpvC interacts specifically with AtMPK6. Interestingly, these results were verified in an independent assay, using the split-YFP technique. Here, 18% of cells co-transformed with YFP_n-MPK6 and YFP_c-SpvC, and 34% of those co-transformed with YFP_n-SpvC and YFP_c-MPK6 revealed a reconstitution of the YFP molecule and indicated an interaction between MPK6 and SpvC (mCherry-transformed cells were used for normalization). Furthermore, it was shown that the effector protein SpvC can be dephosphorylated active MAPKs, which are phosphorylated at the threonine and

tyrosine residues of their activation loop (TEY). To prove this, total plant proteins were incubated with the purified SpvC protein, and the phosphorylation state of *At*MPK6 and *At*MPK3 was disclosed using specific antibodies directed against the phosphorylated version of the activation loop (pTEpY) of the kinase. Contrarily to the results obtained with the control samples, in the presence of SpvC no signal of phosphorylated *At*MPK6 or *At*MPK3 were detected, indicating that SpvC deactivated these kinases.

To further investigate the role of the SpvC effector protein during plant infection, the *Salmonella* Δ*spvC* mutant and the *Salmonella* Typhimurium wild type strain 14028s (as control) were infiltrated in four-week-old *Arabidopsis* leaves, and the pathogen proliferation was evaluated during four days. The results showed that SpvC plays an important role for successful bacterial colonization since the bacterial CFU/leaf disc in leaves infiltrated with *S*. Typhimurium 14028s wild type strain increased by two $\log_{10}$ units, whereas the *S*. Typhimurium Δ*spvC* mutant was unable to proliferate. In summary, SpvC is required for full virulence of *Salmonella* in plants, and conserves its phosphothreonine lyase function to inactivate MAPKs, in particular AtMPK6, and thus inhibit the plant defense signaling.

3.5.2 *Salmonella* effector *AvrA* and *SptP* were cloned to study their translocation into plant cells

To comprehend better how *Salmonella* colonizes plants, two additional effector proteins, AvrA and SptP, were selected to analyze their expression and translocation into plant cells. For this purpose, the open reading frames (ORF) coding for respective protein were amplified, digested with *Bam*HI and cloned into the unique *Bam*HI site of the pIZ1886 plasmid [60], which is a derivative of pSIF003-R1 and pQE32 [86, 87]. These plasmids, expressing the effector-cyaA fusions, were transformed into *E. coli* TP610 cells [88], which should turn red in MacConkey-maltose agar plates when the cyaA fusions were inserted in the correct orientation. Subsequently, the correct plasmids were transfected into the intermediate host *Salmonella* LB5010, a galE$^-$ derivative that is r-m+ for all three DNA restriction-modification (R-M) systems [89]. For translocation studies, the plasmids were finally

transfected into *Salmonella* Typhimurium wild type strain 14028s (WT) and *Salmonella ΔprgH* mutant carrying a mutation in the Type 3 Secretion System 1 (T3SS-1) (Figure 13A).

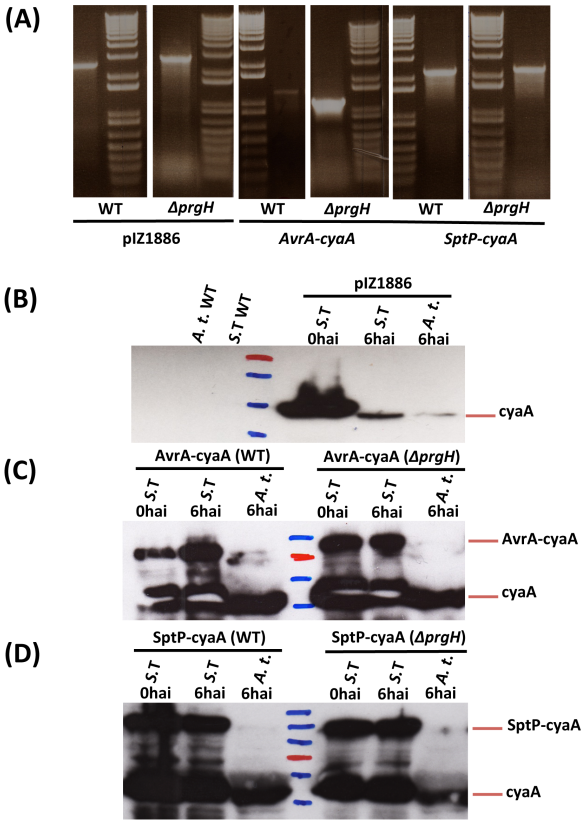

Figure 13. Expression of AvrA- and SptP-cyaA fusion proteins in *Salmonella* wild type and *Salmonella ΔprgH* strains was positive. (A) AvrA and SptP effector ORF were cloned into the –cyaA fusion pIZ1886 plasmid (courtesy of Ramos-Morales) to be expressed in *Salmonella* Typhimurium *14028s* (WT) and *Salmonella 14028s ΔprgH* strains. **(B)** The catalytic domain of cyaA was expressed in *S.* Typhimurium WT (*S.* T.) and in *Arabidopsis thaliana* (*A.t.*) leaves infiltrated with the bacteria carrying the empty plasmid. **(C)** AvrA-cyaA fusion protein was expressed in both bacterial strains soultions, and in leaves infiltrated with *S.* Typhimurium WT 6 hai but not with *Salmonella ΔprgH*. **(D)** The detection of the Sptp-cyaA fusion protein was only positive in *Salmonella* infiltration solutions 6hai; the fusion was not visible in infected leaves at any time point.

To test the expression of the effector-cyaA-fusion proteins, *Salmonella* strains were grown under SPI-1 inducing conditions (*see Materials and Methods*) before infiltration into five-week-old *Arabidopsis thaliana* leaves. The fusion protein expression in bacterial culture and in inoculated leaves (6 hai) is shown in Figure 13B-D. The SPI-1-inducing conditions subserved for the expression of AvrA-cyaA and SptP-cyaA proteins in both *Salmonella* strains (wild type and *ΔprgH*) even after 12 hours. In Figure 13B, *Salmonella* Typhimurium wild type lacking the pIZ1886 plasmid and uninfected *A. thaliana* leaves showed no signal with the cyaA-specific antibody.

On the other hand, cyaA signals were detected in leaves infiltrated with *Salmonella* WT conveying the empty pIZ1886 vector 6 and 12 hai; the presence of the catalytic domain of cyaA was also detected 6 hai in the bacterial suspension that was used for *Arabidopsis* infiltration. The expression of AvrA-cyaA fusion protein (Figure 13C) was positive in the two *Salmonella* strains solutions (wild type and *ΔprgH* mutant) and *Arabidopsis* leaves infiltrated with *Salmonella* WT 6 hai and 12 hai (data not shown), however no signal was identified for the fusion protein in *Salmonella ΔprgH*-infected leaves, suggesting that the T3SS-1 is necessary for the presence of the effector protein *in planta*. SptP-cyaA fusion proteins were not present in infected leaves 6 or 12 hai despite their expression in the bacterial cultures.

To confirm translocation of effector protein in plants, samples from *Salmonella* infected leaves were taken 6 or 12 hai accordingly to the previous results, however the translocation of the effectors remains to be determined via a specific ELISA assay.

3.5.3 Expression of the AvrA effector protein in *Arabidopsis* protoplasts does not inactivate MPK3, MPK4 nor MPK6

In mammalian cells, the YopJ protein superfamily inactivates the MAPK and NF-κB signaling pathway by targeting the MAPKs and IκBs kinases (IKK) [90]. Moreover, the members of this group require the eukaryotic cofactor phytic acid (IP6) for their full activation in host cells [91]. Since AvrA is a YopJ homologue in *Salmonella*, the ability of this effector to inactivate plant MAP kinases was tested in *Arabidopsis* protoplasts expressing AvrA. The activities of *At*MPK6, *At*MPK3 and *At*MPK4 were triggered with the bacterial PAMP flg22 in the presence (or not) of phytic acid, as shown in Figure 14. Transformed protoplasts expressing *35s::*3xHA-*AvrA* did not displayed any differences when compared to control protoplasts transformed with the empty vector pAUL17 [57]. Also, the addition of 100 nM phytic acid to enhance the acetyltransferase activity of the effector protein resulted in no differences in activity. The detection of the 3xHA tag confirmed the presence of AvrA in transformed protoplasts. In accordance to the results, it is suggested that the *Salmonella* effector protein AvrA does not interfere with the plant defense signaling at the level of

MAPKs, but it might rather be involved in ubiquitin-related pathways due to its additional deubiquitinase activity.

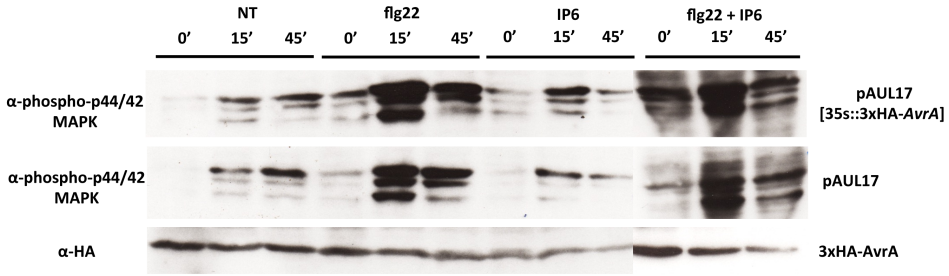

Figure 14. Expression of AvrA effector protein in protoplasts does not inhibit MAPK activation. *Arabidopsis thaliana* Col-0 protoplasts were transformed with pAUL17 expressing 3xHA-AvrA; empty vector was used as control and 3x-HA tag was detected to assure transformation using a specific antibody. Immunodetection of active MAPKs was performed 15 and 45 min after treatment with the bacterial peptide flg22 in the presence of the phytic acid (IP6) for full activation of the effector protein. In all treatments, no differences were distinguished between protoplast expressing AvrA and control protoplasts, indicating that AvrA effector protein do not disturb the MAPKs immune responses despite its acetyltransferase activity. The experiment was repeated at least three times.

4 DISCUSSIONS

4.1 *Sinorhizobium meliloti's* oxo-C14-HSL induces resistance in plants

The interactions between higher organisms and bacteria are based on inter-kingdom and bacterial communication systems. Quorum sensing (QS) is a mechanism regulating gene expression dependent on and controlled by bacterial cell density [92]. In this cell-to-cell communication, Gram-positive and Gram-negative bacteria produce signaling molecules (autoinducers or bacterial pheromones) to regulate processes like bioluminescence, biofilm formation, expression of virulence factors, or symbiosis. [93]. The autoinducer molecules can be classified depending on their structure: Gram-negative bacteria produce acylated homoserine lactones (AHLs), Gram-positive give rise to signaling peptides, and for intra- and interspecies communications both bacterial groups use a furanosyl boronated diester molecule termed AI-2 [47]. In addition, usually the QS system requires a synthase, receptor and response regulator to regulate specific genes belonging to the QS regulon and responsible for the synthesis of the autoinducer molecule.

The Gram-negative rhizobacteria *Sinorhizobium meliloti* produces five different AHLs with long acyl chains, ranging from 12 to 18 carbon atoms [94]. Following an increment in population density, AHLs can attain a threshold value of 1nM resulting in activation of the transcription factor ExpR, which allows the expression of the SinI, the synthase responsible for the production of AHLs. ExpR is also involved in the negative regulation of the transcription factor SinR when AHLs reach a concentration of 40nM, wherein a negative feedback loop is switch on because *SinI* activation is absolutely dependent on SinR [95]. Furthermore, the number of carbons in the AHL acyl side-chain have an impact on the binding strength of ExpR to DNA, where the 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)-tetradecanamide (oxo-C14-HSL) produced by *S. meliloti* Rm2011 strain was demonstrated to induce maximal activation of *SinI* [70, 71].

The Sin-ExpR system is responsible for the biosynthesis of galactoglucan, which is one of the three symbiotically important active exopolysaccharides to establish symbiotic relationships. Mutants that are unable to synthesize galactoglucan (EPS II)

generate feeble root nodules that are destitute of bacteria [70]. ExpR-derived ESP are also involved in sliding motility (but not in swarming) of *S. meliloti* strains [96, 97].

Furthermore, *S. meliloti* quorum sensing molecules have an impact on the defense of plants. The growth of *Pseudomonas syringae* DC3000 (*Pst*) was suppressed when the rhizosphere of *Arabidopsis thaliana* plants was supplied with the "repaired" *S. meliloti* Rm2011 strain producing oxo-C14-HSL ($expR^+$) but not with the transgenic AHL negative *S. meliloti attM* [71]. Other studies also support the involvement of QS regulation in plant defense. For example, the AHL-producing *Serratia plymuthica* strain HRO-C48 confers resistance against the damping-off disease-causing pathogen *Phytium apharnidermatum* in cucumber plants, and induces systemic resistance in bean and tomato plants against *Botrytis cinerea* [51]. Contrarily, the results obtained with the *S. plymuthica* mutant *splI*$^-$ AHL-4 were comparable to the control and wild type treatments [51]. Another example is when tomato roots are colonized with C4-HSL- and C6-HSL-producing *S. liquefaciens* MG1, enhanced systemic resistance against *Alternaria anternata* can be observed but not with the AHL negative mutant *S. liquefaciens* MG44 [53].

Experimental data of this work showed that the transgenic *S. meliloti* $expR^+$ strain was responsible for induced resistance in crop plants. The development of *Blumeria graminis* mycelium was more advanced in barley plants treated with $MgSO_4$ or inoculated with the transgenic *S. meliloti attM* (AHL^-) than in plants inoculated with the AHL-producing *S. meliloti* $expR^+$. Additionally, tomato plants pretreated with oxo-C14-HSL-producing *S. meliloti* strain exhibited only first stage symptoms of late blight disease, while the plants inoculated with the AHL-negative *S. meliloti* strain or control treatments were 20% less effective in constraining pathogen damage [72]. The mechanism of AHL-induced resistance produced by *S. meliloti* is discussed below, it comprises ROS production, cell wall reinforcement and stomata closing.

4.1.1 ROS signaling in oxo-C14-HSL priming

Production of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet-}$), hydroxyl radical (OH^-) and singlet oxygen ($^1\Delta O_2$) is one of the initial

responses of plant cells to abiotic or biotic stress. According to ROS titer, these compounds have a dual role in plant cells by acting as toxic byproducts or signaling molecules for growth, development or defense. Oxidative damage to DNA, proteins, lipids and cell structures are derived from ROS overproduction, however at a lower and controlled concentration, ROS behave as signaling components that are freely diffusible across cellular membranes and compartments. Antioxidant systems consisting of non-enzymatic and enzymatic scavengers balance ROS titer to activate immune mechanisms. In addition, the simultaneous production and scavenging of ROS determine the intensity, duration and localization of ROS signals that can involve feedback loops [98, 99].

Following a pathogen infection, production of hydrogen peroxide has been associated with both oxidative burst and initiation of defense signaling mechanisms. At the infection site, the disruption of the metabolic balance in the membrane leads to an oxidative burst, or rapid accumulation of ROS, that might result in a localized cell death known as hypersensitive response (HR), preventing thereby the progression of pathogen infection [99]. In Hernández-Reyes *et al*., we demonstrated that barley and wheat plants infected with powdery mildew or stem rust fungus (respectively) increased significantly the production of ROS, in particular H_2O_2, resulting in augmented hypersensitive response when plants rhizosphere was pretreated with oxo-C14-HSL or with the *S.meliloti* strain producing the AHL molecule [72].

NADPH-dependent oxidases, peroxidases, mitochondria, peroxisomes and chloroplasts are the main sources of ROS. Peroxisomes migrate beneath the point of contact following a pathogen attack, and have an accumulation rate similar to chloroplasts of 30 to 100 times more than mitochondria [98, 100]. Peroxidases are divided in three classes, where class III peroxidases are important for several plant metabolic processes in the cytosol, vacuole, apoplast and cell wall [99]. Furthermore, class III peroxidases are activated by biotic stress and they are responsible for either H_2O_2 production (oxidase cycle) or H_2O_2 scavenging (peroxidase cycle), depending on the cell physiological condition and on the presence of reducing agents. For a full oxidative burst in the apoplast, which is the space outside the plasma membrane within the boundaries of plant cell walls, the production of H_2O_2 in plant species like

carrots, *Prunella vulgaris* and *Arabidopsis* is generated by cell wall-anchored peroxidases and it is complemented by NADPH oxidases [100]. Barley plants pretreated with oxo-C14-HSL significantly enhanced the expression of vacuolar peroxidase 7 (*HvPRX7*) 24 hours after *Blumeria graminis* attack [72]. Multivesicular bodies (MVBs) and wall-associated paramural bodies (PMBs) recruit HvPRX7 in the vacuole for its redirection to the apoplast upon fungal infection [65].

NADPH oxidases are respiratory burst oxidase homologs (RBHO), and are controlled by a class of Rho-like proteins (ROPs) [98]. Likewise, lipid signaling plays a key role in the activation of NADPH oxidases after MAMP or effector recognition; for example, fatty acids including linoleic and linolenic acid accumulate in the apoplast after MAMPs treatment [100]. Furthermore, phospholipids comport as signaling molecules in many cellular processes e.g., cytoskeletal rearrangement and vesicular trafficking, and they can be further processed into phosphatidic acid (PA) via activation of phospholipase C (PLC) or phospholipase D (PLD); PLC-derived PA is thought to act as a positive regulator of effector-triggered immunity [25], whereas PA originated from PLD is involved in MAMP-triggered immunity (MTI) [25]. After MAMP or effector recognition, PA formation in the plasma membrane via PLC involves the hydrolysis of phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P) by PLC to produce two secondary compounds: diacylglycerol (DAG), which is further hydrolyzed into PA by DAG kinase (DNK), and inositol-1,4,5-triphosphate ($Ins(1,4,5)P_3$) that diffuses to the cytosol releasing cytosolic Ca^{2+} from intracellular stores like the vacuole and ER, thereby regulating Ca^{2+}- and Calmodulin (CaM)-dependent enzymes and channels [101, 102]. Activation of Ca^{2+} influx by $Ins(1,4,5)P_3$ is presume to involve ligand-gated Ca^{2+} channels but their molecular identity is unknown [103]. Microarray data from plants treated with different AHLs exhibited differential regulation in many Ca^{2+}-related genes after priming with oxo-C14-HSL and subsequent flg22 treatment; however, oxo-C14-HSL-treated *Arabidopsis* transgenic seedlings expressing the Ca^{2+} sensitive enzyme aequorin did not displayed significant differences in free cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$), which is directly proportional to the luminescence after triggering Ca^{2+} responses with flg22. Accordingly, this result would suggest that $[Ca^{2+}]_{cyt}$ might not enhanced or be superior in the early stages of AHL-induced resistance, but does not exclude the

possible involvement of CaM as pointed in the microarray evidence. CaM genes (*AtCaM1-9*) were shown to regulate primary root elongation in oxo-C6-HSL-treated plants, where the expression in all genes significantly increased three to six hours after treatment with the short AHL; the effect was not present after treatment with long-chain AHLs (C10-HSL or C12-HSL) [104]. For this reason, it is proposed that CaM genes are associated with short-chain AHL molecules to induce distinct plant responses.

On the other hand, generation of PA through PLC and/or PLD prompts a rapid and transient first ROS burst within the first hour after MAMP recognition. As a precautionary measure, PA binds to phosphatidylinositol-4- kinases (PI4K), which control a feedback loop to avoid inappropriate activation of MTI. Active PLC or PLD pathways stimulate the induction of the phosphoinositide-dependent kinase 1 (PDK1), which subsequently activate the serine/threonine protein kinase OXI1. Interestingly, downstream of OXI1 are the mitogen-activated protein kinase 3 (MPK3) and MPK6, followed by the transcription factors WRK22 and WRK29 [99, 105]. Schikora *et al*. stated that especially these kinases and transcription factors were strongly activated in oxo-C14-HSL-primed plants [62]. Moreover, the first upsurge of ROS, originated from the lipid signaling, triggers salicylic acid biosynthesis and potentiates ROS signaling through a positive feedback loop; as a consequence, a second ROS burst is induced two hours after MAMP/effector perception, and generates HR [25, 106]. SA-induced Pathogenesis-Related proteins have been linked to H_2O_2 accumulation and HR. *Arabidopsis PR-1* and its barley homologue (*HvPR-1b*) were significantly up-regulated in plants treated with pure oxo-C14-HSL or *S.meliloti* expR+ strain, and challenge with flg22 or *Blumeria graminis*, respectively. It is suggested that in crop plants or in the model plant *Arabidopsis thaliana,* AHLs might participate in the amplification of the PA-ROS-SA signaling due to the strong production of ROS, enhanced activation of MAPK3/6 and increased expression of SA-related genes in the presence of oxo-C14-HSL. However, further experiments are required to confirm the involvement of PA signaling.

4.1.2 Cell wall fortification

In plants, the first line of defense resides in the cell wall, where three tactics are employed to inhibit pathogen intrusion: suppression of pathogen-derived cell wall-degrading enzymes, cell wall structural remodeling and production of antimicrobial compounds [107]. Genes related to the production of secondary metabolites e.g., phytoalexins and flavonoids were up-regulated in plants treated with AHLs [63]. Phytoalexins are antimicrobial and antioxidant substances produced to inhibit pathogen colonization, and protect the tissue against compatible pathogen interactions. Accordingly, the stronger and prolonged activation of *At*MPK6 in oxo-C14-HSL treated plants might contribute to the biosynthesis of important phytoalexins, such as camalexin [62]. This important indolic secondary compound in *Arabidopsis* is synthesized after MPK3/6 activation, and requires both ROS production and SA signaling for its accumulation [100, 108].

Moreover, it was previously discussed that cell wall-bound class III peroxidases, belonging to the PR-proteins 9 subfamily, are highly activated to confine pathogen infection through the massive production of reactive oxygen (nitrogen) species [109]. In this biochemical process, the ferric enzyme is converted into compound I after the transfer of an oxygen atom from H_2O_2. Subsequently, a reducing substrate (or reductant) is required to add one electron to produce compound II, and a second electron to return the ferric enzyme to its ground state. Phenolic compounds are the best cell wall reductants because peroxidases have an evident specificity for these compounds [100]. After wounding or pathogen attack, plants exploit this biochemical process as a rapid protective response before the transcription of defense genes. For instance, peroxidases utilize H_2O_2 to generate free radicals from lignin phenolic monomers, known as monolignols, with the purpose of forming a rigid three-dimensional heteropolymer (lignin) that reinforces the cell wall following a pathogen penetration attempt [109]. This immune response was shown to be present in oxo-C14-HSL-induced resistance since primed barley plants infected with *Blumeria graminis* f.sp. *hordei* significantly expressed *HvPRX7*, a vacuolar peroxidase associated with cell wall appositions (CWAs) beneath an infection site [65, 72]. In addition, *Arabidopsis* plants pretreated with oxo-C14-HSL considerably increased

their concentration of soluble phenolics, cell wall-bound phenolics and lignins 24 hours after elicitor treatment. For this reason, it is assumed that oxidative cross-linking is boosted for strengthening the cell wall in AHL priming [63].

Moreover, accumulation of H_2O_2, phytoalexins, papillae, and cross-linking of hydroxyproline-rich glycoproteins (HRGPs) was observed in sorghum plants infected with the fungus *Colletotrichum sublineolum*, which affirms that these defense responses are interconnected to reinforce plant cell wall as a response to biotic stress [110]. Biotrophic fungi obtain nutrients from host cells via specialized feeding structures termed haustoria. As a protection response, plants extend their plasma membrane to form an extrahaustorial membrane containing distinct layers of plant cell wall components, which in turn encompass the fungal extrahaustorial matrix (Figure 15) [111]. Wheat and barley plants infected with stem rust and powdery mildew, respectively, accumulated threonine-hydroxyproline-rich glycoproteins (THRGPs), which are well-characterized cell wall structural proteins, between the extrahaustorial matrix and extrahaustorial membrane [111]. Other cell wall components from the hydroxyproline-rich glycoproteins (HRGPs) family, such as *Expansins* (*At2g03090*, *At5g39270*), *Arabinogalactan Protein 3* (*At4g40090*) and *Proline-rich Proteins* (*At3g62680, At1g54970*) were up-regulated in plants treated with oxo-C14-HSL [112]. As phenolic compounds, soluble extensins are secreted to the apoplast and interact ionically with pectins, which serve as anchors for peroxidases, to form an insoluble network in an H_2O_2-dependent process [109]. Correspondingly, arabinogalactan proteins (AGPs) play a role in plant cell to-cell communication and interact with the pectin network with the aim of limiting pathogen colonization [107]. Callose depositions are commonly found along cells where cross-linkage of cell wall compounds occurred, and especially in *Arabidopsis* leaves that were primed with the oxo-C14-HSL molecule [63]. Callose is β-1,3-glucan linear polymer synthesized at the plasma membrane, and is a structural component of the plasmodesmata, obstruct the intercellular exchange of molecules by forming papillae. These cell wall thickenings can slow and restrain invading pathogens in a higher degree when *Arabidopsis* and barley plants are pretreated with the pure oxo-C14-HSL molecule, while an oxidative burst leading to HR is more prevalent as a blocking method in barley plants pretreated with the oxo-C14-HSL-producing *S.*

meliloti strain [72].

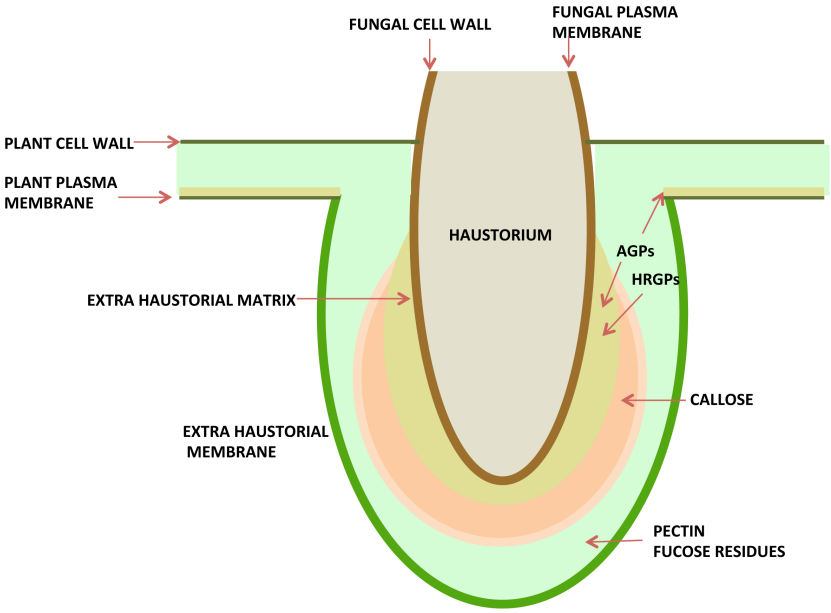

Figure 15. Representation of haustorium blockade by cell wall proteins. Cell wall proteins, such as arabidogalactans (AGPs), extensins and hydroxyproline-rich glycoproteins (HRGPs) accumulate beneath the area of fungal penetration. Peroxidases catalyze the cross-linking of cell wall components to form a physical barrier against fungi. AGPs bind to β–glucans, the forming compounds of callose, and allow attachment of polysaccharides to the cell wall matrix. In consequence, fungal hasutorium is encased by cell wall components and thus limit pathogen colonization. Modified from Starkunau *et al*. (1995)

4.1.3 SA/oxylipin signaling pathway regulates stomata defense response

The phyllosphere, especially the phylloplane, is estimated to host 10^6-10^7 bacteria per square centimeter of leaf surface. These bacteria must fend against nutrient availability, ultraviolet radiation, high or low water fluctuations and antimicrobial compounds produced by plants and other microorganisms [113]. Stomata, which are microscopic pores that regulate transpiration and gas exchange, as well as hydathodes in leaves, can provide an opportunity for bacteria to enter the interior of leaf tissue for acquiring nutrients; therefore, plant pathogens have developed ways to manipulate plant immune responses such as stomatal opening. After one hour incubation of *Arabidopsis* leaves with *Pseudomonas syringae* (min. 1 x 10^7 CFU/mL), the average width of stomatal aperture decreases significantly due to the action of defense responses; however, the orifice diameter of stomata reverts to the pre-bacterial treatment state 3hai even when the pathogen is surrounding the pore [114]. This effect can be attributed to a polyketide toxin known as coronatine (COR) that mimics jasmonoyl isoleucine (JA-Ile), the active form of jasmonic acid (JA), to

allow entry to the apoplastic region [115, 116]. JA-Ile and COR are recognized by the F-box protein and jasmonate receptor Coronatine-insensitive 1 (COI1) that is stabilized by the Skp1/Cullin1/F-box protein complex (SCF^{COI1}). Moreover, jasmonate ZIM-domain (JAZ) is incorporated into the complex as a substrate that is ubiquitinated and degraded to induce JA-related genes transcriptional activation when JA signaling is required [117]. Melotto *et al.* demonstrated that the main function of COR is to suppress defense responses to maintain stomata open, and recent discoveries have as well involved the conserved NAM-ATAF-CUC2 (NAC) transcription factors ANAC019, ANAC055, and ANAC072 as regulators of JA-induced stomatal opening [114, 118]. Furthermore, an *Arabidopsis* plasma membrane H^+ATPase (AHA1) induces stomatal opening by interacting with RIN4, an immune activator that binds to the *P. syringae* effector protein AvrB. The inflow of K^+ ions and increase of turgor pressure cause stomata to open as these changes disturb the plasma membrane potential [119].

During abiotic stress, stomatal closure is regulated by the plant hormone abscisic acid (ABA) [5]. This comprises the activation of the guard-cell specific OST1 kinase, in addition to the production of NO and H_2O_2 [114]. COR inhibits ABA-induced stomatal closure in a NO-independent and COI1-dependent manner [68, 114]. Notwithstanding that COR-inhibiting effect was not directly studied in AHL priming, COI1 independency in AHL-induced resistance was shown in *coi1-16* mutant plants, where the induced resistance related to oxo-C14-HSL was abolished [63].

On the other hand, plants may use a JA-independent pathway to close the stomata after pathogen attack. Montillet *et al*. describe a new signaling pathway involving the precursors of JA but independent of this hormone upon pathogen recognition and activation of the MPK3 and MPK6 kinases [120]. Hormone measurements in oxo-C14-HSL-treated plants disclose the accumulation of SA and JA precursor (cis-OPDA) but not of JA or its active form (JA-Ile), suggesting that a different pathway might control stomatal defense responses in AHL-primed plants. Phyto-oxylipins function as signaling molecules whose function is to protect against different sort of stress like a pathogen attack. These compounds originate from the oxidation of polyunsaturated fatty acids (PUFAs), especially linoleic acid (C18:2) and linolenic acid (C18:3), to form hydroperoxides aldehydes when the pathogen-induced oxygenase

(PIOX) couples two oxygen molecules to PUFAs after harpin, wounding, JA treatment or plant-insect interactions. Additionally, fatty acid hydroperoxides are formed when lipoxygenases (LOX) insert molecular oxygen into PUFAs at the 9 or 13 C position (9-LOX and 13-LOX, respectively) with the requisite of functional 1,4-pentadienyl [121, 122]. Linoleic acid and linolenic acid were able to induced stomatal-defense response after *Pst* treatment but failed in *Arabidopsis* plants compromised in the 9-lipoxigenase LOX1. Fatty acid hydroperoxides are transformed via the LOX pathway and further reduced by a peroxygenase into epoxy-hydroxy derivatives like reactive electrophile species (RES) oxylipins that are highly reactive molecules able to activate stomatal closure at lower concentrations than H_2O_2 [68, 121]. Furthermore, RES oxylipins modify the redox state by adjoining to thiol- or amine-containing compounds such as glutathione (GSH), and target the transcription factors TGA2, TGA5 and TGA6 [120]. For this reason, the expression of *Glutathione S-transferase 6* (*GST6*) and *Heat Shock Protein 70* (*HSP70*), two OPDA-dependent but JA-independent genes, was evaluated in plants treated with oxo-C14-HSL to confirm the involvement of oxylipins in AHL priming. Both genes presented enhanced expression 24 hours after infection with *Pst* when plants are pretreated with long-chain AHLs. Likewise, proliferation of the same pathogen was tested in the *Arabidopsis* triple mutant *tga2/5/6* and in the *lox2-1* mutant, where AHL-induced resistance was not detected in both cases. Furthermore, the defense-related hormone jasmonic acid is formed in the peroxisomes from 12-oxo-phytodienoic acid (OPDA), an oxylipin produced by the successive action of plastid-localized 13-LOX, allene oxide synthase (AOS) and allene oxide cyclase (AOC) [122]. OPDA was shown to accumulated 24 hours after *Pst* infection in oxo-C14-HSL treated plants and displayed strong activity on stomata [63, 68]. Together, these results suggest the involvement of cis-OPDA, the lipoxygenase 2-1 and the transcription factors TGA2, TGA5 and TGA6 in enhanced resistance by the QS molecule oxo-C14-HSL [63].

Additionally, salicylic acid (SA) was found to act downstream of RES oxylipins, and since this response is compromised in *NahG* transgenic plants and in the SA-biosynthetic mutant *eds16-2,* it was suggested that SA is involved in stomatal closure [68, 114]. To understand the involvement of SA in AHL-induced resistance, the expression of *GST6* and *HSP70* was studied in the *bsmt1* mutant and *med4*-1

knockdown line, which are important for SA signaling and Systemic Acquired Resistance [67], respectively [67]. The observed expression of *GST6* and *HSP70* genes did not resemble the results obtained in wild type *Col-0* plants, strengthening the hypothesis that conversion between SA and MeSA and accumulation of SA are required for AHL defense responses [63].

Hao *et al*. reported that SA accumulation increased endogenous concentration of ABA in relation to stomatal closure responses in cucumber cotyledons [123]. In the early stages of plant-pathogen interactions, the accumulation of NO in guard cells can be observed ten minutes after triggering the closing of stomata with flg22 or LPS; however, ABA is not produced when stomata close and LOX1 is controlling the stomatal defense response. For AHL-induced resistance, oxylipins and not ABA appears to control stomata closure because ABA-specific genes including *RD22*, *RAB18, RD29b*, *ABI1* and *ABI2* were not up-regulated before or after *Pst* treatment. Correspondingly, the OST1 kinase and different MAPKs (MPK9 and MPK12) participate in ABA- but not in flagellin-induced signaling, demonstrating that two pathways control stomata responses [68, 120]. However, ABA and SA/oxylipins signaling mechanisms converge at the anion channel SLAC1 to achieve the same purpose [68]. These results suggest that AHL-induced resistance function via the SA/oxylipin signaling and attend stomatal defense response.

Considering the above results, the augmented hypersensitive response in guard cells of wheat plants treated with oxo-C14-HSL, could be explained as the joint action of ROS, lipid-derived and SA signaling defense mechanisms to prevent the entry of *Puccinia graminis* through stomata [72]. To induce resistance in crop plants with bacterial derivative AHLs or synthetic AHLs requires the generation of ROS and signaling amplification through oxylipin/SA pathways.

4.2 oxo-C14-HSL perception

Bacterial communication systems are commonly associated to the regulation of traits such as virulence factors, degrading enzymes, exopolysaccharides and biofilm formation, among others to establish interactions with their surrounding organisms. From one point of view, since bacteria are more frequently found in rhizosphere

than in bulk soil, plants can snoop bacterial communication and interfere with their quorum sensing. Proteomic studies in *Medicago truncatula* revealed that plant defense-related proteins, metabolic enzymes, auxin and cytokinin recognition proteins respond to bacterial quorum sensing molecules like AHLs. The perception of AHLs in plants could be similar to the recognition of bacterial Nod factors involved in the formation of symbiotic relationships between plants and *Rhizobia*. Nod factors are structurally diverse short chitooligosaccharides, or lipochitooligosaccharides (LCOs) with a reducing (2-O-methylfucose) or a non-reducing (N-acetylated lipid chain) end. Their receptors feature high binding affinity and chemical specificity for Nod factors. Lysine motif (LysM)-containing receptor-like kinases (RLKs), abbreviated as LYKs, are Nod factor receptors that possess an extracellular domain (with 1-3 lysine motifs), a transmembrane domain and an intracellular serine/threonine domain [124]. In compatible interactions, like between *Sinorhizobium meliloti* and *Medicago truncatula* plants, the arrest of infection during nodule development shares several features with defense responses against pathogens like the increased levels of defense-related proteins and peroxidases [125]. Nodule formation and bacterial infection can be genetically distinct but Nod factor receptors are necessary for both processes. In addition, Nod factors recognition share similarities with chitin perception because chitin receptors belong as well to the LYK family. Some short-chain chitooligosaccharides may suppress plant innate immunity in legumes and non-legumes, while long-chain chitooligosaccharides may induce MTI [126, 127]. Here is worth to mention that diverse effects have been reported according to the length of AHLs. Microarray data from *Arabidopsis* seedlings exhibited putative RLKs (receptor-like kinases) and TIR-NBS-LRR receptor proteins that responded to oxo-C14-HSL treatments [63]. Accordingly, At2g19210 (RLK) and At5g41540 (TIR-NBS-LRR) were up-regulated upon treatment with AHLs, and might be good candidates for AHL perception. The mode of action of RLKs comprises an extracellular ligand-binding domain that induces conformational changes within the receptor protein, and that subsequently triggers downstream signaling events. The largest subgroup of RLKs contains LRR domains, which are normally associated to the recognition of microbial surface structures or compounds [126].

White clover (*Trifolium repens* L.) roots treated locally with AHLs induced expression of CHS3 (chalcone synthase), which is the first enzyme of the flavonoid biosynthetic pathway. Furthermore, responses were observed in inner cortical cells but not in epidermal or outer cortex cells underlying the site of treatment [52]. These data suggest that AHL responses are specific to certain cell types. Similarly, *Lotus japonicus* Nod factors in epidermal and/or cortical cells can be involved in nodule formation or bacterial infection according to their position. These two processes can be distinguished by calcium spiking and calcium influx, respectively. However, LYK receptors, such as *Mt*LYK3 and LjNFR5 lack kinase activity despite their importance in recognition of chitin and its monomers (N-acetylglucosamine) [126]. Therefore, a very likely scenario could be that two receptor proteins are required to AHL-induced cell signaling.

G-proteins can intermediate cell signaling, and are a highly conserved mechanism in all eukaryotes. In plants, the G-protein-couple receptor 1 (GCR1) and GPA1 have been related to the perception of short-chain AHLs [128]. Another G-protein related to AHLs is the human IQ-motif-containing GTPase-activating protein (IQGAP1), which interacts with N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL) from *P. aeruginosa* [78]. In consequence, an IQGAP1 homologue in *Arabidopsis thaliana,* At2g47500, was cloned and the three domains of the homologue protein were successfully detected via western blot. Nevertheless, AHL-protein interactions studies are still in process and a reliable proof for the role of those proteins in AHL perception is missing.

Alternatively, Palmer *et al.* suggested that AHL-effects (e.g., growth promotion) were independent of intact AHL molecules and suggested to be caused by L-homoserine, a product of the hydrolysis of the amide bond of AHL molecules. The hydrolysis is presumably executed by a fatty acid amide hydrolase (FAAH), which is a membrane-associated enzyme located in root cells [129]. However, Ortiz-Castro *et al*. reported that hydrolysis of C10-HSL by FAAH has an effect on AHL-promoting root growth and structure in *Arabidopsis*, showing the dependency of intact AHLs [130].

In *Arabidopsis*, it was demonstrated that short C6-HSL is transported systematically within the plant, while the long oxo-C14-HSL is not [62]. In barley plants, where lactonases have not yet been found, it was shown that AHLs are taken up actively

and transported systematically mainly through the central cylinder, and probably involve ABC transporters. Longer acyl-side chains, such as oxo-C14-HSL, are more lipophilic and presented a lower rate of transportation due to membrane partitioning [77].

Moreover, two bacterial AHL-synthases were expressed in tobacco plants to examine if AHLs could be created *in planta*: *Yersinia's YenI* synthase, which generates 1:1 the quorum sensing molecules C6-HSL and oxo-C6-HSL, and *LasI* synthase from *Pseudomonas aeruginosa* producing oxo-C12-HSL. The results showed that both transgenic tobacco lines produced physiological levels of all AHLs, and oxo-C14-HSL was surprisingly also produced in *LasI*-expressing plants. Furthermore, YenI was translocated to the chloroplasts allegedly by the abundance of AHL-precursor compounds in these organelles. Plant-derived AHLs were extracted from roots, stems and leaves, indicating that AHLs could freely diffuse across plastids and plasma membranes of these tissues [131, 132]. These results indicate that long-AHLs from bacteria could be distributed within specific plant cells and further metabolized in plants. For example, essential amino acids like threonine can be synthesized from aspartate in a *de novo* biosynthetic pathway, where homoserine and homoserine kinases, which belong to the GHMP kinase superfamily, are required as key intermediates to convert L-homoserine to O-phospho-homoserine before the conversion to threonine [133].

4.3 oxo-C14-HSL impacts the growth of the human pathogen *Salmonella* Typhimurium

Bacterial quorum sensing molecules may induce diverse responses in plants. Certain QS molecules produced by bacteria can contribute to plants welfare by acting as priming agents, i.e. these molecules stimulate plant immunity against particular pathogens preventing propagation of the pathogen. Rhizobacteria can enhance crop yield by influencing nitrogen fixation, production of siderophores and different phytohormones, reduce stress, and attenuate plant pathogenic QS signals [67]. Similarly, endophytes and epiphytes, such as *Pantoea agglomerans* and *Erwinia toletana* may release AHL molecules to modulate the virulence of the pathogen

Pseudomonas savastanoi pv. *savastanoi* (*Psv*) [134]. The effect of oxo-C14-HSL produced by *S. meliloti* was shown not only to encompass plant pathogens but also human pathogens like *Salmonella enterica* serovar Typhimurium. Pretreatment of *Arabidopsis thaliana* plants with the AHL-producing *S. meliloti* strain expR$^+$ reduced significantly *Salmonella* Typhimurium disease symptoms and growth, compared to a transgenic *S. meliloti* strain unable to accumulate AHLs [72]. For this reason, our research purposes expanded to an additional level to understand if oxo-C14-HSL-induced resistance can prevent plant colonization of human pathogens, especially during *Salmonella*-plant interactions [19].

Salmonella enterica subsp. *enterica* comprises 1,454 serotypes that colonize warm-blooded animals, and some of them have been linked to infection in plants [135]. For example, GFP-tagged *Salmonella enterica spp.* such as Montevideo, Michigan and Poona were found to colonize the interior of tomato plants grown hydroponically [136]. In addition, Salmonella *enterica* serovar Dublin can colonize endophytically and epiphytically lettuce plants by a three-phase process: root colonization, infection of the vascular parenchyma and invasion of xylem [137]. Internalization of *S. enterica* ser. Typhimurium SL1344 was also demonstrated in arugula but the same serovar was only found on parsley leaf surfaces [138].

Phytopathogens may exploit their quorum sensing mechanisms to induce virulence. The blackleg disease caused by *Erwinia caratovora* ssp. *atroseptica* depends on cell wall-degrading enzymes e.g., pectinases and cellulases, whose production rely on the bacterial density and production of the AHL molecule oxo-C6-HSL [139]. Similarly, the plant and human pathogen *Pseudomonas aeruginosa* generates C4-HSL and 3-oxo-C12-HSL to control the expression of virulence factors and secondary metabolites, such as chitinase, lectins and superoxide dismutase that contribute to its growth *in planta* [140]. *Salmonella* species contain at least two quorum-sensing systems: Autoinducer-2 (AI-2)-depending system that serves as a cell-to-cell signaling molecule between serovars, and an AHL-induced system. No acyl homoserine lactones have hitherto been discovered for *Salmonella,* which is in line with the lack of an AHL synthase. However, *Salmonella* detects AHL molecules produced by other bacteria. The *Salmonella SdiA* gene, a LuxR homologue, detects nanomolar concentrations of oxo-C4-HSL, C6-HSL (from *Aeromonas hydrophila*), oxo-C6-HSL,

oxo-C8-HSL, oxo-C10-HSL and oxo-C12-HSL from other species. In the presence of AHLs, *Salmonella* SdiA activates the expression of six genes within the *rck* operon, a putative effector protein srgE and the *Salmonella* invasion regulator SirA, which coordinates the *Salmonella* Pathogenicity Island 1 (SPI-1) [135, 141, 142]. Gene clusters located in pathogenicity islands encode Type 3 Secretion System (T3SS) that enable *Salmonella* to invade eukaryotic cells [118, 135]. More than thirty effector proteins derived from SPI-1 and SPI-2 manipulate progressively key functions of host cells including signal transduction and membrane trafficking [143].

Moreover, this human pathogenic bacterium may benefit from the presence of other phytopathogens to induce virulence. *Salmonella* Typhimurium is able to detect 3-oxo-C6-HSL produced by the soft rot bacteria *Pectobacterium carotovorum* when both pathogens are incubated *in vitro*. Additionally, this AHL enhanced the expression of the putative *Salmonella* effector *srgE* in a *SdiA*-dependent manner. In spite of the lack of *SdiA* expression inside tomato fruit or tomato surfaces infected with *P. carotovorum* (presumably to a low pH and compounds produced by plants), *Salmonella* growth was benefited by the presence of the soft rot bacteria during the conditions of tomato fall-winter production, demonstrating the opportunistic abilities of *Salmonella* for plant colonization [144]. Mechanical damage e.g., cut surfaces during food production and handling increases contamination risk, and exemplifies as well the opportunistic behavior of *Salmonella* [145].

On the other hand, the presence of symbiotic bacteria may impede *Salmonella* plant colonization as occurred in *Arabidopsis* plants pretreated with *S. meliloti*. In this work, I postulate that *S.* Typhimurium virulence might be affected by the presence of oxo-C14-HSL from *Sinorhizobium meliloti* because *in vitro* growth of *Salmonella* was delayed when oxo-C14-HSL was added to the culture medium. Until now, I mentioned the possibility to induce resistance of *Arabidopsis* plants against *Salmonella* by pretreating the plants with an AHL-producing *S. meliloti* strain; however, there is no information about AHL-induced resistance against *Salmonella* when *S. meliloti* is forming a symbiotic relationship with its native host plant. To investigate this, we looked at the defense mechanisms induced by *S. meliloti*'s oxo-C14-HSL against *Salmonella* Typhimurium in *Medicago sativa* (alfalfa). Seedlings

were pretreated with the rhizobacteria producing AHLs and three days later infected with *S*. Typhimurium.

Medicago sativa sprouts have been linked to salmonellosis outbreaks, especially with *Salmonella enterica* serovars Stanley, Bovismorbificans, Barelly and Weltevreden. These pathogenic strains can proliferate fast during sprout manufacturing processes, and has significantly better attachment to sprouts than for example the human pathogen *E. coli* O157:H7 [146]. *Salmonella enterica* species preferentially colonize roots and root hairs, probably because root exudates contain carbon sources that enable the bacteria to proliferate [147]. Conversely, alfalfa plants form a symbiotic relationship with *Sinorhizobium meliloti*. These plants secrete flavonoids, vitamins, choline, stachydrine, trigonelline and homoserine compounds that attract *S. meliloti* towards roots [148]. Once the bacteria come in contact with the roots, plants recognize bacterial nodulation factors (lipochitooligosaccharides) that lead to curling of root hairs, and after to the formation of infection threads (IT) that result from the hydrolysis of local cell wall and infolding of plasma membrane. Infection threads can also be considered as tubular ingrowths of the plant cell wall that contain growing and diving bacteria. Root hair infection by *S. meliloti* causes the activation of peroxidases and cross-linking of extensins localized in the legume's extracellular matrix and lumen of IT via an H_2O_2-dependent process that do not cause oxidative stress to the rhizobacteria. At the end of the nodulation process, bacteria are enclosed in non-dividing and nitrogen-fixing bacteroids in the host cell cytoplasm, where they reduce atmospheric nitrogen into nitrogenized compounds utilizable by the plant. In addition, galactosides are secreted from plants three to five days after initiation of *S. meliloti* interaction to support bacterial growth [82, 149].

During early stages of *S. meliloti-Medicago* interaction, several genes encoding repetitive proline-rich proteins (PRPs) and peroxidases (Rip1) are involved in the cross-linking of cell wall components to facilitate the infection process, or for the formation of novel cell wall structures. Conversely, cell wall remodeling may also function to restrict rhizobial infection in an analogous mechanism to that presented in plant-pathogen interactions [125]. Gene expression analyses of *Medicago truncatula* plants infected with a cocktail of five *Salmonella enterica* serovars

(Schwarzengrund, Enteritidis, Mbandaka, Havana and Cubana) demonstrated that PR proteins, peroxidases, cell wall modification components and plant hormones are differentially expressed in response to *S. enterica* [150]. These transcriptional changes may be due to the adaptation process experienced by plants 10 dai, but it is worth to mention the plant responses similarities during AHL-induced resistance and *S. enterica* infection. As in oxo-C14-HSL-induced resistance, *Salmonella*-infected roots of *M. truncatula* up-regulated two peroxidases, a cellulose synthase, an arabinogalactan-protein and a pectin esterase involved in modifying plant cell walls. Additionally, leucine-rich repeat receptor kinases, lectin protein kinase family proteins and two lipoxygenases (LOX1 and LOX5) were induced by this human pathogen [150].

The above events suggested that *S. meliloti expR*$^{+}$ could induce resistance against *S. enterica* during the establishment of a symbiotic relationship; however, alfalfa plants pretreated with oxo-C14-HSL-producing *S. meliloti* strain (*expR*$^{+}$) three days before *Salmonella* Typhimurium infection did not present enhanced resistance as previously shown in *Arabidopsis* plants. The results may not show differences between treatments but they are in accordance to the observation that alfalfa sprouts support the growth of *S. enterica* strains during the initial 72 hours of germination, and thereafter is observed a reduction in size of the maximum population [151]. The absence of AHL-induced resistance in *Medicago* seedlings could be explained first by the saprophytic growth of *Salmonella* during the germination process. This bacterium can metabolize the organic compounds released by germinating seeds and use them as a nutrient source, allowing *S. enterica* strains to utilize irrigation water as a growth medium. A 10-fold reduction in *Salmonella* growth can be detected 72 hai, which is correlated to the decrease of nutrient efflux from germinated seeds [151]. To study AHL-induced resistance in alfalfa seedlings, *Salmonella* Typhimurium were inoculated into the alfalfa growing medium 5 days after initiation of germination, which might have been not the optimal time point for *Salmonella's* growth. However, it should be mentioned that AHL priming requires three days prior pathogen infection, and that this discrepancy should be considered in future experiments.

In spite of the up-regulation of common plant genes involved in *Salmonella* defense and AHL-induced defense, another reason for the absence of AHL-induced resistance in *Medicago* plants could be the limited amount of AHLs and the low number of genes that are differentially expressed after inoculation with the quorum sensing mutant (*S. meliloti ΔsinI*) or the AHL-producing strain (*S. meliloti expR*$^{+}$) during nodule formation. The ExpR/SinI system is dramatically downregulated inside nodules, suggesting that quorum sensing loses its role after invasion is completed [82]. Jamet *et al*. showed that a high number of colonized curled root hairs and extended infection threads can be observed 5 days after *S. meliloti* Rm1021 inoculation [149]. These observations indicate that the production of AHL molecules in *S. meliloti* might have been altered during the infection process, and in consequence the AHL-induced resistance.

Moreover, alfalfa exudates may interfere with the expression of quorum sensing-regulated genes in *S. meliloti*, and hence the AHL-induced resistance effect. Quorum quenching is a process where QS signaling is inhibited by plant- or other microbial-derived molecules or enzymes [67]. An example of quorum-quenching molecule found in alfalfa sprouts is L-canavanine, an arginine analog found in legume seeds and serves as the principal metabolite for nitrogen storage. L-canavanine does not compete directly with AHLs but is incorporated as an arginine analog in the predicted protein sequence of ExpR, which contains 9% arginine residues. The incorporation of L-canavanine results in a negative effect on ExpR structure and function [152].

In summary, oxo-C14-HSL enhanced resistance during symbiotic relationships requires further studies in order to fully understand the possibilities and the limitations that might exist in respect of future agricultural applications.

4.4 *Salmonella enterica* effector-driven manipulation of plant cells.

Conserved molecules of microbial surface structures or cell wall components, better known as microbe-associated molecular patterns (MAMPs), can trigger MAMP-triggered immunity (MTI), which is the first line of defense against pathogens. Flagellin, lipopolysaccharides (LPS) and chitin are recognized by diverse plant

extracellular receptors, or pattern-recognition receptors (PRRs), that initiate a cascade of intracellular signals to arrest pathogen invasion [153]. Plants might recognize some human pathogens more effectively than others; for example, *E. coli* O157:H7 triggered stomatal closure even under high relative humidity, while *Salmonella enterica* SL1344 induced only transiently the same stomatal response [154]. The 22-amino acid sequence of the N-terminal part of flagellin (flg22) from *Salmonella enterica* serovar Typhimurium 14028s is recognized in *Arabidopsis*, tobacco and tomato plants [21, 80]. Furthermore, *Salmonella* Typhimurium colonization in *Medicago truncatula* plants ensue the induction of SA-independent and SA-dependent defense responses e.g., the induction of PR1 by flagella [22]. Non-flagellar T3SS (NF-T3SS) are macromolecular complexes responsible for the delivery of bacterial effectors, and consist mainly of a transmembrane export apparatus, an extracellular needle (or pilus) and a translocon. Seven distinct families of NF-T3SS have been identified, where Hrp1 and Hrp2 are associated to plant-bacteria interactions due to the presence of longer and more flexible pili as an adaptation to the thickness of the plant cell wall [155]. However, the normally animal-associated *Salmonella* SPI-1 can modulate the needle length through the deregulation of *invJ* "switch" that causes the continue secretion of PrgI, and thus formation of elongated needles [156]. Long flexible pilus and harpins may help *Salmonella* to overcome the great challenge of delivering effectors across the cell wall [155]. These complexes are required to surmount defense surveillance and counteractions in plant cells. Living Salmonella did not triggered the production of an oxidative burst while heat killed or chloramphenicol treated *S*. Typhimurium were significant elicitors, indicating that the pathogen actively suppress the plant response. Furthermore, Infiltration of *S*. Typhimurium T3SS mutant *invA* but not wild type, elicited strong HR-like symptoms in *Nicotiana tabacum* leaves, suggesting that T3SS are involved in the suppression of HR-like responses [157].

In mammalian cells, the delivery of SPI-1 effectors serve to induce membrane deformation and rearrangement of the actin cytoskeleton, in addition to the induction of bacterial internalization into *Salmonella*-containing vacuole (SCV) [143]. *Salmonella* Pathogenicity Island 2 (SPI-2) is involved in the replication of intracellular bacteria within the SCVs. In plant cells, the involvement of the SPIs is still

controversial. In *Arabidopsis* plants, lower proliferation rates and enhanced HR-related symptoms were observed in two isogenic T3SS mutants, *prgH* (encoded by SPI-1) and *ssaV* (encoded by SPI-2), when compared to wild type. Moreover, transcriptional analysis revealed that highly conserved *Arabidopsis* genes involved in defense were up-regulated upon infection exclusively with the *prgH* mutant, indicating that *Salmonella* Typhimurium has the ability to repress plant defense mechanisms [158]. Conversely, two *Salmonella* SPI-1 deletion mutants, one with a mutation in a transcriptional regulator of the T3SS (*hilA*::Tn) and another necessary for the assembly of the T3SS (*prgH*), were used to show that the SPI-1-encoded T3SS is not required for *Salmonella* growth on alfalfa sprouts but SPI-2 may have a role during alfalfa sprout colonization. Transcriptional analysis of *Salmonella enterica* serovar Weltevreden showed that genes involved in carbohydrate metabolism, amino acid metabolism and *Salmonella* Pathogenicity Island 2 (SPI-2) were significantly up-regulated, and that five genes encoding effector proteins (SifA, SseE, SopD, PipB and SseL) were significantly higher expressed in the presence of sprouts [146, 151]. Other results implying the involvement of SPI-2 in *Salmonella*-plant interactions were recently published using the model plant *Arabidopsis thaliana*. The effector SpvB and SpvC, which are required for full virulence in mice, are contained in the *spv* (*Salmonella* plasmid virulence) operon and further translocated into host cells by SPI-2 T3SS [84, 159]. SpvC was demonstrated in plants to impede with MTI signaling by down regulating the expression of the flg22-induced receptor-like kinase 1 (*FRK1*), the transcription factor *WRK17,* and the protein-transport protein *Sec61* when *Arabidopsis* protoplasts were transformed with the effector protein. Moreover, pull-down and split-YFP assays showed that SpvC interacts and dephosphorylates active *At*MPK6, confirming that this effector protein has a phosphothreoninelyase activity in animals and plants and that this activity inhibits cell MAPKs by removal of phosphate and modification of target threonine [85, 160].

On the other hand, the second branch of the plant immune system, the effector-triggered immunity [158], is able to recognize effector proteins whose function is to suppress outputs of MTI; for example, the *Salmonella* effector protein SseF elicits HR-like symptoms in *Nicotiana bentamiana*. Loss of symptoms upon silencing of the suppressor *SGT1* (Suppressor of G2 allele of Skip1) and the plasma membrane NDR1

(non-race-specific disease resistance 1) indicates that SseF is recognized by an NLR (nucleotide-binding leucine-rich repeat receptor) carrying a N-terminal coiled-coil domain that activates ETI [19, 24]. Nevertheless, some type 3 effectors (T3Es) are capable of suppressing both plant immune systems (MTI and ETI) by targeting shared components or by having multiple targets [161].

It is important to note, however, that translocation of effectors into plant cells by *Salmonella* remains to be demonstrated. For this reason, two effectors were selected in this work to analyze their secretion and translocation into plant cells. *Salmonella* is known to modulate the actin cytoskeleton for invasion, and this mechanism might be also used in plant cells in order to block SNARE regulatory proteins, which consequently can inhibit mRNA transport by depolarization of the actin cytoskeleton. Colonization of *Lactuca sativa* cv. Tamburo plants by *S. enterica* sv. Dublin were shown to induce the expression of *Sec1* and *Sec6* transport proteins, which are genes involved in the regulation and formation of the actin cytoskeleton [137]. The SPI-1 effector SptP is suggested to regulate the plant actin cytoskeleton by mimicking GTPase activating protein (GAP) that results in returning the host cytoskeleton to the resting state upon bacterial entry [162].

The effectors SpvB, SpvC, AvrA and SseL operate together to induce late apoptosis in both human and murine macrophages infected with *S*. Typhimurium as a cell-to-cell spreading mechanism [160]. Yet for this to happen, the SPI-1 AvrA effector protein inhibits first inflammation and represses apoptosis in mammalian cells by modifying critical serine and threonine residues of MKK4 and MKK7, and thereby blocking their phosphorylation and further activation of the JNK signaling that leads to inflammation and cell death responses [160, 163]. Correspondingly, AvrA blocks the NF-κB pathway downstream of IKK activation while its homologue YopJ (from *Yersinia pestis*) blocks it at a point upstream of IKK activation, despite their 86% amino acid sequence similarity [90]. According to these data, a protein blast was performed in this work to propose putative interactors of AvrA in *Arabidopsis thaliana*. The results suggested that *At*MKK1 and *At*MKK4, which belong to the *At*MPK4 and *At*MPK3/6 signaling pathway respectively, could be good candidates as homologues of human MKK4 and MKK7. However, the activation of *At*MPK6, *At*MPK3 and *At*MPK4 in protoplasts expressing AvrA was not different from the

control when flg22 was used to trigger activation. Similar results were obtained even when the cell suspension was supplemented with phytic acid, which is a eukaryotic co-factor required for the full activation of the acetyltransferase activity [164]. These results suggest that this *Salmonella* effector protein does not interfere with the defense signaling at the level of MAPKs, but may be involved in other immune pathways involving ubiquitinination or inhibition of the plant secretion pathway. The *P. syringae* homologue of AvrA, the effector HopZ1a, has the ability to block secretion of secGFP into the apoplast of *Nicotiana benthamiana* leaves via a constitutive secretion pathway, and destroy microtubule networks [91].

For translocation studies, SptP and AvrA were fused to the catalytic domain of *cyaA* from *Bordetella pertussis*, which is a calmodulin [49]-dependent adenylate cyclase. Bacteria are deficient in CaM, but following the translocation of cyaA-fusion proteins into plant cells, adenylate cyclase is activated to catalyze cyclic AMP (cAMP) via CaM. The expression, secretion and translocation of *Salmonella* effector SteA was demonstrated in murine macrophages and human epithelial cells by measuring cAMP levels [60]. Until now, the two effector-fusions were detected in bacterial suspensions, and AvrA-cyaA was detected in *Arabidopsis* leaves infiltrated with *Salmonella enterica* wild type 14028s but not in *Salmonella ΔprgH*-infected leaves. The results indicate that the T3SS-1 is necessary for the expression of the effector protein in plants. To confirm translocation of both effector proteins in plants, final experiments need to be performed in near future.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis in combination with our published results, attempted to understand the role of quorum-sensing molecules in plant-pathogen interactions and their impact on plant defense. The presented results provide strong evidences that the long *N*-acyl homoserine lactone, oxo-C14-HSL, enhances resistance in the model plant *Arabidopsis thaliana,* as well as in crop plants with economical importance such as barley, wheat and tomato. AHL-induced resistance against the biotrophic and hemibiotrophic pathogens *Pseudomonas syringae*, *Blumeria graminis*, *Puccinia graminis*, and *Phytophthora infestans* was observed but not against *Xanthomonas campestris*. Transcriptional analyses in oxo-C14-HSL-primed plants showed that genes related to biotic stress belong to the most outstanding category after triggering MTI with flg22. Furthermore, this AHL molecule, which is produced by the rhizobacterium *Sinorhizobium meliloti*, was shown to enhance ROS production, callose depositions and cross-linking of phenolic compounds (all important for cell wall reinforcement) and HR reactions after pathogen challenge. In addition, systemic responses were driven by a SA/oxylipin-dependent signaling that culminated in stomata defense reactions as another immune response to inhibit pathogen entry.

Moreover, the proliferation of *Salmonella enterica* serovar Typhimurium was reduced when *Arabidopsis* roots were pretreated with the AHL-producing strain of *S. meliloti* to induce defense. However, AHL-induced resistance against this human pathogen was not observed when the rhizosphere of *Medicago sativa* was inoculated with *S. meliloti*, suggesting that early stages of symbiosis have an impact on oxo-C14-HSL-derived defense responses. These results offer new insights in AHL-priming and offer new strategies for plant protection. Notwithstanding the potential of AHL molecules as biocontrol agents, the perception of these molecules in plants is still an open question, and the studies started in this work need to be further developed.

Furthermore, *Salmonella* was shown to require the effector protein SpvC for full virulence on plants. In plant cells, SpvC targets MPK3 and MPK6 in order to deactivate and suppress the early MTI signaling. This shows that the phosphothreonine lyase activity of this effector is conserved in animal and plants. On

the other hand, AvrA effector protein did not inactivate MPK3, MPK4 or MPK6 in protoplast even with the presence of the eukaryotic co-factor phytic acid, which is required for full activation of AvrA in animal cells. Thi suggest that some effector protein may have different functions in animal and plant cells. Translocation of *Salmonella* effector proteins, like AvrA and SptP, were initiated in this thesis; however, their translocation needs to be further determined.

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7 APPENDIX

7.1 Supplemental Table 1. List of Primers

35s promoter		5’-TTCGCAAGACCCTTCCTCTATA-3’
AvrA	CP001363.1	5’-AAAGGATCCATGATATTTTCGGTGCAGGAGC-3’
		5’-TTTGGATCCGCATAACGGCATTGTTATCG-3’
GHMP kinase	At5g14470	5’-GGAGATAGAACCATGGATCCGAATCCTAAACCGGC-3’
		5’-TCCACCTCCGGATCMTGTTTTTGATAATGTCTTAATATCAGAACTAC-3’
DFR	At5g42800	5’-CAAACGCCAAGACGCTACTCAC-3’
		5-‘ACGGTCTTTGCCTTAACACATGC-3’
DNR5		5’-CTGGCAGTTCCCTACTCTCG-3’
DNR3		5’- GATGGTCGGAAGAGGCATAA-3’
HvPR1b	Z21494.1	5’-GGACTACGACTACGGCTCCA-3’
		5’-GGCTCGTAGTTGCAGGTGAT-3’
HvPRX7	AJ003141.1	5’-TACCTCTCACATGTCAGCGGC-3’
		5’-TACTACTTCGACCTGATCGCG-3’
HvUBQ60	M60175.1	5’-CAGTAGTGGCGGTCGAAGTG-3’
		5’-ACCCTCGCCGACTACAACAT-3’
CH domain	At2g47500	5’-GGAGATAGAACCATGGCGGCTACTGCGACGG-3’
		5’-TCCACCTCCGGATCMCTTTTCGTCAAGAATCTGACTATTG-3’
K + CC domain	At2g47500	5’-GGAGATAGAACCATGAAAACTCGCCAATTTAAACAACTG-3’
		5’-TCCACCTCCGGATCMTGCTTCTTTTCTCGCGAGTGC-3’
C terminus	At2g47500	5’-GGAGATAGAACCATGGAGTCACAGCAGAACAAC-3’
		5’-TCCACCTCCGGATCMATGTCTGGCGTTAGTAGCTTTCC-3’
AtMKK1	At4g26070	5’-GTTAGCAAGTGGGGGAATCAAAG-3’
AtMKK4	At1g51660	5’-TGTGGTTGGAGAAGAAGACG-3’
pQE32		5’-GTATCACGAGGCCCTTTCGTCTTC-3’
rrb1		5’-GTCTTTCGACTGAGCCTTTCG-3’
SptP		5’-AAAGGATCCATGCTAAAGTATGAGGAGAGAAAATTG-3’
		5’-TTTGGATCCGCTTGCCGTCGTCATAAGC-3’
TIR-NBS-LRR	At5g41540	5’- GGAGATAGAACCATGGCTTCTTCTTCGACCC-3’
		5’-CTCAAGTGATCGACAATGGTTTGC-3’
		5’- CTGTTGTGGGTTCATCTTTGCGT-3’
		5’- TCCACCTCCGGATCMTAGTCTCCATCTCCAGCATTCG-3’
T7		5’- TAATACGACTCACTATAGGG-3’
TLP5	At1g43640	5’-GAAAGAGGAAGAAGGAAAGGT-3’
		5’-CAGAAGCTACAGTCACTCTC-3’
U5		5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATG-3’
U3		5’-AGATTGGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCTCCGGATC-3’