Insights into β-glucan biology in mutualistic plant-microbe interactions

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Philipp Fesel

geboren am 20.02.1989 in Meißen

Köln, 2018

Erstgutachter: Frau Prof. Dr. Alga Zuccaro
Zweitgutachter: Herr Prof. Dr. Paul Schulze-Lefert

Tag der mündlichen Prüfung: 19.12.2017

Ort und Jahr der Veröffentlichung: Köln, 2018

Zusammenfassung

Pflanzen sind fortwährend von pathogenen und mutualistischen Mikroorganismen, zum Beispiel Pilzen, umgeben. Um sich gegen pilzliche Infektionen zu schützen, vertrauen Pflanzen auf die Immunität jeder einzelnen Zelle. Sobald über Oberflächenrezeptoren mikroorganismenspezifische Signalmoleküle, so genannte MAMPs, wahrgenommen werden, wird eine Abwehrreaktion ausgelöst. Dabei werden z.B. verzweigte β-1,3/1,6-Glukane aus der Pilzzellwand als MAMPs von Pflanzenrezeptoren detektiert, da diese die Pilzzelle als fremdartige Struktur identifizieren. Die Pilzzellwand ist ein hochdynamischer Zellbestandteil, dessen Struktur und Zusammensetzung sich permanent verändert, um sowohl eine pflanzliche Abwehrreaktion zu vermeiden als auch die Stabilität zu gewährleisten.

Das Ziel dieser Untersuchung war die Identifizierung von Proteinen des mutualistischen Wurzelendophyten *Serendipita indica* und dessen Wirtspflanze *Arabidopsis thaliana*, die beim Schutz und der Erkennung von β-Glukanen eine Rolle spielen. Das *S. indica* Lektin WSC3 ist pflanzen- und pilzresponsiv transkriptionell induziert und lokalisiert an die *S. indica* Zellwand. In der Hefe *Pichia pastoris* wird WSC3-His in die Zellwand integriert und erhöht dort die Widerstandsfähigkeit gegenüber den zellwandschädlichen Substanzen Kongorot und Calcofluor Weiß. Es konnte weiterhin gezeigt werden, dass WSC3-His verzweigte β-1,3/1,6-Glukane binden kann und so zur Agglutination von Pilzzellen führt und zur Unterdrückung von β-1,3/1,6-Glukan-induzierten Abwehrreaktionen in Pflanzen beiträgt. Zusätzlich konnte gezeigt werden, dass das β-1,6-Glukan bindende Lektin FGB1 in der Lage ist, die von verschiedenen pilzlichen und bakteriellen MAMPs ausgelösten Abwehrreaktionen in Pflanzen zu unterdrücken. Bei der Untersuchung der β-1,3/1,6-Glukan-induzierten Abwehrreaktion konnten beachtliche Unterschiede, hinsichtlich der Stärke der induzierten Abwehrreaktionen, zwischen natürlichen *A. thaliana* Ecotypen festgestellt werden, die durch eine genomweiten Assoziationsstudie zur Identifizierung von möglichen Kandidaten genutzt wurden.

Zusammenfassend kann festgehalten werden, das WSC3 möglicherweise ein strukturgebender Komponent ist, der zur Stabilisierung der *S. indica* Zellwand beiträgt, während FGB1 als universeller Suppressor von MAMP-induzierten Abwehrreaktionen agiert. Die beachtlichen Unterschiede zwischen den *A. thaliana* Ecotypen hinsichtlich der Reaktion auf Laminarin und die Ergebnisse der genomweiten Assoziationsstudie legen Nahe, dass mehrere Gene an dieser Abwehrreaktion beteiligt sind.

Summary

Plants are constantly surrounded by pathogenic and mutualistic microbes, for example fungi. To defend against fungal invaders plants rely on the innate immunity of each cell which is activated in response to the perception of microbe-associated molecular patterns, so called MAMPs, by specific plant surface receptors. Fungal cell wall derived polysaccharides like branched β-1,3/1,6-glucans are perceived as MAMPs and identify a fungal cell as non-self. The fungal cell wall is a highly dynamic organelle that is constantly reshaped to tailor its structure and composition to circumvent the activation of the plant defense while ensuring cell wall integrity especially during plant colonization.

This study aimed to characterize proteins of the mutualistic root endophyte *Serendipita indica* and its experimental host plant *A. thaliana* that are engaged in β-glucan biology. The WSC-domain containing lectin WSC3, a member of a multigene family in *S. indica* is transcriptionally induced during plant colonization and in contact with a second fungus. The fusion protein WSC3-GFP localizes to the cell wall of *S. indica*, reduces the exposure of β-1,3-glucan and increases cell wall stress resistance in the yeast *Pichia pastoris*. Isothermal titration calorimetry revealed that WSC3-His binds the MAMP laminarin, a branched β-1,3/1,6-glucan and is able to agglutinate various fungal cells. Furthermore WSC3-His efficiently competes with the unknown plant receptor and thus prevents the induction of the production of reactive oxygen species (ROS) by laminarin but does not increase colonization of barley roots by *S. indica*. Additionally the secreted β-1,6-glucan specific lectin FGB1 of *S. indica* was shown to universally suppress ROS production in barley in response to the MAMPs laminarin, chitin and flg22. The investigation of the laminarin-induced ROS production in *A. thaliana* revealed great natural variation among 100 tested accession. A genome wide association screen identified several genetic loci that are potentially involved in laminarin-triggered defense responses in *A. thaliana*.

Collectively these results suggest that WSC3 might act as a structural component of the fungal cell wall to maintain cell wall integrity while FGB1 is acting as universal suppressor of MAMP-triggered ROS production. Both proteins underline the pivotal role of the fungal cell wall in general and especially of β-glucans during plant-microbe interactions. The responsiveness of *A. thaliana* to laminarin is subject of great natural variation and potentially represents a multigenetic trait.

List of abbreviations

AF594	Alexa Fluor 594
AGI	Arabidopsis genome initiative
bp	base pair
Blast	basic local alignment search tool
CM	complex medium
Col-0	*Arabidopsis thaliana* accession Columbia-0
d	day
dd	double distilled
DEPC	diethyl pyrocarbonate
DIC	differential interference contrast
DNA	deoxyribose nucleic acid
dpi	days post inoculation
DTT	dithiothreitol
ELC	enhanced chemiluminescence
FGB1	fungal glucan-binding 1
flg22	22 amino acid peptide of bacterial flagellin
GFP	green fluorescent protein
GO	gene ontology
GWAS	genome wide association screen
His	histidine
hpi	hours post inoculation
HRP	horseradish peroxidase
ITC	isothermal titration calorimetry
kb	kilobase
K_D	dissociation constant
kDa	kilodalton
LysM	lysin motif
MAMP	microbe-associated molecular pattern
Mb	megabase
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NTA	nitrilotriacetetic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
qRT-PCR	quantitative real-time PCR
RLU	relative luminescence units
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
TAIR	The Arabidopsis Information Resource
TEMED	tetramethylethylen-diamide
v/v	volume per volume
WSC	cell wall stress-responsive component
w/v	weight per volume

Table of Contents

1. Introduction 1

1.1 The plant immune system – repelling the bad, accommodating the good 1

1.2 Chitin as an example for the complexity of pattern recognition and fungal counter strategies 4

1.3 β-glucan as a MAMP and its elusive perception 6

1.4 *Serendipita indica* as a tool to disentangle β-glucan biology in plant-microbe interactions 10

1.5 Spotlight on the WSC tool kit of *S. indica* 13

1.6 Aim of this thesis 16

2. Results 17

2.1 Functional characterization of a WSC-lectin of *S. indica* 17

2.1.1 WSC3 is induced during root colonization and during contact with a plant pathogenic fungus and localizes to the *S. indica* cell wall 17

2.1.2 Recombinant WSC3-His increases *P. pastoris* cell wall stress resistance .. 22

2.1.3 WSC3-His binds the branched β-1,3/1,6-glucan laminarin but does not protect it from hydrolysis 26

2.1.4 WSC3 is able to agglutinate fungal cells 30

2.1.5 WSC3-His suppresses β-glucan triggered plant defense responses but does not increase root colonization 32

2.2 Identification of laminarin-responsive defense components by genome wide association screen 38

2.2.1 Natural variation among *A. thaliana* accessions regarding their ability to recognize chitin and laminarin as MAMPs 38

2.2.2 GWA screen to identify and classify genetic loci involved in chitin- and laminarin- responsive ROS product 41

2.2.3 Identification of genes involved in laminarin-triggered ROS production..... 44

3. Discussion 48

3.1 WSC3 is an integral cell wall component increasing cell wall stability 48
3.2 WSC3 specifically binds the branched β-1,3/1,6-glucans laminarin 52
3.3 WSC3 agglutinates fungal cells but is not involved in broad spectrum immune suppression 55
3.4 Summary: WSC3 as β-glucan adhesive? 58
3.5 Identification of defense components potentially involved in laminarin-triggered plant defense 59
3.6 General conclusion 63
4. Materials and sources of supply 65
4.1 Materials and suppliers 65
4.1.1 Chemicals and "kits" 65
4.1.2 Buffers, solutions and media 65
4.1.3 Enzymes 65
4.1.4 Oligonucleotides 66
4.2 Bacterial and Fungal Strains 68
4.2.1. *Escherichia coli* strains 68
4.2.2 *Pichia pastoris* strains 68
4.2.3 *Ustilago maydis* strains 69
4.2.4 *Bipolaris sorokiniana* strains 69
4.2.5. *Serendipita indica* strains 69
4.3 Plant cultivars and accessions 70
4.3.1 *Hordeum vulgare* cultivars 70
4.3.2. *Arabidopsis thaliana* accessions 70
4.4 Vectors 74
4.4.1 Vectors for protein expression in *P.pastoris* 74
4.4.2 Vectors for transformation of *S. indica* 74
4.5. Bacterial, fungal and plant cultivation 75
4.5.1. *E. coli* cultivation 75

4.5.2 *P. pastoris* cultivation 75
4.5.3 *U. maydis* cultivation 75
4.5.4 *B. sorokiniana* cultivation 75
4.5.5. *S. indica* cultivation 76
4.5.6. *A. thaliana* cultivation 77
4.5.7 *H. vulgare* cultivation 78
4.6. Microbiological methods 79
4.6.1 Preparation of chemocompetent *E. coli* cells 79
4.6.2 Heat shock transformation of *E. coli* 80
4.6.3 Transformation of *P. pastoris* 81
4.6.4. Collection of S*. indica* chlamydospores 81
4.6.5. Collection of B*. sorokiniana* chlamydospores 82
4.6.6. S*. indica – H. vulgare* interaction studies 82
4.6.7. *S. indica – A. thaliana* Col-0 interaction studies 83
4.6.8 *S. indica - B. sorokiniana* interaction on soil 83
4.6.9. S*. indica* protoplastation and PEG-mediated transformation 84
4.6.10 Assessment of fungal growth phenotype in presence of WSC3 86
4.6.11 Adhesion of *S. indica* spores to barley roots 87
4.6.12 Cell wall stress resistance assay 87
4.7. Molecular and biochemical methods 88
4.7.1. DNA isolation 88
4.7.2. RNA isolation 89
4.7.3. DNase digestion and cDNA synthesis 90
4.7.4. Quantitative real-time PCR 90
4.7.5 Purification of FGB1 from *S. indica* culture supernatant 91
4.7.6 Expression and purification of WSC3 in *P. pastoris* 91
4.7.7 Isothermal Titration Calorimetry 93
4.7.8 TCA precipitation of proteins 93

4.7.9 SDS PAGE 94
4.7.10 Western Blot 95
4.7.11 Laminarin protection assay 96
4.7.12 Protein pull-down with cell wall preparations 97
4.7.13 Enzymatic extraction of cell wall proteins 98
4.7.14 Preparation of the chitin suspension 99
4.7.15 Quantification of reactive oxygen species (ROS) 100
4.7.16 Quantification of MAMP-induced Ca^{2+}-fluxes 100
4.8. Microscopy 101
4.8.1 Confocal microscopy 101
4.8.2 Transmission electron microscopy 102
4.8.3 Light microscopy 103
4.9 Bioinformatics 103
4.9.1 Genome wide association screen - GWAS 103
4.9.2 agriGO 104
4.9.3 Mega7 104
5. References 105
6. Acknowledgements / Danksagungen 121

1. Introduction

1.1 The plant immune system – repelling the bad, accommodating the good

Plants are constantly interacting with microbes present in their surrounding. These microbes can be classified based on the nature of the interaction in beneficial, detrimental or neutral microbes. Past research on plant-microbe interactions strongly focused on the investigation of the molecular mechanisms underlying the interaction of one (mostly pathogenic) microbe with its host plant and resulted in the creation of the zig-zag model illustrating the different layers of the plant defense and corresponding microbial counter strategies (Jones & Dangl, 2006). In brief, an invading microbe is primarily recognized by conserved molecules that are not present in the plant and thus identified as non-self and potentially harmful. Such microbial molecules, designated as MAMPs for microbe-associated molecular patterns, are known for bacteria, fungi and oomycetes and are perceived by specific plant receptors, so called pattern-recognition receptors or PRRs (Boutrout & Zipfel, 2017, Yu *et al.*, 2017). The recognition of MAMPs leads to several different cellular defense responses including Ca^{2+}-influx into the cytoplasm, callose depositions into the cell wall and the production of reactive oxygen species (ROS) in order to repel the invader (Yu *et al.*, 2017). This basal and undirected state of immunity, designated as MAMP-triggered immunity or MTI, successfully fights and repels non-adapted microbes (Yu *et al.*, 2017). In turn adapted microbes employ specific, highly variable and often non-conserved proteins, so called effectors, to overcome MTI and to successfully colonize the host plant, a state called effector-triggered susceptibility or ETS (Lo Presti *et al.*, 2015). As a consequence of the co-evolution of plants and adapted microbes the plants in turn developed a second arsenal of immune receptors, called NB-LRR receptors for nucleotide-binding site and leucine-rich repeat receptors, that specifically recognize microbial effector-proteins leading to effector-triggered immunity (ETI) often accompanied by a hypersensitive response (De Wit *et al.*, 2009). Consequently microbes evolved effectors that are targeted against the NB-LRRs to suppress and circumvent their activity leading again to ETS. But also these effectors are potentially recognized by plant immune receptors, which restore the status of ETI. Over the last decade since the zig-zag model was introduced research on plant-microbe interactions accelerated calling for an update of the model (Pritchard & Birch, 2014; Cook *et al.*,

2015). For example the integration of endogenous plant molecules that are released upon a microbial invasion and thus were termed danger-associated molecular patterns (DAMPs), is missing in the current model. DAMPs, for example extracellular ATP or elicitor peptides (PEPs), are often present in parallel to classical MAMPs but only partially activate the same defense responses (Yamaguchi & Huffacker, 2011; Choi *et al.*, 2014; Tanaka *et al.*, 2014). Furthermore the zig-zag model falls short in explaining how plants orchestrate the different layers of immunity considering the presence of a microbiome consisting of a myriad of microbes with various lifestyles. The microbiome of a plant is defined as the entity of all microbes that live in or on a plant and together with the plant collectively form a holobiont (Vandenkoornhuyse *et al.*, 2015). The composition of the microbiome is not only shaped by the environment but also greatly influenced by the plant itself that for example recruits so called hub microbes that stabilize the microbiome (Coleman-Derr *et al.*, 2016; Bai *et al.*, 2015; Agler *et al.*, 2016). This process of direct and active shaping of the own microbiome to fulfill the current abiotic and biotic necessities was for a long time only known from animal systems but in the future will also massively influence the way of our understanding of the plant immune system (Ezenwa *et al.*, 2012; Hacquard *et al.*, 2017). The microbiome represents a novel layer that needs to be considered when describing the complexity of the plant immune system and was recently included into an updated version of the model (Hacquard *et al.*, 2017). To enable such a scenario a sophisticated crosstalk between plants and the desired microbes is required to ensure that only the detrimental or neutral microbes are repelled and the beneficial microbes are accommodated. The communication of beneficial microbes and plants potentially is the result of million of years of co-evolution and may have its origin in the ancient arbuscular mycorrhiza (AM) symbioses between fungi of the division *Glomeromycota* and 80 % of the land plants (Redecker *et al.*, 2000; Brundrett, 2009). Plants attract AM fungi for example by the production and release of strigolactones, a class of phytohormones that induces hyphal branching in AM fungi (Al-Babili & Bouwmeester, 2015; Pandey *et al.*, 2016). AM fungi in turn secrete lipochitooligosaccharides designated as Myc-factors and short-chain chitin oligomers that induce lateral root formation and the components of the SYM-pathway needed to establish AM symbiosis (Maillet *et al.*, 2011; Genre *et al.*, 2013). The plant receptors perceiving these AM-signals are conserved and share striking similarities with the receptors perceiving bacterial Nod-factors as signal to induce nodule formation a crucial step in the establishment of legume-rhizobium

symbiosis (Liang *et al.*, 2014). It was hypothesized that the perception of the Myc -and Nod-factors share the same evolutionary origin which might be found in some ancient immune receptors of algae (Liang *et al.*, 2014). Thus this intimate crosstalk between plants and symbiotic microbes relies on the integrity of a few plant components that are crucial for the perception of symbiotic signals and finally for the discrimination between beneficial and detrimental microbes. It was recently hypothesized that plants even safeguard these components with specific NB-LRRs that prevent the exploitation of these pathways by microbial cheaters (Hacquard *et al.*, 2017). Nevertheless there are symbiotic microbes that confer beneficial effects to their host plant and act independently of the SYM pathway (Banhara *et al.*, 2015). Furthermore not only pathogenic microbes employ effector proteins to suppress or modulate the plant immune system for successful colonization. Recent examples are the effector protein SP7 of the AM fungus *Glomus intraradices* that prevents induction of pathogenesis-related genes by interaction with the transcription factor ERF19, the mycorrhiza-induced small secreted protein MiSSP7 of the ectomyccorhizal fungus *Laccaria bicolor* that interferes with jasmonic acid signaling in *Populus* and the β-glucan-binding lectin FGB1 of the root endophyte *Serendipita indica* which suppresses MAMP-triggered immunity (Kloppholz *et al.*, 2011; Plett *et al.*, 2014; Wawra *et al.*, 2016).

Thus only a fine tuned plant immune system is able, to on the one hand side, allow the interaction with beneficial microbes that are crucial for plant fitness and on the other hand side to fight and restrict detrimental microbes that developed sophisticated strategies to overcome this barriers. The first layer of the immune system is the recognition of MAMPs by PRRs, which already by definition raises the question how a plant can distinguish between beneficial and detrimental microbes if conserved microbial molecules are sensed? Additionally the fungal cell wall constituent chitin exemplifies that the difference between a MAMP triggering a defense response and a symbiotic signaling molecule like a Myc- or Nod-factor might just be an acetyl-group or another chemical modification. Despite the fact that for example the *A. thaliana* genome encodes more than 600 receptor-like kinases for the perception of various signals, the receptors perceiving the antithetic chitin-signals belong to the same receptor family (Shiu & Bleeker, 2001; Liang *et al.*, 2014). Thus understanding the function of plant PRRs as well as the microbial counter strategies is crucial to build an integrated picture of the plant immune system.

1.2 Chitin as an example for the complexity of pattern recognition and fungal counter strategies

Chitin is a complex polysaccharide consisting of β-1,4-linked *N*-acetylglucosamine units and constitutes between 10 to 20 % of the dry mass of the cell wall of filamentous fungi (Bowman & Free, 2006). Chitin is not present in the plant tissue but as a crucial cell wall building block it is a potential MAMP strongly eliciting defense responses in plants and also a target for plant chitinases fighting fungal invaders. The relevance of chitin as a MAMP is underlined by the fact that *A. thaliana* possesses several receptors able to recognize chitin which mostly belong to a common receptor family designated as LysM-containing receptor-like kinases (LYKs). Nevertheless first steps to elucidate the mechanism of chitin-perception were made in rice (*Oryza sativa)* were the chitin oligosaccharide elicitor binding protein (*Os*CEBiP) was shown to form a heteromeric receptor complex with the chitin elicitor receptor kinase 1 (*Os*CERK1) in response to chitin-binding of *Os*CEBiP to elicit a defense response (Kaku *et al.*, 2006; Shimizu *et al.*, 2010; Hayafune *et al.*, 2014). A similar mechanism was later also described in *A. thaliana*. The *Os*CERK1 homolog CERK1 (or LYK1) forms a chitin-induced receptor complex with high-affinity chitin-binding receptor LYK5 which leads to an induction of immune signaling by the intracellular kinase-domain of CERK1 (Miya *et al.*, 2007; Cao *et al.*, 2014). Additionally to its function in chitin-induced immune signaling CERK1 was recently shown to also mediate the recognition of bacterial peptidoglycan by the formation of a receptor complex with the LysM-containing receptors LYM1 and LYM3 (Figure 1; Willmann *et al.*, 2011). Thus current models describe CERK1 as central hub in immune signaling of *A. thaliana* by translating extracellular MAMP-signals that are perceived by high-affinity receptors into an intracellular activation of various defense responses (Rövenich *et al.*, 2016; Couto & Zipfel, 2016). Even though the CERK1/LYK5 complex seems to be the main route for chitin perception and activation of defense responses there are more receptors shown to act in response to chitin. Another member of the LYK family, LYK4 is involved in chitin-induced defense signaling even though the mechanism is still elusive (Wan *et al.*, 2012). Furthermore LYM2, the *A. thaliana* homolog of *Os*CEBiP acts independent of CERK1 and regulates plasmodesmata flux a secondary immune response (Faulkner *et al*, 2013; Narusaka *et al.*, 2013).

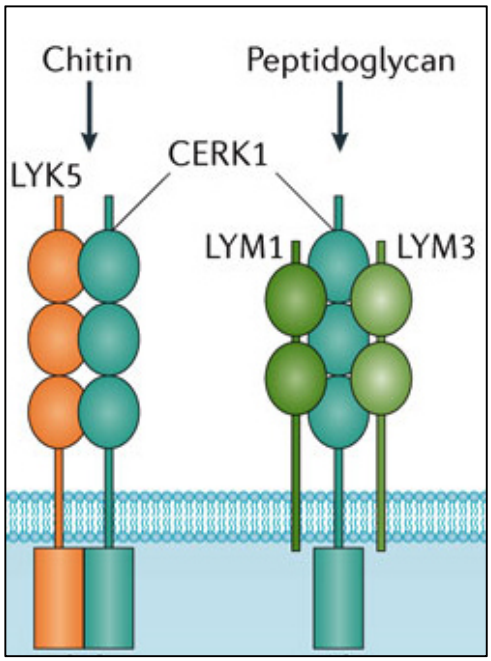

Figure 1: CERK1 is involved in chitin- and peptidoglycan-perception in *A. thaliana*. Upon binding of the fungal MAMP chitin the transmembrane chitin elicitor receptor kinase 1 (CERK1) forms a heteromeric receptor complex with the LysM-containing receptor-like kinase 5 (LYK5) to induce intracellular immune signaling mediated by the kinase-domain of CERK1. In the same way CERK1 is involved in the perception of bacterial peptidoglycan by binding of the MAMP to the LysM domain proteins 1 and 3 (LYM1 and LYM3) which upon binding form a signaling complex with CERK1. Ellipse – LysM domain, stalk – protein backbone and transmembrane domain, rectangle – kinase domain. Figure adapted from Couto & Zipfel, 2016.

LysM-containing plant receptors are not only involved in immune signaling upon chitin perception but do also act in response to the Myc-factors of AM fungi and Nod-factors of rhizobacteria to regulate symbiotic interactions (Den Camp *et al.*, 2011; Liang *et al.*, 2013; Liang *et al.*, 2014; Buendia *et al.*, 2016). In addition recent evidence suggests that the CERK1 receptor of rice is not only involved in defense signaling but has a dual function and also mediates the perception of Myc- and Nod-factors (Miyata *et al.*, 2014; Zhang *et al.*, 2015). The complexity and multiplicity of the chitin-responsive receptor families illustrates on the one hand side the importance of chitin and chitin-derivatives in various plant processes and on the other hand side might be a results of a common evolutionary history (Liang *et al.*, 2014).

As multifaceted as the plant components perceiving and transducing chitin-signals are the fungal counter strategies. One possible way to avoid recognition of chitin is the modification of the cell wall for example through deacetylation of chitin to chitosan (El Gueddari *et al.*, 2002). Another strategy was described in *Magnaporthe oryzae* where cell wall chitin is masked by α-1,3-glucan which protects it from the hydrolysis by plant-derived chitinases (Fujikawa *et al.*, 2012). In general protection of chitin from hydrolysis or inhibition of the hydrolytic enzyme activity seem to represent efficient strategies. The fungal effector Avr4 from *Cladosporium fulvum* binds to the chitin components of the cell wall and protects them from hydrolysis by chitinases (van den Burg *et al.*, 2006). Direct targeting of chitinases mostly involves the cleavage of the enzymes by dedicated proteases (Naumann & Wicklow, 2013; Jashni *et al.*, 2015). Due to the high binding affinity of the plant receptors, which was shown to be as low as 1 µM for LYK5

even extremely low concentrations of chitooligosaccharides are sufficient to trigger a defense response. Thus another fungal strategy is the sequestration of soluble chitin oligomers from the apoplast like described for the *C. fulvum* effector protein Ecp6 (de Jonge *et al*, 2010). Ecp6 consists of three LysM domains, which are also responsive for chitin-binding of the plant receptors but outcompetes the receptors with a much higher binding affinity in the picomolar range due to an intrachain dimerization of two of the LysM domains (Sanchez-Vallet *et al.*, 2013). The sequestration of free chitin oligomers from the apoplast seems to be a very powerful strategy since homologous proteins to Ecp6 proposed to fulfill a similar mechanism were described in *Zymoseptoria tritcii*, *Magnaporthe oryzae* and *Colletotrichum higginsianum* (Marshall *et al.*, 2011; Mentlak *et al.*, 2012; Takahara *et al.*, 2016).

Chitin as a potent MAMP illustrates that both plants and fungi employ complex toolkits either to sense the invader or to prevent recognition and hydrolysis in order to successfully colonize the host. In the cell walls of the few fungi analyzed the most abundant fungal cell wall constituent is β-glucan which constitutes between 50 and 60 % of its dry mass (Bowman & Free, 2006, Latgé, 2007; Latgé *et al.*, 2017). β-glucans were described as potent elicitors of plant defense responses originating from the oomycete cell wall, which does not contain chitin (Fesel & Zuccaro, 2016a). Despite its higher abundance in the fungal cell wall and its potential to induce defense responses in plants its role in plant-microbe interactions is less understood than for chitin.

1.3 β-glucan as a MAMP and its elusive perception

The fungal cell wall was for a long time believed to form a rigid armor which is encapsulating the cytoplasm. Recently this view changed towards the description as highly dynamic organelle whose structure and compositions is constantly changing according to the cell type and the growth condition (Gheoghegan *et al.*, 2017; Latgé *et al.*, 2017). Especially during biotrophic interactions the remodeling of the cell wall architecture is crucial because the cell wall is mostly the first cellular structure which is getting into contact with the hosts immune system and thus represents the primary battleground (Gheoghegan *et al.*, 2017; Latgé *et al.*, 2017). As initially described fungal cell wall derived polysaccharides are potent elicitors of immune responses and thus their exposure needs to be prevented while maintaining cell wall integrity. Whereas chitin structure and content seems to be vastly similar in most of the analyzed fungi,

the glucan content and especially composition varies greatly between species (Bowman & Free, 2006; Latgé, 2007; Gheoghegan *et al.*, 2017). The most abundant cell wall polysaccharides are β-glucans whereas between 65 % and 90% of the β-glucans present in the cell wall are linked by β-1,3-glycosidic linkages forming long fibrils consisting of up to 1,500 glucose units interconnected with each other with β-1,6-glycosidic linkages which account for 3 % to 10 % and confer rigidity to the cell wall (Figure 2; Shahinian & Bussey, 2000; Bowman & Free, 2006; Latgé, 2007). The linkage between the β-glucan network and chitin is mostly assembled by β-1,4-glycosidic linkages (Latgé, 2007). Besides β-glucans also α-glucans are part of the fungal cell whose content massively varies constituting for example up to 40 % of the *A. fumigatus* cell wall where it acts as cement to fill the pores (Ruiz-Herrera, 1991, Bowman & Free, 2006; Latgé *et al.*, 2017). Whereas chitin is located directly adjacent to the plasma membrane the β-glucan fibrils are primarily firmly bound to the rest of the cell wall constituent but were also found to be present around the hyphae as a matrix (Ruel & Joseleau, 1991; Ruiz-Herrera, 2012).

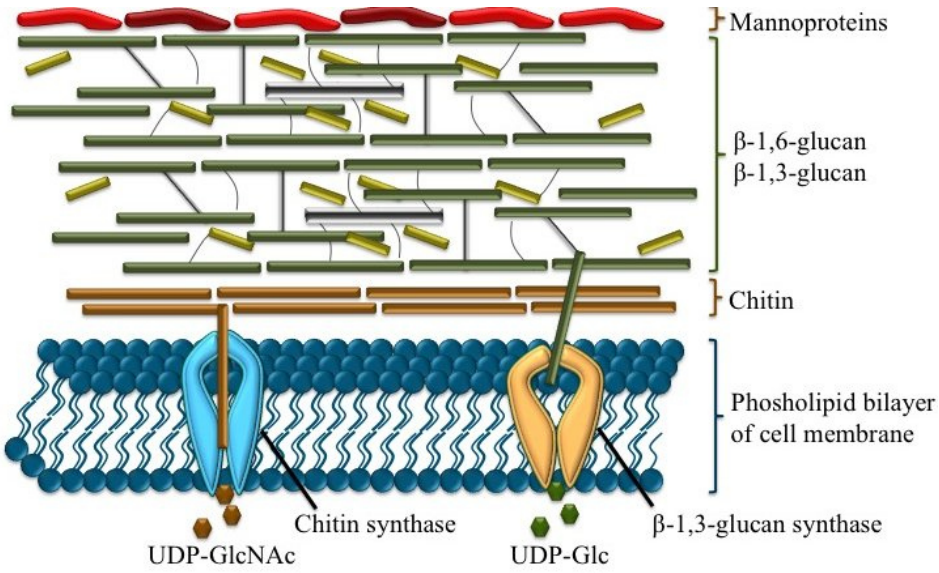

Figure 2: Simplified model of the fungal cell wall and its main constituents. The fungal cell wall is located in the extracellular space directly adjacent to the phospholipid bilayer of the cell membrane (dark blue). The innermost layer consists of chitin fibrils (brown), which are synthesized by transmembrane chitin synthases (light blue) that shuttle uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc, brown hexagons) trough the membrane and attach it to preexisting chitin fibrils. The most abundant cell wall polysaccharide is β-glucan, mostly β-1,3-glucan (dark green) and β-1,6-glucan (light green), which are synthesized in a similar way like the chitin fibrils by transmembrane β-1,3-glucan synthases (yellow) from uridine diphosphate-glucose units (UDP-Glc, green hexagons). The outermost layer consist of mannoproteins (red). Figure adapted from Fesel & Zuccaro, 2016a.

The mechanism of β-1,3-glucan synthesis is not fully understood but most probably is accomplished by transmembrane β-1,3-glucan synthases that attach intracellularly supplied uridine diphosphate-glucose (UDP-Glc) to preexisting β-glucan fibrils present in the extracellular space (Douglas, 2001; Ruiz-Herrera, 2012). Nevertheless the sheer abundance of β-glucan does not make it a potential MAMP especially since β-1,3-glucan is also present in plant cell walls as callose reinforcements and thus is even discussed as potential DAMP (Beffa *et al.*, 1996; Klarzynski *et al.*, 2000). In contrast β-1,6-glucan is specific to fungi and oomycetes and not present in plants except of the some members of the phylum chromista, which makes it a potential MAMP (Bartnicki-Garcia, 1968; Sietsma *et al*., 1969; Fesel & Zuccaro, 2016a).

Accordingly the elicitor activity of β-glucan fragments originating from fungal and oomycete cell walls is known for a long time whereas the β-1,6-glycosidic linkages are crucial for the recognition (Ayers *et al.*, 1976, Ebel *et al.*, 1976; Albersheim & Valent, 1978, Sharp *et al.*, 1976a, b; Anderson 1978, 1980). Interestingly and contrasting to chitin the ability to recognize β-glucans as elicitors is not universal but varies greatly between different plant species and families (Albersheim & Valent, 1978 Anderson 1978; Yamaguchi *et al.*, 2000). Thus there is potentially a high degree of plant and microbe species specificity, which argues for several independent evolutionary events or is the result of persistent co-evolution between plant and microbes. As a result the non-physiological mixed β-1,3/1,6-glucan laminarin from the brown algae *Laminaria digitata* is widely used in research because it elicits various defense responses in different plants (Klarzynski *et al*, 2000; Aziz *et al.*, 2003; Menard *et al.*, 2004). Additionally not only structure and composition of the β-glucans seem to be important for recognition but also chemical modifications like sulfatations might make a difference (Menard *et al.*, 2004, Menard *et al,* 2005; Gauthier *et al.*, 2014). Nevertheless most of the studies in the past trying to identify the potential β-glucan receptor(s) were performed with mixed β-1,3/1,6-glucan released from *Phytophtora* cell walls by acid hydrolysis and yielded first evidence for the presence of a putative component necessary for β-glucan perception in soybean (Yoshikawa *et al.*, 1983). The identified soluble glucan-binding protein (GBP) from soybean was further characterized revealing a particularly low binding affinity to the β-glucan ligand compared to the known chitin receptors and a specificity for β-1,6-glycosidic linkages (Schmidt & Ebel, 1987; Cosio *et al.*, 1990; Cheong & Hahn 1991; Cheong *et al.*, 1992). Intriguingly subsequent studies of the GBP revealed that it does not possess the characteristics of

a classical receptor or receptor-like protein but consists of the expected glucan-binding domain and additionally a glucosidase domain exhibiting β-1,3-glucanase activity (Umemoto *et al.*, 1997; Fliegmann *et al.*, 2004). The GBP was further shown to bind and hydrolyze the oomycete-derived mixed β-1,3/1,6-glucan and also laminarin but can possibly be inhibited by direct competition by a cyclic β-1,3/1,6-glucan from a symbiotic bacterium (Mithöfer *et al.*, 1996; Fliegmann *et al.*, 2004). Furthermore homologs of the GBP are present not only in the Fabaceae as initially suspected but in nearly all plant families and were postulated to be part of the receptor complex that perceives the β-glucan signals (Fliegmann *et al.*, 2004). In summary the GBP is a highly interesting candidate being essential for the tailoring and thus the perception of oomycete and potentially also fungal β-glucan MAMPs but does not perceive the signal directly. Thus the potential β-glucan receptor is still elusive. In general the research on β-glucan recognition is greatly hindered by the availability of suitable substrates. Neither the algal polysaccharide laminarin nor the oomycete-derived heptaglucoside, which was released from the cell wall by acid hydrolysis are physiological substrates that are present during the initial contact of plants and microbes. Despite their limitations both substrates as well as linear β-glucans are useful tools to study β-glucan recognition in plants.

Even though the identity of the β-glucan receptor(s) and the mechanism of perception is unknown the recently discovered fungal strategies to avoid β-glucan recognition are pointing towards its importance as a MAMP. It was shown for the maize pathogen *Colletotrichum graminicola* that the expression of the β-1,3-glucan- and β-1,6-glucan-synthases and thus the β-glucan content is reduced in the biotrophic hyphae colonizing living cells potentially to evade recognition (Oliveira-Garcia & Deising, 2013; Oliveira-Garcia & Deising, 2016). Additionally expression of both genes and thus the β-glucan content was massively increased in the appressoria and necrotrophic hyphae to withstand the high turgor pressure and to enable rapid growth of the hyphae, respectively. Even though it is not clear how the decreased β-glucan content is compensated in the biotrophic hyphae, this mechanism seems to be an efficient way to balance between cell wall rigidity and the evasion of the detection by the plant immune system. Another mechanism was recently described involving the first fungal effector protein directly targeting β-glucan-induced immunity. The β-1,6-glucan specific lectin FGB1 of the root endophyte *S. indica* is suppressing β-glucan triggered immunity

in plant and additionally is altering the cell wall composition potentially to increase resistance to external stressors (Wawra *et al.*, 2016).

These examples clearly highlight the importance of β-glucan as crucial cell wall constituent to ensure cell wall stability and integrity that needs to be protected from hydrolysis and recognition. Nevertheless the mechanism of β-glucan recognition and the key players involved in this process on the plant side as well as the microbial strategies to evade recognition and to maintain cell wall integrity remain nebulous. The mutualistic root endophyte *S. indica* seems to be a valuable tool to dissect fungal mechanisms involved in β-glucan biology and additionally might help to understand the plant perception machinery.

1.4 *Serendipita indica* as a tool to disentangle β-glucan biology in plant-microbe interactions

Fungal endophytes represent a powerful tool to investigate the defense mechanisms shared between but also specific for the response to beneficial and detrimental microbes respectively since they just recently diverged from their pathogenic ancestors or potentially mark an intermediate state during the evolution from a saprotrophic to a biotrophic lifestyle (Xu *et al.*, 2013; Hacquard *et al.*, 2016; Fesel & Zuccaro, 2016b). Especially the well-characterized mutualistic root endophyte *S. indica* (syn.: *Piriformospora indica*) may be of great interest for this purpose as in the past years it was developed as a model system for the order Sebacinales. Sebacinales represent one of the most basal orders within the basidiomycetes with members covering a large spectrum of fungal lifestyles from saprotrophs to ectomycorrhizal fungi with the exception of pathogens (Kohler *et al.*, 2015; Garnica *et al.*, 2016; Weiß *et al.,* 2016). Sebacinales fungi are ubiquitously found in nearly all terrestrial ecosystems interacting with a large variety of plants including the model plant *A. thaliana* and might represent hidden players that have been overlooked for a long time probably due to the fact that they are in particular prone to conventional agriculture (Weiß *et al.,* 2011; Oberwinkler *et al.,* 2013; Riess *et al.,* 2014; Verbruggen *et al.,* 2014). *S. indica* potentially marks an intermediate state during the evolution from saprotrophic nutrition to (obligate) biotrophic nutrition which for example is reflected by the sustainment of an arsenal of plant cell wall degrading enzymes which allow the feeding on senescent plant tissue and synthetic growth medium and whose loss is considered as a hallmark of the

evolution of biotrophy (Schulz & Boyle, 2005; Veneault-Fourrey & Martin, 2011; Zuccaro *et al.*, 2011; Kohler *et al.,* 2015; Lahrmann *et al.,* 2015). *S. indica* undergoes a transcriptomic and nutritional reprogramming during root colonization accompanied by the massive colonization of dead root tissue (Zuccaro *et al.,* 2011; Lahrmann & Zuccaro, 2012; Lahrmann *et al.,* 2013). Before switching to the cell death associated colonization phase *S. indica* hyphae are predominantly found within living root epidermal and cortex cells representing the initial biotrophic colonization phase (Zuccaro *et al.,* 2011; Lahrmann & Zuccaro, 2012). Even though the colonization of plant roots is accompanied by the suppression of the plant immune system and the induction of programmed cell death *S. indica* confers a myriad of beneficial effects on its host plants potentially due to the fact that colonization is preferentially found in the maturation zone of the roots (Waller *et al.*, 2005; Jacobs *et al.,* 2011; Qiang *et al.,* 2012). These beneficial effects include increased nutrient uptake, growth, yield and resistance to biotic and abiotic stresses (Peskan-Berghöfer *et al.,* 2004; Sherameti *et al.,* 2005; Waller *et al.,* 2005; Shahollari *et al.,* 2007; Sherameti *et al.,* 2008; Yadav *et al.,* 2010; Lahrmann *et al.,* 2015). Sequencing of the *S. indica* genome not only reflects the biphasic lifestyle combining initial biotrophic and subsequent saprotrophic nutrition but also uncovered a unique expansion of genes encoding proteins with carbohydrate binding properties (Zuccaro *et al.,* 2011; Lahrmann & Zuccaro, 2012). The physiological relevance of this expansion of carbohydrate binding proteins which might be to compensate the lost ability to produce secondary metabolites is further substantiated by the fact that a large set of these genes is transcriptionally induced during root colonization (Zuccaro *et al.,* 2011; Lahrmann & Zuccaro, 2012; Lahrmann *et al.,* 2015; Fesel & Zuccaro, 2016b). Beneath the chitin-binding LysM proteins known as extinguishers of host immunity and the cellulose-binding CBM1 proteins potentially involved in loosening of the plant cell wall, WSC proteins are remarkably enriched in the genome of *S. indica* even though their function in plant-microbe interaction has not been reported so far (Gaulin *et al.*, 2002; Saloheimo *et al.*, 2002; DeJonge & Thomma, 2009). Proteins with WSC domains have been initially described in the yeast *Saccharomyces cerevisiae* as transmembrane sensors activating the cell wall integrity (CWI) pathway and as part of a β-1,3-glucanase of *Trichiderma harzianum* and thus are thought to bind β-1,3-glucans (Verna *et al.*, 1997; Cohen-Kupiec *et al.*, 1999; Lodder *et al.*, 1999). In total the genome of *S. indica* encodes 35 WSC proteins with 22 of these being differentially expressed during colonization of plant roots (Zuccaro

et al., 2011; Lahrmann & Zuccaro, 2012). In total 28 of the WSC domain-containing proteins display a lectin-like structure meaning that they are devoid of other (enzymatic) domains expect of the WSC domain and a predicted signal peptide (Goldstein *et al.*, 1980; Gabius *et al.*, 2002).

This highlights the importance of WSC proteins during plant colonization but raises the questions why *S. indica* needs so many proteins potentially involved in β-glucan biology? Thus studying the biological function of these WSC proteins and understanding their role during plant-microbe interaction might shed light on fungal mechanisms involved in β-glucan biology Beyond the vast number of proteins potentially involved in β-glucan biology the biphasic lifestyle of *S. indica* might require a remodeling of the cell wall to suit the needs of different nutritional strategies and cellular environments. In the hemibiotrophic maize pathogen *Colletotrichum graminicola* the synthesis and exposure of β-1,3- and β-1,6-glucan is tightly balanced to adapt the cell wall composition to the different needs during the biotrophic and necrotrophic colonization phase (Oliveira-Garcia & Deising, 2013; Oliveira-Garcia & Deising, 2016). The lifestyle switch observed in *S. indica* may also demand special strategies to tailor the cell wall composition and structure to the different colonization phases like described before for phytopathogenic fungi (Geoghegan *et al.*, 2017). Furthermore the broad host range of *S. indica* might be the result of a universal strategy to prevent β-glucan recognition by the plant immune system which due to the uniqueness of the different β-glucan structures recognized by different plant species is hardly conceivable. Thus *S. indica* either is targeting a molecular hub involved in β-glucan perception that is conserved among plant families or evolved various molecular tools to prevent recognition of the released β-glucan molecules which are employed according to the current plant host. In both cases studying these strategies might be of great value to better understand β-glucan biology on both sides of the interaction. Collectively these features turn *S. indica* into an interesting system to study β-glucan biology in plant-colonizing fungi. Due to the unique expansion of WSC proteins encoded in the *S. indica* genome which was later on also observed in the closely related sebacinoid fungus *S. vermifera* a closer look onto this protein family might give first insight in *S. indica* β-glucan biology.

1.5 Spotlight on the WSC tool kit of *S. indica*

The WSC domain was first found as part of the cell **w**all **s**tress-responsive **c**omponents (WSC) in *S. cerevisiae* which act as transmembrane sensor proteins activating the cell wall integrity (CWI) pathway (Verna *et al.*, 1997; Lodder *et al.*, 1999). The CWI pathway acts *via* MAP kinase cascade and activates cellular responses as a reaction to osmotic or pH-stress, or in response to cell growth (Verna *et al.*, 1997; Klis *et al.*, 2002; Tong *et al.*, 2016). The WSC domain itself consists of up to eight conserved cysteine residues and is located at the N-terminus of the WSC1-4 (Verna *et al.*, 1997; Futagami *et al.*, 2011; Jendretzki *et al.*, 2011). WSC1-4 act as mechanosensors and possess a nanospring-like behavior that relies on the anchoring of the C-terminus of the protein at the plasmamembrane by its transmembrane domain and of the N-terminus at the cell wall by the WSC domain (Dupres *et al.*, 2009; Jendretzki *et al.*, 2011). When the integrity of the complex consisting of the plasmamembrane and the cell wall is disrupted the CWI pathway is activated leading to cell wall remodeling (Heinisch *et al.*, 1999; Levin 2005). Beneath anchoring WSC1-4 at the cell wall the WSC domain was furthermore shown to be crucial for the clustering of the WSC protein for example at sides of cell growth and thus was proposed to be additionally involved in the mediation of protein-protein interactions (Heinisch *et al.*, 2010; Jendretzki *et al.*, 2011). The WSC1-4 are not only present in *S. cerevisiae* but were also found to be involved in the activation of the CWI pathway in *Kluyveromyces lactis*, the filamenteous fungi *Aspergillus nidulans*, *Neurospora crassa*, and *Beauveria bassiana* and even in the algae *Fuccus serratus* (Rodicio *et al.*, 2007; Futagami *et al.*, 2011; Maddi *et al.,* 2012; Herve *et al.*, 2015; Tong *et al.*, 2016). Additionally to the described function as CWI sensors WSC domains are also present in fungal β-1,3-glucanases for example of *T. harzianum* or in several proteins of the nematode-trapping fungus *Monacrosporium haptotylum* (Cohen-Kupiec *et al.*, 1999; Andersson *et al.*, 2013). The WSC domain-containing proteins of *M. haptotylum* which interestingly show a comparable expansion like in *S. indica* are proposed to be involved in cell adhesion (Andersson *et al.*, 2013). A phylogentic clustering of the concatenated amino acid sequences of the 35 WSC domain-containing proteins of *S. indica* reveals that those proteins cluster together that display a similar domain architecture (Figure 3). For example the proteins that cluster together in the lower part of the tree mostly display a lectin-like structure, a more or

less similar number of WSC domains per protein and a similar overall length of the protein.

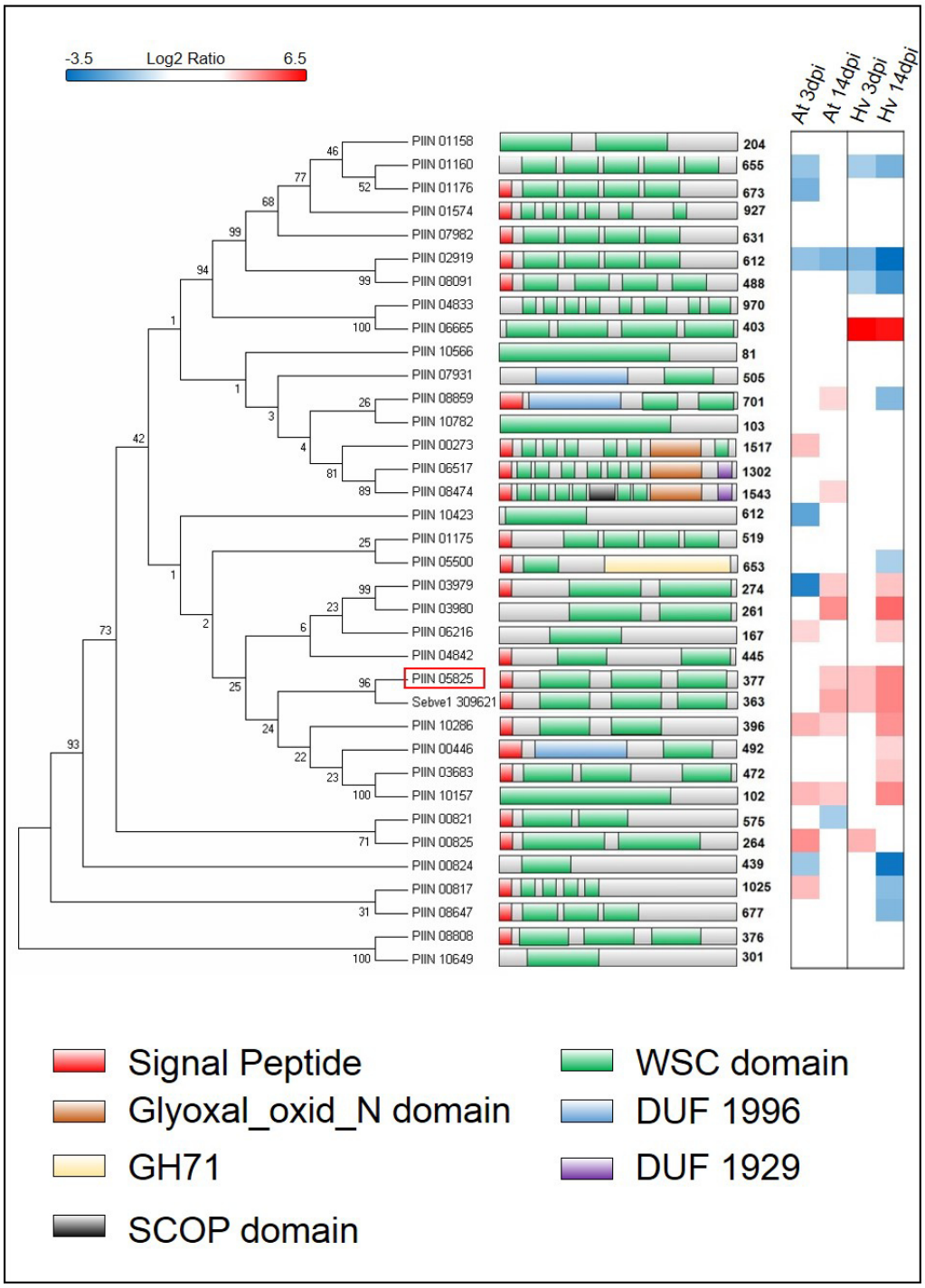

Figure 3: Phylogenetic classification of the WSC proteins in S*. indica* and expression of the WSC proteins during Arabidopsis and barley colonization. The concatenated protein sequences of *S. indica* WSC proteins were aligned using the MUSCLE algorithm and subsequently phylogenetic clustering was performed using the maximum parsimony method with 1000 bootstraps in Mega7 (Kumar et al., 2016). Additionally the closest homologs of the *S. indica* gene *PIIN_05825* (highlighted in red and subject of further analysis) in *Serendipita vermifera* (*Sebve1_309621*) was included into the analysis.

The overall domain architecture of the WSC proteins is depicted next to the phylogenetic tree with the length of the amino acid sequence in bold letters next to it. The respective Pfam domains are color coded like following: signal peptide – red, WSC domain – green, Glyoxal_oxid_N domain – brown, DUF1996 domain – blue, GH71 domain – yellow, DUF1929 domain – purple and SCOP domain – black. The heatmap shows the log2-fold changes for the expression of the WSC proteins during colonization of *A.thaliana* at 3 dpi (1st column, At 3dpi) and 14 dpi (2nd column, At 14dpi) and *H. vulgare* roots at 3 dpi (3rd column, Hv 3dpi) and 14 dpi (4th column, Hv 14dpi), compared to cultivation on PNM. Transcriptome data for *S. indica* and *S. vermifera* resulted from microarray experiments published in Lahrmann et al., 2015 and data deposited at GEO (*S. indica*: GSE60736; *S. vermifera*: GSE60736).

Interestingly those proteins form also the active tool kit of WSC domain-containing proteins which were shown to be transcriptionally induced during barley and Arabidopsis root colonization (Zuccaro *et al.,* 2011; Lahrmann & Zuccaro, 2012). Within this active tool kit is the lectin-like WSC domain-containing protein encoded by the *S. indica* gene *PIIN_05825* (highlighted in red) which is further termed WSC3. WSC3 consists of three WSC domains and a predicted signal peptide which resembles the domain structure of the immune-suppressive LysM lectins of *C. fulvum*, *Z. tritcii*, *M. oryzae* and *C. higginsianum* (De Jonge *et al.*, 2010; Marshall *et al.*, 2011; Mentlak *et al.*, 2012; Takahara *et al.*, 2016). Additionally *WSC3* is among the most highly transcriptionally induced genes during colonization of barley and Arabidopsis roots and thus might be generally needed during plant colonization. The importance of WSC3 is further substantiated by the fact that the homologues protein Sebve1_309621 of the closely related sebacinoid fungus *S. vermifera*, which is also included in the phylogenetic analysis and clusters together with WSC3, shows a similar (up)regulation pattern during plant colonization (Lahrmann *et al.,* 2015).

WSC domain-containing proteins are promising candidates to fill the existing gap in our understanding of fungal strategies to cope with β-glucan as an activator of plant immunity and to maintain β-glucan integrity to ensure cell wall stability. WSC domain-containing proteins are predicted to bind to β-1,3-glucan but their role in plant-microbe interactions is elusive. The *S. indica* lectin-like protein WSC3 is transcriptionally induced during colonization of plant roots and thus might be involved in mastering the requirements to hide and/or protect the β-glucan components of the *S. indica* cell wall.

1.6 Aim of this thesis

The aim of this thesis was to gain a deeper insight into β-glucan biology during the interaction of the sebacinoid root endophyte *S. indica* with its experimental host plants *H. vulgare* and *A. thaliana*. Especially the expanded family of WSC domain-containing proteins of *S. indica* was further investigated using the candidate protein WSC3. WSC3 was overexpressed in *S. indica* as GFP-fusion protein to study its localization and its effect on cell wall structure. Furthermore the recombinant expression in the yeast system *Pichia pastoris* was established to first biochemically characterize the purified protein and second to assess the impact of WSC3 on cell wall stress resistance in an organism devoid of WSC-lectins. The purified protein was biochemically analyzed in regards of its binding specificity and kinetics to several oligo- and polysaccharide ligands and tested for its impact on plant colonization and fungal growth. Ultimately WSC3 and the novel β-1,6-glucan specific lectin FGB1 were tested for their ability to suppress MAMP-triggered immunity in plants.

To also shed light onto the plant components involved in β-glucan induced immunity a genome wide association screen was performed making use of the natural variation regarding the laminarin-induced ROS production. Therefore 100 *A. thaliana* accessions were assayed for their laminarin-induced reactive oxygen production. This data was used to identify genetic loci that can be linked to this trait in *A. thaliana.*

Collectively these two approaches will help to understand how fungal derived β-glucans are involved in plant-microbe interactions and which key components and strategies are employed by fungi and plants to cope with these molecules.

2. Results

2.1 Functional characterization of a WSC-lectin of *S. indica*

2.1.1 WSC3 is induced during root colonization and during contact with a plant pathogenic fungus and localizes to the *S. indica* cell wall

The WSC-domain is proposed to bind to β-1,3-glucans but a function in plant microbe interactions has not been described so far. Therefore the WSC-protein encoded by the gene *PIIN_05825*, further termed as WSC3, was chosen for functional characterization. The microarray and RNAseq data collected during colonization of *H. vulgare* and *A. thaliana* were verified by qRT-PCR and additionally it was tested if WSC3 is transcriptionally induced during confrontation with the plant pathogenic ascomycete *Bipolaris sorokiniana*. Plants were inoculated with *S. indica* spores and roots were harvested after 1, 3, 7 and 14 days of growth on synthetic media under sterile conditions. For the assessment of transcriptional induction of WSC3 by the presence of a second fungus *S. indica* and *B. sorokinana* mycelia were mixed and grown for 2 days. The RNA was extracted from all the samples and reverse transcribed into cDNA, which was used for qRT-PCR with primers specific for the WSC3 encoding gene *PIIN_05825* and the *S. indica* gene *PIIN_03008* encoding the transcription elongation factor Tef routinely used as house keeping gene.

Expression of WSC3 is highest at 1 dpi during colonization of *H. vulgare* roots (8.7-fold up-regulated), then declines to 3.7-fold up-regulation at 7 dpi and finally is 6.7-fold up-regulated at 14dpi (Figure 4a). This expression pattern is in line with the microarray data, which showed an up-regulation of WSC3 at all time points of the interaction (Figure 3). The timing of expression suggests that WSC3 is relevant at all colonization stages but is particularly relevant during the initiation of the interaction. A similar expression pattern was observed for the interaction with *A. thaliana* even though at lower levels which is in line with the results of the microarrays. WSC3 is highest up-regulated at 1 dpi (2.6-fold) and the expression decreased over time to 1.5-fold induction after 14 dpi. During the confrontation with *B. sorokiniana* in soil WSC3 was 3.2-fold up-regulated compared to growth of *S. indica* in soil without the presence of *B. sorokiniana*. This suggests that WSC3 is not only involved in plant colonization but is also required during contact with a second fungus.

Since WSC-protein were proposed to bind to β-1,3-glucan and WSC3 harbors a signal peptide it was hypothesized that WSC3 may localize to the cell wall of *S. indica.* Therefore it was initially investigated if β-1,3-glucans are exposed during growth of *S. indica* in complex medium by visualization of the β-glucan components of the *S. indica* cell wall at an ultrastructural level using transmission electron microscopy. *S. indica* mycelium was grown for five days in liquid CM medium and used for immunogold labelling with WGA to visualize chitin and with a β-1,3-glucan-specific antibody (Figure 4b). Transmission electron microscopy (TEM) was realized in collaboration with Dr. Ulla Neumann at the Central Microscopy Facility (CeMic) of the Max-Planck-Institute for Plant Breeding Research in Cologne. Gold particles conjugated to WGA localizing to areas where chitin is exposed are predominately found at the cell wall (CW, black arrow) of *S. indica* hyphae (Figure 4b pictures A and B). In the septa (black arrowhead) of *S. indica* and the dolipore (black asterisk), a basidiomycete-specific structure within the septa that allows exchange of the cytoplasm and nuclei between the two compartments, chitin is completely absent. In contrast the β-1,3-glucan-specific antibody localizes to the cell wall (CW, black arrow) but also to the septa (black arrowhead) and the dolipore (black asterisk) of *S. indica* hyphae (Figure 4b pictures C and D). Thus it was concluded that β-1,3-glucans are present in the cell wall of *S. indica* and accessible for the specific antibody especially at the septa.

To study the subcellular localization of WSC3 the protein was expressed as fusion with a C-terminal GFP. A similar vector construct was used for the investigation of the subcellular localization of FGB1 (Wawra *et al.*, 2016). The FGB1 promoter is not only induced during *in planta* growth but also during growth in synthetic complex medium (Wawra *et al.,* 2016). Thus abundance and secretion of WSC3-GFP was investigated in the mycelium and culture filtrate of five transformants grown in complex by western blot using an anti-GFP antibody (Figure 4c). WSC3-GFP is solely present in the protein extract of the mycelial samples (upper blot) and was found in highest amounts in the transformants T1 and T3. Additionally to the growth in CM medium the transformants were also grown in liquid MYP medium, another synthetic, rich medium, which was recently shown in our group to increase secretion of other GFP fusion proteins (unpublished data). Nevertheless growth in MYP proofed to be less effective for the secretion of WSC3-GFP and even led to the cleavage of the fusion protein visible as free GFP in the protein extracts of the mycelium of transformant T1 and T3.

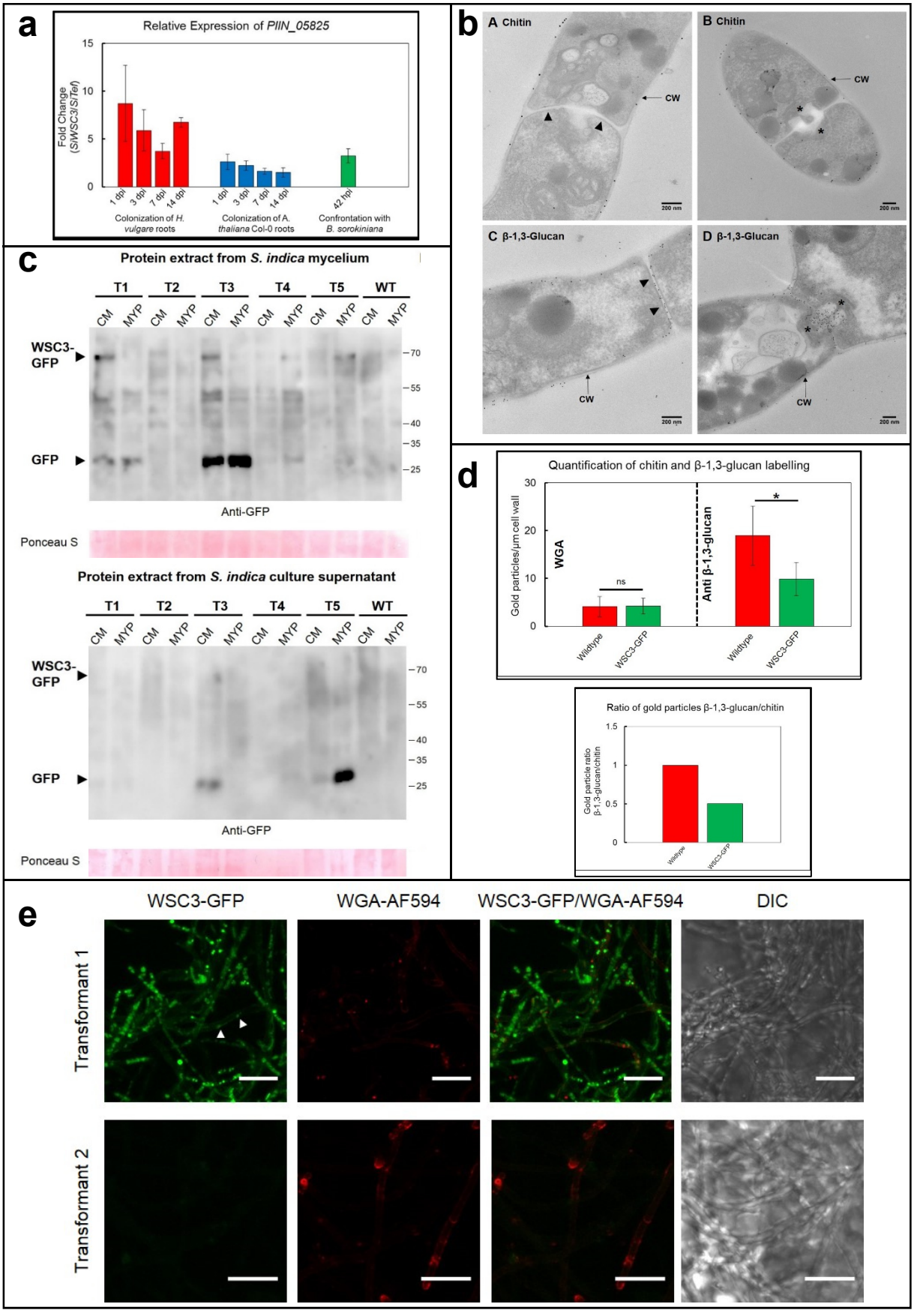

Figure 4: WSC3 is transcriptionally induced during root colonization and during contact with a root pathogen and localizes to the *S. indica* cell wall. a) Expression of WSC3 was quantified by qRT-

PCR during the colonization of *H. vulgare* (red bars) and *A. thaliana* (blue bars) roots or during contact with the root pathogenic fungus *B. sorokiniana* (green bars). For plant colonization RNA was extracted 1, 3, 7 or 14 days post inoculation (dpi) and for fungus-fungus interaction 42 hours post inoculation (hpi), reverse transcribed into cDNA and subsequently used for qRT-PCR. The expression of *WSC3* was calculated using the $2^{-\Delta\Delta Ct}$ method relative to the expression of *SiTef* and normalized to growth on PNM medium for 5 days (for expression in *H. vulgare*), on ½ MS medium for 7 days (for expression in *A. thaliana*) or to growth in soil without a second fungus (for expression with *B. sorokiniana).* Error bars indicate standard error of the mean calculated from four biological replicates. b) Chitin (A and B) was visualized by WGA conjugated with gold particles (visible as black dots) directly and is predominantely found in the cell walls (CW, black arrow) of *S. indica* hyphae but is completely absent in septa (black arrowheads) and in the dolipore (asterisk). β-1,3-glucan (C and D) was visualized with a monoclonal antibody produced in mouse and an anti-mouse antibody conjugated with gold particles (visible as black dots). β-1,3-glucan is also present in the cell walls (CW, black arrow) of *S. indica* hyphae and additionally in septa (black arrowheads) and in the dolipore (asterisk). Scale bar: 200 nm. c) Five *S. indica* transformants (T1 to T5) harboring the fusion protein WSC3-GFP driven by the FGB1 promoter were grown in CM medium or MYP medium before proteins were extracted from the mycelium (upper blot) or by precipitation from the culture filtrate (lower blot) and finally probed with an anti-GFP antibody and detected by a secondary HRP-conjugated anti-mouse antibody. As a control the untransformed wild type *S. indica* strain (WT) was processed in parallel. The band at 70 kDa corresponds to the WSC3-GFP fusion protein (highlighted in the blot) whereas the band visible at around 30 kDa corresponds to free GFP after cleavage of the fusion protein. Before detection the blots were stained in Ponceau S to verify equal loading of all samples. For detection of the proteins blot were incubated in standard ECL solution and chemiluminescence was detected for 20 min. d) The gold particles conjugated to the chitin-specific lectin WGA (upper plot, left part) and to the anti β-1,3-glucan antibody (upper plot, right part) were quantified from the TEM images taken from the dicaryotic *S. indica* reference strain (red bar) and the WSC3-GFP overexpressing transformant 3 (green bar). The total length of the fungal cell wall was measured from the images and used to calculate the number of gold particles per µm of cell wall. Whereas the number of gold particles specifically labelling chitin in the cell wall does not significantly differ between both strain, the number of gold particles labelling β-1,3-glucan is significantly reduced in the WSC3-GFP strain. The calculated β-1,3-glucan-to-chitin ratio (lower plot) was reduced to 50 % in the WSC3-GFP strain. Gold particles were count on 50 images each and error bar corresponds to the standard deviation calculated thereof. Asterik indicate significant differences ($p < 0.05$) calculated using an unpaired Students t-test. e) The *S. indica* transformant T1 and T2 were investigated by confocal laser scanning microscopy for the subcellular localization of the WSC3-GFP fusion protein. In T1 WSC3-GFP (green signal) is found in intracellular vesicles, potentially of the endoplasmatic reticulum or golgi apparatus (white arrowheads), which moved along the fungal hyphae towards the hyphal tip and at the periphery of the cell. The chitin-specific counterstain WGA-AF594 (red) labels the fungal cell wall and partially co-localizes with WSC3-GFP. In T2 where no protein was found in the western blot no signal was observed for WSC3-GFP indicating the specificity of the GFP-signal. Several z-stacks captured of the hyphae are combined in a maximal projection to cover the whole space of the hyphae. Error bars indicate 10 µm.

Free GFP (30 kDa) was also detectable in the mycelium samples of T1 and T3 grown in CM, which suggests that also in this medium the fusion protein is partially cleaved. In the protein extracts of the culture filtrate (lower blot) no WSC3-GFP fusion protein was detectable whereas free GFP was present in the filtrates of T1 grown in CM and T5 grown in MYP.

Since overexpression of WSC3-GFP led to the accumulation of the protein within the mycelium the impact on the chitin- and β-1,3-glucan-specific immunogold-labelling was assessed by TEM. Mycelia of the dicaryotic wildtype strain of *S. indica* and the WSC3-GFP expressing transformant T3 were labelled with WGA- and the β-1,3-glucan-specific antibody both conjugated to gold particles. The number of gold particles was count from the TEM images and used to calculate the number of gold particles per µm of cell wall. The chitin-specific immunogold labelling with WGA was not altered by the overexpression of WSC3-GFP whereas the β-1,3-glucan-specific labelling was significantly reduced (Figure 4d). Consequently the β-1,3-glucan-to-chitin ratio is reduced to 50 % in the cell walls of the WSC3-GFP strain. This could be indicative of the localization of WSC3-GFP to the *S. indica* cell wall potentially leading to an altered exposure or content of β-1,3-glucan.

Accordingly it was investigated if the fusion protein WSC3-GFP localizes to the cell wall of the *S. indica* transformants. The transformants T1, which showed the highest abundance of the uncleaved fusion protein and T2, which did not show any accumulation of the fusion protein in the western blot were grown in liquid CM medium and visualized by confocal laser scanning microscopy. The hyphae were counterstained with WGA conjugated with the fluorescence dye Alexa Fluor 594 (AF594). Microscopical investigation of transformant T1 showed that WSC3-GFP was found in intracellular vesicles potentially of the endoplasmatic reticulum or the golgi apparatus (white arrowheads), which are moving along the fungal hyphae towards the hyphal tip and at the fungal cell wall (Figure 4e upper panel). In T1 co-localization of WSC3-GFP and the cell wall specific stain WGA-AF594 is visible which might indicate that WSC3-GFP localizes to the *S. indica* cell wall. The GFP signal is completely absent in T2 which is in line with the findings of the western blot (Figure 4e lower panel). Taken together the results of the western blot and microscopical investigation suggest that WSC3-GFP is secreted into the extracellular space, where it is partially cleaved resulting in the detected free GFP, but to a big proportion is also attached to the *S. indica* cell wall leading to a co-localization with WGA-AF594.

2.1.2 Recombinant WSC3-His increases *P. pastoris* cell wall stress resistance

WSC3 consists of three WSC-domain each of them harboring eight conserved cysteine residues potentially involved in the formation of disulfide bonds. According to the NetNGlyc 1.0 and NetOGly 4.0 online tool WSC3 is predicted to contain two potential *N*-glycosilation sites and 19 potential *O*-glycosilation sites. Thus a eukaryotic expression system was chosen which favors the formation of the disulfide bonds. The yeast *P. pastoris* is a well-established expression system, which for example was successfully used to produce the cysteine-rich LysM-lectin Ecp6 from *C. fulvum* (DeJonge *et al.*, 2010). Furthermore the genome of *P. pastoris* does not encode WSC-lectins which opens the possibility to access the effect of overexpression of WSC3. The vector construct used for the expression of Ecp6 was provided as a kind gift from Bart Thomma (Wageningen University). The coding sequence of Ecp6 was replaced by WSC3 and the expression was driven by the methanol-inducible alcohol oxidase 1 (AOX1) promoter. The produced WSC3 containing an N-terminal 6x His-tag was secreted into the culture medium guided by the α-secretion signal of *S. cerevisiae*. Thus WSC3-His was purified from the culture supernatant by its His-Tag.

The culture supernatant was pre-purified by ion-exchange chromatography using TMAE anion-exchange material and SO^{3-} cation-exchange material. For the specific purification of WSC3-His the flow through of the ion-exchange columns was subjected to a Ni^{2+}-NTA column which has a high affinity to the His-tag. WSC3-His is predicted to have a molecular weight of 40 kDa and was detected in the elution fractions 1 and 2 with a molecular weight of more than 50 kDa (Figure 5a). This difference of the molecular weight might be explained by posttranslational modifications like for example glycosylation of WSC3-His. Accordingly deglycosilation of the recombinantely produced WSC3-His yielded a second protein band on the SDS-PAGE at a size of 40 kDa potentially corresponding to the unglycosilated protein (Figure 5a right gel). Interestingly the SDS-PAGE analysis of the elution fractions revealed the occurrence of two bands exhibiting slightly different molecular weights. To rule out that a second protein was co-purified both bands were excised from the gel and analyzed by liquid chromatography coupled to a mass spectrometer (LC-MS) at the CECAD Proteomics facility in cooperation with Dr. Tobias Lamkemeyer. According to the LC-MS analysis only WSC3-His was present in both bands with 21 unique peptide matches that covered 60 % of the amino acid sequence highlighted as underlined letters (Figure 5b).

The LC-MS analysis verified the cleavage of the α-secretion signal, the presence of the 6x His-Tag and the presence of all three WSC-domains. Furthermore, no secondary protein was detected in a comparably high abundance like WSC3-His, which rules out the possibility of an unspecific co-purification of a protein of similar size like WSC3-His. Thus the two bands might correspond to different glycosylation patterns or the partial cleavage of the 6x His-tag.

The expression of WSC3 fused to GFP in *S. indica* revealed that the protein is secreted and localizes to the cell wall. Since the *P. pastoris* cell wall is potentially composed in a similar way like the *S. indica* cell wall the question arose if a portion of WSC3-His is residing at the yeast cell wall. Thus a sequential fractionation of the cell wall proteins by different enzymatic and non-enzymatic treatments was performed like described for *Candida albicans* (Pitarch *et al.,* 2002). The cells were mechanically disrupted by crushing in liquid nitrogen and the material was extensively washed to remove all soluble cytoplasmic components. The resulting insoluble cell wall material was treated with SDS and DTT to remove all non-covalently linked proteins residing at the cell surface. Subsequently the insoluble cell wall material was washed again to remove the SDS and DTT prior to the non-enzymatic and enzymatic treatments. The cell wall material was either treated with mild alkali conditions (30 mM NaOH) to remove proteins that are directly linked to β-1,3-glucan with alkali-labile bonds like *O*-glycosyl side chains (Pitarch *et al.,* 2002). For the enzymatic extraction either a β-1,3-endoglucanase or a β-1,3-exoglucanase was employed to remove proteins that are indirectly linked to β-1,3-glucan but get released by hydrolysis of the β-1,3-glucan fibrils. Furthermore a chitinase was used to extract chitin-linked proteins and a commercial *Trichoderma* Lysing Enzyme mix that contains various glucanases and chitinases. For all the treatments the solubilized supernatant and the remaining insoluble cell wall pellet was analyzed on a SDS-PAGE and finally probed with an anti-His antibody (Figure 5c). The highest amounts of WSC3-His were detected in culture supernatant (marked with an asterisk). A faint band corresponding to WSC3-His was detectable in the *P. pastoris* crude cell extract sample which corresponds to the cell material directly taken after the mechanical disruption. The sample corresponding to the SDS-extraction step contained a small amount of WSC3-His visible as a faint band. The alkali treatment with NaOH did neither solubilize WSC3-His nor made it accessible for the antibody in the remaining cell wall material pellet. Thus it might be concluded that WSC3-His is not directly linked to β-1,3-glucan with an alkali-labile bond.

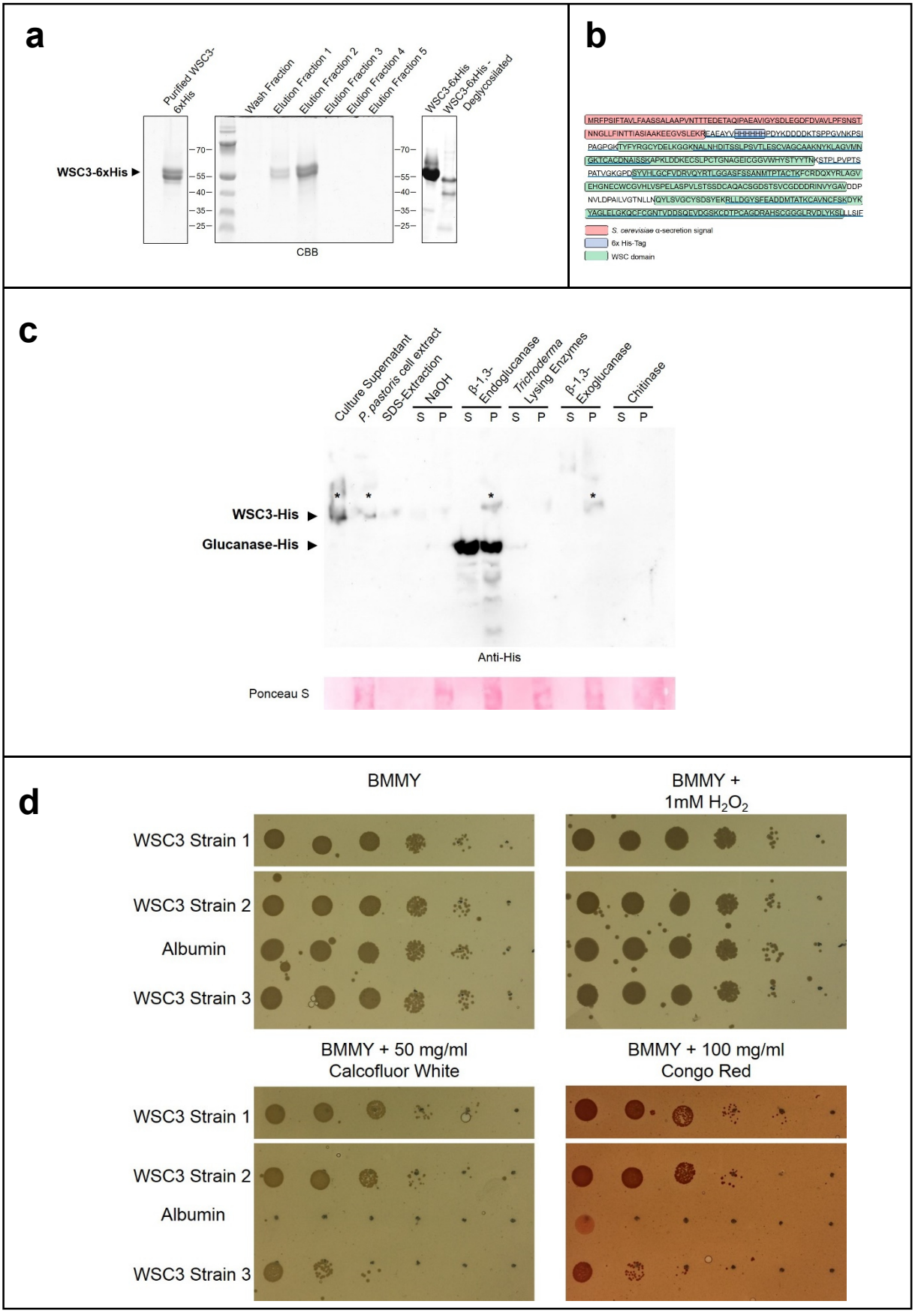

Figure 5: WSC3 was recombinantely produced in *Pichia pastoris* and increased cell wall stress resistance against Calcofluor White and Congo Red. a) Expression of WSC3 with an N-terminal 6x

His-tag was driven by the methanol-inducible AOX1 promoter and the fusion protein was secreted into the supernatant due to the presence of the α-secretion signal of *S. cerevisiae*. WSC3-His was purified from the culture supernatant with a Ni^{2+}-NTA column exhibiting specific affinity to the 6x His-tag. WSC3-His was eluted in 5 fractions whereas the fractions 1 and 2 contained the vast majority of the protein. The wash and elution fractions were analyzed on a 10 % Bis-Tris gel and stained with Coomassie brilliant blue. The predicted molecular mass of WSC3 is 40 kDa but the recombinantely produced WSC3-His is running at a height corresponding to 50 kDa. Deglycosilation with a commercial deglycosilation mix yielded a second protein band at a size of 40 kDa. b) Since two protein bands of similar size were observed on the SDS-Page the purified WSC3-His was analyzed by LC-MS. Both protein bands were excised from the gel and were identified according to 21 unique peptides as WSC3-His. The WSC3-His amino acid sequence consists of the α-secretion signal of *S. cerevisiae* (red box) which is cleaved during secretion, the N-terminal 6x His-Tag (blue box) and three WSC-domains (green boxes). LC-MS identified 21 unique peptide matching the amino acid sequence of WSC3-His, which covered 60 % of the protein sequence after cleavage of the secretion signal and are underlined in blue. c) Cells of the WSC3-His expressing *P. pastoris* strain were mechanically disrupted and the water insoluble cell wall fraction was treated with a SDS extraction buffer. The remaining insoluble cell wall pellet was again washed and subsequently treated with NaOH, a β-1,3-endoglucanase, a commercial *Trichoderma* Lysing Enuyme Mix, a β-1,3-exoglucanase and a chitinase in parallel. The solubilized supernatant (S) and the remaining insoluble pellet (P) were loaded onto a SDS-PAGE, separated by size and finally probed with an anti-His antibody. The protein band corresponding to WSC3-His runs at a height corresponding to a molecular mass of 50 kDa (marked with asterisk). The intense bands corresponds to the β-1,3-endoglucanase which also contains a His-Tag and thus is detected by the anti-His antibody. Ponceau S staining of the western blot membrane was performed to verify equal loading of the supernatant and pellet samples. d) Drop dilution series of 6 successive dilutions of a starting cell suspension with an OD_{600} value of 1 (from left to right) corresponding to three independent WSC3-His expressing strains (1-3) and an albumin expressing reference strain. The strains were grown for 2 days on BMMY medium (top left), BMMY medium supplemented with 1 mM H_2O_2 (top right), supplemented with 50 mg/ml Calcofluor White (lower left) or supplemented with 100 mg/ml Congo Red (lower right).

Among the enzymatic treatments only the β-1,3-endoglucanase and the β-1,3-exoglucanase made WSC3-His accessible for the anti-His antibody whereas for both treatments the protein remained within the cell wall pellet and was not solubilized. The two major bands at a size of 35 kDa correspond to the commercial β-1,3-endoglucanase which also contains a His-Tag. These results indicate that WSC3-His is not only secreted but also incorporated into the cell wall of *P. pastoris* where it is buried within the β-1,3-glucan fibrils and only made accessible for the antibody by hydrolysis of the β-1,3-glucan fibrils.

To test if the incorporation into the cell wall has an effect on cell wall stress resistance three independent *P. pastoris* strains were used in a drop dilution test on media

containing 1 mM H_2O_2, 50 mg/ml Calcofluor White or 100 mg/ml Congo Red. The base medium supplemented with these stressors contained 2 % methanol, which induces the expression driven by the AOX1 promoter. As control a reference strain was picked that produces and secretes Albumin into the growth medium in a methanol-inducible manner to ensure that potential differences regarding the cell wall stress resistance do not originate from the expression of a heterologous protein in general. Albumin is an often-used reference protein that increases solubility of various compounds and is not known to be affecting cell wall stress resistance. All four strains were grown to an OD_{600} value of 0.6 to 0.8, pelleted by centrifugation and resuspended in an appropriate volume of sterile water to an OD_{600} value of 1. The four independent strains were inoculated onto the different media in a drop dilution series starting from the solution with the OD_{600} value of 1 followed by five successive 1:10-dilutions. After two days of growth the cell wall stress resistance assay revealed that the WSC3-His expressing strains are more resistant to Calcofluor White and Congo Red (Figure 5d). Growth on BMMY and BMMY supplemented with 1 mM H_2O_2 was similar for all tested strains. Growth on Calcofluor White was strongly increased in the strains 1 and 2 and to a lesser extent also in strain 3. The albumin reference strain did not grow at all on the Calcofluor White medium. On Congo Red the WSC3 strains 1 and 2 exhibited the highest growth rate. The WSC strain 3 showed again a lower growth rate compared to strain 1 and 2 but still grew considerably better than the albumin control where none of the dilutions grew under this conditions. Thus it can be concluded that WSC3-His is incorporated into the *P. pastoris* cell wall and the production and secretion of WSC3-His increases the resistance to the cell wall stressors Calcofluor White and Congo Red.

2.1.3 WSC3-His binds the branched β-1,3/1,6-glucan laminarin but does not protect it from hydrolysis

After verification of its purity and integrity WSC3-His was used for isothermal titration calorimetry (ITC) to determine its specificity to different oligo- and polysaccharide ligands. To focus the biochemical analysis of WSC3-His and to select the most probable oligo- and polysaccharide ligands for subsequent ITC a protein pull-down experiment with the insoluble polysaccharide fractions of *H. vulgare* and *S. indica* cell wall was performed. The insoluble, protein-free cell wall preparations were incubated with WSC3-His and subsequently analyzed *via* SDS-PAGE. WSC3-His co-precipitates

with the cell wall polysaccharides of *S. indica* but stayed in the supernatant fraction after incubation with the *H. vulgare* cell wall (Figure 6a). This suggests a specificity of WSC3-His for an oligo- or polysaccharide of fungal origin.

Accordingly the mixed β-1,3/1,6-glucan laminarin, the linear β-1,3-glucan laminarihexaose, the β-1,6-linked glucose dimer gentiobiose and chitooctaose were included into the survey. Laminarin is an algal derived polysaccharide which is often used as a surrogate for branched β-1,3/1,6-glucans exhibiting a degree of polymerization of 30 with β-1,6-glycosidic side branches approximately at every tenth glucose molecule. Laminarihexaose is a synthetic, linear β-1,3-glucan consisting of six glucose units. Gentiobiose was also chemically synthesized and consists of two glucose molecules joined by a β-1,6-glycosidic linkage. Furthermore chitooctaose was tested which is the octamer of β-1,4-linked *N*-acetylglucosamine. ITC relies on the release or absorption of heat upon binding of a protein to a ligand. The ligand, in this case the oligo- and polysaccharides, is stepwise titrated into the protein containing sample cell. If binding occurs the temperature of the sample cell is altered and a reference cell is heated or cooled in parallel to keep both cells at an equal temperature. The resulting peaks correspond to an altered temperature within the sample cell, which is compensated in the reference cell what leads to the return of the signal to its starting point. The area of all the resulting peaks is integrated, plotted against the molar ratio of protein to ligand and fitted to a binding model to calculate the dissociation constant K_d as a measure of the affinity, the stoichiometry of the interaction n and the enthalphy ΔH of the reaction. The ITC measurements were performed and analyzed by Dr. Stephan Wawra who kindly provided the data and agreed on publication in this thesis. It was shown that WSC3-His binds to laminarin (black) but not to laminarihexaose (blue), gentiobiose (red) and chitooctaose (green; Figure 6b). The data were baseline corrected and the results from the control titration of the ligand into water were subtracted. Only upon binding of laminarin to WSC3-His heat was released in an exothermal reaction, which is visible as downward peaks in the left plot corresponding to the raw data of the measurement prior to fitting. For laminarihexaose, gentiobiose and chitooctaose not alteration of the temperature was detected which indicates that no binding of the protein to the ligand occurred. Since only laminarin was bound specifically by WSC3-His this dataset was fitted in single-binding-site-model visualized in the right plot. According to the manufacturers information concerning the length of the laminarin molecule and the degree of polymerization a molecular mass of 5 kDa

was assumed for laminarin and used to calculate the parameters of the reaction. The ITC measurement revealed a stoichiometry of the reaction of 1:3 meaning that one WSC3-His molecule binds three laminarin molecules which suggests that each of the three WSC-domains of WSC3-His is able to bind one laminarin molecule. The fact that WSC3-His did not bind to laminarihexaose indicates that the β-1,6-glycosidic side branches of laminarin are crucial for the binding which was also shown for FGB1 (Wawra *et al.*, 2016). Whereas FGB1 was able to bind to the disaccharide gentiobiose, WSC3-His showed no affinity indicating either the need of a mixed β-1,3/1,6-glucan for binding or purely the need of a bigger ligand. A K_d value of 12.5 µM ± 8.8 µM was calculated for the binding of WSC3-His to laminarin

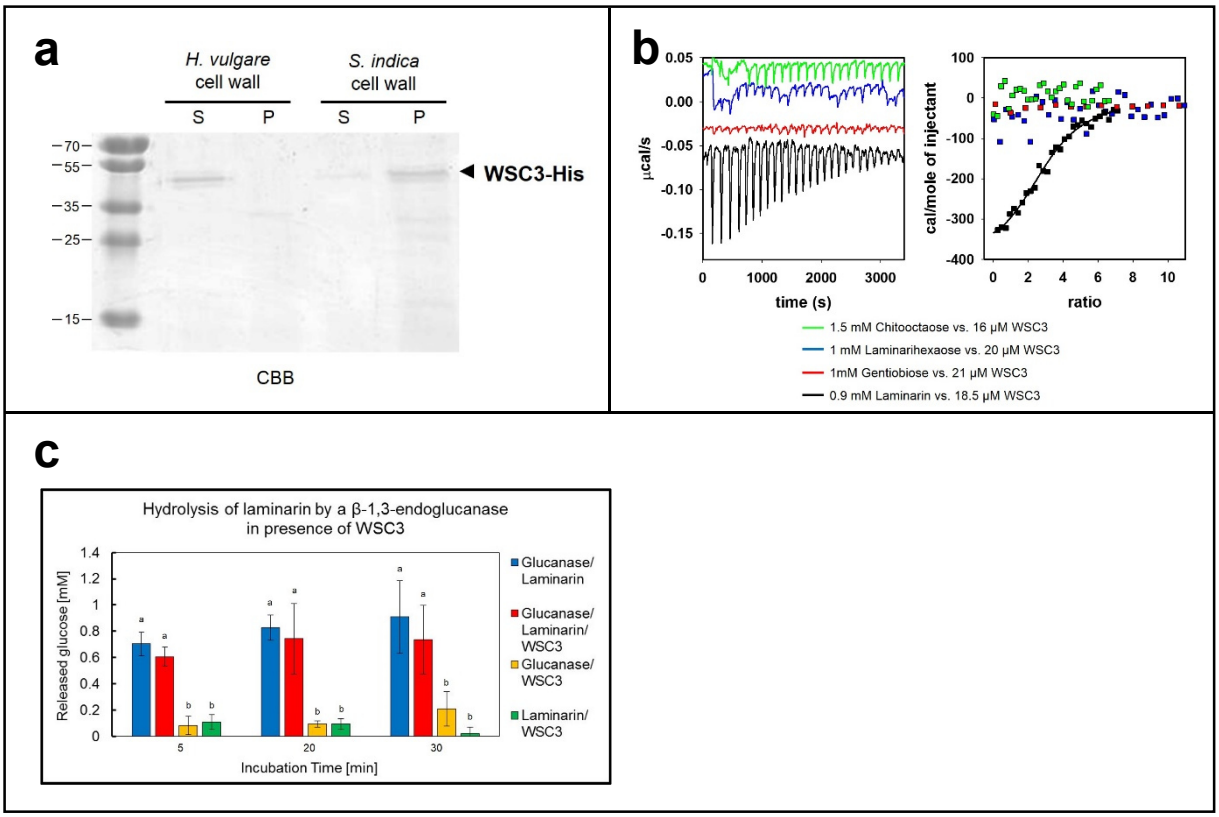

Figure 6: WSC3-His binds to the branched β-1,3/1,6-glucan laminarin but does not protect it from hydrolysis. a) A protein pull-down assay was performed to assess the affinity of WSC3-His to insoluble, protein-free cell wall preparations of *H. vulgare* and *S. indica*. After incubation with 20 µM WSC3-His the cell wall preparations were collected by centrifugation and the presence of WSC3-His in the pellet fraction (P) and the supernatant (S) were analyzed on a SDS-PAGE by Coomassie Brilliant Blue (CBB) staining. WSC3-His co-precipitates with the *S. indica* cell wall preparation but not with those of *H. vulgare* roots. b**)** Raw data of the ITC measurements for the titration of 1.5 mM chitooctaose into 16 µM WSC3-His (green), 1 mM of laminarihexaose into 20 µM WSC3-His (blue), 1 mM of gentiobiose into 21 µM of WSC3 (red) and 0.9 mM laminarin into 18.5 µM WSC3-His (black) are depicted in the left plot. Downward peaks for the titration of laminarin indicate an exothermal binding to WSC3-His. The data for

the binding of WSC3-His to laminarin was fitted in a single-binding-site-model (right plot) and used to calculate following parameters for the reaction assuming a molecular mass of laminarin of 5 kDa: n = 3.1 ± 0.0967; K = 8 x 10^4 ± 1.13 x 10^5 M^{-1}, K_d = 12.5 µM ± 8.8 µM, ΔH = -407.3 ± 18.25 cal/mol. All solutions were prepared in water and data were baseline corrected and results of the corresponding control titrations of the ligand into water were subtracted. All measurements, analysis of the data and calculation of the reaction parameters were performed by Dr. Stephan Wawra who kindly provided the figures and agreed on publication in this thesis. c) A commercial barley β-1,3-endoglucanase (0.125 U/ml) was incubated with laminarin (5 mg/ml; blue), with WSC3 (10 µM; yellow), with laminarin and WSC3 (red) or as a control WSC3 with laminarin alone (green). The released glucose was quantified with a BCA assay after 5, 20 and 30 min of incubation at 42 °C. Error bars represent standard error of the mean of four replicates and significance was calculated with an unpaired Students t-test (p < 0.05).

The fact that WSC3-GFP was found attached to the *S. indica* cell wall prompted us to test if it acts in a similar way than the *C. fulvum* chitin-binding lectin Avr4, which was previously shown to protect chitin from hydrolysis by plant-derived chitinases (van den Burg *et al.*, 2006). In order to test this the ability of WSC3-His to prevent hydrolysis of laminarin by a barley β-1,3-endoglucanase was assessed in a BCA-assay that quantifies the amount of released glucose. Therefore the β-1,3-endoglucanase was incubated with laminarin (blue) or with laminarin and WSC3-His (red). As controls the β-1,3-endoglucanase was incubated solely with WSC3-His (yellow) or WSC3-His was incubated with laminarin alone (green; Figure 6c). After incubation for 30 min 1 mM of glucose was released from the 5 mg/ml laminarin by 0.125 U/ml of the β-1,3-endoglucanase. The enzyme also released a slight amount of glucose from the WSC3-His protein if both were incubated together without laminarin. This could potentially be explained by the partial hydrolysis of the attached glycosilations from the protein. On the other hand side the WSC3-His protein alone did not hydrolyze laminarin which was tested in parallel to rule out that WSC3-His possesses also a glucanase activity under the assay conditions. Interestingly WSC3-His did not or only to a marginal degree decrease the amount of the released glucose. Neither after 5 min, nor after 30 min a significant difference in the amount of released glucose could be detected between the sample with and without WSC3-His. Thus there is no detectable effect of WSC3-His which is in contrast to the mode of action described for Avr4 (van den Burg *et al.*, 2006) but similar to FGB1 which also specifically binds laminarin but is not protecting it from hydrolysis (Wawra *et al.*, 2016).

2.1.4 WSC3 is able to agglutinate fungal cells

Since the expression of WSC3 is not only induced during plant colonization but is also responsive to the presence of a second fungus it was tested if addition of WSC3-His has an effect on the growth of *S. indica*, *B. sorokiniana* and *U. maydis*. As controls the 6.2 kDa *S. indica* lectin FGB1 and the antifungal plant lectin wheat germ agglutinin conjugated with the fluorescence dye AlexaFluor 594 (WGA-AF594) were included (Mirelman *et al.*, 1975; Wawra *et al.*, 2016). The growth phenotype of the fungi was assessed microscopically after overnight growth for *S. indica* and *B. sorokiniana* and after 4 h of growth for *U. maydis*. Remarkably WSC3-His displayed a strong agglutination effect on all tested fungi whereas FGB1 did not and WGA-AF594 only partially led to the formation of aggregates (Figure 7a). The germination rate of the spores was not considerably decreased according to visual inspection when WSC3-His was present. The phenotype and growth rate of *S. indica* and *U. maydis* was not different if the spores were treated with FGB1. Interestingly FGB1 seemed to have a negative effect on the germination and growth of *B. sorokiniana*. The amount of fungal filaments was decreased if FGB1 was present and the filaments showed shorter and swollen hyphae indicative of stress and cell death. WGA-AF594 is also agglutinating *S. indica* and *U. maydis* but to a lesser degree than WSC3-His. For *B. sorokiniana* no difference in the growth phenotype was visible between mock and WGA-AF594 treatment. The minor effect of WGA-AF594 could potentially be explained by the fluorescence conjugate, which might interfere with the agglutination properties of WGA. Since the filamentous growth of *S. indica* and *B. sorokiniana* complicates the quantification of the agglutination effect of WSC3-His the agglutination was quantified for *U. maydis* by calculating the percentage of cells being included in such aggregates relative to the total number of cells (Figure 7b). WSC3-His led to an increase from 13% agglutinated cells observed for the mock treatment to more than 50 % agglutinated cells. Addition FGB1 agglutinated 15% of the cells whereas WGA-AF594 agglutinated 45 % of the cells. The agglutinating effect of WSC3-His on fungal cells raises the question if it is additionally involved in the attachment of the spores to the root surface. To assess the adhesion of the spores to barley roots the roots were incubated in *S. indica* spore solution either containing water, native FGB1 or WSC3-His. The spores were visualized by staining with WGA-AF594 and counted manually on pictures taken

by confocal laser scanning microscopy. Addition of WSC3-His had no positive effect on the adhesion of *S. indica* spores to the surface of barley roots (Figure 7c).

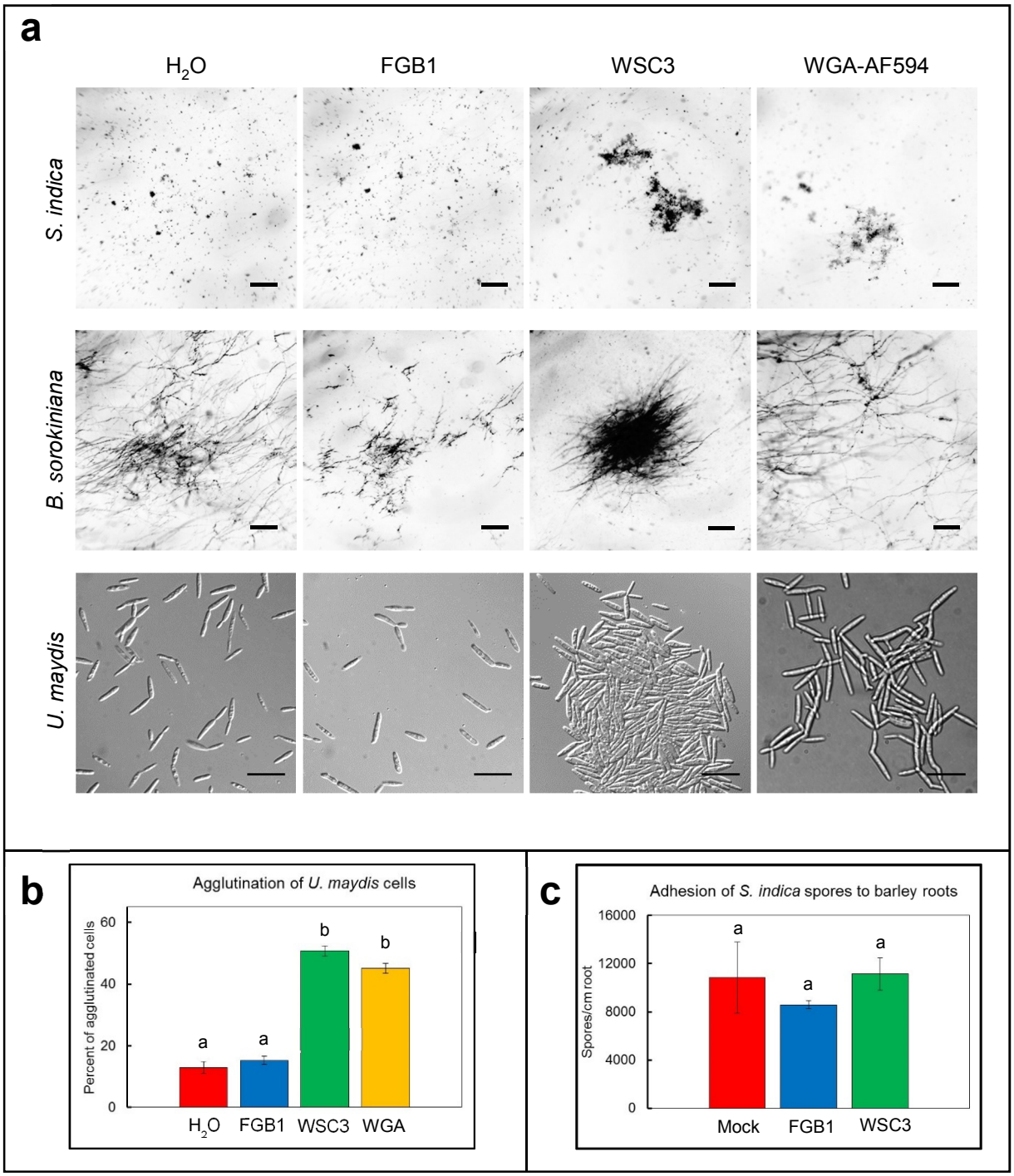

Figure7: WSC3-His leads to the formation of multicellular aggregates of *S. indica, B. sorokiniana and U. maydis*. (a) *S. indica* (1st row), *B. sorokiniana* (2nd row) and *U. maydis* (3rd row) were grown in the presence of water (1st column), 10 µM native FGB1 (2nd column), 10 µM WSC3-His (3rd column) or 10 µM WGA-AF594 (4th column) in rich medium. Pictures of *S. indica* and *B. sorokiniana* were taken after 16 h or growth using a Leica M165 FC stereo microscope and scale bars are indicative of 500 µm. *U. maydis* was grown for 4 h before pictures were taken using a Leica DM2500 light microscope and scale bars represent 25 µm. (b) The degree of aggregation was quantified by calculating the percentage

of aggregated cells relative to the total number of cells in presence of water (red bar), 10 µM native FGB1 (blue bar), 10 µM WSC3-His (green bar) or 10 µM WGA-AF594 (yellow bar). Error bars indicate standard error of the mean of at least four biological replicates. No significant difference was observed between mock- and FGB1-treatment (a) whereas WSC3-His and WGA-AF594 treatment significantly increased aggregates (b) according to an unpaired Students t-test ($p < 0.05$). c) Adhesion of *S. indica* spores was calculated by counting the number of spores that attached to the roots surface from microscopical images acquired by confocal laser scanning microscopy. *S. indica* spore solution was either mixed with water (Mock, red bar), 10µM FGB1 (blue bar) or 10µM WSC3-His (green bar). Error bars represent the standard error of the mean of 3 biological replicates. No significant difference between the treatments was observed using an unpaired Students t-test ($p < 0.05$, indicated by the letter a)

Both treatments resulted in average in 11,000 spores per cm of the roots. Only the addition of FGB1 had a mild but not significant negative effect onto the spore adhesion lowering the number by around 20 % to in average 9,000 spores per cm of the root. As a conclusion WSC3-His has the ability to agglutinate fungal cells of basidiomycete and ascomycete fungi without affecting the adhesion of *S. indica* spores to barley roots. The effect of this agglutination phenotype on spore germination and growth rate seems to be of minor relevance. Since β-glucans released from the fungal cell wall are potent elicitors of plant defense responses the next step aimed to investigate the ability of WSC3 and FGB1 to suppress β-glucan triggered ROS production and if WSC3 is able to increase the colonization rate of *S. indica*

2.1.5 WSC3-His suppresses β-glucan triggered plant defense responses but does not increase root colonization

To test the ability of WSC3-His to efficiently compete for the binding of β-glucan with the plant receptor an assay was performed monitoring the β-glucan triggered accumulation of reactive oxygen species (ROS). The production of ROS is one of the earliest plant defense responses which can be quantified in a luminol-based assay (Yu *et al.*, 2017). Since the reaction to different β-glucans is less well studied than for example chitin and also the defense eliciting activity of different β-glucans greatly varies first attempts were made with different potential β-glucan molecules that might lead to the production of ROS in *H. vulgare* and *A. thaliana* Col-0 leaves. As positive control a crude suspension of shrimp shell chitin was used. Furthermore the β-glucans laminarin, laminarihexaose and gentiobiose were tested for their ability to elicit the

accumulation of ROS in *H. vulgare* and *A. thaliana* Col-0, the two best studied experimental host plants of *S. indica*. Laminarin is constantly used as mixed β-glucan defense elicitor either in its natural form or as sulfated derivative and was shown to trigger accumulation of reactive oxygen species (ROS) in *Nicotiana tabacum*, *Vitis vinifera* and *H. vulgare* (Klarzynski *et al.*, 2000; Aziz *et al.*, 2003; Menard *et al.*, 2004; Gauthier *et al.*, 2013; Wawra *et al.*, 2016). Secondly laminarihexaose a linear β-1,3-glucan consisting of six glucose molecules was tested for its ability to trigger ROS accumulation. Linear β-1,3-glucans have not been systematically studied as elicitors of ROS production but are described to induce various defense responses in *Oryza sativa* and *Nicotiana tabacum* (Inui *et al.*, 1997; Klarzynski *et al.*, 2000). Thirdly a disaccharide consisting of two β-1,6-glycosidic linked glucoses molecules, called gentiobiose, was synthesized by the group of Prof. Dr. Jürgen Seibel (Julius Maximilian University of Würzburg) and tested for the ability to trigger ROS production. Interestingly both laminarin and laminarihexaose showed immune-elicitation activity in *H. vulgare* (Figure 8a) whereas only chitin triggered ROS production in *A. thaliana* Col-0 (Figure 8b).

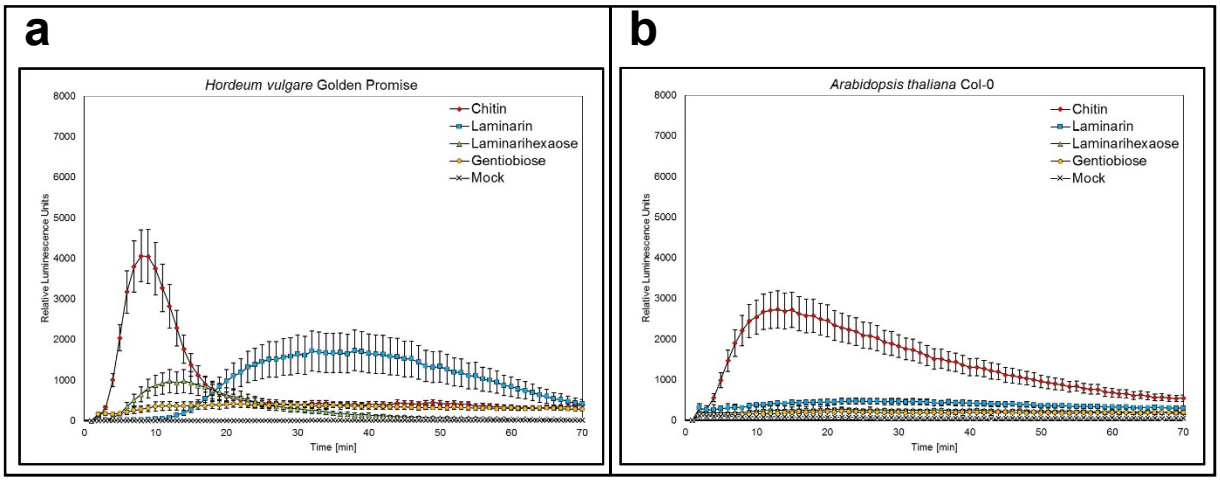

Figure 8: Potential fungal MAMPs differentially elicit ROS-production in *H. vulgare* (a) and *A. thaliana* Col-0 (b). ROS production was quantified in a luminol-based assay by the amount of emitted chemiluminescence. Plants were treated with 0.8 mg/ml chitin suspension (red diamond), 3.3 mg/ml laminarin (blue square), 20 µM laminarihexaose (green triangle), 20 µM gentiobiose (yellow circle) or with water as mock control (black cross). Values are mean of 12 replicates ± standard error of the mean.

Elicitation with the chitin suspension led to a rapid increase of the chemiluminescence signal reaching the peak value after 10 min. In contrast to chitin addition of laminarin

led to a rather slow increase of the chemiluminescence reaching the peak nearly 40 min after addition of the solution. Laminarihexaose yielded the lowest luminescence signal which peak is already reached 12 min after addition of the suspension. Addition of gentiobiose did not lead to a measurable increase in luminescence. In *Arabidopsis thaliana* Col-0 only the chitin suspension was able to trigger ROS production leading to a peak signal 13 min after elicitation. The addition of laminarin, laminarihexaose and gentiobiose led to nearly no increase of the signals.

As a consequence the ability of WSC3-His and FGB1 to suppress β-glucan triggered defense responses was tested in *H. vulgare* with laminarin as elicitor. Immediately before the addition of laminarin 10 µM of either WSC3-His or native FGB1 were added and the production of ROS was recorded. The laminarin-triggered ROS accumulation was almost completely suppressed when 10 µM the native WSC3-His or FGB1 were added (Figure 9a). Remarkably neither WSC3-His nor FGB1 trigger the ROS production in barley. Thus it can be assumed that the proteins are not recognized as effector proteins and thus do not lead to ROS production as part of an effector-triggered immunity (ETI) response (Jones & Dangl, 2006). Hence it can be concluded that WSC3-His and FGB1 are able to suppress the laminarin-triggered ROS production in barley. Since also the flg22- and chitin-induced ROS production is suppressed in *S. indica* colonized plants it was also tested if WSC3-His and FGB1 are able to suppress ROS production triggered by chitohexaose and flg22.

10 µM chitohexaose elicited a rapid increase of luminescence corresponding to the production of ROS, with a peak reached after 8 min (Figure 9b). Addition of 10 µM of FGB1 again almost completely suppressed the ROS production. Even though WSC3-His also reduced the luminescence signal after the addition of chitohexaose the barley plants still reacted with a rapid ROS accumulation that reached around 30 % of the signal intensity recorded for chitohexaose alone. The pattern of the recorded ROS production and the velocity of the reaction are similar to that without WSC3-His present. Thus WSC3-His is able to partially suppress the ROS-production after chitohexaose elicitation whereas FGB1 is again completely damping the reaction.

Since laminarin and chitohexaose both are oligosaccharides the bacterial peptide flg22 was also tested to rule out that the immune suppressive effect of WSC3-His and FGB1 purely rely on unspecific interactions with oligosaccharides. Flg22 is a conserved 22 amino acid peptide of the bacterial protein flagellin and strongly induces plant defense responses and also the production of ROS.

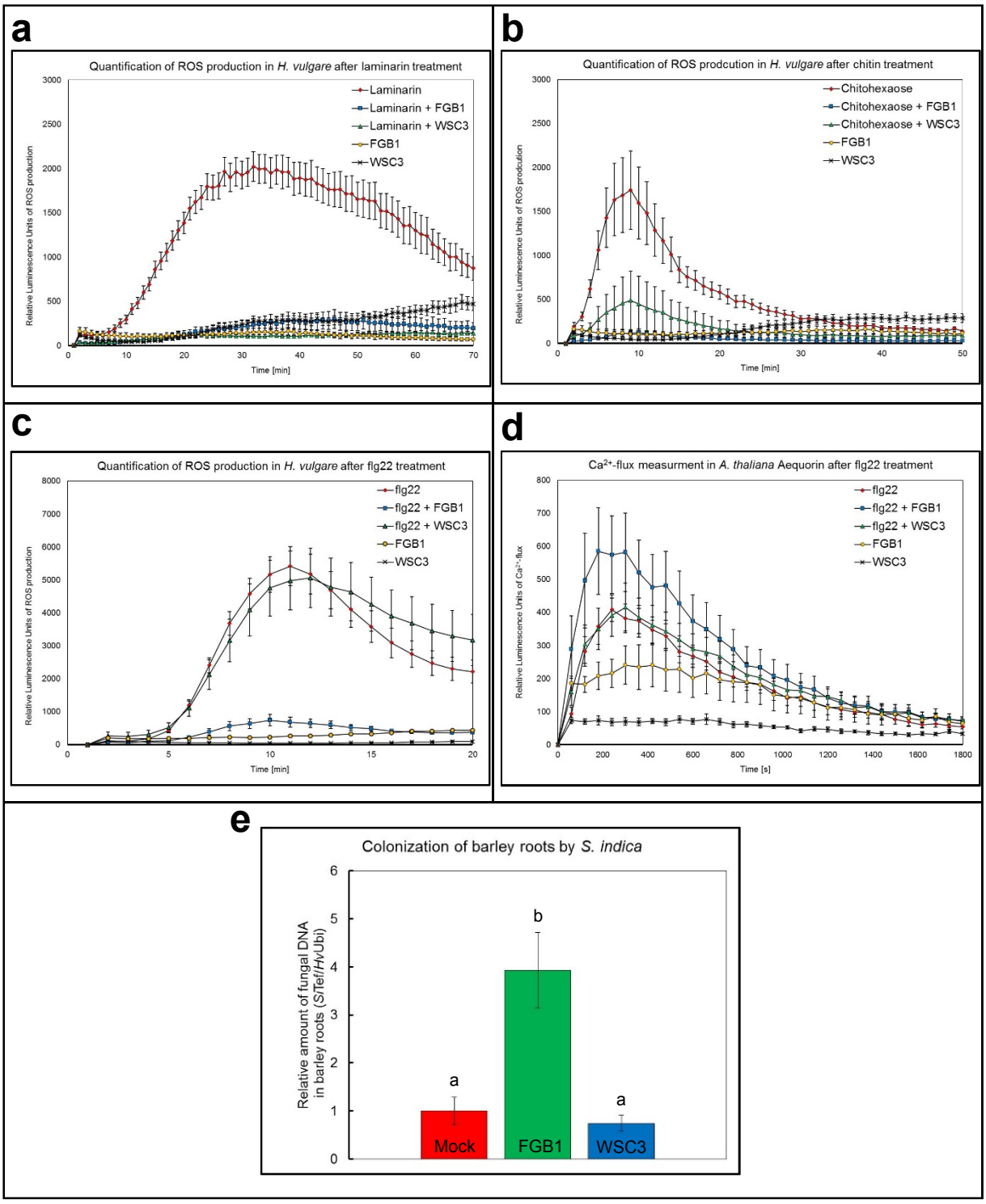

Figure 9: Both WSC3-His and FGB1 suppress MAMP-triggered ROS production in barley but only FGB1 has a positive effect on barley root colonization by *S. indica*. ROS production was quantified in a luminol-based assay by the amount of emitted chemiluminescence. *H. vulgare* Golden Promise plants were treated with elicitor solution (red diamond), elicitor solution and 10 µM FGB1 (blue square), elicitor solution and 10 µM WSC3-His (green triangle), 10 µM FGB1 alone (yellow circle) and 10 µM WSC3-His alone (black cross) before the luminescence was recorded. Values are mean of at least 6 (mostly 12) replicates ± standard error of the mean. As elicitor solution a) 3.3 mg/ml laminarin, b) 10 µM

chitohexaose or c) 20 nM flg22 were used. d) Ca^{2+}-influx was quantified in a transgenic *A. thaliana* reporter line expressing cytoplasmic aequorin which oxidizes coelenterazine upon binding of Ca^{2+}-ions which leads to the emission of chemilumindescence. *A. thaliana* aequorin plants were grown under sterile conditions for 5 to 7 days before the Ca^{2+}-influx was induced by addition of 1 µM of flg22 (red diamond). Neither simultaneous addition of 10 µM FGB1 (blue square) nor the addition of 10 µM of WSC3-His (green triangle) reduced the chemiluminescence signal. WSC3-His alone (black cross) did not induced a Ca^{2+}-influx whereas FGB1 alone (yellow circle) induced a slight increase of chemiluminescence. Values are mean of at least 6 replicates ± standard error of the mean. e) *S. indica* spore solution was either mixed with water (Mock, red bar), 10µM FGB1 (green bar) or 10µM WSC3-His (blue). The colonization rate was quantified by qRT-PCR measuring the relative amount of fungal gDNA (*SiTef*) compared to plant gDNA (*HvUbi*). The colonization rate is normalized to the mock treatment which accordingly was set to 1. Quantification for the FGB1 treatment was performed by Dr. Stephan Wawra and data were taken from Wawra *et al.*, 2016. Error bars represent the standard error of the mean of at least 4 biological replicates. No significant difference between the mock and WSC3-His treatment was observed (a) whereas FGB1 significantly increased the colonization rate (b) according to an unpaired Students t-test ($p < 0.05$)

20 nM of the synthetic peptide flg22 are sufficient to trigger the ROS production in barley. The ROS production was again almost completely suppressed when 10 µM of FGB1 were added whereas the same concentration of WSC3-His did not affect the flg22-triggered ROS production (Figure 9c). Thus the effect of WSC3-His to suppress the ROS production in barley is restricted to laminarin and chitohexaose whereas FGB1 suppresses the ROS production for all three tested MAMPs including the bacterial peptide flg22.

The production of ROS is one of the earliest MAMP-triggered plant defense responses but acts downstream of the influx of Ca^{2+}-ions from the apoplast or internal storages into the cytoplasm (Yu *et al.*, 2017). The Ca^{2+}-influx can be quantified as chemiluminescence using a transgenic *A. thaliana* line that expresses cytoplasmic aequorin. Upon binding of Ca^{2+}-ions aequorin oxidizes its substrate coelenterazine which is externally supplied in the reaction buffer leading to the emission of luminescence. The ability of WSC3-His and FGB1 to prevent MAMP-triggered Ca^{2+}-influx was exemplarily tested for flg22 in the *A. thaliana* aequorin reporter line.

Interestingly neither 10 µM of WSC3-His nor 10 µM of FGB1 were able to suppress the flg22-triggered influx from Ca^{2+}-ions into the cytoplasm which occurred immediately after addition of 1 µM of flg22 (Figure 9d). WSC3-His alone did not lead to an increase of the luminescence signal whereas addition of FGB1 slightly induced Ca^{2+}-influx. Consequently the addition of FGB1 and flg22 induced the highest luminescence signal

which equals the added signals of both molecules. This could either be due to the recognition of FGB1 as an effector protein triggering Ca^{2+}-influx as an ETI response or due to the presence of Ca^{2+}-ions in the FGB1 protein solution. For WSC3-His the results of the flg22-triggered Ca^{2+}-influx are in line with the findings from the quantification of ROS after flg22 elicitation. Interestingly the flg22-induced Ca^{2+}-influx is not suppressed by FGB1 whereas flg22-induced ROS production is effectively suppressed which might suggest a direct effect of FGB1 on the production of ROS.

To test if the immune-suppressive properties of WSC3-His and FGB1 have a positive effect on root colonization of *S. indica* it was tested if addition of either of the two proteins increases the colonization rate in barley roots. Barley plants were inoculated with *S. indica* spores either supplemented with 10 µM FGB1 or WSC3-His and grown for 4 days under sterile conditions. Subsequently gDNA was extracted and the colonization rate was measured by quantification of the relative amount of fungal DNA by qRT-PCR. The effect of FGB1 onto the colonization rate was assessed before by Dr. Stephan Wawra and revealed a four-fold increased colonization rate in presence of FGB1 compared to the mock treatment (Wawra *et al.*, 2016). For simplicity reasons this data is also depicted in figure 9e which shows that addition of the same amount of WSC3-His has no effect on the colonization rate of *S. indica* (Figure 9e). Thus it can be concluded that the immune-suppressive properties of FGB1 positively affect the colonization of barley roots by *S. indica* whereas the *in vitro* suppression of the laminarin- and chitohexaose triggered ROS production of WSC3-His have no direct effect on the colonization rate.

2.2 Identification of laminarin-responsive defense components by genome wide association screen

2.2.1 Natural variation among *A. thaliana* accessions regarding their ability to recognize chitin and laminarin as MAMPs

The initial survey of different potential glucan elicitors revealed that *A. thaliana* Col-0 plants did not react to the branched β-1,3/1,6-glucan laminarin with the production of ROS. This was somewhat surprising since it was described before that laminarin or laminarin sulfate trigger the induction of *PR-1* and *PDF1.2* in *A. thaliana* Col-0 (Menard *et al.*, 2004). On the other hand side natural variation among *A. thaliana* accessions in terms of MAMP recognition was described before for flg22 (Vetter *et al.*, 2012). Therefore the responsiveness to laminarin was tested for 100 *A. thaliana* accessions in order to conduct a genome wide association (GWA) screen which could help to connect the potential phenotypic variation to their genetic basis. GWA screens were initially designed to uncover the genetic basis of human diseases but in recent years have gained raising interest in plant sciences. The study of natural variation within the *A. thaliana* population got kick-started with the release of the complete *A. thaliana* genome sequence in 2000 and got further accelerated by the initial description of the pattern of polymorphisms in 2005 and the development of the genotyping array containing 250,000 SNPs in 2007 (Kaul *et al.*, 2000; Nordborg *et al.*, 2005; Kim *et al.*, 2007). This 250K SNP chip set the foundation for a collection containing more than 1,300 genotypes of globally distributed *A. thaliana* accessions which due to their publically availability build the basis to conduct genome wide association (GWA) screens in *A. thaliana* (Horton *et al.*, 2012). GWA screens correlate the position of SNPs to a certain phenotype to enable the identification of genetic loci causal for the phenotype. GWA screens proofed their power identifying known flowering time and pathogen resistance genes (Aranzana *et al.*, 2005; Atwell *et al.*, 2010). To date GWA screens have been successfully applied to identify genes involved in salt tolerance, regulation of flowering time, regulation of glucosinolate levels, shade avoidance, heavy metal tolerance and the response to growth-promoting rhizobacteria (Baxter *et al.*, 2010; Li *et al.*, 2010; Chan *et al.*, 2011; Filiault & Maloof, 2012; Chao *et al.*, 2012; Wintermans *et al.*, 2016).

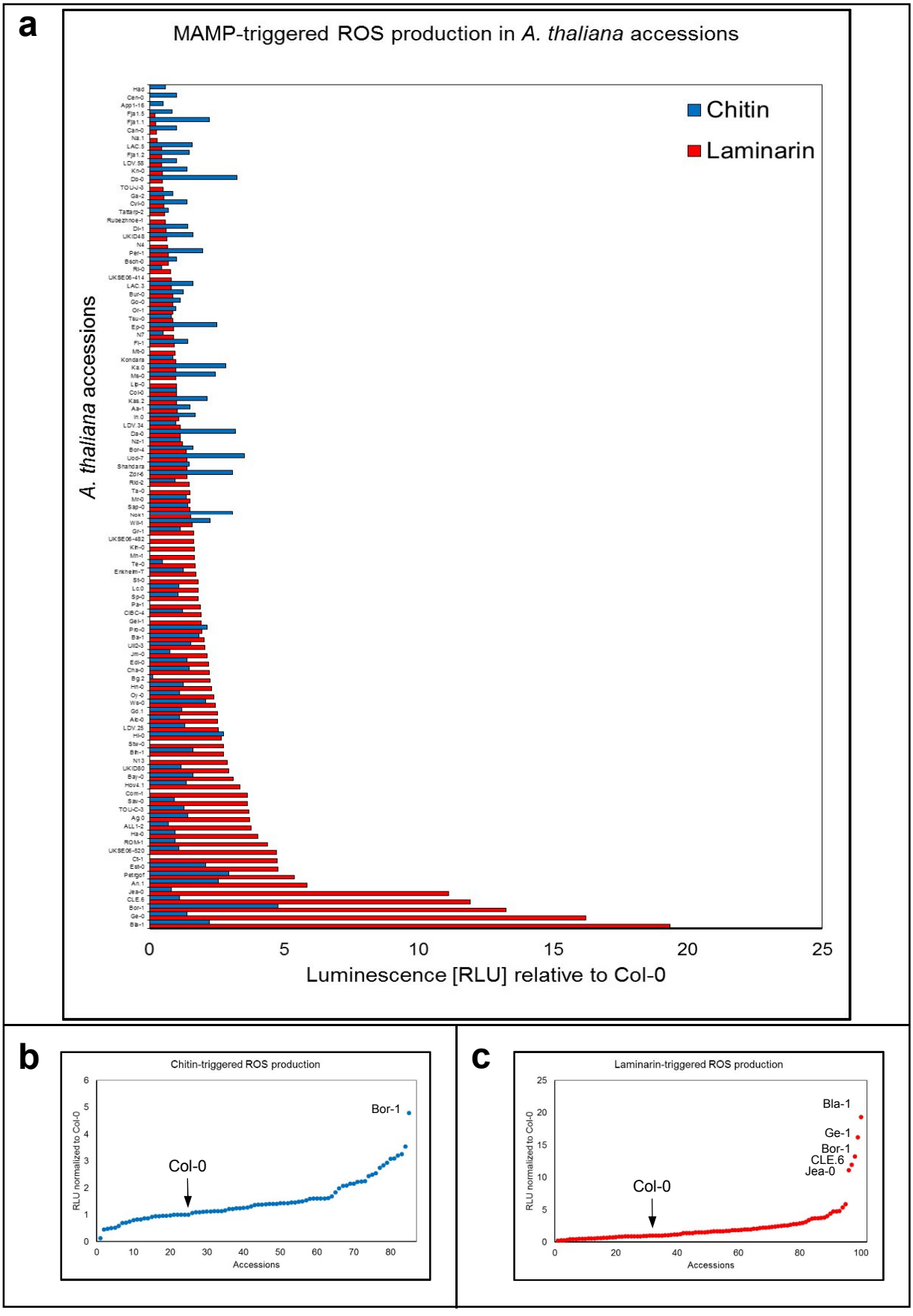

Figure 10: Natural variation in the responsiveness to the fungal-derived MAMPs chitin and laminarin among *A. thaliana* accessions. a) The responsiveness to chitin and laminarin was

quantified in a luminol-based assay measuring the production of ROS. The highest measured luminescence signal for each individual accession was normalized to the reaction of the accession Col-0 which was always measured in parallel as control and respectively set to 1. The reaction to chitin (blue bars) was measured for 85 accessions in total and to laminarin (red bars) for 100 accessions. The tested accessions are depicted in the order of their responsiveness to laminarin. b) The accessions are sorted by the strength of their luminescence signal after chitin treatment starting from the weakest-reacting accession to the strongest-reacting accession (left to right). Col-0 is marked by a black error and Bor-1 is highlighted as outlier. c) The accessions are sorted by the strength of their luminescence signal after laminarin treatment starting from the weakest-reacting accession to the strongest-reacting accession (left to right). Col-0 is marked by a black error and Jea-0, CLE.6, Bor-1, Ge-0 and Bla-1 are highlighted as outlier showing an extreme phenotype after laminarin treatment.

100 natural *A. thaliana* accessions including a nested core collection, which represents 95 % of the genetic diversity of all sequenced *A. thaliana* accessions were tested (McKhann *et al*., 2004). The seeds were provided by Prof. Dr. Adam Schikora (JKI Braunschweig) and Prof. Dr. Stanislav Kopriva (University of Cologne). The complete list of all tested accessions as well as their origin can be found in section 4.3.2 (Table 2). To ensure that a accession which does not or only weakly react to laminarin-elicitation is not showing any ROS production due to a general defect in immunity but due to a specific impairment in laminarin perception we included chitin as a positive control. The plants were stratified for three days at 4°C before they were grown under short day conditions for three to four weeks and harvested before bolting. The ROS production was triggered by the addition of 3.3 mg/ml laminarin and 0.8 mg/ml of a chitin suspension. To compare all measurements the peak luminescence signal obtained for each individual accession was normalized to the peak luminescence signal of Col-0. This method of normalization was possible since the speed and the timing of the reaction was equal for the individual accessions.

In this way the responsiveness to chitin was quantified for 85 accessions (blue bars) and to laminarin for 100 accessions (red bars; Figure 10a). Among the accessions treated with chitin Bor-1 showed the highest luminescence peak being 4.78-times higher than the luminescence peak measured for Col-0. The accession Bg.2 exhibited the lowest luminescence peak after chitin treatment with 0.12-times the signal recorded for Col-0. The difference between weakest and strongest reaction to laminarin was interestingly much higher ranging from 0.21-times recorded for Fja1.5 to 19.34-times for Bla-1. Especially the number of accessions with extremely strong reactions to laminarin-treatment was much higher than for the chitin-treatment. This fact gets even

more obvious if the tested accessions are sorted by their signal strength (Figure 10b and c). For the chitin-treatment (Figure 10b) there was only one accession (Bor-1) which showed an extreme reaction which was remarkably higher than the other accessions. For the laminarin-treatment the number of “extreme” accessions, which are remarkably more reactive was much higher (Figure 10c). In total there were five accessions (Jea-0, CLE.6, Bor-1, Ge-0 and Bla-1) which showed at least a 10-fold stronger luminescence signal, and for Bla-1 a nearly 20-fold stronger signal than recorded for Col-0.

At this stage it could be already concluded that the natural variation is higher regarding their responsiveness to laminarin than to chitin among *A. thaliana* accessions. Furthermore there is potentially not only one loci which is determining if a accessions is reacting to laminarin or not otherwise the difference between the different accessions would not be steadily increasing but one would expect two groups of accessions without intermediate states. Thus there might be several genetic loci that are quantitatively contributing to the elevated responsiveness of the more reactive accessions.

2.2.2 GWA screen to identify and classify genetic loci involved in chitin- and laminarin-responsive ROS product

Both datasets were subsequently used for a GWA screen using the GWAPP online resource (Seren *et al.*, 2012). The chitin dataset was square root transformed and the laminarin dataset was box-cox transformed to ensure a normal distribution of the measured values. Furthermore the box-cox transformation of the laminarin dataset limited the influence of the highly reactive accessions because it minimized the difference between those five accessions and all the others. Afterwards both datasets were analyzed for marker–trait associations with an accelerated mixed model algorithm (amm) like described in Seren *et al.* 2012. The results are displayed in a Manhattan plot highlighting the position of the top 10,000 SNPs per chromosome, their location in the *A. thaliana* genome and the corresponding $-log$(p-value) specifying the significance of the association. The marker–trait associations for the chitin-treated plants revealed that none of the displayed SNPs is reaching the Bonferroni-Hochberg corrected false discovery rate (FDR) of 5 % depicted as purple dashed line (Figure 11a and b).

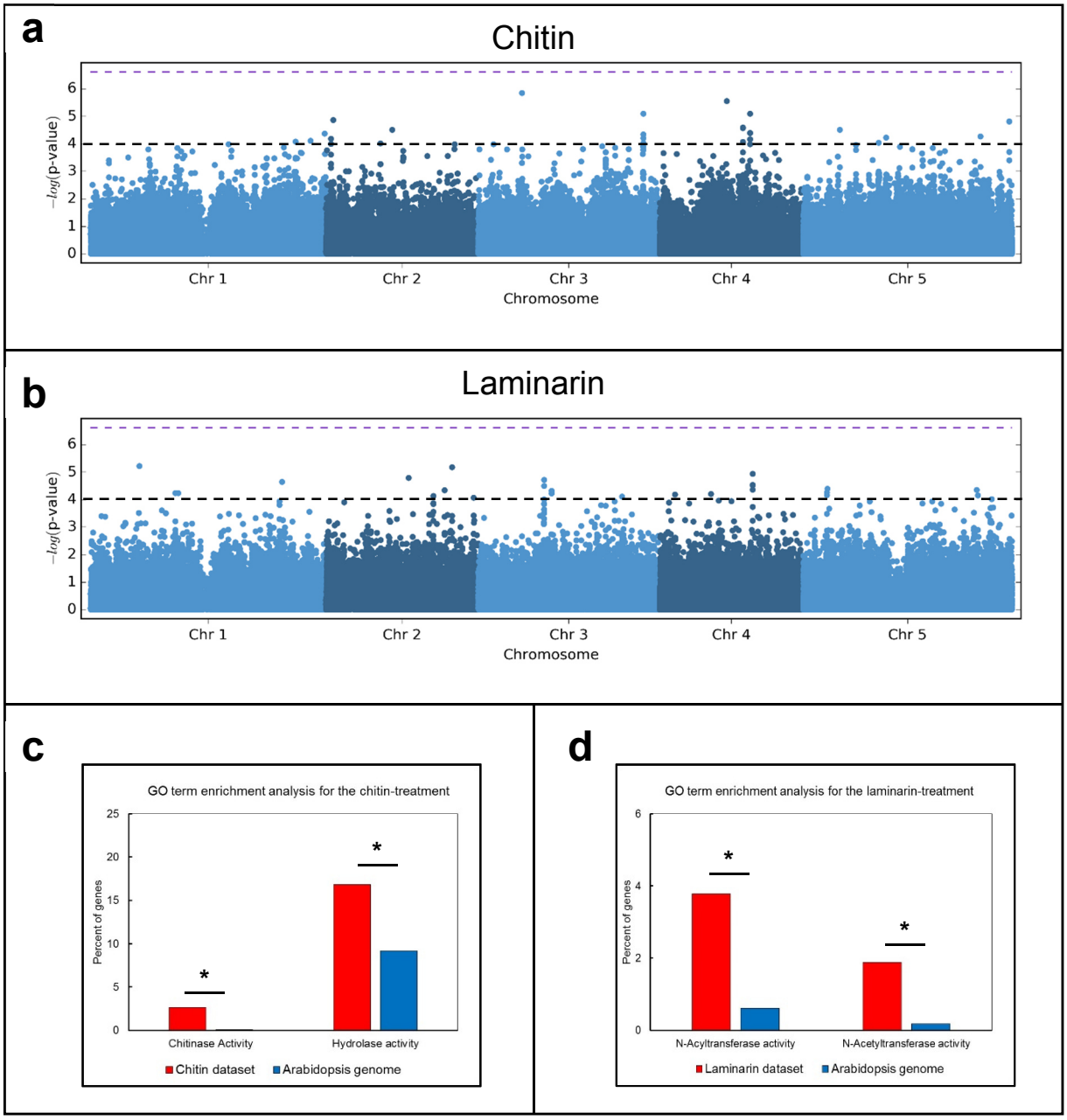

Figure 11: GWA screen for MAMP-triggered ROS production in *A. thaliana* accessions and GO term enrichment analysis for the identified gene sets. Manhattan plots for −*log*(*p*-value) SNP marker-trait associations are shown for chitin (a) and laminarin (b) treatment. Dashed purple line depict the nominal −*log*(p-value) of 6.5 which equals a FDR of 5 % after Bonferroni-Hochberg correction whereas the dotted black line highlights −*log*(p-value) of 4 which was used as threshold for the selection of genetic loci. The different shades of blue mark the five chromosomes of the *A. thaliana* genome from chromosome 1 (Chr 1) to chromosome 5 (Chr 5). SNPs with a minor allele count (MAC) of 5 are depicted as dots. The datasets were analyzed using the accelerated mixed model (amm). c) A list of 204 genes was retrieved from a GWA screen of 82 *A. thaliana* accessions tested for their responsiveness to chitin. The dataset was subjected to a single enrichment analysis (SEA) using the agriGO online database. Two GO terms corresponding to chitinase and hydrolytic activity were significantly enriched in the chitin dataset (red bars) compared to the *A. thaliana* genome used as reference (blue bars). Asteriks indicate

significant differences ($p < 0.05$) calculated using the Fisher significance test with a Yekutieli multi-test adjustment method. d) A list of 277 genes was retrieved from a GWA screen of 97 *A. thaliana* accessions tested for their responsiveness to laminarin-treatment and was subjected to a single enrichment analysis (SEA) using the agriGO online database. The two GO terms N-acyltransferase and N-acetyltransferase activity were significantly enriched in the laminarin dataset (red bars) compared to the *A. thaliana* genome used as reference (blue bars). Asteriks indicate significant differences ($p < 0.05$) calculated using the Fisher significance test with a Yekutieli multi-test adjustment method.

If moderate marker–trait associations with a $-log$(p-value) of 4 are taken into account (dashed black line) 22 SNPs were found. The highest overall marker-trait association was found on chromosome three with a $-log$(p-value) of 5.85. There are several genetic hot spots with multiple SNPs present which supports the assumption that the genetic loci in this regions represent true marker-trait associations.

For the laminarin-treated plants there were also no marker-trait associations reaching a Bonferroni-Hochberg corrected FDR of 5 % (Figure 11b). 26 SNPs were found showing moderate marker-trait associations with the highest $-log$(p-value) of 5.22 on chromosome one. Also for the laminarin-dataset there were genetic hotspots with several SNPs located in close proximity to each other. The initial statement that the responsiveness to both MAMPs is rather a multi-genetic than a mono-genetic trait is supported since several SNPs with moderate marker-trait associations were identified by the GWA screen.

To use the GWA screen for the identification of genetic loci potentially involved in perception or processing of MAMP signals a $-log$(p-value) of 4 was defined as threshold for relevant marker-trait associations which is in accordance to previous studies and accounts for the fact that there are no *a-priori* candidate genes which could have been be used to estimate the power of the GWA screen (Atwell *et al.*, 2010; Wintermans *et al.*, 2016). Furthermore the perception of laminarin as a MAMP is potentially not only mediated by a single trait but rather an interplay of several factors. Another critical factor evaluating the outcome of a GWA screen is defining the area surrounding relevant SNPs to retrieve potentially affected genes from. An area of 20 kb up- and downstream of each relevant SNP was analyzed which is in accordance with previous GWA screens and accounts for the linkage disequilibrium described for the *A. thaliana* population (Kaul *et al.*, 2000; Atwell *et al.*, 2010; Filiault & Maloof, 2012). The genes located within 20 kb up- and downstream of the 22 SNPs obtained from the GWA screen after chitin-treatment were selected and analyzed for the enrichment of gene ontology (GO) terms. A list of in total 204 genes was used for a single enrichment

analysis (SEA) identifying significantly (p = 0.05) enriched GO terms in the dataset using the agriGO online resource (Tian *et al.*, 2017). In total two of the 190 identified GO terms were found to be significantly enriched in the dataset compared to the whole *A. thaliana* genome (Figure 11c). The two significantly enriched GO terms were chitinase activity (2.6 % in the chitin dataset *vs.* 0.06 % in the *A. thaliana* genome) and hydrolytic activity (16.8 % in the chitin dataset *vs.* 9.2 % in the *A. thaliana* genome). This means that the genes potentially affected by the SNPs with the highest marker-trait association score identified by the GWA screen are more often involved in those molecular activities than the genes in the *A. thaliana* genome in general. In other words the phenotypic difference between the 82 tested accessions in terms of the ROS production triggered by chitin is mostly explained by the ability of the plants to hydrolyze chitin or compounds with glycosyl-linkages in general.

From the GWA screen dataset for the laminarin-triggered ROS production in 97 *A. thaliana* accessions 26 SNPs with a marker-trait association score higher than a $-log$(p-value) of 4 were identified. From an area of 20 kb up- and downstream of these SNPs all genes were manually retrieved yielding a list of in total 277 genes. This dataset was again used for a single enrichment analysis (SEA) identifying significantly (p = 0.05) enriched GO terms in the dataset. In total two of the 265 identified GO terms were found to be enriched in the dataset compared to the whole *A. thaliana* genome (Figure 11d). The two enriched GO terms were N-acyltransferase activity (3.8 % in the laminarin dataset *vs.* 0.6 % in the *A. thaliana* genome) and N-acetyltransferase activity (1.9 % in the laminarin dataset *vs.* 0.17 % in the *A. thaliana* genome). Gene products classified as those types of transferases are generally involved in the transfer of acyl- and especially of acetyl-groups from specialized donor molecules predominantely onto proteins and polysaccharides. The contribution of those transferases to β-glucan triggered defense responses is not known so far but a general involvement in various defense-related processes was described before (DeFraia *et al.*, 2010; DeFraia *et al.*, 2013).

2.2.3 Identification of genes involved in laminarin-triggered ROS production

The scope of the GWA screen was the identification of candidate genes, which are potentially involved in the perception of laminarin or the defense reactions induced by it. For the identification of such genetic loci the complete gene list containing 277 genes

which were retrived from an area of 20 kb up- and downstream of the SNPs with a marker-trait association score higher than a $-log$(p-value) of 4, were manually searched for those potentially involved in defense reactions according to the gene annotation in the TAIR9 database (www.arabidopsis.org). The first identified gene is located in proximity to a SNPs located on chromosome 1 which exhibits a $-log$(p-value) SNP marker-trait association score of 5.22 which is the highest score in this GWA screen (Table 1). The SNP is located 0.3 kb upstream of the transcription start of the gene *LRK10* (AT1G18390) encoding a Leaf rust 10 disease-resistance locus receptor-like protein kinase-like 1.1. The encoded receptor is predicted to be localized in the cell membrane and is annotated to harbor an intracellular serine/threonine kinase domain potentially involved in signal transduction of ABA-mediated signals and drought resistance (Lim *et al.*, 2015). The second gene was found in close vicinity to a SNP located on chromosome 2 which has a $-log$(p-value) marker-trait association of 5.18. The SNP is located 1.1 kb upstream of the gene *NATA1* (AT2G39030) encoding the protein N-acetyl-transferase activity 1. NATA1 is annotated as ornithine *N*-delta-acetyltransferase involved in the formation of the defense compound *N*-delta-acetylornithine (Adio *et al.*, 2011). Additionally on chromosome 5 a SNP with a $-log$(p-value) marker-trait association of 4.39 was identified in the GWA screen that is located in an intron of the gene *PROPEP4* (AT5G09980) that encodes the precursor of the elicitor peptide PROPEP4. PROPEP4 is cleaved to release the danger peptide PEP4, which belongs to a group of peptides that are activating pattern-triggered immune responses upon cleavage (Bartels *et al.*, 2013). These three genes were selected from the complete gene list generated by the GWA screen with 97 accessions that have been investigated for their ability to recognize laminarin as a MAMP and to react to this stimulus with the production of ROS. Based on the gene annotation and ontology these genes might be involved in the perception of laminarin as a MAMP (LRK10), in the activation of pattern-triggered immunity (PROPEP4) or in fighting a potential invader (NATA1). Nevertheless none of the three genes is directly influenced by the SNP since none of the SNPs is causing a non-synonymous mutation within the coding sequence. Hence also all non-synonymous SNPs with a $-log$(p-value) marker-trait association of more than 3 were manually searched for those SNPs that are located within genes potentially involved in defense reactions according to the gene annotation in the TAIR9 database.

Table 1: Candidate genes identified from the GWA screen of 97 accessions tested for their laminarin-triggered ROS production.

Candidate gene (AGI)	Gene annotation	Gene ontology	−*log*(p)	SNP-position
AT1G18390	Leaf rust 10 receptor- like protein kinase (LRK10)	Receptor-like protein, potentially involved in stress resistance	5.22	0.3 kb upstream
AT2G39030	*N*-acetyltransferase activity 1 (NATA1)	Involved in formation of defense compound *N*-delta-acetylornithine	5.18	1.1 kb upstream
AT5G09980	Elicitor peptide 4 precursor (PROPEP4)	Precursor of the immunity activating danger peptide PEP4	4.39	intron
AT3G23650	Kinase-like protein	Related to protein kinase	3.51	Coding sequence (non-syn.)
AT3G44400	TIR-NBS-LRR protein	Disease resistance protein with receptor activity	3.09	Coding sequence (non-syn.)
AT4G02690	Bax inhibitor-1 family protein (LFG3)	Suppressor of cell death potentially involved in disease resistance	3.58	Coding sequence (non-syn.)
AT5G17970	TIR-NBS-LRR protein	Disease resistance protein with receptor activity	3.04	Coding sequence (non-syn.)

As additional criteria only genes were selected were the mutated allele variant is found in the highly reactive accessions Jea-0, Bor-1, Ge-0 and Bla-1 and not in the non-responsive accessions and Col-0. This approach identified four more genes containing a Kinase-like protein (AT3G23650), two TIR-NBS-LRR receptors (AT3G44400 and AT5G17970) and a Bax inhibitor-1 protein (AT4G02690). All the identified proteins bear the potential to be directly involved in plant defense responses either by perception of microbial signals like the TIR-NBS-LRRs, by transduction of a perceived

signal like the Kinase-like protein or by inhibition of the Bax-induced cell death like the Bax inhibitor-1.

Taken together the data from the GWA screen suggest that there is great natural variation within the *A. thaliana* population regarding the laminarin-triggered ROS production. The relatively small dataset of 97 accessions already identified several SNPs with moderate and high marker-trait associations and led to the identification of seven genes that have the potential to be involved in laminarin-triggered defense responses. As a conclusion from the GWA screen the ability to recognize and to react to laminarin elicitation is not a mono-genetic trait in *A. thaliana* that is detectable as a qualitative difference between reactive and non-reactive accession but rather a multi-genetic trait that is characterized by quantitative differences between the accessions. The selected genes are subjected to further analysis for functional characterization to assess their influence on the laminarin-triggered ROS production in *A. thaliana*.

3. Discussion

β-glucans are the main building block of the fungal cell walls analyzed to date and crucial for its function with known elicitor-activity on plant defense responses. The aim of this study was to obtain first hints on the molecular components of the root endophytic fungus *S. indica* involved in maintenance of β-glucan integrity and suppression of β-glucan induced immunity as well as the search for putative plant modules crucial for β-glucan signal perception and defense. Therefore the selected lectin WSC3, a member of the expanded protein family of WSC domain-containing proteins was functionally characterized regarding its participation in β-glucan biology.
WSC3 was shown to be transcriptionally induced during plant colonization and during contact with a soil-borne root pathogen and resides at the *S. indica* cell wall where it affects cell wall structure (Figure 4). Expression of WSC3 in the yeast *P. pastoris* led to the incorporation of the protein into the β-glucan compound of the cell wall and drastically increased cell wall stress resistance (Figure 5). The recombinant protein WSC3-His was shown to bind the branched β-1,3/1,6-glucan laminarin but was not able to protect it from enzymatic hydrolysis (Figure 6). The exogenous addition of WSC3 to different fungi led to the formation of cellular aggregates but had no effect on adhesion of the *S. indica* spores to plant roots (Figure 7). The laminarin-triggered production of ROS was suppressed by WSC3 and FGB1, whereas FGB1 additionally suppressed the chitin- and flg22-induced ROS production leading to an enhanced colonization of *S. indica* which was not the case for WSC3 (Figure 9).
For the identification of plant genes involved in β-glucan induced defense 100 *A. thaliana* accessions were tested for their ability to recognize laminarin as a MAMP which revealed remarkable natural variation of this trait (Figure 10). The performed GWA screen identified several genetic loci which might be involved in β-glucan induced defense and led to the selection of candidate genes (Figure 11 and table 1).

3.1 WSC3 is an integral cell wall component increasing cell wall stability

The results of the western blot and the cytological analysis suggest that WSC3-GFP is secreted, but resides at the fungal cell wall and is not released into the extracellular space. The fact that free GFP was detectable in the culture filtrate for some of the generated transformants may be the result of proteolytic cleavage of the fusion protein,

a phenomenon that has been observed for other secreted fusion proteins of *S. indica* like FGB1-GFP (Wawra *et al.*, 2016). In contrast to FGB1-GFP were also the full length fusion protein was present in the extracellular space, WSC3-GFP could not be detected in the culture filtrate according to the western blot analysis. This most probably means that the GFP is cleaved from the cell wall bound WSC3-GFP and thus diffuses into the extracelluar space. The results of the western blot are in line with the microscopical investigation of the WSC3-GFP expressing transformants. The occurrence of GFP-signals within intracellular vesicles moving towards the hyphal tip might be seen as an additional hint that WSC3-GFP is secreted into the extracellular space since this is in line with the mechanism of conventional protein secretion predominantly occurring *via* golgi vesicles at the hyphal tip (Peberdy, 1994; Casadevall *et al.*, 2009; Rodrigues *et al.*, 2011). The idea that WSC3-GFP is secreted by the conventional secretion pathway *via* the golgi apparatus is furthermore not only supported by the occurrence of the fusion protein in such vesicles but also by the presence of a proline residue at the +2 position after the predicted signal peptide cleavage site. This amino acid motif was recently described as ER export signal and is enriched in secreted proteins of *S. indica* (Tsukumo *et al.*, 2009; Zuccaro *et al.,* 2011; Lahrmann & Zuccaro, 2012). The secreted fusion protein most probably localizes to the *S. indica* cell wall considering the overlap of the GFP signal with the signal of the chitin-specific fluorescence counter stain WGA-AF594 and the proofed affinity for branched β-1,3/1,6-glucans. The presence of WSC3-GFP at the cell wall of transformant T3 significantly decreased the labelling of cell wall β-1,3-glucans whereas the labelling of chitin was not altered compared to the wildtype strain. This could be a hint for a similar mechanism like described for FGB1 where deregulation leads to an alteration of the cell wall composition (Wawra *et al.*, 2016). Another explanation could be that WSC3-GFP binds the exposed β-1,3-glucan moieties in the cell wall and in that way prevents detection by the β-1,3-glucan-specific antibody. Hence the cell wall composition would not altered but the exposure of β-1,3-glucans would be blocked which would be in line with the affinity of WSC3 for branched β-1,3/1,6-glucans. To gain a deeper insight into the effects of WSC3 on the cell wall composition of *S. indica* a cell wall linkage analysis like performed for the FGB1-GFP expressing strain might be also performed for the WSC3-GFP strain. Furthermore the expression of WSC3-GFP driven from the native WSC3 promoter might be also tested in the future. This would ensure the right timing of the expression and would allow the investigation of the

localization of WSC3 during plant colonization of *S. indica*. First attempts to use the native promoter of WSC3 did not yield detectable amounts of WSC3-GFP. Considering the high portion of the fusion protein where the GFP is cleaved the use of another protein tag might be encountered. For example a rather short His-tag which could be detected by a specific antibody and visualized by TEM could prevent cleavage of the tag to ensure detection of the fusion protein even if produced in only low amounts.

The fact that the genome of *S. indica* encodes multiple WSC-proteins with similar domain architecture and expression suggest a high degree of redundancy which might complicate the investigation of overexpression and knock-out effects in *S. indica*. Therefore *P. pastoris* was used as model since it i) might also possess a cell wall consisting of β-glucans, ii) as eukaryotic expression system is able to form disulfide bonds and add glycosylation which might both be highly relevant for the folding and function of WSC3 and iii) is devoid of WSC-lectins except of the WSC1-4 homolgs known from *S. cerevisiae*. The recombinant WSC3-His protein produced in *P. pastoris* has a molecular weight of approximately 55 kDa according to the SDS-PAGE and is secreted. The predicted molecular weight of the full length WSC3-His protein based on its amino acid sequence is approximately 40 kDa after cleavage of the α-secretion signal. The deglycosilation of the recombinant protein with a commercial deglycosilation mix revealed that the unglycosilated protein exhibits the expected molecular weight of 40 kDa which suggests that the difference of the molecular weight at least partially originates from glycosylations. After deglycosilation of the recombinant WSC3-His a portion of the protein still exhibit a molecular weight of 55 kDa. This could argue for an incomplete removal of the glycosylation or the presence of an additional post-translational modification other than the glycosylation. Nevertheless it can be assumed that glycosylation sites within the *S. indica* protein are recognized by the *P. pastoris* glycosylation machinery. It is still elusive if the glycosylation pattern of the recombinant protein is identical to the native one especially regarding the known process of hyperglycosilation in yeast cells (Hamilton & Gerngross, 2007). The fact that the enzymatic protein extraction from the *P. pastoris* cell wall preparation revealed the accumulation of the recombinantely produced protein at the cell wall highlights that this potential difference in the glycosylation pattern does not influence the protein localization. The additional presence of WSC3-His in the culture filtrate of *P. pastoris* might be the result of the higher amount of expressed protein compared to the expression of WSC3-GFP in *S. indica*. This potentially could result in the release of the

secreted protein into the medium after all available binding sites for WSC3 within the cell wall are saturated with the protein. In the samples of the cell wall preparations treated with the β-1,3-Endo-or Exoglucanase an accumulation of WSC3-His was detectable on the western blot. WSC3-His even after the glucanase treatment predominately remained in the pellet fraction and was not solubilized to the supernatant of the enzymatically treated cell wall preparations. This might suggest that WSC3-His is not covalently linked to the β-glucans but most probably deeply buried within the β-glucan fibrils. The enzymatic treatment would hence simply loosen the β-glucan structure and make WSC3-His accessible for the antibody. This would be in agreement to earlier studies describing proteins found within the fungal cell wall which are liberated by glucanases but are not covalently linked to the β-glucan moieties (Bernard *et al*., 2002; Latgé, 2007). Additionally those proteins described to be covalently linked to the cell wall mostly possess a GPI anchor which is crucial for the connection to the β-1,3/1,6-glucans but not present in WSC3 (Kapteyn *et al.*, 1996; Kollár *et al.*, 1997; Fuji *et al.*, 1999; Yin *et al.*, 2005). A similar enzymatic cell wall protein extraction was attempted from the *S. indica* WSC3-GFP strain with subsequent detection with an anti-GFP antibody. Unfortunately the amount of detectable fusion protein was low and due to the cleavage of the GFP, only free GFP was detectable in the culture filtrate.

The WSC3-His expressing *P. pastoris* strains 1 to 3 showed a tremendously increased resistance to Calcofluor White and also Congo Red compared to the albumin-expressing reference strain. Calcofluor White and also Congo Red are commonly associated with the synthesis and interconnection of cell wall chitin and β-1,3-glucan (Ram *et al.*, 2006; Kopecka & Gabriel, 1992). These results suggest that WSC3 strengthens the fungal cell wall against external stresses probably acting as a proteinaceous glue reinforcing the cell wall. Most of the proteins found within the cell wall which were hypothesized to be involved in cell wall reinforcement and stability were shown to be mannosylated *via* O-linked glycosylation which indeed might also be the case for WSC3 containing 19 predicted O-glycosilation sites. Thus WSC3 or even WSC proteins in general could represent a novel class of integral cell wall proteins that act as stabilizers of the β-1,3-glucan compound. Such cell wall reinforcing proteins are known from *S. cerevisiae* were they for example were found as part of the so called flexible building block of the cell wall consisting of β-1,3-glucan, β-1,6-glucan and chitin

establishing the core structure of the cell wall polysaccharides (Kollár *et al.*, 1997; Yin *et al.*, 2005).

Taking the localization data of WSC3-GFP, the altered β-1,3-glucan-to-chitin ratio in *S. indica* and the increased cell wall stress resistance in *P. pastoris* by integration of WSC3-His into cell wall into account, it is tempting to speculate that WSC3 is involved in increasing the cell wall stability by reinforcing the β-glucan structure during such demanding conditions like the colonization of plant roots or the direct contact with a second fungus. Under such conditions were cell wall stability is crucial but highly challenged by the extreme conditions for example the presence of hydrolytic enzymes like chitinases and glucanases an integral proteinaceous glue reinforcing the cell wall would be valuable. Thus the expanded WSC protein family of *S. indica* might be an adaption to various external stresses which demand an additional reinforcement of the cell wall.

3.2 WSC3 specifically binds the branched β-1,3/1,6-glucans laminarin

WSC3-His exhibits a preference for oligo- and polysaccharides originating from the fungal cell wall which is in line with the proposed function of the WSC domain as cell wall anchor in yeast or as part of an antifungal β-1,3-glucanase (Cohen-Kupiec *et al.*, 1999; Lodder *et al.*, 1999). Interestingly WSC3-His showed no affinity to laminarihexaose a hexamer of glucose units joined by β-1,3-glycosidic linkages which could have been expected from the afore mentioned reports proposing a binding specificity of the WSC domain to β-1,3-glucans. Instead only a binding to the branched β-1,3/1,6-glucan laminarin consisting of more than 30 glucose molecules was detected. Thus the performed ITC measurements represent the first experimental proof that not β-1,3-glucans but mixed β-1,3/1,6-glucans are the preferred target of WSC domain-containing proteins. Nevertheless an affinity of WSC3-His to linear β-1,3-glucan cannot be completely ruled out at this stage since only a rather short ligand was tested in ITC which as reported for chitin-binding proteins might have a considerable impact on the binding affinity (deJonge *et al.*, 2010; Liu *et al.*, 2012). Still it is unlikely that WSC3-His would exhibit a binding to a longer linear β-1,3-glucan. Otherwise at least a weak binding of WSC3-His to laminarihexaose could have been expected in the ITC measurement which was not the case and also co-precipitation of WSC3-His with the plant cell wall preparation might have been observed in the pull

down experiments. WSC3-His binds laminarin with a K_d value of 12.5 µM ± 8.8 µM and a molar ratio of 1:3. Thus each of the three WSC-domains of WSC3 is potentially able to bind one molecule of laminarin or each of the three WSC-domains binds three distinct sites within one laminarin molecule. Both mechanisms would be supportive to the proposed function of WSC3 as cell wall β-glucan stabilizer assuming that one molecule of WSC3 joins three β-1,3/1,6-glucan fibrils or layers together. Also the fungal cell agglutination effect of WSC3 potentially relies on the connection of the β-glucan fibrils of neighbouring cells which might depend on the binding of at least two β-glucan molecules. The binding affinity of WSC3 to laminarin is in the same range like reported for the *C. fulvum* chitin-binding lectin Avr4 which binds chitohexaose with a K_d value of 6.3 µM and the *A. thaliana* chitin-receptor CERK1 with a reported K_d value of 45 µM (van den Burg *et al.*, 2004; Liu *et al.*, 2012). On the other hand side the immune suppressive effector Ecp6 of *C. fulvum* which exhibits a similar domain architecture like WSC3 binds chitin with a much higher K_d value of 280 pM due to a novel intrachain LysM domain dimerization upon chitin binding (Sanchez-Vallet *et al.*, 2013). Due to its high affinity Ecp6 outcompetes the plant chitin receptor and thus prevents stimulation of the plant immune system by free chitin oligomers (deJonge *et al.*, 2010; Sanchez-Vallet *et al.*, 2013). The number of biochemically characterized β-glucan binding lectins from fungi is greatly limited. Only for the recently discovered β-1,6-glucan specific lectin FGB1 from *S. indica* the affinity to laminarin was calculated revealing an high affinity binding site with a K_d value of 80 nM (Wawra *et al.*, 2016). The best characterized β-glucan specific receptor is the mammalian receptor Dectin-1 which was reported to bind to laminarin with a K_d value of 10 µM (Brown, 2006; Brown *et al.*, 2007). Further examples for β-glucan binding proteins are known from insects where for example in the cockroach *Blaberus discoidales* a β-1,3-glucan binding lectin is involved in recognition and immunity against fungi (Chen *et al.*, 1999). The cockroach lectin also possess three distinct binding sites each binding one molecule of laminarin and was shown to effectively agglutinate yeast cell which is a striking structural and mechanistic similarity tor WSC3 (Chen *et al.*, 1999).

Concluding from that WSC3-His specifically binds the branched β-1,3/1,6-glucan laminarin with an affinity that is comparable to other lectins suggesting a physiologically relevant function of this association. Thus the cell wall reinforcing properties of WSC3 could rely on the connection of several β-1,3/1,6-glucan branch points within the cell wall. This would also explain the decreased labelling of β-1,3-glucans within the cell

wall and would rather argue for a reduced exposure than for a reduction of the overall β-1,3-glucan content. The example of Ecp6 not only illustrates the sophisticated mechanisms evolved by plant colonizing fungi to prevent recognition but also the technical limitations of the ITC determination of the dissociation constant K_d. When produced in *P. pastoris* the high affinity binding pocket of Ecp6 is already pre-occupied by a chitin molecule present in yeast cells. Thus only the binding of the second chitin oligomer with a lower affinity is detectable by ITC and thus a K_d value of 6.4 µM was initially calculated for binding of chitohexaose (deJonge *et al.*, 2010). Only if Ecp6 is produced in a mammalian cell culture system devoid of chitin the protein is produced in its native state and both chitin-binding events can be detected by ITC (Sanchez-Vallet *et al.*, 2013). Similar limitations might be expected for WSC3 since also β-glucans are present in yeast cells and could pre-occupy a potential high affinity binding site. A second limitation is represented by the availability of suitable β-glucan ligands. Laminarin is widely used as a surrogate for branched β-1,3/1,6-glucans representing polysaccharides of fungal and oomycete origin. Nevertheless as a natural substrate the molecule length, the degree of polymerization and the purity differs between various batches. An alternative strategy would be the use of a chemically synthesized substrate like described for FGB1 (Wawra *et al.*, 2016). Due to the highly challenging chemical procedure to synthesis pure and defined β-glucans (only the dimer gentiobiose was used for FGB1) and the limited availability of (solube) β-glucans laminarin is still used as a substrate. Though it has to be always considered that laminarin is not the physiological relevant ligand of WSC3 (and FGB1) since it only represents fungal derived branched β-glucans of similar structure. This might also explain why laminarin is not protected by WSC3 from hydrolysis by the plant β-1,3-glucanase. The use of chemically synthesized oligosaccharides as potential ligands for WSC3 is currently attempted in a high throughput glycan array in cooperation with the group of Dr. Sebastian Pfrengle at the MPI of colloids and interfaces at Potsdam. This allows the testing of a broad spectrum of potential ligands, also such that are associated with potential glycosylation patterns of proteins, and avoids the bias introduced by the targeted testing of selected oligo- and polysaccharides. The revealed affinity of WSC3 to branched β-1,3/1,6-glucans seems plausible since it fits to the observed localization at the fungal cell wall, the ability of WSC3 to agglutinate fungal cells and the ability to prevent laminarin-triggered ROS production. Neverthless the presence of WSC-domains in other *S. indica* proteins for example in combination with

a glyoxal oxidase domain known from enzymes involved in lignin degradation in *Phanerochaete chrysosporium* or with the GH71 domain known from an α-1,3-glucanase from *L. bicolor* opens up the possibility that branched β-1,3/1,6-glucans are not the only targets of WSC-proteins (Whittaker *et al.*, 1999); Martin *et al.*, 2008).

3.3 WSC3 agglutinates fungal cells but is not involved in broad spectrum immune suppression

The agglutinating properties of WSC3 are highly interesting since the relevance of aggregate formation for propagation and infection is known for a long time from bacteria but less described for fungi (Hall-Stoodley *et al.*, 2004). Especially biofilms, a loose aggregation of cells which are embedded in a mostly polysaccharide matrix are highly relevant for bacterial infections and have been also described for fungi like *Aspergillus niger* (Fleming & Wingender, 2010; Priegnitz *et al.*, 2011). The role of fungal biofilms in plant-microbe interactions are largely unknown but recent evidence suggests that *S. indica* might form such biofilms during plant colonization because microscopical investigations found FGB1-GFP localizing to a gel-like matrix surrounding the fungal hyphae, indicating the presence of β-glucans (S. Wawra, unpublished data). The presence of glucan-binding proteins in bacterial biofilms as structural component was shown before and thus it is tempting to speculate that WSC3 might be involved in the process of biofilm or aggregate formation (Lynch *et al.*, 2007). In such a scenario WSC3 would be not completely buried within the cell wall but protruds out of the cell wall into the external space in a similar way like described for adhesins from *S. cerevisiae* and *C. albicans* (Verstrepen *et al.*, 2005; Latgé, 2007). In summary the agglutination of fungal cells might be considered as another hint that WSC3 could act as a fungal glue that connects β-1,3/1,6-glucans with each other. When incorporated into the cell wall like shown for *P. pastoris* WSC3 might act as proteinaceous cement that joins the β-1,3/1,6-glucan compounds of the cell wall. It needs to be considered that the external application of WSC3-His might not represent the physiologically relevant localization of the protein. Hence the agglutination of fungal cells could be seen as an additional proof for the ability of WSC3 to bind (exposed) β-glucans in the fungal cell wall.

Taking the affinity of WSC3-His to laminarin (K_d value of 12.5 µM) into account the binding affinity could potentially be sufficient to compete with the unknown β-glucan

responsive receptor presuming a similar affinity of the receptor to its ligand like described for other β-glucan or chitin binding receptors. Thus the suppression of the laminarin-triggered ROS production most probably relies on the sequestration of laminarin preventing the recognition by the unknown β-glucan receptor. The fact that 10 µM of WSC3-His are sufficient to prevent recognition of 600 µM of laminarin points either towards the hypothesis that the commercial laminarin already contains trace amounts of shorter, more active β-glucans that are causal for the ROS production and also bound by WSC3-His. Alternatively it could be possible that the laminarin molecule itself is not the defense-activating component but that it is tailored by hydrolysis through plant-derived glucanases into shorter, more active parts. This would also explain why the plant defense response to laminarin is remarkably delayed compared to chitin- and flg22 elicitation. Like mentioned before the external addition of WSC3-His might lead to an unusual localization of WSC3. Thus the suppression of the laminarin-induced ROS production by WSC3-His might be seen as another line of evidence that WSC3 is able to bind to branched β-1,3/1,6-glucans like laminarin. This is underlined by the fact that addition of WSC3-His together with *S indica* spores had no positive effect on the colonization rate.

In contrast to WSC3, FGB1 seems to be a universal suppressor of MAMP-triggered ROS production which is achieved independently from binding to the MAMP translating into increased plant colonization. Hence FGB1 might directly interact with a so far unknown shared co-receptor or generally co-regulator which is involved in the perception of various MAMPs. The existence of such universal PTI regulators is illustrated by the existence of the receptor-like cytoplasmic kinase BIK1 which is involved in perception and activation of immunity of the bacterial MAMPs flg22 and elf18, the fungal MAMP chitin and the plant derived DAMP AtPep1 (Couto & Zipfel, 2016). The cytoplasmic receptor-like kinase BIK1 might not be targeted by the apoplastic effector FGB1 but the mechanistic concept of shared components raises the possibility that also other so far unknown plant proteins exist that fulfill similar regulatory functions. Furthermore it is known from plant colonizing bacteria that these regulatory co-receptors are targeted by secreted effectors to deregulate plant PTI responses (Macho & Zipfel, 2015). Beneath tackling a shared co-receptor also a common motif or pattern present in different MAMP receptors, like a common glycosylation pattern might be the target of FGB1. It was recently reported that mutation of the α-1,3-manosyltransferase ALG3 of *A. thaliana* that is responsible for

the addition of a core oligosaccharide to several surface localized immune receptors leads to an altered localization and also a compromised signaling capacity of those receptors (Trempel *et al.*, 2016). Thus it is not unthinkable that a fungal lectin-like effector protein like FGB1 is able to suppress the recognition of several completely different MAMPs by interacting with a common glycosylation pattern added to all these unrelated receptors. Another possibility is the direct targeting of the NAPDH oxidases or peroxidases at the plant plasmamembrane or cell wall, respectively, which are responsible for the generation of extracellular ROS (Yu *et al.*, 2017). This would explain why the flg22-induced Ca^{2+}-flux is not suppressed by FGB1 which is described to be crucial for subsequent ROS production (Ranf *et al.*, 2011). Conceivable targets would be the respiratory burst oxidase homolg D (RBOHD) or the apoplastic peroxidases PRX33/34 which are the major enzymes involved in generation of ROS (Nuhse *et al.*, 2007; Daudi *et al.*, 2012). Another MAMP-induced plant response that acts downstream of the Ca^{2+}-flux but upstream of the generation of ROS are ion fluxes across the plant plasmamembrane. These ion fluxes and the responsible ion channels are crucial for the generation of extracellular ROS and thus might be a potential target for FGB1 to prevent MAMP-triggered ROS production (Jabs *et al.*, 1997).

In general WSC3 and FGB1 seem to vary greatly regarding their function even though both were shown to specifically bind to laminarin. Whereas WSC3 agglutinates fungal cells, suppresses the laminarin-induced ROS production but is not increasing colonization of plant roots, FGB1 has no obvious effect on fungal cells but has the ability to prevent ROS production after stimulation with various MAMPs which translates into an increased plant colonization rate. The reason for these difference might be the different protein structure and localization which might be the determinant for their function. WSC3 is a rather big protein and has the ability to bind three long polysaccharide molecules which is potentially the key for its cell wall reinforcing and fungal cell agglutinating properties. Furthermore WSC3 seems to reside at or within the fungal cell wall and thus its addition to fungal spores has no effect on the plant colonization even though it theoretically is able to prevent laminarin recognition. Contrastingly FGB1 is an extremely short protein that only binds one laminarin molecule or even the β-1,6-glycosidic linked dimer gentiobiose with an ultrahigh affinity to outcompete a potential β-glucan receptor. Furthermore it is not only present at the cell wall, but also released into the extracellular space where it may act directly at the plant cell as a universal PTI suppressor. Both proteins are β-glucan binding lectins

involved in plant colonization but potentially due to their contrasting characteristics fulfill completely different functions which nicely illustrate the dual relevance of β-glucans as crucial components maintaining fungal cell wall integrity and at the same time potent elicitors of plant immunity.

3.4 Summary: WSC3 as β-glucan adhesive?

WSC3 is the first biochemically characterized WSC lectin with proofed specificity to the branched β-1,3/1,6-glucan laminarin. WSC3 localizes to the cell wall of *S. indica* and seemingly is even incorporated into it were it might be buried within the β-glucan fibrils. First hints point toward a possible function in connecting neighbouring β-1,3/1,6-glucan moieties with each other potentially to reinforce the *S. indica* cell wall during growth under stressful conditions. Thus WSC3 might act as a proteinaceous β-glucan adhesive that due to its localization within the cell wall serves as a structural component stabilizing the cell wall. Further investigations should test more potential polysaccharide ligands, the exact localization and association of WSC3 to the cell wall β-glucans and how and if cell wall composition and structure of *S. indica* is altered through WSC3 overexpression. The existence of the two lectins WSC3 and FGB1 both exhibiting affinity to branched β-1,3/1,6-glucans but fulfilling contrasting biological tasks illustrates the importance of β-glucans as essential components of the fungal cell wall that need to be fostered to prevent recognition while maintaining their integrity. Thus the expansion of WSC proteins within the *S. indica* genome might be seen as an adaption to the biphasic lifestyle of *S. indica* including a morphological and nutritional switch during the colonization of a broad range of different plant host without losing its capabilitiy allowing survival in an extremely competitive environment like the soil. An expanded WSC toolkit could enable *S. indica* to encounter these diverse requirements which are extremely challenging for the cell wall. Nevertheless the additional analysis of other members of the WSC protein family is needed which potentially are expressed under other growth conditions of *S. indica* than WSC3 to gain a deeper insight into the role of WSC proteins and to understand the reason for their expansion. The diverse features of WSC3 already give a small insight into the potential of WSC-proteins and the diverse functions they possibly fulfill.

3.5 Identification of defense components potentially involved in laminarin-triggered plant defense

The number of GWA screen studies using *A. thaliana* has tremendously increased over the past five years but most of them investigated well studied biological traits like the regulation of flowering. The advantage of the investigation of such traits is that *a priori* candidate genes are known that can be used first to judge if the present GWA screen has the power to identify true positive associations and second to use SNPs located in or in close proximity to the *a priori* candidate genes to refine the analysis (Filiault & Maloof, 2012). Due to the fact that no such *a priori* candidates are available the initial GWA screen was conducted with the relatively small number of 100 accessions. In general since the knowledge about the plant components that are potentially involved in β-glucan signal perception and processing is restricted, chitin as well-studied fungal MAMP was tested in parallel. The mechanisms of chitin signal perception and processing are not only well understood but potentially also well-conserved and might even date back to one ancient ancestor (Liang *et al.*, 2014). This not only allowed the evaluation of the actual ROS measurement in terms of overall responsiveness of the tested accessions but also serves as training for the applied assumptions and performed analysis. Furthermore it was observed that the response to laminarin seemed to be partially dependent on plant development. The enmeshment of developmental processes and immunity in plants is so far only partially understood but nicely illustrated by the receptor-like kinase BAK1, a universal co-regulator of PTI which is simultaneously also involved in processing of developmental signals (Postel *et al.*, 2010). The use of chitin as second MAMP thus ensured that the tested accession were not generally impaired in immunity. The GO term enrichment analysis after chitin-treatment was thus performed to estimate the power of the dataset. The genes that are located in proximity to the SNPs with a marker-trait association higher than a $-log$(p-value) of 4 are significantly enriched for hydrolases especially chitinases. Taking into account that a crude chitin suspension that is prepared by mechanical disruption of complex chitin was used to elicit the ROS production the enrichment of chitinases that are in charge to further hydrolyze the chitin oligomers to produce more or more active immune-stimulating molecules is not surprising. This at first glance seems trivial but highlights that the ability of a plant to properly defend against an invading fungus not only depends on the perception of chitin as a MAMP but also on

the release and the tailoring of the MAMP. Additionally since none of the known chitin-responsive defense receptors was identified by the GWA screen as hot spot for natural variation it might be concluded that these components are indeed conserved and potentially crucial for the defense against invading fungi. Together this suggests that the GWA screen is despite the limited number of tested accession powerful enough to identify genetic loci involved in defense after chitin elicitation and might also yield meaningful associations for the laminarin-treated plants.

The GO term enrichment analysis of the genes identified by the GWA screen after laminarin-treatment revealed a significant enrichment of N-acetyltransferases. N-acetyltransferases are involved in a myriad different cellular processes for example post-translational modification of proteins through the addition of acetyl-groups. Interestingly in *A. thaliana* the N-acetyltransferase Elongator subunit 3 (Elp3) is acetylating histones and thus positively regulates and accelerates the activation of various defense genes involved in basal immunity (DeFraia *et al.*, 2010; DeFraia *et al.*, 2013). One of the candidate genes identified by the GWA screen also encodes an N-acetyltransferase namely *NATA1*. NATA1 is an ornithine *N*-delta-acetyltransferase involved in the formation of the defense compound *N*-delta-acetylornithine (Adio *et al.*, 2011). Furthermore NATA1 acetylates putrescine and thus prevents the conversion to spermidine and spermine which serve as substrates for polyamine oxidases which are producing H_2O_2 in response to microbial attack (Lou *et al.*, 2016). Interestingly several accessions lack *NATA1* and thus to not produce N-acetylputrescine which makes it highly conceivable that natural variation at this genetic locus might contribute to the altered production of ROS in response to laminarin (Lou *et al.*, 2016). The relevance of N-acetyltransferases during induction of plant immunity is further elaborated by the fact that several of these enzymes including a histone N-acetyltransferase and also NATA1 are significantly induced at transcriptional level at least at one time point during the interaction of *A. thaliana* with *S. indica* or *S. vermifera*, respectively (Lahrmann *et al.*, 2015). Like *NATA1* where the identified SNP is located within an intron and thus could impact processing of the transcribed mRNA, *LRK10* and *PROPEP4* represent two more candidate genes where the identified SNPs are not directly altering protein structure but rather impact protein expression. *LRK10* encodes a plasmamembrane located receptor-like kinase first described in wheat conferring resistance to leaf rust disease (Feuillet *et al.*, 1997). In *A. thaliana* LRK10 is present in two isoforms controlled by two different promoter regions and reported to be involved in drought stress and

ABA-mediated signaling (Lim *et al.*, 2015; Shin *et al.*, 2015). LRK10 harbors an extracellular WAK-domain which is described to be involved in carbohydrate binding and is part of the WAK1 receptor which activates defense signaling after perception of the plant DAMP oligogalacturonides (Brutus *et al.*, 2010). Since LRK10 represents the only candidate gene with receptor-like properties, an extracellular carbohydrate binding domain and is also induced during *S.indica*/*S. vermifera* interaction with *A.thaliana* it is tempting to speculate that it is the elusive β-glucan responsive receptor (Lahrmann *et al.*, 2015). The third candidate gene is *PROPEP4* a member of the AtPEP family of DAMPs. It is the precursor of the endogenous danger signal Pep4 which is known to be involved in the amplification of external danger signals and the induction of innate immunity including the production of ROS (Huffaker *et al.*, 2006; Huffaker & Ryan, 2007). Thus it might potentially be also involved in the response to laminarin and therefore is an interesting candidate especially since *PROPEP4* is mostly expressed in plant roots where it could act in response to soil-borne fungi like *S. indica*. The WAK-domain of the LRK10 and Pep4 have in common that they are known to be either involved in DAMP perception or even functioning as DAMP. This raises the question if laminarin which only differs from the plant derived β-glucan callose by its β-1,6-glycosidic side branches is recognized rather as a DAMP than as a MAMP? NATA1 and Pep4 are both transcriptionally induced by the plant defense hormone jasmonic acid which production is strongly upregulated in plants in response to *S. indica* colonization (Fesel & Zuccaro, 2016b). Hence NATA1, LRK10 and Pep4 are highly interesting candidates which might be involved in laminarin-induced defense responses even though none of them is structurally altered due to the corresponding SNPs identified by the GWA screen.

Encountering also SNPs with a marker-trait association higher than a $-log$(p-value) of 3 but only taking those SNPs into account i) that lead to non-synonymous mutations directly affecting the amino acid composition, ii) that are predominantly found in the highly reactive accessions and iii) that are transcriptionally induced in response to biotic stresses according to the Arabidopsis eFP browser (bar.utoronto.ca; Winter *et al.*, 2007) four more genetic loci could be identified. These assumptions were made to increase the possibility that the genetic loci identified in this way are representing meaningful candidates despite the lower marker-trait association threshold. One of these candidate genes (*AT3G23650*) encodes an intracellular kinase-like protein which potentially could mediate the transduction of the extracellular signal. Hence the natural

variation in regards of laminarin-triggered ROS productioncwould also originate from the ability of the plant to activate downstream defense responses like the accumulation of ROS. Like the kinase-like protein the two TIR-NBS-LRR proteins which were identified by the GWA screen are potentially also not directly involved in perception of laminarin as a MAMP. NBS-LRR proteins are a highly diverse class of plant proteins that intracellularly monitor and guard defense components that are targeted by mircrobial proteins to comprise plant defense responses (Marone *et al.*, 2013). NBS-LRR proteins directly or indirectly recognize specific microbial effector proteins as part of the adaptive plant immune system and are often the result of a co-evolution between plants and microbes (Dodds & Rathjen, 2010). This suggests that there are plant defense components that are either specific for laminarin-triggered defense responses or in general are activated upon microbial attack that are worth to be guarded by NBS-LRRs. Thus knowing the guarded components or the corresponding microbial effectors would shed further light on the components involved in laminarin-induced defense. Nevertheless neither the kinase-like protein nor the TIR-NBS-LRRs might directly influence the laminarin-triggered ROS like for example NATA1. The fourth candidate gene which was identified by the GWA screen and contains a non-synonymous mutation is the LIFEGUARD 3 (LFG3) protein and belongs to the Bax inhibitor-1 (BI1) family. LFG proteins were shown to be involved in inhibition of cell death in plants and furthermore are supporting infection by plant pathogenic microbes (Wei *et al.*, 2013). The exact cellular function of LFG proteins and how they influence microbial infections is still unknown but it was suggested that the elevated susceptibility might be connected to the ability of LFG proteins to regulate Ca^{2+}-fluxes and thus to also prevent production of ROS (Eichmann *et al.*, 2006; Weis *et al.*, 2013). Hence there might be a direct connection between and altered structure and function of LFG3 in some of the tested *A. thaliana* accessions and their ability to produce ROS in response to laminarin perception.

In summary the conducted GWA screen investigating the natural variation of 100 *A. thaliana* accessions regarding their responsiveness to the branched β-1,3/1,6-glucan laminarin identified several candidate genes that belong to well-described classes of defense-related plant proteins. This further highlights the importance of β-glucans as potent elicitors of plant defense responses. To further understand if these genes are specifically involved in laminarin-triggered defense or barely represent general defense components needs to be assessed by the functional characterization of these genes.

This will also help to understand their potential function but might be complicated by the fact that the largest collection of T-DNA knock-out lines was generated in Col-0 background an accession that hardly shows any ROS production in response to laminarin. Furthermore the responsiveness to laminarin seems to be a multigenetic trait with several genetic loci that quantitatively contribute, a fact that greatly complicates the interpretation of the results and the identification of meaningful SNPs by GWA screens (Filliaut & Maloof, 2012). Prior to the initiation of the GWA screen a more simple genetic architecture of the trait was assumed, proposing that the presence or functionality of a laminarin-responsive receptor determines the ability of the accessions to initiate a defense response. The results obtained after the investigation of 100 accessions suggest a more complex genetic architecture with several genetic loci with small effect sizes that are also only present in a small number of accessions. A scenario like this demands a higher number of accessions which would increase the power of the GWA screen to also identify rare genetic loci with a small effect size (Korte & Farlow, 2013). Nevertheless the presented GWA screen yielded valuable insights into the mechanisms of laminarin-perception and response in *A. thaliana*. The following strategy could include i) the functional characterization of the so far identified candidates, ii) the investigation of more accession to increase the power of the GWA screen and iii) the additional QTL mapping approach after crossing of one strongly and one weakly reacting accession.

3.6 General conclusion

This work was conducted to shed light on the molecular strategies employed by *S. indica* to prevent activation of the plant immune system by cell wall derived β-glucans while maintaining cell wall integrity and to identify plant proteins potentially involved in β-glucan triggered defense responses. WSC3, a member of a multi-protein family was shown to specifically bind to the branched β-1,3/1,6-glucan laminarin to potentially reinforce the fungal cell wall during growth in challenging environments while FGB1 prevents the activation of the plant immune response by the universal suppression of ROS production. A GWA screen revealed that the responsiveness to laminarin is a multigenetic trait in *A. thaliana* and identified several plant proteins that have the potential to be involved in laminarin-triggered defense responses. This all together underlines the importance of β-glucans as cell wall constituents and MAMPs that are

guarded in the mutualistic root endophyte *S. indica* by sophisticated mechanisms involving specific fungal proteins. These insights might help to engineer more resistant plants and to increase crop yield by transferring β-glucan responsive elements into plant species that are lacking the ability to recognize β-glucans as MAMPs. Furthermore a better understanding of the fungal cell wall structure and maintenance might help to develop effective and highly specific fungicides that directly compromiseh cell wall function and integrity.

4. Materials and sources of supply

4.1 Materials and suppliers

4.1.1 Chemicals and "kits"

Chemicals and microbiological growth media components used in this thesis were purchased predominately from Carl Roth (Karlsruhe, Germany), Sigma Aldrich (Taufkirchen, Germany) and VWR (Darmstadt, Germany). Components of plant growth media were purchased at Duchefa (Haarlem, The Netherlands).
Extraction of DNA from agarose gels was performed with the PROMEGA Wizard® SV Gel and PCR Clean-Up System (Mannheim, Germany). For the extraction of plasmids from *E. coli*, the DNA QIAprep® Spin Miniprep Kit (QIAGEN, Hiden, Germany) and the PROMEGA PureYield® Plasmid Midiprep system (Mannheim, Germany) were used.

4.1.2 Buffers, solutions and media

All buffers, media and solutions were autoclaved at 121°C for 15 min if not indicated differently. Heat sensitive solutions, media components and antibiotics were sterile filtrated through 0.22 µm Rotilabo ® syringe filters (Carl Roth, Karlsruhe, Germany) if volume was less than 50 ml or through 0.22 µm bottle top vacuum filtration systems (VWR, Darmstadt, Germany).

4.1.3 Enzymes

Restriction enzymes were purchased at New England Biolabs (Frankfurt/Main, Germany). For the amplification of DNA fragments the GoTaq G2 flexi *Taq* polymerase (Promega, Mannheim, Germany) and the Q5 Hifi *Pfu* polymerase (New England Biolabs, Frankfurt/Main, Germany) were used. Ligation of DNA fragments was performed using the T4 DNA Ligase purchased from New England Biolabs (Frankfurt/Main, Germany). For quantitative real time PCR experiments the GoTaq qPCR master mix (Promega, Mannheim, Germany) was used.

4.1.4 Oligonucleotides

All oligonucleotides used as primers for PCR experiments were synthesized by Sigma Aldrich (Taufkirchen, Germany). The sequences of the used primers are listed in table 2.

Table 2: List of oligonucleotides used in this thesis

Name	Sequence (5´- 3´)	Purpose
05825 qPCR fw 05825 qPCR rv	CGGACAGCTACGAAAAGAGG GCTTGCCTAACTCCAATCCA	Expression analysis of *S. indica* gene PIIN_05825
SiTEF qPCR fw SiTEF qPCR rv	GCAAGTTCTCCGAGCTCATC CCAAGTGGTGGGTACTCGTT	Reference for gene expression analysis in *S. indica* (*PIIN_03008* transcription elongation factor, Tef)
SiTEF qPCR Bs rv	ACCCTTGCCCTCGGTCTTCT	Reverse primer for *PIIN_03008 (*transcription elongation factor, Tef) used in *S. indica-B. sorokiniana* interaction study
BsTEF qPCR fw BsTEF qPCR rv	TGCGTTGAGGCTTTCACTG GACGACGGACTTGATGACAC	Reference for gene expression analysis in *B. sorokiniana* (*COCSADRAFT_29 782,* transcription elongation factor, Tef)

Name	Sequence (5´- 3´)	Purpose
AtUBI qPCR fw AtUBI qPCR rv	CCAAGCCGAAGAAGATCAAG ACTCCTTCCTCAAACGCTGA	Reference for gene expression analysis in *A. thaliana* (A*T3G62250*, Ubiquitin5, UBI)
HvUBI qPCR fw HvUBI qPCR rv	CAGTAGTGGCGGTCGAAGTG ACCCTCGCCGACTACAACAT	Reference for gene expression analysis in *H. vulgare* (*MLOC_16862*, Ubiquitin5, UBI)
05825 trunc. fw 05825 trunc. rv	ACCCGGGGTCAACAAGCCATCTATCCC GACTGAATTCTCAGAATATGGAGAGCAATAGGC	Cloning of PIIN_05825 as *XmaI+EcoRI* fragment into pPIC9 vector for expression of N-terminally truncated WSC3 in *P. pastoris*
05825 wSP fw 05825 w/o Stop rv	GTCGCTAGCATGCTTTCCCTCAACTTGC CGGAAGCTTGAATATGGAGAGCAATAGGC	Cloning of PIIN_05825 as *NheI+HindIII* fragment into pGoGFP vector for expression of WSC3 as GFP fusion with His/HA tag in S. *indica*
pPIC9 Seq fw pPIC9 Seq rv	TACTATTGCCAGCATTGCTGC GCAAATGGCATTCTGACATCC	Sequencing primers for *P. pastoris* pPIC9 vector to verify correct insertion of cloned fragments

Name	Sequence (5´- 3´)	Purpose
HD 1.2 fw	AGATATCCGGAGGCGAGTTT	Primer for the S. *indica* mating type locus HD 1.2 (PIIN_09977)
HD 1.2 rv	CCTGAATCTGCTGTTCGTCA	
HD 2.2 fw	ACATCTGGCTCCCATTTACG	Primer for the S. *indica* mating type locus HD 2.2 (PIIN_09978)
HD 2.2 rv	GTTGAGCTTTGGCTCGTTTC	
HD 2.1 fw	ATGAGTACGATTGCCCAAGG	Primer for the S. *indica* mating type locus HD 2.1 (PIIN_09916)
HD 2.1 rv	TCGTCTCGTAGGCGACTTTT	
HD 1.1	CGATACCTACCCGCCTACAA	Primer for the S. *indica* mating type locus HD 1.1 (PIIN_09915)
HD 1.1	CTTTTTAAGCGGTGCTGGAG	

4.2 Bacterial and Fungal Strains

4.2.1. *Escherichia coli* strains

For all DNA cloning procedures described in this thesis, the *E. coli* strain Top10 (Invitrogen, Karlsruhe, Germany) was used. *E. coli* strain Top10 has the following genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-.

4.2.2 *Pichia pastoris* strains

For the expression of WSC3 the *P. pastoris* strain GS115 was used (Invitrogen, Karlsruhe, Germany). *P. pastoris* GS115 has the *his4* genotype and the Mut^+ phenotype. As a reference for successful protein expression and secretion the *P. pastoris* strain GS115 Albumin was used. *P. pastoris* GS115 Albumin has the *HIS4* genotype and the MutS phenotype, secreting Albumin into the medium. For the

expression of WSC3 three independent *P. pastoris* strains were created in this thesis displaying *HIS4* genotype and the MutS phenotype.

4.2.3 *Ustilago maydis* strains

For all experiments described in this thesis the haploid solopathogenic *U.maydis* strain SG200 (Kämper *et al.*, 2006) derived from the FB1 strain was used.

4.2.4 *Bipolaris sorokiniana* strains

For all experiments described in this thesis the *B. sorokiniana* strain ND90Pr was used (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

4.2.5. *Serendipita indica* strains

The dicaryotic *S. indica* strain DSM11827 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was used for all experiments described in this thesis and thus was designated as wild type. As second reference strain a homocaryotic *S. indica* strain generated by regeneration of protoplasts was used. The homokaryotic reference strain carries a geneticin resistance and was described in Wawra *et al.*, 2016.
As control strain for transgenic *S. indica* transformants the *S. indica* GoGFP strain harboring the pGPD::oGFP-tnos, pTEF::HYG-tnos construct and expressing cytosolic oGFP was used (Hilbert *et al.*, 2012).
The *S. indica* strain WSC3-GFP is derived from DSM11827 and was generated by random integration of the construct pFGB1::PIIN_05825::oGFP::HA/His-tnos, pTEF::Hyg-tnos into the genome of *S. indica.*

4.3 Plant cultivars and accessions

4.3.1 *Hordeum vulgare* cultivars

The *H. vulgare* strain Golden Promise was used for all experiments performed in this thesis.

4.3.2. *Arabidopsis thaliana* accessions

The list of the *A. thaliana* accessions used in this thesis and their origin can be found in table 3.

Table 3: List of used *A. thaliana* accessions and the source of supply

Name	ID	Source
Aa-1	7000	SK
Ag.0	6897	SK
Alc-0	6988	AS
ALL1-2	1	SK
An.1	6898	SK
Ba-1	7014	SK
Bay-0	6899	SK
Bg.2	6709	SK
Bla-1	7015	AS
Blh-1	7034	AS
Bor-1	5837	SK
Bor-4	6903	SK
Bsch-0	7031	SK
Bur-0	5719	AS
Can-0	8274	AS
Cha-0	7069	SK
CIBC-4	6729	SK
CLE.6	78	SK
Com-1	7092	SK
Ct-1	6910	AS

Name	ID	Source
Cvi-0	6911	AS
Da-0	7094	SK
Db-0	7100	SK
Di-1	7098	SK
Edi-0	6914	AS
Enkheim-T	7121	AS
Ep-0	7123	SK
Est-0	7128	SK
Fi-1	7139	SK
Fja1.1	8422	SK
Ep-0	7123	SK
Est-0	7128	SK
Fi-1	7139	SK
Fja1.1	8422	SK
Fja1.2	6019	SK
Fja1.5	6020	SK
Ga-2	7141	SK
Gd.1	8296	SK
Ge-0	8297	AS
Gel-1	7143	SK
Gie-0	7147	SK
Go-0	7151	SK
Gr-1	8300	AS
Ha-0	7163	SK
Hi-0	7167	SK
Hn-0	7165	SK
Hov4.1	8306	SK
In.0	8311	SK
Jea-0	91	AS
Jm-0	8313	AS
Ka.0	8314	SK
Kas.2	8424	SK
Kin-0	6926	SK

Name	ID	Source
Kn-0	7186	AS
Kondara	6929	AS
LAC.3	94	SK
LAC.5	96	SK
Lc.0	8323	SK
LDV.34	126	SK
LDV.58	149	SK
Lip-0	8325	AS
Mh-1	7256	AS
Mr.0	7522	SK
Mrk.0	6937	SK
Ms-0	6938	AS
Mt-0	6939	AS
N13	7438	AS
N14	7446	AS
N7	7449	AS
NC.6	8246	SK
Nd.1	6942	SK
Nok1	7270	AS
Nz-1	7263	SK
Or.1	6074	SK
Oy-0	6946	AS
Pa-1	8353	AS
Pan-10	7526	SK
Per.1	8354	SK
Petergof	7296	SK
Pro.0	8213	SK
Rak.2	8365	SK
Ren.1	6959	SK
Ri-0	7317	AS
Rld-2	7457	AS
ROM.1	267	SK
Rubezhnoe-1	7323	AS

Name	ID	Source
Sap-0	8378	AS
Sav-0	8412	AS
Shahdara	6962	AS
Sp-0	7343	AS
St-0	8387	AS
Stw-0	8388	AS
Ta-0	8389	AS
Tottarp.2	6243	SK
TOU-C-3	362	SK
TOU-J-3	383	SK
Tsu-0	7373	AS
UKID48	5753	SK
UKID80	5785	SK
UKSE06-414	5202	SK
UKSE06-482	5245	SK
UKSE06-520	5264	SK
Ull2.3	6973	SK
Uod.7	6976	SK
Var2.1	7516	SK
Wil.1	8419	SK
Ws-0	6980	SK
Wt.5	6982	SK
Zdr.6	6985	SK

SK – Prof. Dr. Stanislav.Kopriva (University of Cologne)

AS – Dr. Adam Schikora (Julius-Kühn-Institute Braunschweig)

Additionally for the quantification for MAMP-induced Ca^{2+}-fluxes an *A. thaliana* Col-0 reporter line expressing cytoplasmic aequorin was used (Knight *et al.*, 1991).

4.4 Vectors

4.4.1 Vectors for protein expression in *P.pastoris*

For the expression of WSC3 in *P.pastoris* the vector pPIC9 (Invitrogen, Karlsruhe, Germany) was used. pPIC9 contains the β-lactamase gene conferring transformed *E.coli* cells resistance to ampicillin. For selection of transformed *P. pastoris* cells the vector contains the *HIS4* gene enabling growth on histidine-deficient medium. *WSC3* was amplified without introns from *S. indica* cDNA with a proof-reading *Pfu* polymerase and ligated after restriction enzyme digestion of vector and insert with *XmaI* and *EcoRI*. The expression of WSC3 is driven by the methanol-inducible *AOX1* promoter. The secretion of WSC3 is ensured by the *Saccharomyces cerevisiae* α-factor secretion signal located N-terminally of the inserted sequence. For subsequent purification of the secreted WSC3 the protein is expressed with an N-terminal 6xHis tag. The correct insertion was verified by sequencing of the generated vector using the pPIC9 sequencing primers (Invitrogen, Karlsruhe, Germany; Table 2) at GATC Biotech (Cologne, Germany).

4.4.2 Vectors for transformation of *S. indica*

For the transformation of *S. indica* for subsequent protein localization studies a modular vector derived from the pGoGFP vector (Hilbert *et al.*, 2012) was used. The vector contains the β-lactamase gene conferring transformed *E.coli* cells resistance to ampicillin. For selection of transformed *S. indica* protoplasts the vector contains the hygromicin resistance gene which expression is driven by the *TEF* promoter. *WSC3* was amplified without introns from *S. indica* cDNA with a proof-reading *Pfu* polymerase and ligated after restriction enzyme digestion of vector and insert with *NheI* and *HindIII*. The expression of WSC3 is driven by the *FGB1* promoter (Wawra *et al.*, 2016). For subsequent localization of WSC3 the protein is expressed with a C-terminally fused, codon optimized GFP and 6xHis/HA tag. The correct insertion is verified by sequencing of the generated vector at GATC Biotech (Cologne, Germany; Table 2).

4.5. Bacterial, fungal and plant cultivation

4.5.1. *E. coli* cultivation

E. coli was cultivated on low salt lysogeny broth (LB) medium (0.5 % (w/v) yeast extract, 1 % (w/v) trypton, 0.5 % (w/v) NaCl) supplemented with 1.5 % (w/v) agar for the preparation of solid medium. For selection purposes 100 µg/ml ampicillin were added to the medium after autoclaving. The cultures were propagated at 37°C and for liquid cultures agitated at 250 rpm.

4.5.2 *P. pastoris* cultivation

P. pastoris was cultivated on YPD medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose) supplemented with 1.5 % (w/v) agar for the preparation of solid medium. The cultures were grown at 28°C and liquid cultures were agitated at 220 rpm. The detailed cultivation conditions for the expression of WSC3 are described in section 4.7.6.

4.5.3 *U. maydis* cultivation

U. maydis was grown on solid potato-dextrose-agar (PDA) medium at 28°C. For cultivation in liquid cultures *U. maydis* was grown in YEPS light medium (0.4 % (w/v) yeast extract, 0.4 % (w/v) peptone, 2 % (w/v) sucrose) with 220 rpm agitation.

4.5.4 *B. sorokiniana* cultivation

B. sorokiniana was grown on solid complex medium (CM-Bs) at 28°C. For cultivation in liquid medium *B. sorokiniana* was grown in MYP medium (0.7 % (w/v) malt extract, 0.1 % (w/v) peptone, 0.05 % (w/v) yeast extract) at 28°C with 120 rpm of shaking.

CM-Bs medium	1 % (v/v) Solution A
	1 % (v/v) Solution B

CM-Bs medium (continued)	0.05 % (v/v) Srb's micronutrients
	0.1% (w/v) yeast extract
	0.05% (w/v) peptone
	0.05% (w/v) Casamino acids
	1 % /w/v) glucose
	1.5% (w/v) Agar
	in dH_2O
	autoclaved for 15 min at 121°C
Solution A	10% (w/v) $Ca(NO_3)_2$ x $4H_2O$
	in dH_2O
	autoclaved for 15 min at 121°C
Solution B	2 % (w/v) KH_2PO_4
	2.5 % (w/v) $MgSO_4$ x $7H_2O$
	1.5 % (w/v) NaCl
	in ddH_2O
	adjust pH to 5.3 with NaOH
	filter sterilized
Srb's micronutrients	0.006 % (w/v) MnSO4 x H_2O
	0.0057 % (w/v) H_3BO_3
	0.049% (w/v) $ZnSO_4$ x $7H_2O$
	0.0013% (w/v) KI
	0.0037 (w/v) $(NH4)_6M0_7O_{24}$ x $4H_2O$
	0.039% (w/v) $CuSO_4$ x $5H_2O$
	in dH_2O
	autoclaved for 15 min at 121°C

4.5.5. *S. indica* cultivation

S. indica was cultivated on complex medium (CM) at 28°C. For cultivation in liquid culture *S. indica* was grown in CM medium at 120 rpm shaking. Transformed strains were selected for hygromicin resistance (80 µg/ml final concentration).

CM medium	5 % (v/v) 20x Salt solution
	0.1 % (v/v) Microelements

CM medium (continued)	0.2 % (w/v) Peptone 0.1 % (w/v) Yeast extract 0.1 % (w/v) Casamino acids 1.5 % (w/v) Agar (for solid medium only) in dH_2O autoclaved for 15 min at 121°C after autoclaving add 2 % (w/v) Glucose (filter sterilized)
20x Salt solution	12 % (w/v) $NaNO_3$ 1.04 % (w/v) KCl 1.04 % (w/v) $MgSO_4 \times 7H_2O$ 3.04 % (w/v) KH_2PO_4 in dH_2O autoclaved for 15 min at 121°C
Microelements	0.6 % (w/v) $MnCl_2 \times 4H_2O$ 0.15 % (w/v) H_3BO_3 0.265 % (w/v) $ZnSO_4 \times 7H_2O$ 0.075 % (w/v) KI 0.24 ‰ (w/v) $Na_2MO_4 \times 2H_2O$ 0.013 % (w/v) $CuSO_4 \times 5H_2O$ in dH_2O autoclaved for 15 min at 121°C

4.5.6. *A. thaliana* cultivation

A. thaliana was grown on green house substrate supplemented with a *Bacillus thuringiensis israelensis* suspension for seed propagation or for oxidative burst measurement (see section 4.7.15). In both cases *A. thaliana* seeds were directly sown in round 9 cm pots and stratified for 3 days at 4°C in the dark. For seed propagation pots were subsequently transferred into the green house and grown for 11 days before plants were separated (5 plants per 9 cm pot with fresh substrate) and grown under green house conditions until siliques were formed and aerial parts of the plants started to dry. Seeds were collected through a sieve with a pore size of 450 µm (VWR, Darmstadt) and stored in paper bags at room temperature.

For oxidative burst measurements the pots were transferred into a phytochamber and plants were grown under short day conditions (8 h light, 16 h dark) with 47 µmol m^{-2} s^{-1} of light at 24°C for 11 days before plants were separated (1 plant per 6 cm pot with fresh substrate). After separation plants were grown for 7 to 14 more days until leaves had a diameter of ≥ 5 mm but were harvested before bolting.

For the quantification of Ca^{2+}-fluxes a transgenic *A. thaliana* line expressing cytoplasmic aequorin was used. The seeds were surface sterilized for 10 min with 70 % ethanol and 7 min with 100 % ethanol. After sterilization seeds were transferred onto half-strength Murashige and Skoog medium (including vitamins, pH 5.7) supplemented with 1 % (w/v) sucrose and 0.4 % (w/v) Gelrite and stratified for 3 days at 4°C in the dark. Subsequently the plants were transferred into a phytochamber and grown for 5 to 7 days under short day conditions (8 h light, 16 h dark) with 47 µmol m^{-2} s^{-1} of light and 24°C.

For *A. thaliana – S. indica* interaction studies plants were grown under sterile conditions after surface sterilization. After stratification plates were transferred to the phytochamber and grown for 11 days under short day conditions (8 h light, 16 h dark) with 47 µmol m^{-2} s^{-1} of light and 24°C. Before inoculation with *S. indica* 20 plants per square petri dish plants were transferred onto half-strength Murashige and Skoog medium (including vitamins, pH 5.7) supplemented with 0.4 % (w/v) Gelrite.

4.5.7 *H. vulgare* cultivation

H. vulgare plants were either grown on greenhouse substrate supplemented with a *Bacillus thuringiensis israelensis* suspension for oxidative burst measurement (see section 4.7.15) or sterilely for interaction studies with *S. indica*. In both cases seeds were surface sterilized with 12 % sodium hypochlorite for 1.5 h and subsequently washed 5 times for 20 min with sterile water. For oxidative burst measurements seeds were directly transferred into round 9 cm pots (3 plants per pot) and grown for 5-7 days under long day conditions (16 h light, 8 h dark) with 60% humidity and 190 µmol m^{-2} s^{-1} at 22°C/18°C in a phytochamber.

For interaction studies with *S. indica* seeds were transferred onto wet filter paper after surface sterilization and pre-germinated at room temperature for 3 days in the dark. The germinated seeds were transferred into 1062 ml Weck® jars (Weck, Wehr-Öflingen, Germany) containing 100 ml 1/10 PNM medium and grown for the indicated

times in presence of *S. indica* under long day conditions (16 h light, 8 h dark) with 60% humidity and 190 µmol m^{-2} s^{-1} at 22°C/18°C in a phytochamber. 1/10 PNM medium was composed like following

1/10 PNM	0.5 mM KNO_3
	0.0005 % KH_2PO_4
	0.00025 % K_2HPO_4
	2 mM $MgSO_4$ x H_2O
	0.2 mM $Ca(NO_3)_2$
	0.25% (v/v) Fe-EDTA
	0.00025 % NaCl
	0.4% (w/v) Gelrite
	pH adjusted to 5.7 with KOH
	in dH_2O
	autoclaved
	after autoclaving add: 10 mM MES pH 6.0 (filter sterilized)
Fe-EDTA	0.55 % (w/v) $FeSO_4$ x $7H_2O$
	0.75% (w/v) Na_2EDTA x $2H_20$
	boiled in dH_2O and stirred for 30 min

4.6. Microbiological methods

4.6.1 Preparation of chemocompetent *E. coli* cells

Chemocompetent *E. coli* cells were prepared according to the RbCl/$CaCl_2$ method and used for the propagation and multiplication of cloned vector constructs. 1 ml of an *E. coli* Top10 liquid culture grown in low salt LB-medium at 37°C was used to inoculate 600 ml fresh low salt LB-medium supplemented with 10 mM $MgSO_4$ and 10 mM $MgCl_2$. The culture was grown at 37°C until OD_{600} reached 0.4 to 0.6 which corresponds to *E. coli* cells in the exponential growth phase. The culture was cooled on ice for 20 min before the cells were harvested by centrifugation at 3,000 rpm for 15 min. The supernatant was discarded and the cells were resuspended in 100 ml cold RF I solution with a pre-chilled glass pipet. The cells were incubated in the RF I solution for 60 min

on ice before they were pelleted again like described above. After discarding the supernatant the cell pellet was resuspended in 6 ml RF II solution and incubated for 15 more min on ice. For long time storage the chemocompetent cells, were split into 50 µl aliquots which were flash frozen in liquid nitrogen and stored at -80°C.

RF I solution	100 mM RbCl
	50 mM $MnCl_2$ x $4H_2O$
	30 mM Potassium acetate
	10 mM $CaCl_2$ x $2H_2O$
	15 % (w/v) glycerol
	pH adjusted to 5.8 with acetic acid
	in ddH_2O
	filter sterilized
RF II solution	10 mM MOPS buffer
	10 mM RbCl
	75 mM $CaCl_2$ x $2H_2O$
	15 % (w/v) glycerol
	pH adjusted to 6.8 with NaOH
	in ddH_2O
	filter sterilized

4.6.2 Heat shock transformation of *E. coli*

The chemocompetent *E. coli* cells were used for heat shock transformation of vector constructs. An aliquot of the chemocompetent cells was thawed on ice before up to 5 µl of the ligation mixture or purified plasmid were added and the cells were incubated for 20 min on ice. The heat shock was conducted subsequently for 45 s at 42°C and the cells were immediately cooled down on ice for 2 min afterwards. For the regeneration of the transformed cells 500 ml low salt LB-medium was added and the cells were incubated for 45 min at 37°C. Prior to plating the cells on selection plates containing 100 µg/ml ampicillin the cells were pelleted for 1 min at 13,300 rpm and the supernatant was discarded only leaving 50 µl, which was used for the resuspension of the cell pellet. The plates were incubated over night at 37°C. On the next day cell material of colonies potentially containing the right vector construct were used for a

colony PCR to verify the presence of transformed plasmid. Transformed colonies were used to inoculate 5 ml liquid cultures containing 100 µg/ml ampicillin which were grown over night at 37°C prior to extraction of the plasmid DNA. The plasmid DNA pre-mixed with suitable primers was sequenced by GATC Biotech (Cologne, Germany) to ensure the correctness of the vector cinstruct.

4.6.3 Transformation of *P. pastoris*

Transformation of *P. pastoris* was performed using the *Pichia* EasyComp® Kit (Invitrogen, Karlsruhe). For the selection the transformed cells were plated on RDB medium deficient of histidine.

RDB plates	1 M sorbitol 2% (w/v) Agar in dH_2O autoclaved for 15 min at 121°C after autoclaving add: 1.34% (w/v) Yeast Nitrogen Base (without amino acids, with $(NH_4)_2SO_4$) $4x10^{-5}$% (w/v) biotin 2% (w/v) dextrose 0.005% (w/v) of each L-glutamic acid, L-methionine, L-lysine, L-leucine, L-isoleucine all solutions filter sterilized

4.6.4. Collection of S*. indica* chlamydospores

Three to four weeks old *S. indica* plates were covered with 5 ml water containing 0.002% Tween20 and spores were harvested by scratching the surface of the plate with a spatula and a scalpel. Filtration of the solution through a Miracloth filter (pore size 22-25 µm; Merck Millipore, Darmstadt, Germany) was performed, to remove residual fungal mycelium and medium. The spores, which went through the filter, were spun down at 3,500 rpm for 7 min and washed twice with 10 ml Tween water. Finally

the spores were resuspended in sterile water and counted using a Neubauer Improved counting chamber with 0.1 mm depth and set to a concentration of 500,000 spores/ml. For long time storage the spore solution was supplemented with 40 % (v/v) glycerol, frozen in liquid nitrogen and stored at -80°C.

4.6.5. Collection of B*. sorokiniana* chlamydospores

B. sorokiniana chlamydospores were collected in a similar way as *S. indica* spores with some minor adaptions. The surface of a 10 to 12 days old plate was covered with 5 ml water containing 0.002% Tween20 and only scratched with a spatula to avoid extensive disruption of the mycelium. The spore solution was filtrated through a Miracloth filter and the spores, which went through the filter were pelleted at 4,000 rpm for 10 min and subsequently washed twice with 10 ml sterile water. After washing the spores were resuspended in sterile water, counted in a Neubauer Improved counting chamber with 0.1 mm depth and set to a concentration of 5,000 spores/ml.

4.6.6. S*. indica – H. vulgare* interaction studies

For S*. indica – H. vulgare* interaction studies three pre-germinated *H. vulgare* seedlings were transferred into one Weck® jar containing 100 ml 1/10 PNM medium and 1 ml of *S. indica* spore solution containing 500,000 spores was added. For the mock controls 1 ml of sterile water was added to the jars. The roots of the plants were harvested after 1, 3, 7 and 14 days of inoculation by cutting 4 cm of the roots starting 0.5 cm below the seed, washing the roots thoroughly in sterile water and flash freezing the material in liquid nitrogen. The material of 4 jars per time point was collected as biological replicates. The material was stored at -80°C until RNA was extracted (see section 4.7.2).

To assess the effect of recombinant WSC3, 9 µM of the protein were mixed to the spore solution prior to addition of the spores to the roots. As control spores without protein was added to the roots. The experiment was performed twice with three biological replicates each. The material was harvested like described above and stored at -80°C until DNA was extracted (see section 4.7.1).

4.6.7. *S. indica – A. thaliana* Col-0 interaction studies

For *S. indica – A.thaliana* interaction studies 20 *A. thaliana* plants were transferred from half-strength Murashige and Skoog medium (including vitamins, pH 5.7) supplemented with 1 % sucrose to ½ MS plates without sucrose and 0.3 ml of a *S. indica* spore solution containing 500,000 spores per ml was added to the roots of each plant. For the mock controls 0.3 ml of sterile water was added. The roots of the plants were harvested after 1, 3, 7 and 14 days of inoculation by cutting 4 cm of the roots starting 0.5 cm below the seed, washing the roots thoroughly in sterile water and flash freezing the material in liquid nitrogen. The material of 3 plates per time point was pooled as one biological replicate and 4 biological replicates were collected per time point. The material was stored at -80°C until RNA was extracted (see section 4.7.2).

4.6.8 *S. indica* - *B. sorokiniana* interaction on soil

S. indica - *B. sorokiniana* confrontation experiment was performed to investigate the expression of the WSC3 encoding gene *PIIN_05825* in response to direct contact to a second fungus. *S. indica* GoGFP and *B. sorokiniana* were grown in liquid MYP medium at 28°C and 120 rpm of shaking for four or three days, respectively. The mycelium of both cultures was filtered through a Miracloth filter and washed three times with sterile ddH_2O before disrupting the mycelial aggregates like described in section 4.6.9. The crushed mycelium of both fungi was regenerated for one day at 28°C with 120 rpm of shaking in fresh MYP medium. For the confrontation assay the mycelium of both fungi was filtered through a Miracloth filter again and 0.5 g of *S. indica* GoGFP mycelium and 0.5 g of *B. sorokiniana* mycelium were mixed well, resuspended in 5 ml sterile ddH_2O and added to 30 g of autoclaved Cologne land soil (CAS10, kindly provided by Prof. Dr. Paul Schulze-Lefert). As a control 1 g of filtered *S. indica* GoGFP mycelium was resuspended in 5 ml sterile ddH_2O and mixed with 30 g of the same soil. After 42 h of incubation at 28°C the fungal mycelium was harvested from the surface of the soil, flash frozen in liquid nitrogen and stored at -80°C until RNA was extracted (see section 4.7.2). The confrontation assay was performed by Debika Sarkar who kindly provided the material for RNA extraction.

4.6.9. S*. indica* protoplastation and PEG-mediated transformation

For the transformation of *S. indica* a 250 ml culture in liquid CM medium was inoculated with 1 ml of a spore solution set to a concentration of 2.5 x 10^4 spores/ml and grown for 7 days at 28°C with 120 rpm of shaking. After 7 days the mycelium was collected by filtration through a Miracloth filter and washed with 50 ml 0.9 % NaCl solution before it was resuspended in 20 ml fresh CM medium. The mycelial aggregates were disrupted using a Microtron® MB550 homogenizer (Kinematica, Luzern, Switzerland) for 10 s and the homogenized culture was regenerated for 3 days at 28°C with 120 rpm of shaking in a total volume of 150 ml fresh CM medium. After the regeneration the young mycelium was filtered again through a Miracloth filter and washed with 50 ml of 0.9 % NaCl solution to remove spores and residual medium. For the protoplastation the mycelium was resuspended in 20 ml SMC supplemented with 2 % (w/v) *Trichoderma harzianum* lysing enzymes (Sigma Aldrich, Taufkirchen, Germany) which was sterile filtrated prior to use. The mycelium was protoplasted for 2 h at 32°C at 100 rpm of shaking. The protoplastation was checked microscopically with a Leica DM2500 light microscope (Wetzlar, Germany) and the reaction was stopped by filtration through a Miracloth and addition of an equal volume of cold STC solution. The protoplasts which went through the filter were pelleted at 4,000 rpm for 10 min and washed for three times in total with cold STC solution. The protoplasts were finally resuspended in an appropriate volume of cold STC and stored on ice until the transformation.

50 µg of the plasmid DNA to be transformed were linearized on the day before by overnight incubation with 20 Units of the restriction enzyme *BsaAI* and precipitated for 10 min at room temperature by addition of 0.5 volumes of 7.5 M ammonium acetate and 2 volumes of 100 % isopropanol. The plasmid DNA was pelleted by centrifugation for 30 min at 13,300 rpm, washed twice with 75 % ethanol, dried for 5 min at room temperature and finally resuspended in 10 µl sterile ddH_2O. The linearized plasmid DNA was stored on ice until the transformation.

For the transformation 70 µl of the protoplast solution were mixed with 10 µl of the linearized vector, 1 µl of a 15 mg/ml heparin solution and 10 Units *BsaAI* and incubated on ice for 10 min. Prior to another incubation step for 15 min on ice 0.5 ml of a freshly prepared, filter sterilized (0.45 µm filter) STC solution supplemented with 40 % (w/v) PEG 3350 (Sigma Aldrich, Taufkirchen, Germany) were added to the protoplast

solution. Finally the protoplast solution was mixed with 5 ml of the top medium which was kept at 45°C to prevent solidification and distributed on 20 ml solidified bottom medium containing 80 µg/ml hygromycin for selection of positive transformants. After solidification of the top medium the plates were incubated at 28°C As a positive control protoplasts were plated on bottom medium without hygromycin to assess if protoplasts were able to regenerate while protoplasts plated on bottom medium with hygromycin were used as negative control to ensure that the selection with hygromycin prevents regeneration of untransformed protoplasts.

First colonies emerging 14 d after transformation were transferred to new CM plates supplemented with 80 µg/ml of hygromycin and to liquid CM medium for microscopy. All clones that were further used were checked whether they are homo- or dicaryotic. Therefore genomic DNA was extracted like described in section 4.7.1 and 100 ng of the DNA was used as template for a PCR amplifying unique regions of the four mating type loci HD 1.1 (*PIIN_09915*), HD 2.1 (*PIIN_09916*), HD 1.2 (*PIIN_09977*) and HD 2.2 (*PIIN_09978*). Dicaryotic transformants possess all four loci whereas homokaryotic transformants only possess HD 1.2 and HD 2.2.

SMC	1.33 M sorbitol
	50 mM $CaCl_2$ x $2H_2O$
	20 mM MES
	pH adjusted to 5.8
	in ddH_2O
	autoclaved for 15 min at 121°C
STC	1M sorbitol
	50 mM $CaCl_2$ x $2H_2O$
	10 mM Tris.HCl
	ph adjusted to 7.5
	in ddH_2O
	autoclaved for 15 min at 121°C
Bottom medium (MYP)	0.7 % (w/v) malt extract
	0.1 % (w/v) peptone
	0.05 % (w/v) yeast extract
	1.2 % (w/v) agar
	0.3 M sucrose

Bottom medium (MYP, continued))	80 µg/ml hygromycin in dH_2O autoclaved for 15 min at 121°C
Top medium (MYP)	0.7 % (w/v) malt extract 0.1 % (w/v) peptone 0.05 % (w/v) yeast extract 0.6 % (w/v) agar 0.3 M sucrose in dH_2O autoclaved for 15 min at 121°C keep at 45°C until use

4.6.10 Assessment of fungal growth phenotype in presence of WSC3

The ability of WSC3 to agglutinate fungal cells/spores was investigated for the fungi *U. maydis*, *B. sorokiniana* and *S. indica*. *U. maydis* was grown over night in a liquid 5 ml YEPS light culture and the OD_{600} of the culture was measured subsequently. The culture was diluted with fresh YEPS light to an OD_{600} of 0.4. *B. sorokiniana* and *S. indica* spores were collected like described above (see 4.6.4 and 4.6.5). *B. sorokiniana* spores were diluted to 500 spores/ml in MYP medium and *S. indica* spores were diluted to 50,000 spores/ml in CM medium. 100 µl of the cell/spore solutions were transferred to individual wells of a 96-well plate. The recombinant WSC3 was sterile filtrated through a 0.22 µm filter and 10 µM were added to the individual wells. As controls 10 µM wheat germ agglutinin conjugated with an AlexaFluor 594 fluorescence probe (WGA-AF594; Invitrogen, Karlsruhe, Germany) or 10 µM native FGB1 were added to the individual wells. As mock control sterile ddH_2O was used. To each well sterile ddH_2O was added to a final volume of 150 µl. Germination and phenotype of *U. maydis* was assessed microscopically after 4 h of incubation at 28°C with 250 rpm of shaking and for *B. sorokiniana* and *S. indica* after overnight incubation at 28°C with 120 rpm shaking. Pictures were taken using a Leica M165 FC stereo microscope or a Leica DM2500 light microscope (Wetzlar, Germany). For *U. maydis* the agglutination effect was quantified by counting the aggregated cells relative to the total number of cells. An aggregate was defined as structure with more than 2 cells being in direct contact with each other but not connected, as for example dividing cells.

4.6.11 Adhesion of *S. indica* spores to barley roots

The ability of WSC3 and FGB1 to foster the adhesion of *S. indica* spores to barley roots was investigated. Therefore barley seeds were germinated for 5 d on wet filter paper at room temperature in the dark. First 4 cm of the roots starting 0.5 cm below the seed were cut into 1 cm pieces and incubated in 1 ml *S. indica* spore solution set to 500,000 spores/ml in 100 mM Tris buffer pH 8.0 for 1 min. Three individual spore solutions collected from three individual plates were used. The spore solutions were supplemented with 10 µM WSC, 10 µM FGB1 or sterile ddH_2O as mock control. All solutions furthermore contained 5 µg/ml of the chitin-specific lectin WGA conjugated with the fluorescence probe AlexaFluor594 to specifically stain the spores. Pictures of the roots pieces were taken using a Leica TCS SP8 confocal microscope (Wetzlar, Germany) with an excitation wavelength of 561 nm and detection between 610-650 nm. 10 µm wide z-stacks of the root pieces were recorded to completely image all layers of the root before counting the spores and measuring the dimensions of the root piece using the Fiji software (Schindelin *et al.*, 2012).

4.6.12 Cell wall stress resistance assay

The cell wall stress resistance assay was performed to investigate if *P.pastoris* transformants expressing WSC3-6xHis display elevated resistance to the cell wall stressors Congo Red, Calcoflour White and H_2O_2. Three independent WSC3-6xHis expressing *P.pastoris* transformants and an Albumin expressing reference strain were grown overnight in liquid YPD medium, diluted the next day to OD_{600} of 0.2 and grown for 2 h at 28°C with 250 rpm of shaking to an OD_{600} of 0.6 to 0.8. The cells were subsequently pelleted by centrifugation at 3,500 rpm for 10 min and washed once with 1 ml sterile ddH_2O. The cells were resuspended in an appropriate volume of sterile ddH_2O corresponding to an OD_{600} of 1. From this solution 5 consecutive serial dilutions from 1:10 to 1: 100,000 were prepared and 6 µl of each solution were dropped onto BMGY and BMMY plates (see section 4.7.6) supplemented with either 100 µg/ml Congo Red, 50 µg/ml Calcoflour White (Sigma Aldrich, Taufkirchen, Germany), 1 mM H_2O_2 (Sigma Aldrich, Taufkirchen, Germany) or without any supplements and grown for 2 d at 28°C before pictures were taken.

4.7. Molecular and biochemical methods

4.7.1. DNA isolation

Genomic DNA was extracted from *S. indica* colonized *H. vulgare* roots to quantify fungal colonization. The colonized roots were harvested like described before (see section 4.6.6) and ground in liquid nitrogen using mortar and pestle. Approximately 200 mg of the ground material were mixed with 1 ml of the extraction buffer at room temperature for 10 min on a Stuart SB3 rotator (Cole-Parmer, Staffordshire, UK). Before centrifugation at 10,000 x g for 20 min, 1 ml of a chloroform:isoamylalcohol (24:1) mixture was added and mixed for 5 min. After centrifugation the upper phase was transferred into a new tube and mixed with 0.2 volumes of 100 % ethanol for 5 min at room temperature. Before the samples were centrifuged again for 20 min at 10,000 x g, 1 ml of a chloroform:isoamylalcohol (24:1) mixture was added and mixed for 5 min. The DNA was precipitated from the upper phase by addition of an equal volume of 100% isopropanol and overnight incubation at 4°C. The DNA was pelleted by centrifugation for 20 min at 5,000 x g and subsequently washed with 900 µl 70 % ethanol. The washed pellet was air dried, dissolved in TE buffer (pH 8.0) containing 1µl RNAse A (10 mg/ml pH 7.4) and incubated for 60 min at 37°C

Extraction buffer	100 mM Tris-HCl (pH 7,5)
	50 mM EDTA (pH 8)
	1,5 M NaCl
	2% (v/v) Cetrimonium bromide
	25 mM ß-Mercaptoethanol
TE buffer	10 mM Tris-HCl
	1 mM EDTA (pH 8.0)
	ph adjusted to 8.0
	in ddH_2O
	autoclaved for 15 min at 121°C

4.7.2. RNA isolation

The RNA was either extracted from *S. indica* colonized *A.thaliana* or *H. vulgare* roots, from *S. indica* grown in axenic culture or from mixed *S.indica – B. sorokiniana* mycelium grown on soil to assess the expression of the *S. indica* gene *PIIN_05825* encoding the WSC3 protein. The material was harvested like described before (see section 4.6.6, 4.6.7 and 4.6.8) and ground in liquid nitrogen using mortar and pestle. For RNA-extraction from liquid culture, the mycelium was collected by filtration through a Miracloth filter, washed with 0.9 % NaCl and also ground in liquid nitrogen using mortar and pestle. For the extraction of RNA from *S. indica* grown on plate, the mycelium was carefully scratched from the plate using a scalpel and subsequently also ground in liquid nitrogen using mortar and pestle. 200 mg of the ground material were mixed with 1 ml TRIzol (Invitrogen, Karlsruhe, Germany) and thoroughly vortexed at 1,500 rpm using a vibrax shaker (IKA, Staufe, Germany) until the material was completely suspended. Prior to centrifugation of the samples at 13,300 rpm for 30 min at 4°C 200 µl chloroform were added and the samples were again vortexed on the vibrax shaker at 1,500 rpm for 20 s. 500 µl of the upper aqueous phase were transferred to a new tube and mixed with 500 µl 100 % isopropanol followed by precipitation of the RNA at 4°C for 1 h. The precipitated RNA was pelleted by centrifugation at 13,300 rpm for 30 min at 4°C and washed with 1 ml of 75 % ethanol diluted water supplemented with 0.1 % DEPC. After washing the ethanol was discarded, the pellet was dried at room temperature and resuspended in 30 µl nuclease-free water (Promega, Mannheim, Germany) at 65°C for 5 min with gentle shaking. The integrity of the RNA was checked by agarose gel electrophoresis on a 1 % 1 x TAE gel and RNA concentration was measured on a NanoDrop 2000c (ThermoScientific, Schwerte, Germany) at a wavelength of 260 nm. The RNA was stored at -80°C or directly used for cDNA synthesis (see section 4.7.3).

50 x TAE buffer	2 M Tris-HCl 2 M acetic acid 50 mM EDTA (pH 8) in ddH_2O autoclaved for 15 min at 121°C

4.7.3. DNase digestion and cDNA synthesis

Prior to the synthesis of cDNA from RNA genomic DNA was removed by a DNase I digestion (Thermo Scientific, Schwerte, Germany). 1µg of the RNA was mixed with 1 µl 10 x DNase I Buffer, 1 µl DNase I (1 U/µl) and the reaction volume was brought to 10 µl in total with nuclease-free H_2O. After 30 min of incubation at 37°C the reaction was stopped by addition of 1 µl 50 mM EDTA and heating to 65°C for 10 min.
The DNA-free RNA was directly transcribed into cDNA using the 1st strand cDNA synthesis kit supplied by Thermo Scientific (Schwerte, Germany) according to the manufacturer's instructions. In brief, 9 µl of the DNase I-digested RNA were mixed with 1 µl Oligo-dT, 1 µl random hexamer primers and heated for 5 min at 65°C. After chilling the RNA on ice 4 µl 5 x reaction buffer, 1 µl Ribolock RNase inhibitor (20 U/µl), 2 µl 10 mM dNTPs and 2 µl MMLV reverse transcriptase were added and the reaction mixture was incubated for 5 min at 25°C. The reverse transcription was performed at 42°C for 1 h prior to heat inactivation of the enzyme at 70°C for 5 min. The cDNA was diluted to 2.5 ng/µl and stored at -80°C for later use.

4.7.4. Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was either performed to quantify the colonization of *H. vulgare* roots by *S. indica* in presence or absence of WSC3 using genomic DNA as template for the reaction or to analyze the expression of the *S. indica* gene *PIIN_05825* during colonization of *H. vulgare* and *A. thaliana*, during the confrontation of *S. indica* with *B. sorokiniana* or during growth of *S. indica* in axenic culture, in all cases using RNA transcribed into cDNA as template. The qRT-PCR was performed using the GoTaq qPCR mastermix (Promega, Mannheim, Germany) which already contained all reaction components and only was supplemented with the template DNA and 0.5 µM of the appropriate forward and reverse primer. For the gene expression analysis during the *S. indica* - *B. sorokiniana* confrontation experiment 20 ng of cDNA were used as template in a reaction volume of 20 µl. For all other experiments 10 ng of template DNA/cDNA were used in a reaction volume of 15 µl. The PCR reaction was performed in a BioRad CFX96 Touch cycler (BioRad, Munich, Germany) using following program: initial denaturation for 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 20 s at 59 °C, 30 s at 72 °C and a melt curve analysis from

65°C to 95°C with an increment of 0.5°C each 0.05 s. Four biological replicates were analyzed in parallel on one plate with three technical replicates per sample. The relative DNA amount or relative expression was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.7.5 Purification of FGB1 from *S. indica* culture supernatant

The detailed purification protocol for FGB1 is published in Wawra *et al.*, 2016. In brief, native FGB1 was purified from the culture supernatant of a 7 d *S. indica* culture grown in CM. After removal of the mycelium the supernatant was filtrated through a 0.22 µm filter, diluted 1:1 with dH_2O and the pH was raised with sodium acetate buffer to 5. The supernatant was then applied to columns containing EMD TMAE Hicap and Fractogel EMD SO^{3-} ion exchange material (both Merck, Darmstadt, Germany) which were subsequently washed with 10 mM sodium acetate buffer pH 5 to remove unbound proteins. FGB1 and all the other proteins bound to the SO^{3-} column were removed by stepwise elution with 10 mM sodium acetate buffer pH 5 supplemented with 1.5 M NaCl. The elution fractions containing the crude FGB1 concentrate were further purified by centrifugation in a Spin-X UF spin concentrator with a cutoff of 30 kDa (Corning, Wiesbaden, Germany), which led to a separation into a ≥ 30 kDa fraction that is not passing the filter and a ≤ 30 kDa fraction that is passing through the filter and contains FGB1. The ≤ 30 kDa fraction was then applied to a Spin-X UF spin concentrator with a cutoff of 5 kDa (Corning, Wiesbaden, Germany) to concentrate the purified FGB1. FGB1 was finally dialyzed once for 3 h against 3 L of water and and for a second time overnight against 3 L of water.

4.7.6 Expression and purification of WSC3 in *P. pastoris*

WSC3 was expressed in the methanotrophic yeast *P. pastoris* and subsequently purified *via* its 6x His-Tag from the culture supernatant. 500 ml BMGY were inoculated with the WSC3-expressing transformant and grown for 3 days at 28°C with 220 rpm shaking in baffled flasks. Subsequently the cells were pelleted by centrifugation at 4,000 rpm for 25 min, resuspended in a total volume of 2 L BMMY medium and grown for 24 h at 28°C with 220 rpm of shaking. Afterwards the cells were removed from the medium by centrifugation at 4,000 rpm for 30 min and remaining cells were further

discarded from the supernatant by filtration through a 0.45 µm filter. The cell-free supernatant was diluted 1:1 with water, supplemented with 1 mM of PMSF and 1 mM of $NiSO_4$ and the pH was raised above pH 7 through addition of 1 M sodium phosphate buffer pH 7.3. Prior to the application of the supernatant to the ion exchange columns the solution was again filtrated through a 0.45 µm filter to remove precipitated salts. The supernatant was first applied to the EMD TMAE Hicap material and the flow through was applied to the Fractogel EMD SO^{3-} ion exchange material (both Merck, Darmstadt, Germany) with a flow rate of 7 ml/min. Since WSC3 neither binds to the TMAE material nor the SO^{3-} material at this pH the flowthrough was applied to a column containing Ni^{2+}-NTA sepharose beads (Qiagen, Hilden, Germany) with a flow rate of 3 ml/min. The Ni^{2+}-NTA column was washed with 10 volumes of 10 mM sodium phosphate buffer pH 7.3 supplemented with 10 mM imidazole (Merck, Darmstadt, Germany) before WSC3 was eluted stepwise with 10 mM sodium phosphate buffer pH 7.3 containing 300 mM imidazole. The collected protein fractions were analyzed on a SDS-PAGE (see section 4.7.9) and those containing pure WSC3 protein were pooled and dialyzed against 3 L of water overnight at 4°C. The dialyzed protein was concentrated using Spin-X UF spin concentrators with a cutoff of 30 kDa (Corning, Wiesbaden, Germany). To investigate if WSC3 is glycosilated by *P. pastoris* the protein was deglycosilated using the protein deglyosilation mix II of NEB (Frankfurt/Main, Germany) according to the manufacturers protocol.

BMGY	1% (w/v) Yeast Extract 2% (w/v) Peptone in dH_2O autoclaved for 15 min at 121°C after autoclaving add: 100 mM Potassium Phosphate pH 6.0 1.34% (w/v) Yeast Nitrogen Base (without amino acids, with $(NH_4)_2SO_4$) $4x10^{-5}$% (w/v) biotin 1% (w/v) glycerol all solutions filter sterilized
BMMY	1% (w/v) Yeast Extract 2% (w/v) Peptone

BMMY (continued)	in dH_2O autoclaved after autoclaving add: 200 mM Potassium Phosphate pH 6.0 2.7 % (w/v) Yeast Nitrogen Base (without amino acids, with $(NH_4)_2SO_4$) 8 x 10^{-5} % (w/v) biotin 2 % (w/v) methanol all solutions filter sterilized

4.7.7 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed and analyzed like described in Wawra *et al.*, 2016 to investigate the affinity of recombinantely produced WSC3-His to different oligo- and polysaccharides. All ITC measurements were performed by Dr. Stephan Wawra and data were kindly provided for publication in this PhD thesis. In brief, ITC quantifies the binding affinity of a protein to a ligand by measurement of the heat that is released when binding of the ligand to the protein occurs. As ligands Laminarin (Sigma Aldrich, Taufkirchen, Germany) at a concentration of 0.9 mM, laminarihexaose (Megazyme, Bray, Ireland) at a concentration of 1 mM, chitooctaose (Isosep, Tullinge, Sweden) at a concentration of 1.5 mM and gentiobiose (provided by Prof. Dr. Jürgen Seibel, Julian-Maximilians University of Würzburg) at a concentration of 1 mM were used. WSC3 and all used ligands were dissolved in water and concentrations of WSC3 are given in the result part (Figure 6). All measurements were conducted at 20°C and the data were baseline corrected and data from the control titration of the ligands into water has been subtracted.

4.7.8 TCA precipitation of proteins

To analyze the secreted protein fraction of *S. indica* and *P. pastoris* proteins were precipitated from the culture supernatant using trichloric acid (TCA). 20 ml of the respective culture supernatant or protein solution were mixed with 5 ml of 95 % (w/v) TCA and incubated overnight at 4°C. Subsequently 20 ml acetone were added and the protein precipitate was collected by centrifugation at 8,000 x g for 30 min. The protein

pellet was washed twice with acetone and shortly dried at 99°C to evaporate residual acetone. The pellet was resuspended in 100 µl Laemmli SDS buffer, heated for 10 min at 99°C and proteins were analyzed on SDS-PAGE (see section 4.7.9).

4.7.9 SDS PAGE

SDS-PAGE was performed in order to separate proteins according to their size and charge. Samples were mixed with an appropriate volume of Laemmli SDS buffer and boiled for 10 min at 99°C. Subsequently samples were loaded onto self-prepared 10 % Bis-Tris gels (stacking gel 5 %) and run in 1 x MES-SDS running buffer containing 5 mM $Na_2S_2O_5$ for 45 min at 180 V in a BioRad Mini-PROTEAN tetra cell electrophoresis chamber (BioRad, Munich, Germany). After electrophoresis the gels were shortly washed in dH_2O and stained for 1 h in Coomassie staining solution with gentle shaking. Gels were destained in destaining solution for approximately 3 h and then kept in dH_2O. Size of the proteins was estimated using the protein marker Page Ruler Prestained (ThermoScientific, Schwerte, Germany).

Laemmli SDS buffer	0.1 M Tris (pH 6.8) 2 M thiourea 8 M Urea 8 % (w/v) SDS 2 % (v/v) β-mercaptoethanol in dH_2O
10 % Bis-Tris gel	333 mM Bis-Tris pH 6.4 10 % (w/v) arcylamide/bisacrylamide (30 % / 0.8 %) 0.1 % (w/v) ammonium persulfate 0.001 % (v/v) TEMED in ddH_2O
20 x MES-SDS Running buffer	1 M MES 1 M Tris 20 mM EDTA (pH 8) 2 % (w/v) SDS
Coomassie staining solution	30 % (v/v) methanol

Coomassie staining solution (continued)	17.5 % (v/v) ethanol 10 % (v/v) acetic acid 2 % (w/v) Coomassie R-250 0.2 % (w/v) Coomassie G-250 in ddH_2O
Destaining Solution	50 % (v/v) methanol 7 % (v/v) acetic acid in ddH_2O

4.7.10 Western Blot

Western blot analysis was performed for the direct detection of recombinant WSC3-6xHis in *P. pastoris* cultures or of WSC3-GFP in *S. indica* transformants. Loading volume of the analyzed samples was estimated from a Coomassie-stained SDS-PAGE according to the signal intensity of prominent protein bands. For the transfer of the proteins to a nitrocellulose membrane the gel, the membrane and two Whatman filter papers were equilibrated in transfer buffer and assembled like following from bottom to top: filter paper – membrane – gel – filter paper. The proteins were blotted using the semidry Fastblot B43 from Biometra (Göttingen, Germany) for 45 min at 375 mA. After blotting the gel was washed for 5 min in membrane wash buffer and subsequently stained for 5 min in Ponceau S solution. The excessive Ponceau S solution was washed away with 1 x PBS and the membrane was scanned. After the membrane was completely destained in PBS the membrane was incubated with the primary antibody in 1 x PBS supplemented with 2.5 % (w/v) milk powder and 0.1 % (v/v) Tween20 for 1 h at room temperature. For the detection of WSC3-6xHis an anti-His antibody directly conjugated to HRP (Qiagen, Hilden, Germany) was used as 1:1,000 dilution and for the detection of WSC3-GFP an anti-GFP antibody (Roche, Mannheim, Germany) was used as 1:1,000 dilution. After the primary antibody solution was removed the membrane was washed three times for 5 min each in 1 x PBS before the secondary antibody was added in 1 x PBS supplemented with 2.5 % (w/v) milk powder and 0.1 % (v/v) Tween20 and incubated for 1 h at room temperature. For the detection of the anti-GFP antibody a secondary anti-mouse antibody conjugated to HRP (Sigma Aldrich, Taufkirchen, Germany) at 1:1,000 dilution was used. The membrane was finally washed again for three times 5 min in 1 x PBS before the Pierce ECL Western blot

substrate (ThermoScientific, Schwerte, Germany) was added and the chemiluminescence was detected using the Gel Doc XR+ device from BioRad (Munich, Germany).

Transfer buffer	48 mM Tris 20 mM HEPES 1 mM EDTA (pH 8) 1.3 mM $Na_2S_2O_5$ 1.3 mM dimethylformamide in ddH_2O
Membrane wash buffer	0.1 M maleic acid 0.15 M NaCl 0.3 % (v/v) Tween20 in ddH_2O
Ponceau S solution	0.1 % (w/v) Ponceau S 1 % (v/v) acetic acid in ddH_2O
20 x PBS	2.76 M NaCl 54 mM KCl 202.8 mM Na_2HPO_4 x $2H_2O$ 35.2 mM KH_2PO_4 in ddH_2O

4.7.11 Laminarin protection assay

An enzyme protection assay was performed to investigate if WSC3 is able to protect laminarin (Sigma Aldrich, Taufkirchen, Germany) from the hydrolysis by an *endo*-1,3-β-D-glucanase from barley (Megazyme, Bray, Ireland). Therefore an BCA-assay was performed which relies on the reduction of Cu^{2+}- to Cu^{+}-ions by the reducing end of mono- and oligosaccharides which are generated by the hydrolysis of laminarin. The Cu^{+}-ions are chelated by the bicinchoninic salt (BCA) which leads to the formation of a complex that absorbs light at a wavelength of 540 nm. This complex can be used for the colorimetric quantification of the released reducing sugars using glucose solutions at different concentrations as calibration curve.

Following reactions were performed with four replicates in parallel in 100 mM sodium acetate buffer pH 5 in a total volume of 125 µl at 40 °C with 900 rpm of shaking in a TS1 thermoshaker (Biometra, Göttingen, Germany):

(A) 0.125 U/ml *endo*-1,3-β-D-glucanase + 5 mg/ml laminarin

(B) 10 µM WSC3 + 5 mg/ml laminarin

(C) 0.125 U/ml *endo*-1,3-β-D-glucanase + 10 µM WSC3

(D) 0.125 U/ml *endo*-1,3-β-D-glucanase + 10 µM WSC3 + 5 mg/ml laminarin

10 µl sample were taken out of the reaction mixture right after mixing (0 min) and after 5 min, 10 min, 15 min, 20 min, 30 min and 40 min of incubation, mixed with 90 µl of ddH_2O and heated for 5 min at 99°C to inactivate the enzyme and stop the reaction. The samples were kept on ice until all samples were collected and 50 µl of each sample was mixed with 50 µl of the BCA working solution containing 77.7 % (v/v) BCA solution A, 2.3 % (v/v) BCA solution B and 20 % (v/v) ethanol. The samples were subsequently incubated for 1 h at 80°C before the OD_{540} was measured in 96-well plates in a Tecan Spark 10M plate reader (Tecan, Männedorf, Switzerland). To generate the calibration curve 12 glucose solutions at concentration ranging from 0 mM to 1.4 mM were treated in a similar way.

BCA solution A	3.9 mM Bicinchoninic acid disodium salt 588 mM Na_2CO_3 in ddH_2O
BCA solution B	175 mM aspartic acid 314 mM Na_2CO_3 45 mM $CuSO_4$ x $5H_2O$ in ddH_2O

4.7.12 Protein pull-down with cell wall preparations

The ability of WSC3-His to bind protein-free cell wall preparations from *S. indica* mycelium and *H. vulgare* roots was investigated. Therefore, *S. indica* mycelium from a seven day old liquid culture grown in CM and root material from ten day barley plants grown in 1/10 PNM medium were collected and ground in liquid nitrogen using a pestle and mortar. The ground material was dissolved in 1 ml PBS containing 0.1 mg/ml Proteinase K (ThermoScientific, Schwerte, Germany). After incubation for 1.5 h at

55°C the Proteinase K was inactivated by 10 min incubation at room temperature with 1 mM PMSF. The cell wall material was spun down at 13,300 rpm for 3 min, washed three times with sterile H_2O and dried over night at room temperature. After drying the material was ground again for 1 min and resuspended in 500 µl sterile H_2O and sonificated for 30 s (duty cycle 40%, output control 4; Branson Sonifier 250, Branson Ultrasonics, Danbury, USA). 5 mg of the ground cell wall material was incubated with 20 µM WSC3-His in a total volume of 800 µl. The samples were incubated for 3 h at room temperature or overnight at 4°C on a rotating shaker. The insoluble fraction was centrifuged down at 13,300 rpm for 5 min and the supernatant was collected. The pellet fraction was washed three times with sterile H_2O and resuspended in Laemmli SDS buffer. The proteins were precipitated from the supernatant with TCA like described in 4.7.8 and also resuspended in Laemmli SDS buffer. All samples were boiled for 10 min at 99°C prior to loading on a SDS-PAGE like described in 4.7.9.

4.7.13 Enzymatic extraction of cell wall proteins

An enzymatic extraction of cell wall localized proteins was performed to investigate if WSC3-6xHis is covalently bound to the cell wall of the *P. pastoris*. *P. pastoris* transformants were grown like for the expression and purification of WSC3 and cells were collected by centrifugation after growth in BMMY (see section 4.7.6). 20 ml of the *P. pastoris* culture supernatant was precipitated with TCA (see section 4.7.8). The *P. pastoris* cell pellet was crushed in liquid nitrogen using mortar and pestle and around 50 mg of the crude extract were heated in 150 µl Laemmli SDS buffer for 10 min at 99°C. The remaining material was washed 5 x with cold ddH_2O, 3 x with cold 5 % NaCl, 3 x with cold 2 % NaCl, 3 x with 1 % NaCl and 3 x with 1 mM PMSF to remove residual medium, loosely bound proteins and other contaminants like polysaccharides. The remaining pellets were heated to 99°C for 20 min in 500 µl SDS extraction buffer. Afterwards the cell wall material was collected by centrifugation at 3,000 x g for 20 min and washed 5 x with 100 mM sodium acetate buffer (pH 5) supplemented with 1 mM PMSF while the supernatant was transferred to a fresh tube, mixed with an equal volume of Laemmli SDS buffer and heated for 10 min to 99°C. The pellets were split into 5 fractions after washing and treated like following overnight:

(A) 30 mM NaOH at room temperature

(B) 25 U/ml *endo*-1,3-β-D-glucanase from barley in 100 mM sodium acetate buffer(pH 5) at 37 °C

(C) 20 mg/ml *Trichoderma harzianum* lysing enzymes in 100 mM sodium acetate buffer(pH 5) at 37 °C

(D) 5 U/ml *exo*-1,3-β-D-glucanase from *Trichoderma virens* (Megazyme, Bray, Ireland) in 100 mM sodium acetate buffer(pH 4.5) at 37 °C

(E) 9 mg/ml chitinase from *Trichoderma viride* (Sigma Aldrich, Taufkirchen, Germany) in 100 mM sodium acetate buffer(pH 5) at room temperature

After the treatment the cell wall material was pelleted by centrifugation at 3,000 x g for 20 min and the pellets were washed twice with ddH_2O while the supernatant was mixed with an equal volume of Laemmli SDS buffer and heated for 10 min to 99°C. The washed cell wall pellets were resuspended in 150 µl Laemmli SDS buffer and heated to 99°C for 10 min. All samples were loaded on 10 % Bis-Tris gels (see section 4.7.9) and further analyzed towards the presence of WSC3-6xHis by Western blot (see section 4.7.10).

SDS extraction buffer	50 mM Tris-HCl (pH 8) 100 mM EDTA (pH 8) 2 % (w/v) SDS 10 mM DTT in ddH_2O

4.7.14 Preparation of the chitin suspension

A chitin suspension was prepared from water insoluble shrimp shell chitin (Sigma Aldrich, Taufkirchen, Germany) to elicit the ROS-production in *A. thaliana* and *H. vulgare* leaves (see section 4.7.15). 50 mg chitin were homogenized in 5 ml of sterile ddH_2O using mortar and pestle for 5 min. The suspension was heated to 40 s in a microwave at maximum watt, transferred to a 15 ml Falcon tube and the volume was filled to 5 ml with sterile ddH_2O. The suspension was subsequently sonificated for 2 min at power level 5 and a duty cycle of 30 % to further homogenize the solution. The water insoluble fraction was collected by centrifugation for 6 min at 3,000 rpm and the supernatant was diluted 1:1 in sterile ddH_2O to obtain the working solution at an approximate concentration of 5 mg/ml.

4.7.15 Quantification of reactive oxygen species (ROS)

The production of ROS as early plant defense response was quantified using a luminol-based assay that relies on the oxidation of the luminol-derivative L-012 (Wako Chemicals, Neuss, Germany) ROS, mostly H_2O_2, produced by the plant for example in response to MAMPs is decomposed by a supplemented HRP (Sigma Aldrich, Taufkirchen, Germany) and the generated oxygen reacts with the luminol molecule leading to a conformational change, which in turn leads to the emission of chemiluminescence.

ROS production was measured in *A. thaliana* and *H. vulgare* plants that have been grown like described in 4.5.6 and 4.5.7, respectively. Leaf discs with a diameter of 5 mm were cut out the rosette leaves of the *A. thaliana* plants or the second and third leaf of the *H. vulgare* plants using a Biopsy puncher (A. Hartenstein, Würzburg, Germany). The leaf discs were carefully transferred to white, flat bottom 96-well plates (Biozym, Hessisch Oldendorf, Germany) and incubated in 200 µl tap water for 16 h at room temperature in the dark. Prior to the ROS measurement the water was replaced by 200 µl 10 mM MOPS buffer pH 7.4 supplemented with 10 µM L-012 and 10 µg/ml HRP. The background luminescence was measured for 10 cycles with 1 min per cycle and an integration time of 450 ms per well. Prior to the injection of the MAMP-solution the indicated proteins and buffers were added by hand at the indicated final concentration. As MAMPs Laminarin (Sigma Aldrich, Taufkirchen, Germany), a chitin suspension (see section 4.7.12), laminarihexaose (Megazyme, Bray, Ireland), chitohexaose (Isosep, Tullinge, Sweden), gentiobiose (provided by Prof. Dr. Jürgen Seibel, Julian-Maximilians University of Würzburg) and flg22 (Genscript, Piscataway, USA) were used and injected with 200 µl/s into the wells. The luminescence was recorded for 100 cycles with 1 min per cycle and an integration time of 450 ms per well using a Tecan Spark 10M or Tecan M200 Pro plate reader (Tecan, Männedorf, Switzerland).

4.7.16 Quantification of MAMP-induced Ca^{2+}-fluxes

The influx of Ca^{2+}-ions into the plant cytoplasm is one of the earliest defense responses after recognition of MAMPs. The Ca^{2+}-influx can be quantified using an *A.thaliana* Col-0 reporter line expressing cytoplasmic aequorin which upon binding of Ca^{2+}-ions is

oxidizing coelenterazine (Promega, Mannheim, Germany) and thus produces a chemiluminescence signal proportional to the amount of Ca^{2+}-ions. The plants were grown like described in 4.5.6 and 6 plants of similar size per treatment were transferred into white, flat bottom 96-well plates (Biozym, Hessisch Oldendorf, Germany) containing 50 µl reconstitution buffer and stored overnight in the dark at room temperature. The next day the background luminescence was measured for 5 cycles with 1 min per cycle using a Tecan Spark 10M or Tecan M200 Pro plate reader (Tecan, Männedorf, Switzerland). Prior to the injection of 1 µM flg22 (Genscript, Piscataway, USA) to induce the Ca^{2+}-influx 10 µM of either FGB1 or WSC3-His were added to the wells by hand. As mock control 2 mM MES pH 5.7 was added. The emitted luminescence was recorded for 30 cycles with 1 min per cycle.

Reconstitution buffer	2 mM MES (pH 5.7)
	10 mM $CaCl_2$
	10 µM coelenterazine

4.8. Microscopy

4.8.1 Confocal microscopy

Confocal microscopy was performed using a Leica TCS SP8 confocal microscope (Wetzlar, Germany) to visualize the localization of WSC3-GFP in *S. indica* transformants. The transformants were grown for 6 to 14 days in liquid CM medium at 28°C with 120 rpm of shaking. Some of the fungal mycelium was transferred to a 1.5 ml Eppendorf tube containing 1 ml tap water and 5 µg/ml of WGA-AF594 (Invitrogen, Karlsruhe, Germany). Prior to microscopy 100 µl of 1 M Tris pH 8 were added, raising the pH of the solution to increase the GFP fluorescence signal. The GFP-fluorescence was excited using an Argon laser at a wavelength of 488 nm and the emitted light was detected at a wavelength range of 500-540 nm using a hybrid detector (HyD). The WGA-AF594 signal was excited with the DPSS laser at a wavelength of 561 nm and the emission was detected at a wavelength range of 605-660 nm using a photomultiplier (PMT). To also detect unspecific background signal a diode at 405 nm was used and emission was detected at 420-460 nm using a hybrid detector (HyD). Pictures were taken at a resolution of 1024 x 1024 pixels with a line average of 2 to 6

and a frame accumulation of 2 to 4. For the analysis of the pictures the Fiji software (Schindelin *et al.*, 2012) was used.

4.8.2 Transmission electron microscopy

Transmission electron microscopy was realized in collaboration with Dr. Ulla Neumann at the Central Microscopy Facility (CeMic) of the Max-Planck-Institute for Plant Breeding Research in Cologne to visualize the chitin and β-1,3-glucan components in the *S. indica* cell wall at the ultrastructural level. The homokaryotic reference strain *S. indica* GenR was grown for 5 d at 28°C with 120 rpm of shaking in liquid CM, the mycelium was filtered through a Miracloth filter, and homogenized like described above (see section 4.6.9). The homogenized mycelium was regenerated for 2 d in liquid CM at 28°C with 120 rpm of shaking before further processing at the MPIPZ. For TEM analysis, samples were fixed in 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.05 M sodium cacodylate buffer, pH 6.9, for 2 h at room temperature followed by an overnight incubation at 4°C. After thorough rinsing with 0.05 M sodium cacodylate buffer, the samples were post-fixed for 1 h on ice with 0.5 % OsO4 in 0.05 M sodium cacodylate buffer, pH 6.9, supplemented with 0.15 % potassium ferricyanide. Thereafter the samples were again thoroughly rinsed with 0.05 M sodium cacodylate buffer and dehydrated in an ethanol series from 10 % to 100 %, then in different ethanol:acetone mixtures and finally in 100 % acetone. Subsequently, samples were infiltrated with 25% Araldite 502/EmBed 812 (EMS, Hatfield, USA) in acetone. Further resin infiltration and final embedding was performed with the help of the EMS poly III, an evaporation-controlled automated embedding and polymerization device (EMS, Hatfield, USA). Ultrathin sections (70 – 90 nm) were prepared like described before (Micali *et al.*, 2011; Kleemann *et al.*, 2012). For the detection of β-1,3-glucan, the samples were immunogold labelled like described in Micali *et al.*, 2011 using a 1:100 dilution of the mouse monoclonal anti-β-1,3-glucan antibody (Biosupplies Australia Pty., Parkville, Australia). Detection of chitin was performed using undiluted WGA conjugated with 10 nm gold particles (EY Laboratories, San Mateo, USA) for 3 h at room temperature, followed by thorough rinsing with TRIS buffer and water. Transmission electron microscopy was performed using a Hitachi H-7650 TEM (Hitachi, Krefeld, Germany) operating at 100 kV. The acquired pictures were further analyzed using the Fiji software (Schindelin *et al.*, 2012).

For the quantification of chitin and β-1,3-glucan within the fungal cell wall the corresponding gold particles after staining with WGA for chitin and an anti-β-1,3-glucan antibody for β-1,3-glucan were counted from at least 50 pictures. The dicaryotic *S. indica* wildtype strain and the *S. indica* WSC3-GFP transformant number 3 were grown on solid CM plates for three weeks. Agar blocks were cut from the outer edge of the culture and processed like described above. After counting the gold particles the length of the cell wall was quantified from the taken pictures using the Fiji software and the number of gold particles per µm of cell wall was calculated. The average of gold particles/µm cell wall was calculated from all pictures and subsequently the β-1,3-glucan-to-chitin ratio was calculated and normalized to the the dicaryotic *S. indica* wildtype strain which accordingly was set to 1.

4.8.3 Light microscopy

Light microscopy was performed using a Leica M165 FC stereo microscope or a Leica DM2500 light microscope (Wetzlar, Germany). The acquired pictures were further analyzed using the Fiji software (Schindelin *et al.*, 2012).

4.9 Bioinformatics

4.9.1 Genome wide association screen - GWAS

The genome wide association (GWA) screen was performed to correlate the phenotypic differences between *A. thaliana* accession regarding their responsiveness to the MAMPs laminarin and chitin to genotypic differences. The peak luminescence values measured in the oxidative burst assay (see section 4.7.15) were normalized to those of the reference accession Col-0 (set to a value of 1) and subjected to a GWA screen using the GWAPP online resource (Seren *et al.*, 2012). The laminarin dataset was box-cox transformed and the chitin dataset was square root transformed to ensure a normal distribution, analyzed using the amm-algorithm and finally used to create a Manhattan plot of the $-log$(p-value) SNP marker–trait associations with a minor allele count (MAC) of 5. SNPs with a $-log$(p-value) of 4 and all genes being located 20 kb up- and downstream of these SNPs were subjected to further analysis. Gene annotations and ontologies were retrieved from TAIR9 (www.arabidopsis.org).

4.9.2 agriGO

The agriGO toolkit and database was used for a GO-term enrichment analysis of the *A. thaliana* gene list created from the genome wide association (GWA) screen to generate a functional profile of the genes identified by the GWAS. The agriGO version 2.0 was used and a single enrichment analysis (SEA) was performed (Tian *et al.*, 2017).

4.9.3 Mega7

A phylogenetic analysis of the 35 WSC-encoding genes was conducted using Mega7 (Kumar et al., 2016). Additionally the closest homolog of the WSC3-encoding gene *PIIN_05825* of the close relative *Serendipita vermifera* (*Sebve1*_01124) was included. The homolog was identified by a blastp search against the non-redundant protein sequences database (nr) with the full length WSC3 protein sequence as query. The 36 concetanated amino acid sequences of the WSC domains were aligned using the MUSCLE algorithm (Edgar, 2004) with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method. The alignment was subjected to a phylogenetic clustering generating a maximum parsimony tree using the Subtree-Pruning-Regrafting (SPR) algorithm with 1,000 bootstraps.

5. References

Adio, A. M., Casteel, C. L., De Vos, M., Kim, J. H., Joshi, V., Li, B., ... & Jander, G. (2011). Biosynthesis and defensive function of Nδ-acetylornithine, a jasmonate-induced Arabidopsis metabolite. *The Plant Cell*, *23*(9), 3303-3318.

Agler, M. T., Ruhe, J., Kroll, S., Morhenn, C., Kim, S. T., Weigel, D., & Kemen, E. M. (2016). Microbial hub taxa link host and abiotic factors to plant microbiome variation. *PLoS biology*, *14*(1), e1002352.

Al-Babili, S., & Bouwmeester, H. J. (2015). Strigolactones, a novel carotenoid-derived plant hormone. *Annual review of plant biology*, *66*, 161-186.

Albersheim, P. & Valent, B. S. (1978). Host-pathogen interactions in plants. Plants, when exposed to oligosaccharides of fungal origin, defend themselves by accumulating antibiotics. *J. Cell Biol.;(United States)*, *78*.

Anderson, A. J. (1978). The isolation from three species of Colletotrichum of glucan containing polysaccharides that elicit a defense response in bean (Phaseolus vulgaris). *Phytopathology*, *68*, 189-194.

Anderson, A. J. (1980). Studies on the structure and elicitor activity of fungal glucans. *Canadian Journal of Botany*, *58*(22), 2343-2348.

Andersson, K. M., Meerupati, T., Levander, F., Friman, E., Ahrén, D., & Tunlid, A. (2013). Characterization of the proteome of the nematode-trapping cells of the fungus Monacrosporium haptotylum. *Applied and environmental microbiology*, AEM-01390.

Aranzana, M. J., Kim, S., Zhao, K., Bakker, E., Horton, M., Jakob, K., ... & Toomajian, C. (2005). Genome-wide association mapping in Arabidopsis identifies previously known flowering time and pathogen resistance genes. *PLoS genetics*, *1*(5), e60.

Atwell, S., Huang, Y. S., Vilhjálmsson, B. J., Willems, G., Horton, M., Li, Y., ... & Jiang, R. (2010). Genome-wide association study of 107 phenotypes in Arabidopsis thaliana inbred lines. *Nature*, *465*(7298), 627-631.

Ayers, A. R., Ebel, J., Valent, B. & Albersheim, P. (1976). Host-pathogen interactions X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of Phytophthora megasperma var. sojae. *Plant Physiology*, *57*(5), 760-765.

Aziz, A., Poinssot, B., Daire, X., Adrian, M., Bézier, A., Lambert, B., Joubert, J.-M. & Pugin, A. (2003). Laminarin elicits defense responses in grapevine and induces protection against Botrytis cinerea and Plasmopara viticola. *Molecular Plant-Microbe Interactions*, *16*(12), 1118-1128.

Bai, Y., Müller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., ... & Hüttel, B. (2015). Functional overlap of the Arabidopsis leaf and root microbiota. *Nature*, *528*(7582), 364.

Banhara, A., Ding, Y., Kühner, R., Zuccaro, A., & Parniske, M. (2015). Colonization of root cells and plant growth promotion by Piriformospora indica occurs independently of plant common symbiosis genes. *Frontiers in plant science*, *6*.

Bartels, S., Lori, M., Mbengue, M., van Verk, M., Klauser, D., Hander, T., ... & Boller, T. (2013). The family of Peps and their precursors in Arabidopsis: differential expression and localization but similar induction of pattern-triggered immune responses. *Journal of experimental botany*, *64*(17), 5309-5321.

Bartnicki-Garcia, S. (1968). Cell wall chemistry, morphogenesis, and taxonomy of fungi. *Annual Reviews in Microbiology*, *22*(1), 87-108.

Baxter, I., Brazelton, J. N., Yu, D., Huang, Y. S., Lahner, B., Yakubova, E., ... & Vitek, O. (2010). A coastal cline in sodium accumulation in Arabidopsis thaliana is driven by natural variation of the sodium transporter AtHKT1; 1. *PLoS genetics*, *6*(11), e1001193.

Beffa, R. S., Hofer, R. M., Thomas, M., & Meins, F. (1996). Decreased Susceptibility to Viral Disease of [beta]-1, 3-Glucanase-Deficient Plants Generated by Antisense Transformation. *The Plant Cell*, *8*(6), 1001-1011.

Bernard, M., Mouyna, I., Dubreucq, G., Debeaupuis, J. P., Fontaine, T., Vorgias, C., ... & Latge, J. P. (2002). Characterization of a cell-wall acid phosphatase (PhoAp) in Aspergillus fumigatus. *Microbiology*, *148*(9), 2819-2829.

Bolton, M.D., van Esse, H.P., Vossen, J.H., de Jonge, R., Stergiopolous, I., Stulemeijer, I.J.E. et al. (2008). The novel Cladosporium fulvum lysine motif effector Ecp6 is a virulence factor with orthologues in other fungal species. Mol. Microbiol. 69(1)

Boutrout, F., & Zipfel, C. (2017). Function, Discovery, and Exploitation of Plant Pattern Recognition Receptors for Broad-Spectrum Disease Resistance. *Annual Review of Phytopathology*, *55*(1).

Bowman, S. M., & Free, S. J. (2006). The structure and synthesis of the fungal cell wall. *Bioessays*, *28*(8), 799-808.

Brown, G. D. (2006). Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nature reviews. Immunology*, *6*(1), 33.

Brown, J., O'Callaghan, C. A., Marshall, A. S., Gilbert, R. J., Siebold, C., Gordon, S., ... & Jones, E. Y. (2007). Structure of the fungal β-glucan-binding immune receptor dectin-1: Implications for function. *Protein Science*, *16*(6), 1042-1052.

Brutus, A., Sicilia, F., Macone, A., Cervone, F., & De Lorenzo, G. (2010). A domain swap approach reveals a role of the plant wall-associated kinase 1 (WAK1) as a receptor of oligogalacturonides. *Proceedings of the National Academy of Sciences*, *107*(20), 9452-9457.

Buendia, L., Wang, T., Girardin, A., & Lefebvre, B. (2016). The LysM receptor-like kinase SlLYK10 regulates the arbuscular mycorrhizal symbiosis in tomato. *New Phytologist*, *210*(1), 184-195.

Cao, Y., Liang, Y., Tanaka, K., Nguyen, C. T., Jedrzejczak, R. P., Joachimiak, A. & Stacey, G. (2014). The kinase LYK5 is a major chitin receptor in Arabidopsis and forms a chitin-induced complex with related kinase CERK1. *Elife*, *3*, e03766.

Casadevall, A., Nosanchuk, J. D., Williamson, P., & Rodrigues, M. L. (2009). Vesicular transport across the fungal cell wall. *Trends in microbiology*, *17*(4), 158-162.

Chan, E. K., Rowe, H. C., Corwin, J. A., Joseph, B., & Kliebenstein, D. J. (2011). Combining genome-wide association mapping and transcriptional networks to identify novel genes controlling glucosinolates in Arabidopsis thaliana. *PLoS biology*, *9*(8), e1001125.

Chao, D. Y., Silva, A., Baxter, I., Huang, Y. S., Nordborg, M., Danku, J., ... & Salt, D. E. (2012). Genome-wide association studies identify heavy metal ATPase3 as the primary determinant of natural variation in leaf cadmium in Arabidopsis thaliana. *PLoS genetics*, *8*(9), e1002923.

Chen, C., Rowley, A. F., Newton, R. P., & Ratcliffe, N. A. (1999). Identification, purification and properties of a β-1, 3-glucan-specific lectin from the serum of the cockroach, Blaberus discoidalis which is implicated in immune defence reactions. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *122*(3), 309-319.

Cheong, J. J., Birberg, W., Fügedi, P., Pilotti, A., Garegg, P. J., Hong, N., Ogawa, T. & Hahn, M. G. (1991). Structure-activity relationships of oligo-beta-glucoside elicitors of phytoalexin accumulation in soybean. *The Plant Cell*, *3*(2), 127-136.

Cheong, J. J. & Hahn, M. G. (1991). A specific, high-affinity binding site for the hepta-beta-glucoside elicitor exists in soybean membranes. *The Plant Cell*, *3*(2), 137-147.

Choi, J., Tanaka, K., Cao, Y., Qi, Y., Qiu, J., Liang, Y., ... & Stacey, G. (2014). Identification of a plant receptor for extracellular ATP. *Science*, *343*(6168), 290-294.

Cohen-Kupiec, R., Broglie, K.E., Friesem, D., Broglie, R.M., Chet, I. (1999) Molecular characterization of a novel β-1,3-exoglucanase related to mycoparasitism of Trichoderma harzianum. Gene 226 (2): 147–154

Coleman-Derr, D., Desgarennes, D., Fonseca-Garcia, C., Gross, S., Clingenpeel, S., Woyke, T., ... & Tringe, S. G. (2016). Plant compartment and biogeography affect microbiome composition in cultivated and native Agave species. *New Phytologist*, *209*(2), 798-811.

Cook, D. E., Mesarich, C. H., & Thomma, B. P. (2015). Understanding plant immunity as a surveillance system to detect invasion. *Annual review of phytopathology*, *53*, 541-563.

Cosio, E. G., Frey, T., Verduyn, R., van Boom, J. & Ebel, J. (1990). High-affinity binding of a synthetic heptaglucoside and fungal glucan phytoalexin elicitors to soybean membranes. *FEBS letters*, *271*(1), 223-226.

Couto, D., & Zipfel, C. (2016). Regulation of pattern recognition receptor signalling in plants. *Nature Reviews Immunology*, *16*(9), 537-552.

Daudi, A., Cheng, Z., O'Brien, J. A., Mammarella, N., Khan, S., Ausubel, F. M., & Bolwell, G. P. (2012). The apoplastic oxidative burst peroxidase in Arabidopsis is a major component of pattern-triggered immunity. *The Plant Cell*, *24*(1), 275-287.

DeFraia, C. T., Wang, Y., Yao, J., & Mou, Z. (2013). Elongator subunit 3 positively regulates plant immunity through its histone acetyltransferase and radical S-adenosylmethionine domains. *BMC plant biology*, *13*(1), 102.

DeFraia, C. T., Zhang, X., & Mou, Z. (2010). Elongator subunit 2 is an accelerator of immune responses in Arabidopsis thaliana. *The Plant Journal*, *64*(3), 511-523.

De Jonge, R., & Thomma, B. P. (2009). Fungal LysM effectors: extinguishers of host immunity?. *Trends in microbiology*, *17*(4), 151-157.

De Jonge, R., van Essen, H.P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R. et al. (2010) Conserved Fungal LysM Effector Ecp6 Prevents Chitin-Triggered Immunity in Plants. *Science* 329: 953-955

De Wit, P. J., Mehrabi, R., van den Burg, H. A., & Stergiopoulos, I. (2009). Fungal effector proteins: past, present and future. *Molecular plant pathology*, *10*(6), 735-747.

Den Camp, R. O., Streng, A., De Mita, S., Cao, Q., Polone, E., Liu, W., ... & Bisseling, T. (2011). LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia. *Science*, *331*(6019), 909-912.

Dodds, P. N., & Rathjen, J. P. (2010). Plant immunity: towards an integrated view of plant-pathogen interactions. *Nature reviews. Genetics*, *11*(8), 539.

Douglas, C. M. (2001). Fungal β (1, 3)-D-glucan synthesis. *Medical mycology*, *39*(1), 55-66.

Dupres, V., Alsteens, D., Wilk, S., Hansen, B., Heinisch, J. J., & Dufrêne, Y. F. (2009). The yeast Wsc1 cell surface sensor behaves like a nanospring in vivo. *Nature chemical biology*, *5*(11), 857-862.

Ebel, J., Ayers, A. R.,& Albersheim, P. (1976). Host-pathogen interactions XII. Response of suspension-cultured soybean cells to the elicitor isolated from Phytophthora megasperma var. sojae, a fungal pathogen of soybeans. *Plant physiology*, *57*(5), 775-779.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research*, *32*(5), 1792-1797.

Eichmann, R., Dechert, C., KOGEL, K. H., & Hueckelhoven, R. (2006). Transient over-expression of barley BAX Inhibitor-1 weakens oxidative defence and MLA12-mediated resistance to Blumeria graminis f. sp. hordei. *Molecular plant pathology*, *7*(6), 543-552.

El Gueddari, N. E., Rauchhaus, U., Moerschbacher, B. M., & Deising, H. B. (2002). Developmentally regulated conversion of surface-exposed chitin to chitosan in cell walls of plant pathogenic fungi. *New Phytologist*, *156*(1), 103-112.

Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M., & Xavier, J. B. (2012). Animal behavior and the microbiome. *Science*, *338*(6104), 198-199.

Faulkner, C., Petutschnig, E., Benitez-Alfonso, Y., Beck, M., Robatzek, S., Lipka, V., & Maule, A. J. (2013). LYM2-dependent chitin perception limits molecular flux via plasmodesmata. *Proceedings of the National Academy of Sciences*, *110*(22), 9166-9170.

Fesel, P. H., & Zuccaro, A. (2016a). β-glucan: crucial component of the fungal cell wall and elusive MAMP in plants. *Fungal Genetics and Biology*, *90*, 53-60.

Fesel, P. H., & Zuccaro, A. (2016b). Dissecting endophytic lifestyle along the parasitism/mutualism continuum in Arabidopsis. *Current opinion in microbiology*, *32*, 103-112.

Feuillet, C., Schachermayr, G., & Keller, B. (1997). Molecular cloning of a new receptor-like kinase gene encoded at the Lr10 disease resistance locus of wheat. *The Plant Journal*, *11*(1), 45-52.

Filiault, D. L., & Maloof, J. N. (2012). A genome-wide association study identifies variants underlying the Arabidopsis thaliana shade avoidance response. *PLoS Genetics*, *8*(3), e1002589.

Flemming, H. C., & Wingender, J. (2010). The biofilm matrix. *Nature reviews. Microbiology*, *8*(9), 623.

Fliegmann, J., Mithöfer, A., Wanner, G., & Ebel, J. (2004). An ancient enzyme domain hidden in the putative β-glucan elicitor receptor of soybean may play an active part in the perception of pathogen-associated molecular patterns during broad host resistance. *Journal of Biological Chemistry*, *279*(2), 1132-1140.

Fujikawa, T., Sakaguchi, A., Nishizawa, Y., Kouzai, Y., Minami, E., Yano, S., ... & Nishimura, M. (2012). Surface α-1, 3-glucan facilitates fungal stealth infection by interfering with innate immunity in plants. *PLoS pathogens*, *8*(8), e1002882.

Fujii, T., Shimoi, H., & Iimura, Y. (1999). Structure of the glucan-binding sugar chain of Tip1p, a cell wall protein of Saccharomyces cerevisiae. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1427*(2), 133-144.

Futagami, T., Nakao, S., Kido, Y., Oka, T., Kajiwara, Y., Takashita, H., ... & Goto, M. (2011). Putative stress sensors WscA and WscB are involved in hypo-osmotic and acidic pH stress tolerance in Aspergillus nidulans. *Eukaryotic cell*, *10*(11), 1504-1515.

Gabius, H. J., André, S., Kaltner, H., & Siebert, H. C. (2002). The sugar code: functional lectinomics. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1572*(2), 165-177.

Garnica, S., Riess, K., Schön, M. E., Oberwinkler, F., & Setaro, S. D. (2016). Divergence times and phylogenetic patterns of Sebacinales, a highly diverse and widespread fungal lineage. *PloS one*, *11*(3), e0149531.

Gaulin, E., Jauneau, A., Villalba, F., Rickauer, M., Esquerré-Tugayé, M. T., & Bottin, A. (2002). The CBEL glycoprotein of Phytophthora parasitica var-nicotianae is involved in cell wall deposition and adhesion to cellulosic substrates. *Journal of Cell Science*, *115*(23), 4565-4575.

Gauthier, A., Trouvelot, S., Kelloniemi, J., Frettinger, P., Wendehenne, D., Daire, X., Joubert, J.-M., Ferrarini, A., Delledonne, M., Flors, V. & Poinssot, B. (2014). The sulfated laminarin triggers a stress transcriptome before priming the SA-and ROS-dependent defenses during grapevine's induced resistance against Plasmopara viticola. *PloS one*, *9*(2).

Genre, A., Chabaud, M., Balzergue, C., Puech-Pagès, V., Novero, M., Rey, T., ... & Barker, D. G. (2013). Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca2+ spiking in Medicago truncatula roots and their production is enhanced by strigolactone. *New Phytologist*, *198*(1), 190-202.

Geoghegan, I., Steinberg, G., & Gurr, S. (2017). The Role of the Fungal Cell Wall in the Infection of Plants. *Trends in Microbiology*.

Goldstein, I. J., Hughes, R. C., Monsigny, M., Osawa, T., & Sharon, N. (1980). What should be called a lectin?. *Nature*, *285*(5760), 66-66.

Gómez-Gómez, L., Felix, G., & Boller, T. (1999). A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. *The Plant Journal*, *18*(3), 277-284.

Hacquard, S., Kracher, B., Hiruma, K., Münch, P. C., Garrido-Oter, R., Thon, M. R., ... & Henrissat, B. (2016). Survival trade-offs in plant roots during colonization by closely related beneficial and pathogenic fungi. *Nature communications*, *7*.

Hacquard, S., Spaepen, S., Garrido-Oter, R., & Schulze-Lefert, P. (2017). Interplay Between Innate Immunity and the Plant Microbiota. *Annual Review of Phytopathology*, (0).

Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature reviews. Microbiology*, *2*(2), 95.

Hamilton, S. R., & Gerngross, T. U. (2007). Glycosylation engineering in yeast: the advent of fully humanized yeast. *Current opinion in biotechnology*, *18*(5), 387-392.

Harrison, M. J. (1999). Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. *Annual review of plant biology*, *50*(1), 361-389.

Hayafune, M., Berisio, R., Marchetti, R., Silipo, A., Kayama, M., Desaki, Y., ... & Molinaro, A. (2014). Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization. *Proceedings of the National Academy of Sciences*, *111*(3), E404-E413.

Heinisch, J. J., Lorberg, A., Schmitz, H. P., & Jacoby, J. J. (1999). The protein kinase C-mediated MAP kinase pathway involved in the maintenance of cellular integrity in Saccharomyces cerevisiae. *Molecular microbiology*, *32*(4), 671-680.

Heinisch, J. J., Dupres, V., Wilk, S., Jendretzki, A., & Dufrêne, Y. F. (2010). Single-molecule atomic force microscopy reveals clustering of the yeast plasma-membrane sensor Wsc1. *PLoS One*, *5*(6), e11104.

Hilbert, M., Voll, L. M., Ding, Y., Hofmann, J., Sharma, M., & Zuccaro, A. (2012). Indole derivative production by the root endophyte Piriformospora indica is not required for growth promotion but for biotrophic colonization of barley roots. *New Phytologist*, *196*(2), 520-534.

Hiruma, K., Gerlach, N., Sacristán, S., Nakano, R. T., Hacquard, S., Kracher, B., ... & Schulze-Lefert, P. (2016). Root endophyte Colletotrichum tofieldiae confers plant fitness benefits that are phosphate status dependent. *Cell*, *165*(2), 464-474.

Horton, M. W., Hancock, A. M., Huang, Y. S., Toomajian, C., Atwell, S., Auton, A., ... & Nordborg, M. (2012). Genome-wide patterns of genetic variation in worldwide Arabidopsis thaliana accessions from the RegMap panel. *Nature genetics*, *44*(2), 212-216.

Huffaker, A., Pearce, G., & Ryan, C. A. (2006). An endogenous peptide signal in Arabidopsis activates components of the innate immune response. *Proceedings of the National Academy of Sciences*, *103*(26), 10098-10103.

Huffaker, A., & Ryan, C. A. (2007). Endogenous peptide defense signals in Arabidopsis differentially amplify signaling for the innate immune response. *Proceedings of the National Academy of Sciences*, *104*(25), 10732-10736.

Jabs, T., Tschöpe, M., Colling, C., Hahlbrock, K., & Scheel, D. (1997). Elicitor-stimulated ion fluxes and O2− from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proceedings of the National Academy of Sciences*, *94*(9), 4800-4805.

Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Lipka, V., ... & Schäfer, P. (2011). Broad-spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the fungus Piriformospora indica. *Plant Physiology*, *156*(2), 726-740.

Jashni, M. K., Dols, I. H., Iida, Y., Boeren, S., Beenen, H. G., Mehrabi, R., ... & de Wit, P. J. (2015). Synergistic action of a metalloprotease and a serine protease from Fusarium oxysporum f. sp. lycopersici cleaves chitin-binding tomato chitinases, reduces their antifungal activity, and enhances fungal virulence. *Molecular Plant-Microbe Interactions*, *28*(9), 996-1008.

Jendretzki, A., Wittland, J., Wilk, S., Straede, A., & Heinisch, J. J. (2011). How do I begin? Sensing extracellular stress to maintain yeast cell wall integrity. *European journal of cell biology*, *90*(9), 740-744.

Jones, J. D., & Dangl, J. L. (2006). The plant immune system. *Nature*, *444*(7117), 323-329.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., ... & Shibuya, N. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Sciences*, *103*(29), 11086-11091.

Kämper, J., Kahmann, R., Bölker, M., Ma, L. J., Brefort, T., Saville, B. J., ... & Perlin, M. H. (2006). Insights from the genome of the biotrophic fungal plant pathogen Ustilago maydis. *Nature*, *444*(7115), 97-101.

Kapteyn, J. C., Montijin, R. C., Vink, E., de la Cruz, J., Llobell, A., Douwes, J. E., ... & Klis, F. M. (1996). Retention of Saccharomyces cerevisiae cell wall proteins through a phosphodiester-linked β-1, 3-/β-l, 6-glucan heteropolymer. *Glycobiology*, *6*(3), 337-345.

Kaul, S., Koo, H. L., Jenkins, J., Rizzo, M., Rooney, T., Tallon, L. J., ... & Town, C. D. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *nature*, *408*(6814), 796-815.

Kim, S., Plagnol, V., Hu, T. T., Toomajian, C., Clark, R. M., Ossowski, S., ... & Nordborg, M. (2007). Recombination and linkage disequilibrium in Arabidopsis thaliana. *Nature genetics*, *39*(9), 1151-1155.

Klarzynski, O., Plesse, B., Joubert, J. M., Yvin, J. C., Kopp, M., Kloareg, B. & Fritig, B. (2000). Linear β-1, 3 glucans are elicitors of defense responses in tobacco. *Plant Physiology*, *124*(3), 1027-1038.

Kleemann, J., Rincon-Rivera, L. J., Takahara, H., Neumann, U., van Themaat, E. V. L., van der Does, H. C., ... & Schmelzer, E. (2012). Sequential delivery of host-induced virulence effectors by appressoria and intracellular hyphae of the phytopathogen Colletotrichum higginsianum. *PLoS Pathog*, *8*(4), e1002643.

Klis, F. M., Mol, P., Hellingwerf, K., & Brul, S. (2002). Dynamics of cell wall structure in Saccharomyces cerevisiae. *FEMS microbiology reviews*, *26*(3), 239-256.

Kloppholz, S., Kuhn, H., & Requena, N. (2011). A secreted fungal effector of Glomus intraradices promotes symbiotic biotrophy. *Current Biology*, *21*(14), 1204-1209.

Knight, M. R., & Campbell, A. K. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature*, *352*(6335), 524.

Kohler, A., Kuo, A., Nagy, L. G., Morin, E., Barry, K. W., Buscot, F., ... & Colpaert, J. (2015). Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. *Nature genetics*, *47*(4), 410-415.

Kollár, R., Reinhold, B. B., Petráková, E., Yeh, H. J., Ashwell, G., Drgonová, J., ... & Cabib, E. (1997). Architecture of the yeast cell wall β (1→ 6)-glucan interconnects mannoprotein, β (1→ 3)-glucan, and chitin. *Journal of Biological Chemistry*, *272*(28), 17762-17775.

Kopecká, M., & Gabriel, M. (1992). The influence of Congo red on the cell wall and (1→ 3)-β-d-glucan microfibril biogenesis in Saccharomyces cerevisiae. *Archives of microbiology*, *158*(2), 115-126.

Korte, A., & Farlow, A. (2013). The advantages and limitations of trait analysis with GWAS: a review. *Plant methods*, *9*(1), 29.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, msw054.

Lahrmann, U., & Zuccaro, A. (2012). Opprimo ergo sum—evasion and suppression in the root endophytic fungus Piriformospora indica. *Molecular Plant-Microbe Interactions*, *25*(6), 727-737.

Lahrmann, U., Ding, Y., Banhara, A., Rath, M., Hajirezaei, M.R., Döhlemann, S., von Wirén, N., Parniske, M., Zuccaro, A. (2013) Host related metabolic cues affect colonization strategies of a root endophyte. Proc. Natl. Acad. Sci. doi:10.1073/pnas.1301653110.

Lahrmann, U., Strehmel, N., Langen, G., Frerigmann, H., Leson, L., Ding, Y., ... & Zuccaro, A. (2015). Mutualistic root endophytism is not associated with the reduction of saprotrophic traits and requires a noncompromised plant innate immunity. *New Phytologist*, *207*(3), 841-857.

Latgé, J. P. (2007). The cell wall: a carbohydrate armour for the fungal cell. *Molecular microbiology*, *66*(2), 279-290.

Latgé, J. P., Beauvais, A., & Chamilos, G. (2017). The Cell Wall of the Human Fungal Pathogen Aspergillus fumigatus: Biosynthesis, Organization, Immune Response, and Virulence. *Annual Review of Microbiology*, (0).

Levin, D. E. (2005). Cell wall integrity signaling in Saccharomyces cerevisiae. *Microbiology and molecular biology reviews*, *69*(2), 262-291.

Li, Y., Huang, Y., Bergelson, J., Nordborg, M., & Borevitz, J. O. (2010). Association mapping of local climate-sensitive quantitative trait loci in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, *107*(49), 21199-21204.

Liang, Y., Cao, Y., Tanaka, K., Thibivilliers, S., Wan, J., Choi, J., ... & Stacey, G. (2013). Nonlegumes respond to rhizobial Nod factors by suppressing the innate immune response. *Science*, *341*(6152), 1384-1387.

Liang, Y., Tóth, K., Cao, Y., Tanaka, K., Espinoza, C., & Stacey, G. (2014). Lipochitooligosaccharide recognition: an ancient story. *New Phytologist*, *204*(2), 289-296.

Lim, C. W., Yang, S. H., Shin, K. H., Lee, S. C., & Kim, S. H. (2015). The AtLRK10L1. 2, Arabidopsis ortholog of wheat LRK10, is involved in ABA-mediated signaling and drought resistance. *Plant cell reports*, *34*(3), 447-455.

Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., ... & Zhou, J. M. (2012). Chitin-induced dimerization activates a plant immune receptor. *Science*, *336*(6085), 1160-1164.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, *25*(4), 402-408.

Lodder, A.L., Lee, T.K., Ballester, R. (1999). Characterization of the Wsc1 Protein, a Putative Receptor in the Stress Response of Saccharomyces cerevisiae. Genetics 152

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., ... & Kahmann, R. (2015). Fungal effectors and plant susceptibility. *Annual review of plant biology*, *66*, 513-545.

Lou, Y. R., Bor, M., Yan, J., Preuss, A. S., & Jander, G. (2016). Arabidopsis NATA1 acetylates putrescine and decreases defense-related hydrogen peroxide accumulation. *Plant physiology*, pp-00446.

Lynch, D. J., Fountain, T. L., Mazurkiewicz, J. E., & Banas, J. A. (2007). Glucan-binding proteins are essential for shaping Streptococcus mutans biofilm architecture. *FEMS microbiology letters*, *268*(2), 158-165.

Macho, A. P., & Zipfel, C. (2015). Targeting of plant pattern recognition receptor-triggered immunity by bacterial type-III secretion system effectors. *Current opinion in microbiology*, *23*, 14-22.

Maillet, F., Poinsot, V., Andre, O., Puech-Pagès, V., Haouy, A., Gueunier, M., ... & Martinez, E. A. (2011). Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature*, *469*(7328), 58.

Marone, D., Russo, M. A., Laidò, G., De Leonardis, A. M., & Mastrangelo, A. M. (2013). Plant nucleotide binding site–leucine-rich repeat (NBS-LRR) genes: active guardians in host defense responses. *International journal of molecular sciences*, *14*(4), 7302-7326.

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K. E., ... & Rudd, J. J. (2011). Analysis of two in planta expressed LysM effector homologs from the fungus Mycosphaerella graminicola reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology*, *156*(2), 756-769.

Martin, F., Aerts, A., Ahrén, D., Brun, A., Danchin, E. G. J., Duchaussoy, F., ... & Salamov, A. (2008). The genome of Laccaria bicolor provides insights into mycorrhizal symbiosis. *Nature*, *452*(7183), 88.

McKhann, H. I., Camilleri, C., Bérard, A., Bataillon, T., David, J. L., Reboud, X., ... & Brunel, D. (2004). Nested core collections maximizing genetic diversity in Arabidopsis thaliana. *The Plant Journal*, *38*(1), 193-202.

Ménard, R., Alban, S., de Ruffray, P., Jamois, F., Franz, G., Fritig, B., Yvin, J.-C. & Kauffmann, S. (2004). β-1, 3 glucan sulfate, but not β-1, 3 glucan, induces the salicylic acid signaling pathway in tobacco and Arabidopsis. *The Plant Cell*, *16*(11), 3020-3032.

Ménard, R., de Ruffray, P., Fritig, B., Yvin, J. C., & Kauffmann, S. (2005). Defense and resistance-inducing activities in tobacco of the sulfated β-1, 3 glucan PS3 and its synergistic activities with the unsulfated molecule. *Plant and cell physiology*, *46*(12), 1964-1972.

Mentlak, T. A., Kombrink, A., Shinya, T., Ryder, L. S., Otomo, I., Saitoh, H., ... & Talbot, N. J. (2012). Effector-mediated suppression of chitin-triggered immunity by Magnaporthe oryzae is necessary for rice blast disease. *The Plant Cell*, *24*(1), 322-335.

Micali, C. O., Neumann, U., Grunewald, D., Panstruga, R., & O'Connell, R. (2011). Biogenesis of a specialized plant–fungal interface during host cell internalization of Golovinomyces orontii haustoria. *Cellular microbiology*, *13*(2), 210-226.

Mirelman, D., Galun, E., Sharon, N., & Lotan, R. (1975). Inhibition of fungal growth by wheat germ agglutinin. *Nature*, *256*(5516), 414-416.

Mithöfer, A., Bhagwat, A. A., Feger, M. & Ebel, J. (1996). Suppression of fungal β-glucan-induced plant defence in soybean (Glycine max L.) by cyclic 1, 3-1, 6-β-glucans from the symbiont Bradyrhizobium japonicum. *Planta*, *199*(2), 270-275.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., ... & Shibuya, N. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proceedings of the National Academy of Sciences*, *104*(49), 19613-19618.

Miyata, K., Kozaki, T., Kouzai, Y., Ozawa, K., Ishii, K., Asamizu, E., ... & Akiyama, K. (2014). The bifunctional plant receptor, OsCERK1, regulates both chitin-triggered immunity and arbuscular mycorrhizal symbiosis in rice. *Plant and Cell Physiology*, *55*(11), 1864-1872.

Narusaka, Y., Shinya, T., Narusaka, M., Motoyama, N., Shimada, H., Murakami, K., & Shibuya, N. (2013). Presence of LYM2 dependent but CERK1 independent disease resistance in Arabidopsis. *Plant signaling & behavior*, *8*(9), e25345.

Naumann, T. A., & Wicklow, D. T. (2013). Chitinase modifying proteins from phylogenetically distinct lineages of Brassica pathogens. *Physiological and molecular plant pathology*, *82*, 1-9.

Ning, J., Zhang, W., Yi, Y., Yang, G., Wu, Z., Yi, J. & Kong, F. (2003). Synthesis of β-(1→ 6)-branched β-(1→ 3) glucohexaose and its analogues containing an α-(1→ 3) linked bond with antitumor activity. *Bioorganic & medicinal chemistry*, *11*(10), 2193-2203.

Nordborg, M., Hu, T. T., Ishino, Y., Jhaveri, J., Toomajian, C., Zheng, H., ... & Jakobsson, M. (2005). The pattern of polymorphism in Arabidopsis thaliana. *PLoS biology*, *3*(7), e196.

Nühse, T. S., Bottrill, A. R., Jones, A. M., & Peck, S. C. (2007). Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. *The Plant Journal*, *51*(5), 931-940.

Oberwinkler, F., Riess, K., Bauer, R., Selosse, M. A., Weiß, M., Garnica, S., & Zuccaro, A. (2013). Enigmatic sebacinales. *Mycological Progress*, *12*(1), 1-27.

Oliveira-Garcia, E., Deising, H.B. (2013). Infection structure-specific expression of b-1,3-glucan synthase is essential for pathogenicity of Colletotrichum graminicola and evasion of b-glucan–triggered immunity in maize. *Plant Cel*l 25 (6), 2356–2378.

Oliveira-Garcia, E., Deising, H.B. (2016). Attenuation of PAMP-triggered immunity in maize requires downregulation of the key b-1,6-glucan synthesis genes KRE5 and KRE6 in biotrophic hyphae of Colletotrichum graminicola *Plant Journal* doi: 10.1111/tpj.13205

Pandey, A., Sharma, M., & Pandey, G. K. (2016). Emerging roles of strigolactones in plant responses to stress and development. *Frontiers in plant science*, *7*.

Peberdy, J. F. (1994). Protein secretion in filamentous fungi—trying to understand a highly productive black box. *Trends in biotechnology*, *12*(2), 50-57.

Peškan-Berghöfer, T., Shahollari, B., Giong, P. H., Hehl, S., Markert, C., Blanke, V., ... & Oelmüller, R. (2004). Association of Piriformospora indica with Arabidopsis thaliana roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmic reticulum and at the plasma membrane. *Physiologia Plantarum*, *122*(4), 465-477.

Pitarch, A., Sánchez, M., Nombela, C., & Gil, C. (2002). Sequential fractionation and two-dimensional gel analysis unravels the complexity of the dimorphic fungus Candida albicans cell wall proteome. *Molecular & Cellular Proteomics*, *1*(12), 967-982.

Plett, J. M., Daguerre, Y., Wittulsky, S., Vayssières, A., Deveau, A., Melton, S. J., ... & Martin, F. (2014). Effector MiSSP7 of the mutualistic fungus Laccaria bicolor stabilizes the Populus JAZ6 protein and represses jasmonic acid (JA) responsive genes. *Proceedings of the National Academy of Sciences*, *111*(22), 8299-8304.

Postel, S., Küfner, I., Beuter, C., Mazzotta, S., Schwedt, A., Borlotti, A., ... & Nürnberger, T. (2010). The multifunctional leucine-rich repeat receptor kinase BAK1 is implicated in Arabidopsis development and immunity. *European journal of cell biology*, *89*(2), 169-174.

Priegnitz, B. E., Wargenau, A., Brandt, U., Rohde, M., Dietrich, S., Kwade, A., ... & Fleißner, A. (2012). The role of initial spore adhesion in pellet and biofilm formation in Aspergillus niger. *Fungal genetics and biology*, *49*(1), 30-38.

Pritchard, L., & Birch, P. R. (2014). The zigzag model of plant–microbe interactions: is it time to move on?. *Molecular plant pathology*, *15*(9), 865-870.

Qiang, X., Zechmann, B., Reitz, M. U., Kogel, K. H., & Schäfer, P. (2012). The mutualistic fungus Piriformospora indica colonizes Arabidopsis roots by inducing an endoplasmic reticulum stress–triggered caspase-dependent cell death. *The Plant Cell*, *24*(2), 794-809.

Ram, A. F., & Klis, F. M. (2006). Identification of fungal cell wall mutants using susceptibility assays based on Calcofluor white and Congo red. *Nature protocols*, *1*(5), 2253.

Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J., & Scheel, D. (2011). Interplay between calcium signalling and early signalling elements during defence responses to microbe-or damage-associated molecular patterns. *The Plant Journal*, *68*(1), 100-113.

Redecker, D., Kodner, R., & Graham, L. E. (2000). Glomalean fungi from the Ordovician. *Science*, *289*(5486), 1920-1921.

Riess, K., Oberwinkler, F., Bauer, R., & Garnica, S. (2014). Communities of endophytic sebacinales associated with roots of herbaceous plants in agricultural and grassland ecosystems are dominated by Serendipita herbamans sp. nov. *PloS one*, *9*(4), e94676.

Rodrigues, M. L., Nosanchuk, J. D., Schrank, A., Vainstein, M. H., Casadevall, A., & Nimrichter, L. (2011). Vesicular transport systems in fungi. *Future microbiology*, *6*(11), 1371-1381.

Rovenich, H., Zuccaro, A., & Thomma, B. P. (2016). Convergent evolution of filamentous microbes towards evasion of glycan-triggered immunity. *New Phytologist*, *212*(4), 896-901.

Ruel, K., & Joseleau, J. P. (1991). Involvement of an extracellular glucan sheath during degradation of Populus wood by Phanerochaete chrysosporium. *Applied and environmental microbiology*, *57*(2), 374-384.

Ruiz-Herrera, J. (1991). *Fungal cell wall: structure, synthesis, and assembly*. CRC press.

Ruiz-Herrera, J. (2012). Fungal cell wall: structure, synthesis, and assembly (Second Edition). *CRC Press,* Chapter 6: 73-95

Saloheimo, M., Paloheimo, M., Hakola, S., Pere, J., Swanson, B., Nyyssönen, E., ... & Penttilä, M. (2002). Swollenin, a Trichoderma reesei protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. *The FEBS Journal*, *269*(17), 4202-4211.

Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D. J., Thomma, B. P., & Mesters, J. R. (2013). Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *Elife*, *2*, e00790.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., ... & Tinevez, J. Y. (2012). Fiji: an open-source platform for biological-image analysis. *Nature methods*, *9*(7), 676-682.

Schmidt, W. E. & Ebel, J. (1987). Specific binding of a fungal glucan phytoalexin elicitor to membrane fractions from soybean Glycine max. *Proceedings of the National Academy of Sciences*, *84*(12), 4117-4121.

Schulz, B., & Boyle, C. (2005). The endophytic continuum. *Mycological research*, *109*(6), 661-686.

Seren, Ü., Vilhjálmsson, B. J., Horton, M. W., Meng, D., Forai, P., Huang,. S., ... & Nordborg, M. (2012). GWAPP: a web application for genome-wide association mapping in Arabidopsis. *The Plant Cell*, *24*(12), 4793-4805.

Shahinian, S., & Bussey, H. (2000). β-1, 6-Glucan synthesis in Saccharomyces cerevisiae. *Molecular microbiology*, *35*(3), 477-489.

Shahollari, B., Vadassery, J., Varma, A., & Oelmüller, R. (2007). A leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus Piriformospora indica in Arabidopsis thaliana. *The Plant Journal*, *50*(1), 1-13.

Sharp, J. K., McNeil, M. & Albersheim, P. (1984a). The primary structures of one elicitor-active and seven elicitor-inactive hexa (beta-D-glucopyranosyl)-D-glucitols isolated from the mycelial walls of Phytophthora megasperma f. sp. glycinea. *Journal of Biological Chemistry*, *259*(18), 11321-11336.

Sharp, J. K., Valent, B. & Albersheim, P. (1984b). Purification and partial characterization of a beta-glucan fragment that elicits phytoalexin accumulation in soybean. *Journal of Biological Chemistry*, *259*(18), 11312-11320.

Sherameti, I., Shahollari, B., Venus, Y., Altschmied, L., Varma, A., & Oelmüller, R. (2005). The endophytic fungus Piriformospora indica stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and Arabidopsis roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. *Journal of Biological Chemistry*, *280*(28), 26241-26247.

Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V. M., Nitz, I., ... & Oelmüller, R. (2008). PYK10, a β-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between Arabidopsis thaliana and the endophytic fungus Piriformospora indica. *The Plant Journal*, *54*(3), 428-439.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., ... & Shibuya, N. (2010). Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *The Plant Journal*, *64*(2), 204-214.

Shin, K. H., Yang, S. H., Lee, J. Y., Lim, C. W., Lee, S. C., Brown, J. W., & Kim, S. H. (2015). Alternative splicing of mini-exons in the Arabidopsis leaf rust receptor-like kinase LRK10 genes affects subcellular localisation. *Plant cell reports*, *34*(3), 495-505.

Shiu, S. H., & Bleecker, A. B. (2001). Plant receptor-like kinase gene family: diversity, function, and signaling. *Sci stke*, *113*(113), re22.

Sietsma, J. H., Eveleigh, D. E., & Haskins, R. H. (1969). Cell wall composition and protoplast formation of some oomycete species. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *184*(2), 306-317.

Takahara, H., Hacquard, S., Kombrink, A., Hughes, H. B., Halder, V., Robin, G. P., ... & Shibuya, N. (2016). Colletotrichum higginsianum extracellular LysM proteins play dual roles in appressorial function and suppression of chitin-triggered plant immunity. *New Phytologist*, *211*(4), 1323-1337.

Tanaka, K., Choi, J., Cao, Y., & Stacey, G. (2014). Extracellular ATP acts as a damage-associated molecular pattern (DAMP) signal in plants. *Frontiers in plant science*, *5*.

Tian, T., Liu, Y., Yan, H., You, Q., Yi, X., Du, Z., ... & Su, Z. (2017). agriGO v2. 0: a GO analysis toolkit for the agricultural community, 2017 update. *Nucleic Acids Research*.

Tong, S. M., Chen, Y., Zhu, J., Ying, S. H., & Feng, M. G. (2016). Subcellular localization of five singular WSC domain-containing proteins and their roles in Beauveria bassiana responses to stress cues and metal ions. *Environmental microbiology reports*, *8*(2), 295-304.

Trempel, F., Kajiura, H., Ranf, S., Grimmer, J., Westphal, L., Zipfel, C., ... & Lee, J. (2016). Altered glycosylation of exported proteins, including surface immune receptors, compromises calcium and downstream signaling responses to microbe-associated molecular patterns in Arabidopsis thaliana. *BMC plant biology*, *16*(1), 31.

Tsukumo, Y., Tsukahara, S., Saito, S., Tsuruo, T., & Tomida, A. (2009). A Novel Endoplasmic Reticulum Export Signal PROLINE AT THE+ 2-POSITION FROM THE SIGNAL PEPTIDE CLEAVAGE SITE. *Journal of Biological Chemistry*, *284*(40), 27500-27510.

Umemoto, N., Kakitani, M., Iwamatsu, A., Yoshikawa, M., Yamaoka, N., & Ishida, I. (1997). The structure and function of a soybean β-glucan-elicitor-binding protein. *Proceedings of the National Academy of Sciences*, *94*(3), 1029-1034.

van den Burg, H. A., Spronk, C. A., Boeren, S., Kennedy, M. A., Vissers, J. P., Vuister, G. W., ... & Vervoort, J. (2004). Binding of the AVR4 elicitor of Cladosporium fulvum to chitotriose units is facilitated by positive allosteric protein-protein interactions: the chitin-binding site of AVR4 represents a novel binding site on the folding scaffold shared between the invertebrate and the plant chitin-binding domain. *Journal of Biological Chemistry*, *279*(16), 16786-16796.

van den Burg, H. A., Harrison, S. J., Joosten, M. H., Vervoort, J., & de Wit, P. J. (2006). Cladosporium fulvum Avr4 protects fungal cell walls against hydrolysis by plant chitinases accumulating during infection. *Molecular Plant-Microbe Interactions*, *19*(12), 1420-1430.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., & Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist*, *206*(4), 1196-1206.

Veneault-Fourrey, C., & Martin, F. (2011). Mutualistic interactions on a knife-edge between saprotrophy and pathogenesis. *Current opinion in plant biology*, *14*(4), 444-450.

Verbruggen, E., Rillig, M. C., Wehner, J., Hegglin, D., Wittwer, R., & Heijden, M. G. (2014). Sebacinales, but not total root associated fungal communities, are affected by land-use intensity. *New Phytologist*, *203*(4), 1036-1040.

Verna, J., Lodder, A., Lee, K., Vagts, A., Ballester, R. (1997) A family of genes required for maintenance of cell wall integrity and for the stress response in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 94

Verstrepen, K. J., Jansen, A., Lewitter, F., & Fink, G. R. (2005). Intragenic tandem repeats generate functional variability. *Nature genetics*, *37*(9), 986.

Vetter, M.M., Kronholm, I., He, F., Häweker, H., Reymond, M., Bergelson, J. et al. (2012). Flagellin perception varies quantitatively in Arabidopsis thaliana and its relatives. *Mol Biol Evol*. 29(6):1655-67

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., ... & Franken, P. (2005). The endophytic fungus Piriformospora indica reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(38), 13386-13391.

Wan, J., Tanaka, K., Zhang, X. C., Son, G. H., Brechenmacher, L., Nguyen, T. H. N., & Stacey, G. (2012). LYK4, a lysin motif receptor-like kinase, is important for chitin signaling and plant innate immunity in Arabidopsis. *Plant physiology*, *160*(1), 396-406.

Wawra, S., Fesel, P., Widmer, H., Timm, M., Seibel, J., Leson, L., ... & Zuccaro, A. (2016). The fungal-specific β-glucan-binding lectin FGB1 alters cell-wall composition and suppresses glucan-triggered immunity in plants. *Nature Communications*, *7*.

Weis, C., Hückelhoven, R., & Eichmann, R. (2013). LIFEGUARD proteins support plant colonization by biotrophic powdery mildew fungi. *Journal of experimental botany*, *64*(12), 3855-3867.

Weiß, M., Sýkorová, Z., Garnica, S., Riess, K., Martos, F., Krause, C., ... & Redecker, D. (2011). Sebacinales everywhere: previously overlooked ubiquitous fungal endophytes. *Plos one*, *6*(2), e16793.

Weiß, M., Waller, F., Zuccaro, A., & Selosse, M. A. (2016). Sebacinales–one thousand and one interactions with land plants. *New Phytologist*, *211*(1), 20-40.

Whittaker, M. M., Kersten, P. J., Cullen, D., & Whittaker, J. W. (1999). Identification of catalytic residues in glyoxal oxidase by targeted mutagenesis. *Journal of Biological Chemistry*, *274*(51), 36226-36232.

Willmann, R., Lajunen, H. M., Erbs, G., Newman, M. A., Kolb, D., Tsuda, K., ... & Jehle, A. K. (2011). Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. *Proceedings of the National Academy of Sciences*, *108*(49), 19824-19829.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V., & Provart, N. J. (2007). An “Electronic Fluorescent Pictograph” browser for exploring and analyzing large-scale biological data sets. *PloS one*, *2*(8), e718.

Wintermans, P.C.A., Bakker, P.A.H.M. and Piterse, M.J. (2016). Natural genetic variation in Arabidopsis for responsiveness to plant growth-promoting rhizobacteria. *Plant Molecular Biology*, 90, 623–634

Xu, X. H., Su, Z. Z., Wang, C., Kubicek, C. P., Feng, X. X., Mao, L. J., ... & Zhang, C. L. (2014). The rice endophyte Harpophora oryzae genome reveals evolution from a pathogen to a mutualistic endophyte. *Scientific reports*, *4*.

Yadav, V., Kumar, M., Deep, D. K., Kumar, H., Sharma, R., Tripathi, T., ... & Johri, A. K. (2010). A phosphate transporter from the root endophytic fungus Piriformospora indica plays a role in phosphate transport to the host plant. *Journal of Biological Chemistry*, *285*(34), 26532-26544.

Yamaguchi, Y., & Huffaker, A. (2011). Endogenous peptide elicitors in higher plants. *Current opinion in plant biology*, *14*(4), 351-357.

Yamaguchi, T., Yamada, A., Hong, N., Ogawa, T., Ishii, T. & Shibuya, N. (2000). Differences in the recognition of glucan elicitor signals between rice and soybean: β-glucan fragments from the rice blast disease fungus Pyricularia oryzae that elicit phytoalexin biosynthesis in suspension-cultured rice cells. *The Plant Cell*, *12*(5), 817-826.

Yin, Q. Y., de Groot, P. W., Dekker, H. L., de Jong, L., Klis, F. M., & de Koster, C. G. (2005). Comprehensive proteomic analysis of Saccharomyces cerevisiae cell walls identification of proteins covalently attached via glycosylphosphatidylinositol remnants or mild alkali-sensitive linkages. *Journal of Biological Chemistry*, *280*(21), 20894-20901.

Yoshikawa, M., Keen, N. T. & Wang, M. C. (1983). A receptor on soybean membranes for a fungal elicitor of phytoalexin accumulation. *Plant Physiology*, *73*(2), 497-506.

Yu, X., Feng, B., He, P., & Shan, L. (2017). From Chaos to Harmony: Responses and Signaling Upon Microbial Pattern Recognition. *Annual review of phytopathology*, (0).

Zhang, X., Dong, W., Sun, J., Feng, F., Deng, Y., He, Z., ... & Wang, E. (2015). The receptor kinase CERK1 has dual functions in symbiosis and immunity signalling. *The Plant Journal*, *81*(2), 258-267.

Zuccaro, A., Lahrmann, U., Güldener, U., Langen, G., Pfiffi, S., Biedenkopf, D., ... & Murat, C. (2011). Endophytic life strategies decoded by genome and transcriptome analyses of the mutualistic root symbiont Piriformospora indica. *PLoS Pathog*, *7*(10), e1002290.

6. Acknowledgements / Danksagungen

First of all I want to thank Alga Zuccaro for her support and encouragement over the past five years. Thanks for your trust to give me the space to develop myself and to face ambitious challenges independently.

Furthermore I want to thank Prof. Dr. Paul Schulze-Lefert for his scientific advices and support and his willingness to review this thesis as well as Prof. Dr. Jan Riemer for being part of the examination board.

I also want to thank CEPLAS for funding and all CEPLAS members for creating an enthusiastic and creative working atmosphere.

A special thanks is dedicated to Dr. Stephan Wawra for his constant scientific support, the sincere and constructive cooperation during the the Glucan-project and the results and analysis that he has provided to this thesis.

Furthermore I want to thank all current and former members of the Zuccaro lab, namely Lisa Leson, Heidi Widmer, Debika Sarkar, Dr. Shadab Nizam, Dr. Gregor Langen, Dr. Ganga Jeena, Dr. Hanna Rovenich, Alan Wanke, Leona Kesseler, Nyasha Charura, Anna Wendel, Lucas Hüttebräucker, Petra Dhein, Margaret Kox, Dr. Urs Lahrmann, Dr. Magdalena Hilbert, Dr. Yi Ding, Robin Nostadt and Elmar Meyer for her support during the last years and the creation of a positive and collaborative atmosphere.

Additionally I want to thank Dr. Tobias Lamkemeyer (formerly Proteomics facility of the CECAD), Dr. Adam Schikora (Julius-Kühn-Institute, Braunschweig), Prof. Dr. Jürgen Seibel and Malte Timm (Julius-Maximilians University Würzburg) and Dr. Ulla Neumann (Central Microscopy facility of the MPIPZ Cologne) for their scientific cooperations that greatly supported my research.

Ich möchte außerdem meinen Eltern, meinem Bruder, meinen Großeltern, Tante Ilse, meinen Schwiegereltern und meiner Schwägerin danken, für die Hilfe und Unterstützung während meines gesamten Studiums und meiner Promotion. Alle von

euch haben einen unermesslichen Anteil an dieser Arbeit, die ohne euch nicht möglich gewesen wäre, die ich aber leider nicht mehr mit allen von euch feiern kann.

Den größten Dank möchte ich an meine Frau Sabine richten. Ohne deine Unterstützung, deine Liebe, deinen Trost und deine Aufmunterung hätte ich es mit Sicherheit nicht bis hierhin geschafft. Du hast nicht nur jeden kleinen Sieg mit mir gefeiert und mich aufgebaut wenn ich deprimiert war, sondern mir auch unsere beiden Töchter Paula Sophie und Anna Isabelle geschenkt. Ihr habt mich immer daran erinnert auf was es wirklich im Leben ankommt. Ich liebe und danke euch von Herzen.

Erklärung zur Dissertation

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.
Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von *Frau Prof. Dr. Alga Zuccaro* betreut worden.

Nachfolgend genannte Teilpublikationen liegen vor:

Wawra, S.*, Fesel, P.*, Widmer, H., Timm, M., Seibel, J., Leson, L., Kesseler, L., Nostadt, R., Hilbert, M., Langen, G. & Zuccaro, A. (2016). The fungal-specific β-glucan-binding lectin FGB1 alters cell-wall composition and suppresses glucan-triggered immunity in plants. *Nature Communications*, *7*.

Fesel, P. H., & Zuccaro, A. (2016). Dissecting endophytic lifestyle along the parasitism/mutualism continuum in Arabidopsis. *Current opinion in microbiology*, *32*, 103-112.

Fesel, P. H., & Zuccaro, A. (2016). β-glucan: crucial component of the fungal cell wall and elusive MAMP in plants. *Fungal Genetics and Biology*, *90*, 53-60.

* geteilte Erstautorenschaft

Promotionsbeginn: Februar 2014

Promotionsende: Dezember 2017

Ich versichere, dass ich alle Angaben wahrheitsgemäß nach bestem Wissen und Gewissen gemacht habe und verpflichte mich, jedmögliche, die obigen Angaben betreffenden Veränderungen, dem Dekanat unverzüglich mitzuteilen.

....

Datum Unterschrift