Functional characterization of *Phytophthora infestans* RXLR effector AVR2

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Table of contents

Table of contents I

List of figures V

List of tables VII

Abbreviations VIII

1. INTRODUCTION 1

1.1. The plant immune system 1

1.1.1 Recognition of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) 3

1.1.2 Effector-triggered immunity (ETI) 5

1.2 Hormones in plant immunity 7

1.2.1 Crosstalk between brassinosteroid signaling and plant immune responses 10

1.3 Effector-triggered susceptibility (ETS) 14

1.3.1 Bacterial effectors 15

1.3.2 Oomycete effectors 16

1.3.2.1 Apoplastic effectors 16

1.3.2.2 Cytoplasmic effectors 17

1.4 The RXLR effector AVR2 from Phytophthora infestans 18

1.5 Objective of the thesis 19

2 MATERIAL AND METHODS 21

2.1. Material 21

2.1.1.1 Chemicals 21

2.1.1.2 Media and antibiotics 21

2.1.2 Antibodies 22

2.1.3 Vectors 23

2.1.4 Bacterial strains 24

2.2 Methods 25

2.2.1 Growth conditions 25

2.2.1.1 Arabidopsis thaliana growth 25

2.2.1.2 Growth of Escherichia coli 25

2.2.1.3 Growth of Agrobacterium tumefaciens 25

2.2.1.4 Growth of Pseudomonas syringae 26

2.2.2 Molecular methods 26

2.2.2.1 Isolation of plasmid DNA from E.coli 26

2.2.2.2 Genomic DNA extraction 26

2.2.2.3 Total RNA extraction 27

2.2.2.4 Nucleic acid quantification 27
2.2.2.5 Restriction digests of plasmid DNA 27
2.2.2.6 Agarose gel electrophoresis 27
2.2.2.7 DNA gel extraction 28
2.2.2.8 Reverse transcription polymerase chain reaction (RT-PCR) 28
2.2.2.9 Polymerase chain reaction (PCR) 28
2.2.2.10 Quantitative Real-Time PCR (qRT-PCR) 30
2.2.2.11 Gateway site-specific recombination cloning of amplified DNA fragments 31
2.2.2.12 Site-directed mutagenesis 32
2.2.2.13 Generation of Gateway compatible binary destination vectors for expression of N-terminal KozakMYC and KozakHA fusion proteins 33
2.2.2.14 Sequencing 33
2.2.2.15 Preparation of chemically competent DH5α E.coli cells 33
2.2.2.16 Transformation of E.coli 34
2.2.2.17 Preparation of electrocompetent Agrobacterium tumefaciens 34
2.2.2.18 Transformation of Agrobacterium tumefaciens 35
2.2.3 Protein Biochemistry 35
2.2.3.1 Protein extraction from leaf tissue 35
2.2.3.2 SDS-PAGE 35
2.2.3.3 Western blotting 35
2.2.3.4 Immunoprecipitation 36
2.2.3.5 Phosphatase assay 37
2.2.3.6 MAPK activation 37
2.2.4 Plant Methods 37
2.2.4.1 Agrobacterium-mediated transient expression in tobacco leaves 37
2.2.4.2 Generation of stable transgenic lines of Arabidopsis thaliana 38
2.2.5 Biological responses 38
2.2.5.1 Oxidative burst assay 38
2.2.5.2 Ethylene assay 38
2.2.5.3 Infection assay with Pseudomonas syringae 38
2.2.5.4 Infection assay with Alternaria brassicicola 39
2.2.5.5 Infection assay with Phytophthora capsici 39
2.2.5.6 Hormone and phytoalexin measurements 40
2.2.5.7 Cell death assay 40
2.2.6 Differential interference contrast microscopy 40
2.2.7 Statistics 40

3 RESULTS 41
3.1 AVR2 associates with BSU1-like phosphatases StBSL1, 2a and 2b and homologs of Arabidopsis 41
3.2 Identification of the molecular determinants of the AVR2-AtBSL2 interaction 45
3.2.1 The hinge region and the putative phosphatase domain of AtBSL2 are required for the interaction with AVR2 45
3.2.2 The C-terminus of the effector domain of AVR2 is critical for the association with AtBSL2 47
3.3 Effect of AVR2 on the phosphatase activity of AtBSU1-like family members 48
3.3.1 In vivo phosphatase assay 48
3.3.2 The catalytic activity of AtBSU1-like family members is BL independent 49
3.3.3 AVR2 does not affect the phosphatase activity of AtBSU1-like family members 50
3.4 Effect of AVR2 on BL-dependent responses in N. benthamiana 52
3.4.1 AVR2 does not affect BL-dependent phosphorylation status of AtBES1 expressed in N. benthamiana 52
3.4.2 AVR2 does affect the expression level of BL-regulated genes in N. benthamiana. 53
3.5 Effect of AVR2 on BL-dependent responses in A. thaliana 55
3.5.1 AVR2 expressing Arabidopsis plants display characteristic BR mutant phenotypes 55
3.5.2 AVR2 affects stomata development and patterning 56
3.5.3 AVR2 modulates the protein expression level of AtGSK3-like kinases 57
3.5.4 AVR2 does not affect the phosphorylation status of AtBES1 60
3.6 Effect of AVR2 on MTI/PTI responses in N. benthamiana 62
3.6.1 AVR2 does not suppress flg22-induced early immune responses in N. benthamiana . 62
3.6.2 AVR2 does not suppress β-megaspermin- induced early immune responses in N. benthamiana 64
3.6.3 AVR2 suppresses INF1-induced cell death in N. benthamiana 66
3.7 Effect of AVR2 on MTI/PTI responses in Arabidopsis thaliana 67
3.7.1 AVR2 does not suppress flg22-induced early immune responses in Arabidopsis 67
3.8 AVR2 affects the resistance/susceptibility to pathogens in Arabidopsis 69
3.8.1 AVR2 enhances Arabidopsis susceptibility to Pseudomonas syringae pv tomato DC3000 69
3.8.2 AVR2 enhances Arabidopsis susceptibility to Phytophthora capsici 70
3.8.3 AVR2 enhances Arabidopsis resistance to Alternaria brassicicola 71
3.9 AVR2 expressing Arabidopsis plants display higher level of JA 72
3.10 AVR2 expressing Arabidopsis plants display large transcriptome changes 75
4 DISCUSSION 79
5 SUMMARY 91
6 Zusammenfassung 93

7 REFERENCES 95
8 APPENDIX 120

List of figures

Figure 1 **The plant immune system (Dangl et al., 2013).** 2
Figure 2 **Brassinosteroid signaling pathway (Clouse, 2011).** 12
Figure 3 **Sequence alignment of AVR2 from P. infestans T30-4 and 06_3928A.** The black boxes indicate signal peptide, K/N polymorphism at residue 31, , RXLR and EER motif respectively. 19
Figure 4 **Phylogenetic tree of Arabidopsis thaliana and Solanum tuberosum BSU1-like phosphatases** 42
Figure 5 **Co-Immunoprecipitation of myc-tagged S. tuberosum BSL1, 2a and 2b and GFP-AVR2 in N. benthamiana** 42
Figure 6 **Co-Immunoprecipitation of myc-tagged Arabidopsis thaliana BSL1-3 together with HA-AVR2.** 43
Figure 7 **Co-Immunoprecipitation of myc-tagged Arabidopsis thaliana BSL1, 2 and 3 together with HA-AVR2 in N. benthamiana.** 44
Figure 8 **Two distinct domains located in the hinge region and phosphatase domain of AtBSL2 are required for association with AVR2 in vivo.** 46
Figure 9 **The 17 amino acids at the C terminus of Avr2 are required for the association with AtBSL2 in vivo.** 48
Figure 10 **AtBSL2 is a functional phosphatase in N. benthamiana.** 49
Figure 11 **BL treatment has no effect on the phosphatase activity of the AtBSU1-like family members.** 50
Figure 12 **Phosphatase activity of AtBSU1-like family members in association with AVR2.** 51
Figure 13 **Phosphorylation status of AtBES1-GFP in presence of GFP-AVR2 in N. benthamiana.** 52
Figure 14 **QRT-PCR analysis showing that AVR2 affects the expression of BR responsive genes in N. benthamiana.** 55
Figure 15 **Phenotype of GFP-AVR2 expressing A. thaliana plants.** 56
Figure 16 **GFP-AVR2 expressing Arabidopsis plants show abnormal stomata patterning.** 57
Figure 17 **The stomata phenotype in GFP-AVR2 expressing plants is partially reversible upon bikinin treatment.** 59
Figure 18 **Avr2 modulates the protein expression level of ASKδ, ASKI and ASKζ in planta.** 60
Figure 19 **Phosphorylation status of AtBES1 in GFP-AVR2 expressing Arabidopsis seedlings.** 61
Figure 20 **AVR2 does not suppress flg22-induced MTI/PTI responses in Nicotiana benthamiana.** ... 64
Figure 21 **GFP-AVR2 does not suppress β-megaspermin-induced MTI/PTI-responses in Nicotiana benthamiana.** 65

Figure 22 **Effect of deletions in AVR2 effector protein on INF1 induced cell death in Nicotiana benthamiana.** ... 67

Figure 23 **GFP-AVR2 does not suppress flg22-induced MTI/PTI responses in Arabidopsis.** ... 69

Figure 24 **Arabidopsis GFP-AVR2 expressing plants are more susceptible to Pto DC3000.** ... 70

Figure 25 **Arabidopsis GFP-AVR2 expressing plants are more susceptible to P. capsici LT263.** ... 71

Figure 26 **Arabidopsis GFP-AVR2 expressing plants are more resistant to Alternaria brassicicola.** .. 72

Figure 27 **Hormone levels in Arabidopsis GFP-Avr2 plants before and after infection with Pto DC3000.** ... 75

Figure 28 **Transcriptome analysis of GFP-AVR2 expressing plants.** ... 77

List of tables

Table 1 **Media used in this study.** 21

Table 2 **Antibiotics used in this study.** 22

Table 3 **Antibodies used in this study.** 22

Table 4 **Vectors used in this study.** 23

Table 5 **Bacterial strains used in this work.** 24

Table 6 **Selected genes and their expression levels in GFP-AVR2 expressing plants.** 78

Table 7 **List of primers used in this study.** 120

Table 8 **MS analysis of Immunoprecipitation of GFP-AVR2.** 122

Abbreviations

aa	Amino acid
ASK	Arabidopsis SHAGGY-related protein kinase
Avr	Avirulence factor
bHlH	Basic helix loop helix
BL	Brassinolide
bp	Base pair
BR	Brassinosteroid
CDPK	Calcium-dependent protein kinase
Col-0	*Arabidopsis thaliana* Columbia-0 ecotype
DNA	Deoxyribonucleic acid
EF-Tu	Elongation factor Tu
Elf18	immunity-inducing 18 amino acid peptide from EF-Tu
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
Flg22	immunity-inducing 22 amino acid peptide from Flagellin
GFP	Green fluorescent protein
GSK-3	Glycogen synthase kinase 3
HR	Hypersensitive response
INF1	Infestin-1
JA	Jasmonic acid
kDa	KiloDalton
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAMP	Microbe-associated molecular pattern

Abbreviations

MAPK	Mitogen-activated protein kinase
NB	Nucleotide-binding
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PP	Protein phosphatase
PPKL	Protein phosphatase with Kelch-like domain
PR	Pathogenesis-related
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
Pto	*Pseudomonas syringae* pv. tomato
qRT-PCR	Quantitative real-time PCR
R	Resistance protein
RFP	Red fluorescent protein
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
WT	Wild type

1. INTRODUCTION

1.1. The plant immune system

Plants are sessile organisms, which are continuously exposed to various biotic stresses. These include viruses, bacteria, fungi, herbivorous insects and nematodes. As a result of million years of coevolution, plants have developed a sophisticated immune system to adapt and to resist to pathogens (Asai and Shirasu, 2015 Wang et al., 2016). Unlike animals, plants do not have specialized defense cell lineages but they rely on the capacity each cell has to recognize pathogenic microorganisms through the presence of intra- and extracellular receptors (Ausubel, 2005, Jones and Dangl, 2006). The understanding of the mechanisms underlying plant resistance and effective disease prevention is of fundamental importance and gained increasing attention of plant biologists (Nishimura and Dangl, 2010). A better knowledge of the nature of the receptors, signaling pathways and their components and how different pathways cope to build an efficient resistance to pathogens will help to evolve new strategies for pest management.

In a general view, the plant immune system comprises two layers of defense (Figure 1). The first one comprises transmembrane pattern recognition receptors (PRRs) that detect conserved microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) such as bacterial flagellin or elongation factor Tu and leads to MAMP or PAMP-triggered immunity (MTI or PTI). The second layer acts inside the plant cell and uses nucleotide binding, leucine reach repeat (NB-LRR) proteins also known as resistance (R) proteins which, directly or indirectly, recognize pathogen virulence molecules called effectors and this recognition leads to effector-triggered immunity (ETI) (Jones and Dangl, 2006, Zipfel, 2008, Dodds and Rathjen, 2010).

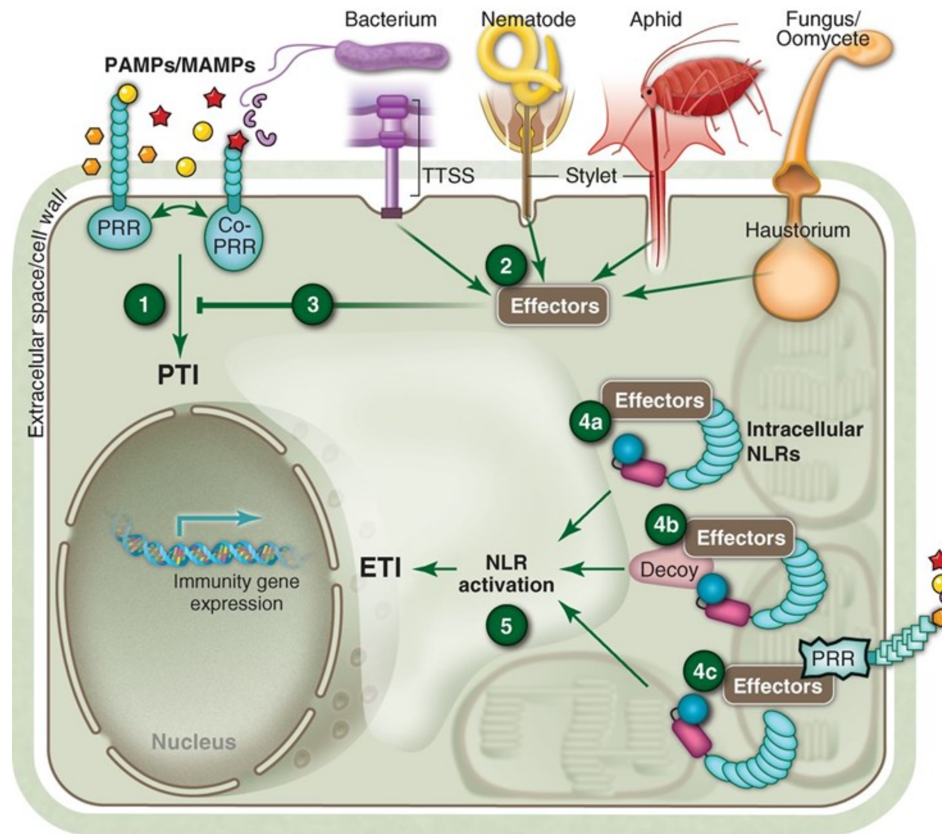


Figure 1 **The plant immune system (Dangl et al., 2013).** Plants perceive pathogens through the recognition of MAMPs/PAMPs via extracellular PRRs and initiate PAMP/MAMP-mediated immunity (MTI/PTI; step 1). Pathogens deliver effectors through specialized structure inside the plant cell to favor colonization (step 2). These effectors act in different subcellular compartments and one main role is to suppress MTI/PTI and facilitate virulence (step 3). Intracellular NLR receptors can sense effectors in three principal ways: first, by direct receptor ligand interaction (step 4a); second, by sensing effector-mediated alteration in a decoy protein that structurally mimics an effector target, but has no other function in the plant cell (step 4b); and third, by sensing effector-mediated alteration of a host virulence target, like the cytosolic domain of a PRR (step 4c).

MTI/PTI as well as ETI induce similar defense mechanisms but ETI is considered as one of the higher amplitude and typically leads to hypersensitive response (HR) and occurrence of a programmed cell death (PCD) around the infection site, while MTI/PTI does not trigger these responses (Jones and Dangl, 2006). MTI/PTI responses are considered as sufficient defense mechanism against non-adapted pathogens and cause non-host resistance, whereas effector recognition by R proteins is effective against adapted pathogens (Doodds and Rathjen, 2010). Several recent studies indicate, however, that the dichotomy between MTI and ETI, as well as between MAMPs and effectors, PRRs and R proteins, is much more ambiguous (Thomma et al., 2011). The discrimination between MAMPs and effectors has become in recent years much more elusive. Several MAMPs display characteristics of effectors and vice

versa. In this manner, the conserved flg22 motif of the flagellin from *Pseudomonas syringae* pv. tabaci and *P. aeruginosa* is also able to induce HR in its non-host Arabidopsis, which is a characteristic of ETI (Naito et al., 2007, Naito et al. 2008). One of the main characteristic of MAMPs is that they are highly conserved among microbial pathogens. The *Cladosporium fulvum* LysM effector Ecp6 binds to chitin, preventing its recognition through the CEBiP receptor in rice (de Jonge et al., 2010). Since LysM effectors are highly conserved among fungi, they can be classified as MAMPs. Vice versa, some MAMPs were shown to be only narrowly distributed among microbial species, a feature characteristic to effectors. For instance, Pep-13, a surface-exposed fragment within calcium-dependent transglutaminase (TGase) is found only in *Phytophthora* species (Brunner et al., 2002). Plant PRRs are defined as surface-localized receptors that bind MAMPs and activate MTI responses and R proteins are intracellular proteins that recognize effectors in the cytoplasm. However, recent studies showed that some R proteins display typical properties of PRRs (Thomma et al., 2011). For example, the extracellular Avr4 effector from *C. fulvum* binds to chitin oligomers and protects fungi against tomato chitinases and is recognized by tomato Cf proteins, what further triggers HR symptoms. Therefore Cf proteins can be classified as PRRs and Avr4 as a MAMP (van den Burg et al., 2006, van Esse et al., 2007).

1.1.1 Recognition of microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs)

MAMPs are highly conserved molecular patterns which are perceived by surface-localized PRRs. Plants recognize a variety of MAMPs (or PAMPs), among them are components of the bacterial or fungal cell wall such as the lipopolysaccharide (LPS) and peptidoglycan (PGN) from bacteria, chitin from fungi as well as intracellular and extracellular proteins such as the aforementioned bacterial elongation factor Tu and flagellin, respectively (Boller and Felix, 2009, Zipfel et al., 2009, Monaghan and Zipfel, 2012). The known plant PRRs comprise two receptor classes: transmembrane receptor-like kinases (RLKs) and receptor-like proteins (RLPs). RLKs consist of an extracellular domain, a single transmembrane domain, and an intracellular kinase domain. RLPs have an extracellular domain, transmembrane domain and a short cytoplasmic region, which lacks a kinase domain. Most of the known RLKs and RLPs have characteristic leucine reach repeat (LRR) motives in their extracellular domain (Ma et

al., 2016, Monaghan and Zipfel, 2012). The bestcharacterized plant PRRs are the RLKs FLAGELLIN-SENSING2 (FLS2) and ELONGATION FACTOR-Tu RECEPTOR (EFR) (Gomez-Gomez and Boller, 2000, Zipfel et al., 2006). The flg22 peptide derived from flagellin is recognized by FLS2 in most plant species (Felix et al., 1999). The first 18 amino acids localized in N-terminus region of EF-Tu are perceived by EFR in *Brassicaceae* (Zipfel et al., 2006). Peptidoglycan, is recognized by lysine motif-containing RLPs: LYM1, LYM3 and the lysine motif containing RLK CERK1 (Willmann et al., 2011). The formation of heterodimer between LysM RLPs and LysM RLKs appears to be central for the perception and signaling of sugar-containing MAMPs (Willmann et al., 2011). CERK1 was also showed to play a key role in chitin perception in Arabidopsis (Wan et al., 2008, Miya et al., 2007). Also other LRR-RLK receptors such as FLS2 and EFR form a complex with other RLKs (Chinchilla et al., 2007, Roux et al., 2011). In this manner EFR and FLS2 receptor interact with the LRR-RLK BAK1 (which is also a co-receptor in brassinosteroid signaling pathway, which regulates plant growth and developmental processes (Chinchilla et al., 2007, Roux et al., 2011). Upon treatment with flg22, FLS2 interacts with BAK1. Further transphosphorylation events lead to dissociation of BOTRYTIS INDUCED KINASE 1 (BIK1) from the complex with FLS2. Activated FLS2 triggers early immune responses,which include calcium burst, production of reactive oxygen species (ROS) and activation of mitogen-associated protein kinases (MAPKs). One of the earliest response upon MAMP perception is influx of extracellular Ca^{2+}, H^{+} in the cytosol and efflux of K^{+}, Cl^{-} and $NO3^{-}$, which leads to membrane depolarization and extracellular alkalinisation (Bigeard et al, 2015, Jeworutzki et al., 2010, Felix et al., 1999). Influx of Ca^{2+} ions leads to CALCIUM DEPENDANT PROTEIN KINASE (CDPK)-dependent phosphorylation and activation of plant NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RbohD), responsible for the MAMP-induced ROS accumulation (Dubiella et al., 2013, Ranf et al., 2011). ROS play multiple functions in plant immune responses, participating in cell wall strengthening and acting as signaling molecules in the induction of the expression of defense-related genes and programmed cell death (PCD) (reviewed in Torres et al., 2006).

Perception of MAMPs leads also to activation of MAPK cascades, which regulate the expression of several defense-related genes. Two Arabidopsis MAPK cascades are known to be activated upon flg22 perception. The first cascade consists of MEKK1, MKK4/MKK5 and MPK3/MPK6. The second one is comprised of MEKK1, MKK1/MKK2 and MPK4 (Meng and Zhang, 2013). Plant specific WRKY transcription factors including WRKY22 and WRKY29,

which bind to W-box DNA elements found in promoters of many defense-related genes, have been implicated as targets of MAP kinases (Asai et al., 2002). Similarly WRKY33 was shown to act as a transcriptional activator downstream of MPK3/MPK6 promoting ethylene accumulation and the induction of the expression of camalexin biosynthetic genes (Li et al., 2012, Mao et al., 2011). VirE2-INTERACTING PROTEIN1 (VIP1) has been identified as a target of MPK3, which upon phosphorylation is re-localized into the nucleus and regulates the expression of PATHOGENESIS-RELATED (PR) protein 1 (Djamei et al., 2007). MAPKs can however act as negative regulator of plant immune responses and it was shown that MPK4 negatively regulates salicylic acid (SA)-dependent defense responses (Peterson et al., 2000, Brodersen et al., 2006).

Late MTI-responses include callose deposition, a β-1,3-glucan to reinforce the cell wall at the site of the infection (Nishimura et al., 2003, Luna et al., 2010), expression of PR proteins, known for their antimicrobial properties (Uknes et al., 1992, Zhang et al., 2010, Durrant and Dong, 2004), accumulation of antimicrobial secondary metabolites such as camalexin in Arabidopsis thaliana and other phytoalexins (Thomma et al., 1999).

1.1.2 Effector-triggered immunity (ETI)

Numerous effectors produced by microorganisms have been described in the literature as trigger of host immunity following direct or indirect recognition by a cognate resistance (R) protein. The recognized effector is termed an avirulence (Avr) protein (Jones and Dangl, 2006). The direct recognition is exemplified by flax L5, L6 and L7 R proteins that interact with the flax rust fungus AvrL proteins and elicit necrotic responses (Dodds et al., 2006).

However, several R proteins recognize the effectors indirectly by detecting the products of their action on the host targets, what is described as “guard model”. The effector-induced modification is monitored (guarded) by the corresponding R protein and will result in its activation, leading to ETI (van der Hoorn and Kamoun, 2008). A classic example of such mechanism of action is the recognition of Arabidopsis plasma membrane associated protein RIN4, which modulates stomatal aperture during pathogen attack (Liu et al., 2009), by *P. syringae* type III effectors AvrRpm1 and AvrRpt2. The degradation of RIN4 caused by AvrRpt2 or its hyperphosphorylation, in case of AvrRpm1, is perceived by RPS2 and RPM1,

respectively, and further triggers plant immune responses (Axtell and Staszkiewicz, 2003, Kim et al., 2005). Since the role of many effector targets in host defense or susceptibility remains unknown, a “decoy” model was proposed in which the target has no biological function other than to trap the effector. These targets could arise through gene duplication event or are another splice variant of a genuine target (van der Hoorn and Kamoun, 2008, Stuart et al., 2013). An example of such mechanism is the recognition of the tomato Pto kinase by AvrPto. The Pto kinase is structurally similar to the intracellular kinase domain of FLS2, which is a target of AvrPto, and is recognized by Prf upon association with AvrPto (Xing et al, 2007). Since the role of the Pto kinase in tomato remains elusive, it is hypothesized to serve as a decoy (reviewed in van der Hoorn and Kamoun, 2008). The subsequent modification of the decoy recognition is the “bait and switch model”, in which the effector interacts with its target protein, and in the next step, interaction between the effector and NB-LRR takes place. The role of the accessory protein is to bait the effector and to facilitate the subsequent NB-LRR-effector interaction. The above mentioned mechanisms of effector recognition are considered as models, which bring us a step closer in understanding how effector recognition works, nevertheless, none of them is yet fully elucidated (Dodds and Rathjen, 2010, Stuart et al., 2013).

Effector recognition by NB-LRRs in plants leads to inhibition of pathogen growth, which is often accompanied in plants by HR-associated cell death (Coll et al., 2011). However, necrotrophic pathogens, including *Botrytis cinerea* and *Alternaria brassicicola*, require dead tissue for nutrient uptake and they have developed mechanisms promoting cell death onset (Govrin and Levine, 2000, Thomma et al., 1999). The gene-for gene resistance,based on the effector recognition by R proteins, to necrotrophic pathogens is so far poorly understood. Leptosphaeria maculans 3 (RLM3) encodes a putative Toll interleukin-1 receptor-nucleotide binding (TIR-NB) class protein in Arabidopsis. RLM3 was showed to be involved in defense against the fungal hemibiotrophic pathogen *L.* maculans and three necrotrophic pathogens: *Alternaria. brassicicola, Alternaria brassicae* and *Botrytis cinerea.* However, the effector for RLM3 has not been identified (Staal et al., 2008, reviewed in Wang et al., 2014). Biotrophic pathogens on the contrary, obtain nutrients from the living cells. In this manner several *P. syringae* effectors, including AvrRpm1 and AvrRpt2, aim to suppress HR (Guo et al., 2009). The mechanism leading to HR upon effector recognition via plant NB-LRRs is not yet fully elucidated. Two signaling pathways have been proposed so far. NON-RACE-SPECIFIC DISEASE

RESISTANCE 1 (NDR1) mediates the HR responses triggered by coiled-coil (CC)-NB-LRR proteins and contributes to ROS-dependent signaling (Aarts et al., 1998, Rusterucci et al., 2001). ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)/PHYTOALEXIN DEFICIENT 4 (PAD4)/ SENESCENCE ASSOCIATED GENE 101 (SAG101) complex mediates the toll-interleukin 1 (TIR)-NB-LRR HR responses and contributes to SA and ROS generation (Aarts et al., 1998, Wiermer et al., 2005, Coll et al., 2011).

ETI has been associated with many responses similar to PTI, including rise in cytoplasmic calcium, apoplast alkalinization, ROS accumulation, increased NO levels and SA accumulation (Mur et al., 2008). More and more studies implicate that chloroplasts contribute significantly to HR through generation of ROS during the light reaction of photosynthesis (Ambastha et al., 2015).

1.2 Hormones in plant immunity

Plant hormones are a class of small, organic molecules regulating not only plant developmental processes, but also defense responses. Most studies have focused on salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) and highlighted their importance in plant immunity (Pieterse et al., 2009).

SA signaling triggers resistance against biotrophic pathogens. SA was first connected to immune responses, when it has been reported that exogenous application of SA to tobacco plants elevated the resistance against TOBACCO MOSAIC VIRUS (TMV) and induced accumulation of pathogenesis-related (PR) proteins (reviewed in Shigenaga and Argueso, 2016). SA accumulation is also correlated with the onset of systemic acquired resistance (SAR), a long-lasting and broad spectrum-induced disease resistance in distal parts of the plants. SA accumulates not only locally at the site of infection, but also in distant tissues (Pieterse et al., 2009). In tobacco, SA is transported as methyl salicylate (MeSA) to distant tissues, converted to SA and activates SAR (Robert-Seilaniantz et al., 2011). NPR1 protein (NONEXPRESSOR OF PR GENES1) emerged as a positive regulator of SA signaling and co-activator of PR gene expression (reviewed in Robert-Seilaniantz et al., 2011, Pieterse et al., 2009).

JA-dependent responses are known to play a role in defense against herbivores and necrotrophic pathogens. Methyl jasmonate (MeJA), a JA derivative was shown to induce expression of PLANT DEFENSIN 1.2 (PDF1.2) (Thomma et al., 1998). The main components of JA signaling in Arabidopsis include CORONATINE INSENSITIVE 1 (COI1), JASMONATE RESISTANT 1 (JAR1) and JASMONATE INSENSITIVE 1/MYC2 (JIN1/MYC2). COI1 is an F-box protein involved in 26S proteasome degradation of JAZ repressors, which enables the expression of JA-responsive genes. JIN1/MYC2 is a basic helix loop helix transcription factor controlling the expression of JA-responsive genes involved in plant immune responses against herbivorous insects. JAR1 encodes a JA amino acid synthase, which conjugates isoleucine to JA (JA-Ile). JA-Ile is an active form of JA (Robert-Seilaniantz et al., 2011, Bari and Jones, 2008).

The best-studied hormonal crosstalk in plant defense is the interaction between SA-JA signaling (Spoel and Dong, 2008). Several studies have shown that the two signaling pathways are antagonistic, meaning that resistance to biothropic pathogens is often correlated with higher susceptibility to necrotrophs and vice versa (Robert-Seilaniantz et al., 2011).

Ethylene (ET) together with SA and JA is a master modulator of plant immune responses. ET can either act as positive or negative regulator of defense responses (Pieterse et al., 2012). It has been shown that ethylene is required for SA-mediated SAR in tobacco and PR-1 protein accumulation in Arabidopsis (Pieterse et al., 2009). In the case of JA-ET crosstalk, ET was shown to act synergistically with JA in response to necrotrophic pathogens and activate immune responses through the action of transcription factors ETHYLENE RESPONSE FACTORs (ERFs) that induce the expression of PR proteins (Lorenzo et al., 2003). However, ERFs suppress the expression of the MYC branch of JA-responsive genes involved in responses to herbivorous insects (Lorenzo et al., 2004, reviewed in Broekgaarden et al., 2015).

Recently, it has been indicated that also other hormones including auxins, gibberellins, abscisic acid (ABA), cytokinins are involved in plant immune responses (Shigenaga and Argueso, 2016). Indole-3-acetic acid (IAA), the most common naturally occurring auxin in plants, antagonizes SA-mediated defense responses including the suppression of PR1 expression (Park et al., 2007). It has been showed that the perception of bacterial MAMPs,

such as flg22, induce the expression of microRNAs that attenuate the expression of auxin F-box transporters-like auxin receptors TRANSPORT INHIBITOR RESPONSE1 (TIR1), AUXIN SIGNALING F-BOX 1 (AFB2), AUXIN SIGNALING F-BOX 3 (AFB3). The repression of auxin signaling restricts further the growth of Pst DC3000 (Navarro et al., 2006). Interestingly, it has been reported that balance between SA and auxin is regulated through GRETCHEN HAGEN3.5 (GH3.5), an IAA-amido synthase that conjugates amino acids to auxin and regulate auxin homeostasis (Zhang et al., 2007). The expression of GH3.5 is induced during the infection with Pst DC3000, what further derepresses auxin signaling in favor of the pathogen. GH3.5 however can also positively regulate the SA biosynthesis and induce the expression of SA-responsive defense-related genes (Zhang et al., 2007).

ABA is a key phytohormone in the regulation of abiotic stress responses in plants. Recently, ABA was reported to play a role in plant-pathogen interactions (Cao et al., 2011). Pst DC3000 was shown to induce ABA biosynthesis in infected Arabidopsis plants, what negatively regulates SA biosynthesis and SA-mediated defense responses such as PR1 expression (Zabala et al., 2009, Hill et al., 2009). Furthermore, ABA is implicated in JA accumulation and in the induction of resistance to the necrotrophic fungus *A. brassicicola* (Fan et al., 2009). ABA is also a key regulator of stomatal aperture and recognition of bacterial MAMPs leads to ABA-mediated stomatal closure in order to prevent bacterial entry (Melotto et al., 2006).

Cytokinins are plant isoprenoid hormones involved in cell division and were reported to modulate SA signaling. Higher concentrations of cytokinins led to increased defense responses to the oomycete *Hyaloperonospora arabidopsidis* through upregulation of SA-dependent immune responses (Argueso et al., 2012). The cytokinin-activated transcription factor ARR2 regulates SA-mediated expression of PR1 and contributes to resistance to *P. syringae* (Choi et al., 2010).

Gibberelins are known to regulate plant growth, development and life cycle progression. In addition, gibberelins promote proteasomal degradation of DELLA transcription factors and disable JA-mediated resistance to necrotrophs (Harberd et al., 2009, Navarro et al., 2009).

1.2.1 Crosstalk between brassinosteroid signaling and plant immune responses

Brassinosteroids (BRs) are a class of plant steroid hormones involved in numerous aspects of plant growth and development, involving cell elongation and division, photomorphogenesis, flowering, pollen development, fruit ripening, senescence and response to biotic and abiotic stresses (Clouse, 2011; Zhu et al., 2013). The first BRs, including brassinolide (BL), were identified in the 1970s and isolated from *Brassica napus* pollen (Mitchell et al., 1970). Unlike animal steroid hormones, which are perceived by nuclear receptors, BRs are perceived in plants at the cell surface by BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor kinase (Li and Jin, 2006, Kim et al., 2009, Clouse, 2011).

The BRI1 receptor in *Arabidopsis thaliana* has been characterized upon identification of mutants in genetic screen that displayed extreme dwarfism, dark green curly leaves and male sterility, all phenotypes that could not be rescued by exogenous BR treatment (Clouse et al., 1996). BRI1 has a similar molecular structure than FLS2 or EFR, it is a leucine-rich repeat receptor-like kinase (LRR-RLK) with an extracellular domain containing 25 LRRs interrupted by a 70 amino acid island, transmembrane domain and cytoplasmic serine/threonine kinase domain (Figure 2). BRs bind to the extracellular domain of BRI1, leading to the autophosphorylation and activation of the BRI1 kinase domain (Chory et al., 2001). The activated BRI1 associates with BAK1 (Nam and Li, 2002), which is also acting as a co-receptor of FLS2 and EFR in MAMP signaling. Transphosphorylation events between BRI1 and BAK1 lead to activation of the BR-signaling cascade (Clouse et al., 2008). In the absence of BR, BRI1 is in complex with BRI1 KINASE INHIBITOR 1 (BKI1) but upon BR treatment, BRI1 dissociates from BKI1, which enables the BRI1-BAK1 complex formation (Wang and Chory, 2006) (Figure 2). Activated BRI1 phosphorylates BR-SIGNALING KINASE 1 (BSK1) or CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1), a receptor-like cytoplasmic kinase (Tang et al., 2008, Kim et al., 2011). BSKs and CDG1 phosphorylate and activate the BRI1 SUPPRESSOR 1 (BSU1) and BSU1-LIKE PHOSPHATASE 1 (BSL1) (Kim et al., 2009, Kim et al., 2011). BSU1 together with its homologues BSL1, BSL2 and BSL3 form a small family of plant Ser/Thr phosphatases. Recent studies have shown that BSU1 together with BSL1 are positive regulators of BR-signaling (Kim et al., 2009). The role of the two remaining homologues, BSL2 and BSL3, remains unknown. In the protein structure of BSU1 and its homologues we can distinguish N-terminal tandem Kelch domain repeats, important for the protein-protein

interactions and C-terminal phosphatase domain. Through the phylogenetic analysis it has been shown that BSL1 and BSL2/BSL3 belong to two highly conserved evolutionary clades. BSU1 in contrast to BSL1, 2 and 3 evolved later as a divergent paralogue of BSL1 family members and it is found exclusively in *Brassicaceae* (Maselli et al., 2014). Further studies showed that BSU1 and BSL1 act as positive regulators of BR-signaling and they dephosphorylate and inactivate the GSK3-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2) at tyrosine residue in position200 (Kim et al., 2009). BIN2 kinase acts as a negative regulator of BR signaling and was first characterized upon identification of *bin2-1/+* in a genetic screen a gain of function mutant that displayed *bri1*-like severe dwarfism, dark green curly leaves, reduced male fertility and late flowering, insensitive to BR treatment (Li et al., 2001). In the absence of BR, active BIN2 negatively regulates the BR-responsive transcription factors BRASSINAZOL-RESISTANT 1 (BZR1) and BRASSINAZOL-RESISTANT 2/BRI1-EMS SUPPRESOR 1 (BZR2/BES1) through phosphorylation. BZR1 and BZR2/BES1 share 88% of sequence identity (Wang et al., 2013). Dominant-active mutants of BZR1 and BZR2/BES1 in the *bzr1-D* and *bes1-D* mutants, respectively, suppress the dwarf phenotype of *bin2-1/+,* which is an indication that BIN2 functions upstream of BZR1 and BES1 in the BR-signaling cascade (He et al., 2002, Yin et al., 2005). BIN2 not only prevents DNA binding of BZR1/BZR2, but also promotes the binding to 14-3-3 proteins required for cytoplasmic retention (Gampala et al., 2007) and degradation (Yang and Wang, 2017) of these transcription factors. BR treatment on the contrary, induces accumulation of dephosphorylated form of BZR1 and BZR2/BES1 in the nucleus (He et al., 2002, Yin et al., 2005).

Recent studies indicate that 7 (subgroup I, II and GSK32 of subgroup III) among 10 Arabidopsis GSK members interact with the transcription factor family comprising BZR1, BZR2/BES1 and BEH1-4 (Youn et al., 2015, Rohzon et al., 2010, Kim et al., 2009). In presence of BR, BZR1 and BZR2/BES1 regulate the expression of thousands of genes either by activation or repression. Chromatin immunoprecipitation (ChIP) studies have shown that both BZR1 and BZR2/BES1 bind to DNA at the BR-responsive element (BRRE) and at the E-box element and interact with several other transcription factors, including members of the bHlH family, which were recently identified to participate in BR-signaling (Yang et al. 2011, Wang et al. 2014). BZR2/BES1 interacts with BES1 INTERACTING MYC-like protein 1 (BIM1) and MYC30 transcription factor to regulate the gene expression including SMALL AUXIN UPREGULATED RNAs (SAURs) including SAUR-AC1 (Yin et al., 2005, Li et al., 2009). SAURs

belong to early auxin responsive genes and regulate a wide range of cellular, physiological and developmental processes (Ren and Gray, 2015). The molecular mechanism of BR-auxin crosstalk however is yet not fully understood (Goda et al., 2002). BZR1 was shown to interact with bHlH transcription factor PIF4 to mediate hypocotyl elongation (Oh et al., 2012). BZR1 also binds to the promoter of genes of the brassinosteroid biosynthetic pathway such as *CPD*, *DWF4*, in a mechanism of negative feedback regulation occurring upon BR treatment (Kim and Wang, 2010).

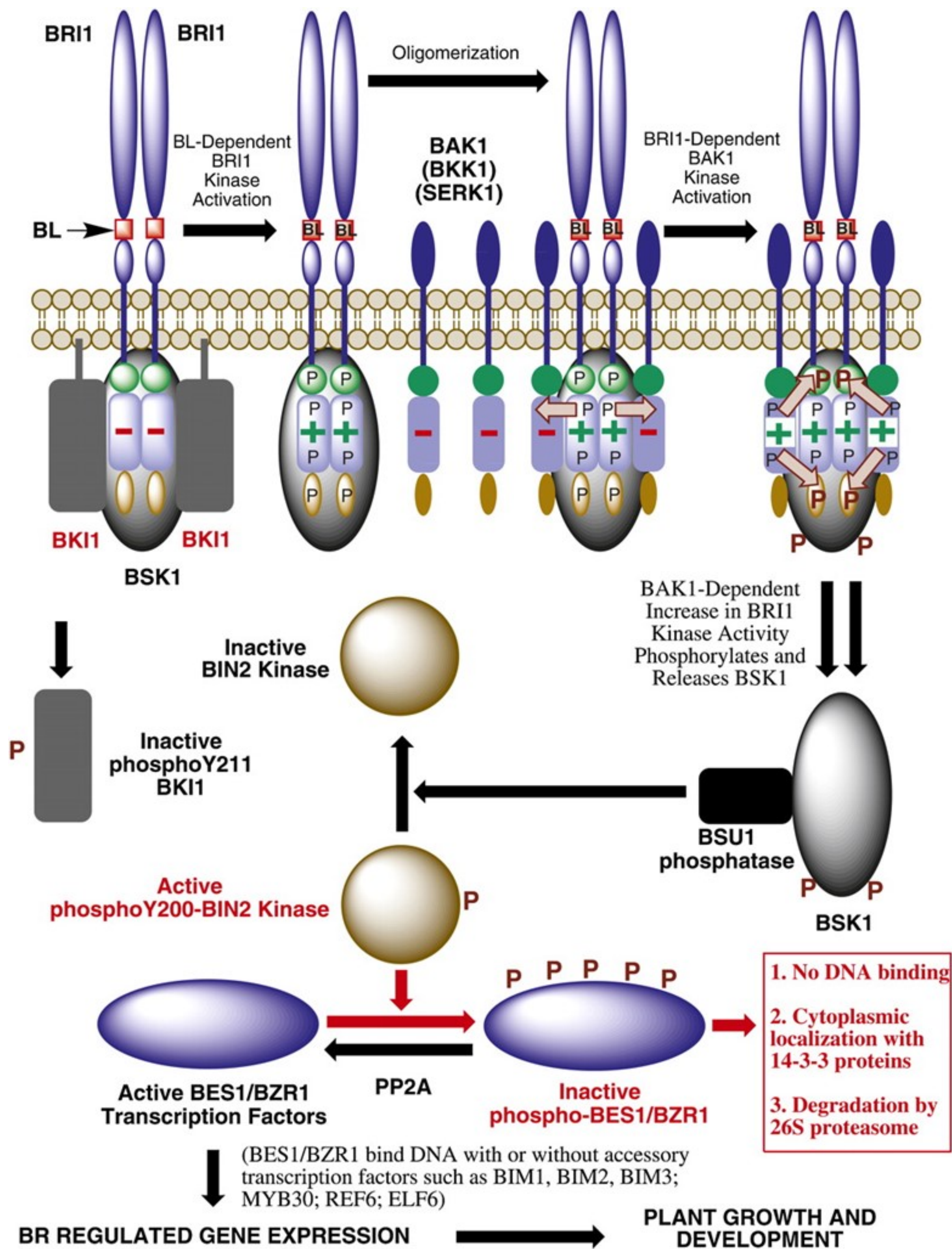


Figure 2 **Brassinosteroid signaling pathway (Clouse, 2011).** Brassinolide binds to the extracellular domain of BRASSINOSTEROID INSENSITIVE 1 (BRI1) kinase and causes the release of BRI1 from the complex with its

inhibitor BRI1 KINASE INHIBITOR1 (BKI1) (Wang et al. 2008). In this stage, activated BRI1 recruits BRI1 ASSOCIATED RECEPTOR KINASE1 (BAK1) (He et al, 2013). The further transphosphorylation events between the two receptors allow BRI1 the activation of BR-signaling cascade via phosphorylation and activation of BRASSINOSTEROID KINASE1 (BSK1) which then subsequently associates and phosphorylates BRI1 SUPPRESSOR1 (BSU1) and BSU1-LIKE PHOSPHATASE 1 (BSL1), a serine/threonine phosphatases resulting in dephosphorylation of GSK3-like kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) at Y200 (Clause, 2011). When the BR levels are low, active BIN2 phosphorylates two closely related transcription factors BRASSINAZOLE INSENSITIVE1 (BZR1) and BRASSINASSOLE INSENSITIVE2 (BZR2) causing their cytoplasmic retention. In presence of BR, BIN2 is inactivated via dephosphorylation by BSU1/BSL1 and unphosphorylated BZR1 and BZR2 relocate into the nucleus and promote the expression of BR-regulated genes (Zhu et al. 2013).

Recent studies have shown that BRs apart from regulating several growth and developmental processes are also acting as modulators of plant immune responses. In the past two decades, BRs were shown to either positively or negatively regulate plant immune responses (reviewed in Vidhyasekaran, 2014). Several research groups have concluded that exogenous application of BRs significantly reduced the level of fungal and oomycete infection in some of the economically important crops including potato, barley and cucumber (Khripach et al., 2000). Field application of BR to potato plants decreased the level of Phytophthora infection (Khripah et al., 1996). It has been also demonstrated that BL induces resistance in tobacco to the viral pathogen tobacco mosaic virus (TMV), the bacterial pathogen *Pseudomonas syringae* pv. tabaci and the fungal pathogen *Oidium* sp. (Nakashita et al., 2003). In rice plants, BL increases resistance to the fungal pathogen *Magnaporthe grisea* and *Xanthomonas oryzae* pv. oryzae (Nakashita et al., 2003). In cucumber, BL was shown to induce systemic stress tolerance and accumulation of transcripts of defense-related genes (Pingfang et al., 2013). The molecular mechanisms underlying the BR-induced resistance is so far poorly understood. Nakashita et al (2003) showed that BR-induced resistance in tobacco and rice was not accompanied by SA accumulation and PR protein gene expression. BR treatment in cucumber seedlings was reported to enhance the NADPH oxidase activity, resulting in the elevation of the H_2O_2 level and the expression of defense-related genes (Xia et al., 2009).

At the opposite, results obtained in recent years in Arabidopsis suggest that activation of the BR pathway inhibits immune responses (Albrecht et al., 2012). It has been shown that that pretreatment with BL suppresses the flg22-induced ROS production and *FRK1* expression in Arabidopsis seedlings, while flg22 treatment does not affected typical BR-responses such as

BES1 phosphorylation or *CPD* expression. Co-immunoprecipitation experiments showed that the amount of BAK1 associated with FLS2 upon flg22 treatment was not affected by BR treatment (Albrecht et al., 2012, reviewed in Lozano-Duran and Zipfel, 2015). Taking together, these data suggest that BR inhibition of MTI responses is BAK1 independent. However, in a parallel study, Belkhadir et al. (2012) have shown that overexpression of BRI1 under the 35S promoter also led to suppression of MTI responses in Arabidopsis, including callose deposition and oxidative burst, and the authors hypothesized that BRI1 titrates BAK1 and that BAK1 is a rate-limiting factor in MTI responses. These two contradictory studies demonstrate that the crosstalk between BR-signaling and immune responses cannot be clearly explained at the receptor level. Recently, it has been shown that treatment with inhibitors of GSK3-like kinases, as well as a triple GSK3-like kinase mutant *Bin2/Bil1*(BIN2-LIKE1)*/Bil2* (BIN2-LIKE2), display reduced flg22 responses, suggesting that regulation between these two networks takes place downstream of BIN2 (Lozano-Duran et al., 2013). In line with the aforementioned results, plants overexpressing constitutive active BZR1 display reduced flg22-induced ROS production, expression of MTI marker genes and resistance to *P. syringae* pv. Tomato DC3000 (Lozano-Duran et al., 2013). Microarray analysis have identified that *WRKY40*, bHLH transcription factor *HBI1* (HOMOLOG OF BRASSINOSTEROID ENHANCED EXPRESSION2 INTERACTING WITH IBH1) and *HBI1* homologues, *BEE2* and *CIB1,* are putative BZR1 targets and their overexpression in Arabidopsis seedlings showed their involvement in the regulation of MTI-BR crosstalk and the inhibition of the flg22-induced immune responses (Fan et al., 2014, Malinovsky et al., 2014).

1.3 Effector-triggered susceptibility (ETS)

If several effectors of microbial origin trigger ETI upon recognition by plant R proteins (see chapter 1.1), their primary function is to promote colonization of the host plant and development of disease symptoms through the mobilization of nutrients or suppression of host immunity by interfering with MTI and ETI, a process globally termed effector-triggered susceptibility (ETS) (Hurley et al., 2014, Jones and Dangl, 2006, Doods and Rathjen, 2010).

1.3.1 Bacterial effectors

Plant pathogens have evolved different strategies of effector delivery. Gram-negative bacterial pathogens translocate the effectors into the host cell via the type III secretion system (T3SS) in order to promote virulence (Block et al., 2008, Schreiber et al., 2016). AvrPto and AvrPtoB from *P. syringae* DC3000 are among the best studied bacterial effectors interacting with several RLKs. AvrPto was shown to target FLS2 kinase domain in order to disrupt plant innate immune responses (Xiang et al., 2008). The exact mechanism how AvrPto suppresses the MTI-responses in yet not fully understood. Shan et al. (2008) showed that AvrPto is also able to interact with BAK1, which is a common co-receptor and regulator for FLS2 and EFR-induced MAMP responses. Xiang et al. (2011), however, showed that FLS2 but not BAK1 is a target of AvrPto . AvrPtoB, which has E3 ligase activity, was reported to ubiquitinate and target FLS2 for degradation (Göhre et al., 2008).

Other studies have revealed that bacterial effectors target not only early stages of MAMP signaling by interfering with PRR function, but they also target downstream components of the plant immune system, which include ubiquitination, hormones , transcription factors, vesicle trafficking, secondary metabolites or gene silencing (Asai and Shirasu, 2015). The effector HopM1 from *P. syringae* targets and destabilizes AtMIN7, an Arabidopsis ADP ribosylation factor guanine nucleotide exchange factor (ARF-GEF), which plays a key role in vesicle trafficking that regulates MTI and SA-mediated immunity (Nomura et al., 2006, Nomura et al., 2011). XopJ from *X. campestris* interacts with the proteasome subunit RPT6 and prevents SA accumulation (Üstün et al., 2013). *P. syringae* effectors HopX1 and HopZ1 promote degradation of JAZ proteins, which are repressors of JA signaling (Jiang et al., 2013, Gimenez-Ibanez et al., 2014). HopU1, another effector from *P. syringae*, targets and blocks several RNA binding proteins including GRP7, which binds to FLS2 and EFR transcripts (Nicaise et al., 2013). It has been further shown that this inhibition correlates with lower protein levels of FLS2 in plants infected with *P.syringae* DC3000 (Nicaise et al., 2013). Two *P. syringae* effectors, AvrRPM1 and AvrRpt2, have been shown to target plasma membrane associated RPM1-INTERACTING PROTEIN 4 (RIN4) for phosphorylation and degradation. How RIN4 degradation contributes to pathogenicity remains unclear, but some studies have shown that RIN4 is a negative regulator of MTI responses (Axtell and Staszkiewicz, 2003, Kim et al., 2005).

1.3.2 Oomycete effectors

While significant progress has been made in understanding the molecular function and recognition of bacterial effectors, yet little is known about effectors from eukaryotic filamentous plant pathogens. Among them are fungi-like oomycetes, which are known to cause most of the devastating diseases among plant species. *Phytophthora infestans,* which causes late blight in potato and tomato, is one the best-studied oomycete. Historically, *P. infestans* was responsible for the Irish great famine in the mid-nineteenth century (Haas et al. 2009).

The recent advances in genome sequencing and bioinformatic tools allowed to identify hundreds of genes in oomycetes that encode putative effectors, classified in two groups targeting distinct cellular compartments in the host plant: the apoplast and the cytosol (Kamoun, 2006). Recently, it has been shown that feeding structures within infected tissues, such as hyphae and haustoria, are sites of effector secretion and eventually translocation into the host cells, although the underlying molecular mechanism for effector delivery is poorly understood so far (Giraldo and Valent, 2013, Petre and Kamoun, 2014).

1.3.2.1 Apoplastic effectors

Apoplastic effectors that are secreted into the extracellular space have been shown to act as inhibitors of host pathogenesis-related (PR) proteins, which accumulate upon pathogen infection. The glucanase inhibitors GIP1 and GIP2 from *Phytophthora sojae* inhibit endo-β-1,3-glucanase EGaseA, which degrades β-1,3/1,6 glucans and release of elicitor-active elicitor oligosaccharides from *P. sojae* cell wall (Rose et al., 2002). *P. infestans* secretes diverse protease inhibitors: the Kazal-like serine protease inhibitors EP1 and EP10 inhibit the activity of pathogenesis-related P69B subtilisin-like serine protease of tomato (Tian et al., 2004, Tian et al., 2005), while the cystatin-like protease inhibitors EPIC1 and EPIC2 inhibit the papain-like extracellular cysteine protease, PHYTOPHTHORA INHIBITED PROTEASE 1 (PIP1) (Tian et al., 2007).

Another set of extracellular effectors display toxin-like activities, exemplified by the PcF phytotoxin or PcF-like proteins, such as the small cysteine rich protein 96 (SCR96) from

Phytophthora cactorum, that induce necrosis in strawberry and *Solanaceae* species, respectively (Orsomando et al., 2001, Chen et al., 2005). NEP1-LIKE PROTEINS (NLPs) are found in eukaryotic as well as prokaryotic pathogens. NLPs not only can induce cell death but also elicit strong immune responses in dicotyledous plants. NLP_{Pp} from *Phytophthora parasitica* was showed to cause necrosis upon infiltration in tobacco leaves (Ottmann et al., 2009).

1.3.2.2 Cytoplasmic effectors

Several identified oomycete effectors have been shown to induce ETI and trigger HR upon recognition by a cognate intracellular R protein, which provides indirect evidence for entry of these effectors into the host cells (Whisson et al., 2007). However, the mechanism of their translocation from the haustorium into the plant cell is not yet fully elucidated. Cytoplasmic effectors from oomycetes have been shown to carry characteristic conserved motifs in their protein sequence, which are crucial for their translocation into the host plant cell. Two classes of oomycete cytoplasmic effectors have been identified so far: RXLR effectors and crinklers (CRNs) (Wawra et al., 2012, Schornack et al., 2009). CRNs contain conserved LXLFLAK motif at the N-terminus required for their translocation into the host plant cell where they accumulate in the nucleus and cause characteristic leaf crinkling and necrotic symptoms (Stam et al., 2013, Schornack et al., 2010).

In the case of RXLR effectors, we can distinguish a N-terminal signal peptide for secretion, a conserved Arg-X-Leu-Arg (RXLR) motif often followed by single or multiple Glu-Glu-Arg (EER) motif and the C-terminal effector domain. The RXLR-EER motif has been shown to be required for a successful translocation of the effectors into the host plant cell (Whisson et al., 2007, Dou et al., 2008, Grouffaud et al., 2008). The RXLR-EER motif seems to have a similar function as the PEXEL motif found in effector proteins from *Plasmodium falciparum*, a human pathogen (Bhattacharjee et al., 2006). It has been demonstrated that RXLR-EER sequence from *P. infestans* effector protein Avr3a fused to GFP enables the translocation of the chimeric protein into erythrocytes (Bhattacharjee et al., 2006, Halder et al., 2006).

The genome of *P. infestans* encodes more than 500 RXLR effectors, whereas approximately 350 were found in *P. sojae* and 150 in *H. arabidopsidis* (Haas et al., 2009, Tyler et al., 2006,

Baxter et al., 2010). One of the biggest obstacle in oomycete effector research is the identification of their host targets. Most of the RXLR effectors do not display any homology to known proteins. Their C-terminal domain is showing a higher level of amino acid polymorphism than the N-terminal part, suggesting that it is responsible for the effector function (Win et al., 2007).

All the RXLR effectors that have been functionally characterized so far suppress plant immune responses. Avr3a is known to target and stabilize host E3 ligase CMPG1, which is a negative regulator of PCD induced by INF1. The effector domain also contains amino acids that are important for the avirulence function and the recognition by the potato resistance protein R3a, leading to the activation of a HR (Bos et al, 2008). *P. infestans* effector Avrblb2 prevents the secretion of the host papain-like cysteine protease C14 (PLCP C14) into the apoplast in order to prevent immune responses (Bozkurt et al., 2011). Another effector from *P. infestans*, Pi03192, interacts with NAC transcription factors NTP1/NTP2 preventing their re-localization from ER into nucleus and activation of immune responses (McLellan et al., 2013). *P. infestans* effector PexRD2 interacts with kinase domain of MAPKKKε, a positive regulator of cell death (King et al., 2014). *H. arabidopsidis* HaRxL44 targets MED19a and attenuates SA-induced plant immune responses (Caillaud et al., 2013). Pi04314 from *P. infestans* targets host PP1c isoforms and down-regulate JA- and SA-responsive genes (Boevink et al., 2016). Several RXLR effectors from *P. sojae* including Avr1b-1 and Avr1k have been also showed to suppress PCD triggered by INF1 and/or mouse pro-apoptic protein BAX (Wang et al., 2011, reviewed in Song et al., 2013).

1.4 The RXLR effector AVR2 from Phytophthora infestans

The *P. infestans* RXLR effector AVR2 is a 116 amino acid protein with a relative short effector domain of approx. 50 amino acids. The gene encoding AVR2 (PITG_22870) was first characterized upon map-based cloning from *P. infestans* T30-4 isolate and identified as an avirulence gene causing ETI in potato plants carrying the resistance gene *R2* (Lokossau et al., 2009, Gilroy et al., 2011). Two alleles of *AVR2* encoding for a protein differing by a single amino acid (N31K) outside of the effector domain were cloned from T30-4 (Figure 3). Both isoforms are giving visible HR symptoms when co-expressed with R2 in *N. benthamiana*. In

accordance with previous observations performed with RXLR effectors bearing an avirulence function, the C-terminal domain of AVR2 is sufficient to trigger R2-mediated cell death. Recently, PCR-based amplification from the *P. infestans* isolate 06_3928A, particularly aggressive on potato cultivars, yielded a sequence which differs in 25 nucleotides/13 amino acids when compared to the sequence of the reference AVR2 isoforms (Figure 3). The *P. infestans* isolates containing only the *AVR2*-like gene were virulent on *R2* potato plants and failed to elicit R2-mediated HR (Gilroy et al., 2011). Moreover, the R2 orthologues (R2, R2-like, Rpi-blb3, Rpi-abpt), that were able to recognize AVR2, failed to recognize the AVR2-like variant Lokossau et al. (2009) (Susane Breen PhD thesis, 2012). In order to identify the host targets of AVR2, a yeast two-hybrid (Y2H) screen analysis was performed and identified potato BSU1-like phosphatase (BSL1) as an interactor of AVR2 (Saunders et al., 2012). Co-immunoprecipitation experiments performed in *N. benthamiana* confirmed that AVR2 associates with potato BSL1 and that this association is required for the recognition of AVR2 by R2 (Saunders et al., 2012). The authors showed that R2 associates only with the BSL1-AVR2 complex and in turn, hypothesized that BSL1 is guarded by R2. To support their hypothesis, the authors report that in *N. benthamiana* BSL1 knock-down lines, transient expression of AVR2 and R2 yields reduced HR and higher level of disease lesions to *P. infestans*.

Figure 3 **Sequence alignment of AVR2** ***from*** **P. infestans T30-4 and 06_3928A.** The black boxes indicate signal peptide, K/N polymorphism at residue 31, , RXLR and EER motif respectively.

1.5 Objective of the thesis

With the beginning of my thesis, proteins containing an RXLR-EER motif were identified as the main class of effectors with a proven virulence and also avirulence function in *P. infestans*. However, the mode of action of many RXLR effectors was largely unknown, although it appeared that a key function was to suppress host immune responses.

The RXLR effector AVR2 triggers ETI in potato cultivars carrying the resistance gene R2. AVR2 associates with potato BSU1-like phosphatase BSL1, a known component of brassinosteroid-induced signaling and a positive regulator of brassinosteroid-dependent growth and development in Arabidopsis.The interaction between AVR2 and BSL1 is prerequisite for the recognition by R2 and activation of the HR response. *P. infestans* isolates with silenced AVR2 were less efficient in colonizing susceptible potato cultivars, suggesting that AVR2 is important for the virulence of *P. infestans*.

The objective of this thesis aimed to characterize and compare the virulence function of AVR2 *in planta* using available genetic and molecular resources in *N. benthamiana* and Arabidopsis, a host and non-host plant species of *P. infestans*, respectively. In the first part, I have studied the interaction specificities between AVR2 and the BSU1-like phosphatases and I have evaluated the influence of the interaction between AVR2 and BSL1-3 on brassinosteroid-regulated signaling components and brassinosteroid-dependent responses. In recent years, activation of the brassinosteroid pathway emerged as negatively regulating MTI and therefore, I have investigated in the second part whether and to what extent the interaction between AVR2 and BSL1-3 affects the activation of plant immune responses triggered by the recognition of MAMPs.

2 MATERIAL AND METHODS

2.1. Material

2.1.1.1 Chemicals

All common chemicals used in this study were purchased from Carl-Roth (Karlsruhe, GER), Sigma-Aldrich (Taufkirchen, GER), Duchefa (Haarlem, NL), and Roche (Grenzbach, GER) unless specified otherwise in the text. The DNA polymerase, ligases, restriction enzymes and respective buffers were obtained from Thermo Fischer Scientific (Waltham, Massachusetts, USA) or New England Biolabs (Beverly, USA). The BP\LR recombination enzymes used in the cloning strategy were obtained from Life Technologies (Darmstadt, GER). The DNA oligonucleotides used in PCR reactions were synthesized by Eurofins MWG Operon (Ebersberg, GER). Antibodies applied in Western blot analysis were purchased from Sigma-Aldrich (Taufkirchen, GER). All solutions and bacterial growth media were prepared with Mili-Q H_2O and were sterilized if necessary for 20 min. at 121°C.

2.1.1.2 Media and antibiotics

All bacteria and plant growth media were prepared with Mili-Q H_2O and were sterilized for 20 min. at 121°C. The appropriate antibiotics were added after cooling the medium to 55°C.

Table 1 **Media used in this study.**

Medium	**Components**	**Species**
Luria-Bertani broth (LB)	10g/l Bacto-tryptone, 5g/l NaCl, 5g/l Yeast extract, 15g/l Agar	*Escherichia coli* *Agrobacterium tumefaciens*
King's Medium B (King's B)	20 g/l Glycerin, 40 g/l Proteose-Pepton 3 10 ml/l 10 % K_2HPO_4 and 10 ml/l 10 % $MgSO_4$ filter sterilized and added after autoclaving)	*Pseudomonas syringae*
0.5X Murashige-Skoog Medium (0.5X MS)	2,2 g/l MS, pH 5.7 (KOH), 10 g/L Select-Agar	*Arabidopsis thaliana*

YEBS	1g/l Yeast extract, 5g/l Beef extract, 5g/l Sucrose, 5g/l Bacto-peptone, 0.5g/l $MgSO_4$, pH 7.0	*Agrobacterium tumefaciens*

Table 2 **Antibiotics used in this study.**

Antibiotic	**Working concentration**	**Solvent**
Carbenicilin	50 µg/ml	H_2O
Cyclohexamid	50 µg/ml	H_2O
Gentamycin	25 µg/ml	H_2O
Kanamycin	50 µg/ml	H_2O
Rifampicin	50 µg/ml	DMSO
Spectinomycin	75 µg/ml	H_2O
Tetracyclin	15 µg/ml	Ethanol
Hygromycin	20 µg/ml	H_2O

2.1.2 Antibodies

Antibodies used in Western blot analysis were purchased from Sigma-Aldrich (Taufkirchen, GER), Acris Antibodies GmbH (Herford, GER), ChromoTek GmbH (Planegg, GER).

Table 3 **Antibodies used in this study.**

Antibody	**Host organism**	**Dilution**	**Company**
pp44/pp42-MAPK	rabbit	1:1000	Sigma-Aldrich
α-MYC	mouse	1:1000	Sigma-Aldrich
α-HA	mouse	1:1000	Sigma-Aldrich

α-GFP	goat	1:5000	Acris Antibodies
α-mouse-AP	rabbit	1:3000	Sigma-Aldrich
α-goat-AP	rabbit	1:3000	Sigma-Aldrich
α-rabbit-AP	goat	1:3000	Sigma-Aldrich
α-goat-HRP	rabbit	1:10000	Sigma-Aldrich
α-mouse-HRP	rabbit	1:7000	Sigma-Aldrich
α-rat-AP	goat	1:3000	Sigma-Aldrich
α-RFP	rat	1:5000	ChromoTek

2.1.3 Vectors

Table 4 **Vectors used in this study.**

Vector	Description	Reference
pDONR201	Donor vector for Gateway cloning	Life Technologies
pDONR221	Donor vector for Gateway cloning	Life Technologies
pDONR207	Donor vector for Gateway cloning	Life Technologies
pDONRZeo	Donor vector for Gateway cloning	Life Technologies
pSOUP	Helper plasmid for *A. tumefaciens*-mediated plant transformation with pGreenII0229	JIC (Norwich, UK)
pGreenII0229	Binary vector for *A. tumefaciens*-mediated plant transformation	JIC (Norwich, UK)
pK7WGF2	Binary Gateway destination vector for expression of fusion proteins with N-terminal GFP in plants	VIB (Gent, BEL)

pB7WGF2	Binary Gateway destination vector for expression of fusion proteins with N-terminal GFP in plants	VIB (Gent, BEL)
pB2GW7	Binary Gateway destination vector for expression of proteins in plants	VIB (Gent, BEL)
pB2kozHAGW7	Binary Gateway destination vector for expression of fusion proteins with N-terminal HA in plants	ZMBP (Tübingen University)
pB2kozMYCGW7	Binary Gateway destination vector for expression of fusion proteins with N-terminal MYC in plants	ZMBP (Tübingen University)
pHm34GW,0	Multisite binary Gateway destination vector for expression of proteins in plants	VIB (Gent, BEL)
pUBN-RFP	Binary Gateway destination vector for expression of fusion proteins with N-terminal RFP in plants	ZMBP (Tübingen University)

2.1.4 Bacterial strains

Table 5 **Bacterial strains used in this work.**

Species	**Strain**	**Genotype**
Escherichia coli	DH5α	F– endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK–mK+), λ–
	TOP10	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 $λ^-$
	DE3.1	F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1

Pseudomonas syringae	*Pto* DC3000	Rif^r
Agrobacterium tumefaciens	GV3101::pMP90	$T\text{-}DNA^-$ vir^+ Rif^r pMP90 $Gent^r$

2.2 Methods

2.2.1 Growth conditions

2.2.1.1 Arabidopsis thaliana growth

Experiments in this study were performed with *Arabidopsis thaliana* Col-0 ecotype. The plants were grown on sterile GS90-soil (Gebr. Patzer) or on 1/2MS medium upon sterilization with chlorine gas and stratification for 1-3 days at 4°C. Afterwards plants were grown under long day conditions (16h light, 8h darkness) or short day conditions (8h light, 16h darkness). The growth chambers provided 40-60% humidity, 22°C and 150umol/m^2s conditions. The seeds of Arabidopsis thaliana were sterilized if necessary in desiccator for 3-5h with chlorine gas formed after mixing 12% sodium hypochlorite with 1.5ml 37% HCl.

2.2.1.2 Growth of Escherichia coli

Escherichia coli culture was grown 15- 18h at 37 °C either on LB-agar plates or in liquid LB-medium at 230rpm shaking. The media contained appropriate selection antibiotics.

2.2.1.3 Growth of Agrobacterium tumefaciens

Agrobacterium tumefaciens culture was grown 1-2 days at 28 °C either on LB-agar plates or in liquid LB-medium at 180rpm shaking. The media contained appropriate selection antibiotics.

2.2.1.4 Growth of Pseudomonas syringae

Pseudomonas syringae Pto DC3000 culture was grown 1-2 days at 28 °C either on King's B-agar plates or in liquid King's B medium at 180rpm shaking. The media contained appropriate selection antibiotics.

2.2.2 Molecular methods

2.2.2.1 Isolation of plasmid DNA from E.coli

The plasmid DNA has been obtained using alkaline lysis method described by Birnboim and Doly (1979). 2-4 ml of overnight LB culture of *E.coli* harboring the plasmid of interest was centrifuged at 14000rpm for 2 min. at 4°C. The bacterial pellet was re-suspended in 200µl solubilisation buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose, 0.1 mg/ml RNase A) and 400µl lysis buffer was added (0.2 M NaOH, 1 % (w/v) SDS). The lysis was carried out for 5 min at RT. The mixture was neutralized after adding neutralization buffer (3 M KAc, 11.5% HAc) followed by centrifugation at 14000rpm for 10 min at room temperature. The supernatant containing plasmid DNA has been transferred into the new Eppendorf tubes and 0.7 volume of isopropanol was added. The mixture has been left for 10 min at room temperature and subsequently centrifuged at 13000g for 30 min at RT. The supernatant was discarded and the DNA pellet has been washed with 500µl 70% Ethanol followed by centrifugation at 13000g for 5 min at RT. DNA was subsequently air dried at room temperature and re-suspended in 50-100µl deionized H_2O.

2.2.2.2 Genomic DNA extraction

DNA from leaf material has been obtained according to the method described by Edwards et al. (1991). Approximately 30 mg of leaf tissue has been homogenized in 200µl of Edwards Buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8, 0.5% SDS). The mixture has been centrifuged at 13000g for 5 min at room temperature. The supernatant containing DNA was transferred to a clean Eppendorf tube and 200µl isopropanol was added. The mixture has been left for 5-30 min. at room temperature and subsequently centrifuged at 13000g for 10 min at 4°C. The supernatant was discarded and the DNA pellet has been washed with 500µl 70% Ethanol followed by centrifugation at 13000g for 5 min at room

temperature. DNA has been air dried in room temperature and re-suspended in 50-100µl deionized H_2O.

2.2.2.3 Total RNA extraction

Total RNA was extracted using TRIZOL reagent (Chomczynski and Sacchi, 1987). 50-100 mg of leaf tissue was homogenized in liquid nitrogen and 1ml of Trizol was added. The mixture was vortexed for 10 sec and incubated at RT for 10 min. 200µl chloroform has been added followed by vigorous shaking for 10 sec and incubation at RT for the next 10 min. Samples were centrifuged at 13000g for 10 min at RT. The upper aqueous phase containingRNA was transferred to a new Eppendorf tube and 500µl isopropanol was added. The mixture was left in RT for 15 min. and centrifuged at 13000g at 4°C for 10 min. The precipitated RNA was washed with 1ml 70% ethanol followed by centrifugation at 13000g at 4°C for 10 min. The pellet has been air dried at RT and re-suspended in deionized H_2O.

2.2.2.4 Nucleic acid quantification

The concentration and purity of nucleic acids was determined using *NanoDrop2000* Spectrophotometer (PeqLab, GER).

2.2.2.5 Restriction digests of plasmid DNA

0.2-0.5µg of plasmid DNA has been digested in 20µl reaction volume using 5U of selected enzymes under recommended buffer conditions. The reaction was carried out at 37°C for 1-2 hours.

2.2.2.6 Agarose gel electrophoresis

DNA fragments were separated via 0.8-2% agarose gel electrophoresis in 1X TE buffer (40mM Tris/Acetate pH 8.0, 100mM EDTA pH 8.0). After dissolving the agarose in TAE buffer ehidium bromide was added to a final concentration of 0.5µg/ml. The DNA samples were mixed with appropriate volume of 6X loading dye (30% glycerol, 0.25% bromophenol blue)

and separated at 120V. The DNA fragments were visualized using UV Transiluminator and 1 kb DNA Ladder (Fermentas, GER) was applied to determine the size of the DNA.

2.2.2.7 DNA gel extraction

The desired DNA fragments were excised from a gel upon gel electrophoresis in 0.8-2% agarose. The DNA purification was done with QIAquick Gel Extraction Kit (QIAGEN, GER) according to manufacturer's specification.

2.2.2.8 Reverse transcription polymerase chain reaction (RT-PCR)

2µg of total RNA in 10µl of H_2O was used for cDNA synthesis. Samples were treated with 1µl of DNAse and incubated at 37°C for 20 min. The DNAse was subsequently inactivated after adding 1µl of 25mM EDTA at 65°C for 10 min. 10 µl of reaction mixture containing 4 µl 5X RT Buffer, 2µl of 2.5mM dNTP mix, 2µl of 30uM oligo-dT, 0.5µl RNase Inhibitor, 1µl of reverse transcriptase was added to RNA samples. The reverse transcription was carried out at 42°C for 90 min followed by 70°C for 10 min.

2.2.2.9 Polymerase chain reaction (PCR)

Standard control PCR reactions were done with home-made *Taq* polymerase. Each 20µl reaction mixture contained:

Taq Buffer (10X)	2µl
Forward Primer (10uM)	1µl
Reverse Primer (10uM)	1µl
2.5mM dNTPs	1µl
Nuclease free H_2O	13.5µl
DNA	1µl
Taq polymerase	0.5µl (1U)

Thermal cycling conditions:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	30 sec	1
Denaturation	94	15 sec	
Annealing	55-60	30 sec	20-35
Extension	72	1 min/kb	
Final extension	72	5-10 min	1

DNA fragments for the cloning were amplified with *Pfu* DNA polymerase (Fermentas, GER).

Each 50uµ reaction mixture contained:

Pfu Buffer (10X)	5µl
Forward Primer (10uM)	1µl
Reverse Primer (10uM)	1µl
2.5mM dNTPs	1µl
Nuclease free H_2O	up to 50µl
DNA	1-2µl
Pfu polymerase	0.5-1µl (2.5U/µl)

Thermal cycling conditions:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	1-3 min	1
Denaturation	95	15 sec	

Annealing	55-60	30 sec	20-35
Extension	72	2 min/kb	
Final extension	72	5-10 min	1

2.2.2.10 Quantitative Real-Time PCR (qRT-PCR)

The gene expression was determined using quantitative real-time PCR. The cDNA was amplified using Maxima SYBR Green (Thermo Scientific, GER) qPCR Master Mix on iQ5 Multicolor Real Time PCR Detection system (BioRad, GER).

Each 20µl reaction mixture contained:

Maxima SYBR Green qPCR Master Mix (2X)	10µl
Forward Primer (10pmol/µl)	1µl
Reverse Primer (10pmol/µl)	1µl
Nuclease free H_2O	7µl
1:4 diluted cDNA	1µl

Thermal cycling conditions:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	
Annealing	55-60	30 sec	40
Extension	72	30 sec	

The gene expression was quantified using the $2^{-\Delta\Delta CT}$ algorithm described by Livak et al. (2001) and normalized to one of the housekeeping genes which was either ef1α or actin.

2.2.2.11 Gateway site-specific recombination cloning of amplified DNA fragments

The full-length coding sequences of selected genes were cloned using Gateway System (Life Technologies). The coding regions of selected genes were amplified in PCR reaction using gene specific primers containing appropriate *att* sites. The amplified DNA fragments were cloned into selected pDONR vector (Life Technologies) in

BP recombination reactions:

attB flanked PCR Product (10-150ng)	1-7µl
Donor Vector (150ng/µl)	1µl
Nuclease free H_2O	up to 8µl
BP Clonase^{TM} Enzyme Mix	2µl

The mixture was incubated 1h at RT. The reaction was stopped after adding 1µl of Proteinase K followed by incubation in 37°C for 10 min. 2-5µl of the reaction mixture was cloned into the competent DH5α competent cells. The entry clones containing full length coding sequences cloned were transferred into the expression vectors in

LR recombination reactions:

Entry Clone (50-150ng)	1-7µl
Destination Vector (150ng/µl)	1µl
Nuclease free H_2O	up to 8µl
LR Clonase^{TM} Enzyme Mix	2µl

The mixture was incubated 1h at RT. The reaction was stopped after adding 1µl of Proteinase K followed by incubation in 37°C for 10 min.

2.2.2.12 Site-directed mutagenesis

The desired mutations were introduced into the entry clones using mutagenic primers containing desired mutation in overlap extension PCR reaction. The 50µl reaction mixture contained:

Pfu Buffer (10X)	5µl
Forward Mutagenic Primer (10µM)	1µl
Reverse Mutagenic Primer (10µM)	1µl
2.5mM dNTPs	1µl
Nuclease free H_2O	up to 50µl
Plasmid DNA (10 ng)	1-2µl
Pfu polymerase	0.5-1µl (2.5U/µl)

Thermal cycling conditions:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	1 min	1
Denaturation	95	30 sec	
Annealing	60	50 sec	18
Extension	60	2 min/kb	
Final extension	60	5-10 min	1

After the PCR reaction 1ul *DpnI* (10U/µl) was added to the reaction mixture in order to digest the parental methylated plasmid DNA. The digestion was carried out overnight in 37°C followed by heat inactivated of the restriction enzyme in 80°C for 20 min. 5-15µl of the reaction mixture was transformed in to the chemically competent DH5α *E.coli* cells. The presence of the mutation in isolated entry clones was verified via sequencing.

2.2.2.13 Generation of Gateway compatible binary destination vectors for expression of N-terminal KozakMYC and KozakHA fusion proteins

10-20µg of pB2GW7 destination vector (VIB Gent, BEL) was digested overnight with *SpeI* and purified with QIAquick Gel Extraction Kit (QIAGEN, GER). 10 µl of 10 X dephosphorylation buffer (10mM $ZnCl_2$, 10mM $MgCl_2$, 100mM Tris-HCl pH 8.3) was added to 90µl of the eluate together with 1U CIAP (Calf Intestial Alkaline Phosphatase, SIGMA) for each 2µg of the plasmid. The mixture was incubated for 15 min at 37°C. The same volume of DCIP was added to the mixture and the reaction was incubated for 45 min at 55°C. The dephosphorylated plasmid was purified with QIAquick Gel Extraction Kit (QIAGEN) and the volume was adjusted to 0.1µg/µl. The adaptor duplexes were generated after mixing 10pmol of forward and reverse adaptor primer in 10µl of ligase buffer (10 mM $MgCl_2$, 10 mM DTT, 0.5mM ATP, 40mM Tris-HCl pH 7.8). The mixture was heated to 95°C for 2 min followed by slowly cooling over a period of 15 min to 50°C. The reaction was further incubated for at 50°C for 5 min and finally cooled to RT. 0.1µg of the digested and dephosphorylated plasmid was mixed wih 0.1 pmol of adaptor duplex in 10µl of the ligase buffer. 1µl of T4 DNA ligase (ThermoFisher Scientific, GER) was added to the mixture and the reaction was incubated overnight at 16°C. The whole reaction volume was transformed into the *ccdb* resistant *E. coli* DB3.1 strain and selection was done on LB plates containing appropriate antibiotic.

2.2.2.14 Sequencing

The generated constructs were sequenced by *GATC Biotech AG* (Konstanz, GER). The provided sequencing mixture contained 5µl of the plasmid DNA (80-100 ng/µl) and 5µl of the sequencing primer (5µM). The sequences were analyzed using *CLC Workbench Main 7* software and compared to appropriate GenBank accessions.

2.2.2.15 Preparation of chemically competent DH5α E.coli cells

Chemically competent DH5α *E.coli* cells were prepared according to Inoue et al. (1990). 250 ml of SOB medium (2% Bacto-Tryptone, 5% Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ pH 7.0) was inoculated with 5 ml overnight bacterial culture. Cells were grown in 18°C under 200rpm shaking until the optical density at 600nm reached approximately 0.6. The culture was cooled on ice for 10 min and centrifuged at 1600g for 10 min at 4°C. The

supernatant was discarded and the bacterial pellet was gently resuspended in cold TB-Buffer (10mM PIPES, 15mM $CaCl_2$, 250mM KCl, 55mM $MnCl_2$ pH 6.7). The bacterial suspension was left for 10 min on ice and subsequently centrifuged at 1600g for 10 min at 4°C. The washing step was repeated two times (as described above) and the pellet gently re-suspended in 20 ml of TB-Buffer. DMSO to the end concentration of 7% was added. 200µl of bacteria suspension were aliquoted in Eppendorf tubes and flash frozen in liquid nitrogen. The competent cell were stored in -80°C up to 6 months.

2.2.2.16 Transformation of E.coli

The aliquot of chemically competent DH5α *E.coli* cells was thawed on ice. The 5-10 µl of ligation mixture or 100ng of plasmid DNA was added to the cells followed by incubation on ice for 20 min. The DNA is delivered into the cells by heat shock at 42°C for 30 sec followed by incubation on ice for 1-3 min. 0.8ml of LB medium was added to the cell suspension which was subsequently incubated at 37°C for 1 hour under 180rpm shaking prior platting.

2.2.2.17 Preparation of electrocompetent Agrobacterium tumefaciens

200ml of LB medium containing rifampicin and gentamycin was inoculated with 2ml overnight culture. Cells were grown in 18°C under 200rpm shaking until the optical density at 600nm reached approximately 0.6. The culture was cooled on ice for 10 min and centrifuged at 1600g for 10 min at 4°C. The supernatant was discarded and the bacterial pellet was gently re-suspended in 20 ml cold sterile deionized H_2O. The bacterial suspension was left for 10 min on ice and subsequently centrifuged at 1600g for 10 min at 4°C. The washing step was repeated two times (as described above) and the bacterial pellet was gently re-suspended in 20 ml of sterile 20% glycerol. 50µl of bacteria suspension were aliquoted in Eppendorf tubes and flash frozen in liquid nitrogen. The competent cell were stored in -80°C up to 6 months.

2.2.2.18 Transformation of *Agrobacterium tumefaciens*

The aliquot of electrocompetent GV3101 *A. tumefaciens* cells was thawed on ice and 1µl of plasmid DNA was added to the cells. The aliquot was transferred to the precooled electrocuvette and pulsed with 1440V for 5 ms. The mixture was immediately transferred on ice and 0.8 ml LB was added. The cells were subsequently incubated for at 28°C for 2 hours under 180rpm shaking prior platting.

2.2.3 Protein Biochemistry

2.2.3.1 Protein extraction from leaf tissue

For each sample, 3 leaf discs (d=5mm) were cut out using a cork borer and homogenized in liquid nitrogen. An equal volume of 5X loading buffer (0.25% bromophenol blue, 0.5M DTT, 50% glycerol, 10% SDS, 0.25M Tris-HCl pH 6.8) was added to the leaf material. The mixture was briefly vortexed and incubated on ice for 10 min. After incubation the probes were centrifuged at 1600g for 10 min at 4°C. The supernatant containing the extracted proteins was transferred to new Eppendorf tubes and subsequently heated at 95°C for 1 min followed by short incubation on ice.

2.2.3.2 SDS-PAGE

The discontinuous SDS polyacrylamide gel electrophoresis was performed according to the method described by Laemelli (1970). The proteins were first condensed in 5% stacking gel and subsequently separated in 10-15% resolving gel depending on the size of the protein of interest. The gels were casted and run using the Mini-PROTEAN system (Bio-Rad). PageRuler™ Prestained Protein Ladder was used to determine the size of separated proteins.

2.2.3.3 Western blotting

After separation in SDS polyacrylamide gel, the proteins were transferred on Hybond ECL nitrocellulose membrane (GE Healthcare, GER) using Mini trans-Blot Electrophoretic Transfer Cell system (Bio-Rad, GER) for 1 hour at 350mA according to the manufacturer’s protocol. The transfer was done in 1X transfer buffer (25mM Tris, 192mM glycine, 1% SDS, 20%

methanol). After the transfer the membrane was incubated in Ponceau S solution (0.1% Ponceau S Red in 5% acetic acid) to evaluate the transfer efficiency and protein loading. To avoid the unspecific biding of the antibodies, the membrane was blocked in 5% milk prepared in 1X TBST buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 0.1 % Tween 20) for 1 hour at RT. After a brief washing step in TBST buffer the membrane was incubated with primary antibody overnight in 4°C. Next, the membrane was washed three times with TBST for 10 min and incubated with secondary antibody for 2 hours at RT. The membrane was washed afterwards three times with TBST buffer for 10 min. The signal from horseradish peroxidase coupled antibodies was developed using ECL™ prime Western Blotting Detection Reagent according to manufacturer's protocol. The chemiluminescent signal was visualized using Amersham Imager 600 (GE Healthcare). The signal from alkaline phosphatase coupled antibodies was detected in BCIP/NBT buffer (15mM Tris-HCL pH 9.5, 0.5mM $MgCl_2$, 10mM NaCl, 0.025mg BCIP, 0.025mg NBT).

2.2.3.4 Immunoprecipitation

For immunoprecipitation, 200mg of leaf material was homogenized in liquid nitrogen. To each sample 1.7 ml of solubilisation buffer (25mM Tris-HCl pH 7.5, 150mM NaCl, 2mM DTT, 1% NP40, protease Inhibitor (Roche), Phosphatase inhibitor (Roche)) was added. The mixture was briefly vortexed and incubated on ice for 10 min. After incubation the probes were centrifuged at 1600g for 20 min at 4°C. 1ml of the supernatant containing the extracted proteins was transferred to new Eppendorf tube containing 10-20µl of agarose affinity matrix coupled with appropriate antibody (equilibrated before with solubilisation buffer). The mixture was incubated at 4°C for 1-2 hours under 10 rpm shaking. After the incubation the beads were centrifuged at 1000g for 2 min at 4°C and washed two times with solubilisation buffer and two times with washing buffer (25mM Tris-HCl pH 7.5, 150mM NaCl, 2mM DTT, protease Inhibitor (Roche), phosphatase inhibitor (Roche)). The proteins were eluted from the beads after adding 50µl 5X SDS loading buffer and heated for 5-10 min at 95°C. The probes were cooled on ice and supernatant was collected after a brief centrifugation at 1600g for 2min at 4°C. The samples were analyzed by Western Blot after electrophoresis with SDS polyacrylamide gel.

2.2.3.5 Phosphatase assay

For phosphatase assay, the RFP-tagged kinases were immunoprecipitated with RFP-Trap A (Chromotek, GER).The precipitated material was additionally washed in phosphatase buffer (40mM Tris-HCl pH 7.5, 0.25mg/ml, 10mM $MgCl_2$) followed by brief centrifugation and re-suspended in 50µl of phosphatase buffer. 25µl of the immunoprecipitated material was used in the phosphatase assay. The phosphatase activity was measured with ProFluor Ser/Thr PPase Assay (Promega, GER) according to the manufacturer's protocol. The remaining 25µl was eluted from the beads after adding 10ul 5X SDS loading buffer and heated for 5-10 min at 95°C. The samples were analyzed by Western Blot after electrophoresis with SDS polyacrylamide gel.

2.2.3.6 MAPK activation

Total plant protein extract was prepared as described in 2.2.3.1 and 15µl of each sample was loaded on the gel and separated in 13% SDS-PAGE, followed by Western blot and detection with primary antibody against p44/p42-MAPK and secondary anti-mouse antibody.

2.2.4 Plant Methods

2.2.4.1 Agrobacterium-mediated transient expression in tobacco leaves

5ml LB medium containing appropriate antibiotics were inoculated with *Agrobacterium tumefaciens* harboring desired plasmids. The bacteria culture was grown overnight at 28°C and 180rpm shaking. The culture was centrifuged at 5000rpm for 10 min. The pellet was gently re-suspended in Infiltration Buffer (10mM $MgCl_2$ solution containing 150µM acetosyringone) and centrifuged at 5000rpm for 10 min. The washing step was repeated one time (as described above) and bacterial pellet was re-suspended again in infiltration buffer and the volume of the inoculum was adjusted to the concentration that gives OD_{600} of 1. The suspension was left at RT for 2-3 hours, mixed (1:1) with bacteria culture carrying p19 construct and diluted in infiltration buffer to adjust the concentration of each culture to the final OD_{600} of 0.1. The mixture was infiltrated into the leaves of *Nicotiana benthamiana* using a 5ml syringe. The expression was checked after 2-3 days after the infiltration by Western blot detection.

2.2.4.2 Generation of stable transgenic lines of Arabidopsis thaliana

The stable transgenic lines of *Arabidopsis thaliana* were generated according to the method described by Davis et al. (2009). 500ml of YEBS medium (1g/l yeast extract, 5g/l beef extract, 5g/l sucrose, 5g/l bacto-peptone, 0.5g/l $MgSO_4$, pH 7.0) containing appropriate antibiotics were inoculated with 50ml overnight culture and grown in 28°C for 10 hours. Afterwards 100-200µl Silwett L77 were added to the medium. The *Arabidopsis thaliana* plants with present immature floral buds were dipped into bacterial suspension and placed afterwards at high humidity conditions for the next 12-24 hours. The transformants were selected on 1/2 MS medium containing appropriate antibiotics or on soil when BASTA (glufosinate) was applied.

2.2.5 Biological responses

2.2.5.1 Oxidative burst assay

Leaves of 4-6 week old *Arabidopsis thaliana* or *Nicotiana benthamiana* plants were cut into 5mm pieces and incubated overnight on water. The leaf pieces were transferred into the 96-well plate containing 10µM luminol, 0.005mg/ml peroxidase solution. The oxidative burst was measured using Centro LB 900 (Berthold Technologies, GER) luminometer after induction with selected MAMPs.

2.2.5.2 Ethylene assay

Leaves of 4-6 week old *Arabidopsis thaliana* or *Nicotiana benthamiana* plants were cut into 5mm pieces and incubated overnight on water. The leaf pieces were transferred into glass vials filled with 0.5 ml H_2O. β- megaspermin to the final concentration of 100nM was added to the tubes followed by incubation for 3-4 hours at RT and 50 rpm shaking. The ethylene production was measured using gas chromatography (GC-14A, Shimadzu, JPN).

2.2.5.3 Infection assay with Pseudomonas syringae

50 ml of King's B medium containing appropriate antibiotics were inoculated with 2-3ml overnight culture and grown at described in 2.2.1.4. The culture was cooled on ice for 10 min. and centrifuged at 4500g for 10 min. at 4°C. The supernatant was discarded and the

bacterial pellet was gently re-suspended in cold 10mM $MgCl_2$. The bacterial suspension was left for 10 min on ice and subsequently centrifuged at 4500g for 10 min at 4°C. The washing step was repeated two times (as described above). The bacterial pellet was gently re-suspended in 10mM $MgCl_2$ and the volume of the inoculum was adjusted to the concentration that gives OD_{600} of 1. Afterwards, an appropriate volume of Silwett L77 was added to the mixture to the final concentration of 0.02%. The 4-6 week old *Arabidopsis thaliana* plants were sprayed with the bacterial suspension. Afterwards plants were grown in short day conditions maintaining high humidity. The bacterial growth was quantified at 0, 3, 6 days after infection. Equal amounts of leaf material were collected, followed by brief surface sterilization in 70% ethanol and washing step in H_2O. The leaf material was homogenized afterwards in 100µl of $MgCl_2$. The extract was diluted if necessary and 10µl were plated on King's B agar plates. The bacteria were grown for 2 days at 28°C. The bacterial growth is expressed in colony forming units (cfu) per cm^2.

2.2.5.4 Infection assay with Alternaria brassicicola

The pathogenity assay with *Alternaria brassicicola* was performed with 4-6 week old *Arabidopsis thaliana* plants grown in short day conditions. 2-3 leaves of 12-16 plants were inoculated with 5µl droplets of spore suspension ($1x10^6$spores/ml). The plants were then kept at high humidity and the fungal growth was monitored 7, 10, 13 days after infection. The severity of the symptoms was quantified according to the infection index described by Kemmerling et al. (2007). "1" – no symptoms, "2" – light brown spots at infection site, "3"- dark brown spots at infection site, "4"- spreading necrosis, "5"- leaf maceration, "6" – sporulation.

2.2.5.5 Infection assay with Phytophthora capsici

The pathogenity assay with *Phytophthora capsici* was performed with 6 week old *Arabidopsis thaliana* plants grown in short day conditions. 8 leaves per transgenic line were inoculated with 15µl droplets of zoospore suspension ($1x10^6$spores/ml). The plants were then kept at high humidity conditions. The leaf discs were collected 2.5 and 3.5 days after infection followed by genomic DNA isolation with DNeasy Plant Mini Kit. The biomass of *P. capsici* was quantified as a ratio of *P. capsici* genomic DNA to *Arabidopsis* DNA.

2.2.5.6 Hormone and phytoalexin measurements

For the hormone measurements, leaf material of 4-6 week old *Arabidopsis thaliana* plants was collected and the concentration of salicylic acid (SA), jasmonic acid (JA), auxin, abscisic acid (ABA) as well as the phytoalexin camalexin was determined with GC-MS according to the method described by Iven et al. (2012).

2.2.5.7 Cell death assay

For the cell death assay, the proteins of interest were transiently expressed in *Nicotiana benthamiana* together with INF1 (infestin 1) via *Agrobacterium tumefaciens*. 2-3 leaves of at least 3 plants were used per replicate. HRs were evaluated at 7 day post-infiltration.

2.2.6 Differential interference contrast microscopy

The differential interference contrast images of stomata were done with fluorescent microscope Axiophot (Zeiss).

2.2.7 Statistics

Statistical analysis were performed with two tailed unpaired Student's t test ($p<0,05$) or with one-way ANOVA ($p<0,01$).

3 RESULTS

In this work, I aimed to characterize the function *in planta* of the AVR2 effector protein from *Phytophthora infestans*. Previous experiments performed in the lab of S. Kamoun (TSL, Norwich, UK) and P. Birch (JHI/UoD, Dundee, UK) revealed that AVR2 associates with the BSU1-like phosphatase BSL1 from potato (StBSL1) and that this interaction is recognized by the resistance protein R2 and triggers ETI (Saunders et al., 2012). The potato genome encodes three BSU1-like phosphatases: StBSL1, StBSL2b and StBSL2a, which are homologous to AtBSL1, AtBLS2 and AtBSL3 of Arabidopsis, respectively. So far no homolog of AtBSU1 has been identified in potato. Since AtBSU1 and AtBSL1 are known to be positive regulators of BR-signaling, I have decided to further investigate the impact of AVR2 on the brassinosteroid-signaling pathway and plant immune responses in both a host (*N. benthamiana*) and a non-host (*A. thaliana*) plant species of *P. infestans*.

3.1 AVR2 associates with BSU1-like phosphatases StBSL1, 2a and 2b and homologs of Arabidopsis

In order to identify potential host targets of AVR2, Yeast Two Hybrid (Y2H) was performed in the lab of Paul Birch (JHI/UoD, Dundee, UK). The potato Y2H library was made of pooled pathogen-infected resistant and susceptible potato cultivars (Susan Breen, PhD thesis). In addition to StBSL1 (Saunders et al. 2012), the initial screen identified StBSL3 as an interactor of AVR2. Primers for cloning *StBSL3* and *StBSL1* have been designed based on ESTs found in *S. lycopersicum* and the coding sequences for StBSL3 and StBSL1 were amplified using *S. tuberosum* cDNA. Using *AtBSL2* and *AtBSU1* sequences as a query, a BLASTX search in *S. lycopersicum* allowed to design additional primers and clone *StBSL2*. No candidate sequence has been identified for *BSU1* in *S. lycopersicum* or *S. tuberosum*. Phylogenetic analysis revealed that *StBSL2* and *StBSL3* are paralogues and have been renamed into *StBSL2b* and *StBSL2a* respectively (Susan Breen, PhD thesis and Figure 1). Although the initial screen did not show that StBSL2b is interacting with AVR2, a pairwise Y2H interaction assay has shown that StBSL2b is also targeted by AVR2 (Susan Breen, PhD thesis).

Figure 4 **Phylogenetic tree of *Arabidopsis thaliana* and *Solanum tuberosum* BSU1-like phosphatases.** UPGMA (Unweighted Pair Group Method with Arithmetic Mean) tree based on nucleotide sequence of At and St BSU1-like phosphatases and Arabidopsis PROTEIN PHOSPHATASE 2A-1(AtPP2A-1). The tree was generated in *CLC Workbench Main 7* using a bootstrapping of 100.

In order to confirm the results obtained in the Y2H assay, we performed association studies in *N. benthamiana*. Myc-tagged StBSL1, 2a and 2b were co-expressed with GFP-tagged AVR2 in *N. benthamiana* leaves and upon immunoprecipitation of GFP-AVR2 followed by immunodetection, we observed that AVR2 associates with the three StBSLs (Figure 5).

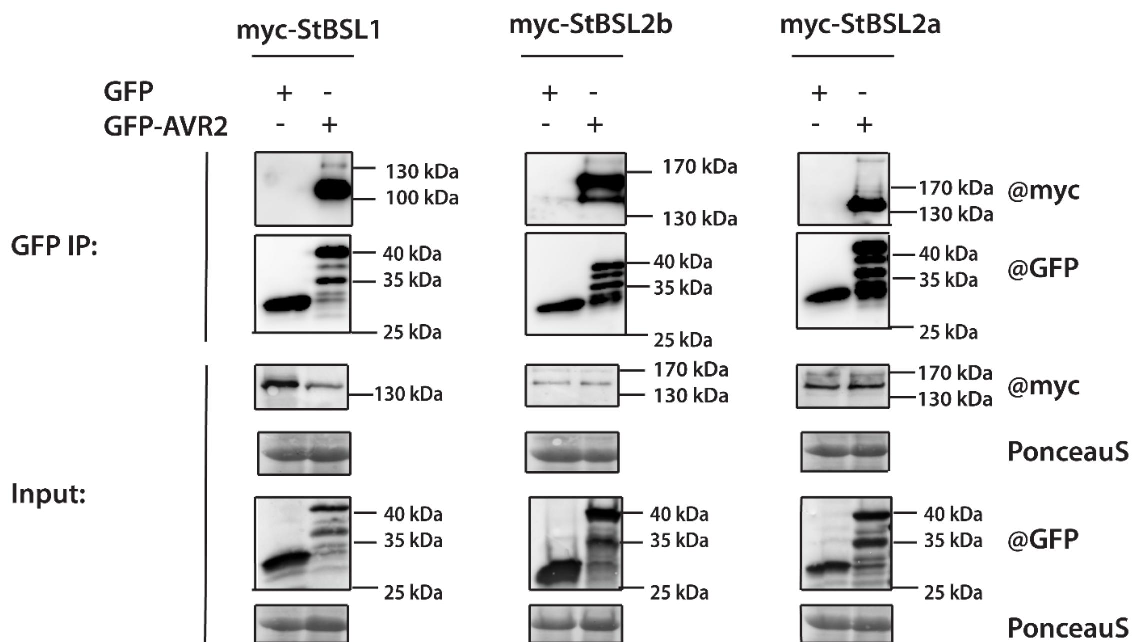


Figure 5 **Co-Immunoprecipitation of myc-tagged *S. tuberosum* BSL1, 2a and 2b and GFP-AVR2 in *N. benthamiana* .** GFP-AVR2 was co-expressed together with myc-tagged StBSL1, 2a and 2b in *N. benthamiana* leaves. GFP was used in control experiments. The immunoprecipitation was performed with anti-GFP agarose and the proteins were analyzed on western blot with anti-myc and anti-GFP antibody. The upper panel (GFP IP:) shows the co-immunoprecipitation results and the lower panel shows expression levels of myc-StBSLs and GFP-AVR2 in *N. benthamiana* extracts.

In the next experiment, we wanted to assess whether AVR2 is also able to interact with BSU1-like phosphatases from Arabidopsis, in the expectation to use eventually the existing genetic and molecular resources in BR signaling in this plant species to fasten the elucidation of the virulence function of AVR2. Immunoprecipitation experiments performed in Arabidopsis protoplasts transiently expressing HA-AVR2 and further liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis revealed that AVR2 interacts also with the Arabidopsis BSL1, 2 and 3 (C. Jäntsch, Annex 2).

The LC-MS/MS results have been confirmed through pairwise interaction studies in Arabidopsis protoplasts. The Arabidopsis *BSU1* and *BSL1-3* genes were amplified from an Arabidopsis cDNA library and cloned into a gateway compatible binary vector designed for N-terminal fusion to myc-tag. HA-tagged AVR2 has been transiently expressed with myc-tagged AtBSU1 and AtBSL1-3. The Co-Immunoprecipitation experiment showed again that AVR2 associates with AtBSL1, 2 and 3. AtBSU1 was not pulled down with AVR2 although AtBSU1 is a divergent paralogue of AtBSL1 (C. Jäntsch, Figure 6).

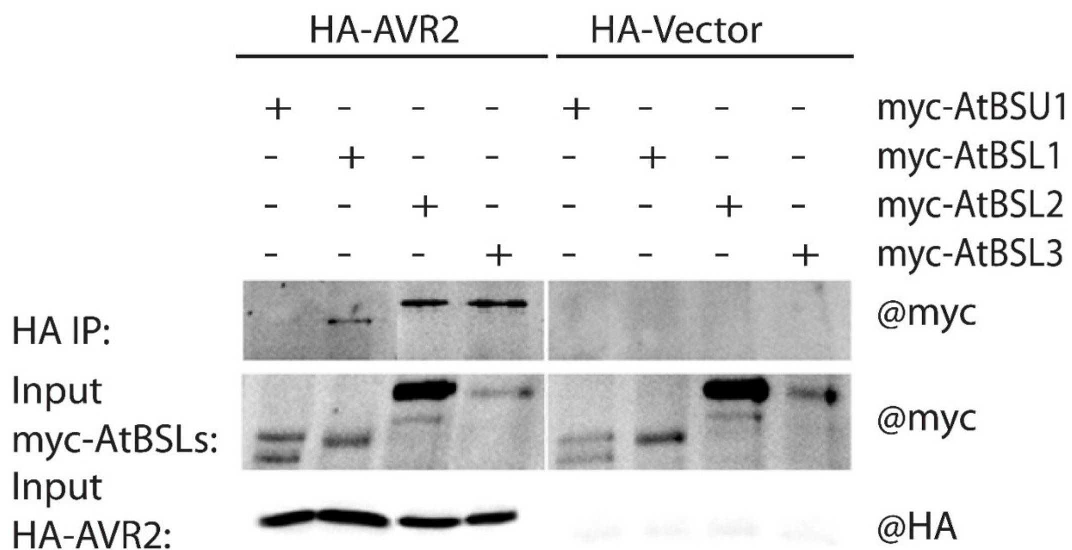


Figure 6 **Co-Immunoprecipitation of myc-tagged *Arabidopsis thaliana* BSL1-3 together with HA-AVR2.** Myc-tagged AtBSL1, 2, 3 and AtBSU1 were transiently co-expressed with HA-tagged AVR2 in Arabidopsis protoplasts. In control experiments HA empty vector was co-expressed together with the myc-tagged AtBSU1-like phosphatases. The immunoprecipitation of HA-AVR2 was performed with anti-HA agarose and the proteins were analyzed in western blot with anti-myc and anti-HA antibody. The upper panel shows the Co-IP results (HA IP), the mid (Input Myc-AtBSLs) and lower (Input HA-AVR2) panels show the expression levels of myc-AtBSLs and HA-AVR2 in Arabidopsis protoplasts extracts (provided by C. Jäntsch).

In an additional experiment, transient co-expression of Arabidopsis myc-tagged BSU1- like phosphatases with HA-AVR2 in *N. benthamiana* showed that AVR2 also associates with AtBSL1-3 but not with AtBSU1 in a heterologous expression system (Figure 7).

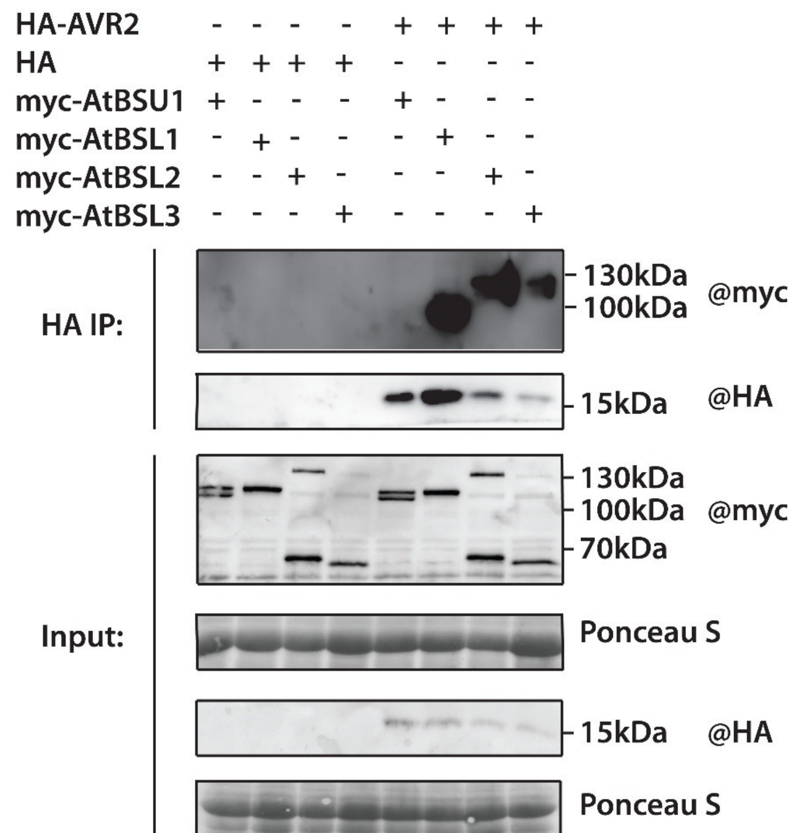


Figure 7 **Co-Immunoprecipitation of myc-tagged *Arabidopsis thaliana* BSL1, 2 and 3 together with HA-AVR2 in *N. benthamiana*.** HA-AVR2 was co-expressed together with myc-tagged AtBSU1 and AtBSL1-3 in *N. benthamiana* leaves. HA empty vector was used in control experiments. The Co-Immunoprecipitation was performed with anti-HA agarose and the proteins were analyzed by western blot with anti-HA and anti-myc antibody. The upper panel shows the Co-IP experiment. The mid and lower panels show expression levels of myc-AtBSLs and HA-AVR2 in *N. benthamiana* extracts.

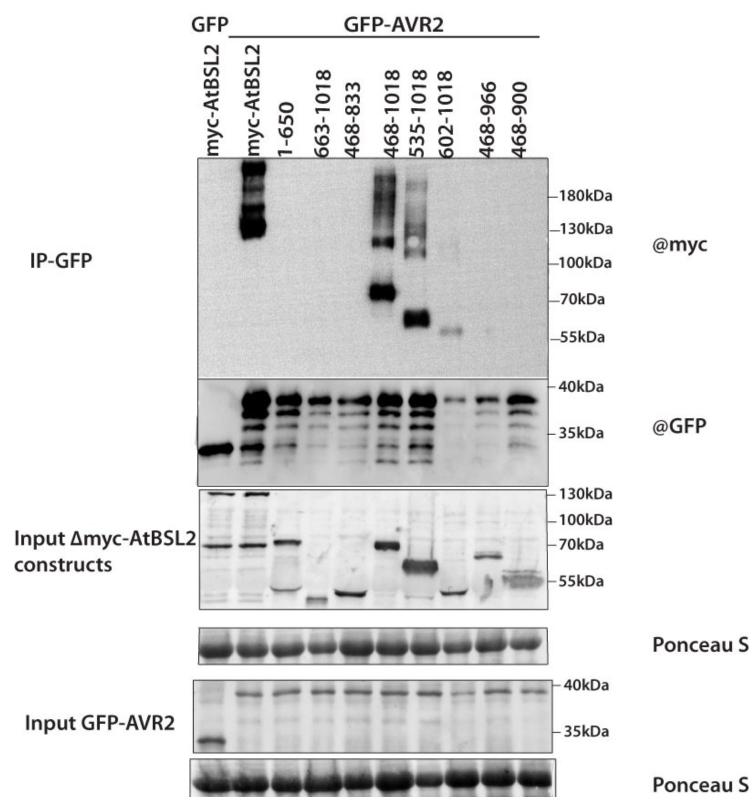
Altogether, the interaction studies performed with the potato or Arabidopsis BSLs and AVR2 suggest the existence of a high level of association specificity. Although AtBSU1 is homolog to the other BSLs (BSU1-BSL1 share 53% sequence identity and BSU1-BSL2/3 43% sequence identity at the amino acid level), it does not associate with AVR2, which could reflect a phenomenon of non-evolved adaptation to a protein that is absent in the natural host plants (potato, *N. benthamiana*) of *P. infestans*.

3.2 Identification of the molecular determinants of the AVR2-AtBSL2 interaction

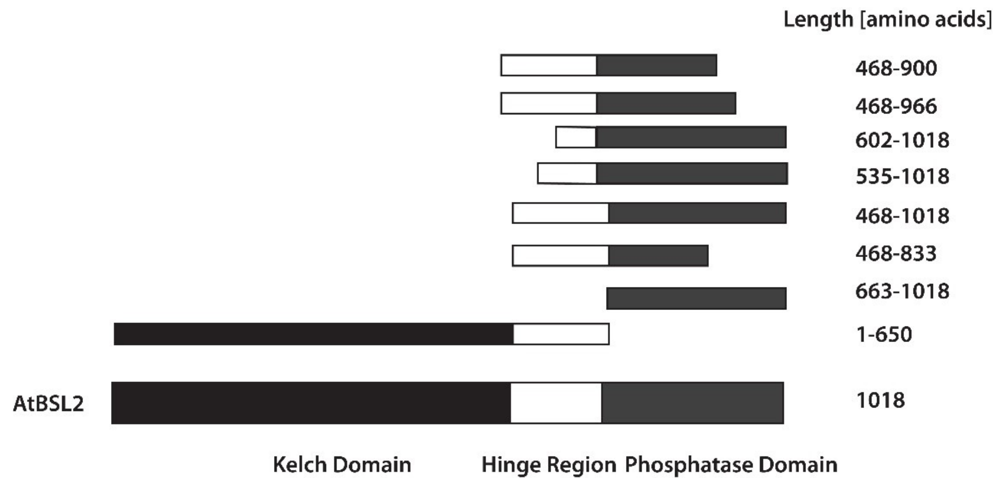
3.2.1 The hinge region and the putative phosphatase domain of AtBSL2 are required for the interaction with AVR2

To determine the critical region of the BSLs required for the interaction with AVR2, we have cloned several deletion constructs in the amino acid sequence of AtBSL2, chosen as a representative member of the BSU1-like phosphatases. We have cloned the region upstream of the putative phosphatase domain, which corresponds to the Kelch domain and hinge region (1-650 aa), sole putative phosphatase domain (663-1018 aa) and several deletion constructs in the hinge region and phosphatase domain (468-833 aa, 468-1018 aa, 535-1018 aa, 602-1018 aa, 602-1018 aa, 468-966 aa, 468-900 aa) (Figure 5B). All cloned fragments have been fused to myc-tag at the N-terminus and subsequently co-expressed together with GFP-AVR2 in *N. benthamiana*. Using Co-IPs it has been demonstrated that two motifs comprising the amino acid residues 602-663 in the hinge region and amino acid residues 901-1018 at the C-termini of the phosphatase domain are required for the interaction with AVR2 *in planta* (Figure 8 A, C).

A:



B:



C:

602 ... 663

IRLHHRAVVVAAETGGALGGMVRQLSIDQFENEGRRVSYGTPESATAARKLLDRQMSINSVPKKVIAHLL

KPRGWKPPVRRQFFLDCNEIADLCDSAERIFASEPTVLQLKAPIKIFGDLHGQFGDLMRLFDEYGSPSTAG

DISYIDYLFLGDYVDRGQHSLETISLLLALKVEYQHNVHLIRGNHEAADINALFGFRIECIERMGERDGIWV

WHRINRLFNWLPLAASIEKKIICMHGGIGRSINHVEQIENIQRPITMEAGSIVLMDLLWSDPTENDSVEGL

901

RPNARGPGLVTFPDRVMEFCNNNDLQLIVRAHECVMDGFERFAQGHLITLFSATNYCGTANNAGAILVL

GRDLVVVPKLIHPLPPALSSPETSPERHIEDTWMQELNANRPATPTRGRPQNSNDRGGSLAWM

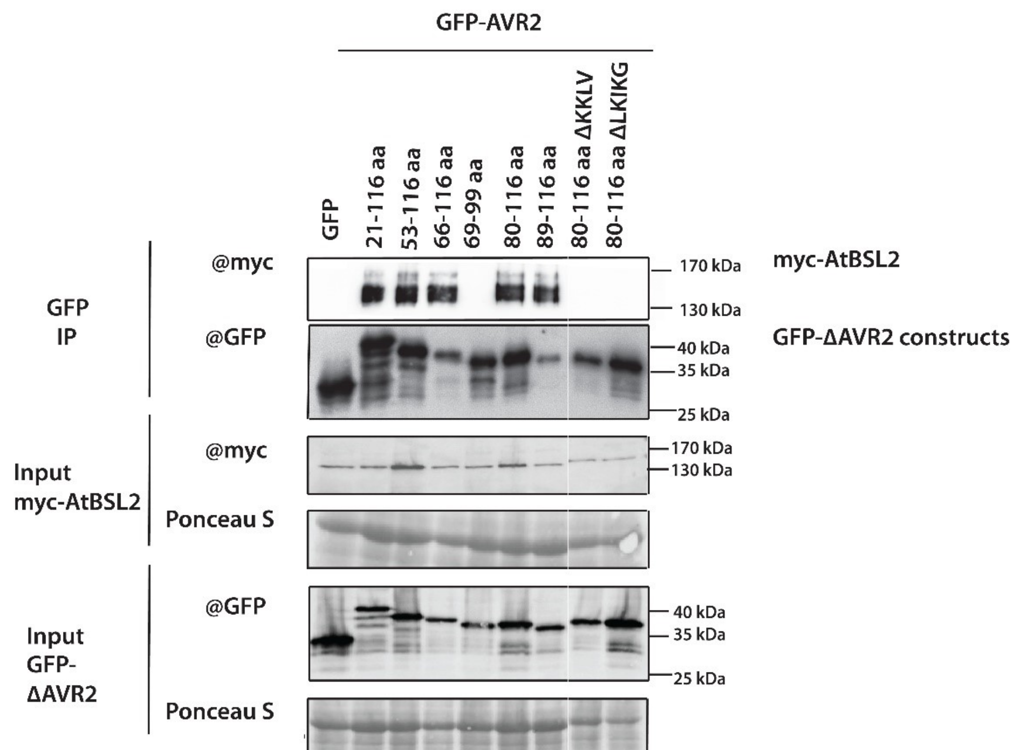
1018

Figure 8 **Two distinct domains located in the hinge region and phosphatase domain of AtBSL2 are required for association with AVR2 in vivo. (A)** Myc-tagged AtBSL2 deletion mutants were co-expressed with GFP-tagged AVR2 in *N. benthamiana* leaves. In control experiments, GFP or GFP-AVR2 were co-expressed with myc-tagged BSL2. Co-Immunoprecipitation was performed with anti-GFP agarose and the proteins were analyzed in western blot with anti-myc and anti-GFP antibody. The upper panel (IP-GFP) shows the Co-IP results. The lower panel (Input) shows the expression levels of myc-tagged AtBSL2 deletion constructs and GFP-AVR2. **(B)** Scheme illustrating the regions of AtBSL2 used to evaluate the interaction with AVR2. Numbers indicate the amino acid positions based on the full-length sequence protein. **(C)** The two amino acid sequences within AtBSL2 (only amino acid residues 602-1018 are presented) that are necessary for the interaction with AVR2 are highlighted in red (sequence within the hinge domain) and blue (sequence within the phosphatase domain).

3.2.2 The C-terminus of the effector domain of AVR2 is critical for the association with AtBSL2

A similar approach as mentioned above was applied to investigate which domain of AVR2 is critical for the interaction with AtBSL2. Several constructs encoding for diverse deletions in the amino acid sequence of AVR2 were cloned and subsequently co-expressed with myc-tagged AtBSL2 in *N. benthamiana* (Figure 9A). The mutated version of AVR2 included N- and/or C-terminal deletions and short amino acid sequence deletions in the C-terminal part of the effector domain (Figure 9B). All cloned fragments were fused to GFP at the N terminus. The co-immunoprecipitation experiments revealed that the conserved RXLR-EER motif is not required for an association of AVR2 with AtBSL2. Our studies revealed that the interaction takes place in the effector domain. The last 17 amino acids at the C terminus of AVR2 are necessary for the interaction with AtBSL2 (Figure 6, see construct 69-99 aa and 89-116 aa). This region contains KKLV (102-105 aa) and LKIKG (108-112 aa) motifs that are known to be present in phosphatase inhibitors (Nadine Wagener, personal communication). A deletion of these amino acid motifs (80-116 aa ΔKKLV and 80-116 aa ΔLKIKG) abolished the interaction of AVR2 with AtBSL2 (Figure 9A).

A:



B:

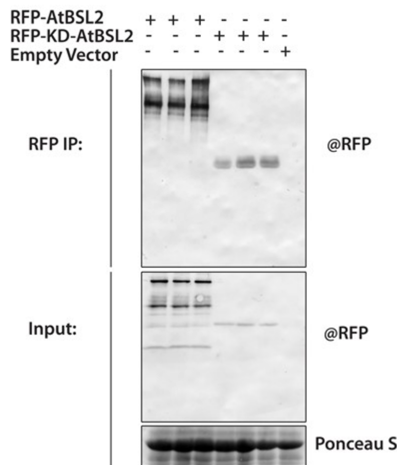
Figure 9 **The 17 amino acids at the C terminus of Avr2 are required for the association with AtBSL2 in vivo. (A)** GFP-tagged AVR2 deletion mutants were co-expressed with myc-tagged AtBSL2 in *N. benthamiana* leaves. In control experiments GFP was co-expressed with myc -tagged AtBSL2. Co-Immunoprecipitation was performed with anti-GFP agarose and the proteins were analyzed in western blot with anti-myc and anti-GFP antibodies. The upper panel (GFP IP) shows the Co-IP results. The lower panel (input) shows the expression levels of GFP-AVR2 deletion mutants and myc-AtBSL2. **(B)** Scheme illustrating the regions of AVR2 used to evaluate the interaction with AtBSL2. Numbers indicate the amino acid positions based on the full-length sequence protein.

3.3 Effect of AVR2 on the phosphatase activity of AtBSU1-like family members

3.3.1 In vivo phosphatase assay

Mora-Garcia et al., (2004) and Kim et al., (2009) have shown that AtBSU1 displays phosphatase activity *in vitro*. The BSU1-like phosphatases display highest homology with the PP1 and PP2a phosphatase families. Using commercially available assays, we next aimed to determine whether BSU1-like family members are functional protein phosphatases. Full length AtBSL2 and the Kelch domain of AtBSL2 (KD-AtBSL2) fused to RFP at the N terminus were expressed in *N. benthamiana* (Figure 10A) and the catalytic activity of immunoprecipitated material was examined. In one of these assays, dephosphorylation of the peptide substrate followed by proteolysis produces a fluorescent signal. Our assay, has revealed an increase of the fluorescence signal, assimilated with an increase of phosphatase activity, only with the full length RFP-AtBSL2, whereas the deletion mutant lacking the phosphatase domain (RFP-KD-AtBSL2), the empty vector and buffer controls, displayed similar level of fluorescence (Figure 10B).

A: **B:**



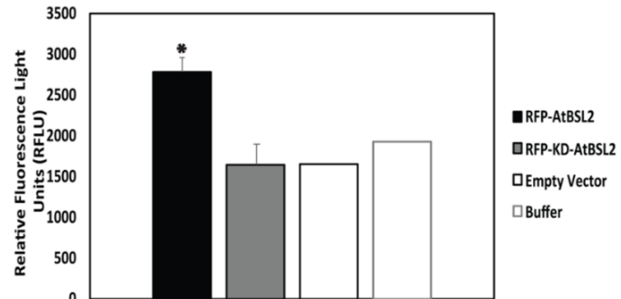


Figure 10 **AtBSL2 is a functional phosphatase in *N. benthamiana*.** **(A)** RFP-tagged AtBSL2 and KD-AtBSL2 were transiently expressed in *N. benthamiana* leaves. Empty vector was used as a control. Immunoprecipitation was performed with anti-RFP agarose and the proteins were analyzed in western blot with anti-RFP antibody. The upper panel (RFP IP) shows the results of the Immunoprecipitation and the lower panel (Input) shows the expression levels of RFP-AtBSL2, RFP-KD-AtBSL2 and empty vector. **(B)** Bar diagram showing phosphatase activity of AtBSL2 and KD-AtBSL2 measured in relative fluorescence light units (RFLU). The phosphatase assay was performed using (ProFluor Ser/Thr PPase Assay, Promega). RFP and buffer without any immunoprecipitated material were used in control experiments. Results are mean values ± SEM from three biological replicates. *denote statistical significance ($p<0.05$) in one-way ANOVA.

3.3.2 The catalytic activity of AtBSU1-like family members is BL independent

Genetic studies have shown that AtBSU1 and AtBSL1 are positive regulators of brassinosteroid-mediated responses, notably the inhibition of the GSK3-like kinase BIN2 through dephosphorylation has been intensively studied (Kim et al., 2009). Therefore, we decided first to investigate whether the phosphatase activity of AtBSU1 and AtBSL2 is regulated by BL. AtBSU1 was included in this experiment as an additional control because it does not associate with AVR2 and therefore, it is very unlikely that its enzymatic activity is modulated by AVR2. We expressed RFP-tagged AtBSU1 and AtBSL2 in *N. benthamiana* and after 48 h, leaves were infiltrated with 10nM and 1µM BL and collected at 0, 15 and 60 min for protein extraction, immunoprecipitation and phosphatase assay (Figure 11). This assay revealed that AtBSU1 as well as AtBSL2 phosphatase activity does not appear to be post-translationally regulated by BL.

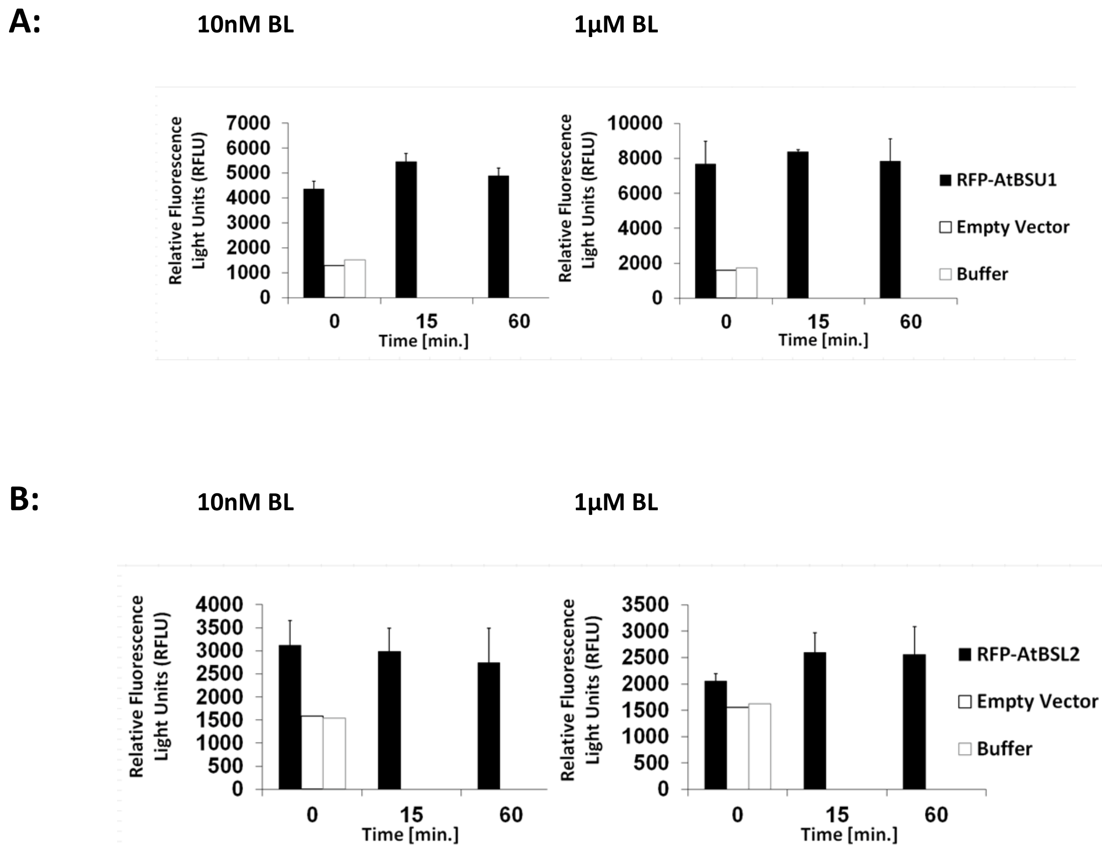


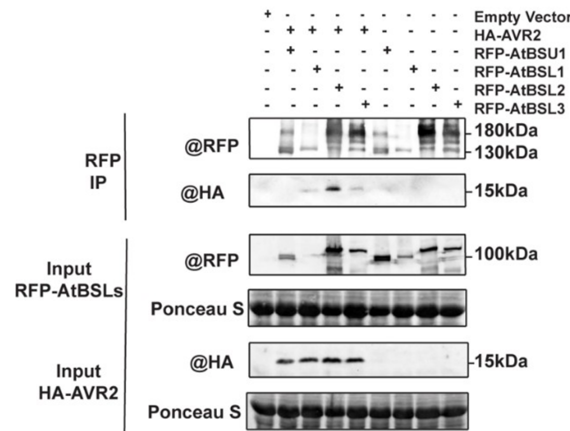
Figure 11 **BL treatment has no effect on the phosphatase activity of the AtBSU1-like family members. (A)** Bar diagram showing phosphatase activity of AtBSU1 measured in relative fluorescence light units (RFLU) upon treatment with 10nM or 1µM BL for 0, 15 and 60 min. RFP-tagged AtBSU1 was expressed and pulled-down from *N. benthamiana* leaf extract and the phosphatase activity has been measured using Ser/Thr Phosphatase Assay (Promega). Results are mean values ± SEM from three biological replicates. No statistical difference has been observed in one-way ANOVA ($p < 0.05$). **(B)** Bar diagram showing phosphatase activity of AtBSL2 measured in relative fluorescence light units (RFLU) upon treatment with 10nM or 1µM BL for 0, 15 and 60 min. RFP-tagged AtBSL2 was expressed and pulled-down from *N. benthamiana* leaf extract and the phosphatase activity has been measured using Ser/Thr Phosphatase Assay (Promega). Results are mean values ± SEM from three biological replicates. No statistical difference has been observed in one-way ANOVA ($p < 0.05$).

3.3.3 AVR2 does not affect the phosphatase activity of AtBSU1-like family members

Since the phosphatase domain of AtBSL2 has been shown to be involved in the association with AVR2, we examined whether the enzymatic activity of the AtBSLs was affected. All four members of the Arabidopsis BSU1-like family were transiently expressed in *N. benthamiana* in presence and absence of HA-AVR2 (Figure 12A). Western blot analysis confirmed the interaction between HA-AVR2 and RFP-tagged AtBSL1-3 but not AtBSU1 (Figure 12A). Further, the Co-Immunoprecipitated material was tested for phosphatase activity and we did

not observe any difference of the catalytic activity of the AtBSLs in the presence/absence of HA-AVR2 (Figure 12B). These results suggest that AVR2 association with AtBSL1-3, though involving the C-terminal part of the phosphatase domain, does not affect their intrinsic enzymatic activity.

A:



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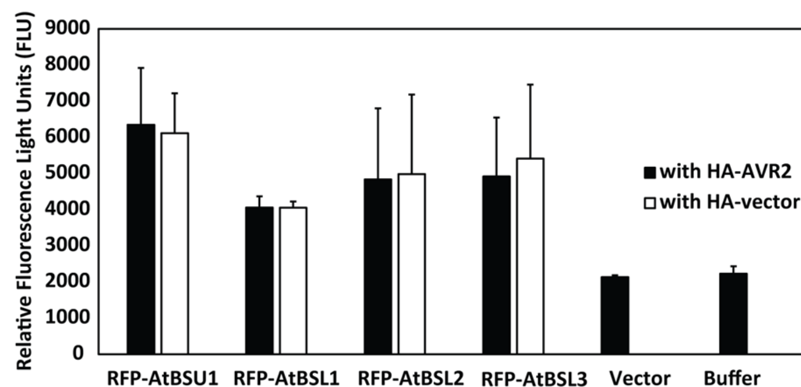


Figure 12 **Phosphatase activity of AtBSU1-like family members in association with AVR2. (A)** RFP-tagged AtBSL1, 2, 3 and AtBSU1 were transiently co-expressed with HA-tagged AVR2 in *N. benthamiana*. In control experiments, RFP-tagged BSU1-like phosphatases were co-expressed with HA empty vector. The co-immunoprecipitation was performed with anti-HA agarose and the proteins were analyzed in western blot with anti-RFP and anti-HA antibody. The upper panel shows the Co-IP results and the lower panel shows the expression levels of RFP-AtBSLs and HA-AVR2. **(B)** Bar diagram showing phosphatase activity of AtBSU1-like family members with/without HA-AVR2 measured in relative fluorescence light units (RFLU). The phosphatase assay was performed using Ser/Thr Phosphatase Assay (Promega). RFP and buffer without any immunoprecipitated material were used in control experiments. Results are mean values ± SEM from two biological replicates. No statistical differences have been seen in one-way ANOVA ($p < 0.05$).

3.4 *Effect of AVR2 on BL-dependent responses in N. benthamiana*

3.4.1 *AVR2 does not affect BL-dependent phosphorylation status of AtBES1 expressed in N. benthamiana*

AtBES1 (AtBZR2) and its close homolog AtBZR1 are two key transcription factors in the regulation of BL-dependent gene expression in Arabidopsis, whose activation is controlled by AtBSU1 and AtBSL1 (Mora-Garcia et al., 2004, Kim et al., 2009). In order to get insight whether and to what extent AVR2-BSLs interaction affects the brassinosteroid pathway, AtBES1-GFP was co-expressed in presence of GFP-AVR2 in *N. benth*amiana leaves (Figure 13). The samples have been collected at 0 and 3 h upon treatment with 1µM BL and total protein extracts have been submitted to SDS-PAGE gel electrophoresis followed by western blotting prior immunodetection with anti-GFP antibody. As already described in Arabidopsis (Yin et al., 2002), BL treatment induced a shift of the apparent molecular mass of AtBES1-GFP from a phosphorylated (BES1-P-GFP) into a de-phosphorylated (BES1-GFP) form (Figure 13). This result indicates that BR signaling is functional in *N. benthamiana*. In this experiment, we also tested whether the presence of GFP-AVR2 does affect AtBES1-GFP phosphorylation status, but we did not detect a significant difference in response to BL treatment in comparison to the GFP control. A possible explanation is that the BL-dependent de-phosphorylation of AtBES1 is not regulated by the endogenous NbBSLs or that AVR2 does not associate with the NbBSLs, although we have observed an interaction of AVR2 with both Arabidopsis and potato BSLs.

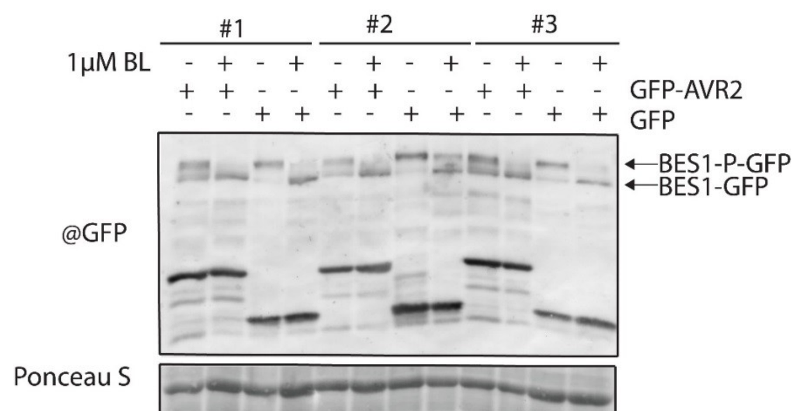


Figure 13 **Phosphorylation status of AtBES1-GFP in presence of GFP-AVR2 in *N. benthamiana*.** AtBES1-GFP was transiently co-expressed with GFP-AVR2 or GFP in *N. benth*amiana leaves. The leaf material was collected at 0 and 3h after treatment with 1µM BL. The total protein extracts have been separated by SDS-PAGE and AtBES1-GFP phosphorylation status has been analyzed by western blot using anti-GFP antibody. Three biological replicates (#1-3) were used in the experiment.

3.4.2 AVR2 does affect the expression level of BL-regulated genes in N. benthamiana.

In contrast to Arabidopsis, the BR signaling pathway and BR-induced responses have been poorly investigated in *Solanaceae.* Based on the analysis of the transcriptome, several candidate marker genes conserved in Arabidopsis, *N. benthamiana* and potato were differentially expressed in potato treated with BL (Paul Birch, UoD/JHI, Dundee, UK, personal communication). Among them, were genes of the BR biosynthetic pathway and genes involved in the control of cell expansion and growth. The expression of *N. benthamiana* homologues of the *Sterol reductase (DWF5), Steroid dehydrogenase (SteroidDH), Xyloglucanendotransglucosylase / hydrolase protein 2 (XTH2)* and *CRYPTOCHROME-INTERACTING BASIC HELIX LOOP HELIX 1 (CIB1/bHlH63)* have been investigated in qRT-PCR analysis upon treatment with BL and in the presence of GFP-AVR2 or GFP as a control. Leaf material was collected at 0, 12 and 24h upon treatment with 1µM BL. In control plants expressing GFP, the expression of *DWF5* and *SteroidDH* is downregulated by BL (Figure 14 A, B), similar to what has been observed in Arabidopsis (Gooda et al., 2002). When GFP-AVR2 is present, the expression of these genes is slightly induced (Figure 14 A, B).

Regarding the expression of genes involved in cell expansion/growth, the effect of GFP-AVR2 was less pronounced. The expression of *XTH2* is induced in both GFP-AVR2 plants and in plants transiently expressing GFP, although to a lesser extent in presence of GFP-AVR2 (Figure 14 C). CIB1 belongs to bHlH transcription factor family, which is induced upon BR treatment and shown to negatively regulate PTI/MTI in Arabidopsis (Malinovsky et al., 2014). In the presence of AVR2, the expression of *CIB1* was down regulated 24h after BL treatment when compared to the GFP control (Figure 14D). Altogether, the expression analysis suggests that AVR2 has an inhibiting effect on BR-regulated gene expression in *N. benthamiana*.

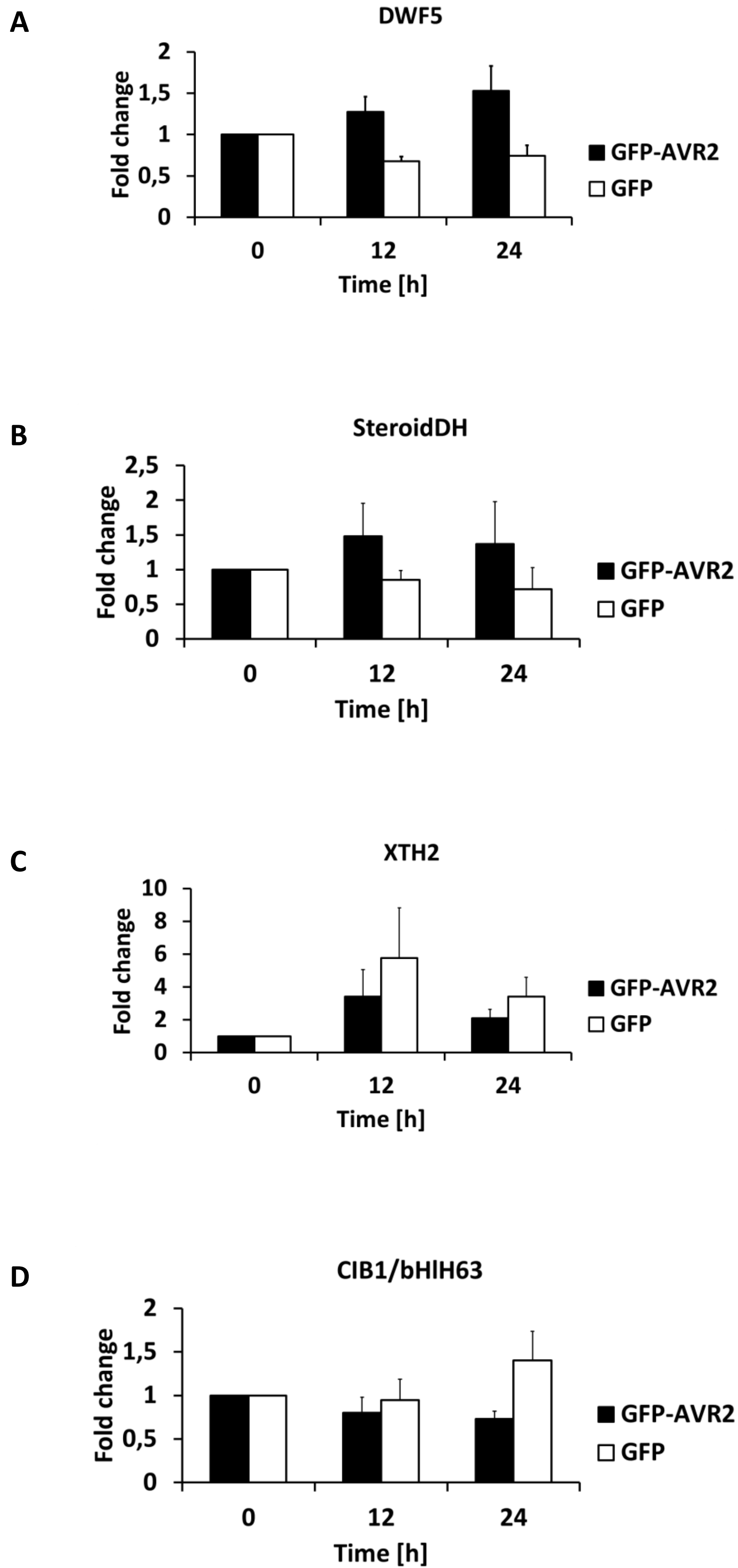

A
DWF5
2
1,5
1
0,5
0
Fold change
0
12
24
Time [h]
GFP-AVR2
GFP
B
SteroidDH
2,5
2
1,5
1
0,5
0
Fold change
0
12
24
Time [h]
GFP-AVR2
GFP
C
XTH2
10
8
6
4
2
0
Fold change
0
12
24
Time [h]
GFP-AVR2
GFP
D
CIB1/bHlH63
2
1,5
1
0,5
0
Fold change
0
12
24
Time [h]
GFP-AVR2
GFP

Figure 14 **QRT-PCR analysis showing that AVR2 affects the expression of BR responsive genes in *N. benthamiana*.** GFP-tagged Avr2 was transiently expressed in *N. benthamiana* leaves. Leaf material was treated with 1µM BL and collected at 0, 12 and 24 h after treatment. Plants expressing GFP were used in control experiments. QRT-PCR data are expressed as fold change (normalization with actin). (**A)** *sterol reductase* (*DWF5*), (**B)** *Steroid dehydrogenase* (*SteroidDH*), (**C*)*** *Xyloglucanendotransglucosylase/hydrolase protein 2* (*XTH2*), (**D)** *CRYPTOCHROME-INTERACTING BASIC HELIX LOOP HELIX 1 CIB1/bHlH63.*

3.5 Effect of AVR2 on BL-dependent responses in A. thaliana

3.5.1 AVR2 expressing Arabidopsis plants display characteristic BR mutant phenotypes

The phylogenetic analysis and interaction studies between the BSLs and GFP-AVR2 in *N. benthamiana* and Arabidopsis protoplasts strongly suggested that the interaction specificities and molecular determinants involved in the association between the effector and the BSL1-3 phosphatases are conserved between the two plant species. This observation prompted us to further investigate the putative function of AVR2 on BR signaling pathway and BR-regulated responses using transgenic *Arabidopsis thaliana* plants expressing constitutively GFP-AVR2. We have obtained phenotypes ranging from severe dwarf to semi-dwarf plants having small and thick leaves with shorter stems and short inflorescence without viable seeds (Figure 15). These characteristics were previously described in BR-insensitive and BR-deficient mutants including *constitutive photomorphogenesis and dwarfism1* (*cpd1)* (Ohnishi et al., 2012), *deetiolated2* (*det2)* (Clouse et al., 1998), *dwarf4* (*dwf4)* (Choe et al., 1998) and *brassinosteroid insensitive1* (*bri1)* (Noguchi et al., 1999) and would be consistent again with AVR2 acting as a negative regulator of BR signaling.

Figure 15 **Phenotype of GFP-AVR2 expressing *A. thaliana plants*.** Pictures represent the phenotype of two independent Arabidopsis transformants (#1 and #2) expressing GFP-AVR2 under the control of the 35S promoter grown in permanent light for 8 weeks. GFP-AVR2 overexpressing plants display severe dwarfism, curly leaves, short stems and reduced fertility when compared to control empty vector (pGreen_0229) plants (provided by C. Jäntsch).

3.5.2 AVR2 affects stomata development and patterning

The leaf structure of the GFP-AVR2 expressing lines was analyzed with differential interference contrast (DIC) microscopy. The leaves of these lines exhibited many clustered stomata with numerous satellite meristemoids (stomata precursor cells) adjacent to guard cells (Figure 16), which is an indication that the orientation of cell division in the stomatal lineage is defective in these lines. The abnormal stomata phenotype of the GFP-AVR2 expressing plants resembles the phenotype previously described in some of gain- and loss-of-function BR mutants (Kim et al., 2012, Gudesblat et al., 2012).

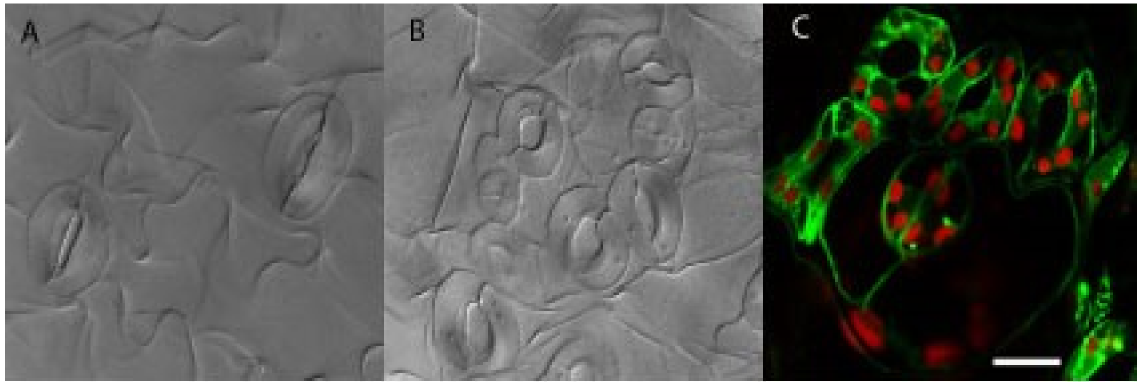


Figure 16 **GFP-AVR2 expressing Arabidopsis plants show abnormal stomata patterning**. Microscopic analysis of **A:** pGreen 0229 Differential-Interference-Contrast (DIC) microscopy, **B:** GFP-AVR2 Differential-Interference-Contrast (DIC) microscopy **C:**Confocal images of GFP-AVR2 lines, 10 µm scale (provided by C. Jäntsch).

Notably, *the brassinosteroid-deficient* (*det2-1*) and brassinosteroid-signaling mutants such as the BRI1 receptor mutant *(bri1-116*), *a quadruple mutant for BSU1-like phosphatases* (*bsu-q*) (Kim et al., 2009), a constitutive active BIN2 mutant (*bin2-1*) as well as BIN2 overexpressing plants (*BIN2-ox*) have abnormal stomata patterning (Kim et al., 2012), phenocopying GFP-AVR2 expressing plants. The authors' conclusion was that BRs negatively regulate stomata development and patterning with the BSU1-like phosphatases having a negative effect, while the BIN2 kinase has a positive effect, independent of BZR1, which again is in line with an inhibition of BSU1-like phosphatases by AVR2.

3.5.3 AVR2 modulates the protein expression level of AtGSK3-like kinases

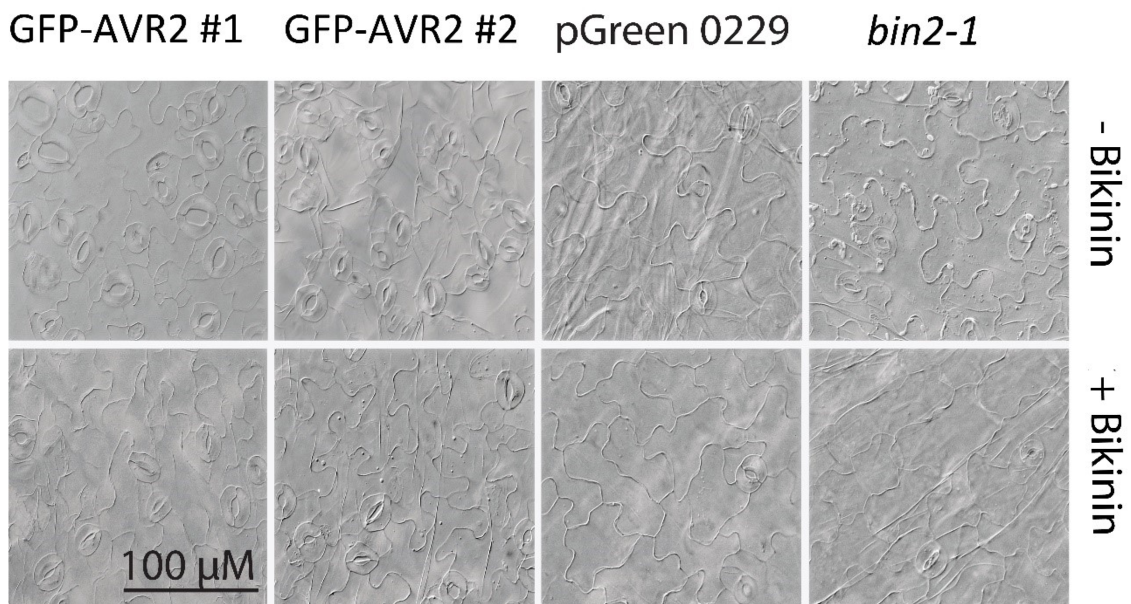
Since the observed stomata clustering in GFP-AVR2 expressing Arabidopsis plants could correlate eventually to an increase of the GSK3-like kinase activity, we have grown GFP-AVR2 expressing Arabidopsis plants on MS agar plates containing 30 µM bikinin, a compound that was previously described as an inhibitor of GSK3-like kinases such as BIN2 and that could partially revert the stomata phenotype in *bin2-1* mutant (Kim et al., 2012). GFP-AVR2 expressing seedlings from two independent lines, GFP-AVR2 #1 and GFP-AVR2#2, showed reduced stomata density when treated with bikinin, similar to the *Bin2-1* mutant or pGreen_0229 control plants (Figure 17A, B).

To further investigate the effect of AVR2 on the GSK3-like kinases, we transiently co-expressed GFP-AVR2 with all 10 members of the Arabidopsis GSK3-like kinase family (kindly provided by C. Jonak, GMI Vienna, Austria) in *N. benthamiana*. The resulting immunoblot

showed that GFP-AVR2 influences the protein expression level of three AtGSK3-like kinases (Figure 18). Myc-ASKδ and Myc-ASKI accumulate whereas the amount of Myc-ASKζ decreases (Figure 18). Unfortunately, this experiment was not conclusive about an effect of AVR2 on the BIN2 (or BIN2-1) level since it was unclear whether these GSKs were expressed at all. The same applies to ASKε (Figure 18).

Based on these results, we propose that AVR2 through its interaction with the BSLs is regulating the interaction with at least some members of the Arabidopsis GSK3-like kinases, resulting in the activation of stomata development and patterning. AVR2 is behaving as an inhibitor of the function of the BSLs, although the molecular mechanisms underlying its mode of action regarding the manipulation of the interaction specificities between the BSLs and GSK3-like kinase in Arabidopsis remains to be elucidated.

A:



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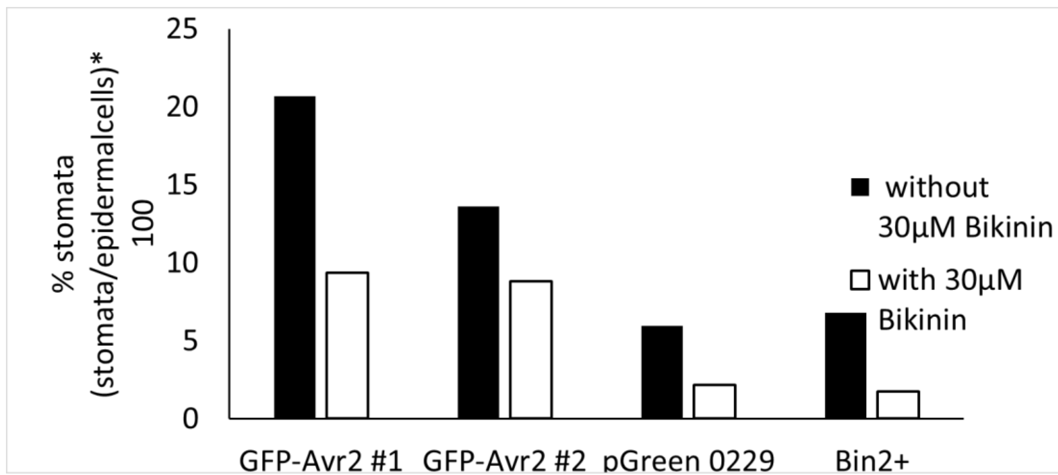


Figure 17 **The stomata phenotype in GFP-AVR2 expressing plants is partially reversible upon bikinin treatment. (A)** Differential interference contrast (DIC) microscopy images of 2 week old GFP-AVR2 seedlings grown on ½ MS medium with and without 30µM bikinin. Empty vector pGreen_0229 line and *bin2-1* mutant expressing a constitutive active BIN2 have been used as controls. **(B)** Stomata count was expressed as % of total epidermal cells counted per 200*200 µm.

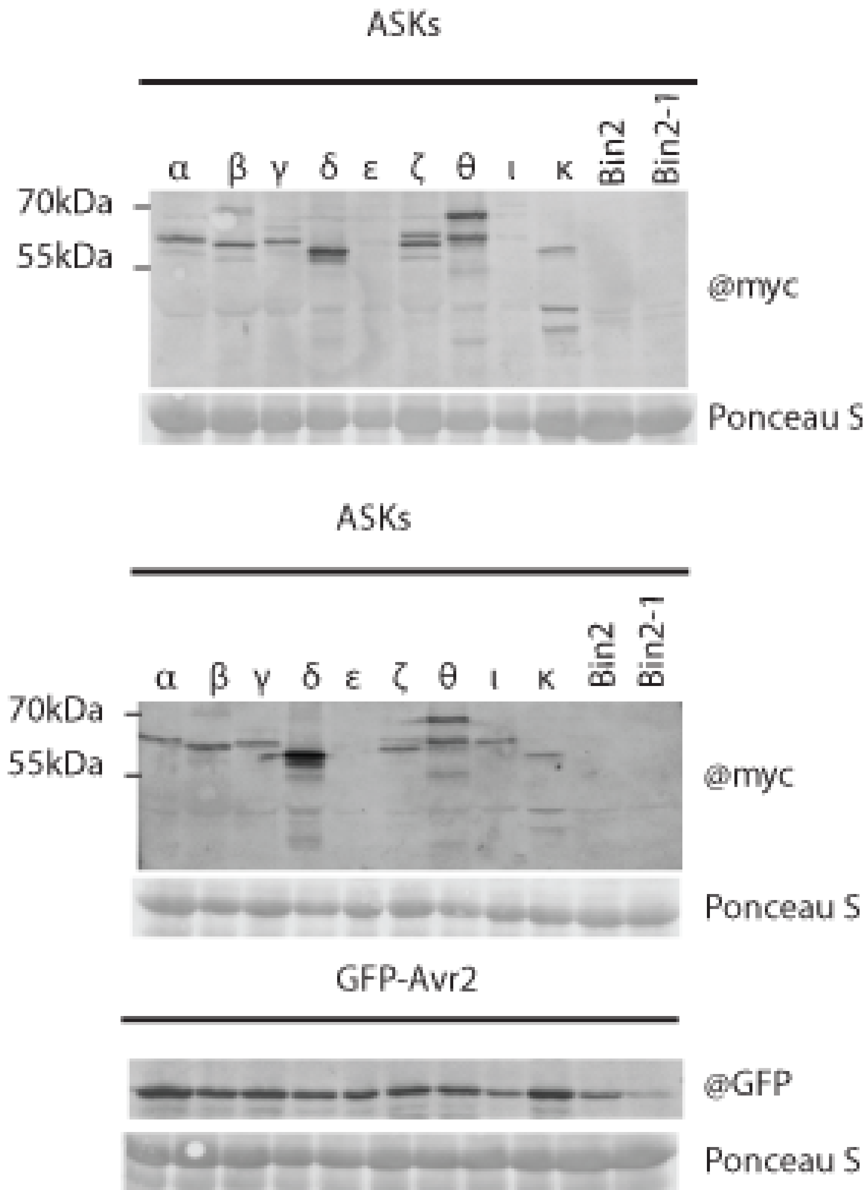


Figure 18 **Avr2 modulates the protein expression level of ASKδ, ASKI and ASKζ *in planta*.** Western blot showing the expression level of Myc-tagged AtGSKs when co-expressed in presence or absence of GFP-AVR2 in *Nicotiana benthamiana* leaves. The leaf material was collected 2 days after *Agrobacterium tumefaciens* infiltration. The total protein extracts have been separated by SDS-PAGE and the expression myc-GSKs and GFP-AVR2 expression has been analyzed by western blot using anti-myc and anti-GFP antibody.

3.5.4 AVR2 does not affect the phosphorylation status of AtBES1

Kim et al., (2009) showed in a Y2H assay that 6 among 10 AtGSKs interact with AtBZR1 and they might be involved in BR-signaling. The effect of AVR2 on the expression level of some of the GSK3-like kinases prompted us to investigate the phosphorylation status of AtBES1 in 2-week old seedlings of the GFP-AVR2 #1 and GFP-AVR2#2 lines. GFP-AVR2 lines were treated

for 2h with 10µM BL and Col-0 seedlings have been used as a control. Phosphorylation status of endogenous AtBES1 has been determined by immunodetection as described in 3.4.1 using an antibody raised against AtBES1. In untreated GFP-AVR2 lines and the wild type control, both a major phosphorylated (BES1-P) and minor de-phosphorylated (BES1) form of AtBES1 were detected in the absence of BL treatment (Figure 19). In BL-treated seedlings the de-phosphorylated form of AtBES1 has accumulated in both GFP-AVR2 and Col-0 plants (Figure 19). These results confirm previous experiments in *N. benthamiana*, suggesting that the association of GFP-AVR2 with AtBSL1-3 has no influence on the BL-regulated phosphorylation status of AtBES1 and that this transcription factor is probably not involved in stomata biogenesis.

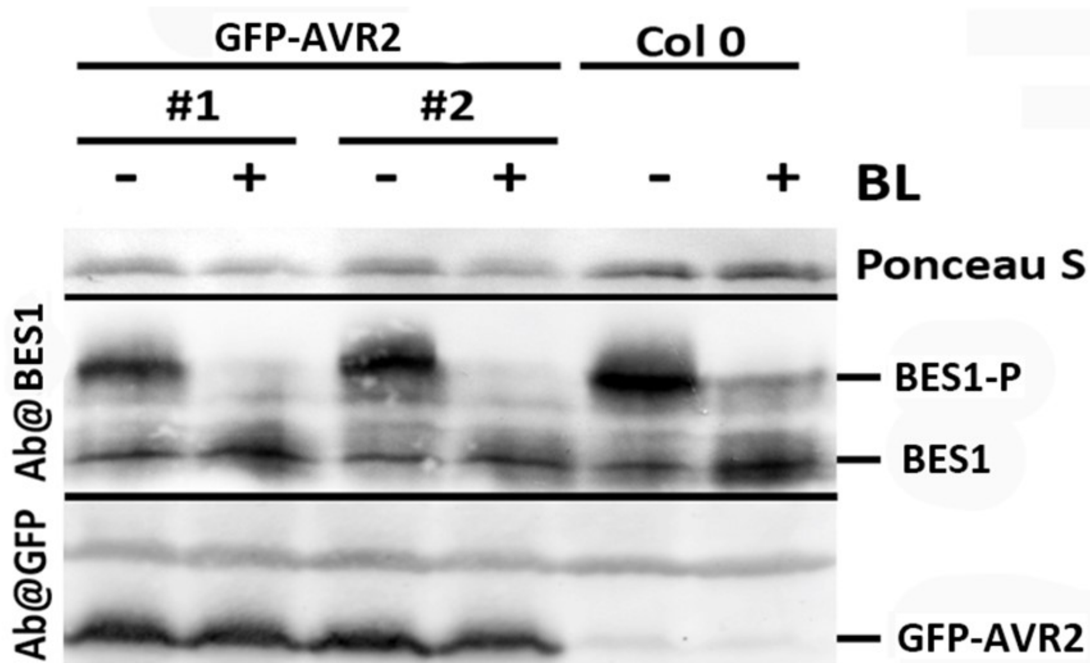


Figure 19 **Phosphorylation status of AtBES1 in GFP-AVR2 expressing Arabidopsis seedlings.** 2 week-old Arabidopsis seedlings were treated with 10µM BL. Leaf material was collected at 0 and 2h after treatment. Protein extract was separated by SDS-PAGE electrophoresis and subsequently analyzed by western blot with anti-BES1 antibody. The expression of GFP-Avr2 was demonstrated with anti-GFP antibody. Wild type Col-0 Arabidopsis seedlings were used as a control (provided by C. Jäntsch).

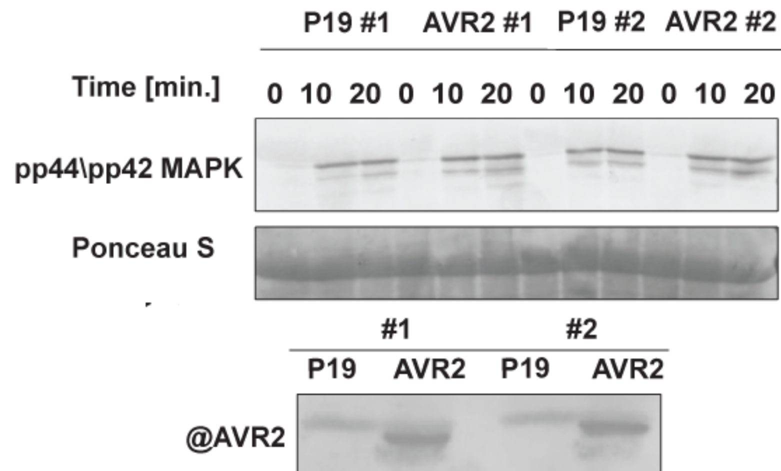
3.6 Effect of AVR2 on MTI/PTI responses in *N. benthamiana*

3.6.1 AVR2 does not suppress flg22-induced early immune responses in *N. benthamiana*

Nearly all the RXLR effector proteins that have been characterized so far block MTI/PTI-responses, which enables successful colonization of the host plant (Stassen and Van den Ackerveken, 2011, Zheng et al., 2014, Anderson et al., 2015). Recently, it has been shown that there is a crosstalk between BR-signaling and plant immune responses in Arabidopsis. BR was shown to negatively regulate MTI/PTI responses. BL pretreatment of Arabidopsis seedlings as well as plants expressing constitutive active BZR1 showed reduced flg22-mediated ROS burst (Albrecht et al., 2011, Lozano-Durán et al., 2013). Since AVR2 interacts with BSU1-like family members involved in BR-signaling, we decided to investigate the impact of AVR2 on plant immune responses in *N. benthamiana*.

For this purpose, we expressed transiently AVR2 in *N. benthamiana* leaves and after elicitation with flg22, a genuine MAMP derived from bacterial flagellin, we measured the post-translational activation of immunity-associated MAP kinases, the induction of the production of Reactive Oxygen Species (ROS) and the expression of MTI/PTI-induced marker genes (Figure 20). No difference in the kinetic and intensity of MAPK activation was observed between plants expressing GFP-AVR2 and the control (Figure 20A). Similarly, AVR2 showed no influence on the flg22-induced ROS burst, comparable to the control (Figure 20B). By contrast, the *P. syringae* effector AvrPto, a well-known suppressor of early MTI/PTI signaling, had a strong suppressing effect (Figure 20B). Several reporter genes for MTI/PTI induction in *N. benthamiana,* including *Pti5* and *Acre31* were previously described (Nguyen et al., 2010). QRT-PCR analysis showed that AVR2 does not influence the expression of *Pti5* and *Acre31* in *N. benthamiana* upon treatment with flg22 (Figure 20C). In parallel to AVR2, empty vector- and AvrPto-expressing plants were used as negative and positive control for the suppression of flg22-induced *Pti5* and *Acre31* expression, respectively (Figure 20C).

A:



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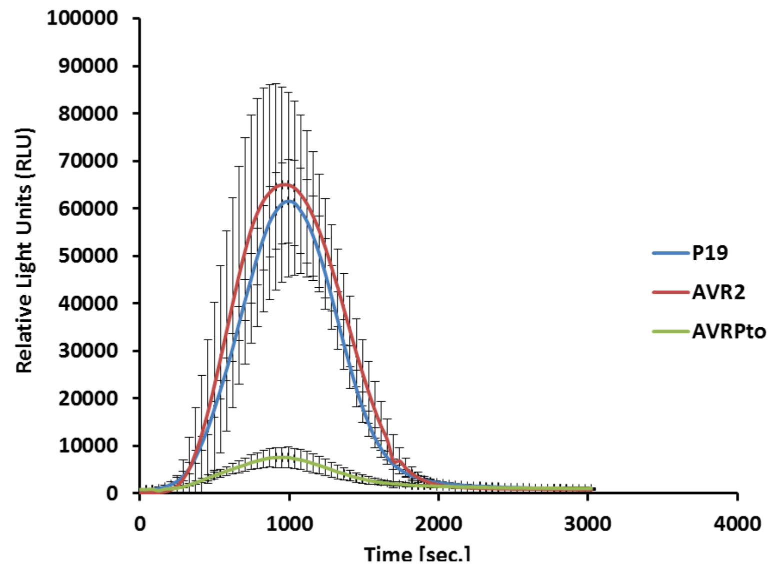

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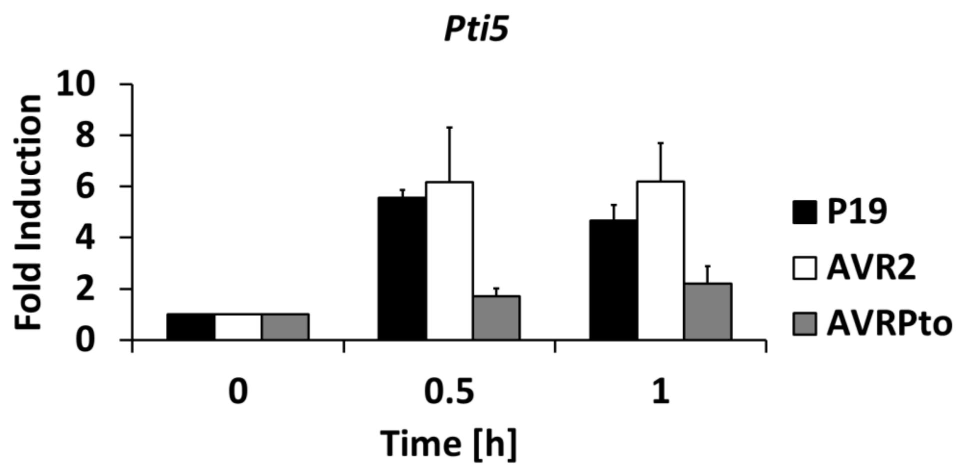

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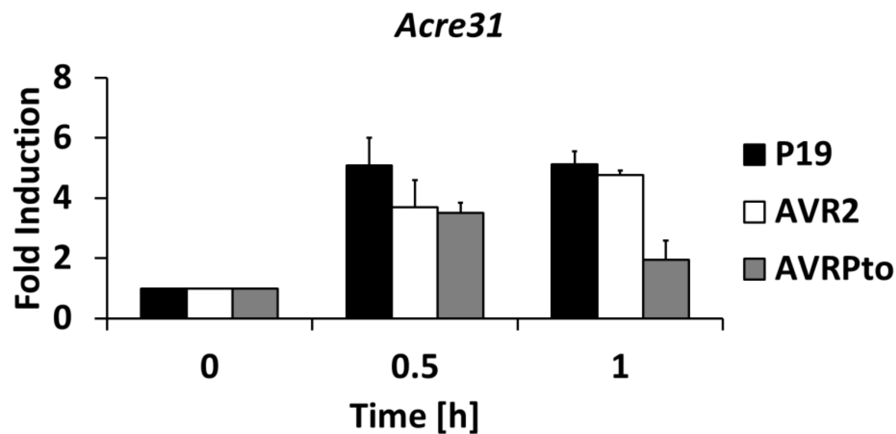


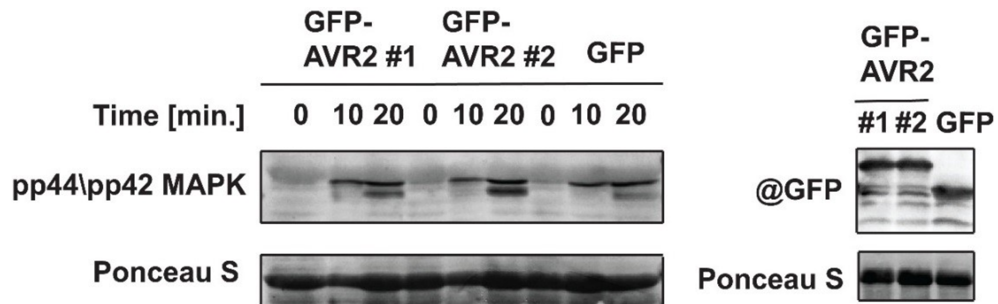
Figure 20 **AVR2 does not suppress flg22-induced MTI/PTI responses in *Nicotiana benthamiana*. (A)** AVR2 and P19 were transiently expressed in *N. benthamiana.* Leaf material was treated with 1µM flg22 and collected at 0, 10 and 20 min after flg22 treatment. Protein extracts were subsequently separated in SDS-PAGE and immunoblotted against anti-pp44/pp42 MAPK antibody. **(B)** Oxidative burst triggered by 100nM flg22 measured in luminol-based assay upon transient expression of AVR2 and empty vector were transiently expressed in *N. benthamiana* leaves. **(C)** AVR2, empty vector and AvrPto were transiently expressed in *N. benthamiana.* Leaf material was treated with 1µM flg22 and collected at 0, 0.5 and 1 h after flg22 treatment. The expression level of *ACRE31* and *PTI5* was analyzed with gene specific primers and normalized to actin.

3.6.2 AVR2 does not suppress β-megaspermin- induced early immune responses in N. benthamiana

Elicitins comprise a family of small, conserved proteins secreted by *Phytophthora* and *Pythium* spp. that induce MTI/PTI in tobacco, often associated with a hypersensitive (HR)-like cell death (reviewed in Kamoun, 2007). Elicitins trigger ethylene production, PR protein accumulation and ROS burst (Derevnina et al., 2016). It was shown that AVR3a, another RXLR effector protein from *P. infestans* suppresses elicitin (INF1)-mediated HR in *N. benthamiana* (Bos et al., 2010, Gilroy et al., 2010). Since flg22 is a bacterial MAMP, and to exclude the possibility that AVR2 is a specific suppressor of early MTI/PTI signaling induced by a MAMP originated from an oomycete, we expressed transiently the effector in *N. benthamiana* and induced early MTI/PTI responses with 100nM β-megaspermin, an elicitin from *Phytophthora megasperma* (Baillieul et al., 1995). However, like with flg22 treatment, we did not observe a difference in the β-megaspermin-induced MAPK activation, ROS and ethylene production between the plants expressing GFP and GFP-AVR2 (Figure 21 A-C).

Altogether, these data suggest that AVR2 does not suppress ubiquitous events of early MTI/PTI signaling in *N. benthamiana*.

A:



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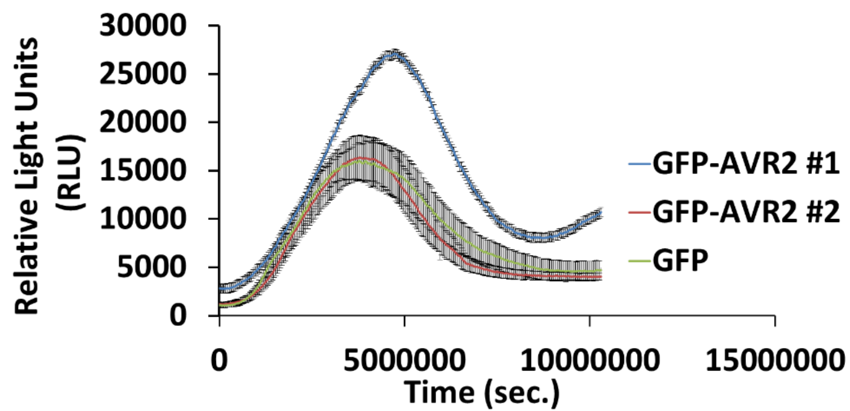

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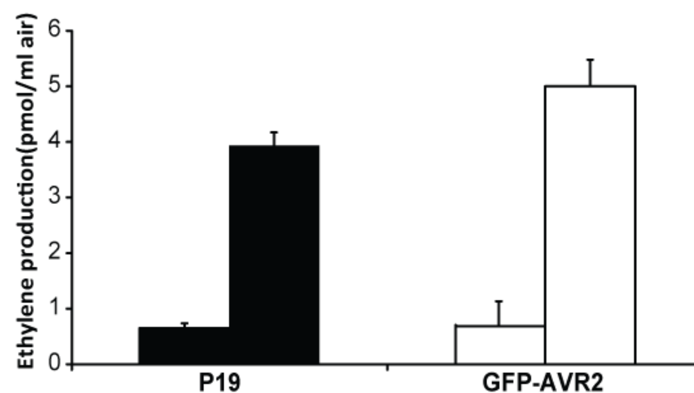

Figure 21 **GFP-AVR2 does not suppress β-megaspermin-induced MTI/PTI-responses in *Nicotiana benthamiana*. (A)** GFP-tagged AVR2 was transiently expressed in *N. benthamiana.* Leaf material was treated with 100nM β-megaspermin and collected at 0, 10 and 20 min after β-megaspermin treatment. Protein extracts were subsequently separated in SDS-PAGE and immunoblotted against anti-p44/p42 MAPK antibody.

Plants expressing GFP were used in control experiment. **(B)** Oxidative burst triggered by 100nM β-megaspermin measured in luminol-based assay upon transient expression of GFP-tagged AVR2 in *N. benthamiana* leaves. Plants expressing GFP were used in control experiments. **(C)** GFP-AVR2 and empty vector (P19) were transiently expressed in *Nicotiana benthamiana* plants, treated with 100nM β-megaspermin for 3 h and tested for ethylene-inducing activity.

3.6.3 AVR2 suppresses INF1-induced cell death in N. benthamiana

We investigated whether GFP-AVR2 can suppress the HR-like cell death triggered by some elicitins. ß-megaspermin revealed to be a poor inducer of cell death in *N. benthamiana* and therefore, we carried out Agrobacterium-mediated co-expression of GFP-AVR2 and INF1, an elicitin from *P. infestans*, and checked for suppression of the HR-like cell death. This assay is routinely used to assess the suppressing activity of RXLR effectors of late-induced MTI/PTI events (Gilroy et al., 2011). GFP-AVR2 significantly suppressed INF1-induced cell death when compared to GFP control (50 vs 85% of necrotic sites) but was not as effective as GFP-AVR3a (<20% necrotic sites), used as a control in this experiment (Figure 22). Importantly, GFP-AVR2 deletion mutants lacking the 18 amino acid at the C-terminus (69-99) or the phosphatase inhibitor motif (80-117 ΔKKLV and 80-117 ΔLKIKG), which did not interact with AtBSL2 (chapter 3.2.2), were also unable to suppress INF1-mediated cell death, providing correlative evidence that the BSLs are involved in the control of this mechanism and that a virulence function of AVR2 consists to interfere with late MTI/PTI responses.

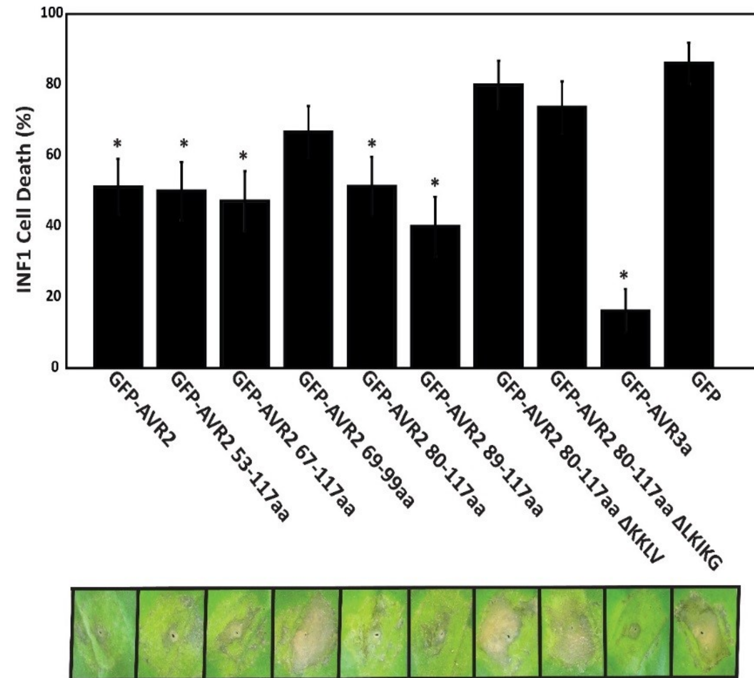

Figure 22 **Effect of deletions in AVR2 effector protein on INF1 induced cell death in *Nicotiana benthamiana*.** The bar diagram shows the percentage of infiltration sites showing clear HR at 7dpi after co-expression of GFP-tagged AVR2 deletion constructs with INF1. GFP-AVR3a or GFP were co-expressed together with INF1 in control experiments. Experiments were repeated 3 times each with no less than 5 plants. Error bars represent SEM. *denote statistical significance ($p<0,01$) in one-way ANOVA. Images represent development of INF1-induced cell death upon co-expression of GFP-AVR2 deletion constructs with INF1.

3.7 Effect of AVR2 on MTI/PTI responses in Arabidopsis thaliana

3.7.1 AVR2 does not suppress flg22-induced early immune responses in Arabidopsis

MAMP-triggered early immune responses were also tested in Arabidopsis transgenic lines expressing GFP-AVR2. MAPK activation, ROS production and *FRK1* expression have been measured in 4 week-old leaves upon induction with 100nM flg22 and, like in *N. benthamiana*, no difference in the activation of these early immune responses was observed between GFP-AVR2 and pGreen_0229 control lines (Figure 23 A-C).

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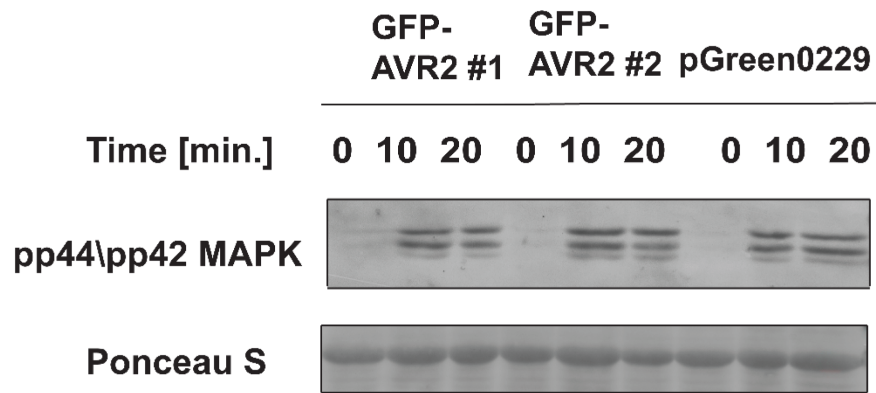

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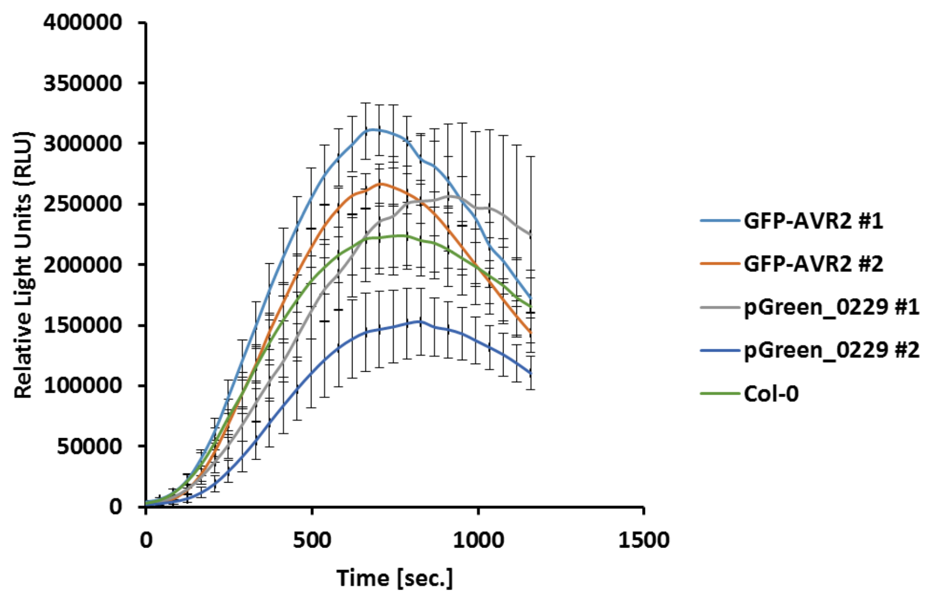

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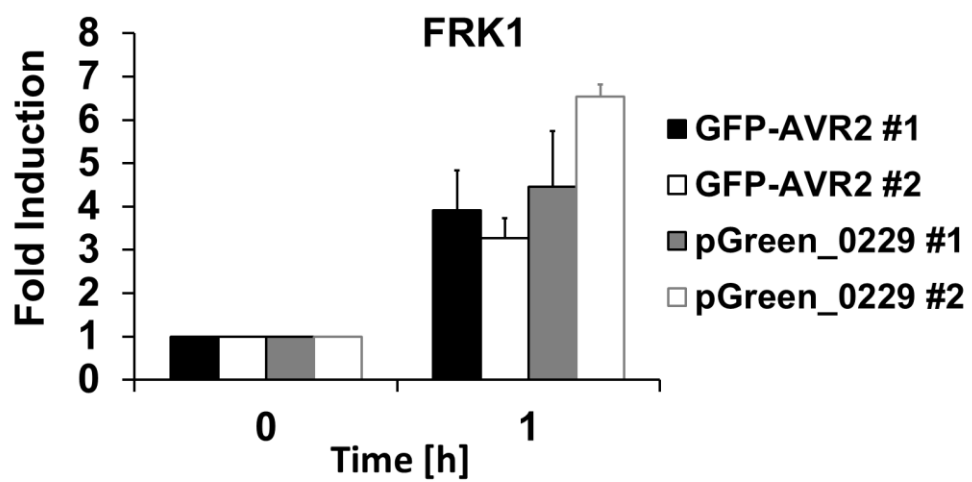


Figure 23 **GFP-AVR2 does not suppress flg22-induced MTI/PTI responses in Arabidopsis. (A)** Arabidopsis plants expressing GFP-AVR2 and pGreen_0229 control plants were treated with 100nM flg22 and leaf material was collected at 0, 10 and 20 min after flg22 treatment. Protein extracts were separated by SDS-PAGE and immunoblotted against anti-pp44/pp42 MAPK antibody. **(B)** Oxidative burst triggered by 100nM flg22 measured in luminol-based assay in GFP-AVR2 expressing plants. Control pGreen_0229 plants were used in control experiments. **(C)** GFP-AVR2 plants and empty vector pGreen_0229 control plants were treated with 1µM flg22 and collected at 0 and 1 h after flg22 treatment. The expression level of *FRK1* was analyzed with gene specific primers and normalized to actin.

3.8 AVR2 affects the resistance/susceptibility to pathogens in Arabidopsis

Several independent studies showed that the expression of many RXLR effector genes is up-regulated during the pre-infection and biotrophic stadium of *P. infestans* infection, at the site where the haustorium is formed (Whisson et al., 2007, Haas et al., 2009, Van Poppel et al., 2009). AVR2 belongs to the category of RXLR effectors that are expressed during early stages of the infection (Gilroy et al., 2011). The expression pattern of AVR2 suggests that it plays an important role in the biotrophic phase of host adaptation. In order to test whether AVR2 expression affects the resistance/susceptibility of Arabidopsis to common pathogenic microorganisms with different life styles, we did infection assays with (hemi)biothrophic and necrothrophic microorganisms.

3.8.1 AVR2 enhances Arabidopsis susceptibility to Pseudomonas syringae pv tomato DC3000

Two independent Arabidopsis lines overexpressing GFP-AVR2 as well as two GFP control lines were spray inoculated as described in § 2.2.5.3 with *P. syringae* pv tomato DC3000 (Pto DC3000), a hemibiotrophic pathogen of Arabidopsis and the bacterial growth was measured at 0 and 3 days after infection. The data showed that GFP-AVR2 expressing plants display increased susceptibility to Pto DC3000 by approximately 1 log cfu/cm^2 leaf disc when compared to the control GFP lines (Figure 24).

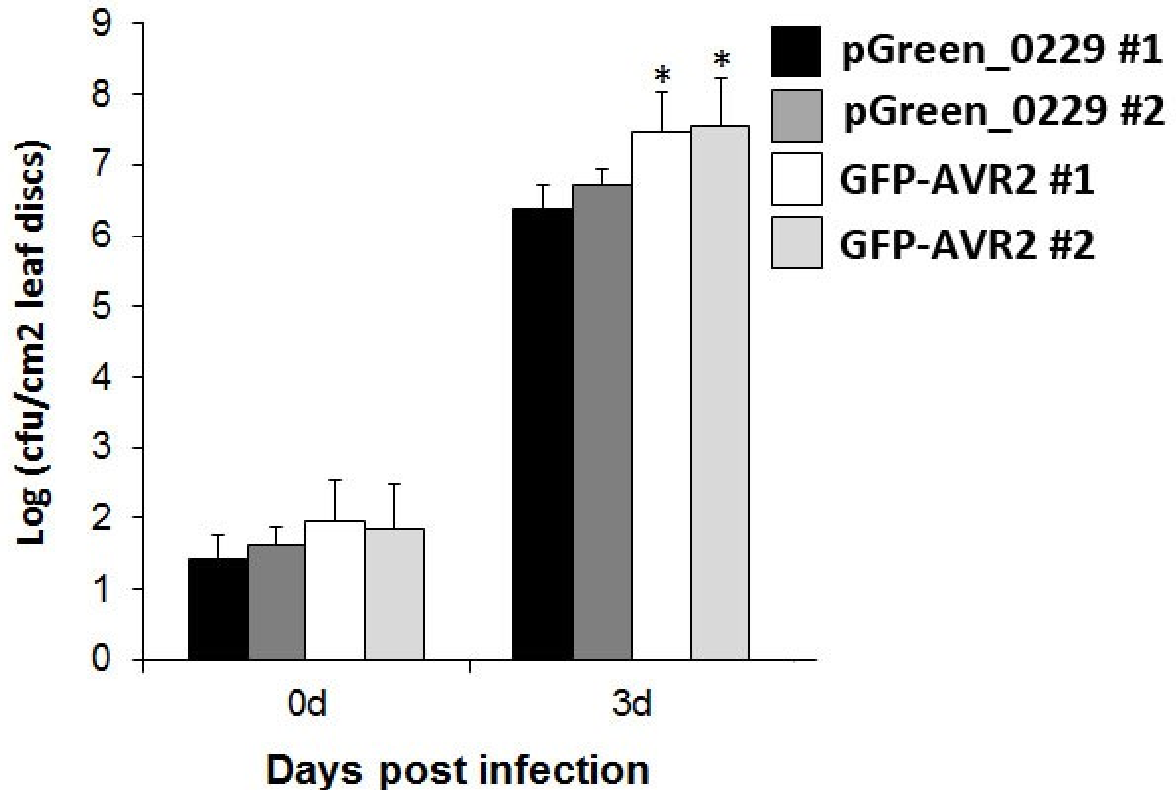


Figure 24 **Arabidopsis GFP-AVR2 expressing plants are more susceptible to Pto DC3000.** Leaves of 4 week old GFP-AVR2 expressing lines and control empty pGreen_0229 control lines were infected with *Pto* DC3000 and bacterial growth was measured at 0 and 3 days post infection and represented as log cfu/cm^2 leaf discs. The experiment was repeated tree times with similar results. Error bars represent SEM. *denote statistical significance (p<0,05) in Student's t-test.

3.8.2 AVR2 enhances Arabidopsis susceptibility to Phytophthora capsici

To gain further insights in the role of AVR2 in resistance/susceptibility in host-pathogen interactions, the GFP-AVR2 expressing lines were infected with *Phytophthora capsici*, another hemibiothrophic pathogen. *P. capsici* is not described as a natural pathogen of Arabidopsis but it has been adapted and can colonize this plant species under highly controlled laboratory conditions (Wang et al., 2013). A PCR-based approach with primers specific to *P. capsici* internal transcribed spacer regions and primers specific to the Arabidopsis *Rubisco* was used to measure the relative biomass of *P. capsici.* PCR quantification revealed that the growth of *P. capsici* was significantly higher in the GFP-AVR2 expressing lines in comparison to the pGreen empty vector lines (Figure 25 C, D). Moreover,

the lesions on GFP-AVR2 lines were larger than on the pGreen_0229 control lines (PG1 and PG2) at both 2.5 and 3.5 days after inoculation (Figure 25 A, B).

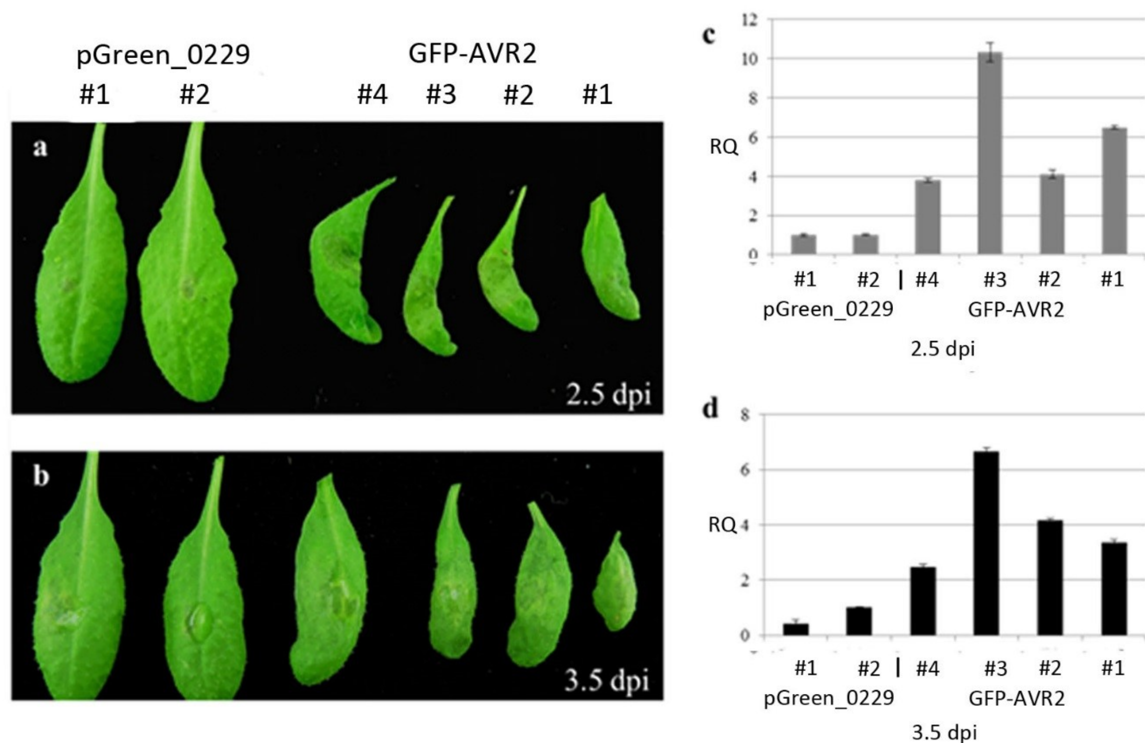


Figure 25 **Arabidopsis GFP-AVR2 expressing plants are more susceptible to *P. capsici* LT263.** 6 week old leaves of four independent GFP-AVR2 expressing lines and two pGreen_0229 control lines (PG1 and PG2) were drop inoculated with *P. capsici* LT263 strain. **(a, b)** Lesions on Arabidopsis leaves at 2.5 and 3.5 days post inoculation (dpi). Relative quantification (RQ) of *P. capsici* biomass was measured at 2.5 dpi **(c)** and 3.5 dpi **(d)** with qRT-PCR. The experiment was repeated twice with similar results (provided by Yan Wang, Francine Goovers lab, Wageningen University, The Netherlands).

3.8.3 AVR2 enhances Arabidopsis resistance to Alternaria brassicicola

The results obtained by testing hemibiotrophic pathogens in Arabidopsis and the observation that AVR2 suppresses INF1-induced cell death in *N. benthamiana*, prompted us to hypothesize that AVR2 has a general effect on delaying or attenuating apoptotic or necrotic processes. In general, such cell death-promoting processes are favoring the growth of necrotrophic microorganisms. Therefore, we infected GFP-AVR2 Arabidopsis plants with the necrotrophic fungal pathogen *Alternaria brassicicola*. Disease symptoms were measured up to 13 days after drop inoculation (Figure 26). The disease index of the GFP-AVR2 expressing lines was significantly lower than empty vector pGreen 0229 lines after 13 days,

indicating that AVR2 is increasing the resistance of Arabidopsis to *A. brassicicola* when compared to control empty vector pGreen 0229 lines. These results together with the previous infection assays with the hemibiotrophic bacteria Pto DC300 and oomycete *P. capsici*, indicate that very likely, AVR2 plays a role in the down-regulation of host-induced mechanisms leading to cell death, providing support for host colonization during the biotrophic phase of the infection.

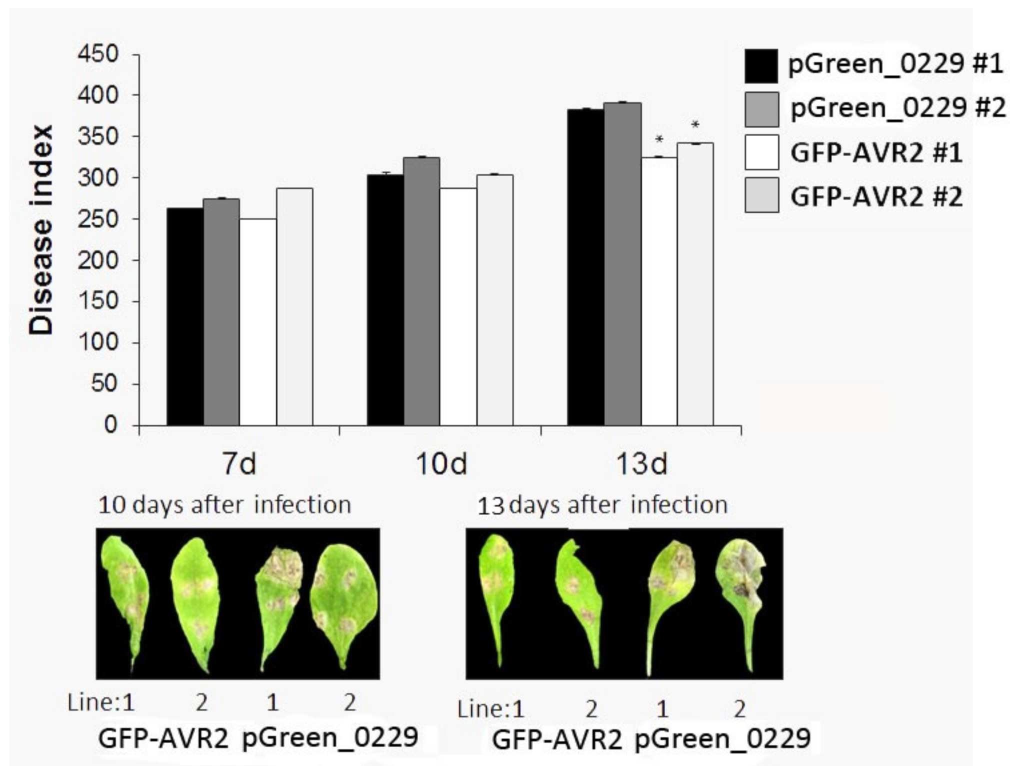


Figure 26 **Arabidopsis GFP-AVR2 expressing plants are more resistant to Alternaria *brassicicola.*** Leaves of 4 week-old GFP-AVR2 expressing lines and two pGreen_0229 control lines were drop inoculated with *A. brassicicola* with 5µl droplets of spore suspension (1x10^{6}spores/ml). Fungal growth was measured at 7, 10 and 13 days upon inoculation and represented as disease index values. The experiment was repeated three times with similar results. Error bars represent SEM. *denote statistical significance (p<0,05) in Student's t-test.

3.9 AVR2 expressing Arabidopsis plants display higher level of JA

Plant hormones are known not only as modulators of developmental processes but also act as signaling molecules in plant defense responses (Shigenaga and Argueso, 2016). The role and importance of salicylic acid (SA), jasmonate (JA) and ethylene (ET) crosstalk in plant defense has been extensively studied and their interactions in disease control become increasingly understood (Spoel and Dong, 2008, Robert-Seilaniantz et al., 2011). A general,

simplified view is that the JA/ET pathway participates to resistance toward necrotrophic pathogen, whereas the SA pathway is more important for the control of biotrophic pathogen. JA is described as an antagonist of SA signaling and a negative regulator of immune responses in regard to biotrophic pathogens (Shigenaga and Argueso, 2016). Our pathoassays showed that AVR2 contributes to susceptibility towards hemibiothrophic pathogens and increased resistance towards necrotrophs. In order to see whether the resistance/susceptibility phenotype was correlated with changes in the profile of plant hormones, we measured the level of SA, JA, IAA (indole-3-acetic acid) and ABA (abscisic acid) in Arabidopsis GFP-AVR2 expressing plants at 0, 1 and 3 days after infection with Pto DC3000.

Technical problems during GC-MS analysis prevented the measurement of accurate and trustful values of the SA level in GFP-AVR2 and pGreen0229 control plants and therefore, it is not possible to establish a possible correlation between reduced levels of SA and a higher susceptibility to PstDC3000 of GFP-AVR2 expressing lines.

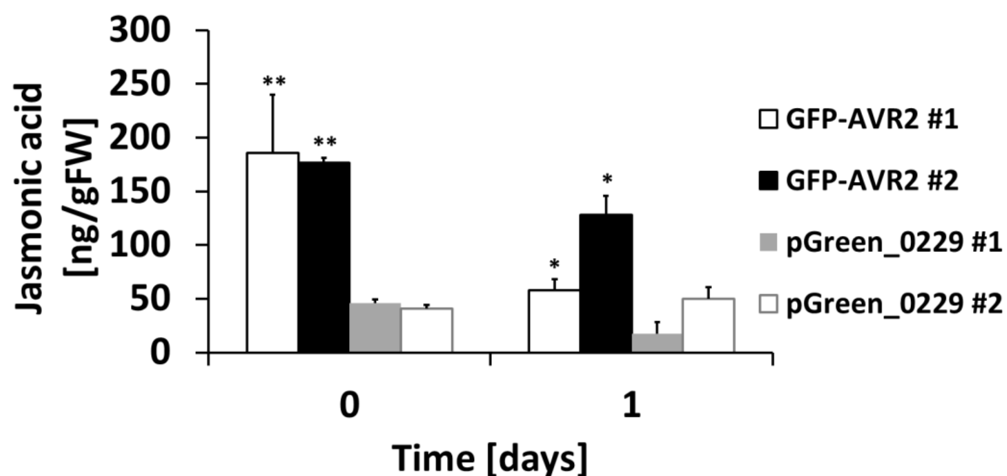
The JA level was 4 x higher in GFP-AVR2 plants (200 ng/g fresh weight) in comparison to empty vector lines (50 ng/g fresh weight) (Figure 27A). However, 1 day after Pto DC3000 infection, we observed a decrease of the JA content in GFP-AVR2 (57-128 ng/g fresh weight) as well as in the control empty vector (17-50 ng/g fresh weight) lines but, the JA level was still 3-6 fold higher in GFP-AVR2 than in GFP plants (Figure 27A). JA accumulation caused by AVR2 expression can be considered as advantageous for (hemi)biotrophic pathogens such as *P. syringae* or *P. infestans* since JA-signaling antagonizes SA-induced defense responses.

GFP-AVR2 had slightly higher IAA level than control pGreen 0229 plants at day 0 (55-57 ng/g FW and 42-46 ng/g FW respectively) (Figure 27B). It has decreased on the 1st day after infection in GFP-AVR2 plants (37-38 ng/g FW), what can be a result of the defense mechanism activation against Pto DC3000. On the 3rd day after infection however, IAA level was significantly higher in GFP-AVR2 expressing plants as in control pGreen 0229 plants (203-206 ng/g FW and 92-102 ng/g FW respectively) (Figure 27B). Auxin accumulation in Arabidopsis plants overexpressing GFP-AVR2 is beneficial for biotrophic pathogens. Biotrophic and hemibiotrophic pathogens such as *P. syringae* are known to up-regulate auxin signaling to promote disease development and suppress SA signaling (Kazan and Manners, 2009, Jones et al., 2011, Kunkel et al., 2013).

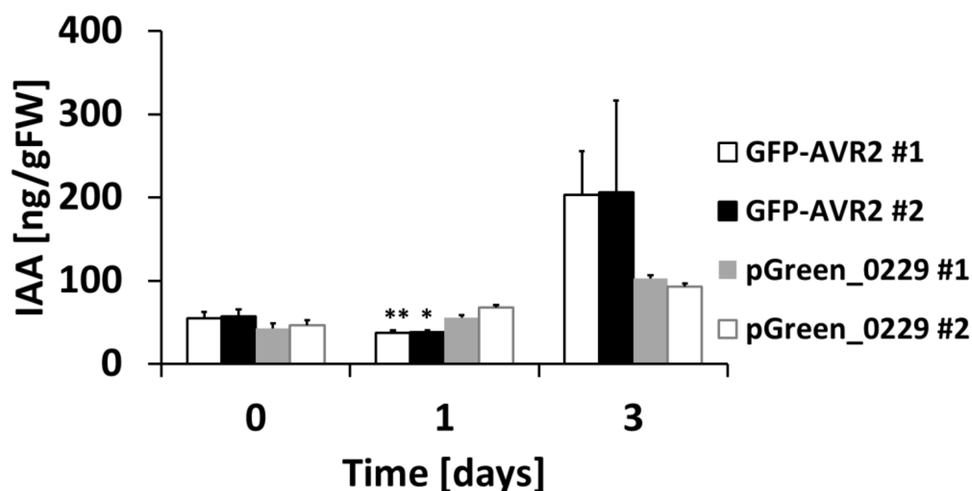
In the case of ABA, both GFP-AVR2 plants as well as control pGreen 0229 lines showed higher levels 3 days after infection (165-188 ng/g FW and 140-144 ng/g FW respectively) (Figure 27C). Zabala et al. (2008) showed that ABA increases in Arabidopsis upon infection with Pto DC3000 and suppresses SA biosynthesis and SA-mediated defense responses. Furthermore Arabidopsis ABA deficient mutant *aba3-1* was shown to be less susceptible to oomycete pathogen *H. arabidopsidis* (Fan et al., 2009).

The hormone studies showed that AVR2 expression in Arabidopsis leads to increased levels of JA, ABA and IAA, which correlate with the higher susceptibility of AVR2 expressing plants to PtoDC300 through a possible attenuation of SA-mediated responses.

A:



B:



C:

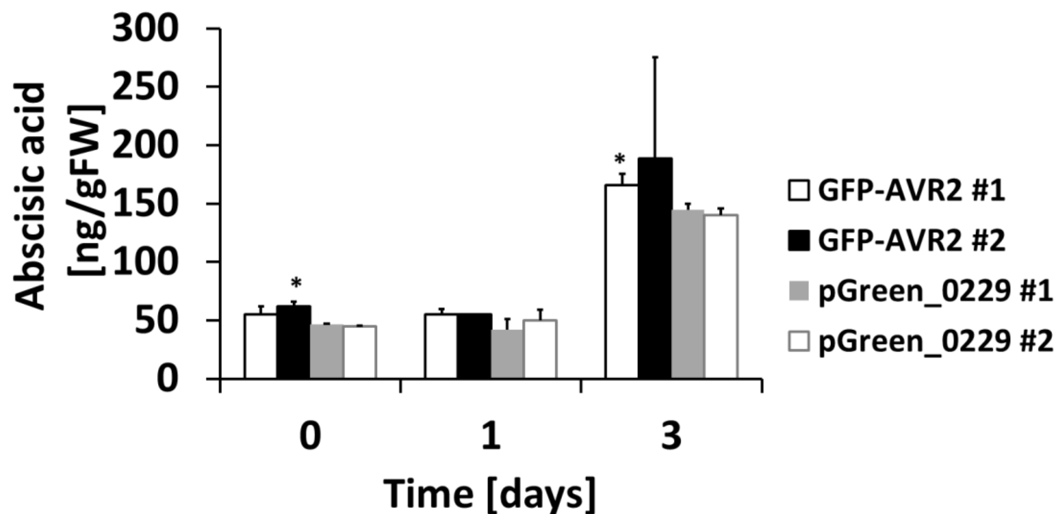


Figure 27 **Hormone levels in Arabidopsis GFP-Avr2 plants before and after infection with Pto DC3000.** 4 week-old GFP-AVR2 expressing and pGreen_0229 control lines were infected with Pto DC3000. Leaf material was collected before and after infection and hormone levels were measured with GC-MS. For every time point and line 3 replicates were performed. Error bars represent SEM. *denote statistical significance ($p<0,05$) in Student's t-test to at least one empty vector line ** denote statistical significance ($p<0,05$) in Student's t-test to both pGreen_0229 lines., **A**: Jasmonic acid, **B**: IAA, **C**: Abscisic acid.

3.10 AVR2 expressing Arabidopsis plants display large transcriptome changes

GFP-AVR2 expressing plants display phenotypes corresponding to BR-insensitive or BR-signaling mutants associated with enhanced susceptibility to (hemi)biotrophic pathogens. In order to better understand which signaling pathways and metabolic networks are affected by AVR2, we performed a transcriptome analysis of the GFP-AVR2 expressing lines.

This analysis revealed that 2060 genes are differentially expressed in GFP-AVR2 plants when compared with the pGreen_0229 control lines (Figure 28). This number includes 1373 genes that are up-regulated and 687 down-regulated genes. The biggest GO annotated groups for which we observed differential expression, contain genes that are related to general RNA and protein metabolism. However these genes are not clearly linked to BR-signaling.

Many of the identified genes encode transcription factors belonging to different families such as WRKY, MYB, bZIP and bHlH transcription factors, which modulate different developmental processes and control responses to biotic and abiotic stresses (Tsuda and

Somssich, 2014). From the selected transcription factors, the one belonging to bHlH family are most abundant. Several of the up-regulated genes in this group are involved in stomata differentiation (table 6). Recently, *SPEECHLESS (SPCH), MUTE* and *FAMA* (*FMA)* have been shown to positively regulate stomata lineage development (Gudesblat et al., 2012). An increase of the expression of these may account for the abnormal stomata patterning, observed in GFP-AVR2 expressing plants. Other up-regulated bHlH transcription factor genes: *BR ENHANCED EXPRESSION 2* (*BEE2), BEE3, BIM3* and PACLOBUTRAZOL RESISTANCE1 (*PRE1)*, are encoding for BR-responsive TFs involved in hypocotyl elongation. Nevertheless, the two master regulators of BR pathway (BZR1 and BZR2/BES1) were not differentially expressed in GFP-AVR2 expressing plants. Also previously examined in *N. benthamiana* brassinosteroid biosynthesis including *DWF5, steroid dehydrogenase (SteroidDH) Xyloglucanendotransglucosylase / hydrolase protein 2 (XTH2)* or brassinosteroid responsive bHlH *CIB1* were not identified in our transcvriptome analysis to be differentially expressed in GFP-AVR2 plants. Furthermore the Arabidopsis GSK3-like kinases were not found in our microarray data analysis although the protein level of some of them was affected in presence of AVR2 in transient expression assays in *N. benthamiana*.

Among up-regulated genes, several are involved in defense responses (Table 6). These include several genes encoding pathogenesis-related proteins and antimicrobial small proteins and peptides such as thionins and defensins (Silverstein et al., 2005, Sels et al., 2008), induced by JA and ET. Several up-regulated defense-associated genes are involved in JA biosynthesis, including *12-oxophytodienoate reductase* and *allene oxide cyclase, JAR1* (*JA-amido synthetase*), which can explain the elevated JA level in GFP-AVR2 expressing plants. The down-regulated genes include AtCYP19 which knock-out mutant has been reported to be more susceptible to *P. syringae* (Pogorelko et al, 2014), a CC-NB-LRR which recognizes the effector ATR39-1 from *H. arabidopsisdis* in Wei-0 ecotype (Goritschnig et al., 2012) and defensing-like (DEFL) family protein which has been previously reported to be down-regulated upon *P. syringae* infection in Arabidopsis (Tesfaye et al., 2013).

The transcriptome analysis in Arabidopsis revealed that AVR2 modulates several signaling cascades on the transcriptional level. The biggest group is represented by TFs which belong to bHlH family and several of them related to brassinosteroid signaling and/or stomata development. AVR2 through association with BSL1-3 and further so far unknown

downstream signaling events affects these TFs. However their role in plant immunity needs further elucidation.

Functional class	Number of differentially expressed genes
Light Perception	5
Major CHO Metabolism	27
Glycolisis	2
Fermentation	1
Gluconeogenesis	1
TCA Cycle	3
Mitochondrial Electron Transport	4
Cell Wall	34
Lipid Metabolism	44
N-Metabolism	1
Amino Acid Metabolism	21
S-assimilation	2
Metal Handling	8
Secondary Metaboism	25
Hormone Metabolism	60
Co-factor Metabolism	8
Tetrapyrole Synthesis	3
Stress Response	79
Redox Status	16
Polyamine Metabolism	3
Nucleotide Metabolism	14
Biodegradation of xenobiotics	4
C1 Metabolism	1
RNA Processing	287
DNA Synthesis	54
Protein Metabolism	250
Signalling	107
Cell Organization	44
miRNA	32
Development	73
Transport	76
Unspecified	675

Figure 28 **Transcriptome analysis of GFP-AVR2 expressing plants.** 35S::GFP-AVR2 plants were grown in short day conditions. Total RNA was isolated from4 weeks old GFP-AVR2 plants and pGreen 0229 control plants grown in short day conditions was sent for transcriptome analysis. Chart shows abundance of differentially expressed (at least 2 fold different than the control pGreen 0229 lines) 35S::GFP-AVR2 expressing grouped into different functional classes. Analysis was performed with MapMan Software.

Table 6 ***Selected genes and their expression levels in GFP-AVR2 expressing plants.***

Gene name	Process	Gene accession	Fold change
MUTE (bHlH TF family)	Stomata formation	at3g06120.1	15,826137
SPCH (SPEECHLESS, bHlH TF family)		at5g53210.1	8,033317
FMA (bHlH TF)		at3g24140.1	3,7594788
POLAR (WRKY TF family)		at4g31805.1	42,93739
BAS1 (PHYB ACTIVATION TAGGED SUPPRESSOR 1)	BR synthesis/signaling	at2g26710.1	43,612133
PRE1 (PACLOBUTRAZOL RESISTANCE1, bHlH TF family)		at5g39860.1	10,251007
SQE2 (squalene epoxidase 2)		at2g22830.1	3,1898513
BEE3 | BEE3 (BR ENHANCED EXPRESSION 3, bHlH TF family)		at1g73830.2	3,160635
BRH1 (BRASSINOSTEROID-RESPONSIVE RING-H2)		at3g61460.1	2,6981974
DWF3, CYP90A1 | CPD (CONSTITUTIVE PHOTOMORPHOGENIC DWARF)		at5g05690.1	2,6618285
BEE2 (BR Enhanced Expression 2, bHlH TF family)		at4g36540.1	2,6279106
BIM3 (BES1-interacting Myc-like protein 3, bHlH TF family)		at5g38860.1	2,2372422
ACS8 | ACS8; 1-aminocyclopropane-1-carboxylate synthase	Ethylene synthesis/signaling	at4g37770.1	30,58587
ATERF-1 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1)		at4g17500.1	8,085126
ATERF15 (Ethylene-responsive element binding factor 15)		at2g31230.1	0,2324255
ACS6 (1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6)		at4g11280.1	0,2674482
MARD1	ABA synthesis/signaling	at3g63210.1	14,451942
AP2		at5g61590.1	12,236939
ABF4 (ABRE BINDING FACTOR 4)		at3g19290.3	2,1088495
ABF2 (ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 2)		at1g45249.3	0,4932573
PIN4	Auxin synthesis/signaling	at2g01420.2	3,2092545
PIN3 (PIN-FORMED 3)		at1g70940.1	2,4156427
AFB3 (AUXIN SIGNALING F-BOX 3)		at1g12820.1	2,0387561
PIN5 (PIN-FORMED 5)		at5g16530.1	0,21552262
JAZ3	Jasmonate synthesis/signaling	at3g17860.1	7,4837193
AtTCP14		at3g47620.1	5,311182
AOC2 (ALLENE OXIDE CYCLASE 2)		at3g25770.1	3,022003
12-oxophytodienoate reductase		at1g17990.1	2,7030356
12-oxophytodienoate reductase		at1g18020.1	2,6666212
JAR1 (JASMONATE RESISTANT 1)		at2g46370.4	0,3951682
THI2.1 (THIONIN 2.1); toxin receptor binding	Defense responses	at1g72260.1	24,746202
pathogenesis-related family protein		at1g78780.2	12,564996
WRKY28		at4g18170.1	10,862265
defensin-like (DEFL) family protein		at1g64195.1	9,49313
PROPEP4 | PROPEP4 (Elicitor peptide 4 precursor)		at5g09980.1	9,011107
systemic acquired resistance (SAR) regulator protein NIMIN-1-related		at4g01895.1	7,981215
PAD4 (PHYTOALEXIN DEFICIENT 4); lipase/ protein binding / triacylglycerol lipase		at3g52430.1	7,80937
MLO6 (MILDEW RESISTANCE LOCUS O 6)		at1g61560.1	5,213156
MKS1 (MAP kinase substrate 1)		at3g18690.1	2,8270638
HLS1 (HOOKLESS 1)		at4g37580.1	2,411596
SEP2 (STRESS ENHANCED PROTEIN 2)		at2g21970.1	0,371724
Encodes a defensin-like (DEFL) family protein		at5g18407.1	0,10996493
PIL5 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 5, bHlH TF family)	Response to light	at2g20180.2	6,4529414
PIL1 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 1, bHLH TF family)		at2g46970.1	3,3035896
PIL6 (PHYTOCHROME INTERACTING FACTOR 3-LIKE 6, bHlH TF family)		at3g59060.2	2,39055

4 DISCUSSION

In this work I have tried to characterize the mode of action in the plant cell of the RXLR-EER effector protein AVR2 from the oomycete pathogen *Phytophthora infestans*. Through different approaches that were performed in different labs, a Y2H screen by our collaborator Paul Birch (JHI, Dundee, UK) and immunoprecipitation experiments in Arabidopsis protoplasts followed by LC-MS-MS, revealed that AVR2 associates with plant ser/thr phosphatases belonging to the BRI1 SUPPRESSOR 1-LIKE PHOSPHATASE (BSL) family. The role of some BSL members as positive regulator of brassinosteroid signaling in plant growth and development is well known. A comprehensive interaction study between AVR2 and the Arabidopsis and *N. benthamian*a BSLs has shown the existence of very specific interaction specificities with the importance of the hinge region and phosphatase domain for the interaction and the absence of interaction with BSU1, a ser/thr phosphatase, only detected in Arabidopsis so far.

In the second part of my thesis, I have examined the effect of AVR2 on typical brassinosteroid and MTI responses in Arabidopsis and *N. benthamiana*. Although, AVR2 expressing Arabidopsis plants can display a very strong developmental phenotype, typical brassinosteroid-regulated markers with the exception of stomata development were not affected. Also early MTI signaling was not diminished, yet AVR2 behaves as a virulence factor, increasing plant susceptibility to bacterial pathogens. By contrast, AVR2 expression In *N. benthamiana*, affects the expression of BR marker genes. Similarly to Arabidopsis, no influence was observed on early MTI signaling responses in *N. benthamiana* but AVR2 inhibits INF1-mediated cell death and promotes P. infestans growth. In my study I provide strong evidence for a physiological relevance of AVR2-BSL interaction but whether and to what extent this interaction impacts Brassinosteroid and/or immune response remains elusive. How the Avr2-BSL interaction is contributing to *P. infestans* infection and the underlying molecular mechanism is still obscure.

AVR2-BSL interaction specificities

BSL1, 2 and 3 belong to the family of Kelch-like protein phosphatases. In Arabidopsis there is a fourth member of PPKL family-BSU1 found exclusively in *Brassicaceae*. No interaction has been observed between BSU1 and AVR2 in our experiments.

Our structure-function analysis performed with AVR2 and several deletion constructs in amino acid sequence of BSL2 revealed that the hinge region and the putative phosphatase domain are required for interaction between BSL2 and AVR2 *in planta*. The alignment between the BSU1-like family members showed that BSU1 lacks a hinge region between the Kelch domain and phosphatase domain, which could explain the lack of the interaction with AVR2. However, further interaction analysis revealed that replacing the phosphatase domain of BSL2 with the phosphatase domain from BSU1 also did not led to the interaction of the chimera protein with AVR2 (data not shown) We have identified amino acid 901-1018 within the putative phosphatase domain of BSL2 to be required for the interaction with AVR2. A sequence comparison of the phosphatase domain of BSU1 and BSL2 revealed significant differences of the amino acid sequence in the C-terminal part.

The interaction specificity analysis showed also that the minimal fragment of AVR2 required for the interaction with BSL2 is located at the C-terminal region of AVR2. This 17 amino acid region comprises two characteristic motifs, KKLV and LKIKG, and deletion of either one of them leads to abolishment of the interaction with BSL2. Recently, Kim et al. (2016) have shown that a similar motif, KKVI, present in BSU1-like family members, promotes their homo-oligomerisation. Further, the authors have shown that the KKVI motif is required for BSU1 function and the effective BIN2 dephosphorylation in vivo. In this work, I have shown that a domain comprising aa 602-663 of BSL2 is required for the interaction with AVR2. However, we cannot rule out that interaction domain may extend beyond this domain, including the KKVI motif which corresponds to aa 664-667 in the sequence of BSL2. However, our interaction studies suggest that the KKVI motif alone would not be sufficient for the interaction with AVR2. We emitted the hypothesis that AVR2, through its interaction with the BSLs, is either stabilizing or preventing the formation of oligomers and therefore affects the activity or substrate interaction but no difference on the oligomerisation between BSU1-like phosphatases was observed upon co-expression with AVR2 (Paul Birch,

personal communication). Still, it would be very interesting to test the interaction between Avr2 and a ΔKKVI BSL2 mutant. Ultimately, a 3D structure of the AVR2-BSL2 complex would give a better insight which amino acid residues are crucial for the interaction, complementing our structure function analysis. An attempt to express and purify recombinant AtBSL2 and AVR2 in *E.coli* for co-crystallization studies had been initiated by N. Wagener in our lab but the expression of AtBSL2 was very difficult. In a future approach, the expression of the minimal sequence of AtBSL2 required for the interaction with AVR2 (aa 602-1018) could help to overcome this problem. In the case of AVR2, a synthetic peptide corresponding to the last 18 amino acid could be sufficient for co-crystallisation.

What influence has AVR2 on BR-signaling?

AtBSU1 and AtBSL1 have been shown to act as positive regulators of brassinosteroid signaling in Arabidopsis (Mora-Garcia et al., 2004, Kim et al., 2009). The role of AtBSL2 and AtBSL3 in Arabidopsis remains unknown. Our investigations showed that AVR2 did not alter the phosphatase activity of the BSU1-like family members when co-expressed in *N. benthamiana*. An additional experiment would consist to measure the phosphatase activity upon treatment with BL. However, no change in AtBSU1 and AtBSL2 phosphatase activity has been observed in response to BL without AVR2 and it is very unlikely that a difference will occur with AVR2.

In presence of BL, BSU1 and BSL1 were shown to dephosphorylate and inactivate the GSK3-like kinase BIN2 (Kim et al., 2009) It is hypothesized that BL increases the affinity of the BSU1-like phosphatases towards BIN2. Further mass spectrometry analysis for post-translational modifications of the BSLs prior and after BL treatment and in the presence or absence of AVR2 would bring us a step closer in understanding how AVR2 is manipulating the biochemical function of the BSLs.

Through its interaction with the BSLs, AVR2 influences BR-dependent formation stomata development and patterning. Kim et al. (2012) proposed a model in which active BIN2 phosphorylates and inactivates YDA, a MAP kinase kinase kinase that is negatively regulating stomata formation. The authors have shown that bikinin is counteracting the effect of BIN2. Similarly, in our study, bikinin was counteracting the effect of AVR2.

Taken together these results indicate that in presence of AVR2, BIN2 activity increases. We have received an antibody from Milipore capable to detect BIN2 phosphorylated at Tyr220, an important residue for BIN2 activity. Unfortunately, we were not able to detect this protein in Arabidopsis (data not shown). The alternative approach which consisted to study the effect of AVR2 on GFP/myc tagged BIN2 in *N. benthamiana* transient assays were inconclusive, also because we struggled to detect BIN2 protein expression. Further experiments should investigate the phosphorylation status of YDA in AVR2 expressing plants to see if it is inactivated, which would correlate with the observed excess of stomata.

The present model of BR- signaling concentrates on BIN2 as a key GSK3-like kinase which negatively regulates BR pathway by phosphorylating and targeting BZR1/2 for degradation. Recent studies however showed that not only BIN2 but also other GSK3-like kinases are involved in the BR signaling pathway. It has been demonstrated that ASKθ directly phosphorylates BZR1, BES1, and BEH2 (Rozhon et al., 2010). ASKγ interacts with BZR1 and BZR2 transcription factors in Y2H screen (Youn et al., 2013). In this work all 10 members of Arabidopsis GSK3-like kinase family have been co-expressed in presence and absence of AVR2 in tobacco leaves. In presence of Avr2 the expression level of ASKδ and ASKI increases and Myc-ASKζ decreases what can be a consequence of AVR2-BSL interaction. AVR2 may influence the interaction between BSU1-like phosphatases and GSK3-like kinases other than BIN2. However no study so far showed that they are substrates of BSU1-like phosphatases. Further in vitro kinase assays upon co-expression of the GSK3-like kinases in presence and absence of AVR2 would clarify the influence of AVR2 on the GSK3-like kinases. Homologues of all Arabidopsis GSKs were found in *N. benthamiana* in BLAST search. The protein sequence identity between Arabidopsis and *N. benthamiana* ranged from 68-90%. Also genetic studies based on the single or multiple gain- and loss-of function GSK3-like kinases mutants would answer the question if AVR2 is activating or suppressing the GSK3-like kinases.

In recent studies, BZR1 has been proposed to play a key role at the cross section of Brassinosteroid and Immune responses (Lozano-Duran et al., 2013). BZR1 and BES1 share 88% of amino acid sequence identity and were often considered as having largely overlapping function. Protein blast search performed with Arabidopsis BZR1 and BES1 protein sequences against *N. benthamiana* database resulted in the identification of

NbBZR1/BES1 homologues which share 87% and 81% similarity with Arabidopsis AtBZR1 and AtBES1, respectively. In light of the role of BZR1 as a positive regulator of brassinosteroid responses and negative regulator of MTI responses in Arabidopsis, it would be necessary to test the phosphorylation status of BZR1 in presence of AVR2 in both Arabidopsis and *N. benthamiana* . In Kim et al (2012), the authors proposed that, when BR levels are low, BIN2 phosphorylates and inactivates YDA which reduces MAP kinase 3 and 6 activity which in turn leads to higher SPCH activity, another bHlH transcription factor, and increased stomata number. The transcriptome analysis of AVR2 expressing Arabidopsis plants indicates that several bHlH transcription factors , among them SPCH, FMA, MUTE, all positive regulators of stomata formation, are upregulated. Therefore, an effect of AVR2 on these transcription factors could eventually help further to understand its mode of action in Arabidopsis.

The stomata phenotype observed in AVR2 expressing Arabidopsis plants is contrasting with the phenotype obtained upon expression of AVR2 in potato plants (Thurnbull et al., 2017). In these plants, the stomata density was clearly reduced compared to non-transgenic plants. Furthermore, the authors interpreted further morphological traits such as twisted stems, curled leaflets and reduced number of leaflets as the result of an activation of the BR signaling pathway, similar to that observed in *BRI1* or *DWF4* overexpressing plants (Thurnbull et al., 2017). This discrepancy in the observed phenotypes suggests that AVR2 interaction with BSU1-like phosphatases has opposite effects in Arabidopsis and potato.

BR pathway is so far poorly understood in *Solanaceae* species. In Thurnbull et al. (2017) several BR-responsive marker genes were identified in potato upon transcriptome analysis of AVR2 expressing plants and control plants treated with EBL. Up-regulated BR-responsive genes included a bHlH-encoding homologue of *AtCIB1* and *AtHBI1*, named *StCHL1*, *Expansin 8* (St*EXP8),* chlorophyll a-b binding protein associated with light harvesting (St*CAB50)*, subtilisin-like protease (St*P69F)* and small auxin upregulated RNA 67 (St*SAUR67*). Also one down-regulated BR responsive gene, gibberellin-2-oxidase (St*GA2ox1),* was identified. The transcriptome analysis indicates that AVR2 causes the activation of BR signaling in potato plants (Turnbull et al., 2017).

In my work, gene expression analysis upon BL treatment were performed in *N. benthamiana* plants transiently expressing GFP-AVR2. Several marker genes have been identified to be

differentially expressed in *N. benthamiana* upon BL treatment. That includes *Sterol reductase (DWF5), Steroid dehydrogenase (SteroidDH), Xyloglucanendotransglucosylase / hydrolase protein 2 (XTH2)* and *CRYPTOCHROME-INTERACTING BASIC HELIX LOOP HELIX 1 (CIB1/bHlH63).* The brassinosteroid biosynthesis genes: *sterol reductase* (*DWF5*) and *Steroid dehydrogenase* (*SteroidDH*) were shown to be downregulated in control plants upon BL treatment what most likely is a result of negative feedback loop similar to that observed in Arabidopsis. Tobacco plants overexpressing GFP-AVR2 on the other hand showed slight increase in transcript level of these genes. Recent papers indicated that several bHlH transcription factors are involved in BR-signaling that includes BEE1-3, CIB1 and HBI1 (Friedrichsen et al., 2002, Lozano-Duran et al., 2013, Wang et al., 2009, Malinowsky et al., 2014). Moreover BEE2, CIB1 and HBI1 appeared to negatively regulate the PTI responses (Malinowsky et al., 2014). Turnbull et al. (2017) showed that AVR2 upregulates the expression of StCHL1 which is a homologous to Arabidopsis HBI1 and CIB1. Transient expression of AVR2 or StCHL1 in *N. benthamiana* enhanced the leaf colonization of *P. infestans* and suppressed INF1-mediated cell death (Turnbull et al., 2017). In our study we showed that AVR2 downregulates the expression of *CIB1* upon BR treatment. Overall, our gene expression analysis indicates that AVR2 has an inhibiting effect on BR-regulated gene expression in *N. benthamiana* what is opposite to the effect described in AVR2 expressing potato and *N. benthamiana* plants in the Thurnbull et al., 2017 paper.

One of our working hypothesis was that, through its interaction with the BSLs, AVR2 disturbs downstream signaling involving BIN2 and the transcription factors BZR1/BZR2 (BES1) and resulting in a change in the expression of BR-responsive genes. In our study we have shown that AVR2 does not influence BL-dependent dephosphorylation of BES1 transcription factor in different experiments performed in Arabidopsis and in *N. benthamiana*. In Arabidopsis, this effect could be explained by the fact that AVR2 does not interact with BSU1, maintaining the signaling pathway operational. This possibility draws into question the soundness of the approach to studying virulence function and mode of action of AVR2 in a plant species in which *P. infestans* is not adapted. Indeed, It is hypothesized that most effectors that are involved in host-specific interactions have evolved together with their targets as a result of strong selection for effective pathogenesis. As a direct consequence, a failure to colonize a plant species may be due to the lack of effectors capable to manipulate or interfere efficiently with plant function, most likely because they are unable to interact with and

properly manipulate host target proteins. Such phenomenon of non-evolved resistance can also serve to explain the non-host resistance of Arabidopsis vs *P. infestans*. In this line, it would be interesting to perform additional experiments in an Arabidopsis *bsu1* mutant

However, the experiments performed in *N. benthamiana* in which AVR2 is able to interact a priori with each member of the BSL family, suggest that BL-dependent activation of AtBES1 is truly not affected or that BES1 activation is independent on BSLs in *N.benthamiana*.

What effect has AVR2 on plant immune responses?

In this study, we aimed to evaluate the influence of AVR2 on typical MTI responses in Arabidopsis and in the host of *P. infestans, N.benthamiana.* In Arabidopsis, no influence on flg22-induced MAPK activation, ROS burst and *FRK1* expression has been observed in AVR2 expressing plants. Albrecht et al. (2012) showed that inhibition of flg22 - induced ROS burst and *FRK1* expression in Arabidopsis was observed only in the case of a 24h pre-treatment with BL. A similar pre-treatment with BL in AVR2- expressing plants followed by flg22 treatment has not been done and this experiment could eventually reveal a suppressing function of AVR2 on MTI signaling. Another explanation is, like in the case of BR-regulated responses, flg22-induced immune responses are not suppressed due to the presence of a functional BSU1 and the absence of its interaction with AVR2.

In *N. benthamiana*, no BSU1 ortholog has been identified and our interaction studies suggest that AVR2 is able to interact with all the members of the BSU1-like phosphatases. Still, none of the tested early MTI-responses induced by flg22 or an elicitin (MAPK activation, ROS burst and expression of MTI genes) are altered in presence of AVR2. As mentioned previously, a pretreatment with BL prior flg22 or β-megaspermin application should be performed in *N. benthamiana* to eventually uncover the effect of AVR2 on early PTI events. Albrecht et al. (2012) showed that BR signaling inhibits flg22-induced signaling downstream of the FLS2-BAK1 complex. More recently, it has been demonstrated that BZR1 and HBI1 are negative regulators of plant immune responses (Fan et al., 2014, Lozano-Duran et al., 2013). BZR1 is involved in suppression of immune signaling by BR and induces the expression of several WRKY TFs that negatively control early immune responses. HBI1 overexpression leads to reduced MAMP-triggered responses and enhanced susceptibility to *Pto* DC3000. In line with

the results in Arabidopsis, Thurnbull et al. (2017) showed that the expression of AVR2 in potato upregulates *StCHL1* and gain-and loss-of-function experiments with *NbCHL1* in *N. benthamiana* have confirmed the role of this transcription factor in promoting disease and in suppressing immunity, illustrated by the inhibition of INF1-induced cell death. Although, expression of CIB1/bHLH63 was down-regulated in my gene expression studies, additional experiments should be performed to determine exactly how the expression of this transcription factor and other BR-responsive bHLH transcription factors is affected in AVR2 expressing plants. It would be also interesting to elucidate if AVR2 affects the activity of BZR1, since it has not been performed in this study.

Further, Thurnbull et al. (2017) showed that *N. benthamiana* plants transiently expressing AVR2 display suppression of MTI marker genes including *NbACRE31* and *NbWRKY7* upon treatment with culture filtrate of *P. infestans*. In our study, flg22 or β -megaspermin-treated *N. benthamiana* plants expressing AVR2 did not show the suppression of *NbACRE31* and *NbPTI5.* The culture filtrate used in Thurnbull et al., is a mixture of different elicitors and it might explain the discrepancy with our results. However, the use of well-defined, individual MAMPs such as flg22 and elicitin should be more favorable for the detection of a suppressing effect than using a complex mixture of elicitors that might trigger different branches of MTI pathway. Another result in Thurnbull et al., was that flg22 and the culture filtrate of *P. infestans* are suppressing the expression of BR-induced genes such as StCHL1, StEXP8, StASAUR67, STCAB50 and StP9F and of BR-biosynthesis-associated genes StDWF4 and StSTDH in potato plants. It would make sense to perform similar analysis in *N. benthamiana* plants expressing AVR2.

In this work, it has been demonstrated that AVR2 is able to suppress INF1-induced PCD in *N. benthamiana*. We have correlative evidence through the structure function analysis with the AVR2 deletion constructs that the interaction between AVR2 and BSLs is important for the suppression of INF1-induced PCD. Besides INF1, it would make sense to test if AVR2 is also suppressing PCD associated with ETI responses. Several RXLR effectors have been shown to interfere not only with MTI but also with ETI including Avh172 and Avh6 from *P. sojae* (Wang et al., 2011).

Another aspect that would be interesting to follow up is related to the avirulence function of AVR2. Saunders et al. (2012) showed that AVR2 associates with StBSL1 in planta and this association is perceived by R2 protein what further triggers HR. The interaction studies performed between AVR2 and Arabidopsis or potato BSL2, revealed that the last 18 amino acid of AVR2 sequence were sufficient for the interaction and for suppression of INF1-induced cell death. A next step would be to determine whether this motif is also sufficient to trigger R2-dependent HR. Moreover, these association studies could also help to better understand how AVR2-like (Lokossau et al., 2009) evades recognition by R2 while keeping its virulence function. AVR2-like is also able to associate with BSL1, 2 and 3 (C. Jantsch, personal communication) but the minimal motif required for interaction is not known. AVR2 and AVR2-like differ only by 13 amino acid residues, 5 of these substitutions are located in the C-terminal part. While the KKLV motif is conserved, the LKIKG motif is replaced by a LQSKG motif in AVR2-like. The hypothesis is that these mutations could play a key role in evasion of R2 recognition.

Our infection assays performed with 35S::GFP-AVR2 expressing plants revealed that these plants are more resistant to the necrotrophic fungus *Alternaria brassicicola* but more susceptible to the hemibiotrophic pathogens *P. syringae* pv tomato DC3000 and *Phytophthora capsici*. The latter one however is not a natural pathogen of Arabidopsis and future work should include infection assays with for example *Hyaloperonospora arabidopsidis*, an oomycete which is a natural pathogen of Arabidopsis. So far, no homologue of AVR2 was found in *Hyaloperonospora arabidopsisdis* by doing a blast search.
Since expression of GFP-AVR2 under the 35S promoter has a severe developmental phenotype it complicates the elucidation of the mode of action of AVR2 in plant susceptibility. For this reason, we have generated GFP-AVR2 expressing lines without any visible developmental phenotype using an estradiol inducible system. Preliminary results showed that these lines turned out to be more susceptible to *P. syringae DC3000* but this effect was much less pronounced and not statistically significant when compared to the 35S expressing plants (data not showed). Since inducible AVR2-expressing lines are better reflecting the natural infection conditions, additional experiments with the same range of pathogens tested with the constitutive AVR2 expressing lines, coupled to transcriptome changes analysis would perhaps help to understand better the mode of action of AVR2.

A main question that remains unanswered is the role of BSLs in plant immunity. My work suggests that AVR2 is inhibiting the function of BSLs. Therefore, BSLs seem to be positive regulators of the resistance against pathogens such as *P. infestans* and *P. syringae.*

In this regard, *bsl* mutants should be more susceptible to these pathogens. The *bsu1*, *bsl1*, *bsl2* and *bsl3* Arabidopsis mutants are available at the Notthingham Arabidopsis Seed Collection (NASC). All the single *bsl* mutants as well as the double *bsu1 bsl1* mutant, kindly provided by Joanne Chory (SALK Institute, San Diego USA), display a normal growth phenotype. In the literature, a *bsu1 bsl1* mutant in which BSL2 and BSL3 expression was downregulated by RNAi-based technique (*bsuq* mutant) showed a severe dwarf phenotype with abnormal stomata patterning (Kim et al., xx) resembling to AVR2 expressing plants. I have generated several double and triple mutant combinations: *bsl1/bsl3, bsl2 heterozygous/bsl3, bsl1/bsl2heterozygous/bsl3.* However, it was impossible to obtain double and triple knock-out mutants with homozygous *bsl2* although, Mora-Garcia et al. (2014) describe the obtention of a *bsl2 bsl3* mutant that displayed a dwarf phenotype, epinastic cotyledons and different degree of symmetry breakdown and cotyledon fusion . These observations suggest that BSL2 and BSL3 can complement the function of BSU1 and BSL1 but BSU1 and BSL1 do not complement the function of BSL2 and BSL3. A first set of pathoassays with *bsl1/bsl2heterozygous/bsl3 and bsl2 heterozygous/bsl3* mutants revealed that these lines are slightly more resistant to *P. syringae* infection, what would support our hypothesis that functional BSLs are positively regulating resistance against hemibiotrophic pathogens.

However, the functional redundancy between BSLs, the difficulty to obtain loss-of-function mutants coupled with the role of BSLs in plant growth and development renders the study of a role of this family of proteins in Arabidopsis immunity very difficult. A possibility to overcome this problem would be to use an inducible-RNAi based approach (Detlef Weigel, MPI for Developmental Biology, Tubingen GER) or newly developed CRISPR-Cas technology (Zhang et al., 2014).

Plant defense-associated hormones are playing an important role in signaling and establishment of resistance toward pathogens with different life style. Our hormone measurements are in agreement with previous studies and the observed susceptibility/resistance phenotype. AVR2 plants showed higher auxin and JA levels, which negatively regulates SA-mediated immune responses during the infection with *P. syringae*

and positively regulates immune responses against necrotrophic pathogens such as *A. brassicicola* (Shigenaga and Argueso, 2016). However, the difference in hormone levels between control and AVR2-expressing plants is not very strong and not obvious in weak AVR2 expressing lines such as line #2. Therefore, we also formulate the hypothesis that the increased susceptibility to *P. syringae* and *P. capsici* in AVR2 expressing lines could be due to the excess of stomata , which are natural entry places for bacterial or fungal pathogens. However, the stomata phenotype in AVR2 expressing plants should equally favor the infection by *Alternaria*, by contrast to our results showing that AVR2 expressing planta were more resistant to this pathogen.

Transcriptome analysis revealed that a variety of defense-related genes are differentially expressed in AVR2 plants when compared to control empty vector lines. This includes several defensin-like, pathogenesis-related and NB-LRR proteins. From selected TFs, BEE2 which is upregulated in AVR2 plants, was shown to negatively regulate the plant immune responses (Malinovsky et al., 2014). It seems that JA-regulated defense genes are up-regulated in AVR2 expressing plants, in association with a higher level of JA and its presumed role in antagonizing SA signaling and favoring *P. infestans* colonization during the biotrophic phase. It has been shown that *H. arabidopsidis* HaRxL44 targets and causes the degradation of MED19a, a subunit of the mediator complex regulating the interaction between TFs and RNA polymerase II in eukaryotes, thereby attenuating SA-induced immune responses while JA/ET signaling is increased and *H. arabidopsidis* growth faster (Caillaud et al., 2013). However, the transcriptome analysis in AVR2 expressing Arabidopsis plants did not produce a clear picture about which immune pathways are affected and how the activation and suppression profile of immunity-associated genes would be beneficial for *P. infestans* growth. Public databases such as the the Arabidopsis transcriptome genome express database (SALK) contain several thousand sets of transcriptome change analyses in Arabidopsis, performed in wild type and mutant plants submitted to different developmental, environmental conditions and treatments. A meta-analysis with the transcriptomic data generated in AVR2 expressing plants could eventually reveal mutants or treatments displaying similar changes in the gene expression profiles and help to better understand which metabolic pathways are targeted by AVR2 and the biological relevance for *P. infestans* disease.

By performing transient co-expression of AVR2 and Arabidopsis GSKs in *N. benthamiana*, I have shown that the amount of BIL1 (BIN2-Like1)/ASKζ decreased while the amount of BIL2 (BIN2-Like2)/ASKι and ASKδ increased. Several studies indicate that GSK3-like kinases may also be involved in regulating plant immune responses. The triple GSK3 mutant bin2/bil1/bil2 was reported to exhibit reduced flg22-induced ROS production (Lozano-Duran et al., 2013). Stampfl et al. (2016) showed that ASKα acts as a positive regulator of PTI. ASKα phosphorylates and activates glucose-6-phosphate dehydrogenase (G6PD), which provides NADPH and is essential for maintaining the cellular redox state. Furthermore, they demonstrate that activation of G6PD enhances early PTI responses. These results provide a preliminary mechanistic link between AVR2-BSLs interaction and plant immunity through the manipulation of the activity of some ASKs.

The effect of flg22 and BL on the activation of the GSKs in the presence/absence of AVR2 should be done in a next step to further confirm the current results and generate novel working hypothesis. In Arabidopsis, further experiments should investigate how these ASKs contribute to plant immunity through generation of loss and gain of function mutants in different combinations. It would also be necessary to perform similar investigation and experiments with *N. benthamiana* GSKs. A BLAST search with Arabidopsis GSKs against the *N. benthamkiana* protein database revealed that there are homologues present in tobacco witch share approximately 80% sequence similarity with AthGSKs. Using native tobacco GSKs as it is a natural host of *P. infestans* would be advantageous in understanding how AVR2 could affect BIN2 and other GSKs and how it contributes to BR-signaling and plant immune responses.

5 SUMMARY

Oomycetes are deep-branching eukaryotes that include *Hyaloperonospora arabidopsidis*, the causal agent of downy mildew on Arabidopsis thaliana, or *Phytophthora infestans* which causes potato and tomato late blight. In silico analysis predicted that the genome of several oomycete species encodes for a large number of so-called RXLR-dEER effectors that are assumed to manipulate host cellular functions as part of their infection strategy. A novel area of research in plants is centered on the antagonism between signaling pathways regulating plant growth and development and plant immunity. These cross-talks are potential targets for the pathogen. The P. infestans effector AVR2 interacts with BSU1-like phosphatases, which are known to be positive regulators of Brassinosteroid signaling. In this thesis, I have used a combination of biochemical, molecular biology and reverse genetic approaches to characterize the molecular function of AVR2 in both a host (*N. benthamiana*) and non-host (Arabidopsis thaliana) plant species of *P. infestans*. The expression of AVR2 in Arabidopsis leads to plants having a dwarf phenotype and abnormal stomata density. The phenotype is consistent with a defect of the brassinosteroid signalling pathway. However, transcriptome change analysis and activation studies of the BR-regulated key transcription factor BES1 and marker genes showed, except for genes involved in stomata biogenesis, no deregulation by AVR2. Only the protein expression level of some GSKs e.g. ASK⍰, ASKI and ASKδ, a family of kinases including members that are acting downstream of BSLs in Brassinosteroid signaling, was modulated. AVR2 did not interfere with the early stages of MAMP-induced immune signaling responses e.g. calcium and oxidative burst, post-translational MAP kinase activation and transcriptional up-regulation of MAMP-inducible genes in Arabidopsis and *N. benthamiana*. However, AVR2 interfered with immunity-associated programmed cell death in *N. benthamiana*, and caused higher susceptibility to biotrophic pathogens including *P. syringae* and *P. capsici* and increased resistance to nectotrophic *A. brassicicola* in Arabidopsis.

Altogether, these results identify AVR2 as the first effector from a filamentous plant pathogen that targets components of the BR signaling pathway and potentially exploit the antagonistic cross talk between plant growth and development and innate immunity. However, the mechanism by which AVR2 manipulates plant immunity through its interaction with BSLs remains elusive. The comparative study of effector activity in the non-host A.

thaliana and the host *N. benthamiana* uncovered apparent differences in the effect of the effector and draws into question the utility of Arabidopsis as a model system to elucidate the virulence function of AVR2. On the other hand, the specific interaction of AVR2 with AtBSL1-3 but not AtBSU1 offers the possibility to use the effector as a molecular probe to decipher fundamental cellular mechanisms regulated by individual BSLs.

6 Zusammenfassung

Oomyceten sind tiefverzweigte Eukaryoten, wie z.B. *Hyaloperonospora arabidopsidis*, der Erreger des Falschen Mehltaus auf Arabidopsis thaliana, oder *Phytophthora infestans*, der Kartoffel- und Tomaten-Krautfäule verursacht. In silico-Analysen sagen voraus, dass das Genom mehrerer Oomycetenspezies für eine große Anzahl von sogenannten RXLR-dEER-Effektoren kodiert, von denen angenommen wird, dass sie die zellulären Funktionen des Wirts als Teil der Infektionsstrategie manipulieren. Ein neuer Bereich der Pflanzenforschung konzentriert sich auf den Antagonismus zwischen Signalwegen zur Regulation von Pflanzenwachstum und Entwicklung und der Pflanzenimmunität. Diese Wechselwirkungen sind mögliche Ziele für mikrobielle Effektoren. Der P. infestans-Effektor AVR2 interagiert mit BSU1-ähnlichen (BSL) Phosphatasen, von denen bekannt ist, dass sie positive Regulatoren des Brassinosteroid (BR)-Signalwegs sind. In dieser Arbeit habe ich eine Kombination aus biochemischen, molekularbiologischen und revers-genetischen Ansätzen verwendet, um die molekulare Funktion von AVR2 sowohl in einer Wirtspflanze (*N. benthamiana*) als auch in einer Nicht-Wirtspflanze (A. thaliana) von P. infestans zu charakterisieren. Die Expression von AVR2 in Arabidopsis führt zu zwergwüchsigen Pflanzen mit abnormaler Stomata-Dichte, einem Phänotypen der auch bei einem Defekt im Brassinosteroid-Signalweg zu beobachten ist. Eine Analyse von Transkriptomänderungen und Aktivierungsstudien des BR-regulierten Schlüsseltranskriptionsfaktors BES1 sowie von BR-Markergenen zeigten jedoch, abgesehen von Genen, die an der Stomata-Biogenese beteiligt sind, keine Deregulierung durch AVR2. Nur das Genexpressionsniveau einiger Glycogensynthase-Kinasen (GSK), einer Familie von Kinasen die unter Anderem unterhalb von BSLs im Brassinosteroid-Signaweg wirken, wurde moduliert wie z.B. ASKζ, ASKI und ASKδ. AVR2 interferierte nicht mit den frühen MAMP-induzierten Immunantworten, z.B. der Akkumulation von Calcium und reaktiven Sauerstoffspezies, der post-translationalen MAP-Kinase-Aktivierung und der transkriptionellen Hochregulation von MAMP-induzierbaren Genen in Arabidopsis und N. benthamiana. Jedoch störte AVR2 den immunitäts-assoziierten programmierten Zelltod in *N. benthamiana* und verursachte in Arabidopsis eine höhere Anfälligkeit für biotrophe Pathogene wie z.B. Pseudomonas syringae *und Phytophthora capsici* sowie eine erhöhte Resistenz gegen den nektotrophen Pilz *Alternaria brassicicola*.

Insgesamt zeigen diese Ergebnisse AVR2 als den ersten Effektor eines filamentösen Pflanzenpathogens auf, der auf Komponenten des BR-Signalwegs abzielt und die antagonistische Wechselwirkung zwischen Pflanzenwachstum und -entwicklung einerseits und angeborener Immunität andererseits ausnutzt. Der Mechanismus, durch den AVR2 die Pflanzenimmunität durch seine Interaktion mit BSLs manipuliert, bleibt jedoch noch unklar. Die hier gezeigte vergleichende Untersuchung der Effektoraktivität in dem *P. infestans*-Nicht-Wirt A. thaliana und dem Wirt *N.*

benthamiana offenbarte offensichtliche Unterschiede in der Wirkung des Effektors und stellt den Nutzen von Arabidopsis als Modellsystem zur Aufklärung der Virulenzfunktion von AVR2 in Frage. Auf der anderen Seite bietet die spezifische Interaktion von AVR2 mit AtBSL1-3, aber nicht AtBSU1, die Möglichkeit, diesen Effektor als molekulares Werkzeug zur Entschlüsselung der durch BSL-regulierten zellulären Mechanismen zu verwenden.

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8 APPENDIX

Table 7 **List of primers used in this study.**

AVR2_for	CCCGAATTCCTGCATGCAGCTCCAGGTGC
AVR2_rev	CCCGCGGCCGCTTAACTCCTCTTGTCACCC
*att*B1	GGGGACAACTTTGTACAAAAAAGCAGGCT
*att*B2	GGGGACCACTTTGTACAAGAAAGCTGGGT
AthBSU1_*att*B1_for	AAAAAGCAGGCTttATGGCTCCTGATCAATCTTATCAATATCC
AthBSU1_*att*B2_rev	AGAAAGCTGGGTtTTATTCACTTGACTCCCCTCGAGC
AthBSL1_*att*B1_for	AAAAAGCAGGCTcaATGGGCTCGAAGCCTTGGCTACATCC
AthBSL1_*att*B2_rev	AGAAAGCTGGGTTTCAGATGTATGCAAGCGAGC
AthBSL2_*att*B1_for	AAAAAGCAGGCTTAATGGATGAAGATTCGTCTATGG
AthBSL2_*att*B2_rev	AGAAAGCTGGGTTTCACATCCAAGCCAGAGAACC
AthBSL3_*att*B1_for	AAAAAGCAGGCTATATGGATTTGGATTCTTCAATGG
AthBSL3_*att*B2_rev	AGAAAGCTGGGTTTTATATCCAAGCAAGAGAGCC
qSAUR-AC for	TGAGGAGTTTCTTGGGTGCT
qSAUR-AC rev	AAGTATGAAACCGGCACCAC
qSAUR-65 for	TGCCCTCAAGAGGAAAAGAA
qSAUR-65 rev	TCAACCGTGTAAACCACGAA
qRT-CPD-F1	TTGCTCAACTCAAGGAAGAG
qRT-CPD-R1	TGATGTTAGCCACTCGTAGC
qRT-DWF4-F1	CATAAAGCTCTTCAGTCACGA
qRT-DWF4-R1	CGTCTGTTCTTTGTTTCCTAA
qAct_for	AGTGGTCGTACAACCGGTATTGT
qAct_rev	GAGGAAGAGCATACCCCTCGTA
qFRK1_for	ATCTTCGCTTGGAGCTTCTC
qFRK1_rev	TGCAGCGCAAGGACTAGAG

Appendix

pDONR-for	TCGCGTTAACGCTAGCATGGATCTC
pDONR-rev	GTAACATCAGAGATTTTGAGACAC
qRT-NbDWF5-for	TGGCGGAGAGTCAGTTGGTA
qRT-NbDWF5-rev	CAGACCCATCGGCAAGAACA
qRT-NbSteroidDH-for	GCTTTTGTTGTATAGCCCTGCAT
qRT-NbSteroidDH-rev	TCAAAGCAGAAGAAAGCAAAGCA
qRT-NbXTH2-for	GGTTCGATCCATCCGCTGAT
qRT-NbXTH2-rev	TGGGTACTTGGTCCACGTAGA
qRT-NbExpA1-for	AGGGTGTTTGCCTGGTTCAA
qRT-NbExpA1-rev	TGGTGAAGGGGAGGATTACAC
qRT-NbCIB1/bHlH63-for	AGTATGGCTCAACGCCAGTT
qRT-NbCIB1/bHlH63-rev	TGATACAGGTGCGCTAGTGG
AVR2-53-116-frw:	AAAAAGCAGGCTTCATGGTCGATAATGGGGAATTTGAG
AVR2-66-116-frw:	AAAAAGCAGGCTTCACCATGGGATTCAGTCTGAAGGAT
AthBIN2-frw	AAAAAGCAGGCTTCATGGCTGATGATAAGGAGATG
AthBIN2-rev	AGAAAGCTGGGTCCTATTAAGTTCCAGATTGATTCAA
AthBIN2-1-frw	CAACTCGAAAAGAAATCCGTTGTATGAAT
AthBIN2-1-rev	GGATTTCTTTTCGAGTTGGTGTACCAAGAA
AthBSL2-663-frw	AAAAAGCAGGCTTCATGGCCCAAAGAAGGTTATAGCTCA
AthBSL2-650-rev	AGAAAGCTGGGTGTTACCTTGCTGCTGTTGCACTTTCT
AthBSL2-468-frw	AAAAAGCAGGCTTCACCATGGCGGCAACAAATTCTCCACCAG
AthBSL2-833-rev	AGAAAGCTGGGTGTTACTTCTCAATCGATGCAGCAAGA
AthBSL2-602-frw	AAAAAGCAGGCTTCACCATGATTCGTCTTCATCATAGAGCT
AthBSL2-535-frw	AAAAAGCAGGCTTCACCATGGTGGAGTATCTTGTTGAAGCA
AthBSL2-900-rev	AGAAAGCTGGGTGTCAGTCAGGCCCAAAAGTAACCAA
AthBSL2-966-rev	AGAAAGCTGGGTGTCAATGAATGAGCTTAGGTACCACTAC

Table 8 **MS analysis of Immunoprecipitation of GFP-AVR2.** (provided by C. Jäntsch)

Name	Function	Uniprot	Peptide identified in	
			14 days old plants	28 days old plants
BSL1	Ser/Thr-Protein Phosphatase	Q8L7U5	7	2
BSL2; BSL3	Ser/Thr-Protein Phosphatase	Q9SJF0; Q9SHS7-1;Q9SHS7-2	12	8

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