Functional characterization of an effector candidate of the root colonizing fungus *Piriformospora indica* during interaction with plants

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List of Abbreviations

aa	Amino acids
AD	Activating domain
AM	Arbuscular mycorrhiza
APS	Ammonium persulfate
BD	Binding domain
BiFC	Bimolecular florescent complementation
bp	Base pair
cDNA	complementary DNA
CM	Complete medium
COP9	constitutive photomorphogenic9
CTAB	Cetyltrimethylammonium bromide
dai	days after inoculation / infection
ddH_2O	double distilled water
DLDH	D-lactate dehydrogenase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
dsp	deleted signal peptide
ECM	Ectomycorrhiza
EtBr	Ethidium bromide
ETI	Effector – triggered immunity
ETS	Effector – triggered susceptibility
GFP	Green fluorescence protein
HR	Hypersensitive response
Hsp	Heat shock protein
Hyg	Hygromycin B
ITS	Internal transcribed spacer
Kb	Kilobase
kDa	kilo Dalton
LB	Lauri – Bertani
LysM	Lysine motif
MAMP	Microbe associated molecular pattern
MAPK	Mitogen-activated protein kinase

min	minutes
MTI	MAMP – triggered immunity
NBS-LRR	Nucleotide binding site – leucine rich repeat
OD_{600}	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PR	Pathogenesis related
PRR	Pattern recognition receptor
PTI	PAMP – triggered immunity
PVDF	Polyvinylidene Fluoride
qRT – PCR	quantitative real time-PCR
RLK	Receptor like kinase
RLP	Receptor like protein
RNA	Ribonucleic acid
ROS	Reactive oxygen specie
rpm	rounds per minute
RT	Room temperature
RT-PCR	reverse transcriptase-PCR
SDS	Sodium dodecyl sulfate
SP	Signal peptide
T3SS	Type III secretion system
Taq	DNA polymerase from *Thermus aquaticus*
TBE	Tris-buffered EDTA
TBS	Tris-buffered Saline
T-DNA	Transfer DNA
TEMED	N,N,N,N-tetramethylethylenediamine
UBQ	Ubiquitin
WGA	Wheat germ agglutinin
WT	Wild type
YEB	Yeast extract broth

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1. Introduction

1.1 Plant-microbe interaction

Similarly to animals, plants are under constant attack from a wide range of microbes including bacteria, fungi, oomycetes, viruses but also from pest such as nematodes and insect herbivores. The outcome of these attacks can either be pathogenic, as is the case with phytopathogens and pests (including, oomycetes, bacteria, nematodes, viruses and filamentous fungi pathogens) or mutualistic as with beneficial microbes (including Rhizobia and mycorrhiza) (Newton, Fitt et al. 2010). Because plants are sessile organisms unable to move to avoid attacks, they have developed a sophisticated defense system to quickly react and fend off would be pathogens while interacting with, and accommodating beneficial microbes. Plant associated microbes have evolved different strategies to infect their respective plant host. While biotrophs require living host tissue during infection for nutrient acquisition, necrotrophs secrete toxin which kill the host cell as they prefer dead tissues to obtain nutrients. On the other hand, hemibiotrophs adopt both strategies with an initial biotrophic life style where they feed on living host tissue and a subsequent switch to necrotrophic growth where they kill the host cell and obtain nutrient from dead tissues (Glazebrook 2005)

1.2 The plant immune system

It is now a widely accepted fact that plants possess a two layered immune system to defend themselves from microbial invaders (Jones and Dangl 2006). Upon microbial perception, the first layer of defense is activated. This layer relies on transmembrane pattern recognition receptors (PRRs) which recognizes and respond to conserved microbial features known as pathogen – or microbe – associated molecular patterns (PAMPs / MAMPs) leading to PAMP- or MAMP-triggered immunity (PTI / MTI) (Chisholm, Coaker et al. 2006, Jones and Dangl 2006, Schwessinger and Zipfel 2008). PAMPs/MAMPs are defined as invariant epitopes within molecules that are crucial to the microbe's fitness, and widely distributed among different microbes, but absent in the host and recognized by a wide array of potential hosts (Schwessinger and Zipfel 2008). PAMPs/MAMPs are conserved microbial structures that are difficult to modify without affecting their functionality and microbial fitness (Boller and He 2009). Some of the well-studied PAMPs include the stretch of 22 amino acids in the N terminus of bacteria flagellin (flg22), lipopolysaccharides and Peptidoglycan (Zipfel and Felix 2005), bacterial elongation factor Tu, (EF-Tu) (Kunze, Zipfel et al. 2004), ergosterol, β-glucan and chitin, a major component of fungal cell wall (Nurnberger, Brunner et al. 2004).

The second layer of plant defense which largely takes place in the cell relies on highly polymorphic resistance (R) proteins which detects microbial molecules termed effector proteins and triggers a more robust immune response known as effector triggered immunity (ETI) (Chisholm, Coaker et al. 2006, Jones and Dangl 2006).

1.2.1 PAMP triggered immunity

In their attempt to penetrate host plants, potential pathogens are confronted with barriers such as a waxy cuticle layer and rigid cell walls. Successful pathogens that overcome such barriers are subject to molecular recognition be specialize plant molecules (Zipfel 2008). At the first line of defense, plants deploy surface localized pattern recognition receptors (PRRs) which perceive PAMPs and respond to the invading pathogens leading to PAMP triggered immunity (Jones and Dangl 2006, Zipfel 2008) (Figure 1). In most cases the activation of PTI leads to intracellular responses including mitogen activated protein kinase (MAPK) activation, rapid ion fluxes across the plasma membrane, production of reactive oxygen species, cell wall reinforcement and changes in gene expression which inhibits pathogen progress (Jones and Dangl 2006, Schwessinger and Zipfel 2008, Zipfel 2008). Several PAMP molecules and their corresponding host receptors have been well characterized. The best characterize PAMP capable of inducing defense response in several plants is flg22, a stretch of 22 amino acid peptide at the N-terminal of bacteria flagellin. In Arabidopsis, flg22 is perceived through direct interaction with the pattern recognition receptor (PRR) FLAGELLIN-SENSING 2 (FLS2), a transmembrane leucine rich repeat receptor like kinase (LRR-RLK) triggering PTI. Upon flg22 stimulation, FLS2 rapidly associates with BAK1, another receptor-like kinase previously known to dimerize with the brassinosteroid receptor BRI1 (Zhou and Chai 2008). However, whereas plants mutated in the flagellin receptor gene, *FLS2* did not respond to flg22 treatment, a strong inhibition of disease development was observed after treatment with crude bacterial extracts suggesting that there exist additional elicitors other than flagellin capable of inducing resistance. (Felix, Duran et al. 1999, Kunze, Zipfel et al. 2004, Chinchilla, Bauer et al. 2006). Other characterized PAMPs include the bacterial elongation factor Tu (EF-Tu), the most abundant protein in bacterial cell capable of acting as a potent elicitor of defense response. Similarly to flg22, a conserved N-acetylated 18 amino acid peptide, elf18 is sufficient to trigger the same response as the full length EF-Tu. In Arabidopsis, EF-Tu is perceived through interaction with the PRR elongation factor receptor (EFR), a leucine rich repeat receptor like kinase (LRR-RLK) of the same subfamily as FLS2 (Kunze, Zipfel et al. 2004, Zipfel 2008). Another well studied MAMP is chitin, a major

component of fungal cell wall that can trigger defense responses in monocots and dicot plants, suggesting the existence of a conserved mechanism to perceive chitin in a wide range of plant species (Shibuya and Minami 2001, Okada, Matsumura et al. 2002). In Arabidopsis, CERK1 (chitin elicitor receptor kinase 1), a plasma membrane protein containing three extracellular LysM motifs and an intracellular Ser/Thr kinase domain binds chitin and triggers defense responses. The ability to respond to chitin elicitor and initiate defense responses was completely lost in *CERK1* knockout mutants of Arabidopsis (Miya, Albert et al. 2007, Shimizu, Nakano et al. 2010). Meanwhile in rice, chitin perception is mediated by CEBiP (chitin elicitor binding protein), a plasma membrane glycoprotein containing two extracellular LysM motifs. However CEBiP does not contain an intracellular domain for signaling and was shown to require a LysM receptor like kinase, OsCERK1, for chitin signaling in rice. Both CEBiP and OsCERK1 were shown to form chitin-induced hetero- or homo-oligomers (Kaku, Nishizawa et al. 2006, Shimizu, Nakano et al. 2010).

1.2.2 Effector triggered immunity

Effector triggered immunity (ETI) was first described in the famous gene-for-gene model proposed by Flor in 1995 during genetic analysis of resistance and virulence using the rust fungus, *Melampsora lini* and its host plant flax (*Linum usitatissimum*) (Flor 1955).

Although PTI inhibits pathogen infection, successful pathogens have evolved strategies to overcome PTI through the secretion of virulence factors known as effectors leading to effector triggered susceptibility (ETS) and pathogen virulence (Figure 1). It has been reported that in order to suppress PTI, pathogen effectors target pattern recognition receptors (PRR) and processes directly downstream of PRR as well as mitogen activated protein kinase (MAPK) cascades (Block, Li et al. 2008). However plants have evolved resistance proteins (R proteins) of the nucleotide binding leucine rich repeat (NB-LRR) class which recognizes effectors either directly, through the gene-for-gene concept or indirectly through the guard hypothesis leading to effector triggered immunity (ETI).(Jones and Dangl 2006, Zhou and Chai 2008) (Figure 1). The outcome of ETI is usually the hypersensitive cell death response (HR) at the site of pathogen infection and is effective against obligate biotrophic pathogens (pathogens that can only grow on living tissues) or hemi-biotrophic pathogens (pathogens requiring initial biotrophic growth and later growth on dead tissues), but not against necrotrophic pathogens (dead tissue colonizers). ETI is an accelerated and amplified form of PTI (Jones and Dangl 2006). Through natural selection, pathogens are driven to again suppress ETI either through diversifying their effector repertoire or by acquiring new effectors. However natural selection

also drives plants to modify their R proteins or acquire new ones such that pathogen effectors can be recognized and ETI can be triggered again (Figure 1).

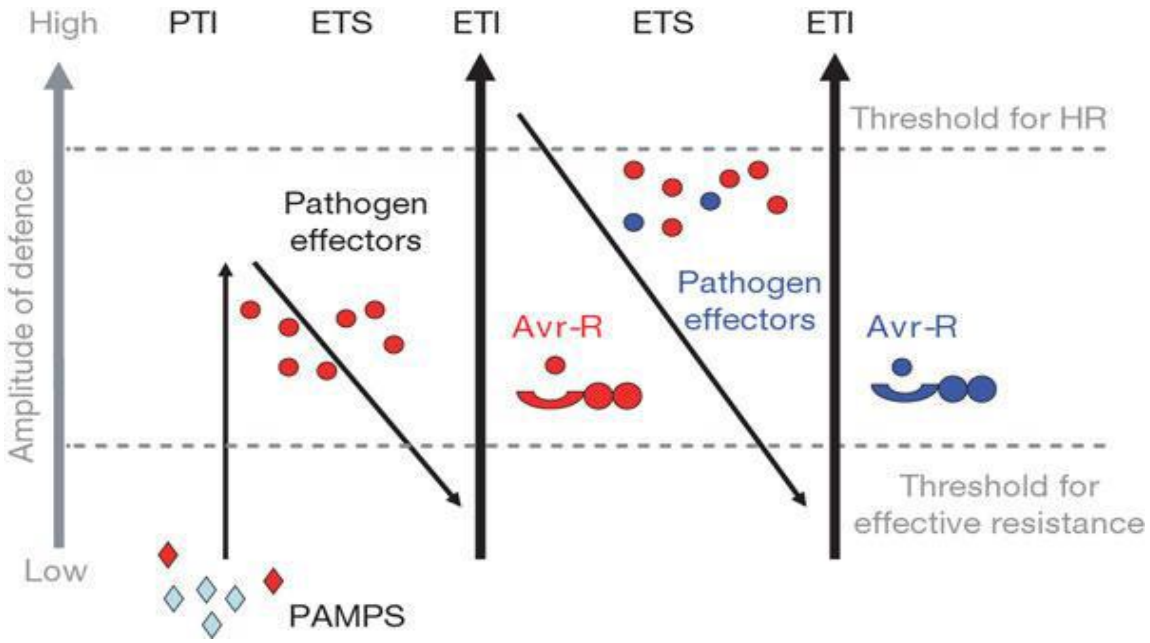


Figure 1. The zigzag model illustrating the two branches of the plant immune system. PAMP-triggered immunity (PTI) is activated by recognition of pathogen associated molecular patterns (PAMPs, red and sky blue diamonds) via pattern recognition receptor (PRR). Pathogens secret effectors (red dots), which suppress PTI and initiates effector triggered susceptibility (ETS). Pathogen effectors are recognized by nucleotide binding-leucine rich repeat (NB-LRR) proteins (Avr-R) resulting in effector-triggered immunity (ETI). Successful pathogens are able to secret modified effectors to suppress ETI, leading again to ETS. But eventually, due to natural selection the plant recognizes these new modified effectors via modified plant NB-LRR proteins and triggers again an ETI (Jones and Dangl 2006).

1.2.3 The oxidative burst

Following successful pathogen recognition through PAMP perception, one of the earliest cellular responses is the rapid production of reactive oxygen specie (ROS) known as the oxidative burst. The recognition of avirulent pathogens through the action of the plant resistance (R) gene triggers a two phase ROS accumulation with an initial transient phase, followed by a much stronger and sustained phase leading to disease resistance. However, virulent pathogens avoid host recognition either by only inducing the much weaker transient first phase of ROS burst or by completely suppressing ROS accumulation through the action of secreted effectors (Torres, Jones et al. 2006). ROS production is a conserved signaling process during plant immunity and generated ROS inhibit pathogen infection by acting as

cross-linkers of plant cell walls or as antimicrobials, but also as local and systemic secondary messenger to trigger immune processes such as gene expression and stomatal closure. In plants the production of ROS is catalyzed by the respiratory burst oxidase homolog (RBOH) family member, NADPH (Nicotinamide adenine dinucleotide phosphate) oxidase (NOX) by first catalyzing the production of superoxide (O_2^-) and subsequent conversion to hydrogen peroxide by peroxidases. RBOH contains 10 members in Arabidopsis and the plasma-membrane-localized RBOHD mediates PAMP induced ROS burst (Torres and Dangl 2005, Nathan and Cunningham-Bussel 2013, Kadota, Sklenar et al. 2014). Genetic evidence for the involvement of NADPH oxidase-RBOH in pathogen-induced ROS production came from the analysis of *rboh* mutants and antisense lines as silencing or knockout of *RBOH* inhibit the formation of extracellular peroxide.(Torres, Jones et al. 2006). The production of ROS has been implicated in several plant immune signaling processes including the hypersensitive response (HR), calcium metabolism, hormone regulation involving salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA) as well as ethylene. The ability of ROS generated by RBOH to mediate diverse cellular process and to act in several hormone signaling pathways underscore its importance in plant defense (Torres and Dangl 2005, 2006, Kadota, Sklenar et al. 2014).

1.3 Effector proteins

Although the term “effector” is widely used in plant-microbe interaction studies, its definition varies. Kamoun (2003) and Huitema, Bos et al. (2004) defined effectors as “molecules that alter the structure and function of host cells, thereby facilitating infection (virulence factors or toxins) and/or triggering defense response (avirulence factors or elicitors)”. In another definition birch and collegues (2014) reffered to effectors in plant pathogen interactions as “any protein synthesized by a pathogen that is exported to a potential host, which has the effect of making the host enviroment beneficial to the pathogen. However the widely accepted hallmarks of effectors include; small secreted proteins (SSPs) with a signal peptide, having low sequence homology to known proteins, no conserved host targeting signal and capable of manipulating and reprograming host metabolism and immunity.

Effector proteins are secreted by plant-associated microbes, including bacteria, fungi, oomycetes, and can either be apoplastic, exerting their functions extracellularly at the microbe-plant interface (apoplast), or cytoplasmic, having molecular targets in the cytosol, nucleus or other subcellular compartment where they modulate plant immunity, physiology

and metabolism in favor of microbial growth (Bos, Kanneganti et al. 2006, Rafiqi, Ellis et al. 2012).

1.3.1 Bacterial effector proteins.

To overcome host defense, phytopathogenic bacteria secrete a suite of effector proteins through the type three secretion system (T3SS), injectisome, into the host cell. The main functon of the T3SS is to ensure the delivery of type three effector proteins (T3Es) across the bacterial and host membranes into the cytosol of the host where they interfere with several host cellular responses including defense responses, alteration of membrane cytoskeleton to enhance bacterial infection and promote disease or in some cases are used to facilitate symbiosis (Tseng, Tyler et al. 2009, Erhardt, Namba et al. 2010). Unlike oomycete and fungi, knowledge on bacterial effectors is well advanced and many bacterial effectors have been characterized and assigned functions. However while many T3Es are known to be enzymes including proteases, phosphorylases, acetyltransferases and E3 ligases, others have no obvious enzymatic activities and although many have a unique biochemical activity, they usually act in a cooperative and redundant manner to target conserved host cellular processes such as vesicle trafficking, defense pathways, transcription, ubiquitination and hormone signaling (Baltrus, Nishimura et al. 2011, Lee, Middleton et al. 2013, Ustun, Bartetzko et al. 2013). In a recent report Ustun, Konig et al. (2014), showed that the *Pseudomonas syringae* effector, HopZ4, a member of the HopZ T3E family from the YopJ superfamily suppresses salicylic acid (SA) mediated defense signaling by targeting the proteasomal subunit, RPT6 at the plasma membrane and inhibiting proteasome function, and that this effect was dependent on its localization and enzymatic activities. Similarly to HopZ4, it was reported that the *Xanthomonas campestris* T3E YopJ also targets the proteasomal subunit, RPT6 to inhibit proteasome function and suppress SA-dependent defense signaling (Ustun, Bartetzko et al. 2013). The *Salmonella* T3E SpvC, a phosphothreonine lyase suppresses host defense by inactivating MAPKs, while SptP another *Salmonella* T3E with tyrosine phosphatase and GTPase (GAP) activities inhibit the activation of the defense related extracellular regulated kinases (ERK) by interfering with the activation of raf-1 thereby enhancing *Salmonella* virulence (Lin, Le et al. 2003, Li, Xu et al. 2007). HopD1, a *Pseudomonas syringae* T3E suppresses ETI by targeting the Arabidopsis transcription factor NTL9 at the endoplasmic reticulum and suppresses genes whose expression are induced by active NTL9 during ETI (Block, Toruno et al. 2014). AvrPtoB, a multifunctional T3E of *Pseudomonas syringae* suppresses signaling following flagellin perception by interacting with the kinase domain of

FLS2 and BAK1, and at the same time promote the proteasomal degradation of FLS2 and possibly also EFR through its C-terminal RING finger and U box E3 ubiquitin ligase domain. AvrPtoB suppresses ETI by ubiquitinating and degrading Fen, a kinase protein of the Pto family able to trigger Prf-mediated immune response in Pto-lacking tomato plants (Goehre, Spallek et al. 2008, Deslandes and Rivas 2012). Another T3E of *Pseudomonas syringae* is HopAI1, a phosphothreonine lyase which suppresses PAMP-triggered gene transcription and cell wall-associated host defenses by irreversibly dephosphorylating the MAPKs, MPK3 and MPK6 (Zhang, Shao et al. 2007)

1.3.2 Oomycete effector proteins

Plant pathogenic oomycetes are fungus-like eukaryotic microbes, causing some of the most destructive plant diseases worldwide (Ariztia, Andersen et al. 1991). Prominent example includes the late blight pathogen, *Phytophthora infestans* causal agent of the devastating potato blight disease in the 1880s. Similarly to phytopathogenic bacteria, oomycete secrete a repertoire of effector proteins to modulate host metabolism and innate immunity thereby enhancing plant infection. Unlike bacteria, oomycetes genome encodes hundreds of effector proteins. With the release of genome wide sequencing of some important plant pathogenic oomycetes including the necrotrophic, *Pythium*(Levesque, Brouwer et al. 2010), the obligate biotrophic downy mildew, *Hyaloperonospora arabidopsidis* (Baxter, Tripathy et al. 2010), and the hemibiotrophic, *Phytophthora sojae*, *Phytophthora infestans and Phytophthora ramorum* (Tyler, Tripathy et al. 2006, Haas, Kamoun et al. 2009) a large and diverse class of effector proteins were identified (Bozkurt, Schornack et al. 2012).

Oomycetes secretes cytoplasmic and apoplastic effectors and most of the cytoplasmic effectors belong to one of two classes: RXLR effectors and/or crinkler (CRN) effectors. The RXLR motif is consist of a conserved N-terminal four amino acid (Arginine, any amino acid, Leucine and Arginine) located after the signal peptide and a C-terminal domain with effector function. Together with the dEER (Asparagine, Gluatmine, Glutamine and Arginine) motif, the RXLR is important and required for the efficient delivery of oomycete effectors into host cells during oomycete infection (Kale and Tyler 2011). Like the RXLR effectors, the CRN effectors all carry an LFLAK motif (Leucine, Phenylalanine, Leucine, Alanine, Lysine) at the N-terminus thought to be important for effector translocation into host cells and a C-terminal domain with effector activities.(Schornack, van Damme et al. 2010). While cytoplasmic effectors target host cellular processes such as gene transcription, defense signaling and ubiquitination pathways, apoplastic effectors have to act against host defense enzymes such as

glucanase, proteases, and chitinases. Some examples include, the extracellular protease inhibitors (EPIs), EPI1 and EPI10 which belong to the family of kazal-like serine protease inhibitors protects several P infestans secreted proteins from degradation by inhibiting the extracellular tomato protease P69B, an enzyme that is upregulated during infection and also implicated in plant defense response (Tian, Huitema et al. 2004). Similarly, the cystatin-like extracellular protease inhibitors, EPIC1 and EPIC2B from *P infestans* were shown to interact with and inhibit the host apoplastic proteases Rcr3 and Pip1 in tomato as well as C14 in *N. benthamiana* (Song, Win et al. 2009, Kaschani, Shabab et al. 2010). *P sojae* sercete the glucanase inhibitor protein, GPI-1 which specifically target and inhibit the soybean EGaseA (a plant endo-β-1,3-glucanase) thereby suppressing endo-glucanase mediated PTI (York, Qin et al. 2004). Cytoplasmic effectors such as the *P infestans* effector CRN8, a host translocated serine/threonine kinase was shown to accumulate in the host nucleus and triggers cell death when expressed in planta thereby facilitating infection. CRN8 nuclear localization requires a functional nuclear localization signal (NLS)(van Damme, Bozkurt et al. 2012). In a recent research, the *P. capsici* effector CRN12_997 was shown to interact with the tomato transcription factor SITCP14-2, an important regulator of immunity, in the nucleus and causes it relocalization in the nucleoplasm and nucleolus. This interaction causes the dissociation of SITCP14-2 from chromatin, altering its sub-nuclear localization and reducing its stability thereby suppressing defense responses and enhances *P. capsici* virulence (Stam, Motion et al. 2013). The *P. infestans* avirulence protein Avr3a is recognized by the potato resistance protein R3a and represented by two alleles encoding effectors, $AVR3a^{KI}$ and $AVR3a^{EM}$ which differ by just two amino acids. Although both effectors suppress program cell death (PCD) induced by the elicitin molecule infestin 1 (INF1), $AVR3a^{KI}$ does so strongly and is recognized by R3a whereas $AVR3a^{EM}$ suppression of PCD is weak and is not recognized by R3a. Avr3a interacts with and stabilizes the potato E3 ubiquitin ligase CMPG1 which is required for cell death triggered by the *P. infestans* elicitin, INF1 and in this way suppresses PTI (Bos, Armstrong et al. 2010). Another *P. infestant* RxLR effector is Avrblb2 which facilitate infection by interacting with the plant papain-like cysteine protease C14 involved in plant immunity and blocks its secretion into the apoplast (Bozkurt, Schornack et al. 2011).

1.3.3 Fungal effector proteins

The genome of phytopathogenic fungi contains hundreds of genes coding for effector proteins which are secreted during plant-pathogen interactions. Similarly to phytopathogenic oomycetes, plant pathogenic fungi also secrete apoplastic and cytoplasmic effectors. However

unlike bacteria which uses T3SS for effector delivery into host and oomycetes where host delivery of effectors is mediated by the conserved RxLR and LFLAK motifs, very little is known about host delivery of fungal effector. Some of the well-studied fungal effectors include Avr4 and Ecp6 (extracellular protein 6), of the biotrophic leaf mold pathogen *Cladosporium fulvum*. Avr4 is a chitin-binding lectin which facilitates host virulence by specifically binding to chitin on fungal cell walls protecting it against hydrolysis by plant chitinases (van den Burg, Harrison et al. 2006). In a similar way, Ecp6 an LysM domain containing effector with three LysM domains inhibit the activation of chitin-triggered immune response by binding and scavenging the chitin oligosaccharides, $(GlcNAc)_{3,}$ $(GlcNAc)_5$ and $(GlcNAc)_3$ (de Jonge, van Esse et al. 2010). Slp1 (Secreted LysM Protein 1) an effector of the hemibiotrophic rice pathogen *Magnaporthe oryzae* contains two LysM domains. Slp1 specifically binds chitin and inhibit chitin-triggered immunity in rice by suppressing chitin induced reactive oxygen specie (ROS) generation and also competes for chitin binding with the rice pattern recognition receptor CEBiP, which is required for chitin-triggered immunity in rice (Mentlak, Kombrink et al. 2012). Another *M. oryzae* effector AvrPiz-t suppresses PTI by interacting with the E3 ubiquitin ligase APIP6 (AvrPiz-t Interacting Protein 6) and suppresses its E3 ligase activity in vitro. In return APIP6 could also ubiquitinate and degrade AvrPiz-t. Expression of the PTI related gene KS4 was suppressed by AvrPiz-t, while flg22 induced ROS generation was completely inhibited, and chitin-triggered ROS generation was reduced to 50% by AvrPiz-t (Park, Chen et al. 2012). AvrPiz-t also suppresses BAX-induced cell death in tobacco and is recognized in the plant cytoplasm by the cognate R protein, Piz-t, indicating that AvrPiz-t is translocated into the plant cytosol where it carries out its functions (Li, Wang et al. 2009). In an attempt to screen the haustorium-specific cDNA library of the obligate biotrophic fungal pathogen, *Melampsora lini*, causal agent of flax rust, several secreted rust effector proteins termed haustorially expressed secreted proteins (HESPs) were identified. However, numerous *R* genes from flax encoding nucleotide binding leucine rich repeat proteins have been clone and can recognize these secreted effector proteins. For example, AvrL567 and AvrM are two effectors secreted by the flax rust fungal pathogen, inside the host cytosol. AvrL567 is recognized by the R proteins L5, L6 and L7 while AvrM is recognized by the R protein M in the cytosol and both recognition triggers HR. Even in the absence of the fungus both AvrL567 and AvrM can translocate into the plant cytosol probably due to cellular uptake signals at the N-terminal of these effectors (Dodds, Lawrence et al. 2004, Rafiqi, Gan et al. 2010). Also, AvrP4 and AvrP123 are two cysteine rich rust effector

proteins recognized by their cognate R proteins, P4 and P1, P2, P3 respectively and recognition leads to HR (Catanzariti, Dodds et al. 2006).

1.3.4 Effector delivery systems

In order to manipulate their respective hosts, plant pathogenic fungi, oomycete, and bacteria have evolved different mechanisms to deliver their effectors inside the host cells. Bacteria for example, use the specialized type three secretion system (T3SS) to deliver their effector proteins inside host cells (Zhou and Chai 2008). Meanwhile, biotrophic fungi and oomycetes use a specialized structure known as the haustoria for effector delivery into host cells. Initially, the haustoria which is invaginated by the plant plasma membrane was thought to function solely for nutrient uptake, but research has shown that it is also involved in effector delivery into host cells (Dodds, Lawrence et al. 2004, Catanzariti, Dodds et al. 2006). However, some fungal effectors including AvrL567 and AvrM have been reported to translocate into host cells independent of the pathogen and uptake was found to be mediated by cellular uptake signals located at the N-terminal regions of each proteins (Rafiqi, Gan et al. 2010). Hemibiotrophic and necrotrophic fungi use specialized invasive hyphae (IH) for effector delivery inside host cells. For example the hemibiotrophic rice pathogen *M. oryzae* accumulate effectors in a lobed structure at the hyphal tip called the biotrophic interfacial complex (BIC) before subsequent delivery into the host cytosol (Khang, Berruyer et al. 2010). Nothing is known about the delivery mechanism used by mutualistic fungi, including mycorrhiza fungi to deliver effectors inside host cells. However, it has been speculated that mutualistic fungi may utilize structures similar to pathogenic fungi such as haustoria or IH for effector delivery. For example, the arbuscules of mycorrhizal fungi known to be the site of nutrient and signal exchange between the plant and the fungus, may also be involved in effector delivery into host cells.

1.4 The COP9 Signalosome complex

The COP9 Signalosome (CSN) is an evolutionary conserved nuclear-enriched multisubunit protein complex that was originally discovered in 1994 as an essential complex regulating light induced development (photomorphogenesis) in Arabidopsis. The CSN is found in all eukaryotes and interacts with a large number of proteins including regulators of the ubiquitin-proteasome system (Wei, Chamovitz et al. 1994, Gusmaroli, Figueroa et al. 2007). It has been detected in all of the organs examined in plants and animals, and seems to accumulate to slightly higher levels in flowers and roots of Arabidopsis (Chamovitz, Wei et al. 1996). The

CSN regulates a variety of plant signaling and developmental processes including plants response to light and hormones, DNA repair gene expression, circadian rhythms, cell cycle as well as defense responses. The CSN also has a central role in regulating the ubiquitin / 26 S proteasome pathway which mediates protein degradation in plants through the activities of three enzymes, E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase (Nezames and Deng 2012). The CSN is composed of eight subunits (Figure 2A) named CSN1-CSN8, with six subunits (CSN1-CSN4, CSN7 and CSN8) containing a PCI (Proteasome, COP9 signalosome and eIF3) domain and two (CSN5A and CSN5B) contain an MPN/MOV34 (Mpr1p and Pad1p N-terminal) domain. The CSN exhibit strong similarities to the proteasome lid which also contain a subunit composed of six PCI and two MPN/MOV34 proteins (Wei and Deng 2003). In Arabidopsis the two MPN subunits (CSN5 and CSN6) are encoded by two highly homologous genes, *CSN5A* and *CSN5B* (Kwok, Solano et al. 1998) (Figure 2B) and *CSN6A* and *CSN6B* (Gusmaroli, Figueroa et al. 2007). The MPN domain of the CSN5 subunit contains a metalloprotease motif called the JAMM (Jab1/MPN domain metalloenzyme) or MPN+ motif which is absent in the other MPN domain subunit of CSN6 (Figure 2B). So far the only known biochemical activity of the CSN is the removal of the ubiquitin-like protein RUB1 (Related to Ubiquitin 1) in plants / NEDD8 (neural precursor cell expressed, developmentally downregulated 8) in mammals from the cullin subunit of the Cullin-RING Ligase (CRL) family of E3 complexes known as derubylation or deneddylation (Gusmaroli, Figueroa et al. 2007). In Arabidopsis CSN interacts with and derubylates several E3 ligase complexes including SCF^{TIR1}, SCF^{COI1}, and SCF^{UFO} which regulate auxin response, jasmonic acid (JA) response and flower development respectively. CSN also deconjugates RUB1 from cullins including cullin1-containing SCF (SKP1-CUL1-F-box protein), cullin2-containing VCB (Von Hippel Lindau-Elongin B-Elongin C), cullin3-containing BCR (BTB/POZ domain-CUL3-RING) and cullin4-containing E3 ubiquitin ligases(Lyapina, Cope et al. 2001, Schwechheimer, Serino et al. 2001, Zhou, Seibert et al. 2001, Groisman, Polanowska et al. 2003) (Figure 2B). The CSN derubylation activity is catalyzed by the JAMM/MPN+ motif of the CSN5 which constitute the catalytic center for cleavage of RUB1-cullin conjugate by the CSN. While rubylation is important for several plant physiological processes including auxin and ethylene responses (Bostick, Lochhead et al. 2004), CSN-mediated derubylation on the other hand is essential for an optimal E3 function *in vivo*. Infact it is known that CSN derubylation activities promote CRL E3 function by blocking the instability of the E3 components triggered by CRL autoubiquitination activity. Therefore cycles of rubylation and derubylation are important to maintain an optimal pool of active

CRLs (He, Cheng et al. 2005, Wee, Geyer et al. 2005, Cope and Deshaies 2006). Although the CSN complex was shown to localize to the nucleus, the monomeric form of CSN5 was reported to localize predominantly to the cytoplasm (Kwok, Solano et al. 1998). In Arabidopsis CSN5A and CSN5A share high sequence similarity at the nucleotide (cDNA), 86 % and at the protein, 88% levels respectively and both isoforms have been reported to play unequal roles in regulating plant root elongation, photomorphogenesis, auxin response, vegetative and reproductive growth.

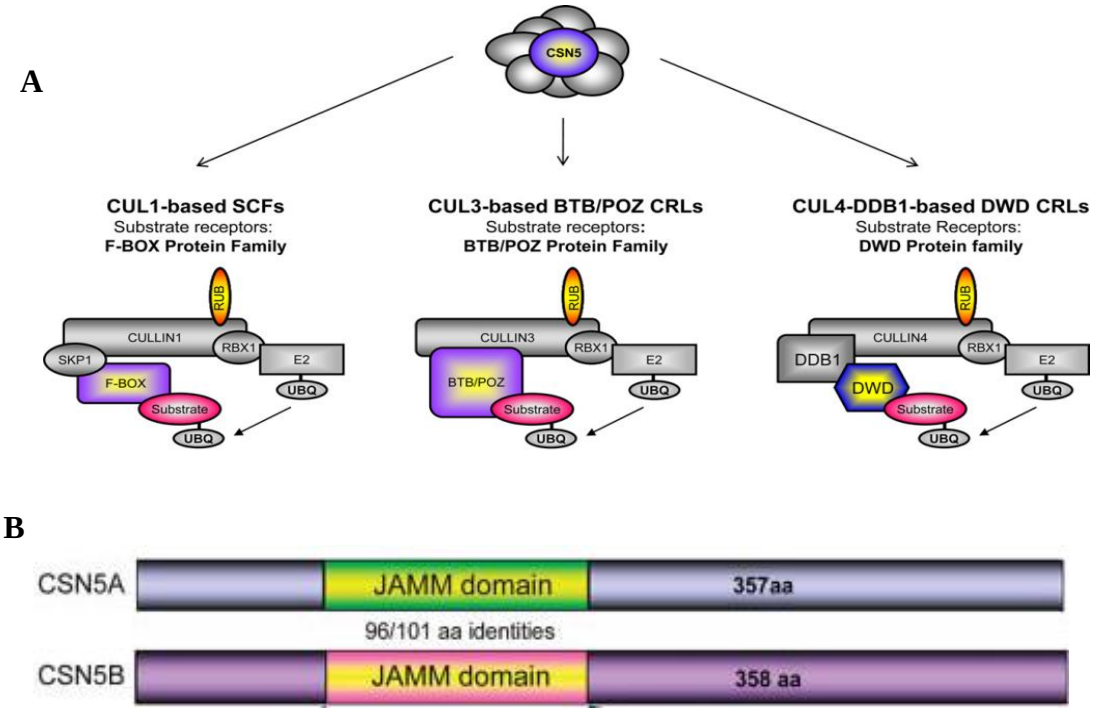


Figure 2. A) shows three plant families of CSN-regulated CULLIN-based E3 ligases (CRLs) (CUL1, CUL3 and CUL4) and their different adaptor proteins. At the top is the multisubunit CSN (CSN5 subunit highlighted) which interacts with all three CRLs. B) schematic representation of the CSN5A and CSN5B subunits showing the catalytic JAMM domain. (Gusmaroli, Feng et al. 2004, Stratmann and Gusmaroli 2012).

1.5 Mutualistic plant-fungus interaction

In nature, every organism interacts with atleast one other organism either directly or indirectly and the outcome of such interactions is very important for the functioning of the ecosystem. One of such interaction termed mutualism, where by all intereacting organisms benefit from each other, is very prevalent in all types of ecosystem. Mutualistic associations contributed significantly to the diversification of life and were crucial for the colonization of land by plants (Traveset and Richardson 2014). The best known mutualistic interaction is that between plant roots and fungi symbionts known as mycorrhiza. There are different forms of

mycorrhiza including ecto-mycorrhiza, endo-mycorrhizal including orchid, ericoid and arbuscular mycorrhiza. Arbuscular mycorrhiza (AM) which is formed by 70-90% of land plants is the most common terrestrial symbiosis dating back 400 million years ago. Root colonization by AM fungi of the Glomeromycota phylum results to the formation of arbuscules (tree-shaped fungal structures) inside root cells which is the site of nutrient exchange between plant host and the AM fungi partner leading to a beneficial interaction. While the AM fungi obtain carbohydrates from the plant, in return the plant receives enhance water uptake and vital nutrients e.g phosphates and nitrogen from AM fungi (Parniske 2008). Root colonization by AM fungi was reported to increase disease resistance in the shoots leading to inhibition of bacterial leaf pathogens (Liu, Maldonado-Mendoza et al. 2007). Although AM fungi have a very wide host range including many crop plants, they cannot be culltured on synthetic medium as they are obligate biotrophs and require a host to complete their life cycle (Parniske 2008). However, *Piriformospora indica*, a root colonizing basidiomycete fungus of the order sebacinales can be cultured on synthetic medium, colonizes the roots a wide variety of monocotyledonous and dicotyledonous plants including the model plant Arabidopsis as well as barley and have several beneficial effects on colonized hosts. Roots colonized by *P. indica*, which is considered an endophyte, share similarities with AM fungi as both interactions leads to beneficial outcome for both partners (Varma, Verma et al. 1999, Peskan-Berghofer, Shahollari et al. 2004, Waller, Achatz et al. 2005, Yadav, Kumar et al. 2010, Qiang, Zechmann et al. 2012). *P indica*, therefore provides an alternative system for the molecular study of the interaction between plant roots and fungal mutualists.

1.5.1 *Piriformospora indica*

Piriformospora indica, a basidiomycete fungus of the order Sebacinales was originally discovered in association with a spore of Glomus mosseae from the rhizosphere of shrubs in the Indian Thar desert (Varma, Verma et al. 1999). *P. indica* displays an endophytic life style and can colonize the roots of a wide range of mono- and dicotyledonous plants including the model plants Arabidopsis and barley (Figure 3) resulting in several beneficial effects to the host and increase biomass (Varma, Verma et al. 1999, Lahrmann and Zuccaro 2012). Unlike mycorrhizal fungi which are obligate biotrophs and cannot grow outside their host, *P. indica* is cultivable and can be cultured on synthetic medium where it produces pear-shaped chlamydospores. The order Sebacinales which includes *P. indica* has been found worldwide (in all continents) and reported to associate with the roots of many different plant species (Selosse, Dubois et al. 2009). The recent sequencing of the *P. indica* genome helped identify

characteristic features associated with saprotrophy including expansion of genes encoding hydrolytic enzymes such as cell wall degrading enzymes (CWDE) and metalopeptidases as well as features associated with biotrophy such as a repertoire of small secreted proteins (SSPs) and impaired nitrogen assimilation (Lahrmann and Zuccaro 2012). The availability of the *P. indica*'s genome coupled with its ability to colonize Arabidopsis roots, makes it a promising model organism to investigate and understand beneficial plant-fungi interaction.

1.5.2 *P. indica* root colonization strategy

Root colonization by *P. indica* has been extensively studied in the model plant arabidopsis as well as barley (Figure 3). However, while colonization of barley root by *P. indica* is biphasic, the colonization strategy used by *P. indica* on Arabidopsis roots still needs to be clearified. In a previous study by Jacobs, Zechmann et al. (2011), the colonization of Arabidopsis roots by *P. indica* was reported to be biphasic with an initial biotrophic phase starting two to three days after inoculation of Arabidopsis roots with chlamydospores followed by germination and production of inter- and intracellular hyphae which begin to penetrate rhizodermal and cortical root cells. Thereafter, intracellular colonization becomes prominent with hyphal branching ocassionally forming whorls. During biotrophic colonization, the cytosol, endoplasmic reticulum (ER), nucleus and plasma membrane of colonized root cells were intact confirming that the cells were alive and intracellular hyphae were invaginated by the plant plasma membrane and could not be stained by wheat germ agglutinin – Alexa Flour 488 (WGA-AF488). At the later colonization phase (7 and 14 dai) external and intracellular sporulation was initiated and more dying or death cells were observed in *P indica* colonized root cells but not in adjacent non colonized cells (Jacobs, Zechmann et al. 2011). The root endophyte interferes with ER stress reponse in Arabidopsis roots triggering a caspase dependent vacuolar cell death program and enhancing root colonization(Qiang, Zechmann et al. 2012)

By contrast, in a recent research Lahrmann, Ding et al. (2013) revealed that colonization of Arabidopsis roots by *P. indica* leads to a long lasting feeding relationship on living host cells with no sign of cell death. Throughout this interaction *P. indica* produced thick bulbous invasive hyphae in epidermal cells which could not be stained by WGA-AF488 even at the later time points because of the presence of a plant derived plasma-membrane and no sign of papillae was observed during hyphal penetration (Lahrmann, Ding et al. 2013) (Figure 3B). Meanwhile, colonization of barley roots by *P. indica* is mainly, biphasic with an initial biotrophic phase (3-5 dai) where fungal hyphae penetrates inter- and intracellularly the

rhizodermis and cortical root cells. Cell wall appositions are observed beneath the site of attempted hyphal penetration of living barley root cells. However, because of natural root cortical cell death (RCD) occuring in the older root zones of barley, *P. indica* switches from biotrophic to a saprophytic (cell death) colonization phase (Figure 3A). During this saprophytic phase, *P. indica* produces secondary thinner hyphae and secretes hydrolytic enzymes (Deshmukh, Hueckelhoven et al. 2006, Lahrmann, Ding et al. 2013). The conserved cell death suppressor protein BAX-IHIBITOR 1 (BI-1) which controls PCD is involved in the regulation of *P indica* root infection during the first three weeks of colonization. Although BI-1 is often induced in plants in response to biotic and abiotic stresses, *P. indica* colonization of barley roots resulted in suppression of BI-1 expression (Deshmukh, Hueckelhoven et al. 2006). Despite the colonization strategy, interaction between *P. indica* and Arabidopsis or barley roots leads to several beneficial outcome.

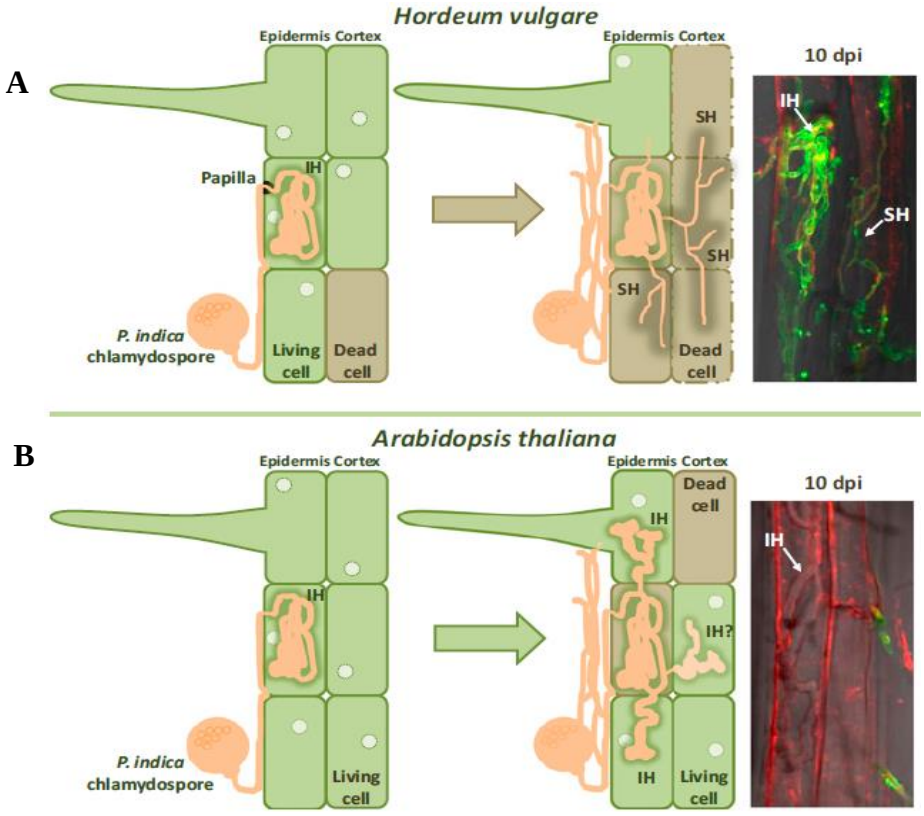


Figure 3. Schematic representation illustrating *P. indica* colonization strategies at different symbiotic stages in barley and in Arabidopsis. A) WGA-stainable invasive hyphae (IH) and secondary thin hyphae (SH) of *P. indica* in barley dead cells (at 10 days after inoculation). B) non-WGA-stainable biotrophic broad invasive hyphae of P indica in Arabidopsis epidermal cells (10 days after inoculation). More dead cells are observed on colonized barley tissues as indicated by WGA-stained IH and SH, but not in Arabidopsis. Fungal structures were stained with WGA-AF488 (green); plant membranes were stained with FM4-64 (red) (Lahrmann, Ding et al. 2013)

1.5.3 *P. indica* host beneficial effects

P. indica which has has been described as a mutualistic root endophyte and classified as a member of the family Sebacinales has the ability to colonize the root cortex cells of a wide range of agriculturally important crop plants including the monocotyledonous barley as well as the dicotyledonous Arabidopsis. The colonization of plants by *P. indica* leads to several beneficial outcome. Several studies have demonstrated that *P. indica* colonization results in increase plant growth of a wide range of crop species (Peskan-Berghofer, Shahollari et al. 2004, Lee, Johnson et al. 2011, Qiang, Zechmann et al. 2012). The growth promoting effects conferred by *P. indica* on barley leads to increased barley grain yield (Achatz, Kogel et al. 2010), enhanced disease resistance against the necrotrophic root pathogen *Fusarium culmorum* as well as the biotrophic leaf pathogen *Blumeria graminis* and also enhance tolerance to mild salt stress. *P. indica* induces higher accumulation of antioxidants in barley roots including ascorbate and key antioxidant enzymes such as dehydroascorbate reductase (DHAR) leading to salt-stress tolerance. *P. indica* induced system resistance in barley was through an unkown mechanism since marker genes indicative of jasmonic acid (JA), *JIP-23*, and salicylic acid (SA) ,*BCI-1* were not upregulated in response to *P. indica* (Waller, Achatz et al. 2005). Similarly, Baltruschat, Fodor et al. (2008) demonstrated that *P. indica* confer salt-stress tolerance in barley root by upregulating ascorbate and antioxidant enzymes including catalase (CAT), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase. The colonization of Arabidopsis roots by *P. indica* resulted in root and shoot growth promotion (Vadassery, Ranf et al. 2009), suppression of defense gene expression (Jacobs, Zechmann et al. 2011) suppresion of MAMP-induced oxidative burts in barley roots as well as induction of ethylene signaling in Arabidopsis and barley (Baltruschat, Fodor et al. 2008, Khatabi, Molitor et al. 2012). Interestingly some plant antioxidant enzymes maybe induced by ethylene during heat stress (Larkindale and Huang 2004) and plants may recruit ethylene signaling for salt tolerance (Cao, Liu et al. 2007). The endophyte also enhances phosphate uptake in maize three weeks after infection of maize roots (Yadav, Kumar et al. 2010)

1.5.4 Effector candidates in the genome of *P. indica*

Until recently, little was known about small secreted proteins coding for putative effectors in *P. indica*. With the recent characterization of effector proteins in mutualistic fungi (Kloppholz, Kuhn et al. , Plett, Kemppainen et al. 2011), it became apparent that such

effector-like proteins exited in other mutualistic fungi including *P. indica*. Generally, the identification of fungal effector candidates has been guided by the following principles: genes that are up-regulated during *in planta* growth, genes that code for predicted small secreted proteins (SSPs) (<300 aa), with unknown functions to proteins in databases(Martin, Aerts et al. 2008, Zuccaro, Lahrmann et al. 2011, Hacquard, Joly et al. 2012). The recent sequencing of the *P. indica* genome (Zuccaro, Lahrmann et al. 2011) helped to identify hundreds of genes coding for small secreted proteins (candidate effectors), several of which were up-regulated *in planta* thereby opening new avenues to investigate the role played by these genes during *P indica* host interaction. *In silico* analysis of the *P. indica* SSPs revealed that some *in planta* expressed SSPs are cysteine rich, where as a regular pattern of alanine and histidine residues was found in others. Motif search using amino acid sequences identified a highly conserved pattern of seven amino acids termed RSIDELD located at the C-terminus of the histidine-alanine rich proteins (designated DELD) (Figure 4.1). The DELD proteins which are small in size, ranging between 100-130 aa do not contain functional domains and the function of the DELD motif is not known,but it is suggested to be involved in effector translocation into the plant cytosol as has been suggested for other conserved motifs, Y/F/WxC motif for powdery mildew effectors (Godfrey, Bohlenius et al. 2010) or shown for the RXLR motif of oomycetes (Kale and Tyler 2011). Among the SSPs rich in histidine and alanine residues, 25 effector candidates contain the conserved C-terminal DELD motif. Just like the DELD motif, the function of the regular pattern of histidine and alanine residues in some SSPs is not known, but has been suggested to be important for correct protein folding and stability in the apoplast. However, similarly to other cysteine rich effector proteins of several fungi such as the poplar rust, the *P. indica* cysteine rich SSPs should be important for the formation of disulfide bridges known to maintain protein structure, function and stability in the apoplast (Duplessis, Cuomo et al. 2011, Zuccaro, Lahrmann et al. 2011).

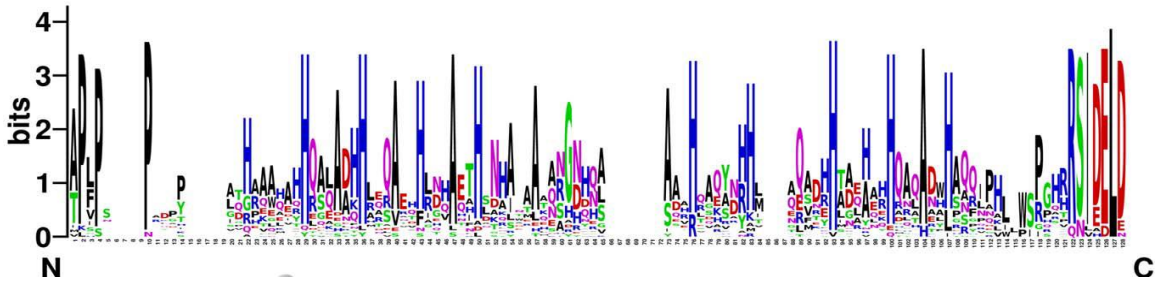


Figure 4.1.) schematic representation of conserved residue positions of the *P. indica* DELD proteins. Regions with regular pattern of histidine (blue) and alanine (black) residues are visible in the consensus alignment. The conserved pattern of seven amino acid termed RSIDELD is visible at the C-terminal. (Zuccaro, Lahrmann et al. 2011).

Using an *in silico* pipeline that excluded protein size but included pfam domain-containing proteins, Rafiqi, Jelonek et al. (2013) further identified effector candidates in *P. indica*. 976 genes predicted to code for proteins containing signal peptide were identified using SignalP. After excluding transmembrane containing proteins, enzymes and mitochondrial proteins but retaining pfam domain-containing proteins 543 secreted proteins designated candidate effectors were identified, of which 389 secreted proteins had unknown functions (Figure 4.2). Out of the 154 secreted proteins identified to carry predicted pfam domians, 64 carried predicted protease activity while 23 contained the carbohydrate-binding protein domain, LysM (Petersen, Brunak et al. 2011, Rafiqi, Jelonek et al. 2013) (Figure 4.2). 14 *P. indica* SSPs showed similarity to predicted secreted proteins of the ectomycorrhizal fungi, *Laccaria bicolor* and recently homologos of the DELD proteins were also found to be present in the genome of the closely related sebacinalean fungus, *piriformospora williamsii* suggesting a possible conservation of these proteins in these fungi family (Rafiqi, Jelonek et al. 2013) (Rafiqi, unpublished).

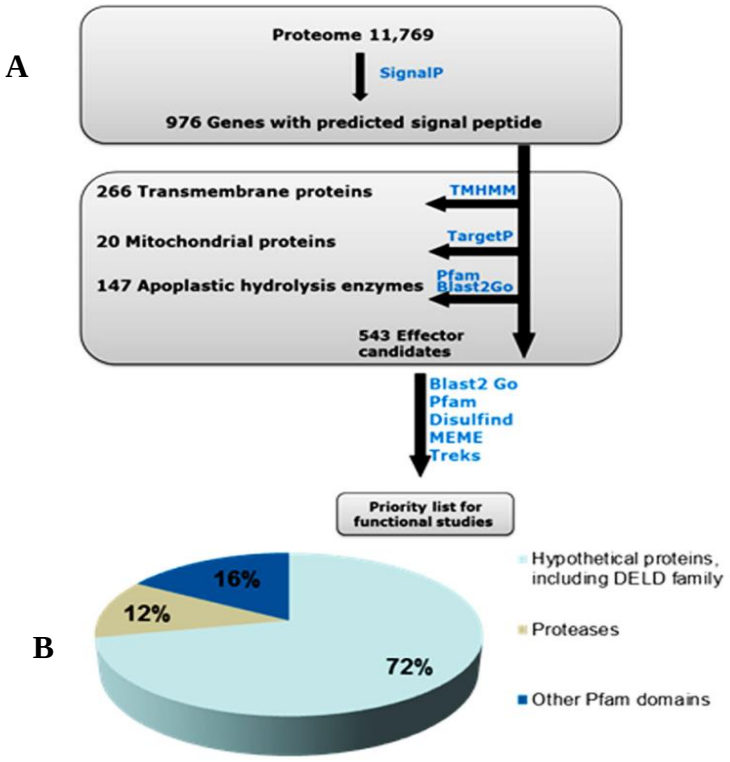


Figure 4.2) Overview of the computational pipeline used to mine the list of effector candidates in the secretome of *P.indica*. A) 976 proteins from 11,769 *P. indica* proteome (secretome) were predicted to contain signal peptide using SignalP. Transmembrane domain-containing proteins and mitochondrial proteins were excluded using TMHMM and TargetP respectively. Apoplastic hydrolysis enzymes such as chitinase and glucanase were excluded based on function and not their size using Pfam and Blast2Go and a final list of 543 candidate effectors were retained. B) 72% of effector candidate are new sequences of unknown function including the DELD proteins, 12% contained predicted protease activity and 16% had other Pfam domains. (Rafiqi, Jelonek et al. 2013).

1.5.5 Effectors of mutualistic fungi

Plants form a variety of mutualistic associations with beneficial fungi of which mycorrhizal symbiosis: an association between plant roots and soil fungi is the most abundant and important for plant growth. About 80% of plants form mycorrhizal symbiosis, with arbuscular mycorrhizal as the most common (Sanders 2011). The symbiotic development of arbuscular mycorrhizal fungi leads to the formation of tree-shaped structures within the plant cell known as arbuscules which are thought to be the site of nutrient exchange between the fungal and host plant (Parniske 2008). Other forms of mycorrhizal symbiosis include ectomycorrhizal, ericoid-mycorrhizal and orchid-mycorrhizal symbiosis. All of these symbiosis are important for both the plants and fungi, as they help plants get access to essential nutrients such as phosphates, inorganic nitrogen and improve plant growth and in return provide carbohydrates to the fungi. The most intriguing question about mutualistic symbiosis is how the fungal symbiont is able to colonize the host plant and establish a symbiotic relationship without setting of the plants complex defense reactions.

However, exciting new research now indicates that similarly to fungal pathogens of plants, mutualistic fungi also secrete effectors into the host cells to suppress host defense and establish symbiosis. MYCORRHIZAL iNDUCED SMALL SECRETED PROTEIN 7 (MiSSP7) an effector of the ectomycorrhizal fungus *Laccaria bicolor* was shown to enter root cells and accumulate in the host nucleus when delivered by transgenic *Laccaria* where it alters host gene transcription. MiSSP7 enters plant cells through lipid raft-mediated endocytosis by binding to the membrane bound phospholipid, phosphotidylinositol 3-phosphate and the C-terminal RALG (Arginine, Alanine, Leucine and Glycine) domain is required for cell uptake (Plett, Kemppainen et al. 2011) (Figure 5B). In a recent study, MiSSP7 was reported to interact with the JAZ domain containing protein JAZ6 of *populus trichocarpa* (PtJAZ6), a repressor of jasmonic acid (JA) induced gene expression and blocks the degradation of PtJAZ6 in the nucleus. Transgenic poplar roots expressing MiSSP7 resulted in suppression of JA-induced gene transcription thereby enhancing the proliferation of *L. bicolor* in plant tissue. RNAi silencing of MiSSP7 resulted in a decreased hartig net formation and a rduction of poplar root cells colonization demonstrating a requirement of this effector for *L. bicolor* root colonization (Plett, Kemppainen et al. 2011, Plett, Daguerre et al. 2014). Similarly, Kloppholz, Kuhn et al. (2011), identified SP7 (SECRETED PROTEIN 7) an effector secreted by the arbuscular mycorrhiza, *Glomus intraradices*. Using a yeast two hybrid screen SP7 was shown to interact with the *Medicago truncatula* pathogensis-related

transcription factor *Mt*ERF19 within the plant nucleus leading to a suppression of defense responses involving ERF19 (Figure 5A). *Colletotrichum trifolii*-mediated induction of *Mt*ERF19 was inhibited in roots expressing SP7 and upregulation of *PR10-1*, a pathogensis related protein induced by *C. trifolii* in *M. truncatula* leaves was also suppressed in roots expressing SP7 leading to enhanced mycorrhiza colonization. Expression of SP7 in the hemibiotrophic rice (*Oryza sativa*) pathogen, *Magnaporthe oryzae* suppressed *M. oryzae*-induced immune responses and resulted in an extend biotrophic growth of *M. oryzae* in roots (Kloppholz, Kuhn et al. 2011). It is now widely accepted that mutualistic fungi carry hundreds of genes in their genome coding for small secreted proteins (SSPs) called effectors. However, only two effectors of mutualistic fungi (SP7 and MiSSP7) has been functionally characterized until now and both were shown to be important for mycorrhizal symbiosis. The identification of a catalogue of small secreted proteins (SSPs) coding for candidate effectors in the genome of *P. indica* and their subsequent characterization would enable us understand the role played by these proteins in root-endophyte symbiosis. It is also highly probable that more of such effectors secreted by plant mutualistic fungi will soon be identified and would help advance our knowledge on how mutualistic fungi establish and maintain symbiotic relationship with their respective hosts.

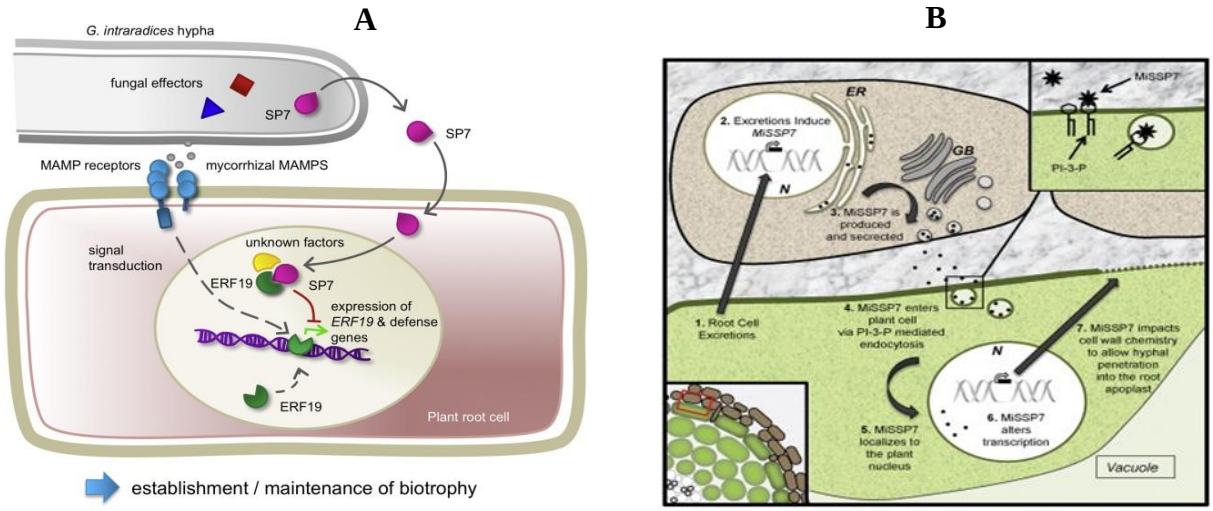


Figure 5. Schematic representation of the fungal effectors SP7 and MiSSP7 during fungal root interaction. A) The effector protein SP7 of the arbuscular mycorrhizal fungi *Glomus intraradices* localizes to the plant nucleus where it inhibit the transcription of the pathogenesis related transcription factor ERF19 thereby promoting root symbiosis. B) Effector MiSSP7 of the ecto-mycorrhizal fungi *Laccaria bicolor* is taken up inside the plant nucleus through lipid raft-mediated endocytosis by binding to membrane bound phosphatidylinositol 3-phosphate (PI-3-P). MiSSP7 alters gene transcription, inhibit the defense related transcription factor JAZ6 and alters the plant cell wall chemistry, thereby promoting symbiosis. (Kloppholz, Kuhn et al. , Plett, Kemppainen et al. 2011).

1.6 Objective

Plant-microbe interactions are influenced by several factors among which are effector proteins. Until now much of the studies about eukaryotic effector proteins has focused on fungal pathogen effectors which have been shown to modulate the plant immunity and suppress defense responses to promote pathogen infection. Although genes coding for efffectors has recently be found in the genome of mutualistic fungi, only two effectors of plant mutualistic fungi has been characterize till date and both were shown to promote symbiosis. These includes, SECRETED PROTEIN 7 (SP7), an effector protein of the arbuscular mycorrhiza, *Glomus intraradices* and Mycorrhizal iNDUCED SMALL SECRETED PROTEIN 7 (MiSSP7), an effector protein of the ecto-mycorrhiza, *Laccaria bicolor*. *Piriformospora indica* is a cultivable root colonizing endophytic fungus with a wide host range including the model plants Arabiodpsis and barley making this fungus a genetic tractable model to study and understand plant-root symbiosis. Root colonization confers several beneficial traits to the host indicating that the fungus can be of huge agricultural value. We took advantage of the recently sequenced genome of *P. indica* which was also shown to contain a repertoire of effector candidates, non of which has been functional characterized. The aim of this study was to use the *P. indica* – plant interaction model to investigate and functionally characterize the biological role of selected *P. indica* effector candidates in plants. First, using a yeast two hybrid screen performed on Arabidopsis and tobacco cDNA library, *in planta* targets were identified for selected *P. indica* effector candidates. One interesting protein identified in this screen was a subunit of the COP9 signalosome, CSN5 which occurs in two isoforms, CSN5A and CSN5B. Both isoforms were found to interact with the *P. indica* effector candidate PIIN_08944. The interaction between PIIN_08944 and CSN5 was further investigated using the bimolecular flourescent complementation assay and both proteins were expressed *in planta* to verify their subcellular localization. Second, RNA interference as well as gene knockout by homologous recombination was performed on *P. indica* to investigate the role played by PIIN_08944 in the virulence of *P. indica*. Third, stable transgenic barley and Arabidopsis plants expressing PIIN_08944 were produced and used for several pathogen infection assays to examine the role played by PIIN_08944 in plant immunity. The ability of PIIN_08944 to interfere with PAMP triggered immunity was also ivestigated by performing a reactive oxygen species (ROS) assay on transgenic barley leaves. Taken together, the general aim of the study was to reveal the molecular function for *P. indica* effector candidates, non of

which has been functionally characterized until now. Understanding the functions of these effector candidates should provide molecular insight into how *P. indica* colonizes plants.

2. Materials and methods

2.1 Chemicals and Kits

Except otherwise stated, all chemicals and kits used throughout this thesis were purchased from the following companies, ROTH (Kalshruhe, Germany), FLUKA (Spain), Sigma Aldrich (Deisenhofen, Germany), MERCK (Darmstadt, Germany), DUCHEFA (RV Haarlem, The Netherlands), and Difco (Augsburg). DNA extraction from *E. coli* was performed using the Wizard® Plus SV Minipreps DNA Purification Systems (Promega, Mannheim), QIAprep® Spin Miniprep Kit, NucleoSpin Plasmid DNA Purification Kit (MACHEREY-NAGEL) and the QIAGEN® Plasmid Midi Kit (QIAGEN, Hilden). For the purification of DNA fragments from agarose gels, the PROMEGA Wizard® SV Gel and PCR Clean-Up System (Promega, Mannheim) were used.

2.1.1 Enzymes

All restriction enzymes were purchased from New England Biolabs (NEB Frankfurt) and Thermo Scientific - Fermentas (Germany). Other enzymes included Taq DNA polymerase (Fermentas, Germany), Phusion® High Fidelity (HF) DNA polymerase (NEB Frankfurt), and the Gateway® LR clonase® II enzyme mix (Invitrogen, Germany).

2.1.2 Antibiotics (selection marker) Stocks

Bacterial	
Ampicillim	100 mg/ml diluted in H_2O
Kanamycin	50 mg/ml diluted in H_2O
Streptomycin	50 mg/ml diluted in H_2O
Spectinomycin	100 mg/ml diluted in H_2O
Rifampicin	50 mg/ml diluted in methanol
Gentamycin	50 mg/ml diluted in H_2O
Fungal	
Hygromycin B	50 mg /ml diluted in H_2O
Plants	
Kanamycin	50 mg/ml diluted in H_2O

2.2 Plants, Fungal, Bacterial strains and growth conditions

2.2.1 Plant materials

2.2.1.1 ***Arabidopsis thaliana***: *Arabidopsis thaliana* ecotype *Columbia*-0 (Col-0 N60000), obtained from the Nottingham *Arabidopsis* Stock Center (NASC) was used as wild-type for all experiments during this thesis excepted otherwise noted. For aseptic cultivation, seeds were surface sterilized with 70% (v/v) ethanol for 1 min followed by treatment with 3% sodium hypochlorite for 10 min. The seeds were washed 5 times with sterile water and let to dry under the clean bench. The seeds were subsequently placed on solid ½ MS or ATS medium in square or round petri dishes (Greiner Bio-One) and grown vertically or horizontally. For soil grown plants, seeds were placed in pots on top of a 3:1 soil – sand mixture (Fruhstorfer Erde Typ P; Quarzsand). In order to synchronize germination, seeds with placed at 4^{o}C in the dark for 48 h before cultivation. The cultivation conditions were as follows: 8 h light (fluorescent cool white, Toshiba FL40SSW/37, 180 µmol m-2 s-1 photon flux density) / 16 h night, 22°C / 18°C, and 60% relative humidity.

2.2.1.2 ***Hordeum vulgare***: Barley (*Hordeum vulgare* cv. Golden Promise) plants were grown in soil and on 1/10 PNM medium. For soil grown plants, seeds were placed in pots containing soil type T (Fruhstorfer Erde Typ T) and cultivated in a growth chamber under long day conditions (24°C, 16 hours light, 180 µmol m^{-2} s^{-1} photon flux density, 80% rel. humidity). After 2 to three weeks, plants were used for further assays. For detached leaf assays, 2 to 3-week old lesion and disease free second leaves were used. For aseptically grown plants, seeds were surfaced sterilized with 70% (v/v) ethanol for 2 min followed by treatment with 6% sodium hypochlorite for 1-2 h. The seeds were washed 5-10 times with sterile water and the seed coat was carefully removed. The seeds were placed on a wetted sterile filter paper and let to germinate for three days in the dark at RT. After germination, the seedlings were transfer red onto 1/10 PNM agar in sterile glass jars and cultivated in a growth chamber under long day conditions.

2.2.1.3 ***Nicotiana benthamiana*:** Tobacco plants were grown on soil (Fruhstorfer Erde Typ T) in a growth chamber under long day conditions (24°C, 16 hours light, 180 µmol m^{-2} s^{-1} photon flux density, 80% rel.humidity). Leaves of 4-6 week old plants were then used for *A. tumefaciens* mediated transient transformation.

2.2.2 Fungi

2.2.2.1 ***Piriformospora indica:*** The *P. indica* isolate DSM11827 (German collection of microorganisms and cell cultures in Braunschweig, Germany) was used in all experiments during this thesis. Chlamydospores of this isolate, kept as glycerol stock at -80^{o}C were used to

prepare master plates for subsequent assays. The chlamydospores were cultured either on CM agar plates at 25^{o}C for 4 to six weeks or in CM liquid medium at 25^{o}C for seven days.

2.2.2.2 *Fusarium graminearum*: strain IFA 65 was used for barley infection assays

2.2.2.3 *Botrytis cinerea*: strain B 05.10 was used for *Arabidopsis* infection assay during this study

2.2.3 Bacteria

2.2.3.1 *Escherichia coli*: DH5α and XL1 Blue was used for preparation of all plasmids during this study except stated otherwise.

2.2.3.2 *Agrobacterium tumefaciens*: strains GV3101 containing the helper plasmid pMP90 and LBA 4404 were used for stable transformation of *A. thaliana* plants and transient expression of constructs in *N. benthamiana* respectively.

Table1. List of Oligonucleotides used throughout this thesis

Designated names	Sequences (5' – 3')	Use
AtUBQ5-4_FW	CCAAGCCGAAGATCAAG	qRT-PCR and RT-PCR
AtUBQ5-4_RV	ATGACTCGCCATGAAAGTCC	
ITS _F	CAACACATGTGCACGTCGAT	
ITS_R	CCAATGTGCATTCAGAACGA	
Pi_UBI_PCR_F	GCAGCTCGAAGATGGTCGCA	
Pi_UBI_PCR_R	ACATGCACGCTTGCGGCAGT	
AtUbQ4_F	GCTTGGAGTCCTGCTTGGACG	
AtUBQ4_R	CGCAGTTAAGAGGACTGTCCGGC	
Tnos_F	TCTCCGGCTGCAGATCGTTCAAACATTTGGC	
Tnos_R	GAGCGATCTAGTAACATAGATGACAC	
M13_F	TATGCCAGGGTTTTCCCAGTCACGACG	
M13_R	GCGAGCGGATAACAATTTCACACAGG	
XmaI_08944_F	ATACCCGGGATGTTCTCTTTCCGAAAAGCCGCA	
NotI_08944_R	TTTGCGGCCGCTGATTCGTGAGTTTTACGCTTTCCG	
KpnI_US_08944_F	TATGGTACCTTCGAGTCCAGGCGACAACG	knockout cloning
KpnI_US_08944_R	GCGGGTACCGTCGATTACGAATTGGTGAAG	
StuI_DS_08944_F	GTCAGGCCTACCTGTAAAGTAACTCCTTCCCCTC	
SacI_DS_08944_R	GTCGAGCTCGGATAAAACCTTCGAAGGACAGTG	

JI Hyg_F	TATCGGCACTTTGCATCGGC	
JI Hyg_R	GATCGGACGATTGCGTCGCA	
T35S_F	GAGATTTTATTGAGAGCAGTAT	
T35S_R	GTGTGCTGATAAATACAAATAC	
CSN5A_F	ATGGAAGGTTCCTCGTCAGCCATCGC	
CSN5A_R	CGATGTAATCATGGGCTCTGGATCTGATGAGTCG	
CSN5B_F	TATATGGAGGGTTCGTCGTCGACGATAGCAAGGAAGAC	
CSN5B_R	ATATGTAATCATAGGGTCTGGATCCGACGAGTCAGTGG	
BiFC_8944_F	CACCATGCCCATCGAAAAAATGATTCTTCCAGACC	**Gateway cloning**
BiFC_8944_R	GTCTGATTCGTGAGTTTTACGCTTTCCGAAGTG	
BiFC_CSN5a_F	CACCATGGAAGGTTCCTCGTCAGCCATCGC	
BiFC_CSN5a_R	CGATGTAATCATGGGCTCTGGATCTGATGAGTCG	
BiFC_CSN5b_F	CACCATGGAGGGTTCGTCGTCGACGATAGCAAGG	
BiFC_CSN5b_R	ATATGTAATCATAGGGTCTGGATCCGACGAGTCAGTGG	
XmaI_GFP_F	ATACCCGGGATGGTGAGCAAGGGCGAGGAGCTG	
NotI_GFP_R	ATAGCGGCCGCACCCTTGTACAGCTCGTC	
XmaI_iCherry_F	TTTCCCGGGATGGTAAGCAAGGGCGAGGT	
NotI_Flcherry_R	TATGCGGCCGCTTACTTGTCATCGTCGTCCTTGTAGTC	
KpnI_dldh_F	TATGGTACCGATGGATATGCTGTAGATCC	**RNAi cloning**
XmaI_dldh_R	CTCCCCGGGTTTTCTGTCTATTTGTTGTTGTCTCTA	
KpnI_Hsp70_F	TATGGTACCACGACTCTGGTCTGTTGAGACC	
XmaI_Hsp70_R	TCTCCCGGGGTTCTGTTTAGAAATCTAGAAACAAGAAAC	
SacI_dldh_F	GACGAGCTCTTCTCAACCTTCTCATCGTC	
NotI_dldh_R	GGTGCGGCCGCTTTTCTGTCTATTTGTTGTTGTC	
HYG_F	ATGAAAAAGCCTGAACTCACCGCGACG	
HYG_R	TTCCTTTGCCCTCGGACGAGTGCTG	
EcoRI_08944dSP_F	CTCGAATTCATGCCCATCGAAAAAATGATTCTTCCA	
BamHI_08944_R	CCCGGATCCTTATGATTCGTGAGTTTTACGCTTTCCG	
GS_08944dSP_F	AGGTTCAGGTTCAATGCCCATCGAAAAAATGATTCTTCCAG	
SmaI_08944_R	ATACCCGGGTTATGATTCGTGAGTTTTACGCTTTCCG	

2.3 Vectors

2.3.1 Plant expression vectors

pUBQ::SIP1::GFP

This vector was used for the ectopic expression of the *P. indica* effector candidate PIIN_08944 (SIP1) in barley. SIP1 without its signal peptide was PCR amplified from *P. indica* cDNA using primers GS-08944dSP-F and SmaI-08944-R while GFP (green fluorescent protein) was amplified using primers SmaI-GFP-F and ATG-GSGFP-R (Table 1). Both PCRs were analyzed on a 1% agarose gel and the products were gel purified. Overlapping PCR was performed using the GFP and SIP1 PCR products as templates to generate a GFP-SIP1 protein fusion. The fusion construct was cloned into the binary vector pUbi and expression of the fusion was under the control of the constitutively active maize *ubiquitin* promoter and the *nos* terminator from *A. tumefaciens.*

p35S::SIP1::GFP

This vector was used for the ectopic expression of the *P. indica* effector candidate SIP1 in *Arabidopsis thaliana*. The *SIP1* gene (without signal petide) fused to GFP (green fluorescent protein) was cloned into the binary vector pICH86988 using SmaI restriction enzyme. Expression of the fusion construct was under the control of the constitutively active *Cauliflower Mosaic Virus 35S* promoter (CaMV 35S) and the *nos* terminator from *A. tumefaciens.*

2.3.2 *P. indica* transformation vectors

pBShhn-Tef and pHSP70

The pBShhn-Tef vector carried the *hygromycin B* gene under the *P. indica* translation elongation factor promoter designed from the pBShhn backbone (Kamper 2004, Zuccaro, Basiewicz et al. 2009). The pHSP70 vector was constructed from the pBShhn-Tef by replacing the TEF promoter with *P indica* heat shock protein 70 (HSP70) promoter at the KpnI and XmaI restriction site by using the primers KpnI-HSP70-F and XmaI-HSP70-R (Table 1). Both vectors contain the nos terminator from *A. tumefaciens*.

pHSP70::GFP and pHSP70::mCherry

These vectors were designed by replacing the *hygromycin B* gene with GFP or mCherry and used for expression studies in *P. indica*. GFP or mCherry were cloned by PCR amplification of the respective DNA constructs using the primers XmaI-GFP-F / NotI-GFP-R and XmaI-iCherry-F / NotI-iCherry-R (Table 1) and ligation using T4 DNA ligase at the XmaI and NotI

restriction sites. Expression was under the control of the heat shock protein promoter (HSP70) which is active *in P. indica* and the *nos* terminator from *A. tumefaciens.*

2.3.3 RNAi vector

pHSP70::SIP1::HDLDp

Gene knock-down in *P. indica* was performed by applying the dual promoter silencing strategy. This vector was used for the expression of the silencing construct targeting the effector candidate SIP1 in *P. indica.*

2.3.3.1 The principle of RNA interference

Basically induced within the cytoplasm, the phenomenon of RNAi was first described in the nematode worm *Caenorhabditis elegans* (Fire, Xu et al. 1998). RNAi is a technique where by double stranded RNA (dsRNA) triggers the degradation of homologous mRNA, thereby suppressing gene expression. This technique is known by other names such as quelling (fungi) and post-transcriptional gene silencing (plants). As shown on Figure 6.1 below, the transcription of the target by both promoters (promoter A and promoter B) leads to the production of double stranded RNA (dsRNA) which is recognized by a multidomain RNAIII enzyme, Dicer. The Dicer cleaves the dsRNA into short fragments (21 -25 nucleotides) known as short interfering RNA (siRNA) (Zamore, Tuschl et al. 2000, Elbashir, Lendeckel et al. 2001). These siRNA strands associates with a multiprotein complex known as the RNA induced silencing complex (RISC), which through ATP unwinds the siRNA into two single stranded RNA (ssRNA) strands, the guide and passenger strands (Nykanen, Haley et al. 2001). While the passenger strand is degraded, the guide strand is retained within the complex and provides sequence specificity for the targeting of corresponding mRNA molecules through Watson-Crick base pairing (Zamore, Tuschl et al. 2000, Martinez, Patkaniowska et al. 2002). A perfectly matched mRNA is degraded by cleavage at a single site in the center leading to a decrease in the synthesis of the corresponding protein and thereby blocking gene expression (Elbashir, Lendeckel et al. 2001, Nykanen, Haley et al. 2001).

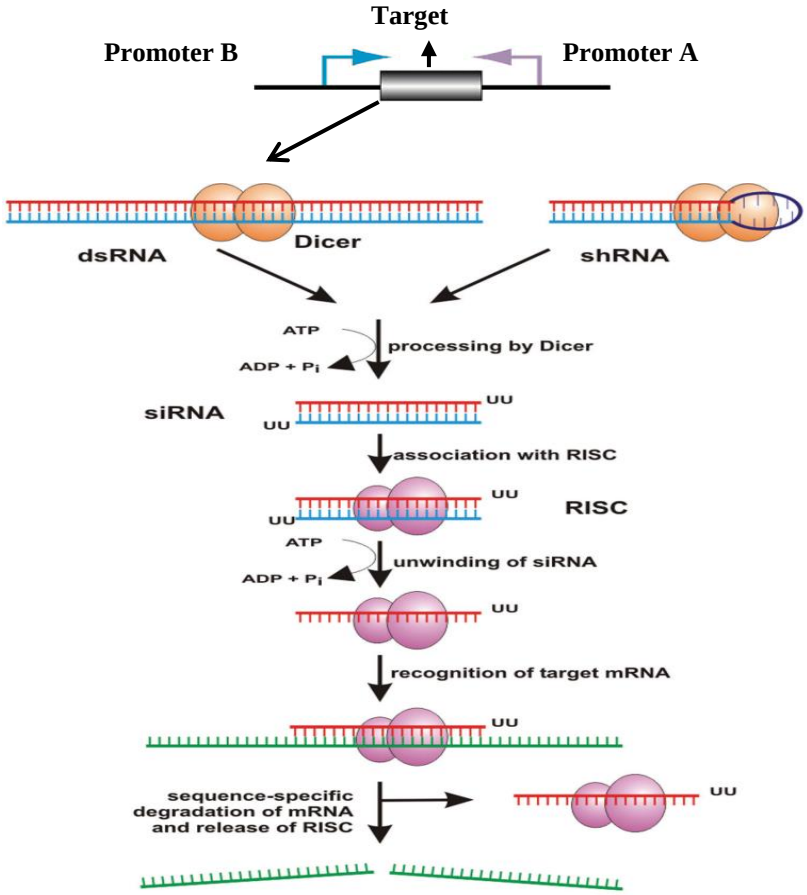


Figure 6.1) Principle of RNA interference. Transcription of the target by the convergent dual promoters (promoter A and promoter B) produces long dsRNA which is processed by the Dicer to short interfering RNA (siRNA). siRNA associates with the RNA induced silencing complex (RISC) to produce a single stranded RNA (ssRNA) known as the guide strand. Guide strand mediates sequence specific degradation of corresponding mRNA molecules by cleaving at a single site in the middle. (*scheme modified* after (Rutz and Scheffold (2004))

2.3.3.2 Preparation of the dual promoter vector

Two promoters, the heat shock protein (HSP70) promoter and the D-lactate dehydrogenase (DLDH) promoter both active in *P. indica* were amplified by PCR and cloned through SacI and KpnI restriction sites in a convergent manner separated by the gene of interest (*SIP1*). The full length gene of interest (~ 400 bp) was PCR amplified from genomic DNA using primers XmaI08944_F and NotI08944_R (Table 1) and cloned at the XmaI / NotI restriction sites of the convergent dual promoter silencing vector pRNAi. The resulting plasmid was transformed into *E. coli* DH5α, verified by colony PCR and confirmed by sequencing after DNA extraction by mini-prep. The silencing cassette consisting of the target gene, sandwiched between the convergent dual promoters was digested using KpnI and SacI

restriction enzymes at 37^{o}C for 1.5 h and purified from agarose gel. Blunting of the purified cassette by fill-in was performed using Klenow fragment (Fermentas) and the cassette was ligated into the pBshhn-Tef vector backbone after it was digested with SacI and filled-in with Klenow fragment. *E. coli* DH5α was transformed, colonies were verified by colony PCR and DNA was extracted from selected positive colonies by mini-prep. The final vector (pRNAi08944) carrying the SIP1-dual promoter silencing cassette and a *hygromycin B* gene (as selection marker), was confirmed by sequencing and used to transform *P. indica*. Silencing efficiency was verified by semi-quantitative RT-PCR. Selected silenced strains were further analyzed for colonization activity during infection of *Arabidopsis* roots.

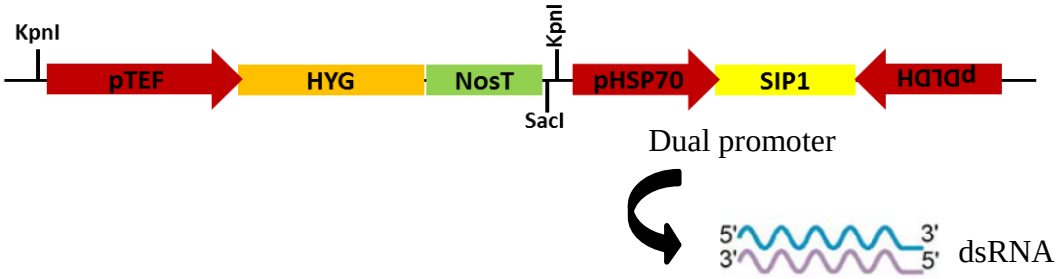


Figure 6.2) Schematic representation of the dual promoter vector (pRNAi08944): The full length gene (yellow box) was cloned in middle of the two convergent dual promoters, *pHsp70* and *pDldh* (red boxes). The silencing cassette was transferred to an expression vector harboring the *hygromycin B* resistance gene under the *TEF* promoter (*pTEF*) and *nopaline synthase* terminator (*nosT*). *SIP1* is cloned between two convergent promoters (pHsp70 and pDLDH) which produce dsRNA after SIP1 transcription by both promoters. HYG: Hygromycin B, HSP70: Heat Shock Protein 70, DLDH: D – Lactate Dehygrogenase, SIP1: Signalosome Interacting Protein 1.

2.3.4 Knockout vector

USSIP1:: pHSP70::HYG::nosT::DSSIP1

Vector was used to replace (Knock-Out) the *P. indica* effector candidate SIP1 through homologous recombination. The hygromycin cassette consisting of the *hygromycin B* gene under the control of the *HSP70* promoter and the *nos* terminator from *A. tumefaciens* was introduced by homologous recombination at the PIIN_08944 (SIP1) locus. The upstream sequence of SIP1 (USSIP1 or 5' UTR (untranslated region)) ~ 180 bps cloned using KpnI and the downstream sequence (DSSIP1 or 3' UTR region) ~ 1000 bps cloned using SacI and StuI, provided the required flanking regions of homologies.

2.3.4.1 *SIP1* knockout by Homologous recombination

The targeted intergration of DNA contructs by homologous recombination in filamentous fungi, leads to gene inactivation by replacement or deletion. Homologous recombination between a target gene and the introduced DNA results in a targeted gene knock-out (Bhadauria, Banniza et al. 2009). Here, the 5'and 3' UTR regions (Left and Right arms) of the target gene (*SIP1*) were PCR amplified from *P. indica* genomic DNA using the appropriate primers (Table 1) and ligated directionally to a *hygromycin B* gene cassette through KpnI and StuI restriction sites. The Left and Right arms provides the flanking homologies required for homologous recombination (Bhadauria, Banniza et al. 2009) (Figure 6.3). The resulting plasmid was transformed into *E. coli* and selected on ampicillin LB agar plates at 37^{o}C overnight. Positive colonies were verified by colony PCR, followed by plasmid extraction from selected colonies and sequencing to confirm the DNA inserts. After confirmation, the plasmid was transformed into *P. indica* using the PEG mediated transformation method (Section 2.6.2).

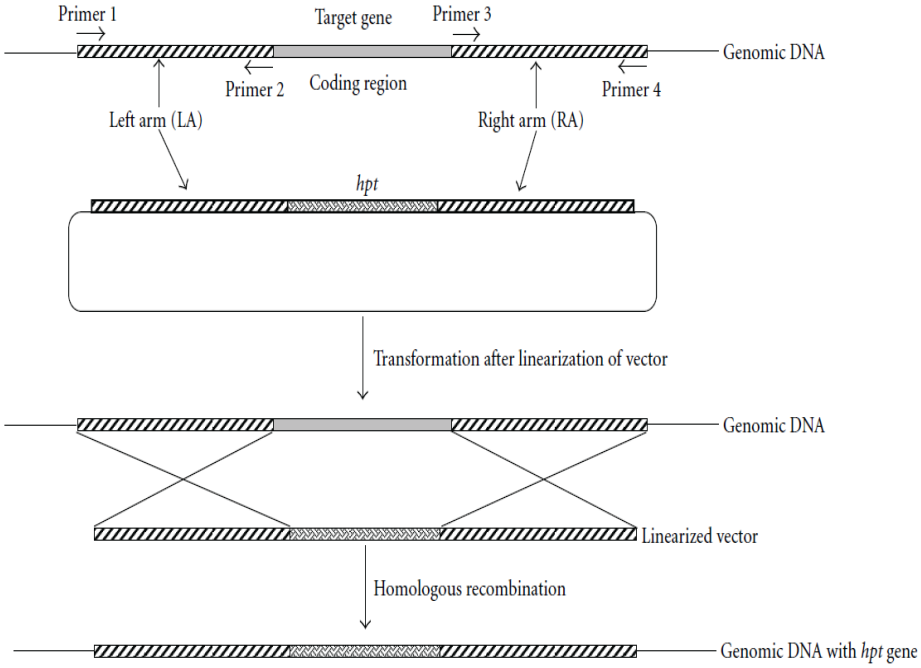


Figure 6.3) The principle of gene replacement by homologous recombination. The 5' and 3' flanking regions (left and right arms) of the target gene are PCR amplified from genomic DNA of the wild-type strain and ligated into a vector containing the *hygromycin phosphotransferase* (*hpt*) gene cassette. The vector is then linearized using appropriate restriction enzyme and introduced into the protoplast of the wild-type strain through PEG mediated transformation. Homologous recombination replaces the gene of interest with the *hpt* gene cassette which also serves as the selection marker for positive transformants.(Bhadauria, Banniza et al. 2009).

2.4.1 DNA extraction (Doyle & Doyle)

DNA extraction was performed following the Doyle & Doyle DNA extraction protocol (Doyle 1987). For this, plant or fungal material was thoroughly crushed to fine powder in liquid nitrogen using a mortar and pestle or tissue lyser II (Retsch® Qiagen®, Germany). The resulting powder was mixed with 700 µl of pre-warm (65°) Doyle & Doyle DNA extraction buffer in 2 ml Eppendorf tubes and incubated for 30-60 min at 65°C. Thereafter, 700 µl of CIA (chloroform;isoamylalcohol 24:1) was added to the samples and mixed gently by inversion for 5 min. The samples were centrifuged for 30 min at 4°C with 13,000 rpm and the aqueous phase collected in new Eppendorf tubes. 600 µl of CIA was added to this, mixed gently by inversion for 5 min and then centrifuged for another 30 min at 4°C with 13,000 rpm. The aqueous phase was again collected in new Eppendorf tubes and mixed with 500 µl isopropanol, 50 µl 10M NH_4OAc and 60 µl 3M NaOAc (pH 5.5) to precipitate the DNA. Precipitation took place by incubation on ice for 1-2 h or at RT. The samples were centrifuged for 10 min at 4°C with 13,000 rpm and the DNA pellet was washed with 500 µl 70% ethanol / 10mM NH_4OAc. After another centrifuge for 10 min at 4°C with 13,000 rpm, the ethanol was discarded, the DNA pellet air dried at RT for ~10-15 min and then dissolved in 50 µl sterile TE buffer (pH 8.0) or ddH_2O. Contaminating RNA was digested by adding 1 µl of RNase A (10 mg / ml) and incubation at 37°C for 1-2 h. DNA concentration was determined using a NanoDrop ND-1000 (PeqLab Biotechnology GmbH, Erlangen Germany) and quality was confirmed on a 1% agarose gel.

Doyle & Doyle extraction buffer	100 mM Tris-HCl (pH 8.0)
	20 mM EDTA (pH 8.0)
	1.4 M NaCl
	2% CTAB
	1% $Na_2S_2O_5$
	0.2% β – mercaptoethanol
TE buffer (pH 8.0)	1 mM EDTA (pH 8.0)
	10 mM Tris – HCl (pH 8.0)

2.4.2 RNA extraction

RNA extraction of plant and fungal material was performed using TRIzol (Invitrogen, Karlsruhe, Germany). Plant or Fungal material was crushed to fine powder in liquid nitrogen

using a mortar and pestle. 1 ml TRIzol was added to 100-200 mg of powdered sample, vortex vigorously to obtain a homogenous mixture. 200µl chloroform was added to the mixture, again vortex vigorously and incubate for 5 min at RT. The samples were centrifuged for 30 min with 14,000 rpm at 4^{o}C and the upper aqueous phase was carefully collected into a new 1.5 ml Eppendorf tube. RNA was precipitated by mixing the supernatant with 500 µl isopropanol, incubation for 10-15 min at RT and centrifuged for 10 min with 14,000 rpm at 4^{o}C. The supernatant was discarded, the pellet washed with 70 % (v/v) ethanol and centrifuged (10 min, 4^{o}C, 14,000 rpm). The ethanol was discarded and the pellet air dried for 10-15 min at RT. 30-50 µl RNase free water was used to dissolve the RNA pellet and the samples were digested with DNase I (Fermentas) to get rid of any residual DNA following the manufacturer's instruction. The amount and quality of RNA was estimated using a NanoDrop ND-1000 (peqLab Biotechnology GmbH, Erlangen, Germany) and on a 1 % agarose gel.

DNase treatment of extracted RNA

2 µg	RNA
1 µl	10X reaction buffer with $MgCl_2$ (Fermentas)
1 u	DNase I, RNase – free (Fermentas)
1 u/µl	Ribolock RNase inhibitor (optional)
To 10 µl	DEPC – H_2O
Incubate at 37 ^{o}C for 30 min	
1 µl	50 mM EDTA
Incubate at 65 ^{o}C for 10 min	
Proceed to cDNA synthesis or store samples at -20^{o}C	

2.4.3 cDNA synthesis (Reverse transcription)

The extracted RNA was transcribed to double cDNA using the qScript ^{TM} cDNA Synthesis Kit (Quanta Biosciences^{TM}) or first strand cDNA was produced using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen^{TM}, Carlsbad CA, USA). All reactions were performed following the manufacturer's instructions. When the reaction was completed, the final concentration of the cDNA was set at 10-20 ng/µl by dilution with H_2O_{DEPC}. cDNA samples were either stored at -20^{o}C or used directly for PCR.

2.4.4 Reverse transcription polymerase chain reaction (RT – PCR)

For quantitative analysis of *P. indica* and plant gene expression, RT-PCR was performed on RNA extracted from *P. indica,* plant roots inoculated with *P. indica* or water inoculated

control. For this, 40-100 ng cDNA was used as template in a 25 µl final reaction volume, 0.5µM forward and reverse primers and a thermal cycling period of 30 cycles. The products were analysed on a 1 % agarose gel.

2.4.5 Quantitative real time PCR (qRT-PCR)

qRT-PCR was performed using a 7500 Fast Real-Time PCR cycler (Applied Biosystems Inc, CA, USA) to quantify the relative amount of fungal genomic DNA on colonized plant roots as well as to analyze the relative expression level of genes in cDNA samples. To quantify the amount of fungal DNA on colonized plant roots, fungal and plant genomic DNA was co-extracted following the Doyle & Doyle extraction protocol and used as template. To analysis gene expression levels, cDNA was prepared from plant extracted RNA and used as template. The reaction was performed in a 20 µl final volume with 40 ng of genomic DNA or cDNA as template using 1 x SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) according to manufacturer's instructions. Each sample was pipetted as a triplicate. The threshold Ct values for specific genes were used to determine the relative expression and relative amount of DNA respectively according to the $2^{-\Delta\Delta Ct}$ method(Livak and Schmittgen 2001).

qRT-PCR reaction mix (20 µl)

10 µl	SYBR green mix
0.5 µl	Forward primer (10 pM) (Eurofins)
0.5 µl	Reverse primer (10 pM) (Eurofins)
5 µl	DNA template (10 ng /µl)
xµl	DNase free H_2O

Cycling Program

95 °C	5 min	Holding stage
95 °C	15 sec	40x cycling stage
60 °C	30 sec	
72 °C	30 sec	
95 °C	15 sec	Melting curve stage
60 °C	60 sec	
95 °C	30 sec	
29 °C	15 sec	

2.4.6 Polymerase Chain Reaction (PCR)

PCR was implored (Taq polymerase) to verify the cloning of gene sequences as well as bacterial and fungal transformants using appropriate primers. While elongation time varied and depended on the size of the amplified DNA fragment, the annealing temperature depended on the primer sequences. A Phusion® High Fidelity DNA polymerase (New England BioLabs, USA) was used if the amplified DNA fragment was use for subsequent cloning. The standard PCR reaction mix used throughout this study is as described below

Standard PCR reaction mix (25 µl).

2.5µl	10x BD buffer	1µl	10pM Reverse primer (Eurofins)
2.5µl	2mM dNTPs (Fermentas)	0.5µl	Taq DNA Polymerase (Fermentas)
1µl	25mM $MgCl_2$ (Fermentas)	20-250 ng	DNA template
1µl	10pM Forward primer (Eurofins)	xµl	Milli Q (H_2O)

Standard PCR program

Initial denaturation	95^oC	5 min	
Denaturation	95 ^oC	30 sec	
Annealing	55-65 ^oC	30 sec	34 cycles
Extension	72 ^oC	2 min	
Final extension	72 ^oC	10 min	

2.4.7 Agarose gel electrophoresis

Agarose gel electrophoresis was implored to separate, amplified DNA fragments, digested plasmid DNA as well as to to assess the quality of isolated DNA and RNA. In general, except otherwise stated 1% agarose was dissolved in 100 ml TBE buffer through heating in a microwave with gentle shaking. The mixture was allowed to cool down at room temperature for about 15 min while stirring. In the meantime, the electrophoresis chamber was assembled. After cooling, ethidium bromide was added and the mixture was poured into the electrophoresis chamber with the comb in place to produce sluts. After solidifying, the agarose was transferred to 1x TBE buffer in a running chamber. DNA / RNA samples were loaded into the sluts and the gel was run for 1 h at 120 V. After the run, bands were visualized under UV light and pictures were made using a digital camera (Canon).

2.4.8 Production of chemically competent *E. coli* DH5α

3 ml LB medium was inoculated with *E. coli* DH5α cells from a glycerol stock and allowed to grow overnight at 37^oC with 220 rpm. The next day, 1 ml overnight culture was transferred to

50 ml fresh LB medium in a 250 ml Erlenmeyer flask and cultivated further at 37^{o}C with 220 rpm for up to 2 h until an OD_{600} of ~0.6 was reached. The culture was collected in a 50 ml falcon tube and centrifuged at 4,000 rpm (4^{o}C) for 10 min. The supernatant was discarded and the pellet carefully resuspended in 3 ml cold TSS buffer. The suspension was incubated on ice for 30 min, followed by 50 µl aliquots preparation in liquid nitrogen and subsequently frozen at -80^{o}C for later use.

TSS buffer	10% (v/v) PEG 6000 (Merck)
	5% (v/v) DMSO (Fluka)
	20 mM $MgSO_4$ x $7H_2O$ (Roth)

2.4.9 Heat shock transformation of chemical competent *E. coli* DH5α

The heat shock transformation was performed by mixing 50 µl of chemical competent *E. coli* DH5α or BL 21 cells with 10 µl of ligation mix in a 1.5 ml Eppendorf tube. The reaction was incubated on ice for 30 min. After 45 sec of heat shock at 42^{o}C, the reaction was again placed on ice for 2 min. 1 ml of LB medium was pipetted into the reaction tube and incubated at 37^{o}C for 1 hours shaking at 150 rpm. Then after, the reaction was briefly centrifuged (2 min at 12,000 rpm) on a microcentrifuge. Supernatant was poured away such that the cell pellet was resuspended in approximately 250 µl of remaining supernatant. For colony selection, the cells were spread on dry LB agar plates supplemented with the appropriate antibiotics. The plates were then incubated at 37^{o}C overnight. Positive colonies were analyzed by colony PCR using the appropriate primers and separated on a 1% agarose gel.

Lysogeny broth (LB) both liquid and agar has been used in this study.

LB medium / Liter

Peptone	10g
Yeast extract	5g
NaCl	10g
Agar agar	10g / liter (for agar plates only)

2.4.10 Transformation and growth of *Agrobacterium tumefaciens*.

In order to transform *Agrobacteria,* 50 µl of electro-competent *Agrobacterium tumefaciens* strains GV310::pMP90 or LBA 4444 was thawed on ice. The cells were then mixed with 1 µl (100 ng) of the candidate recombinant plasmids in a cuvette (0.2 cm gap) making sure to avoid air bubbles. The mixture was placed on ice for 15-20 min. In the meantime, the

electroporation chamber was prepared. The voltage was set to 2400 V, the pulse control unit was adjusted to 25 µF and the resistance on the voltage booster was set to 400 Ω. After incubation on ice, each cuvette was placed onto the electroporation chamber and a pulse of 2.4 kV was applied using the Gene Pulser Mxcell Electroporation system (BioRad). The applied pulse was confirmed by the appearance on the voltage booster meter of the actual peak voltage delivered to the sample. This was performed for all samples. Then 500 µl of SOC or LB medium was given to the cells and incubated at 28^{o}C for 90 min without shaking. 50 µl and 100 µl respectively of the bacteria suspension were plated on LB agar plates supplemented with the appropriate antibiotics. Plates were incubated at 28^{o}C for 2 days and positive colonies were confirmed by colony PCR using appropriate primer sets (Table 1)

2.5 *P. indica* root colonization assay

2.5.1 Isolation of *P. indica* chlamydospores

The isolation of *P. indica* chlamydospores was performed under sterile conditions. For this, 3 to 4-week-old *P. indica* cultured on solid CM agar plate and grown at 24^{o}C in the dark was used. Sterile tween water (0.002 % (v/v) Tween20 in ddH_2O) was added to the plates and chlamydospores were collected by carefully scratching the surface with a spatula. In order to remove residual mycelium, the chlamydospore suspension was filtered through a Calbiochem Miracloth and collected in a sterile 50 ml falcon tube. Thereafter, the suspension was centrifuged at 3500 rpm for 7 min at RT and chlamydospores washed three times with sterile tween water. The spore concentration was adjusted to 500,000 spores / ml using a Fuchs-Rosenthal counting chamber and used for *P. indica* colonization studies.

2.5.2 *Arabidopsis* roots colonization

P .indica-Arabidopsis root colonization was performed under sterile conditions using surface sterilized *Arabidopsis* seeds. *Arabidopsis* seedlings were germinated for 7 days on ATS agar in vertically placed square petri dishes. For colonization studies, 1 ml *P. indica* chlamydospore suspension (5 x 10^5 ml^{-1} in 0.002% TWEEN20) per square petri dish was pipetted on top of the roots and the plates were let to dry for 1 h. The plants were further cultivated in a growth chamber under controlled conditions with a day/night cycle of 16 h light at 20^{o}C and 8 dark at 18^{o}C. Root samples were harvested at different time points (3, 7, 14, and 21 dai). For this, the plants were carefully pulled out of the agar and the roots were cut off at the colonized differentiation zone and immediately frozen into liquid nitrogen for

subsequent DNA extraction. About 5 petri dishes were used for each time point with each petri dish contain approximately 20 plants. All experiments were performed in three independent biological repetitions.

2.5.3 Barley roots colonization

Colonization of barley roots by *P. indica* was performed under sterile conditions using surface sterilized and germinated barley seeds. Barley seeds were germinated for 3 days on sterile wet filter paper in the dark at room temperature. For colonization studies, roots of germinated seedlings were incubated in *P. indica* chlamydospore suspension (5 x 10^5 ml^{-1} in water containing 0.002% TWEEN20) for 30 mins with gentle shaking at room temperature. Seedlings were transferred into sterile jars containing 1/10 PNM medium and further inoculated with 2 ml chlamydospore suspension per jar. Each jar contained four plants. Incubation took place in a growth chamber under controlled conditions with a day/night cycle of 16/8 hours at 24 / 18^{o}C. Roots samples were carefully collected at 3, 7, 14, 21 dai and the colonized differentiation zone immediately frozen into liquid nitrogen for subsequent DNA extraction. All experiments were performed in two to three independent biological repetitions.

Medium for *P. indica* mediated colonization assays

1/10 PNM	1M KNO_3
	0.05g KH_2PO_4
	0.025g K_2HPO_4
	1M $MgSO_4$ x $7H_2O$
	1M Ca $(NO_3)_2$
	2.5ml Fe-EDTA
	0.025g NaCl
	4g Gelrite
	Fill to 1 L with ddH_2O
Fe-EDTA	Add 2.5g $FeSO_4$ x $7H_2O$
	Add 3.36g Na_2EDTA
	Bring to boil
	Stir 30 min while cooling
	Bring to final volume of 450 ml

2.6 Genetic transformation of *P. indica*

2.6.1 Protoplast preparation

P. indica chlamydospores were collected according section 2.5.1. One milliliter of spore suspension was inoculated in 200 ml of CM medium and the culture was cultivated under continuous shaking at 130 rpm (28^{o}C) for 7 days. Thereafter, the culture was filtered through a Calbiochem® Miracloth and the mycelium washed with 0.9 % NaCl. The flow through was discarded and the mycelium collected in a sterile blender (Microtron MB 550, Kinematica AG) and crushed for 10 seconds in 40 ml CM medium. 30 ml of crushed mycelium was transferred into 130 ml of fresh CM medium in a sterile baffled flask and further cultivated for 3 days at 130 rpm (28^{o} C). The young mycelium were again filtered through a Miracloth, washed with 50 ml 0.9% NaCl and dissolved in 10-20 ml pre-chilled enzyme solution (L1412 Sigma lysing enzymes for Trichoderma harzianum, 0.2 g/10ml SMC filter sterilized 0.2 µm) in a sterile 50 ml falcon tube. Protoplastation took place at 37^{o}C with gentle shaking for 45 min-1.5 h. Protoplast formation was verified using a light microscope (Axiostar, Zeiss). When successful, protoplastation was stopped by filtering mycelium through Miracloth to collect protoplast, followed by adding 10-20 ml cold STC through the Miracloth filter to the protoplast sample. Protoplast were then centrifuged 10 min at 4,000 rpm (4^{o}C), supernatant carefully discarded and the pellet washed three times by gently resuspending in 1 ml cold STC. After the last wash, protoplast concentration was adjusted to 10^{7}-10^{9} protoplasts per ml and used for transformation.

2.6.2 PEG mediated transformation of *P. indica* protoplast

For protoplast transformation, 5 to10µg of plasmid DNA was linearized using the appropriate restriction enzyme and temperature overnight. Linearization was verified on agarose gel using 1-2µl of the digest. Afterwards, the linearized plasmid was precipitated using isopropanol, centrifuged at 13,000 rpm (4^{o}C) for 10 min. The pellet was then washed with 70% ethanol and air dry for 15-20 min at RT. Thereafter, the pellet was dissolved in sterile ddH_2O and the concentration was adjusted to 1µg/µl in 10µl by nano-drop measurement. 70µl of freshly prepared protoplasts were mixed with the linearized plasmid (10µg in 10µl). To this, 1µl heparin (15 mg/ml) and 1µl restriction enzyme used for linearizing the plasmid (~20 U) was added. The mixture was incubated on ice for 10 min. Afterwards, 0.5 ml FRESH, cold (filter sterilized 0.45µm) STC/40% PEG 3350 (Sigma-Aldrich) was added, mixed gently by inverting the tube a few times and incubated on ice for another 15 min. The mixture was then

added to 5 ml fresh top medium (MYP + 0.6 pure agar) still liquid but not hot in a 15 ml falcon tube, mixed gently and poured onto plates containing 20 ml of solidified bottom medium (MYP + 1.2% pure agar + 50 mg/ml hygromycin B). The plates were incubated at 28^{o}C for ~14 days until transformants appear. Single isolates were transferred onto new CM agar plates supplemented with hygromycin B (100 mg/ml) for further selection and cultivated at 28^{o}C for 3 to 6 weeks. Fungal material was collected from the edges of actively growing hyphae of transformants as well as wild type control and DNA was extracted (Doyle & Doyle). PCR was performed on the extracted DNA using appropriate primers (Table 1) to verify transformation success and positive isolates were further sequenced to confirm correct DNA.

Solutions / medium / buffers for PEG mediated transformation of *P. indica* protoplast

Enzyme solution	0.2g / 10ml *Trichoderma Harzianum* (L1412 Sigma lysing enzymes) in SMC Filter sterilized (0.2 µm, Roth)
TC	50mM $CaCl_2$ ($CaCl_2$ x $2H_2O$) 10mM TrisHCl, pH 7.5
STC	1M Sorbitol in TC Fill to 1 L with ddH_2O and autoclave
SCS	20mM sodium citrate 1M Sorbitol, pH 5.8
SMC	1.33M Sorbitol 50mM $CaCl_2$ x $2H_2O$) 20mM MES buffer Fill to 1 L with ddH_2O and autoclave
$MgSO_4$	1M $MgSO_4$ ($MgSO_4$ x $7H_2O$)
PEG buffer	40% PEG 3350 (Sigma – Aldrich) in STC Filter sterilized (0.45 µm, Roth)
CM	50ml 20x salt solution 20g glucose 2g pepton 1g yeast extract 1 g casamino acids 1 ml microelements 15g agar (for solid)

	Fill to 1 L with ddH_2O and autoclave
20x salt solution	120g/l $NaNO_3$
	10.4g/l KCl
	10.4g/l $MgSO_4$ x $7H_2O$
	30.4g/l KH_2PO_4
	Fill to 1 L with ddH_2O
Microelements	6g $MnCl_2$ x $4H_2O$
	1.5g H_3BO_3
	2.65g $ZnSO_4$ x$7H_2O$
	750mg KI
	2.4mg Na2Mo_7O4 x $2H_2O$
	130mg $CuSO_4$ x $5H_2O$
	Fill to 1 L with ddH_2O
MYP	0.7% Malz extract
	0.1% Pepton (Soja)
	0.05% Yeast
	0.3M Sucrose
	6g Pure agar
	Fill to 1 L with ddH_2O and autoclave

2.6.3 WGA staining of *P. indica* hyphae during interaction with *Arabidopsis* roots

Arabidopsis roots colonized by *P. indica* were harvested at different time points and fixed in 250 ml chloroform + 750 ml ethanol + 1.5 g trichloraacetic acid (TCA) overnight or longer. Afterwards roots were washed 5 times for 3 to 5 min in distilled water and 1 time in 1 x PBS (pH 7.4). The roots were then transferred to WGA AF 488 (wheat germ agglutinin – Alexa Flour 488, 10µg/ml) in 1x PBS containing 0.02% silwet L-77 and stained by vacuum infiltration at 3 x 25 Hg for 30-120 sec and let to stay in the staining solution for 10 min. The stained roots were later transferred to 1 x PBS and analyzed by microscopy or stored at 4^{o}C in the dark for later use.

WGA stock solution: 1µg/ml (stored at -20^{o}C)

WGA working solution: 50µl stock solution + 4950µl 1 x PBS (pH 7.4)

2.4.6 Reactive Oxygen Species (ROS) assay

2.4.6.1 Analysis of chitin and flagellin induced ROS burst in barley leaves

To examine whether expression of *SIP1* in barley affected reactive oxygen specie (ROS) production after PAMP treatment, soil grown SIP1-transgenic and control barley plants were used. Leaf discs from 3 to 4-week-old barley plants were collected using a sharp 5 mm puncher. The leaf discs were carefully placed into a 96 well microtiter plate containing water and incubated in the dark overnight at RT. Thereafter, water was replaced with aqueous luminol solution (in a dark room), making sure not to stress the leaf discs. The plate was then put into a TECAN infinite® F200 micro plate reader (TECAN, Switzerland) and a blank was measured for 8 cycles. Immediately after, the plate was quickly taken out and the leaf discs were treated with 100 nM flg22, or 200 mg/ml chitin (crab shell) and water control. Then, the plate was quickly returned into the TECAN reader and the relative light units over time as a result of the production of oxygen radicals were measured for 1 h. (~ 50 cycles). At the end of the measurement, the result was analyzed using Microsoft Excel.

Luminol buffer	Stock: 15 mg / ml DMSO (Sigma, A8511-5g) End concentration: 30 µg / ml Dilution factor: 500
Horse radish peroxidase (HRP)	Stock: 10 mg / ml End concerntration : 20 µg / ml Dilution factor: 500 Solution is light sensitive
Luminol solution	40 µl luminol buffer 400 µl HRP (1 mg / ml) 20 ml Aqua dest
Flg 22	Stock: 10 mM End concerntration: 100 nM
Chitin (Crab shell)	200 mg / ml

2.7 Yeast two Hybrid Screen (Y2H)

2.7.1 Looking for interactors of the *P. indica* effector candidate SIP1

The Yeast two-hybrid screen was performed following the yeast protocols handbook and the Matchmaker GAL4 Two hybrid System 3 manual (Clontech, Cat # K1605-A, Heidelberg, Germany) using the yeast reporter strains AH109 and Y187. The PIIN_08944 gene (cDNA) encoding a *P. indica* effector with amino acids (aa) 98 (lacking the signal peptide) was PCR amplified using primers EcoRI-08944dSP-F and BamHI-08944-R (Table 1) and ligated in the pGBT-9 vector through the EcoRI and BamHI restriction sites generating a fusion between the GAL4 DNA-binding domain (BD). The resulting vector was transformed into DH5α *E coli* cells by heat shock and colony PCR was performed on selected colonies using primers GAL4 DBD-F and BamHI-08944-R (Table 1). Plasmids were isolated from selected positive colonies and the desired in-frame insertion was confirmed by sequencing using the GAL4 DBD 5' sequencing primer. The plasmid was then transformed into the yeast strain Y187 and again confirmed by sequencing. The generated yeast strain Y187 carrying the DBD-SIP1 construct was mated with a strain of AH109 pre-transformed with a two-hybrid library from Arabidopsis inflorescence (kindly provided by the Arabidopsis Biological Resource Center) and another AH109 strain pre-transformed with a library derived from tobacco (*N. benthamiana*) source leaves. Cells were plated on synthetic dropout (SD) -Leu, - Trp, and - His supplemented with 4 mM 3 aminotriazole and incubated at 30^{o}C for 3 days. Positive colonies were further tested for *LacZ* reporter gene activity and plasmids were isolated from the yeast cells and transformed into DH5α *E. coli* cells before sequencing of the cDNA insert. To further investigate the direct interaction of two proteins, the respective plasmids were co-transformed in the yeast strain AH109. Transformants were selected on SD -Leu and -Trp at 30^{o}C for 3days and subsequently transferred to SD - Leu, - Trp, and - His for another 3 days at 30^{o}C. The positives and interacting clones were further tested for *LacZ* reporter gene activity.

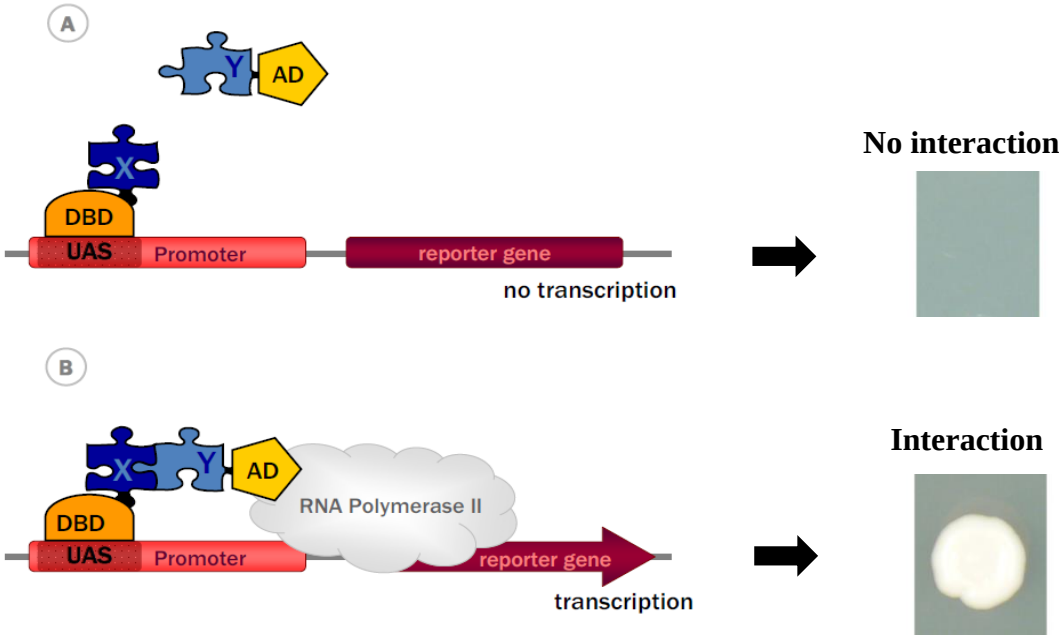


Figure 6.4. The classical yeast two hybrid system: The protein of interest X, for example SIP1 (Bait) is fused to the DNA binding domain (DBD) of a reporter gene. The target protein Y, from a cDNA library (prey) is fused to the activation domain of a reporter gene. A) If the bait (SIP1) and the prey (protein from cDNA library) do not interact with each other, transcription will not occur and the reporter gene will not be activated leading to no yeast cell growth on selective media. B) If the bait and the prey do interact with each other, a fully functional transcription factor is activated through RNA polymerase II leading to activation of the reporter gene thus allowing cells to grow on selective media. AD: activating domain, DBD: DNA binding domain, UAS: upstream activating sequence, X: protein of interest (bait), Y: target protein (prey).

2.7.2 Preparation of plasmids for transient expression in *N. benthamiana*

The binary vectors expressing *SIP1*, *CSN5A* or *CSN5B* genes were constructed according to (Bartetzko, Sonnewald et al. 2009) using the gateway technology (Invitrogen, Karlsruhe, Germany). For this, the entire open reading frame (ORF) of *SIP1*, *CSN5A* and *CSN5B* were PCR amplified using *P. indica* (SIP1) and Arabidopsis (CSN5A and CSN5B) genomic DNA respectively as template. To enable directional cloning, four nucleotide sequences, CACC were added to the ATG 5' end of the forward primers resulting in CACCATG. Through complementarity, the four nucleotides will anneal to the overhang sequence, GTGG, of each pENTR-D/TOPO vectors leading to directional cloning with efficiencies of up to 90%. The resulting PCR fragments were purified and inserted into the pENTR-D/TOPO vector following the manufacturer's instructions (Invitrogen). The resulting plasmids were transformed into *E. coli* DH5α and selected on LB kanamycin agar plates at 37^{o}C overnight.

Positive colonies were verified by colony PCR and plasmid extracted from selected colonies. Directional in-frame-insertion for all three coding regions were further confirmed by sequencing using M13 primers. Thereafter, the *SIP1* cDNA coding region was recombined into the pRB-35S Gateway destination vector using L/R -Clonase (Invitrogen) generating a C-terminal mCherry protein fusion. On the other hand, the *CSN5A* and *CSN5B* coding regions were recombined into pK7WGF2 (Karimi, Inze et al. 2002) using L/R-Clonase (Invitrogen) generating a C-terminal GFP protein fusion. The resulting plasmids were transformed into *E.coli* DH5α and selected on LB spectinomycin / streptomycin agar plates at 37^{o}C overnight. Plasmids were extracted from positive colonies after colony PCR and the respective fusion constructs were confirmed by sequencing using appropriate primers (Table 1). Glycerol stocks were prepared for all sequenced positive colonies and stored at -80^{o}C. The confirmed destination vectors, carrying the different fusion constructs were either used directly for transient expression assays in *N. benthamiana* leaves or stored at -20^{o}C.

2.7.3 *Agrobacterium* – mediated transient transformation of *N. benthamiana* leaves

Positive *agrobacteria* (GV3101) carrying the respective fusion constructs from section 2.7.2 were cultivated in LB medium in the presence of the appropriate antibiotics at 28^{o}C with 140 rpm overnight. As control, untransformed wild type agrobacteria were used. The cultures were harvested by centrifuging for 15 min with 14,000 rpm at RT and the pellet was resuspended in induction medium. The resulting mixture was again centrifuged for 15 min with 14,000 rpm and the pellet resuspended in induction medium. The optical density at OD_{600} was measured using a Biospectrometer (Eppendorf, Germany), adjusted to a final OD_{600} of 1.0 by dilution with induction medium and the cell culture was incubated in the dark at RT for 1 h. After incubation, the culture was infiltrated into the abaxial air space of 4- to 6 week old tobacco leaves using a 1 ml needleless syringe. The infiltrated plants were further cultivated with enough watering and subjected to a16 h light: 8 h dark cycle (25°C : 21°C) at 180 µmol m^{-2} s^{-1} photon flux density, and 80% relative humidity. After 48 h, infiltrated leaves were harvested for microscopy. Fluorescence was detected using confocal laser scanning microscopy on a Leica TCS SP 2.

Induction medium

10 mM $MgCl_2$

10 mM MES

150 µM Acetosyringone (stock: 0.5M in ethanol)

2.7.4 Bimolecular florescence complementation (BiFC) assay

2.7.4.1 Cloning the interactors in split YFP binary vectors using the *Gateway*® system

One of the methods used to investigate the interaction between two proteins *in planta* is the bimolecular florescence complementation assay (BiFC). It is based on the reconstitution of split non-fluorescent yellow fluorescent protein, YFP (a variant of GFP or VENUS) to produce a fluorescent fluorophore (Ghosh, Hamilton et al. 2000). BiFC is a simple but sensitive technique capable of detecting weak and transient interactions mainly due to the stability of the reconstituted YFP complexes (Hu, Chinenov et al. 2002). To construct the vectors used to perform the BiFC assay in this thesis, the generated entry clones of SIP1, CSN5A and CSN5B (section 2.7.2) consisting of the entire coding region of each cDNA were transferred into split VENUS-YFP vector. $VENUS^N$ consist of the N-terminal half (aa 1 to 173) and $VENUS^C$ the C-terminal half (aa 174 to 239) of the 239 amino acid (aa) yellow fluorescent protein (Figure 6.5). By themselves, each of these fragments will not show any fluorescence. But when they are brought into close proximity to each other, a fully functioning YFP is formed and fluorescence is released which can be detected by confocal microscope (Figure 6.5).

Using the LR clonase II enzyme (Invintrogen), the respective entry clones of SIP1, CSN5A and CSN5B were recombined into Gateway compatible split VENUS-YFP vectors (pRB35S-GW-$VENUS^N$ and pRB35S-GW-$VENUS^C$) at the non-fluorescent N-terminal and C-terminal parts of the yellow fluorescent protein, YFP at their respective C-terminin (Supplemental Figure S8). The resulting plasmids were cloned into *E. coli* DH5α and selected on LB agar plates with spectinomycin / streptomycin at 37^{o}C overnight. The resulting colonies were verified by colony PCR, and extracted plasmids from positive clones were sequenced using appropriate primers (Table 1) to confirm the correct ORF orientation. The confirmed BiFC destination vectors containing the different constructs (SIP1-$VENUS^N$, AtCSN5A-$VENUS^C$, AtCSN5B-$VENUS^C$, FBPase-$VENUS^N$ and FBPase-$VENUS^C$) were transformed into *Agrobacterium tumefaciens* GV3101 (Section 2.4.10) and confirmed by colony PCR. To deliver the constructs into tobacco leaf cells, *agrobacteria* carrying the respective coding regions were infiltrated into tobacco leaves (Section 2.7.3). 48 h after infiltration, the BiFC-induced florescence was observed using Leica TCS SP2 confocal laser scanning microscope.

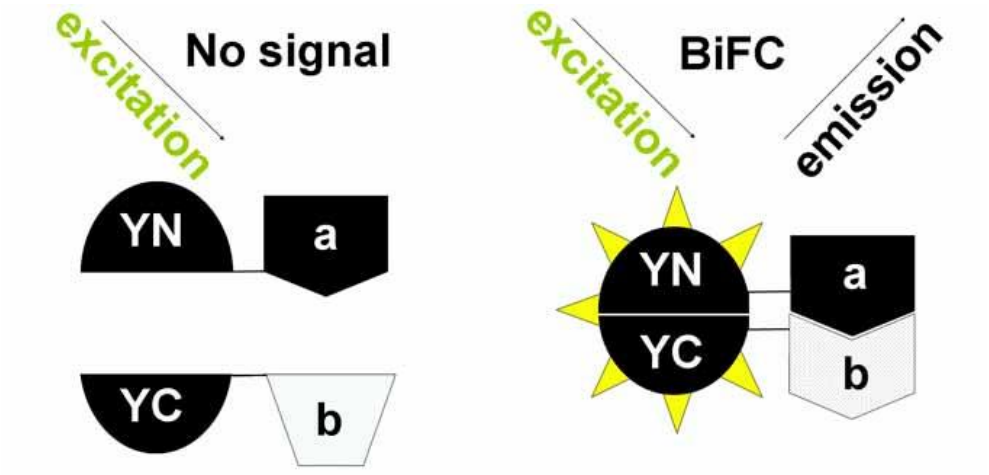


Figure 6.5. The principle of bimolecular fluorescent complementation assay: The non-functional N-terminal (YN) and C-terminal (YC) parts of a yellow fluorescent protein (YFP), (in this case VENUS) are fused to potentially interacting proteins (a and b) and expressed *in planta.* If protein a and b do not interact, the reconstitution of YN ($VENUS^N$) to YC ($VENUS^C$) will not occur and no BiFC signal will be observed. If protein a and b do interact, a fully functioning YFP (YN/YC) will be reconstituted leading to BiFC signal emission which can be observed under the microscope. Diagram (Ohad and Yalovsky 2010)

2.7.4.2 Western blotting

Leaf materials (*N. benthamiana*, and *Hordeum vulgare*) were crushed to fine powder using a tissue lyser II (Retsch® Qiagen®, Germany). The powder was homogenized in 4x laemmli buffer by short vortexing on ice and boiled for 10 min at 95°C. The extracted proteins were separated on SDS-PAGE for 1.5 h at 100 V. To enable protein size verification, a protein marker (PageRuler Prestained Protein Ladder, Fermentas, Germany) was pipetted alongside the samples The separated proteins were blotted onto a PVDF membrane (Roti®-PVDF, pore size 0.4µm, ROTH, Germany) using a semi-dry electrophoretic transfer cell (BioRad) at 12V for ~1 h. Before transfer, the PVDF membrane was cut to fit the size of the gel, activated by dipping in 100% methanol for 5 min and rinsed in 1x Towbin buffer for 15 min.

4x Laemmli buffer

125mM Tris – HCl pH 6.8

50% Glycerol

4% SDS

0.02% Bromophenol Blue,add DTT (30mg/ml) or Mercaptoethanol shortly before use

10x Towbin buffer	**1x Towbin buffer**
0.25M Tris	0.025M Tris
1.92M Glycine	0.192M Glycine
pH 8.6	20% Methanol

SDS – PAGE gels

Resolving gel (12%)	2.5 ml Lower gel 4x buffer
	3.4 ml Water
	4 ml Acrylamide solution (30%)
	100 µl SDS (10%)
	100 µl APS (10%)
	10 µl TEMED
Stacking gel (12%)	600 µl Upper gel 4x buffer
	3.6 ml Water
	1 ml Acrylamide solution (30%)
	50 µl SDS (10%)
	50 µl APS (10%)
	10 µl TEMED
Lower gel 4x buffer	1.5M Tris-HCl, 0.4% SDS, pH 8.8
Upper gel 4x buffer	1M Tris-HCl, 0.4% SDS, pH 6.8
1x Gel running buffer	0.25M Tris base
	0.192M Glycine
	0.1% SDS
	pH adjusted to 8.3

2.7.4.3 Immuno-detection

Once the protein transfer was completed, non-specific binding sites were blocked by incubating the membrane with 5 % (w/v) milk powder (ROTH, Germany) in 1x TBS-T buffer at RT for 1 h with gentle shaking. After washing three times in 1x TBS-T for 5 min each, the membrane was treated with the primary antibody dissolved in 5% milk powder (1: 3,000 dilution) and incubated overnight at 4^{o}C with gentle shaking. Thereafter, the membrane was again washed three times in 1x TBS-T for 5 min each and incubated with the secondary

antibody dissolved in 5% milk powder (1:10,000 dilution) for 1h at RT with gentle shaking. Afterwards, the membrane was washed three times in 1x TBS-T for 5 min each and then transferred into a pre-mixed chemiluminescent substrate (SuperSignal® WestPico, Germany) for 2 min with the protein side towards the solution. Then, the membrane was placed between transparent foils (protein side upwards) and put in a cassette for development on a hyperfilm (Amersham Hyperfilm^{TM} MP, GE Healthcare, Japan) in a photo-laboratory.

10x TBS buffer (1 L)	**1x TBS-T buffer**
60.6 g Tris	100 ml TBS (10x)
87.6 g NaCl	900 ml ddH_2O
pH 7.6 with pure HCl	1 ml Tween20

2.7.5 Southern blotting

The PEG mediated transformation of *P. indica* resulted to several stable transformants which were sub-cultured on selection medium. To verify the genome integration of the transformed vectors in *P. indica,* a southern blot analysis was perform on DNA extracted from pure fungal cultures of the positive transformants. For this, 15-30µg of fungal DNA was digested with an appropriate enzyme (SacI) at 37^{o}C overnight in a final volume of 20 µl. A loading dye was added and the digest was loaded onto a 0.9% agarose gel prepared from 1x TAE buffer. The samples were run at 100 V for 4 h. Afterwards, the DNA was depurinated by placing the gel in 0.25 N HCl for 15 min with gentle shaking and then neutralized in the transfer buffer for another 15 min with gentle shaking. In the meantime, the transfer chamber was assembled. A filter paper bridge (whatmann paper) was cut to fit the size of a glass support with its end dipping in the transfer buffer. Air bubbles were removed by carefully rolling a glass rod on the surface of the bridge. The nylon membrane (Amersham Biosciences Hybond+, GE Healthcare) and 3 pieces of filter paper were cut to fit the size of the agarose gel. The agarose gel was carefully placed on the bridge, followed by the nylon membrane making sure to avoid air bubbles. The edges of both nylon membrane and agarose gel were covered with parafilm. Then, 3 pieces of filter paper were placed on the nylon membrane. To enhance capillary flow, a thick layer of paper towels were added on top and transfer chamber pressed with a 1 kg weight. The chamber was let to stand overnight. After blotting overnight, the chamber was disassembled, the membrane was let to dry for about 10 min and the DNA was cross-linked to the membrane in a GS GENE LINKER UV chamber (BIO-RAD) using an auto cross-linking program (C2, 50mµ Joule).

2.7.5.1 α ^{32}P radioactive DNA probe labelling

Probe labelling was performed following the Prime-a-Gene® Labelling System protocol (Promega). 1µg of purified DNA template obtained from PCR was denatured at 95^{o}C for 2 min and the sample was quickly chilled on ice. The labelling reaction was assembled in a microcentrifuge tube on ice in the order shown below

Components	**Add**	**Final Conc**
Labelling 5X buffer	10 µl	1X
Mixture of the unlabeled dNTPs	2 µl	20 µM each
Denatured DNA template	25 ng	500 ng/ ml
Nuclease – free BSA	2 µl	400 µg/ ml
[α-^{32}P]dNTP, 50µCi, 3,000Ci/ mmol	5 µl	333 nM
DNA Polymerase I Large (Klenow) Fragment	5units	100 U/ ml
Nuclease-Free water to a final volume of 50 µl	___µl	
Final volume	**50 µl**	

The above reaction was mixed gently and incubated at RT for 1 h. Afterwards, the reaction was terminated by heating at 95-100^{o}C for 2 min and subsequently chilling on ice. 20Mm EDTA (pH 8) was added and the labelled nucleotides were removed by size exclusion chromatography using an illustra^{TM} ProbeQuant G-50 Micro Columns (GE Healthcare, Germany) following the manufacturer's instructions. The purified radiolabelled DNA probe was stored at -20^{o}C.

2.7.5.2 Membrane hybridization and exposure

The membrane was hybridized using the Amersham Rapid-hybridization buffer - RPN 1635 (GE Healthcare, Germany). First, the buffer was pre-warmed to 65^{o}C. The membrane was then completely immersed in the buffer in a glass bottle and pre-hybridized with gentle shaking inside a hybridization oven at 65^{o}C for 30 min. After pre-hybridization, the radiolabelled DNA probe was added directly to the buffer and mixed gently. The sample was further incubated in the hybridization oven for 2 h at 65^{o}C with gentle shaking. Afterwards, the hybridization buffer was discarded and the membrane was washed with 50 ml of solution A for 20 min at 65^{o}C with gentle shaking. Solution A was discarded and the membrane was further washed with 50 ml of solution B for another 15 min at 65^{o}C with gentle shaking.

Solution B was discarded and the membrane was carefully pulled out of the glass bottle and let to dry for 10 min. The membrane was checked for radioactivity using a radioactive monitor and wrapped in a SaranWrap^{TM}. The membrane was exposed to a Storage Phosphor Screen BAS-IP MS 2040 E Multipurpose Standard (GE Healthcare, Germany) in a phosphor imager cassette for few hours, overnight or longer and signals were detected using a molecular imager and the Quantity One software (Bio-Rad). The entire experimental procedure was performed behind a screen to shield from the exposure to radioactive materials.

Solutions and buffers used for southern blotting

Solutions / buffers	**components /Final concentrations**
50X TAE buffer	242 g Tris Base
	57.1 ml Glacial acetic acid
	100 ml 0.5 M EDTA
	Fill with ddH_2O to 1 L
1x TAE buffer	20 ml 50X TAE buffer
	980 ml ddH_2O
Transfer buffer	0.4 M NaOH
	0.6 M NaCl
	Fill with ddH_2O to 1 L
20X SSC	3 M NaCl
	0.3 M Na_3Citrate
	pH 7.4 with 1 M HCl
2X SSC	100 ml 20X SSC
	900 ml ddH_2O
1X SSC	50 ml 20X SSC
	950 ml ddH_2O
Solution A	2X SSC
	0.1% SDS
	ddH_2O
Solution B	1X SSC
	0.5 % SDS
	ddH_2O

2.7.6 Plant infection assays

2.7.6.1 Botrytis infection of *Arabidopsis*

Botrytis cinerea strain B 05.10 grown on HA agar medium as described by (Doehlemann, Berndt et al. 2006) was used for this assay. Plates containing 10 to 14-day-old *B. cinerea* conidia grown from glycerol stocks at 22^{o}C in the dark (until actively growing grey mycelium appeared) were washed with 24 g/L potato dextrose broth (PDB, Duchefa Biochemie, Haarlem, The Netherlands) at RT. The spore suspension was collected in a 50 ml falcon tube and centrifuged at 2,500 rpm for 3 min at RT. The pellet was resuspended in PDB and the spore density was adjusted to 2 x 10^5 spores / ml. Five week old disease free Arabidopsis leaves (soil grown) from transgenic lines (T1) and control plants (wild type) were detached and placed in square petri dishes containing 1 % water agar with the petiole embedded in the medium. For each transgenic line as well as control, 15 leaves were harvested from 10 different plants. 3 µl droplets of the spore suspension was pipetted on each side of the middle vein and incubated with a 12 h photoperiod at 22^{o}C. The size of the lesion area was measured 5 days after inoculation (dai) from digital images using the Image J free software program by calculating the percentage of leaf area showing disease symptoms relative to the non – inoculated area.

HA-Agar medium (pH 5.5) (Kretschmer and Hahn 2008)	**½ PDB broth (1L)**
1% malt extract	24 g in ddH_2O
0.4 % yeast extract	
0.4 % glucose	
1.5% agar	

2.7.6.2 *Fusarium* infection of barley leaves and roots

Fusarium graminearum strain IFA 65was grown on SNA agar medium.and conidiospores were harvested using sterile tween water by carefully scratching the surface with a spatula. The spore suspension was filtered through a miracloth and collected in a 50 ml falcon tube. The spore concentration was adjusted to 5 x 10^5 spores / ml by dilution with tween water. Leaves of 2 to 3-week-old barley plants (transgenic lines (T2) and wild type control) were detached and placed in square petri dishes containing 1% water agar with 0.4% benzimidazole to inhibit leaf senescence. Leaves were inoculated by pipetting 20µl spore suspension to the middle vein and the petri dishes were sealed with the lid and incubated at RT. Disease progression was accessed 6 DAI by measuring the lesion size from digital images using

Image J free software program. DNA was extracted and the relative amount of fungal DNA was determined by qPCR.

For root infection, transgenic as well as wild type control barley seedlings were germinated for 3 days on wetted filter paper in the dark. Roots were inoculated with 5 x 10^5 spore / ml in tween water, then transferred to pots (1 seedling per pot) containing a 2:1 mixture of expanded clay (Seramis, Masterfoods, Verden, Germany) and Oil-Dri (Damolin, Mettmann, Germany) and allowed to grow in a growth chamber under long day conditions at 24°C, 16 hours light, 180 µmol m-2 s-1 photon flux density, 80% rel. humidity. Two weeks after inoculation, the root fresh weight and root length were analyzed. The relative amount of fungal DNA on infected leaves was quantified by qRT-PCR.

SNA agar medium

KH_2PO_4	1g	
KNO_3	1g	
$MgSO_4$	0.5g	
KCl	0.5g	
Sucrose	0.2g	
Agar	22g	
NaOH	4M	(150µl)

2.7.7 Antioxidant assay of SIP1 transgenic *Arabidopsis*

To investigate whether SIP1 may alter the antioxidant status and affects salt stress tolerance in Arabidopsis, an antioxidant assay was performed. After germination, seedling of SIP1 transgenic Arabidopsis, csn5a_2, csn5_b and Col-0 control (WT) were transferred to 200 ml pots filled with peat moss soil (4 seedling per pot) (Pindstrup) and grown at 22 / 20 ^oC with 8 h photoperiod. At six-week-old, the plants were irrigated with 250mM NaCl every second day for 14 days, after which the total shoot fresh weight, levels of ascorbate and glutathione were measured. Three pots with a total of 12 plants were used for each line. Ascorbate was determined by using the bipyridyl method (Knorzer, Durner et al. 1996), while glutathione was measured using the method described by (Waller, Achatz et al. 2005).

2.7.8 Sequence analysis and alignment tools

All DNA sequences mentioned in this thesis were sequenced either at LGC Genomics GmbH, Berlin,Germany or at Sequence Laboratories Goettingen (SeqLab), Germany. Basic Local Alignment Search Tool (BLAST) search and sequence analysis were performed on the following sites: NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), The Arabidopsis

Information Resource (TAIR) (http://www.arabidopsis.org) and (http://www.arabidopsis.org/Blast/index.jsp), SignalP 4.1 (Center for biological sequence analysis, Technical University of Denmark, DTU) (http://www.cbs.dtu.dk/services/SignalP/). DNA Sequences were also analyzed using the sequence analysis software Clone Manager suite Ink (Sci-Ed Software).

3. Results

3.1 *In silico* analysis of the *P. indica* effector candidates PIIN_08944, PIIN_06837, and PIIN_04362

3.1.1 PIIN_08944

The sequencing of the *P. indica* DSM 11827 genome in 2011 revealed a wealth of information needed to study and understand the mutualistic and endophytic life style adopted by this root colonizing fungus. The 24.97 Mb (megabase) genome of *P indica* encodes 386 small secreted proteins (putative effectors) among them *Piriformospora indica*_08944 (PIIN_08944) (Zuccaro, Lahrmann et al. 2011). PIIN_08944 is a small secreted protein encoding 120 amino acids (aa) (protein_identity: CCA74964.1) with a 363 nt (nucleotide) (cDNA) coding sequence. In addition to a size cut-off of < 300 aa, the presence of an N-terminal signal peptide has always been used as a criteria for selecting fungal genes coding for putative effectors (Panstruga and Dodds 2009, Zuccaro, Lahrmann et al. 2011). Using SignalP 4.0 (Petersen, Brunak et al. 2011), PIIN_08944 was predicted to contain a 23 aa signal peptide located at the N-terminal (aa 1-23). Therefore, PIIN_08944 encode a 120 aa protein with a predicted 23 aa N-terminal signal sequence which after cleavage will result in a 97aa mature protein lacking cysteine residues (Figure 7). Meanwhile transmembrane domain and mitochondrial signal prediction using the TMHMM 2.0 and TargetP 1.1 softwares (http://www.cbs.dtu.dk/services/TMHMM/) and (http://www.cbs.dtu.dk/services/TargetP/) respectively revealed that PIIN_08944 has neither a transmembrane domain nor a mitochondrial signal. Search for other conserved domains using the NCBI conserved domain database did not reveal any putative conserved domain for PIIN_08944 and PIIN_08944 did not shown any significant homology to known sequences of other organisms. The presence of a signal peptide, coupled with its small size (120 aa) strongly suggest that PIIN_08944 is an effector candidate secreted by *P indica.*

During this thesis, yeast two hybrid screen result revealed that, PIIN_08944 interacts with CSN5A and CSN5B, components of the COP9 signalosome in *Arabidopsis* and tobacco, and with a member of the SEVE IN ABSENTIA family of E3 protein ligase, SINAT3 in tobacco. As a result of this interaction, PIIN_08944 was renamed SIP1 (Section 3.4).

Figure 7. Schematic representation of PIIN_08944. Structure of PIIN_08944 candidate effector consisting of a signal peptide (SP, yellow). (see supplemental data for PIIN_08944 protein sequence).

3.1.2 PIIN_06837

PIIN_06837 encodes a 115 aa (protein identity: CCA72901.1) with a 345 nt (cDNA) coding sequence. Using SignalP 4.0 PIIN_06837 was predicted to contain a 20 aa N-terminal signal peptide (aa 1-20) which after cleavage results in a 95 aa mature protein lacking cysteine residues. No transmembrane domain or mitochondrial signal was predicted for PIIN_06837 using the prediction softwares TMHMM and TargetP 1.1 respectively, while search for other conserved domains using the NCBI conserved domain database also revealed no conserved domains for PIIN_06837. Homology search for PIIN_06837 did not reveal any significant homology to known sequences of other organisms. PIIN_06837 was found to contain a unique seven amino acids, RSIDELD at the C – terminus (Figure 7.1). RSIDELD (or DELD) is a highly conserved pattern of seven amino acids found at the C – terminus of 25 *P. indica* small secreted proteins whose functions are still unknown (Zuccaro, Lahrmann et al. 2011). However it has been suggested that RSIDELD could contribute to effector translocation in a similar way like the well-studied RXLR motifs found in oomycete effectors. Sequence similarity search using the BLASTP non redundant protein database revealed strong similarity between PIIN_06837 and all 25 *P. indica* DELD protein family.

Our yeast two hybrid screen result revealed that PIIN_06837 interacts with a tetratricopeptide repeat containing domain protein (TPR), a basic leucine zipper (bZIP) and reproductive meristem 1 (REM1) all transcriptional regulators in Arabidopsis. The interaction with transcription factors makes PIIN_06837 an interesting effector candidate, since the only two mutualist effectors characterized until now, SP7 of *Glomus intraradices* and MiSSP7 of *Laccaria bicolor* were shown to target the transcription factors ERF19 and JAZ6 to enhance root mycorrhization (Kloppholz, Kuhn et al. 2011, Plett, Daguerre et al. 2014) Meanwhile in tobacco PIIN_06837 interacts with three hypothetical proteins with accession numbers XM_009785891.1, XM_009607415.1, XM_009785897.1.

Figure 7.1. Schematic representation of PIIN_06837. Structure of PIIN_06837 candidate effector consisting of an N-terminal signal peptide (SP, yellow) and a C-terminal RSIDELD motif. (see supplemental data for PIIN_06837 protein sequence).

3.1.3 PIIN_04362

Another *P. indica* effector candidate, PIIN_04362 encodes 393 aa (protein identity: CCA70423.1) with a 1179 nt (cDNA) coding sequence. Although PIIN_04362 has an amino acid sequence above the cut-off size for effector proteins (<300 aa), recent reports have shown that oomycete and fungal effectors can exceed this cut-off size (Rafiqi, Gan et al. 2010, van Damme, Bozkurt et al. 2012). Signal peptide prediction using signal 4.0 revealed an 18 aa N-terminal signal peptide which after cleavage will result in a 375 aa mature protein. No transmembrane domain or mitochondria signal were found after analysis with TMHMM 2.0 and TargetP respectively. However PIIN_04362 was predicted to contain a C-terminal Nuclear Localization Signal (NLS) indicating that PIIN_04362 is targeted to the plants nucleus (Figure 7.2).

Sequence similarity search using BLASTp revealed that PIIN_04362 share 65% identity at the amino acid level with a hypothetical protein from *Serendipita vermifera* MAFF 305830 (KIM 31619.1), 57% with epoxide hydrolase from *Moniliophthora roreri* MCA2997 (XP 007843966.1), 57% with a hypothetical protein of *Gymnopus luxurians* FD-317 M1 (KIK60086.1), 57% with a hypothetical protein of *Hebeloma cylindrosporum* h7 (KIM42734.1), 54% with a predicted protein of *Laccaria bicolor* S238N-H82 (XP 001884358.1), 54%, 53%, and 50% with α/β hydrolase of *Rhizoctonia solani* AG-1 IB (CCO33604.1), *Punctularia strigosozonata* HHB-11173 SS5 (XP 007388433.1), and *Stereum hirsutum* FP-91666 SS1 (XP 007305269.1). Also 52% with epoxide hydrolase of *Heterobasidion irregular* TC 32-1 (XP 009543300.1), and 50% with α/β hydrolase of *Fomitiporia mediterranea* MF3/22 (XP 007268023.1). A Coiled coil region was predicted at amino acid position 356-392 using the SMART software, while search for conserved domains using the NCBI conserved domain database predicted an Esterase and lipase superfamily domain at positions 65-160 aa and α/β hydrolase superfamily or acetyltransferase domain at positions 54-355 aa (Figure 7.2).

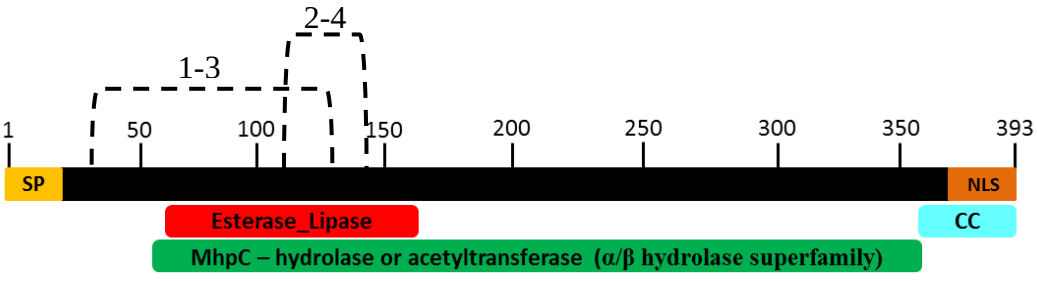


Figure 7.2. Schematic representation of PIIN_04362 showing domains. PIIN_04362 is encoded by 393 aa, contains a signal peptide at 1-18 aa (SP,yellow) and a nuclei localization signal at C-terminal

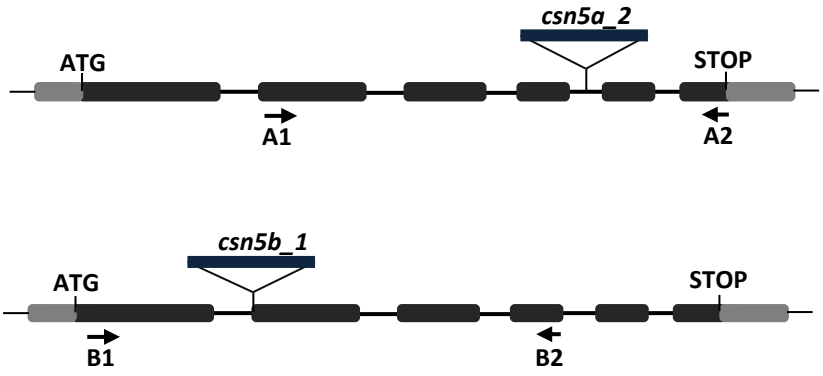
(NLS, brown). Esterase and lipase domains at positions 65-160 aa (red), a coiled coil domain at 356-392 aa (CC, sky blue) and α/β hydrolase / acetyltransferase domain at 54-355 aa (green). Likely connectivity of cysteine residues to form disulfide bonds (dash lines). (See supplemental data for PIIN_06837 protein sequence).

The PIIN_04362 protein also contains 4 cysteine residues at position aa 33, 123, 135, and 145, all of which were predicted to be involved in the formation of disulfide bonds. Using the prediction software DiANNA (Ferre and Clote 2005, Ferre and Clote 2006), the most likely connectivity of the cysteine residues was identified as follows: 1-3, 2-4 (Figure 7.2). Recent research has shown that the formation of disulfide bonds from cysteine residues is important for effector proteins structure and stability in the apoplast (Liu, Zhang et al. 2012).

3.2 Characterization of *csn5a_2* and *csn5b_1* T-DNA insertion mutants

The Arabidopsis CSN5, a subunit of the COP9 Signalosome protein complex is encoded by two conserved genes called *CSN5A* and *CSN5B* (Kwok, Solano et al. 1998). Both *csn5_2a* and *csn5b_1* Arabidopsis mutant lines used in this thesis were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (N527705 – *csn5a_2* and N588593 – *csn5b_1*). Figure 8A shows the positions of the gene specific primers used to amplify the *CSN5A* and *CSN5B* transcripts and also the T-DNA insertion within the *CSN5A* and *CSN5B* locus as identified from the SALK collection (Salk_027705 for *csn5a_2* and Salk_088593 for *csn5b_1*) in the SIGnAL database (Alonso, Stepanova et al. 2003). RT – PCR analysis of the *csn5a_2* mutant line showed a significant reduction of the *CSN5A* transcript compared to WT line, whereas a complete lack of *CSN5B* transcript was observed for the *csn5b_1* mutant (Figure 8B). The Arabidopsis *CSN5A* null mutant (*csn5a_1*) showed severe developmental defects including depleted trichomes at the vegetative stage, dwarf stature and loss of apical dominance at the reproductive stage, shorter roots as well as smaller flowers (Gusmaroli, Figueroa et al. 2007) and therefore could not be used for this study.

A)



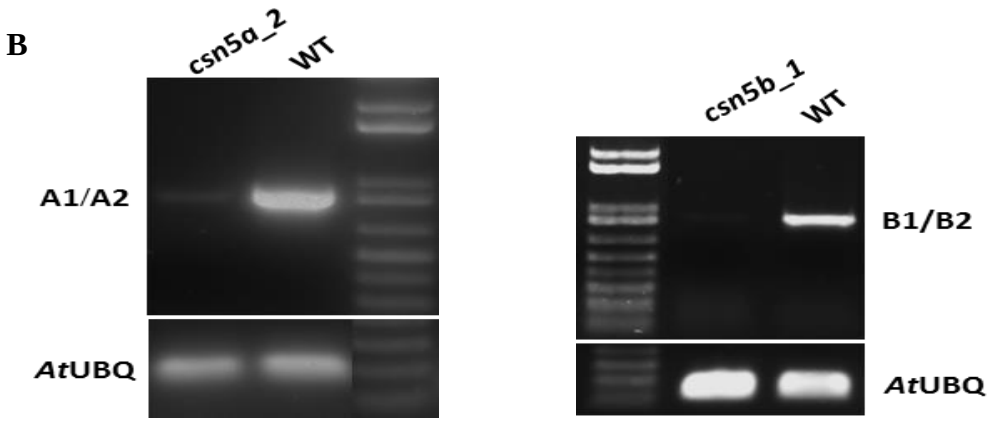


Figure 8. Structural representation and expression analysis of *csn5* mutants: A) Structural representation of the Arabidopsis *CSN5A* (At1g22920) and *CSN5B* (At1g71230) loci and of the T-DNA insertion mutants used throughout this study. Introns are represented by lines while exons are represented by black and gray (untranslated regions) boxes. Arrows indicate the position and orientation of the gene-specific primers (A1, A2, B1, and B2) used for the RT-PCR analysis. (Modified from (Gusmaroli, Figueroa et al. 2007) B) RT-PCR based genotype analysis of Arabidopsis wild type and *csn5a_2* and *csn5b_1* homozygous mutants. Primers A1 and A2 were used to specifically amplify the *csn5a_2* and wild type transcript. Primers B1 and B2 were used to specifically amplify the *csn5b_1* and wild type transcript. The *At*UBQ-5 transcripts were used as positive control for the RT-PCR analysis. (*AtUBQ*: *Arabidopsis thaliana* ubiquitin 5)

3.3 *P. indica* colonization of *Arabidopsis* Col-0 wild type as well as *csn5a_2*, and *csn5b_1* mutants roots increases over time.

To determine the colonization pattern of *P. indica* during interaction with Arabidopsis roots, the relative amount of fungal DNA on colonized root samples of Col-0 (wild type), *csn5a_2* and *csn5b_1* mutants was determined by quantitative real time PCR at different time points. Fungal DNA in root samples increased significantly from 3 to 21 days after inoculation (dai) with a more than 300 fold increase observed for wild type over this period and a 250 and 200 fold increase observed for *csn5a_2* and *csn5b_1* mutants respectively relative to plant DNA (Figure 9). Although the colonization of individual lines increased from 3 to 21 dai, the colonization of wild type plants was significantly higher compared to *csn5a_2* and *csn5b_1* mutant lines (Figure 9). The difference in colonization observed between *csn5a_2* and *csn5b_1* mutants maybe due to the fact that both proteins contribute significantly to several different plant developmental processes and play unequal roles in regulating such processes including photomorphogenesis, root elongation, vegetative and reproductive growth (Gusmaroli, Feng et al. 2004, Gusmaroli, Figueroa et al. 2007).

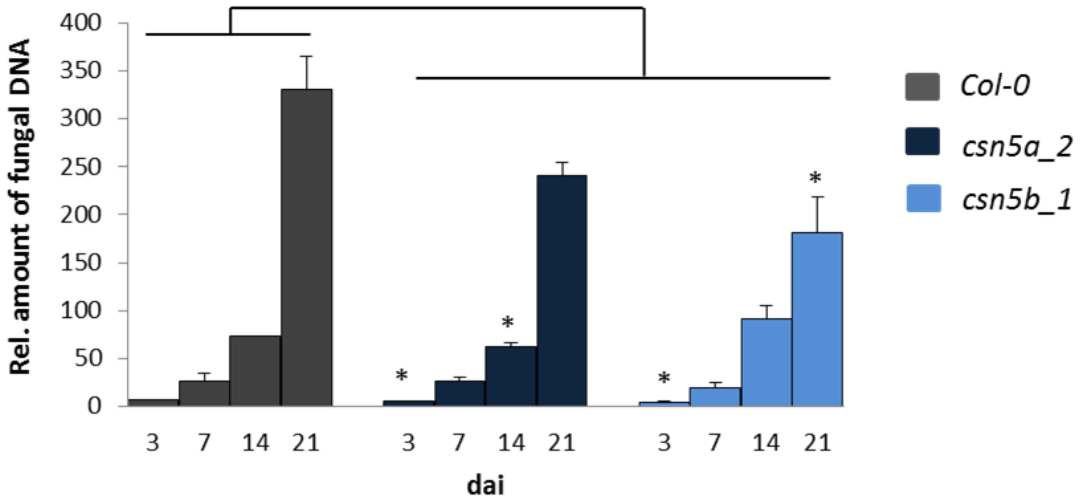


Figure 9. *P. indica* colonization of Arabidopsis roots increases over time. Seven day old Arabidopsis wild type (*Col*-0) roots, as well as the mutants *csn5a_2* and *csn5b_1* were inoculated with *P. indica* chlamydospores. Fungal biomass was determined at 3, 7, 14, and 21 dai, as relative amount of fungal DNA after DNA extraction from colonized roots and subsequent qRT-PCR using fungal and plant specific primers. Data represents the Ct thresholds of Pi-ITS relative to the Ct thresholds of *At*UBQ-4 using the $^{\Delta\Delta}$Ct method (average Ct thresholds of three technical replicates within one biological experiment are presented). The values represent the means with standard errors of one experiment. The experiment was repeated three times with similar results (**Supplemental Fig. S1A and S1B**). Asterisks indicate significance at $P < 0.05$ (*) between Col-0 and csn5a_2, and csn5_1 as analyzed by Student's *t*-test. Pi-ITS: *P indica* Internal Transcribe Spacer, AtUBQ-4: *Arabidopsis thaliana* Ubiquitin-4, dai: days after inoculation. About 100 plants were analyzed per time point for each line.

3.3.1 *P. indica* colonizes Arabidopsis roots and forms multi-lobed extracellular hyphae

Other than qRT-PCR which accurately quantify nucleic acids, one of the methods previously used to quantify root colonization by arbuscular mycorrhiza fungi relied on the staining of colonized roots followed by microscopic analysis (Mcgonigle, Miller et al. 1990).

To confirm the increase *P. indica* colonization of Arabidopsis roots observed from qRT-PCR data, colonized roots were collected from 3 to 21 dai for wild type plants, *csn5a_2* and *csn5b_1* mutants. The roots were stained with wheat germ agglutinin-Alexa Flour 488 (WGA-AF488) and analyzed using confocal laser scanning microscopy (CLSM) on a Leica TCS SP2. During root colonization, *P. indica* forms extensive hyphal network which increased from 3 to 21 dai resulting in increased fungal biomass especially at the late colonization phases (14 and 21 dai) (Figure 3, lower panels). The extracellular stainable hyphae which would eventually produce chlamydospores were irregularly swollen, projecting a multi-lobed structure (Figure 9.1, white arrows). These *P indica* multi-lobed hyphal structures are

reminiscent of the bulbous invasive hyphae (IH) produced by the hemibiotrophic fungal pathogen, *Magnaporthe oryzae* during infection of rice (*Oryza sativa*) cells (Khang, Berruyer et al. 2010). While extracellular hyphae were easy to observe after staining with WGA AF488, intracellular (invasive) hyphae could not be stained due to the presence of a plant-derived membrane which prevents uptake of WGA AF488 (Lahrmann, Ding et al.).

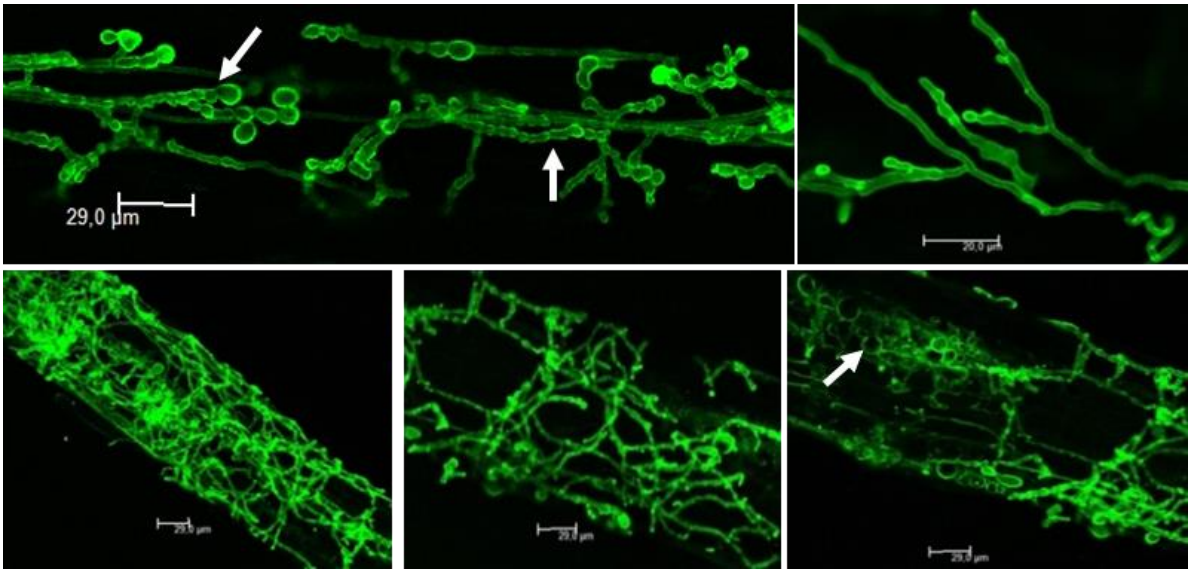


Figure 9.1. *P. indica* forms bulbous extracellular hyphae on colonized Arabidopsis epidermal root cells. Projection of Arabidopsis roots colonized by *P.indica* at 3, 7, 14, and 21 dpi. Heavy colonization of roots with increased amounts of mycelium was observed at late (14 and 21 dai) colonization phases (lower panels) Multilobed extracellular hyphae were formed and observed at both early (upper panels) and late (lower panels) colonization phases. Colonized roots were treated with WGA-AF488 (green) (10 µg/ml) for 10 min which stains extracellular hyphae Root samples were imaged using confocal laser scanning microscopy (CLSM) on a Leica TCS SP2 with excitation at 488 nm.

3.3.2 *P. indica* colonization of barley roots increases over time

Three-day-old barley (Golden Promise) seedlings were inoculated and grown in sterile glass jars on 1/10 PNM medium. The relative amount of fungal DNA on root samples was analyzed at different time points (3, 7, 14 and 21 dai) after DNA extraction and subsequent qRT-PCR. Similarly to the colonization of *Arabidopsis* roots (Figure 2), *P. indica* colonization of barley roots leads to a significant increase in fungal biomass over time from 3 to 21 dai. The amount of fungal DNA on colonized root samples increased more than 20 fold relative to plant DNA (Figure 9.2).

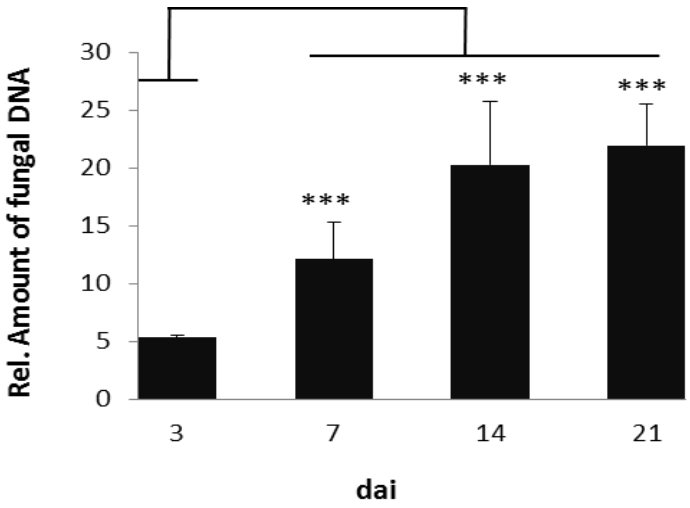


Figure 9.2. *P. indica* colonization of barley roots increases over time. Enhanced colonization of barley (Golden Promise) roots by *P. indica* over time. Roots of three day old germinated barley seedlings were inoculated with *P. indica* under sterile conditions and the plants were grown on 1/10 PNM agar in sterile glass jars. Colonization levels were determined at 3, 7, 14 and 21 dai as the relative amount of fungal DNA after DNA extraction of colonized root samples and subsequent qRT-PCR using barley (*Hv*UBQ-60-Deg) and fungal (Pi-ITS) specific primers. Values represent the mean of two experiments, with error bars indicating standard deviation (SD). Asterisks indicates significance between time points at $P < 0.001$ (***) analyzed using student's *t* test

3.3.3 *P. indica* is amenable to genetic transformation

An effective way to study gene functions including those of filamentous fungi during plant microbe interaction is by over expression, silencing or disruption through transformation. Intracellular hyphal growth and fungal progression can be monitored using reporter genes like Green Fluorescent Protein (GFP) or Red Fluorescent Protein (mcherry) which can be transformed and expressed by filamentous fungi. To visualize *P. indica* hyphal growth during root colonization, a double cassette expression vector was designed carrying the Green Fluorescent Protein (GFP) or the red fluorescent protein (mCherry) under the *P. indica* heat shock protein (HSP70) promoter and *hygromycin B* resistance gene under the *P. indica* translation elongation factor (TEF) promoter. *P. indica* protoplasts were transformed with the GFP or mCherry expression vectors using the PEG (polyethylene glycol) mediated transformation method. Chlamydospores of the transgenic *P. indica* carrying GFP or mCherry were inoculated on barley roots and confocal laser scanning microscopy was performed from 24 hours after inoculation. Three days after inoculation, *P. indica* intercellular invasive (biotrophic) hyphae expressing GFP could be detected on barley epidermal cells (Figure 10A), and five days after inoculation P indica intercellular invasive and extracellular hyphae expressing mCherry were visualized (Figure 10B).

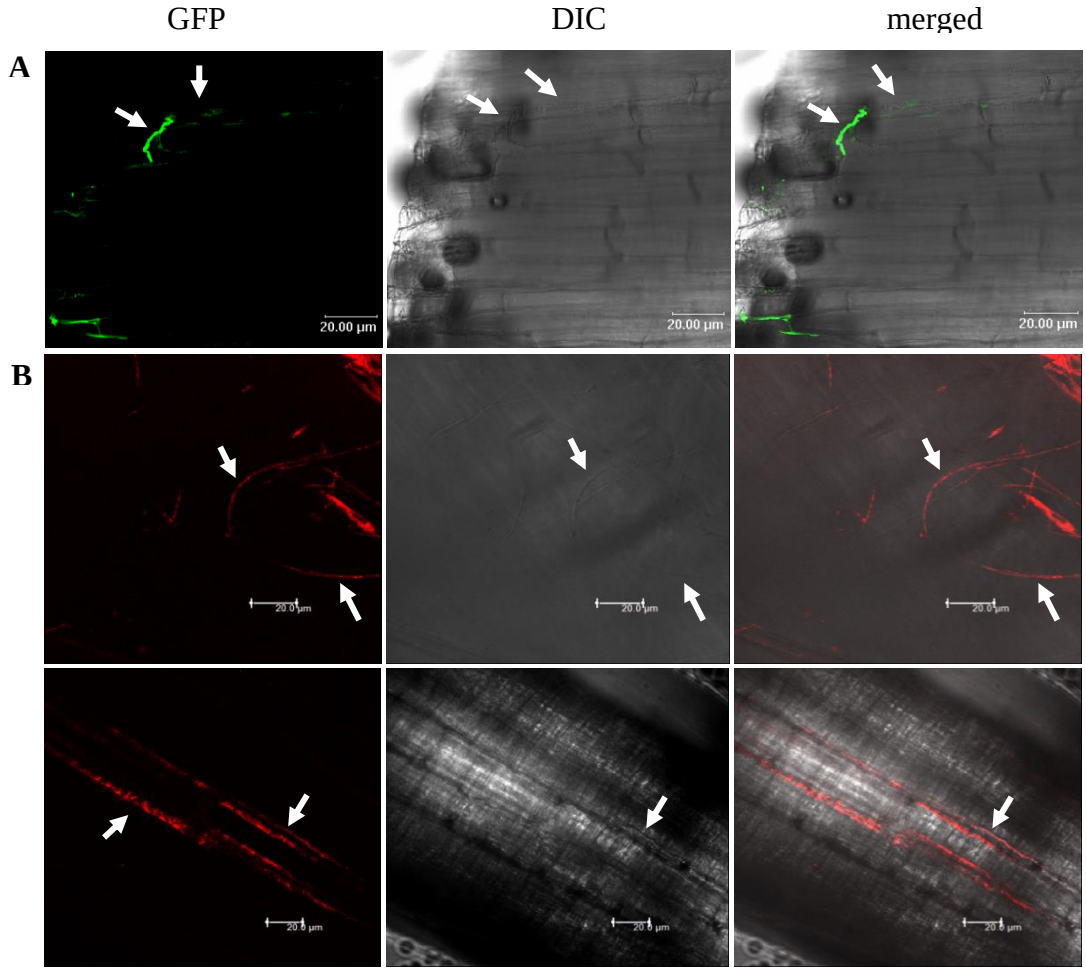


Figure 10. Barley epidermal root cells showing invasive hyphae of *P. indica* expressing GFP and mCherry. Roots of three day-old germinated barley seedlings were inoculated with GFP and mCherry expressing *P indica* strains. A) Biotrophic invasive hyphae expressing GFP were detected 3 dai and B) Expressing mCherry were detected 5 dai using a Leica SP2 confocal laser scanning microcope. Bar = 20 µm

3.4 Identification of PIIN_08944 interacting proteins

In order to identify potential protein targets for the *P. indica* effector candidate PIIN_08944 in plant cells, a yeast two hybrid screen was performed with PIIN_08944 (GAL4-PIIN_08944) as a bait and an Arabidopsis and tobacco (*Nicotiana tabaccum*) cDNA library, respectively as a prey. Because of its very broad host range, with no non-host reported till date, it is highly likely that *P. indica* effector targets are conserved in different plant species. Three candidates were identified to interact with PIIN_08944. Two of the proteins identified as interaction partners of PIIN_08944 in both libraries were CSN5A and CSN5B, components of the COP9 Signalosome protein complex (Figure 11 A and B). Blastn search on the NCBI database reveal strong sequence similarity (more than 90%) between the putative interactor sequence and *CSN5A* and *CSN5B*. In Arabidopsis, *CSN5A* (At1g22920) and *CSN5B* (At1g71230) are two conserved genes encoding two isoforms of the subunit 5 (CSN5) of the COP9

Signalosome (CSN) (Gusmaroli, Feng et al. 2004). CSN5 (COP 9 Signalosome 5) is one of 8 subunits (CSN1-8) which make up the CSN, a nucleus-enriched multisubunit protein complex with high homology to the lid sub-complex of the 26S proteasome (Wei and Deng 2003, Gusmaroli, Figueroa et al. 2007). The *AtCSN5A* and *AtCSN5B* genes are 2338 and 1936 base pair in length respectively, with both genes consisting of six exons and five introns and encoding a protein of 39,270 Da. Based on sequence analysis, the *AtCSN5A* and *AtCSN5B* genes share high sequence similarity at the nucleotide, cDNA (86 %) and the protein (88 %) levels respectively (Gusmaroli, Feng et al. 2004) which likely contributed to the interaction of both proteins with PIIN_08944. The main biochemical function of the CSN is the derubylation or deneddylation (removal of the ubiquitin like protein, RUB1 in plants / NEDD8in animals) of the cullin subunits of cullin-RING ligase (CRL) family of E3 complexes.

The catalytic center responsible for this derubylase activity is found only in the CSN5 subunit, embedded within the JAMM (JAB1/(Cope and Deshaies 2006)/Mov34) or MPN+ motif , making CSN5 the only catalytic subunit of the CSN described till date (Lyapina, Cope et al. 2001, Schwechheimer, Serino et al. 2001, Cope, Suh et al. 2002). The derubylation activities of the CSN enhances the CRL E3 function by blocking the instability of the E3 components caused by the autoubiquitination activity of the the CRL (He, Cheng et al. 2005, Wee, Geyer et al. 2005, Cope and Deshaies 2006). This makes cycles of rubylatioin and derubylation important for the optimal functioning of CRLs (Cope and Deshaies 2006). In plants, rubylation has been shown to be essential for auxin and ethylene responses (Bostick, Lochhead et al. 2004, Larsen and Cancel 2004). In Arabidopsis *CSN5A* and *CSN5B* were shown to regulate root elongation, photomorphogenesis, auxin response as well as vegetative and reproductive growth with *CSN5A* having a more prominent role. A reduction or loss of CSN5A in Arabidopsis seedlings resulted in a decreased number of root hairs in response to jasmonic acid, shorter hypocotyls in the dark and decrease number of lateral roots but not in CSN5B mutants. However both CSN5A and CSN5B Arabidopsis mutants resulted in shorter roots in response to auxin. While *csn5b_1* Arabidopsis loss of function mutant is virtually indistinguishable from wild type at the vegetative stage, *csn5a_2* reduction of function mutants show less sever phenotype. By contrast, *csn5a_1* loss of function mutant show severe phenotype with depleted trichomes and smaller rosette size (Gusmaroli, Figueroa et al. 2007). Because of its interaction with CSN5A and CSN5B, the *P. indica* effector candidate, PIIN_08944 was renamed and from now on would be referred to as Signalosome Interacting Protein 1 (SIP1).

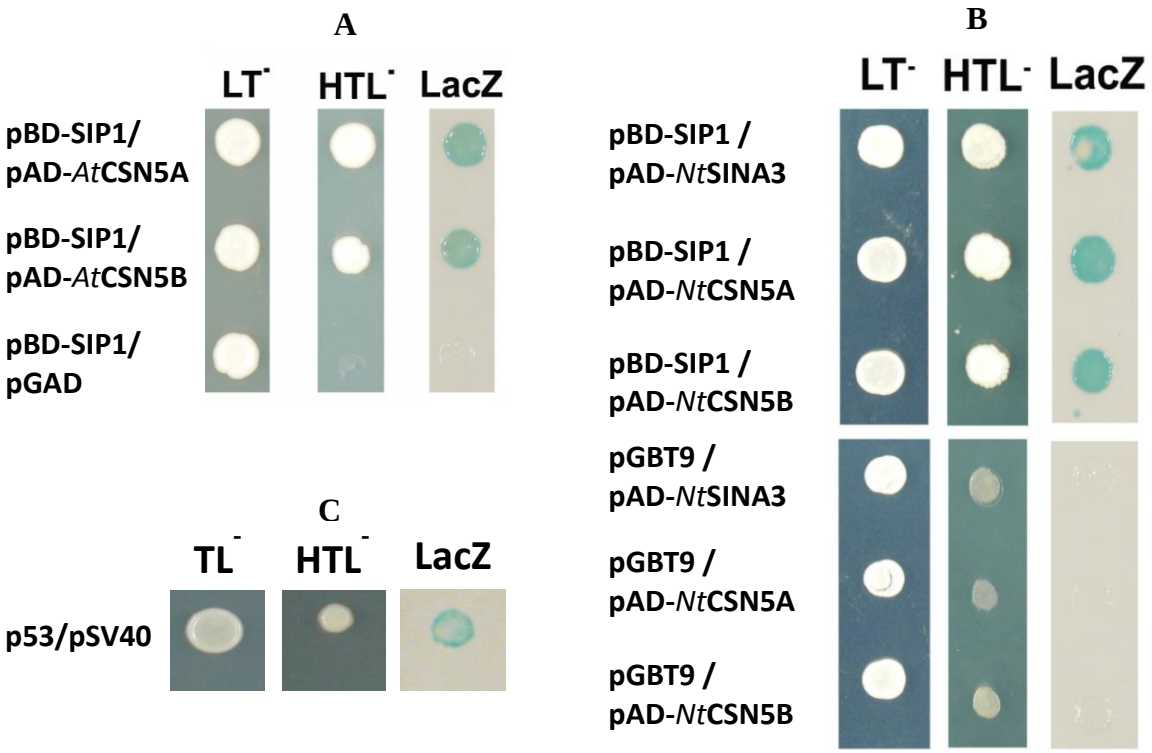


Figure 11. Interaction between SIP1 with CSN5A, CSN5B and SINAT in Yeast Two-Hybrid assay. SIP1 fused to the GAL4 DNA binding domain (BD) was expressed in combination with CSN5A, CSN5B (Fig. A and B) and SINA3 (Fig. B) fused to the GAL4 activation domain (AD) in yeast strain Y190. Cells were grown on selective media before a *LacZ* filter assay was performed. pSV40 / p53 served as positive control (Fig. C) while the empty vectors pGAD and pBD (Fig. A and B) served as negative control. *At*CSN5A = *Arabidopsis thaliana* CSN5A, *At*CSN5B = *Arabidopsis thaliana* CSN5B, *Nt*CSN5A = *Nicotiana tabacum* CSN5A, *Nt*CSN5B = *Nicotiana tabacum* CSN5B, *At*SINA3 = *Arabidopsis thaliana* SEVEN IN ABSENTIA 3 -LT = growth on medium lacking Leu and Trp. -HLT = growth on medium lacking His, Trp and Leu, indicating expression of the HIS3 reporter gene. *LacZ* reporter gene is indicated by LacZ activity.

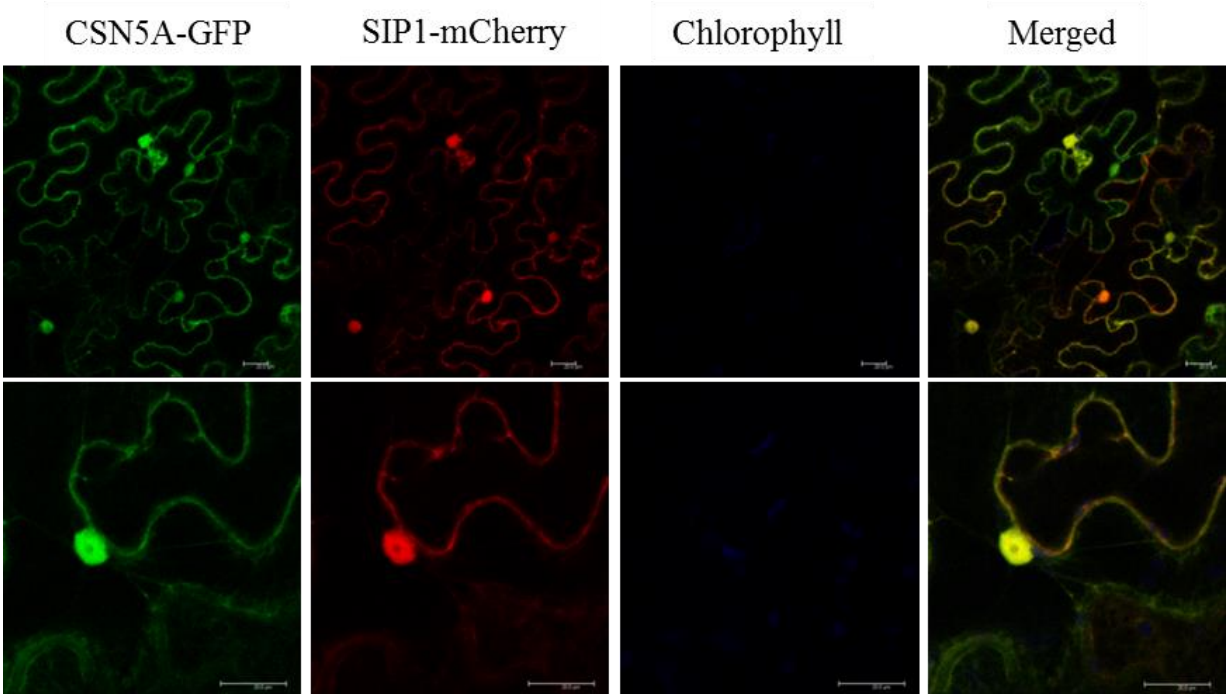
The second protein found to interact with SIP1 belonged to a member of the SEVEN IN ABSENTIA (SINA) family of E3 ligases, SINAT3 – like protein from the tobacco cDNA library (Figure 11B). Blastn search on the NCBI database reveal strong sequence similarity (more than 90%) between the putative interactor and the E3 ubiquitin protein ligase, SINAT3 of *Nicotiana tomentosiformis* (parent specie of tobacco) and *Nicotiana sylvestris* The SEVEN IN ABSENTIA family of E3 ligases was first described in *Drosophila melanogaster* and later in mammals and plants (Carthew and Rubin 1990, Della, Senior et al. 1993). SINA E3 ligase proteins contain an N-terminal RING (Really Interesting New Gene) finger domain, followed by the conserved SINA domain containing two Zn^{2+} finger motifs and involved in substrate binding and dimerization (Hu and Fearon 1999, Depaux, Regnier-Ricard et al. 2006). In mammals, the substrates for SINA E3 protein ligases includes mainly transcription factors and

cytosolic proteins but also membrane proteins and most have been reported to be degraded in a ubiquitin dependent fashion (Habelhah, Laine et al. 2004, Kim, Jeong et al. 2004). Although well characterize in mammals, little is known about the functions and mechanisms of SINA E3 ligases in plants. SINA protein families are highly conserved in plants with six members in Arabidopsis and rice, 10 in poplar (*Populus trichocarpa*) and six in *Medicago truncatula* and *Lotus japonicus* (Den Herder, De Keyser et al. 2008, Wang, Jin et al. 2008, Den Herder, Yoshida et al. 2012). However, only a few have been functional characterized. In Arabidopsis for example, SINAT5 has been shown to regulate lateral root numbers by targeting the NAC1 transcription factor, a member of the NO APICAL MERISTEM / CUP-SHAPED COTYLEDON family) for degradation (Xie, Guo et al. 2002), while SINAT2 was reported to interact with the transcription factor RAP2.2 involve in carotenogenesis (Welsch, Maass et al. 2007). In a recent study by Den Herder, Yoshida et al. (2012), *Lotus japonicus* SINA4 was shown to interact with and destabilizes the Symbiosis Receptor like Kinase (SYMRK) in order to regulate rhizobial infection (Den Herder, Yoshida et al. 2012). E3 ubiquitin ligases and the ubiquitin proteasome pathway have become important targets for fungal effector proteins. However, the outcome of the interaction between SIP1 and SINAT3 E3 ligase needs to be further investigated. As negative control no cell growth or lacZ activity was observed in interaction between the empty vector pGBT9 (binding domain) and CSN5A, CSN5B or SINA3 (Figure 11B). Whereas interaction between pSV40 and p53 served as positive control and resulted in cell growth and a positive lacZ activity (Figure 11C).

3.4.1 Subcellular localization of CSN5A, CSN5B and SIP1 in *planta*

Protein subcellular localization is an important factor to predict and understand protein functions during interaction with other cellular proteins. Because the subcellular localization of each protein varies upon cell entry, it is vital to investigate cellular localization to deduce protein function. It has been reported that gene delivery methods based on Agrobacterium mediated transfer perform significantly better than biolistic in terms of transgene expression, transformation efficiency and inheritance (Rivera, Gomez-Lim et al. 2012). Therefore to examine the subcellular localization of CSN5A, CSN5B and SIP1 and to determine whether they overlap with each other in plant cells, tobacco (*N. benthamiana*) epidermal cells were transiently transformed by Agrobacterium infiltration. The co-localization of both proteins would indicate a possible interaction between the two proteins *in planta*. Through the Gateway® cloning system, the Green Fluorescent Protein (GFP) fused to the C-terminus of *AtCSN5A* and *AtCSN5B* and the SIP1-mCherry fusion constructs all under the control of the

CaMV 35S promoter in the pK7FWG2 and pRB-35S binary vectors were generated (Supplemental figure). The fusion proteins were expressed in 4 to 6-week old *N. benthamiana* leaves through Agrobacterium mediated infiltration (Section 2.7.3). After 48 h, fluorescence signal was investigated on infiltrated leaves using confocal laser scanning microscopy. Fluorescence signals for AtCSN5A-GFP and AtCSN5B-GFP were detected in the nucleus and cytoplasm indicating that AtCSN5A and AtCSN5B are localize in the nucleus and cytoplasm (Figure 12A and 12B). In a previous study, the subcellular localization of CSN5A and CSN5B was reported in the nucleus and cytoplasm (Kwok, Solano et al. 1998). SIP1-mCherry signals were also detected in the nucleus and cytoplasm (Figure 12A and 12B). The co-localization of both AtCSN5A-GFP with SIP1-mCherry and AtCSN5B-GFP with SIP1-mCherry revealed an overlapping fluorescence pattern in the nucleus and cytoplasm, indicating a possible interaction between both proteins *in vivo* (Figure 12A and 12B, merged). A careful observation of the co-localization data would suggest that AtCSN5A-GFP is distributed throughout the cytoplasm and the nucleus, whereas SIP1-mCherry seems to be more confined to the nucleus.



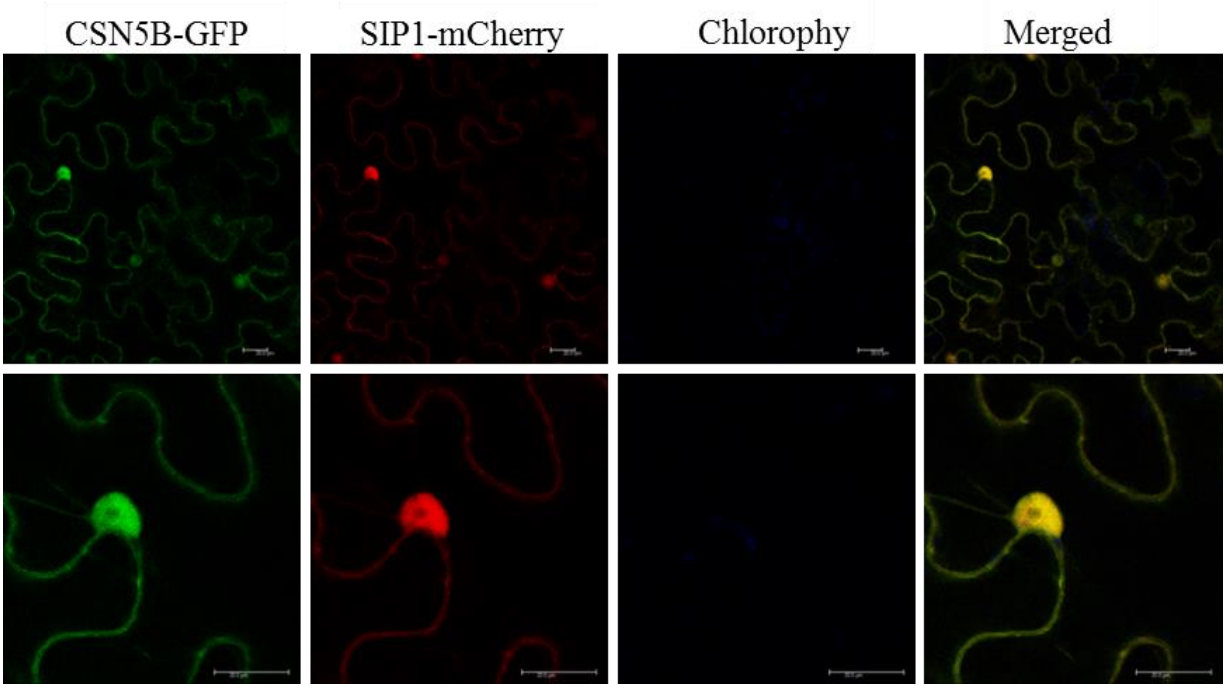


Figure 12. AtCSN5A, AtCSN5B and SIP1 show a nucleo-cytoplasmic localization *in planta*. Subcellular localization of AtCSN5A-GFP, AtCSN5B-GFP and SIP1-mCherry fusion proteins was performed in. 4 to 6-week-old *N. benthamiana* epidermal cells through Agrobacterium mediated transient transformation. A) AtCSN5A-GFP and SIP1-mCherry localized to nucleus and cytoplasm. Both proteins also partially overlapped in the nucleus and cytoplasm during colocalization, an indication of possible interaction between the two proteins *in vivo* B) AtCSN5B-GFP and SIP1-mCherry also localized to the nucleus and cytoplasm and as well displayed an overlap during colocalization, indicating that both proteins could interact *in vivo*. The Green Fluorescence (GFP), Red Fluorescence (mCherry), and chlorophyll autofluorescence (Chl) were monitored separately to prevent cross-talk of the fluorescence channels, and the resulting fluorescence images were merged. Visualization was done using confocal microscopy 48 h after infiltration. Bars = 20 µm

3.4.2 Confirmation of the interaction of SIP1 with AtCSN5A and AtCSN5B *in planta* by bimolecular fluorescent complementation assay

To establish whether the interaction between CSN5A, CSN5B and SIP1 also occur *in planta*, a bimolecular fluorescence complementation (BiFC) assay was performed in *N. benthamiana* (Section 2.7.4). Using the Gateway® cloning system, the full length genes for *AtCSN5A*, *AtCSN5B* and dSP-*SIP1* (deleted signal peptide-dSP) were recombined into a Gateway compatible split VENUS-YFP vector by fusing with the non - fluorescent N-terminal and C-terminal parts of the yellow fluorescent protein ($VENUS^N$ and $VENUS^C$) respectively. Leaves of 4 to 6-week-old *N. benthamiana* plants were infiltrated with *A. tumefaciens* GV3101 carrying the different constructs of interest (Table 2). Two days after infiltration, leaves were collected and immediately observed with a confocal laser scanning microscope (Leica SP2).

The resulting confocal images revealed strong YFP fluorescence in the nucleus and cytoplasm when a combination of SIP1-VENUS^{N} with AtCSN5A-VENUS^{C} was expressed (Figure 13A). In a similar observation, the expression of SIP1-VENUS^{N} with AtCSN5B-VENUS^{C} also produced strong YFP fluorescence in the nucleus and cytoplasm (Figure 13B). These data demonstrated that SIP1 and AtCSN5A as well as SIP1 and AtCSN5B do interact *in planta*, most likely in the nucleus and cytosol. Fructose 1,6-bisphosphatase which forms homodimers in the cytosol served as positive control, as a combination of FBPase-VENUS^{N} and FBPase-VENUS^{C} produced strong YFP fluorescence in the cytosol (Figure 13D). By contrast, the following combinations, FBPase-VENUS^{C} with SIP1-VENUS^{N} or FBPase-VENUS^{N} with AtCSN5A-VENUS^{C} or AtCSN5B-VENUS^{C} which served as negative control produce no fluorescence (Figure 13C)

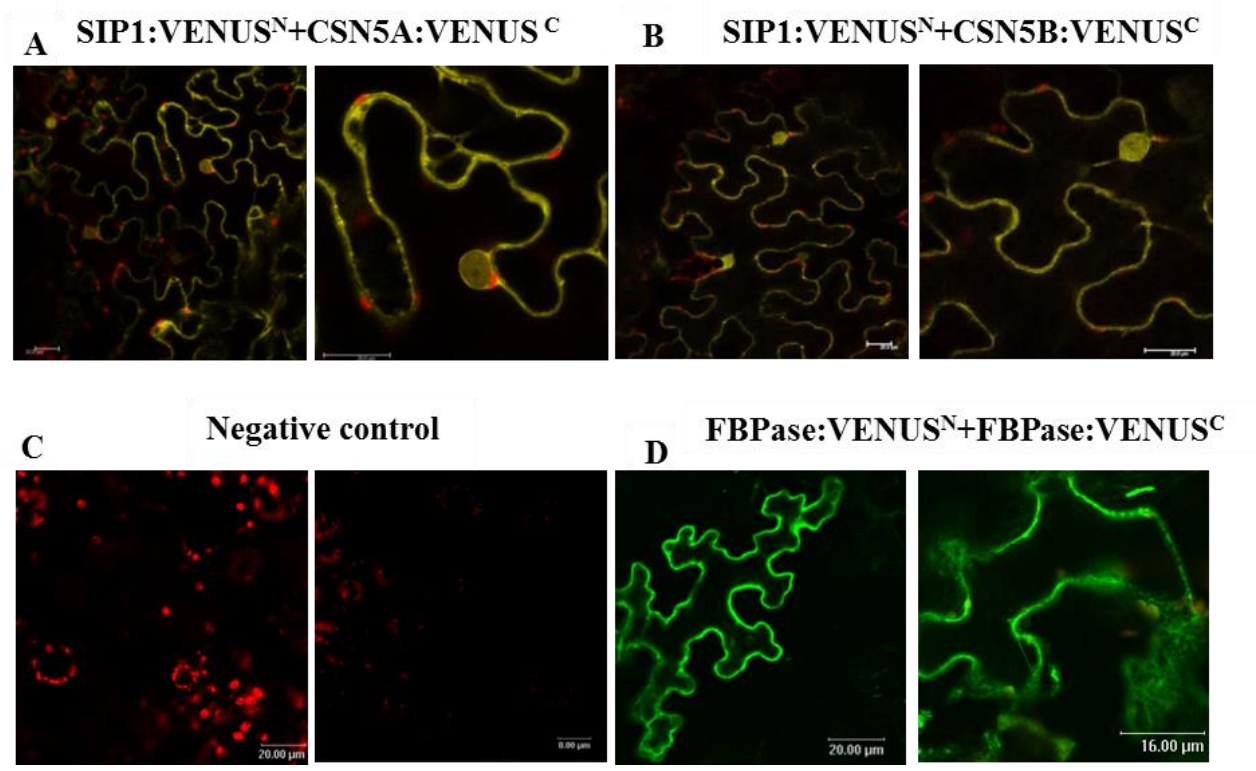


Figure 13. Bimolecular fluorescence complementation (BiFC) analysis of SIP1 interaction with AtCSN5A and AtCSN5B in *Nicotiana benthamiana* cells. Leaves (4 to 6-week-old) were infiltrated with *A. tumefaciens* containing the different YFP contructs and images taken 2 dpi using confocal microscope.Yellow flourescent protein (YFP) confocal microscopy images show tobacco leaf epidermal cells transiently expressing SIP1-YFP^{N} in combination with CSN5A-YFP^{C} **(A)**, and SIP1-YFP^{N} in combination with CSN5B-YFP^{C} **(B)**. Close-up of the same cells show the YFP fluorescence to be nucleo-cytoplasmic. A positive control showing the dimerization of fructose-1,6-bisphosphatase (FBPase) within the cytosol **(C).** Red fluorescence represents Chl autofluorescence. No YFP fluorescence was seen in coinfiltration experiments of SIP1 with CSN5A and CSN5B constructs with the respective FBPase-YFP^{N} or FBPase-YFP^{C} controls. The experiments were repeated several times with similar results. Bars = 20 µm and 8 µm

Table 2 Vector backbone used for BiFC

Gene	Vector backbone	Description
SIP1 (dSP)	pRB35S-Venus^{N173}	N-terminal part of Venus (aa 1 to 173)
	pRB35S-Venus^{C155}	C-terminal part of Venus (aa 174 to 239)
AtCSN5A	pRB35S-Venus^{N173}	N-terminal part of Venus (aa 1 to 173)
	pRB35S-Venus^{C155}	C-terminal part of Venus (aa 174 to 239)
AtCSN5B	pRB35S-Venus^{N173}	N-terminal part of Venus (aa 1 to 173)
	pRB35S-Venus^{C155}	C-terminal part of Venus (aa 174 to 239)
FBPase	pRB35S-Venus^{N173}	N-terminal part of Venus (aa 1 to 173)
	pRB35S-Venus^{C155}	C-terminal part of Venus (aa 174 to 239)

dSP: deleted signal peptide , aa: amino acid. Numbers in bracket indicate amino acid start and stop positions.

3.5 Expression of SIP1 *in planta* as well as in germinated spore

The expression profile of *SIP1* was examined by semi-quantitative RT-PCR in *in-vitro* germinated spore as well as *in-planta*. For this, the transcript level of *SIP1* was quantified from 7 day old *P. indica* spore culture and from *P. indica* inoculated *Arabidopsis* roots at different time points (3, 7, 14, and 21 dai). Transcripts of *SIP1* were detected in *in vitro* germinated *P. indica* spores (Figure 14A), implying that SIP1 is induced during germination, but before host infection has been established and is therefore not dependent on any host signals. In a similar finding, Catanzariti, Dodds et al. (2006) also reported the expression of the Flax rust effector protein, *AvrM* in *in vitro* germinated spores.

During *P. indica* infection of Arabidopsis root, SIP1 transcript was also detected and increased over time (Figure 9B). The absence of transcripts in the early infection stages (3 and 7 dai) is most probably due to less fungal biomass as reflected by transcripts of the *P. indica* housekeeping gene (*PiUBQ*) serving as internal control (Figure 14B). But at the late infection stages (14 and 21 dai), SIP1 transcripts increased significantly probably due to increase in fungal biomass during this period as reflected by transcripts of the *P. indica* housekeeping gene (Figure 14B). The Arabidopsis housekeeping gene (*AtUBQ*) was also used as reference to confirm the quality and quantity of plant cDNA. These results demonstrated that the *P. indica* effector candidate, SIP1 does not require a host signal for induction.

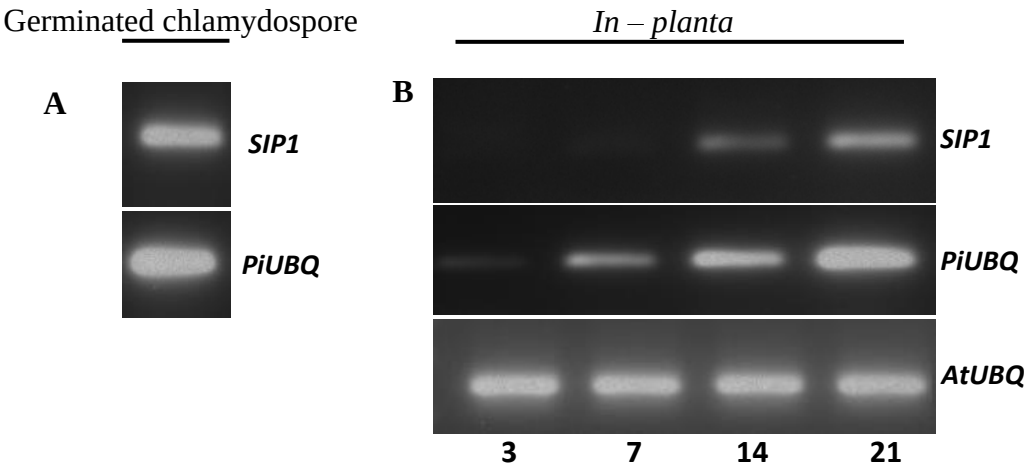


Figure 14. Analysis of *SIP1* expression pattern by semi – quantitative RT-PCR. A)Transcripts of *SIP1* were detected in *in- vitro* germinated *P. indica chlamydo*spores. Spores were grown in CM liquid medium for 7 days and RT-PCT was performed on extracted RNA. **B)** During *P. indica* – root infection, *SIP1* transcript was detected and increased over time (3, 7, 14, and 21 dai) probably due to increasing fungal biomass as reflected by the *P. indica* housekeeping gene (*PiUbiquitin*) used as reference. The Arabidopsis housekeeping gene (*AtUbiquitin*) was also used as control to verify cDNA quality.

3.6 Analysis of *P. indica* RNAi transformants

Generally referred to as RNA interference, post transcriptional gene silencing (PTGS) in plants or gene quelling in filamentous fungi are important and powerful approaches to study gene function through reverse genetics (Waterhouse, Graham et al. 1998). These techniques not only investigate the function of many unknown genes, but also identify core genes which are essential to the growth and survival of the organism (De Backer, Raponi et al. 2002). Through double stranded RNA (dsRNA), small interference RNAs (siRNA) are produced which triggers the degradation of homologous mRNA. In a recent study, Lahrmann, Ding et al. (2013) reported the applicability of RNA interference in the root endophytic fungi, *P. indica* by silencing the high affinity ammonium transporter *PiAMT*. To study the function of SIP1 during *P. indica*-host interaction, *P. indica* RNAi strains were generated carrying silencing construct targeting the *SIP1* gene using the dual promoter RNAi system. Putative RNAi transformants were selected on MYP agar plates containing 50µg/ml hygromycin B and subsequently transferred onto new CM agar plates with 100µg/ml hygromycin B. To verify the success of transformation and the integration of the RNAi construct, PCR was performed on DNA extracted from 3-week-old transformants. Specific primers were used to amplify specific areas of the introduced RNAi construct (Figure 15B, black arrows). Seven positive RNAi transformants were obtained by amplifying the expected amplicon, whereas no

PCR product was amplified from the wild type (WT) control as observed on agarose gel (Figure 15A).

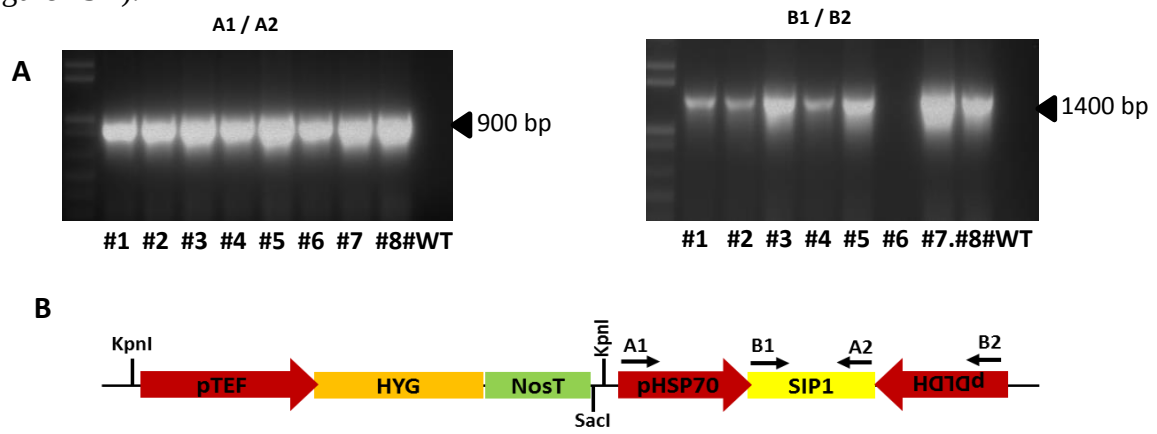


Figure: 15. Analysis of *P. indica* silenced transformants. A) PCR analysis of *P.indica* transformants as well as untransformed wild type (WT) control. Using specific primers, PCR was performed from genomic DNA extracted from *P. indica* transformant and WT control. PCR product was analyzed on 1% agarose gel. Bands corresponding to specific regions on the silencing construct targeting SIP1were detected in 7 transformants, whereas no band was detected in the WT control. B) Structural representation of the dual-promoter silencing vector used to generate *P. indica* RNAi strains. SIP1 is cloned between two convergent promoters (pHsp70 and pDLDH) which produce dsRNA after SIP1 transcription. Black arrows indicates the positions and orientations of the primers used for PCR analysis.

To investigate the silencing efficiency and verify the level of silencing, semi – quantitative RT-PCR was performed on RNA extracted from7-day-old *in vitro* germinated spores of the *P. indica* RNAi strains and wild type control (Figure 16A). The results revealed a decrease in SIP1 transcript accumulation in all RNAi strains as seen by the presence of very weak bands compared to the wild type (WT) control. Although all RNAi strains showed a decrease in SIP1 transcript, the SIP1 expression was found to be dramatically reduced in strains T6 and T8. The *P indica* ubiquitin gene was used as normalization control (Figure 16A).

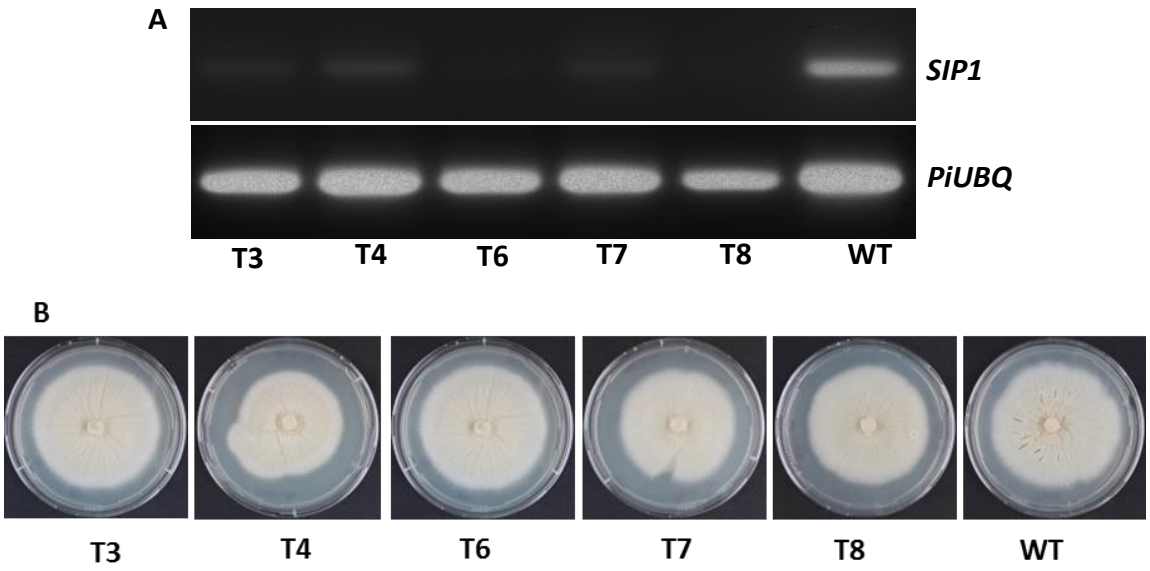


Figure: 16. Relative expression of *SIP1* in spore culture of *P. indica* RNAi strains. A) Transcript accumulation of *SIP1* was determined in 7 day old *in-vitro* germinated chlamydospores of *P. indica* RNAi strains and WT control after RNA extraction, cDNA synthesis and subsequent RT-PCR using SIP1 specific primers. A decrease in SIP1 transcript accumulation was observed in all RNAi strains compared to wild type control. The *P. indica* Ubiquitin housekeeping gene (*PiUBQ*) served as internal control. T3 – T8: *P. indica* RNAi strains, WT: Wild Type. B) The colony morphology and growth rates between *P. indica* RNAi strains and wild type control. Isolates were grown from agar plugs on CM agar plates for 3 weeks at 25^{o}C in the dark.

The colony morphology and growth rates of the *SIP1* silenced transformants were further analyzed on CM agar plates. Mycelial agar plugs of SIP1 silenced transformants and wild type control were placed at the center of CM agar plates and allowed to grow at 25^{o}C for 3 weeks in the dark. Neither abnormal colony morphology nor major difference in the growth rates between *SIP1* silenced transformants and wild type was observed (Figure 16B), indicating that silencing of *SIP1* in *P. indica* does not affect the overall fungal growth.

To investigate *SIP1* silencing during *P. indica*-plant interaction, Chlamydospores of SIP1 silenced transformants and wild type control were inoculated on 7-day-old Arabidopsis roots in square petri dishes under sterile conditions and allowed to grow vertically. Roots were harvested at 3, 7, 14 and 21 dai and semi-quantitative RT-PCR was performed on RNA co-extracted from fungal and plant materials. A decrease in *SIP1* transcript was observed at all the time points for the RNAi strains T7 and T8 (Figure 16.1) compared to the wild type control (Figure 9B). *SIP1* transcript was also reduced at 3, 7 and 14 dai for the RNAi strains T4, T3 and T6 (Figure 16.1) compared to wild type (Figure 9B), but showed a significant accumulation at 21 dai (Figure 16.1). The *P. indica* ubiquitin gene (*PiUBQ*) was used as a normalization control.

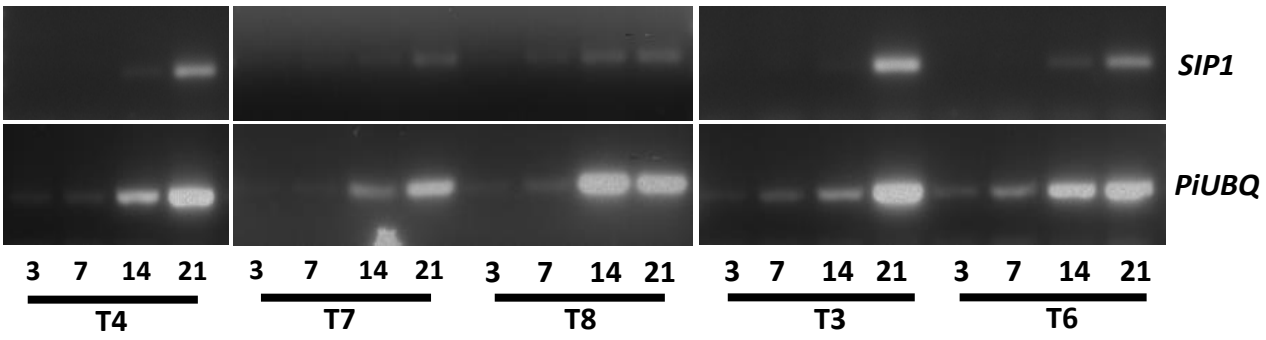


Figure: 16.1. Relative expression of *SIP1* in *P. indica* RNAi strains during interaction with Arabidopsis roots. Seven-day-old Arabidopsis roots were inoculated with *P indica* RNAi strains and WT control. The roots were harvested at 3, 7, 14 and 21 dai and *SIP1* expression was determined after co-extraction of fungal and plant RNA and subsequent RT-PCR using SIP1 specific primer (SIP1). The *P indica UBQ* gene served as internal control. WT: wild type

3.6.1 Analysis of *P. indica* knockout transformants

Unlike RNAi (gene knockdown) where residual mRNA levels of the target gene can still be detected in the silenced organism, gene knockout leads to the complete deletion or replacement of the target gene. Through homologous recombination, *SIP1* knockout *P indica* strains were generated. To verify the integration of the knockout construct in *P. indica*, DNA was extracted from the generated transformants and PCR was performed using specific primers (Table 1). Specific regions were amplified by PCR corresponding to specific areas on the introduced construct (Figure 17B) as revealed by bands on agarose gel (Figure 17A). This result demonstrated that the transformation was successful and the introduced DNA construct was integrated in the genome of *P. indica.*

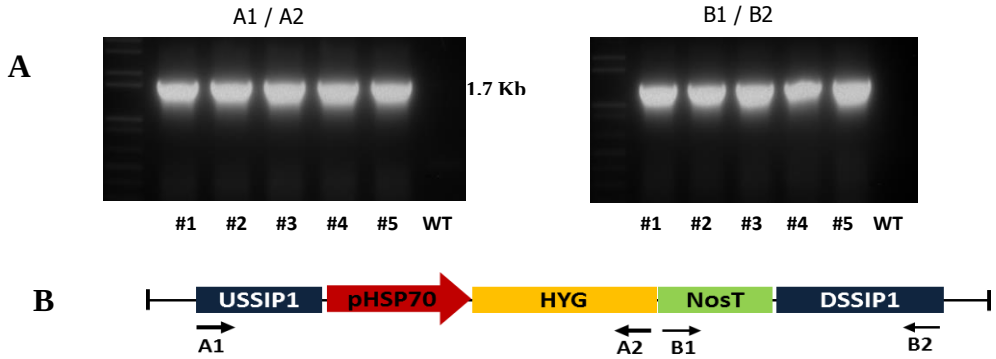


Figure: 17. PCR analysis of *P indica* Knockout transformants. A) DNA was extracted from in vitro germinated spore culture of *P. indica* transformants and wild type. PCR analysis of the extracted DNA revealed bands corresponding to the expected sizes in positive transformants. No band was detected in the WT control. B) Structural representation of the gene replacement construct used to delete *SIP1*. The 5' and 3'flanking regions (USSIP1 and DSSIP1) of SIP1 were PCR amplified and ligated into a vector containing hygromycin B. The vector was linearized and used to transform *P. indica* protoplast. Through homologous recombination SIP1 will be replaced with the hygromycin cassette.

To verify the complete deletion of the *SIP1* gene in the knockout transformants, chlamydospores were collected from selected transformants (Figure 17A) and grown in CM medium for 7 days at 25^{o}C with gentle shaking. RNA was extracted and semi-quantitative RT-PCR was performed to determine *SIP1* expression after cDNA synthesis. RT-PCR results revealed that all five transformants, T1, T2, T3, T4 and T5 showed no accumulation of *SIP1* transcript indicating the deletion of the *SIP1* gene in these transformants (Figure 18A). As expected, *SIP1* transcript could be detected in the wild type control (Figure 18A). No major difference in the colony morphology and growth rates was observed in the transformed strains compared to wild type control. All transformants grew similarly to wild type, except for few

irregular growth patterns which generally occurs during *P. indica* growth (Figure 18B). This data demonstrated that the deletion of *SIP1* in *P. indica* does not affect the overall growth of the fungi.

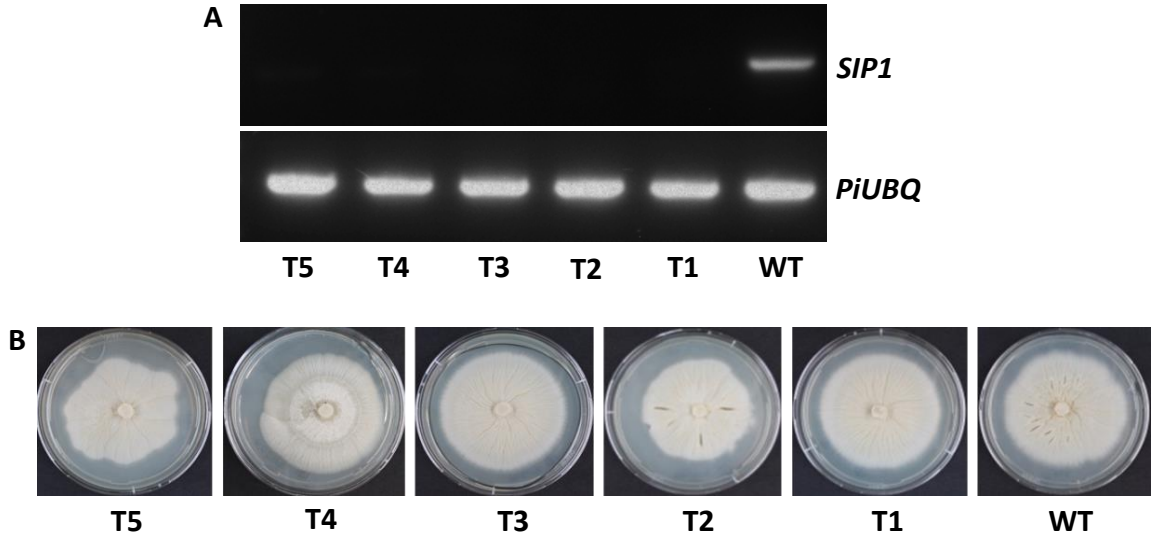


Figure: 18. Analyis of *P. indica* knockout transformants. A) Relative expression of *SIP1* in *in vitro* germinated spores of *P.indica* Knockout strains and WT control by RT-PCR. SIP1 was strongly expressed in the WT compared to the transformants which showed no accumulation of *SIP1* mRNA indicating deletion of *SIP1*. B). Phenotypic analysis of transformants and WT. Fungal growth rates and morphology was similar among transformants and wild type. The *PiUBQ* gene served as internal control.

The deletion of *SIP1* was further investigated during *P. indica in planta* growth. Roots of 7-day-old Arabidopsis seedlings were inoculated with the *P. indica* knockout strains T1 and T2 under sterile conditions. Colonized roots were harvested at 3, 7, 14 and 21 dai, and semi-quantitative RT-PCR was performed on RNA co-extracted from plant and fungal materials. No expression of *SIP1* was observed for strains T1 and T2 at all the analyzed time points (Figure 19.1), demonstrating the complete deletion of *SIP1* and confirming the previous result from Figure 18A. The *P. indica* ubiquitin gene (*PiUBQ*) served as a normalization control (Figure 19.1).

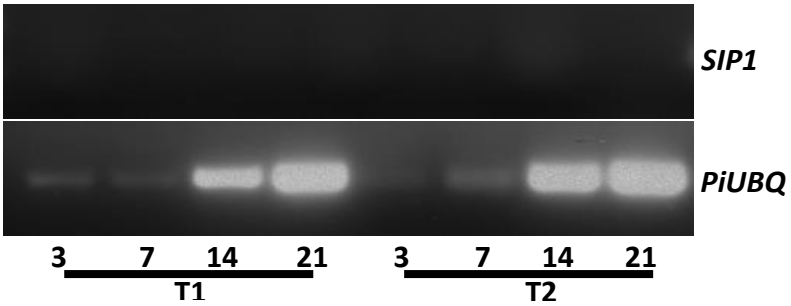


Figure: 19.1. Expression analysis of *SIP1* in *P. indica* KO strains by RT-PCR. Roots of 7-day-old Arabidopsis seedlings were inoculated with chlamydospores of *P. indica* KO strains. The roots were

harvested at 3, 7, 14 and 21 dpi and SIP1 transcript level was determined from extracted RNA by RT-PCR using SIP1 specific primer (SIP1). No *SIP1* transcript accumulated, indicating complete deletion of *SIP1*. The *P. indica Ubiquitin* housekeeping gene (*Pi*UBQ) served as internal control.

3.6.2 Southern blot analysis of *P indica* Knockout transformants

The transformation success and plasmid copy number integration of the knockout strains was further investigated by southern blot. In brief, genomic DNA was extracted from 7-day-old *P. indica* cultures grown in CM medium and digested overnight at 37^{o}C with sacI. The digested DNA was separated on 0.9% TAE agarose gel at 100 V for 4 h and blotted onto a nylon membrane (Amersham Biosciences Hybond+, GE Healthcare) overnight. Hybridization was performed with a ^{32}P radio – labeled hygromycin probe (Prime-a-Gene® Labelling System protocol, Promega). The result obtain indicated that all the transformed strains had one to two copies of T-DNA integration in the genome, except the non-transformed wild type control which had no integration as expected. Transformants T2, T3 and T5 harbored only one copy of T-DNA integration, whereas T1 and T4 had two bands suggesting that two copies of T-DNA existed in the genome of these transformants (Figure 19.2).

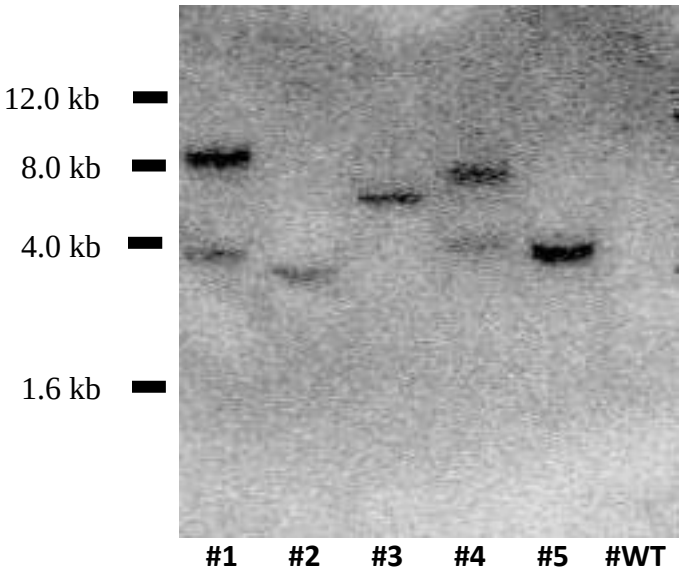


Figure: 19.2. T-DNA copy number analysis in *P. indica* transformants. Southern blot analysis of T-DNA integration for selected P. *indica* knockout transformants. Genomic DNA from *P. indica* transformants and WT was digested with SacI, separated on 0.9% TAE agarose gel, transferred to a nylon membrane (Amersham Biosciences Hybond+, GE Healthcare), and hybridized with a ^{32}P radio-labeled (^{32}P-dCTP) 600 bp fragment of hygromycin B (*hph*). Three out of the five transformants have one copy of T-DNA inserted in the genome, while two have two copies. Lanes 1 – 5: *P. indica* KO transformants, WT*: P. indica* wild type.

3.6.3 Colonization of Arabidopsis roots by *P. indica* RNAi strains

The SIP1 RNAi strains were further investigated for colonization of Arabidopsis roots. Roots of seven-day-old Arabidopsis Col-0, as well as *csn5a_2* and *csn5b_1* mutants were inoculated with *P. indica* RNAi strain and wild type. DNA was extracted at 3, 7, 14 and 21 dai and the fungal biomass was determined as the relative amount of fungal to plant DNA by qPCR. The results revealed a delay in the colonization of the Arabidopsis Col-0, *csn5a_2* and *csn5b_1* roots by the RNAi strain compared to the WT and this delayed colonization was significant at the 3 and 7 dai (Figure 19.3). This data demonstrated that *SIP1* silencing affects *P indica* infection of Arabidopsis roots more especially at the 3 and 7 dai.

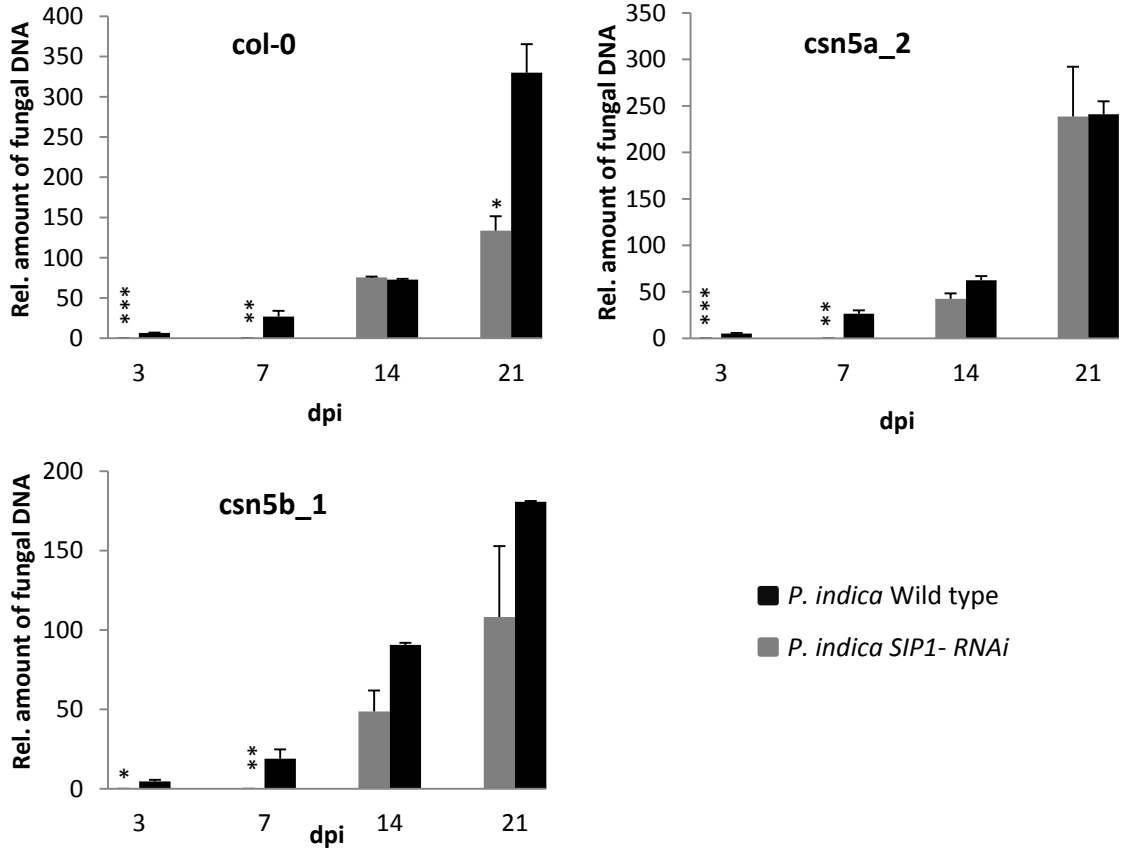


Figure 19.3. *SIP1* silencing delays *P indica* infection of Arabidopsis roots. Seven-day-old Arabidopsis roots were inoculated with *P. indica* RNAi strain or WT control. Fungal biomass was determined at 3, 7, 14, and 21 dai as relative amount of fungal DNA by qPCR using fungal and plant specific primers. Data displays the Ct thresholds of Pi-ITS relative to the Ct thresholds *At*UBQ- 4 using the $^{\Delta\Delta}$Ct method (average Ct thresholds of three technical replicates within one biological experiment are presented). The values represent the means with standard errors of one experiment. Asterisks indicate significance at P < 0.05 (*), 0.01 (**), 0.001 (***) as analyzed by Student's *t*-test. The experiment was repeated three times with similar results (Supplemental Figure S2A and S2B).

3.6.4 Colonization of Arabidopsis roots by *P. indica* Knockout strain

Since the silencing of *SIP1* led to a delayed colonization of Arabidopsis roots by *P. indica*, we questioned whether the deletion of *SIP1* will affect colonization in a similar way. To answer this question, roots of 7-day-old Arabidopsis Col-0, as well as *csn5a_2* and *csn5b_1* mutants were inoculated with *P. indica* knockout strain and wild type. DNA was extracted at 3, 7, 14 and 21 dai and the fungal biomass was determined as the relative amount of fungal to plant DNA by qPCR. Similar to silencing, the deletion of *SIP1* resulted in a significant delay in *P. indica* infection of Arabidopsis roots during all analyzed time points (Figure 19.4).

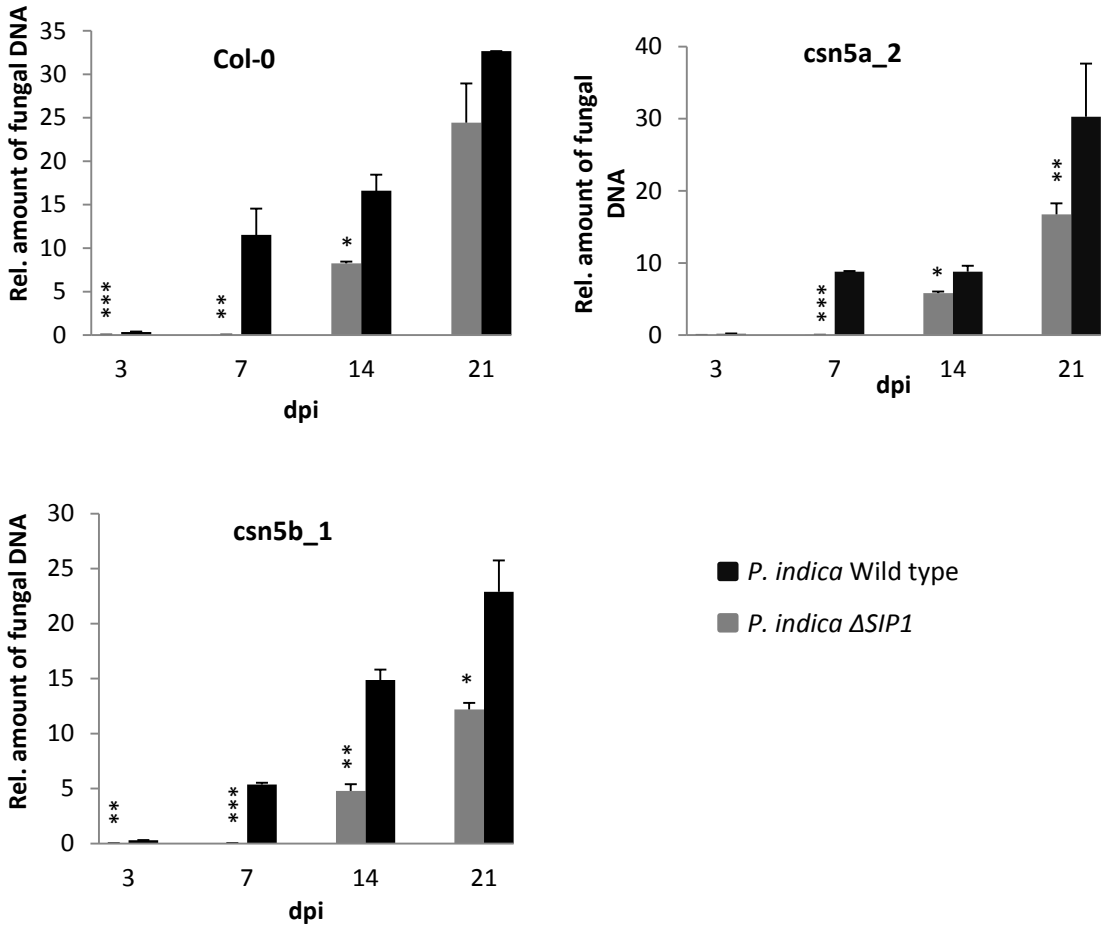


Figure 19.4. Deletion of *SIP1* delays *P. indica* infection of Arabidopsis roots. Seven-day-old Arabidopsis roots were inoculated with *P. indica* deletion strain or WT control. Fungal biomass was determined at 3, 7, 14, and 21 dai as relative amount of fungal DNA by qRT-PCR using fungal and plant specific primers. Data displays the Ct thresholds of Pi-ITS relative to the Ct thresholds *At*UBQ-4 using the $^{\Delta\Delta}$Ct method (average Ct thresholds of three technical replicates within one biological experiment are presented). The values represent the means with standard errors of one experiment. Asterisks indicate significance at P < 0.05 (*), 0.01 (**), 0.001 (***) as analyzed by Student's *t*-test. The experiment was repeated three times with similar results (Supplemental Fig. S3A and S3B)

3.7 Identification of transgenic barley and Arabidopsis expressing *SIP1*

Using the generated plant expression vector (Section 2.3.1), stable transgenic barley plants were produced through the stable root transformation system (STARTS) (Imani, Li et al. 2011). Transgene integration was confirmed by PCR analysis after DNA extraction. Several independent transgenic lines (T0) were selected and self-pollinated to produce T1 lines. Expression of the GFP-SIP1 fusion protein in seedlings of transgenic progenies (T1 or T2 generations) was confirmed by western blot. The result indicated that the fusion protein was correctly expressed with bands corresponding to the expected protein size (Figure 20A). There was no observable difference in plant growth between *SIP1* transgenic lines and GFP or WT control indicating that the expression of *SIP1* did not affect the growth and development of barley under normal conditions.

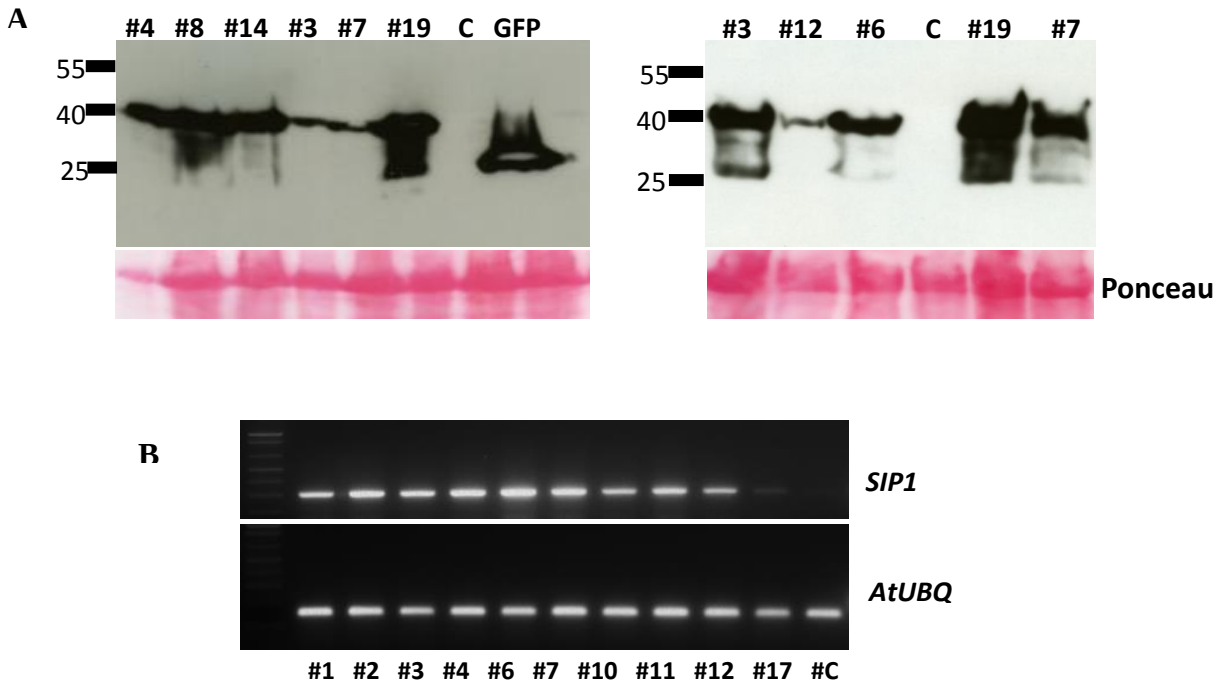


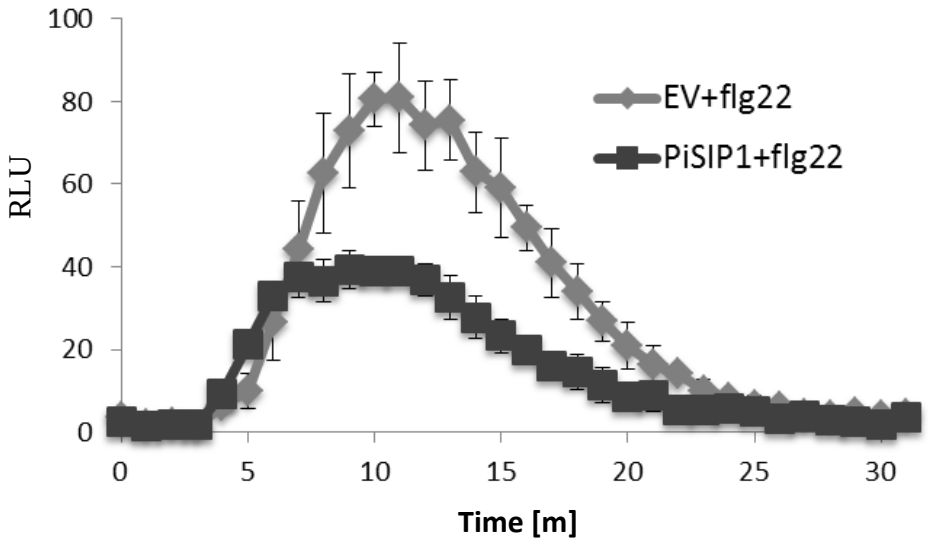
Figure 20. Characterization of *SIP1* transgenic barley and Arabidopsis. A) Western blot analysis of transgenic barley expressing GFP-SIP1 fusion protein using antibody against GFP. No band observed in WT control plants and a lower GFP band observed in the GFP control. B) RT-PCR analysis of independent transgenic Arabidopsis lines (T1). The upper panel represents the gene fragment for SIP1and the lower panel represents the Arabidopsis ubiquitin used as internal control. #1 - #19: independent transgenic lines C: Control (golden promise), GFP: Green Fluorescent Protein.

Stable transgenic Arabidopsis plants expressing *SIP1* were produced through Agrobacterium-mediated floral dip transformation (Clough and Bent 1998). Transgene integration was confirmed by PCR analysis after DNA extraction and T0 transgenic lines were self-pollinated

to produce T1 generation. The expression of *SIP1* in T1 generation was confirmed by RT-PCR after RNA extraction using gene specific primers, whereas no expression was visible in the WT control (Figure 20B). The Arabidopsis *ubiquitin* gene (*AtUBQ*) served to normalize the result. No difference in growth was observed between the *SIP1* transgenic and WT control lines, indicating that *SIP1* expression did not also affect the growth of Arabidopsis under normal conditions.

3.7.1 Heterologous expression of SIP1 in barley suppresses flg22 and Chitin induced ROS burst

To examine whether the expression of *SIP1* in planta will affect ROS generation after elicitor treatment, leaf discs were collected from 2 to 3-week-old stable transgenic barley plants expressing *SIP1* and empty vector control. After incubation with water overnight in the dark, the leaf discs were treated with 100 nM flg22 or 100 mg/ml chitin (crab shell) and water control and the ROS burst was measured through a luminol based assay by measuring the relative light unit over time as a result of the production of oxygen radicals. As expected, control plants exhibited a ROS burst after treatment with flg22 or chitin (Figure 21A and 21B). The chitin induced hydrogen peroxide accumulation was almost completely suppressed (Figure 21B) and the flg22 induced hydrogen peroxide accumulation was reduced by 50 % in transgenic plants expressing *SIP1* relative to control plants (Figure 21A). These data demonstrated that the *P. indica* effector candidate, SIP1 may contribute to *P. indica*-host colonization by interfering with basal defense.



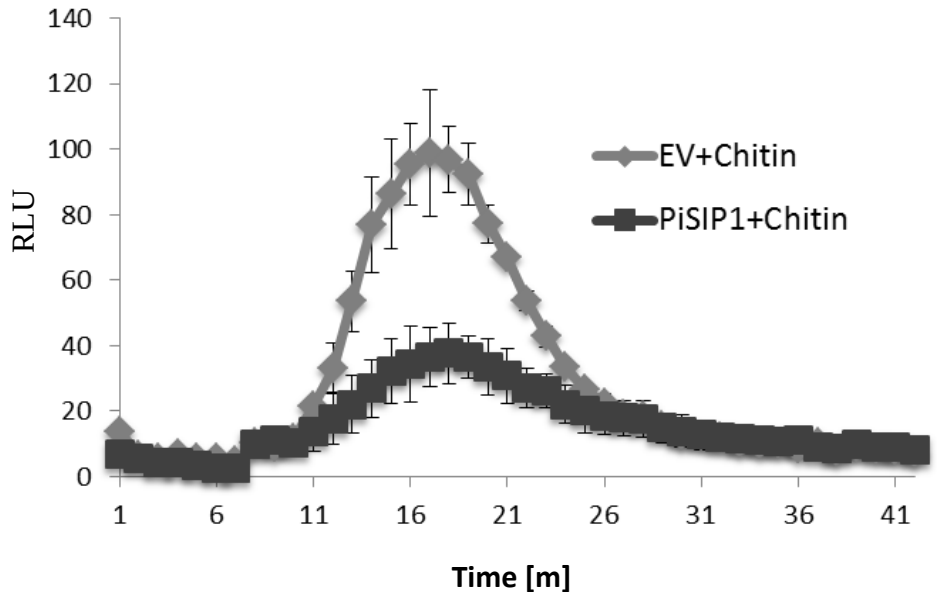


Figure 21. Measurement of flg22 and Chitin induced ROS burst in transgenic (SIP1-Ex) and wild type (EV) barley leaves. Leaf disks from 3-week-old *SIP1*-Ex and EV control barley plants were treated with (A) 100 nM flg22 , and (B) 200 mg/ml crab shell chitin. ROS was detected by measuring the relative light unit over time with a luminol-chemiluminescence assay using a Tecan reader. Error bars represent the ±SE of three independent experiments. (RLU: relative light units, EV: empty vector, *SIP1*-Ex: *SIP1*-expressing barley).

3.7.2 *P. indica* colonization of transgenic barley expressing *SIP1*

Because expression of *SIP1* in barley interfered with PAMP induced ROS production, the question became, could SIP1 contribute to *P. indica* virulence and the mutualistic symbiosis with the host through basal defense suppression? To answer this question, roots of transgenic barley seedlings expressing *SIP1* and wild type control were inoculated with *P. indica* chlamydospores under sterile conditions. Inoculated roots were harvest at 3, 7, 14, and 21 dai and the relative amount of fungal to plant DNA was determined by qRT-PCR after DNA extraction. The results revealed a significant increase in the colonization of the transgenic barley plants expressing *SIP1* at 3, 7 and 14 dai compared to the control plants. However, colonization of *SIP1* transgenic lines dropped at 21 dai compared to control plants (Figure 22). This data demonstrated that SIP1 contribute to *P. indica* virulence during interaction with the plant host.

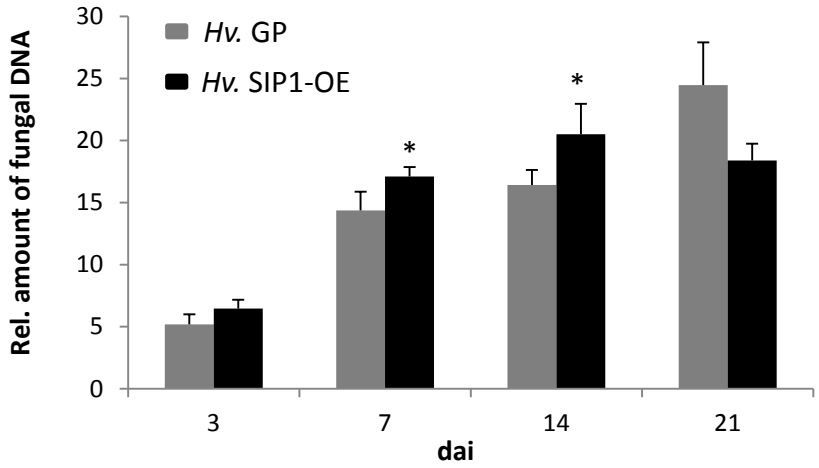


Figure: 22. Enhanced *P. indica* colonization of transgenic barley expressing *SIP1*. Three-day-old transgenic barley seedlings and GP control were inoculated with *P. indica* chlamydospore. Root materials were harvested at 3, 7, 14 and 21 dai, genomic DNA was extracted and the relative amount of fungal biomass was determined by qRT-PCR using plant and fungal specific primers. Data represent the mean of two experiments, with error bars indicating standard deviation (SD). Asterisks indicates significance at $P < 0.05$ (*) analyzed using student's *t* test.

3.7.3 *Fusarium graminearum* infection of transgenic barley expressing *SIP1*

To investigate whether the *SIP1* induced effector triggered susceptible (ETS) could lead to susceptibility to other pathogens, transgenic barley plants expressing *SIP1* were challenged with the necrotrophic fungal pathogen, *F. graminearum* (IFA 65). Briefly, leaves of three-week-old barley plants expressing *SIP1* and empty vector control were detached and placed on water agar containing 0.4% benzimidazole in square petri dishes. Leaves were inoculated with spores of *F. graminearum* (IFA 65) and disease symptoms were analyzed 6 dai. Lesion size was similar between the *SIP1* expressing leaves and empty vector control (Figure 23E). qRT-PCR analysis of extracted DNA revealed that there was no significant difference in the relative amount of fungal biomass on the *SIP1* transgenic and empty vector control leaves (Figure 23D).

In a similar assay, roots of transgenic barley seedlings expressing *SIP1* and empty vector control were inoculated with spores of *F. graminearum* (IFA 65) and transferred to pots containing a 2:1 mixture of seramis and oil-dri. Two weeks after inoculation roots were assessed for disease by measuring the root length and root fresh weight. Results revealed no significant difference in the root length and root fresh weight between the *SIP1*expressing lines and the empty vector control line (Figure 23B and 23C). Phenotypic analysis also revealed no significant difference in root length between *SIP1* transgenic and control line

(Figure 23 A). These data indicated that the SIP1 induced ETS was specific to *P. indica* and does not compromise basal defense to the necrotrophic pathogen, *F. graminearum*.

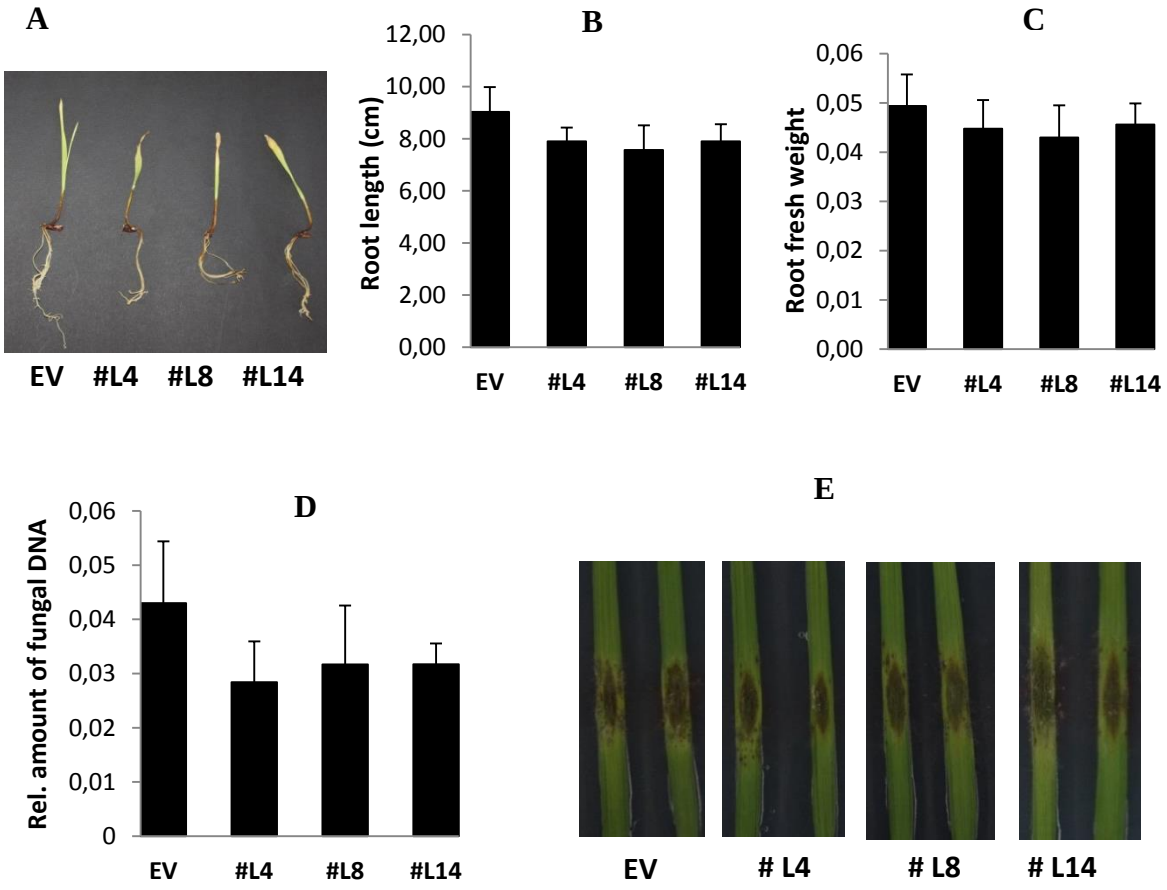


Figure 23. A) Phenotype of *SIP1* transgenic plants inoculated with *F. graminearum.* Roots of transgenic barley (T2 generation) and EV control were inoculated with spores by root dip. Infected seedlings were transferred to pots containing seramis oil-dri (2:1). Photographs were taken 2 weeks after inoculation. B) Average root length of transgenic barley inoculated with *F. graminearum*. Averages were calculated from 6 plants per line. C) Average root fresh weight of transgenic barley inoculated with *F. graminearum*. Averages were calculated from 6 plants per line. These experiments were repeated three times with similar outcome. D). Relative fungal biomass on detached transgenic and EV barley leaves inoculated with *F. graminearum*. E) Detached leaves of 3-week-old transgenic barley plants were inoculated with *F. graminearum* spores. Photographs were taken 6 dai using a canon digital camera. The experiment was repeated three times with similar outcome.

3.7.4 Colonization of *SIP1* transgenic Arabidopsis plants by *P. indica* deletion strains

When roots of Arabidopsis Col-0 plants were inoculated with the *P. indica* Δ*SIP1*deletion strain, a delayed colonization was observed especially during the early biotrophic stages, 3 and 7 dai (Figure 19.4). To investigate whether the deletion of *SIP1* contributed to the delayed

colonization, a complementation assay was performed. Roots of transgenic Arabidopsis plants expressing *SIP1* were inoculated with chlamydospores of *P. indica* Δ*SIP1* deletion strain under sterile conditions. Roots were harvested at 3, 7, 14 and dai and fungal biomass analyzed by qRT-PCR. The results revealed that the colonization of *SIP1* transgenic roots strongly recovered to wild type levels during the early biotrophic stages, 3 and 7 dai, and was significantly enhanced compared to wild type plants during the late biotrophic (cell death colonization) stages, 14 and 21 dai (Figure 24). This data demonstrated that *SIP1* may contribute to the mutualistic symbiosis between *P. indica* and plant roots.

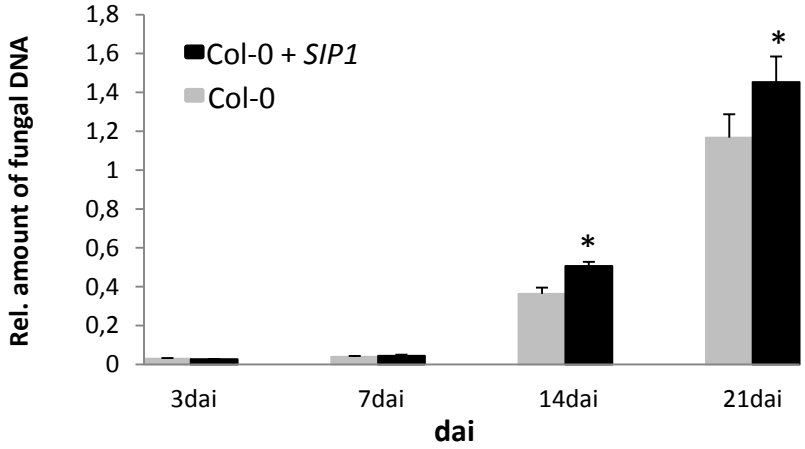


Figure 24. Colonization of *SIP1* transgenic Arabidopsis with *P. indica* Δ*SIP1* deletion strain. Roots of 7-day-old transgenic Arabidopsis seedlings expressing *SIP1*or wild type (Col-0) control were inoculated with *P. indica* Δ*SIP1* deletion strain. Roots were harvested and fungal biomass determined by qRT-PCR at 3, 7, 14 and 21 dai using fungal (ITS) and plant (Ubq-4) pecific primers. Data displays the average Ct thresholds of three technical replicates within one biological experiment of ITS relative to the Ct thresholds of Ubq- 4 using the $^{\Delta\Delta}$Ct method. The values represent the means with standard errors of one experiment. Asterisks indicate significance at $P < 0.05$ (*), as analyzed by Student's *t*-test. The experiment was repeated twice with similar results (Supplemental Figure S3C.)

3.7.5 SIP1 interfere with transcription of flg22 induced defense marker genes

In a previous study, Jacobs, Zechmann et al. (2011) reported that *P. indica* was able to suppress flg22 triggered activation of *WRKY22*, *WRKY33* and *WRKY53*, marker genes for MTI : (Colcombet and Hirt 2008) as well as the salicylic acid marker gene *CBP60g*: (Wang, Tsuda et al. 2009) during colonization of Arabidopsis root. *P. indica* probably recruits JA to counter SA signaling during root colonization as was observed by up-regulation of *VSP2*, a marker gene for JA in *P. indica*-colonized roots. Recent research revealed that secreted protein 7 (SP7), an effector protein secreted by the arbuscular mycorrhizal fungi, *Glomus intraradices* contributed to the biotrophic development of arbuscular mycorrhiza fungi in

roots through its interaction with the pathogenesis-related transcription factor ERF19 in the nucleus leading to suppression of the plant defense (Kloppholz, Kuhn et al. 2011). In a similar way, MiSSP7 (Mycorrhiza-induced Small Secreted Proteins), an effector of the mutualistic fungus *Laccaria bicolor* was reported to enhance fungal colonization by protecting and stabilizing JAZ6, a transcriptional repressor protein, from jasmonic acid (JA) induced degradation and also by inhibiting JA induced gene transcription in *populous trichocarpa* (Plett, Daguerre et al. 2014). Therefore it is likely that, similarly to pathogenic fungi, mutualistic fungi can also use effectors to target the host transcription machinery and hormone signaling to enhance colonization. To investigate whether *P. indica* could deploy SIP1 to interfere with host defense response, we examined the expression levels of defense marker genes after flg22 treatment.

Arabidopsis seedlings were cultured in ½ MS agar containing 1% sucrose for two weeks before carefully transferred to ½ MS medium containing 1% sucrose under sterile conditions. Seedlings were treated with 100 nM flg22 and samples were collected at 0, 2, 6 and 12 hours to analyze the expression levels of *AtWRKY22* and *CBP60g* by qRT-PCR after RNA extraction. Expression of the MTI marker gene, *AtWRKY22* was significantly reduced 2 and 6 hours after flg22 treatment while expression of the salicylic acid marker gene, CBP60g was reduced at 2, 6 and 12 hours after flg22 treatment with a significant reduction observed at 6 hours (Figure 25), suggesting that *P. indica* could deploy effectors to counter host defense during colonization by interfering with host gene transcription.

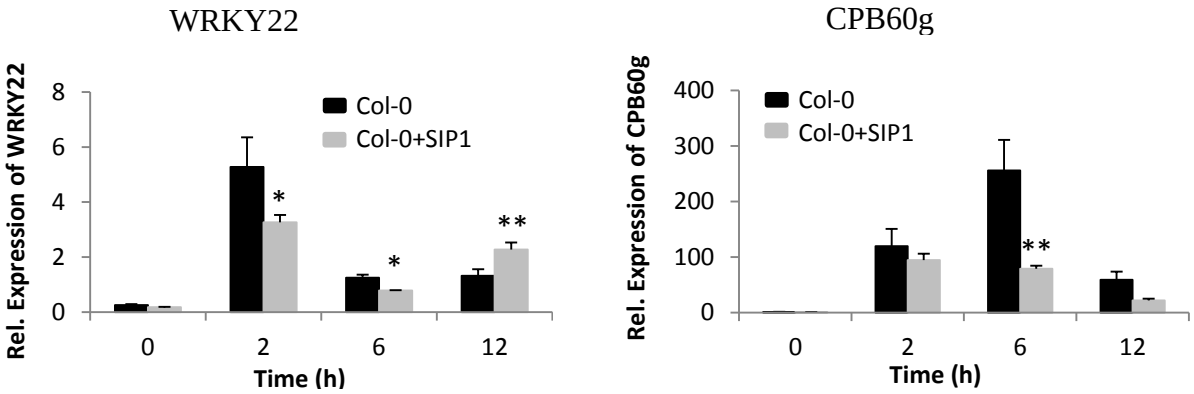


Figure: 25. Suppression of flg22-induced gene transcription as determined by qRT-PCR. Seven to ten-day-old *SIP1* transgenic Arabidopsis seedlings and Col-0 control were treated with flg22 (100 nM) under sterile conditions in 6 well plates containing ½ MS medium. RNA was extracted at 0 (untreated control), 2, 6, and 12 hours after treatment and qRT-PCR performed. Suppression of flg22-induced transcription was observed for *AtWRKY22* (MTI marker), and *CBP60g* (SA marker). Arabidopsis gene *UBQ-4* was used as normalization control. The experiment was repeated twice with similar results (at least 10 – 15 seedlings were used per time point). Asterisks indicate significant differences at P< 0.05 (*), 0.01 (**) as analyzed by student's *t*-test.

3.7.6 *Botrytis cinerea* infection of transgenic Arabidopsis expressing *SIP1*

Using the necrotrophic fungal pathogen, *B. cinerea* 05.10, transgenic Arabidopsis leaves expressing *SIP1* and wild type control were tested for susceptibility to a necrotrophic pathogen. Four to six-week-old leaves of transgenic Arabidopsis expressing *SIP1* and Col-0 control were detached and placed onto 1 % agar in square petri dishes. The leaves were inoculated with spores of *B. cinerea* (2 x 10^5 spores / ml) by pipetting 3 µl spore suspension on both sides of the middle vein. Five days after inoculation disease symptoms analysis revealed no visible difference between *SIP1* expressing and Col-0 control plants (Figure 26A). Determination of the average lesion size using the Image J software also showed no difference in the infected leaf area relative to the uninfected area on both *SIP1* transgenic and Col-0 control lines (Figure 26B). Again indicating that the SIP1 induced ETS was specific to *P. indica* and does not compromise host defense to the necrotroph, *B. cinerea*.

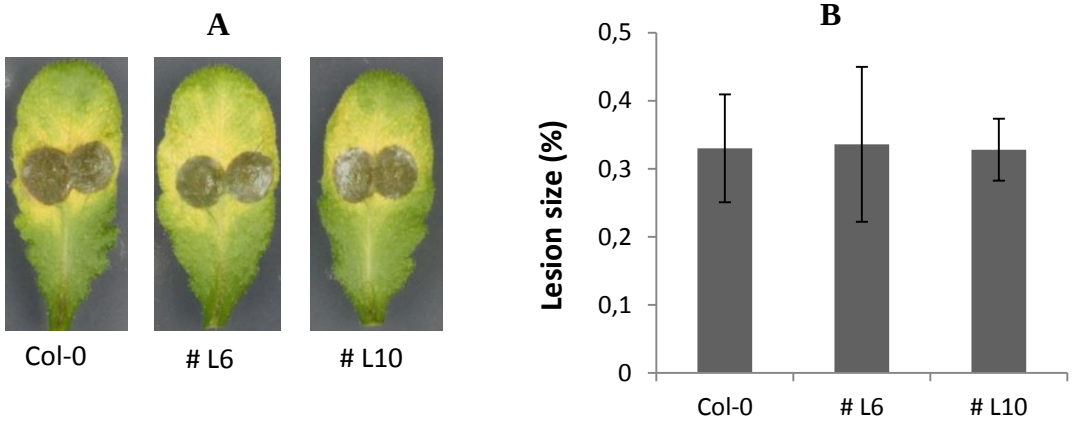


Figure 26. Infection analysis of *SIP1* transgenic Arabidopsis. A) Phenotype of SIP1 transgenic leaves inoculated with *B. cinerea*. Detached leaves of 4 to 6-week-old transgenic Arabidopsis plants were inoculated with *B. cinerea* spores. Photographs were taken 5 dai. B) Average lesion size of transgenic Arabidopsis inoculated with *B. cinerea*. Averages were calculated from 10 – 15 leaves per line. The experiment was repeated three times with similar results.

3.7.7 Salt (NaCl) induce stress response of *SIP1* transgenic Arabidopsis plants

In a previous study Waller, Achatz et al. (2005), reported that *P. indica* protects barley plants from salt stress as well as induces resistance against the head blight fungal pathogen *Fusarium culmorum* and systemic resistance against barley powdery mildew *Blumeria graminis* by altering the antioxidant status of colonized plants. However the molecular mechanism underlining this process has not been elucidated and whether *P. indica* could deploy effectors to interfere with the host antioxidant status has not been shown. Therefore to investigate whether the *P. indica* effector candidate, SIP1 could alter the antioxidative system

of plants and contribute to salt stress tolerance, an antioxidant assay was performed on *SIP1* transgenic Arabidopsis as well as *csn5a_2* and *csn5b_1* mutants to measure levels of the antioxidants glutathione and ascorbate after salt treatment. Upon exposure to NaCl (salt stress) fresh weight of all plants decreased compared to the untreated controls (Figure 27A). The loss of fresh weight in percentage was higher in all lines compared to the wild type control which showed the lowest weight loss (Figure 27B).

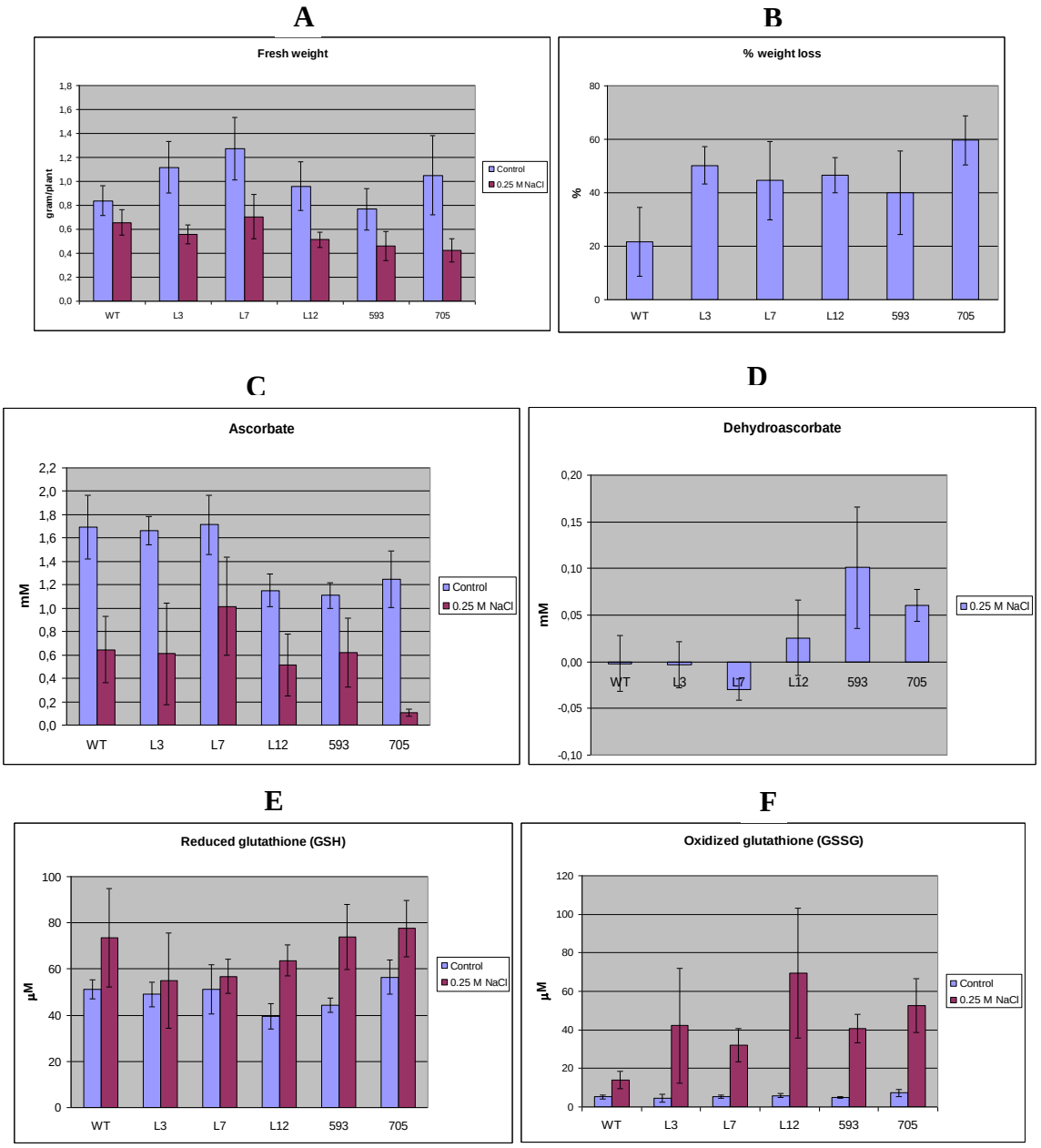


Figure 27. Transgenic Arabidopsis expressing *SIP1* as well as *csn5a_2* and *csn5b* mutants are sensitive to NaCl. Six-week-old plants grown in peat moss soil (4 plants / pot) were irrigated with 250 mM NaCl every second day for up to 14 days and (A) The fresh weight as well as the percentage weight loss were determined. (B) and (C) the Change in the redox balance after salt treatment was analyzed. In general WT plants showed the lowest sensitivity to salt stress compared to all the transgenic and mutant lines. WT: wild type, L3 – L12: SIP1, 593: *csn5b_1*, 527: *csn5a_2*.

Meanwhile the ascorbate content was slightly lower in the lines L12, 593, 705 compared to wild type before treatment with NaCl. After exposure to NaCl, levels of reduced ascorbate decreased in all lines with a strong reduction observed for line 705 making it the most sensitive to salinity among all the lines (Figure 27C). In all untreated lines dehydroascorbate (DHA) was hardly detectable (data not shown), likewise in NaCl treated lines of WT (wild type), L3, L7 and L12. However detection occurred in the lines 593 and 705 (Figure 27 D). Levels of reduced glutathione (GSH) were similar in all lines prior to NaCl treatment and were induced after NaCl treatment (Figure 27E). Oxidized glutathione (GSSG) was induced in salt-treated plants, with the lowest induction observed in the WT control which is in agreement with their weight loss (Figure 27F). These data indicated that wild type plants were less sensitive to salt stress, with the line 705 probably the more sinsitive. However this difference in sensitivity was not significant among the lines. Put together, this data suggestted that SIP1 does not interfer with the antioxidant status *in planta* and probably does not contribute to salt stress tolerance in Arabidopsis.

4. Discussion

Unlike previously thought, it is now widely accepted that similarly to plant pathogenic fungi, plant mutualistic fungi also secrete effector proteins inside plant cells to modulate the host cell and promote symbiosis (Kloppholz, Kuhn et al. 2011, Plett, Kemppainen et al. 2011). The recent release of the *P. indica* genome resulted in the identification of several gene families encoding hydrolytic enzymes such as metallopeptidases and cell wall degrading enzymes (CWDE) indicating saprotrophic characteristics, but also hundreds of genes coding for small secreted proteins (SSPs; <300aa). These proteins are considered candidate effectors, features typically associated with biotrophy (Zuccaro, Lahrmann et al. 2011). However virulence functions of these effector candidates are largely unknown. *P. indica* which exhibits an endophytic life style is capable of colonizing the roots of several plant species and confers various beneficial traits to the host plants including growth promotion, biotic stress resistance, abiotic stress tolerance, higher yield, enhanced nitrate and phosphate assimilation (Peskan-Berghofer, Shahollari et al. 2004, Sherameti, Shahollari et al. 2005, Waller, Achatz et al. 2005, Yadav, Kumar et al. 2010). Recognition of *P. indica* by plants, induces plant defense response, which is swiftly suppressed as soon as the fungus penetrates the root cortex cells and establishes a biotrophic relationship (Jacobs, Zechmann et al. 2011). It is highly likely that *P. indica* deploys effector proteins to suppress and/or evade plant immune response. In this thesis an effector candidate of *P. indica* designated SIP1 (Signalosome interacting protein 1) was functionally characterized *in planta*. The question of whether and how SIP1 contribute to the interaction between *P. indica* and plant roots was addressed.

Until recently, most of the research on fungal effector proteins in plant-microbe interaction has focused mainly on phytopathogenic fungi. However, the recent realization that mutualistic fungi also carry genes coding for putative effectors (Martin, Aerts et al. 2008, Zuccaro, Lahrmann et al. 2011) highlighted the importance of effectors in plant-microbe interaction. Plant associated microbes secrete effector proteins to circumvent and/or evade plant immune responses in order to promote microbial growth. Although a larger number of genes coding for SSPs (putative effectors) has been reported in the genome of *Laccaria bicolor* (Martin, Aerts et al. 2008), at the time of this thesis, only two mutualistic fungal effectors had been functionally characterized. SP7, an effector secreted by the arbuscular mycorrhizal fungus *Glomus intraradices* promote root symbiosis by targeting the pathogenesis related transcription factor ERF19 in the nucleus thereby suppressing plant defense responses (Kloppholz, Kuhn et al. 2011). Similarly, MiSSP7 an effector secreted by

the ectomycorrhizal fungus *Laccaria bicolor* also enters root cells and localize to the nucleus where it inhibits jasmonic acid (JA) induced gene expression by targeting the jasmonic acid repressor protein JAZ6. MiSSP7 also reprograms the transcriptome of the host (*Populus trichocarpa*) by modulating activity of genes implicated in the alteration of root architecture such as *auxin/indole-3-acetic acid (AUX/IAA), glycoside hydrolase 3 (GH3)* and *CLAVATA3/ESR-RELATED5 (CLE5)* thereby promoting symbiosis (Plett, Kemppainen et al. 2011, Plett, Daguerre et al. 2014). Similarly to SP7 of *G. intraradices* and MiSSP7 of *L. bicolor*, the *P. indica* effector candidate, SIP1 does not contain cysteine residues.

4.1 Arabidopsis and barley roots colonized by *P. indica* show increased fungal biomass and multi-lobed extracellular hyphae

The beneficial effects transferred by *P. indica* onto host plants are reflected by the increase in fungal biomass on colonized roots. During root infection by *P. indica*, fungal biomass increases over time as the root endophyte undergoes intracellular and extracellular sporulation, producing new chlamydospores and eventually more hyphal growth (Zuccaro, Lahrmann et al. 2011, Das, Kamal et al. 2012, Pedrotti, Mueller et al. 2013). Therefore, I analyzed the colonization of Arabidopsis and barley roots by *P. indica*. Our data revealed that fungal biomass increased significantly overtime from three- to 21 dai during *P. indica* colonization of barley (Figure 9.2) as well as Arabidopsis roots (Figure 9) which is in line with previous results. Root colonization was further substantiated by confocal laser scanning microscopy on WGA AF-stained colonized roots which could only stain extracellular fungal structures. Similarly to previous reports showing that *P. indica* produce multi-lobed invasive hyphae (Lahrmann, Ding et al. 2013), I observed extensive *P. indica* hyphae on colonized roots which increased over time (Figure 9.1) and these hyphae appeared irregularly swollen in shape with a multi-lobed formation. The formation of multi-lobed hyphae by *P. indica* during root colonization is reminiscent of the bulbous invasive hyphae formed by the hemibiotrophic fungal pathogen *Magnaporthe oryzae* during rice (*Oryza sativa*) infection and are important for the formation of the biotrophic interfacial complex (BIC) which accumulates effector proteins that are subsequently secreted inside plant cells (Khang, Berruyer et al. 2010). During root infection, *P. indica* forms branched intracellular biotrophic hyphae which penetrate root cell walls and are invaginated by the plant plasma membrane (Lahrmann, Ding et al.). These *P. indica* hyphal structures could function as site of effector secretion inside plant cells as reported for phytopathogenic fungi such as *M. oryzae* or as site for nutrients and

signal exchange between the plant host and the fungus similarly to the arbuscules of the arbuscular mycorrhiza fungi (Parniske 2008).

4.2 Small secreted proteins (SSPs) coding for candidate effectors in the genome of *P. indica*

The genome of *P. indica* (25 Mb) contains hundreds of small secreted proteins (SSPs) (<300 aa) considered candidate effectors and about 10% of the genes induced during the colonization of barley roots encode SSPs (Lahrmann and Zuccaro 2012). The high expression level of *P. indica* genes encoding SSPs *in planta* is analogous to many other fungal effector proteins that are upregulated during host colonization and suggest that these SSPs may contribute to the establishment of a symbiotic interaction between *P. indica* and plant roots either by facilitating host penetration or evasion/suppression of host immune response. The identification of motifs on some of *P. indica* SSPs such as a regular pattern of histidine and alanine residues, cysteine residues, and a highly conserved pattern of seven amino acids at the C-terminus "RSIDELD" (called DELD proteins) (Zuccaro, Lahrmann et al. 2011) hinted at possible conserved molecular functions for these sets of SSPs. For example, the cysteine residues in the cysteine-rich SSPs may function in the formation of disulfide bridges which are important for protein stability in the protease-rich apoplastic space as reported for several cysteine-rich effectors of fungal pathogens. Whereas the conserved C-terminal DELD motif maybe important for effector delivery inside plant cell as suggested for the N-terminal conserved Y/F/WXC motifs of barley powdery mildew effectors or as reported for the well-studied RXLR motifs of oomycetes (Godfrey, Bohlenius et al. 2010, Duplessis, Cuomo et al. 2011, Kale and Tyler 2011, Garnica, Upadhyaya et al. 2013). Most of the DELD proteins are less than 135 amino acid in size and do not possess functional protein domains. It is also worth noting that a significant number of *P. indica* SSPs carry either one or a combination of the following carbohydrate binding motifs; LysM (Lysin Motif), WSC (cell Wall integrity and Stress response Component), or CBM1 (Carbohydrate-Binding Module family 1) whose functions in *P. indica* are still unknown. Recent reports showed that the *Cladosporium fulvum*, LysM domain containing effectors; Avr4 and Ecp6 as well as the *Mycosphaerella graminicola* effectors; Mg3LysM and Mg1LysM binds and sequester chitin fragments from the apoplast thereby protecting fungal cell wall from plant secreted chitinases (van den Burg, Harrison et al. 2006, de Jonge, van Esse et al. 2010, Marshall, Kombrink et al. 2011). Considering all that is known about LysM proteins from other fungi, the LysM domain containing SSPs in *P. indica* might play a role in chitin binding and sequestration during *P.*

indica root symbiosis. Not much is known about glucan binding WSC and cellulose binding CBM1 proteins in fungi. However, the WSC domain containing proteins of *S. cerevisiae*, Wsc1, Wsc2 and Wsc3 acts as sensors of cell wall integrity (Rodicio and Heinisch 2010) whereas the CBM1 containing protein of *Trichoderma reesei*, swollenin functions as expansin on cellulose-containing cell walls (Saloheimo, Paloheimo et al. 2002). Similarly to the LysM proteins, the WSC containing SSPs of *P. indica* may also function in glucan binding and sequestration. In addition to hundreds of SSPs encoded in the *P. indica* genome, the expansion domains such as LysM, WSC and CBM1 might also act as important determinants of symbiosis during interaction between this endophyte and plant roots. However, until now the functions of SSPs in *P. indica* can only be hypothesized. Therefore elucidating the functions of SSPs would provide us with molecular insight into the colonization strategies developed by *P. indica* on different hosts.

4.3 The *P. indica* effector candidate SIP1 is expressed in *in-vitro* germinated spores as well as *in planta*

To investigate the expression profile of *SIP1*, RT-PCR was performed on RNA extracted from *in-vitro* germinated *P. indica* chlamydospores as well as from Arabidopsis roots colonized by *P. indica*. Our results demonstrated that the *P. indica* effector candidate, SIP1 is expressed in *in-vitro* germinated spores as well as in planta. Transcripts of SIP1 were detected in RNA of in-vitro germinated *P. indica* spores and also in RNA from Arabidopsis roots infected with *P. indica* (Figure 14). The increase in abundance of SIP1 transcript which could barely be detected at three and seven dai, but then accumulated significantly at 14 and 21 dai, (Figure 14) probably reflects the increase in fungal biomass observed during *P. indica* root colonization. This expression pattern suggests that SIP1 is induced during germination of *P. indica* spores, but before host penetration and biotrophic interaction has been established and induction was not dependent on any host signals. A similar situation was observed with the flax rust effector, AvrM which was also found to be induced not only in *in-vitro* germinated spores, but also *in planta* (Catanzariti, Dodds et al. 2006). The expression of effectors in *in-vitro* germinated spores was also observed for the wheat stripe rust fungal pathogen, *Puccinia striiformis* f.sp. *tritici* (*Pst*) (Garnica, Upadhyaya et al. 2013). Based on this data, we conclude that secretion of the *P. indica* effector candidate, SIP1 is independent of any host signal and also it is possible that SIP1 could be secreted by *P. indica* prior to host cell penetration as a recognition signal and/or to prepare host cells for fungal penetration.

4.4 The *P. indica* effector candidate SIP1 targets the evolutionarily conserved host proteins, CSN5A and CSN5B *in planta*

The identification of the molecular targets of effector proteins is vital in elucidating their functions in planta. In this study, we demonstrated using a yeast two hybrid screen and bimolecular fluorescent complementation assay that the *P. indica* effector candidate, SIP1 interacts with CSN5A and CSN5B, two isoforms representing the CSN5 subunit of the COP9 signalosome (CSN) but also with SEVEN IN ABSENTIA 3 (SINA3) a member of the SINAT family of E3 ligase (Figure 11A and 11B). The CSN is conserved multiprotein complex found in all eukaryotes and composed of eight subunits, CSN1-8. The main function of the CSN is to regulate the cullin-RING ubiquitin E3 ligases (CRLs) which form the largest family of ubiquitin E3 ligases. The CSN regulates the CRLs by cleaving the covalently linked RUB1 (related to ubiquitin 1) protein from the cullin subunit of the CRL E3 complexes in a process known as derubylation. Among the CSN subunits, CSN5 is the only catalytic subunit with an isopeptidase activity which catalyzes the derubylation of cullins. CSN5 is involved in the regulation of several plant developmental processes including flower development, auxin signaling, root elongation, photomorphogenesis and reproductive growth (Gusmaroli, Figueroa et al. 2007, Jin, Li et al. 2014). Targeting of the CSN5 by SIP1 suggest that *P. indica* could secrete effectors which interfere with the ubiquitin-proteasome pathway to promote symbiosis. This would be a first scenario of a mutualistic fungal effector targeting the ubiquitin-proteasome system. However, given the remarkable capacity of *P. indica* to colonize the roots of several plant species, it is reasonable to suggest that the fungus has evolved strategies to target evolutionary conserved host pathways such as the CSN5-mediated ubiquitination pathway. The targeting of the ubiquitin-proteasome pathway has been reported previously for effectors secreted by pathogenic fungal and bacterial. AvrPiz-t, an effector protein secreted by the hemibiotrophic fungal pathogen Magnaporthe oryzae interacts with and destabilizes the rice RING E3 ubiquitin ligase APIP6 during blast infection thereby suppressing APIP6 E3 ligase activity and promoting infection. In turn, APIP6 ubiquitinates AvrPiz-t and target it for degradation through its E3 ligase activity (Park, Chen et al. 2012). On the other, the oomycete effector Avr3a interacts with and stabilizes, *in planta*, the host U-box E3 ligase CMPG1 to prevent INF1mediated host cell death during the biotrophic phase of infection (Bos, Armstrong et al. 2010). Recently, Ustun, Konig et al. (2014) showed that HopZ4, a T3SS effector of the bacterial pathogen Pseudomonas syringae targets the proteasomal subunit RPT6 *in planta* and inhibit proteasome activity during host infection. It is evident that the manipulation of the host ubiquitin-proteasome system by effectors is an

efficient virulence mechanism of phytopathogens specialized on different host plants. Through a systematic protein-protein interaction network, Wessling, Epple et al. (2014), revealed that effector proteins from three evolutionarily highly diverse phytopathogens including the ascomycete *Golovinomyces orontii*, oomycete *Hyaloperonospora arabidopsidis* and eubacterial *Pseudomonas syringae* interacts with CSN5A indicating interspecies convergence of effectors to a common host target and strongly suggest that such common host targets are functionally relevant to the pathogen. In a separate, but similar study Mukhtar, Carvunis et al. (2011) also reported that 29 distinct effectors from *Hyaloperonospora Arabidopsis* and *Pseudomonas syringae* interact with CSN5A and that the N-termini of NB-LRR proteins and the cytoplasmic domains of RLKs also interacts with CSN5A. The geminiviral virulence protein C2 interacts with CSN5, *in planta*, and inhibits ubiquitination by altering the CSN-mediated derubylation of SCF E3 ligase complexes (Lozano-Duran, Rosas-Diaz et al. 2011). Since CSN is a key regulator of several plant developmental processes through its CSN5-mediated rubylation activity and many proteins are substrates for CSN5-dependent degradation, the ability of phytopathogenic effectors but also effectors of mutualistic fungi such as *P. indica* to interfere with or hijack the activities of CSN5 could plausibly alter the protein turnover of the cell immune function in favor of the invading microbe. Targeting of CSN5, an evolutionarily conserved eukaryotic protein involved in several plant developmental processes by the *P. indica* effector, SIP1, might be one of the strategies evolved by this root endophyte to be able to colonize and promote the growth of a very wide spectrum of plant species.

4.5 CSN5A, CSN5B and SIP1 localizes to the nucleus and cytoplasm

The subcellular localization of proteins is very vital in revealing the possible function of a particular protein. Eukaryotic cells are subdivided into various subcellular compartments all of which contain unique proteins and contribute differently to the full functioning of the cell (Lunn 2007). Since proteins have several cellular destinations, in order to elucidate their function, it is important to determine the localization of each protein after entry into the host cell. For this, the full length gene of *CSN5A* and CSN5B were fused at the N-terminal of the green fluorescent protein (GFP) (CSN5A::GFP and CSN5B::GFP), and SIP1 (without its signal peptide) was fused to mCherry (SIP1::mCherry). Using Agrobacterium-mediated transient transformation of *N. benthamiana* leaves and confocal microscopy CSN5A and CSN5B revealed a nucleo-cytoplasmic localization (Figure 12). This result is in line with previous results which also showed that CSN5A and CSN5B were localized to nucleus and

cytoplasm (Kwok, Solano et al. 1998). Similarly, the *P. indica* effector candidate, SIP1 also localized to the nucleus and cytoplasm (Figure 12). Both proteins also co-localized in the nucleus and cytoplasm, indicating that they may interact in these locations. Interaction was subsequently confirmed *in planta* using BiFC (Figure 13). Since CSN5 localizes to the plant cell nucleus and cytoplasm where it carries out its function of derubylation, it is reasonable to suggest that upon secretion the *P. indica* effector candidate, SIP1 enters plant cell and targets CSN5 at these locations. The ability of SIP1 to move to the nucleus would explain its interference with defense marker gene transcription. The interaction between SIP1 and CSN5 could lead to several outcomes. On the one hand SIP1 may inhibit or enhance the derubylation activity of the CSN through its interaction with CSN5 thereby interfering with the functioning of several cullin RING E3 ligases including CUL1 based SCF complexes, CUL2 based VCB, CUL3 based BCR and CUL4 E3 Ubiquitin ligases (Lyapina, Cope et al. 2001, Schwechheimer, Serino et al. 2001, Zhou, Seibert et al. 2001, Groisman, Polanowska et al. 2003). Defects in any of these cullin RING E3 complexes could potentially influence a tremendous amount of plant cellular processes such as, flower development, auxin response, root elongation, and reproductive growth. Meanwhile on the other hand SIP1 itself maybe substrate for CSN5-dependent degradation.

4.6 The role of SIP1 during the colonization of plant roots by *P. indica*

To investigate the functional role of SIP1 during *P. indica*-root interaction, independent transgenic lines of *P. indica* with lowered production of SIP1 were generated using RNA silencing (RNAi) or complete absence of SIP1 were generated using homologous recombination. Reduced accumulation or absence of the SIP1 transcript was confirmed by RT-PCR (Figure 16 and Figure 18). Southern blot analysis revealed one to two transgene integration (Figure 19.2). Silencing of SIP1 resulted to a significant delay in the colonization of Arabidopsis roots by *P. indica* especially at the early biotrophic phase (3 and 7 dai) but also at the later colonization phase (14 and 21 dai) (Figure 19.3). A similar but even stronger effect was observed with the complete loss of function SIP1 mutant (knockout), which did not only delay root colonization at the early biotrophic phase, but also at the later phase (Figure 19.4). When roots of transgenic Arabidopsis plants expressing *SIP1* were inoculated with P. indica knockout strain the delayed colonization was recovered to wild type level at the early biotrophic phase and enhanced at the later phases (Figure 24) indicating that SIP1 was responsible for the delayed colonization. Similarly, transgenic barley plants expressing SIP1 also showed enhanced colonization by *P. indica* (Figure 22). However the reduced

colonization observed for barley at 21 dai (Figure 22), though not significant, might be due to the different colonization strategies evolved by *P. indica* on these hosts. On the one hand a switch to cell death colonization at later time points has been reported in barley; whereas in Arabidopsis *P. indica* seems to establish a long lasting biotrophic relationship (Lahrmann, Ding et al. 2013). This is not the first report of a mutualistic fungal effector contributing to host colonization. MiSSP7 and SP7, two effectors of ectomycorrhizal and arbuscular mycorrhizal fungi respectively were shown to contribute to root mycorrhization (Kloppholz, Kuhn et al. 2011, Plett, Kemppainen et al. 2011). However this would be the first report of a *P. indica* effector protein contributing to the mutualistic symbiosis between *P. indica* and plant host. Nevertheless, the expansion of small secreted proteins (SSPs) considered candidate effectors in the genome of *P. indica*, many of which are induced in Arabidopsis and barley (Lahrmann, Ding et al. 2013) may represent general determinants for the establishment of biotrophy during root colonization by this endophyte. In fact, an important hallmark of biotrophy is fungal effector proteins, which corresponds to the presence of hundreds of candidate effectors in the genome of *P. indica* among which is SIP1. Therefore the data presented here suggest that the *P. indica* effector candidate, SIP1 may indeed contribute to the establishment and maintenance of biotrophy during plant roots colonization by *P. indica*.

4.7 SIP1 suppresses PAMP triggered immunity (PTI) as well as marker genes expression

Among the best studied PAMP molecules are the bacterial flagellin (flg22) and fungal chitin which are recognized by the pattern recognition receptors (PRRs), FLAGELLIN SENSING II (FLS2) and CERK1and/or CEBiP respectively leading to PTI. Previous reports indicated that *P. indica* suppresses PAMP triggered immunity induced by flg22 and Glc8 (an octamer of fungal chitin, *N*-acetylchitooctaose) including oxidative burst and defense gene transcription such as the PTI marker, *WRKY22* and the SA maker *CBP60g* (Jacobs, Zechmann et al. 2011). In this study, barley plants expressing the *SIP1* gene without its signal peptide nearly completely suppressed ROS generation triggered by the PAMP elicitor chitin (crab shell) (Figure 21B), while ROS generation triggered by flg22 was reduced by almost 50% (Figure 21A). In addition, *SIP1* transgenic Arabidopsis seedlings showed a significant reduction of the PTI marker gene *WRKY22* and the SA marker gene *CBP60g* following flg22 induction (Figure 25). Hormonal cross-talk greatly affect plant microbe interaction outcome. While ethylene (ET) and jasmonic acid (JA) activates defense against necrotrophs, salicylic acid activates defense against biotrophs and JA-ET signaling and SA signaling generally act antagonistically to one another following microbial attack (Glazebrook 2005, Spoel and Dong

2008). Earlier reports have shown that *P. indica* inhibits SA signaling and PTI defenses by recruiting JA (Jacobs, Zechmann et al. 2011) which favors our result that SIP1 suppresses the SA marker gene *CBP60g*. Based on these data, *SIP1* could contribute to *P. indica* virulence by inhibiting SA signaling and PTI, thereby suppressing early defense responses to *P. indica* during host infection. We therefore cautiously suggest that the mutualistic root endophyte, *P. indica* secrete effector proteins including SIP1 to facilitate its accommodation within plant roots.

To ascertain whether the *SIP1* mediated basal defense suppression was specific for *P. indica* or would also affect the plant response to pathogenic fungi, pathogen infection assays were performed on SIP1 transgenic plants. For this, transgenic barley plants expressing *SIP1*were challenged with the necrotrophic fungal pathogen *F. graminearum*. After analyzing the root length, root fresh weight, and lesion size no difference in disease symptom development was observed between the *SIP1* transgenic plants and wild type control (Figure 23). In a similar way, transgenic Arabidopsis expressing *SIP1* were challenged with the necrotrophic fungal pathogen, *B. cinerea*. Disease symptom analyses revealed that infection of the transgenic plants were comparable to the wild type control (Figure 26). This observation is in contrast to the effector protein SP7 of the arbuscular mycorrhizal fungus, *G. intraradices* which in addition to suppressing host defense was also found to extend the length of the biotrophic infection phase of the hemibiotrophic fungal pathogen *M. oryzae* by delaying root decay which characterizes the necrotrophic phase (Kloppholz, Kuhn et al. 2011). However, the difference between this study and mine is that, SP7 was expressed in *M. oryzae* which was then used to infect the roots of rice plants (*O. sativa*), whereas *SIP1* was expressed *in planta* and the transgenic plants were challenged with fungal pathogens. Based on these data, I speculate that the observed *SIP1* mediated basal defense suppression was specific for *P. indica* and does not interfere with the plants ability to response to fungal pathogens.

Based on these observations, a schematic model of the mode of action of the effector candidate SIP1 during the interaction between *P. indica* and plant host has been proposed (Figure 28). Following host cell penetration, *P. indica* secretes SIP1 which enters plant cell and targets subunit 5 (CSN5) of the COP9 signalosome (CSN) in the nucleus and cytoplasm. SIP1 interferes with basal defense by suppressing PAMP triggered ROS burst and also inhibits flg22 induced transcription of defense marker genes including the *WRKY22* and *CBP60g*. (Figure 28).

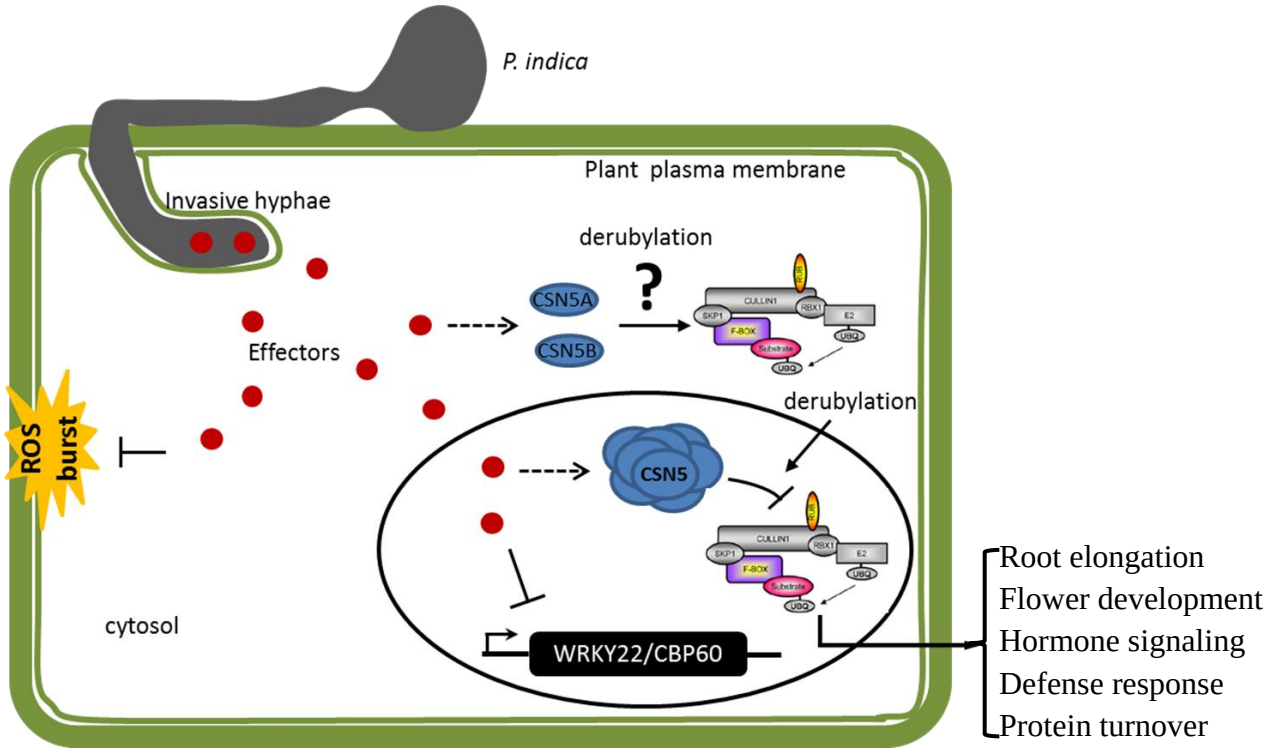


Figure 28. Proposed model for SIP1 function during colonization of plant roots by *P. indica*. *P. indica* penetrates plant cell and extends invasive hyphae which is surrounded by a plant derived plasma membrane. Hyphae secrete effectors (red dots), some of which enter the cytosol through unknown mechanisms. One of these effectors, SIP1 suppresses PAMP triggered ROS burst and also targets CSN5A and CSN5B in the cytosol. SIP1 then moves into the nucleus where it interacts with the CSN5 subunit of the CSN and also inhibits transcription of defense marker genes *WRKY22* and *CBP60g*. CSN derubylates cullin-RING E3 ubiquitin ligases (CRLs) through its CSN5 metalloprotease activity and regulates the ubiquitin / 26S proteasome pathway. Several hundred distinct CRLs exist in plants regulating multiple cellular processes such as root elongation, flower development, hormone signaling, defense responses, gene expression and protein degradation. SIP1 interaction with CSN5 could interfere with these CRL regulate processes or could lead to SIP1 degradation through the ubiquitin proteasome pathway.

4.8 Conclusion and future perspective

The identification and characterization of more effectors is and will increase our knowledge on the concepts of biotrophy and thus will enhance our understanding of plant-microbe interaction. However new questions are also being raised. The present study which aimed at getting molecular insight into the role of SIP1, an effector candidate of the root endophytic fungus *Piriformospora indica* reveal that SIP1 might contribute to the mutualistic symbiosis during root colonization by *P. indica*. In order to contribute to mutualism, SIP1 suppress PAMP induced ROS as well as PAMP induced transcription of defense gene. It also targets the eukaryotic conserved protein CSN5. The fact that *P. indica* would target an evolutionarily conserved plant protein, might constitute a compatibility factor required by *P. indica* to manipulation plant hosts and be able to colonize the roots of wide range of plant. I speculate that SIP1 may indirectly interfere with the stability of CRLs by targeting the CSN5-mediated deneddylation of CRLs or alternatively SIP1 may itself be a substrate manipulated by CSN5 for degradation through the ubiquitin proteasome pathway. However this assumption still needs to be further investigated. In addition, it would be interesting to study the secretion of SIP1 *in planta* using transgenic approaches and also to investigate the mechanism of SIP1 translocation into plant cells.

Given these data, the role played by other *P. indica* effector candidates should be investigated as well as the identity of their plant-based interactors which may also control *P. indica* growth within plant roots. This would reveal how beneficial microbes including the root endophyte,

5. Summary / Zusamenfassung

5.1 Summary

Plants have evolved a sophisticated two-layered immune system to protect themselves from microbial invaders. However, in order to promote infection, plant associated microbes including pathogens as well as mutualists secrete molecules called effectors to suppress the plant's immune response. Mutualistic fungi including the arbuscular mycorrhiza (AM) *Glomus intraradices* and the ectomycorrhiza (ECM) *Laccaria bicolor* secretes effector proteins which contribute to the establishment and maintenance of mutualistic symbiosis by suppressing host immunity during root colonization.

The root endophytic fungus, *Piriformospora indica* is capable of colonizing the roots of a wide variety of different plant species including the monocot barley (*Hordeum vulgare*) and the dicot *Arabidopsis thaliana* and establishes mutualistic symbiosis during colonization. The colonization pattern of *P. indica* is mainly divided into two phases, an initial biotrophic phase and a later cell death-associated colonization phase. Colonization by the fungi which is mostly limited to the root cortex cells leads to several beneficial effects to the host such as growth promotion, increased resistance to biotic and abiotic stresses. The recently released genome of *P. indica* helped to identify hundreds of small secreted proteins (SSPs) coding for candidate effectors and provides an opportunity to investigate the role of these effectors during the interaction between this endophytic fungus with plants. It has been reported that *P. indica* actively suppresses PTI to colonize and establish a mutualistic relationship with plants. However, the deployment of effectors and their role during the interaction of *P. indica* with plants has not been reported.

In this study, PIIN_08944, an effector candidate of *P. indica* was functionally characterized *in- planta*. PIIN_08944 encode a 120 amino acid protein with a predicted 23 amino acid N-terminal signal peptide. Expression profile analysis revealed that *PIIN_08944* was induced in in-vitro germinated *P. indica* chlamydospore as well as *in-planta* during colonization of Arabidopsis roots by *P. indica*. To investigate the functional role of PIIN_08944 in mutualistic symbiosis, transgenic *P. indica* with reduced production or complete loss of PIIN_08944 were generated. RNAi silencing as well as deletion of the gene coding for *PIIN_08944* by homologous recombination resulted in delayed colonization of Arabidopsis roots by *P. indica*. On the hand transgenic Arabidopsis and barley plants expressing PIIN_08944 show enhanced colonization by *P. indica* compared to wild type. Heterologous expression of PIIN_08944 in barley suppresses flg22 and chitin triggered ROS production.

Meanwhile expression of PIIN_08944 in Arabidopsis interfered with the transcription of marker genes for PTI, *AtWRKY22* and salicylic acid, *CBP60g*. However, the expression of *PIIN_08944* in Arabidopsis and barley did not affect the plant's response to the necrotrophic fungal pathogens *Botrytis cinerea* and *Fusarium graminearum* respectively. Yeast two hybrid screen revealed that PIIN_08944 interacts with CSN5A and CSN5B, components COP9 signalosome protein complex, in Arabidopsis and *N. benthamiana* and with SINA3, a member of the SEVEN IN ABSENTIA family of E3 protein ligase in tobacco. Subcellular localization assays performed on *N. benthamiana* leaves revealed that CSN5A, CSN5B and PIIN_08944 localized to the nucleus and cytoplasm. In summary, the fact that PIIN_08944 targets the evolutionarily conserved eukaryotic proteins, CSN5A and CSN5B and interfere with basal defense, indicate that *P. indica* has evolved strategies involving the use of effectors as general compatibility determinants to establish and/or maintain a mutualistic relationship during interaction with plants. Moreover, this might further explain the capability of *P. indica* to colonize a multitude of different plant species.

5.2Zusammenfassung

Pflanzen haben im Laufe der Evolution ein zweischichtiges Immunsystem entwickelt, um sich von mikrobiellen Eindringlingen zu schützen. Auf der anderen Seite können Mikroben einschließlich Krankheitserreger sowie Mutualisten so genannte Effektor-Moleküle sekretieren, um eine Infektion zu fördern und eine Immunantwort der Pflanze zu unterdrücken. Mutualistische Pilze, einschließlich der arbuskulären Mykorrhiza (AM) *Glomus intraradices* und der Ektomykorrhiza (ECM) *Laccaria bicolor,* sekretieren Effektor-Proteine, um Wirtsimmunität bei Wurzelbesiedlung zu underdrücken und somit dienen diese Effektoren zur Etablierung und dem Erhalt der mutualistischen Symbiose.

Der Wurzel endophytische Pilz *Piriformospora indica* ist fähig Wurzeln einer Vielzahl von verschiedenen Pflanzenarten, einschließlich der monokotylen Gerste (*Hordeum vulgare*) und dem zweikeimblättrigen *Arabidopsis thaliana*, zu kolonisieren und etabliert eine mutualistischen Symbiose. Die Besiedlung Strategie von *P. indica* wird hauptsächlich in zwei kolonisierungs Phasen unterteilt. Einer ersten biotrophen und einer späteren Zelltod-assoziierten Phase. Die Kolonisierung durch den Pilz, die meist auf die Wurzel Cortex Zellen begrenzt ist, führt zu mehreren positiven Effekten auf den Wirt, wie Wachstumsförderung und erhöhte Resistenz gegen biotische und abiotische Stressfaktoren. Das kürzlich veröffentlichte Genom von *P. indica* zeigte hunderte von kleinen sekretierten Proteine (SSP), welche Effektor Kandidaten kodieren. Dies bietet die Möglichkeit, die Rolle dieser Effektoren bei der Interaktion zwischen endophytischen Pilz und Wirtspflanze zu untersuchen. Es wurde bereits berichtet, dass *P. indica* aktiv PTI unterdrückt um den Wirt zu kolonisieren. Allerdings ist der genaue Einsatz der Effektoren und deren Rolle bei der Wechselwirkung zwischen *P. indica* und den Wirtspflanzen noch nicht bekannt.

In dieser Studie wurde der Effektor Kandidat von *P. indica* PIIN_08944 funktionell *in- planta* charakterisiert. PIIN_08944 kodiert ein 120 Aminosäuren langes Protein mit einem prognostizierten 23 Aminosäuren N-terminales Signalpeptid. Expressionsanalysen ergaben, dass *in vitro* PIIN_08944 in gekeimten *P. indica* Chlamydospore sowie *in planta* während der Kolonisation von Arabidopsis Wurzeln, erhöht induziert ist. Um die Funktinalität des PIIN_08944 in der mutualistischen Symbiose zu untersuchen, wurde transgener *P. indica* mit reduzierten Produktions oder vollständigen Verlust des PIIN_08944 hergestellt. RNAi-Silencing sowie Deletion von PIIN_08944 gene durch homologe Rekombination führte zu einer verzögerten Besiedlung von *P. indica* an Arabidopsis Wurzeln. Auf der anderen Seite zeigen transgene Arabidopsis und Gerste Pflanzen, die PIIN_08944 exprimieren, verbesserte Besiedlung mit P. indica im Vergleich zum Wildtyp. Die heterologe Expression von

PIIN_08944 in Gerste unterdrückt flg22- und Chitin- ausgelöste ROS-Produktion. Wohingegen Expression von PIIN_08944 in Arabidopsis suppremiert die Transkription von Markergenen für PTI, *AtWRKY22* und Salicylsäure, *CBP60g*. Die Expression von PIIN_08944 in *Arabidopsis* und Gerste hatte jedoch keinen Einfluss auf die Verteidigungsantwort der Pflanze gegen die nekrotrophen Pilzerreger *Botrytis cinerea* und *Fusarium graminearum*. *Yeast two hybrid* Screening ergab, dass PIIN_08944 mit CSN5A und CSN5B Komponent COP9 Signalosom Proteinkomplex, in Arabidopsis und *N. tabaccum* und mit SINA3, ein Mitglied der SEVEN IN ABSENTIA Familie E3-Protein-Ligase, in Tabak interagiert. Subzelluläre Lokalisierungs assays an *N. benthamiana* Blättern zeigten eine Lokalisation von CSN5A, CSN5B und PIIN_08944 im Zellkern und im Cytoplasma. Zusammenfassend zeigt diese Studie, dass PIIN_08944 als Effektor auf die evolutionär konservierte eukaryotischen Proteinen, CSN5A und CSN5B abzielt und somit die basale Abwehr der Wirtspflanze stört. Dadurch wird gezeigt, dass *P. indica* Effektoren als allgemeine Kompatibilitäts Determinanten zur Etablierung und / oder zum Erhalt einer mutualistischen Beziehung innerhalb der Interaktion mit Pflanzen gebraucht. Außerdem könnte dies erläutern, wie es *P. indica* befähigt eine Vielzahl von verschiedenen Pflanzenarten zu besiedeln.

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7. Supplemental figures

7.1 Arabidopsis root Colonization

Colonization of Arabidopsis wild type and csn5 mutant roots by *P. indica*

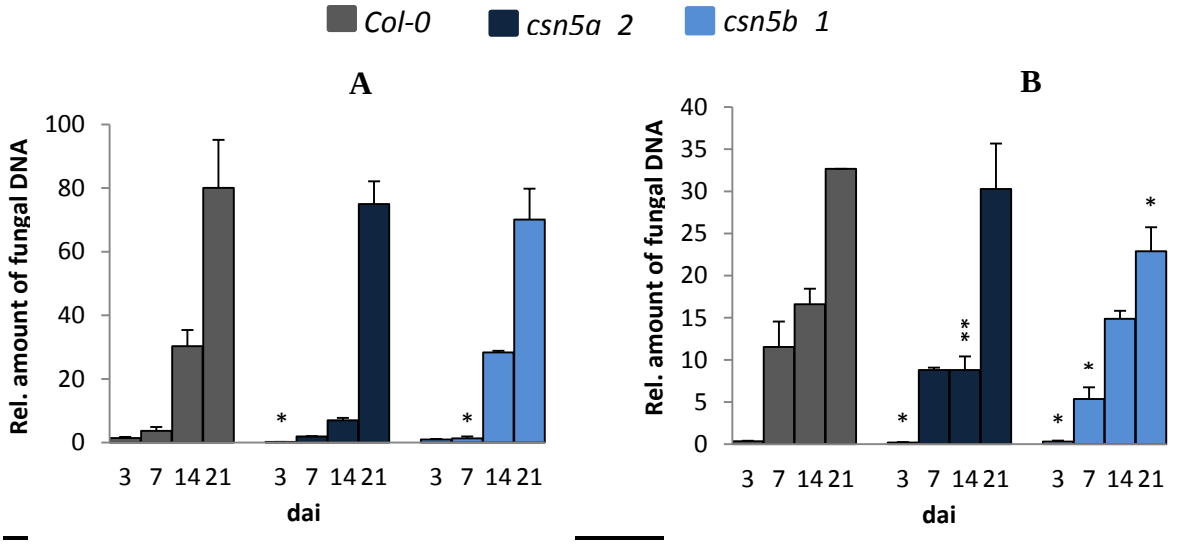
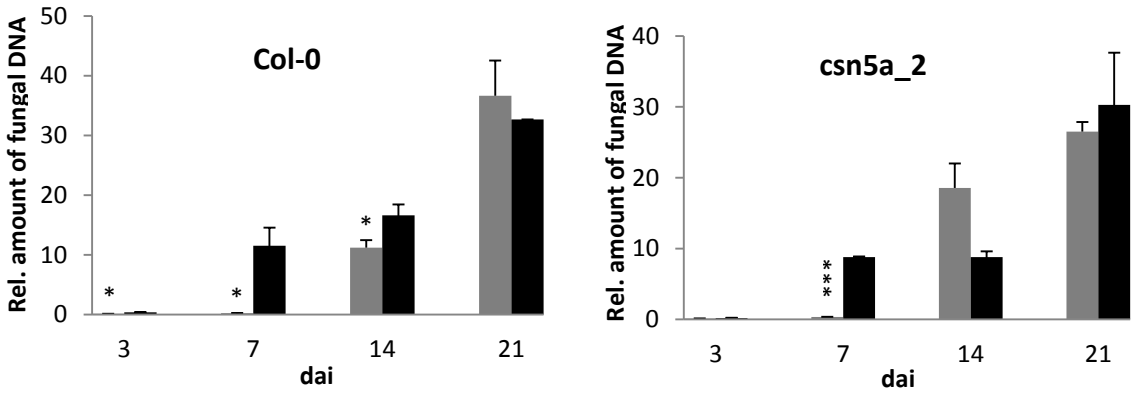


Figure S1A and S1B. ***P. indica*** **colonization of** ***Arabidopsis*** **roots**. Seven day old *Arabidopsis* wild type (*Col*-0), *csn5a_2* and *csn5b_1* roots were inoculated with *P. indica* chlamydospores. Fungal biomass was determined at 3, 7, 14, and 21 dai, as relative amount of fungal DNA after DNA extraction subsequent qRT-PCR using fungal and plant specific primers. Data represents the Ct thresholds of Pi-ITS relative to the Ct thresholds of *At*UBQ-4 using the $^{\Delta\Delta}$Ct method (average Ct thresholds of three technical replicates within one biological experiment are presented). The values represent the means with standard errors of one experiment. The experiment was repeated three times with similar results. Asterisks indicate significance at $P < 0.05$ (*), 0.01 (*) as analyzed by Student's *t*-test. Pi-ITS: *P indica* Internal Transcribe Spacer, *AtUBQ-4*: *Arabidopsis* thaliana Ubiquitin-4, dai: days after inoculation.

Colonization of *Arabidopsis* wild type and csn5 mutant roots by *P. indica* silenced mutant

Figure S2A



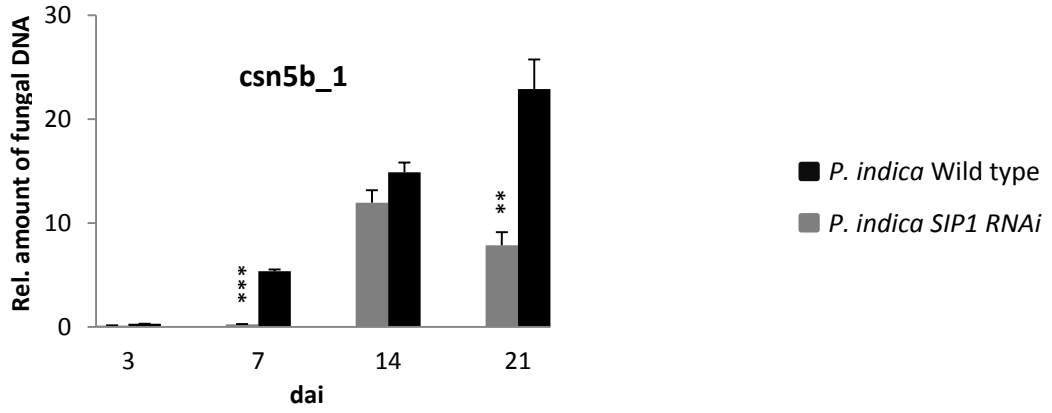


Figure S2B

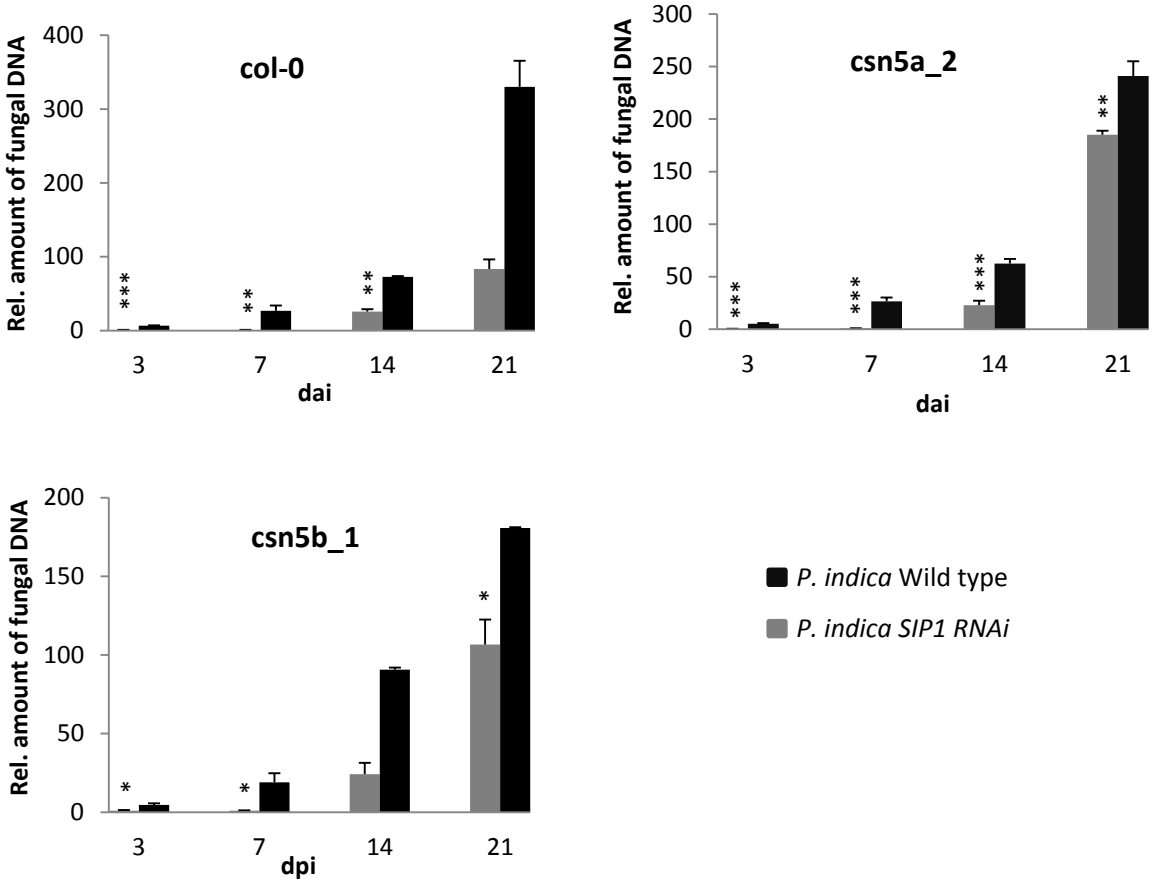


Figure S2A and S2B. SIP1 silencing delays *P indica* infection of *Arabidopsis* roots. Seven day old Arabidopsis roots (Col-0, csn5a_2 and csn5b_1) were inoculated with *P. indica* RNAi strain or WT control. Fungal biomass was determined at 3, 7, 14, and 21 dai as relative amount of fungal DNA by qPCR using fungal and plant specific primers. Data displays the Ct thresholds of Pi-ITS relative to the Ct thresholds *At*UBQ- 4 using the $^{\Delta\Delta}$Ct method (average Ct thresholds of three technical replicates within one biological experiment are presented). The values represent the means with standard errors of one experiment. Asterisks indicate significance at $P < 0.05$ (*), 0.01 (**), 0.001 (***) as analyzed by Student's *t*-test. The experiment was repeated three times with similar results.

Colonization of Arabidopsis wild type and csn5 mutant roots by *P. indica* knockout mutant

Figure S3A

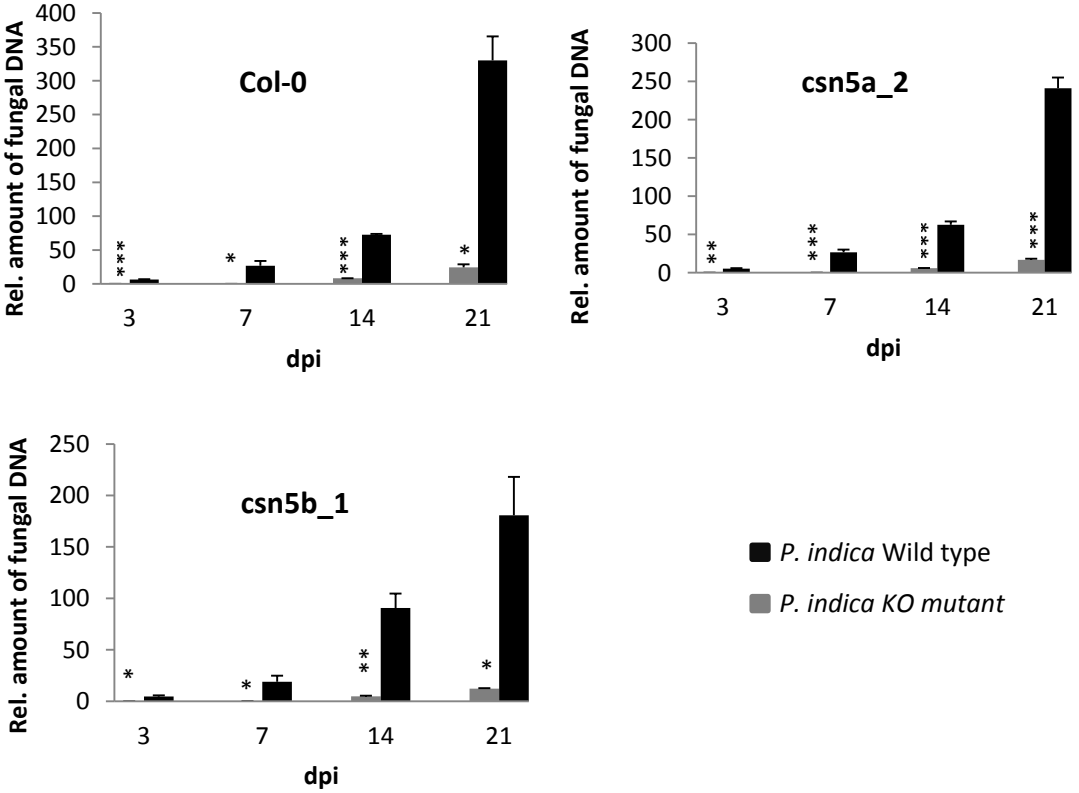


Figure S3B

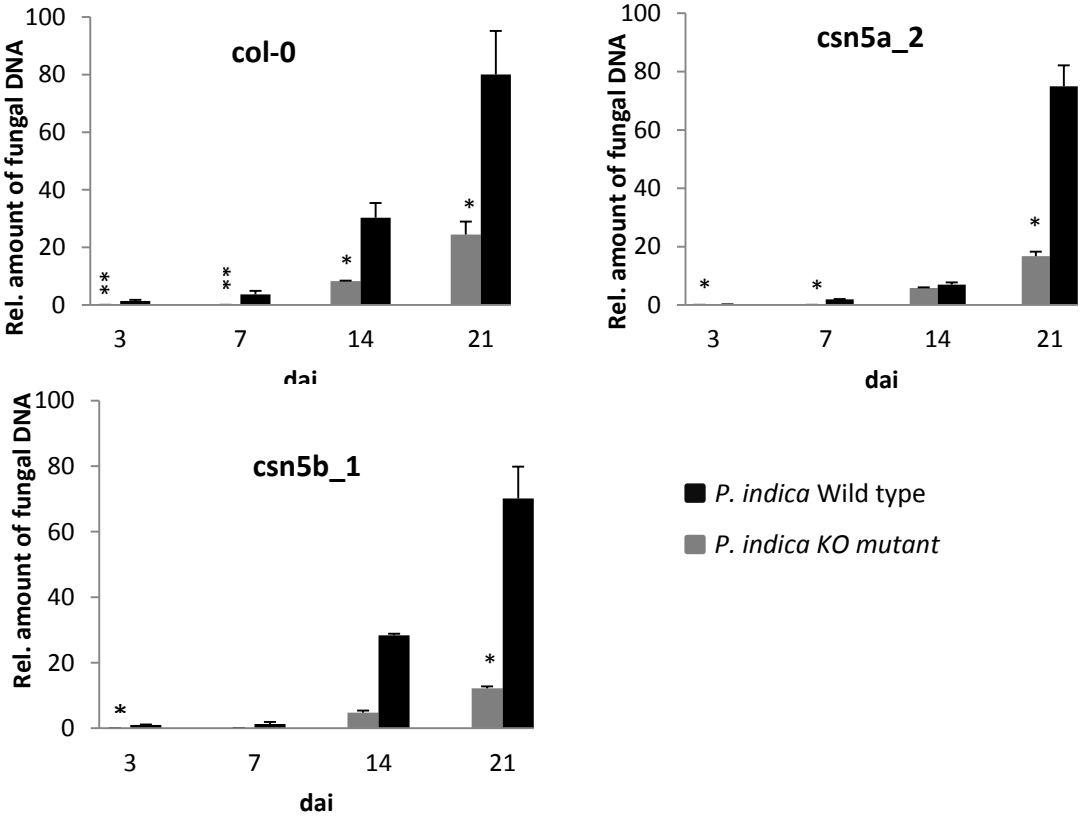


Figure S3A and S3B. Deletion of *SIP1* delays *P indica* infection of Arabidopsis roots. Seven day old Arabidopsis roots (Col-0, csn5a_2 and csn5b_1) were inoculated with *P. indica* deletion strain or WT control. Fungal biomass was determined at 3, 7, 14, and 21 dai as relative amount of fungal DNA by qRT-PCR using fungal and plant specific primers. Data displays the Ct thresholds of Pi-ITS relative to the Ct thresholds *At*UBQ-4 using the $^{\Delta\Delta}$Ct method (average Ct thresholds of three technical replicates within one biological experiment are presented). The values represent the means with standard errors of one experiment. Asterisks indicate significance at P < 0.05 (*), 0.01 (**), 0.001 (***) as analyzed by Student's *t*-test. The experiment was repeated three times with similar results.

Figure S3C

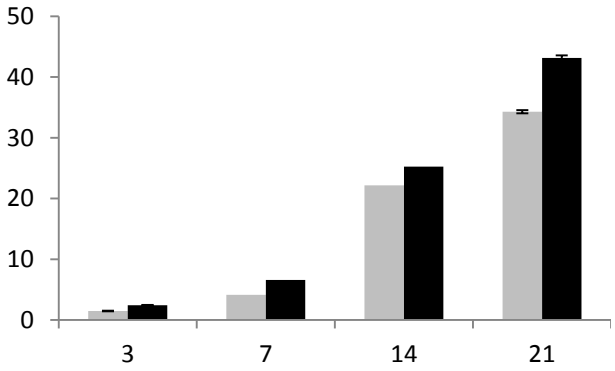
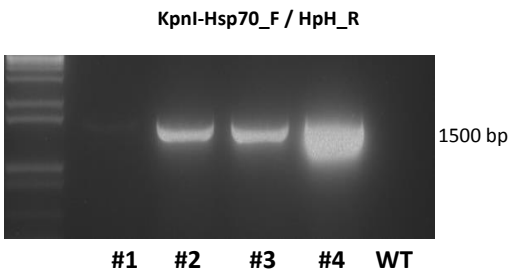
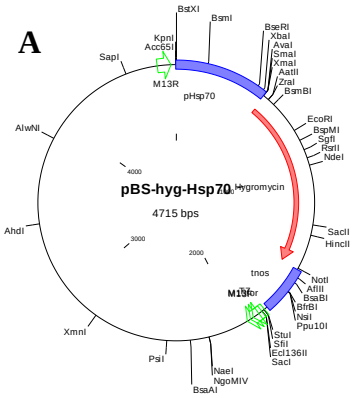


Figure S3C. Colonization of *SIP1* transgenic Arabidopsis with *P. indica* Δ*SIP1* deletion strain. Roots of 7-day-old transgenic Arabidopsis seedlings expressing *SIP1*or wild type (Col-0) control were inoculated with *P. indica* Δ*SIP1* deletion strain. Roots were harvested and fungal biomass determined by qRT-PCR at 3, 7, 14 and 21 dai using fungal (ITS) and plant (UBQ-4) pecific primers. Data displays the average Ct thresholds of three technical replicates within one biological experiment of ITS relative to the Ct thresholds of Ubq- 4 using the $^{\Delta\Delta}$Ct method. The values represent the means with standard errors of one experiment. Asterisks indicate significance at P < 0.05 (*), as analyzed by Student's *t*-test.

7.2 Maps of vectors used for *P. indica* transformation





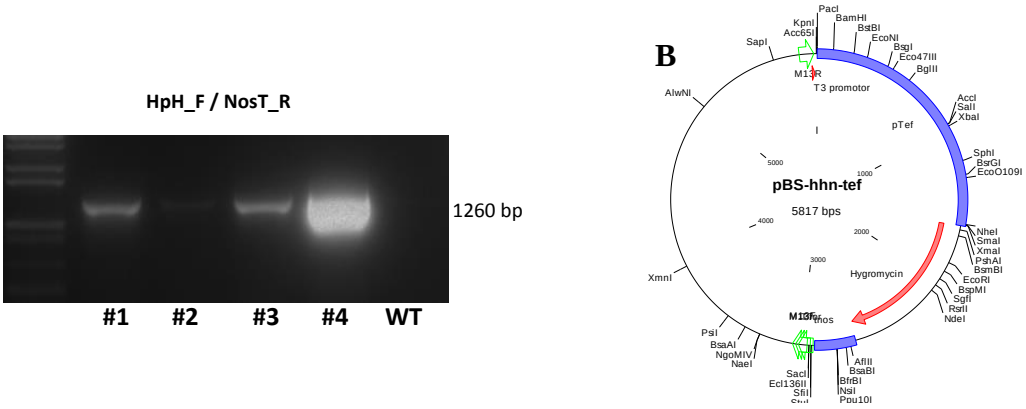


Figure S4. Both vectors were designed carrying the hygromycin B resistance gene and used to transform *P indica* to confirm promoter activity of **A)** Hsp70 promoter and **B)** Tef promoter**.** Agarosel gel bands represent positive transformants obtained after *P indica* transformation with the respective vectors.

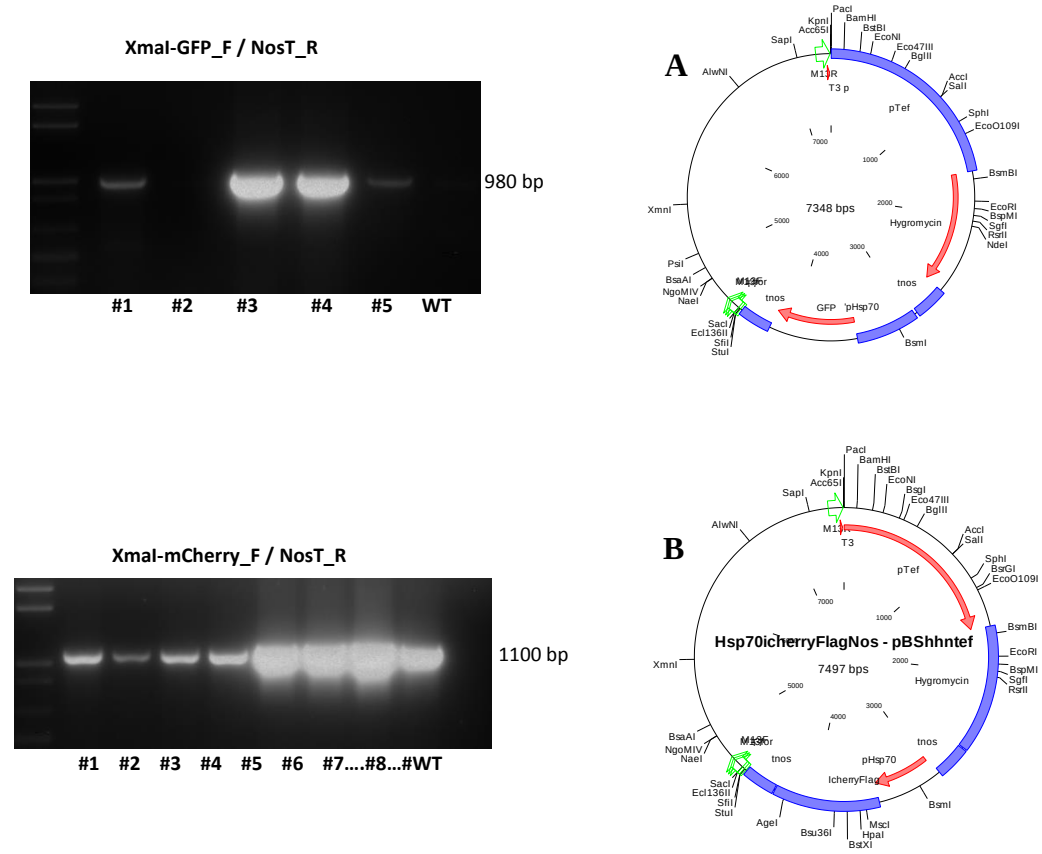


Figure S5. A) Dual cassette vector carrying a hygromycin B cassette and green fluorescent protein (GFP) used for expression studies in *P indica*. **B)** Dual cassette vector carrying a hygromycin B gene under the *P indica* tef promoter and mCherry under the *P indica* Hsp70 promoter. Agarosel gel bands represent positive transformants obtained after *P indica* transformation with the respective vectors.

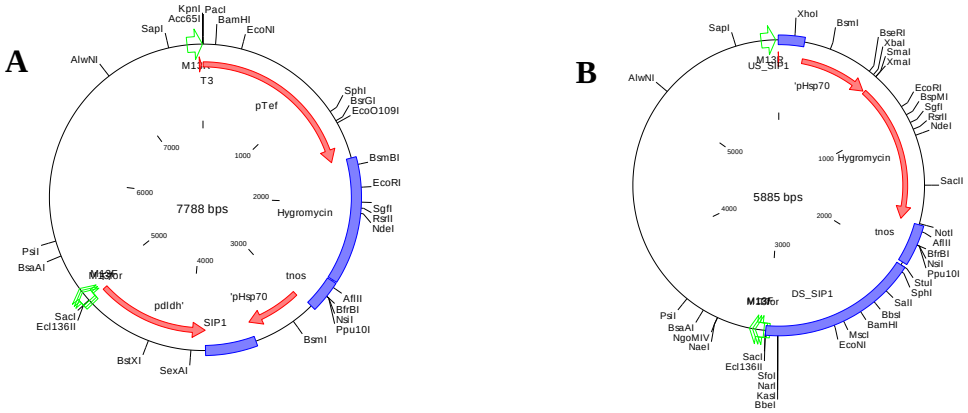


Figure S6. A) Dual promoter vector used for the silencing (RNAi) of SIP1. The hygromycin B gene is under the control of the *P indica* tef promoter (pTef) and nos terminator from *A. tumefaciens*. **B)** Vector used for the knockout of SIP1 by homologous recombination.

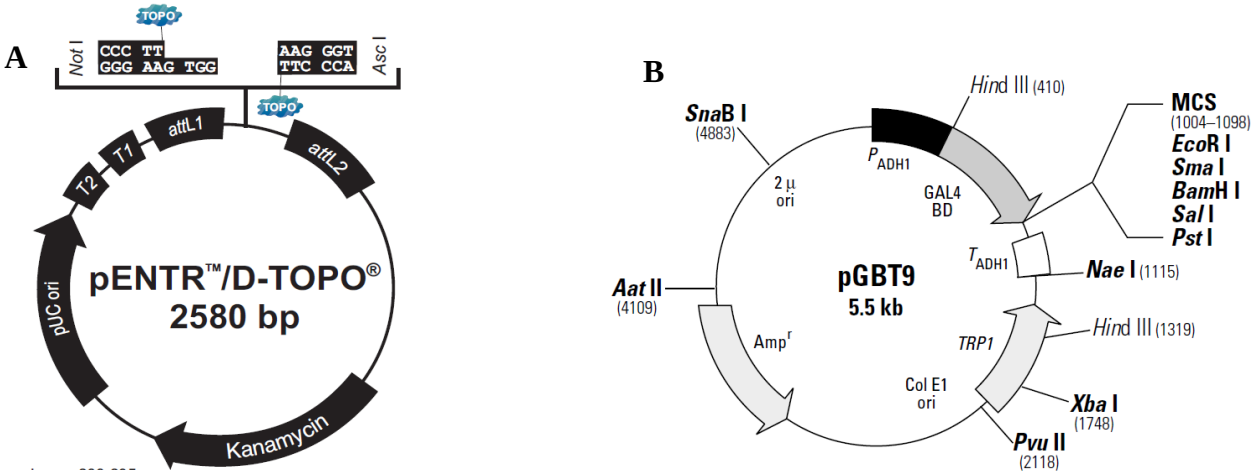
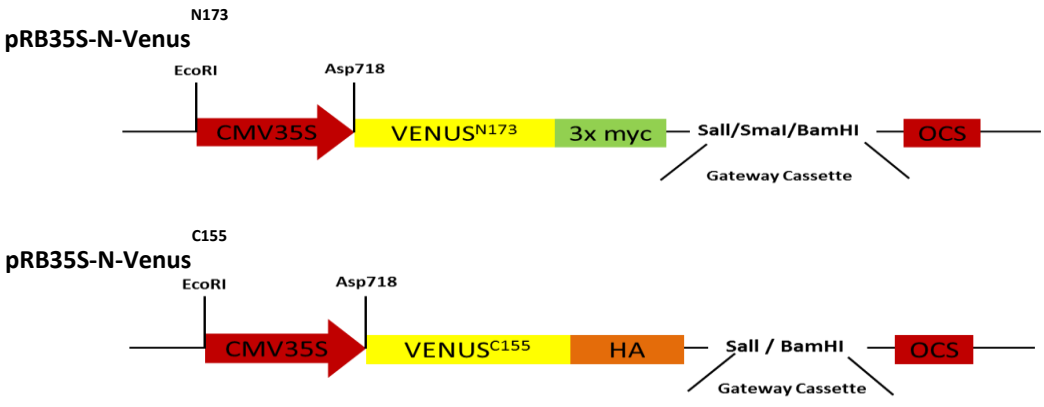


Figure S7. A) Vector used to generate entry clones for the Gateway cloning system (Invitrogen). **B)** Vector used to clone *SIP1*, contains the GAL4 binding domain (BD) and served as bait for the yeast two hybrid (Clontech).



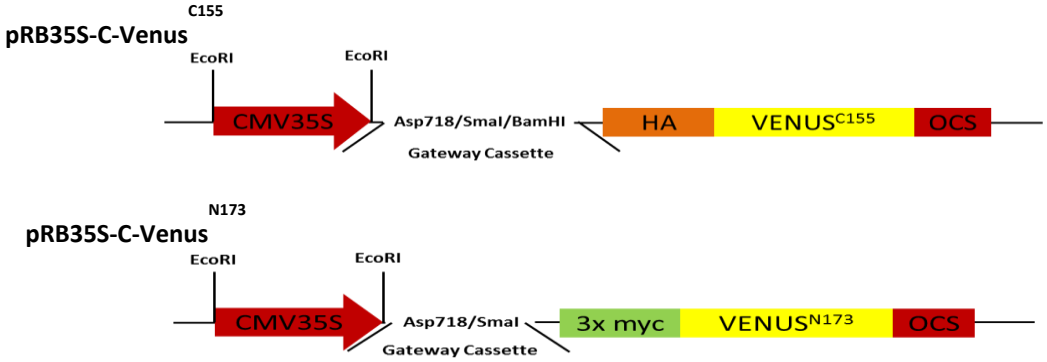


Figure S8. Schematic representation of the different N – and C – terminal fusions of the venus fluorescent protein used for bimolecular fluorescent complementation assay. Vectors are under the 35S promoter and OCS terminator as well as carry HA or Myc tags.

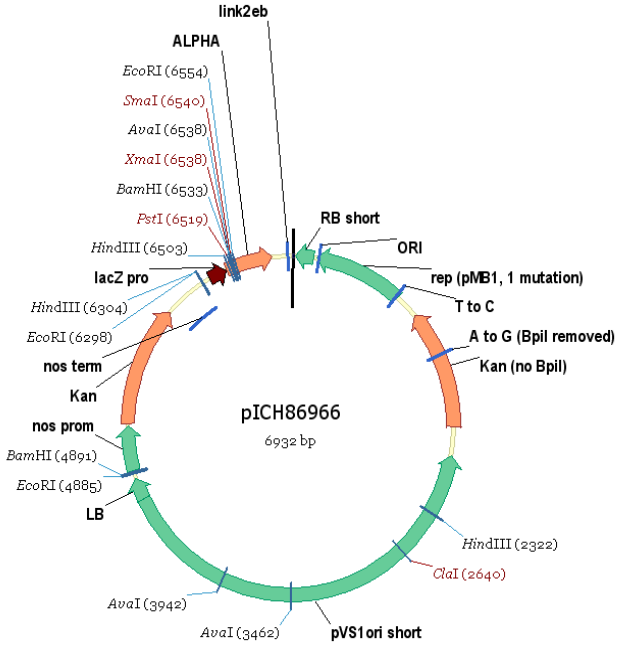


Figure S9 Binary vector map of pICH86966. This expression vector was used to generate SIP1 transgenic Arabidopsis plants

7.3 Gene sequences

PIIN_08944 (SIP1) cDNA sequence showing signal peptide in bold

ATGTTCTCTTTCCGAAAAGCCGCAACTTTCGTTTTCTTCGCTGGTCCCTTCCT CCTCGCAAGCGCTGTTCCCATCGAAAAAATGATTCTTCCAGACCTTACAGAGCA TTTCGGCAAGCGCGAAAAGCTGGTGCTACCAGATCTCACCGAGCACTTTGGAAGA CGGGACAAACTCATATTGCCAGATCTCACCGAGCATTTCGGAAAGCGCGATAAAC TTGTCTTGCCAGATCTCACTGAACACTTTGGAAAACGGGAGAAGCTCGTCTTGCC AGATCTCACCGAGCACTTTGGGAGGAGGGACAAGCTCGTCTTGCCTGATCTTACC GAACACTTCGGAAAGCGTAAAACTCACGAATCATAA

PIIN_08944 (SIP1) amino acid showing signal peptide in bold and underlined

MFSFRKAATFVFFAGPFLLASAVPIEKMILPDLTEHFGKREKLVLPDLTEHFGRRDK
LILPDLTEHFGKRDKLVLPDLTEHFGKREKLVLPDLTEHFGRRDKLVLPDLTEHFGKR
KTHES

PIIN_06837 cDNA sequence showing signal peptide in bold

**ATGCGCTTCGGATTCTTCGCCATAATTTTTGCTGCATCCGCTCTTCTGGTGTC
CGCTACT**CCGGTCCCTAGAATCGATCACCATGTGGAGCATGCACATCACCAAGC
ACAGGCAGACCACCATCTACAGCAAGCAGAAACCCACCTGGGTCATGCAGAGAC
ACATGCAAATCATATCCACGCGGCAGAACAAAATGGTAACCAACAGCTCGCTGC
GGCACACCAGGTTCATTACGATCATCATATGCAACAAGTGGATCATCATACGAAC
CTTCATCAGGAACACCAAGCCCAGGCTGACTACCATGCTCGGTTTATTCACCATC
GGAGCATAGACGAGTTGGAT

PIIN_06837 amino acid showing signal peptide in bold and underlined and RSIDELD motif in red

MRFGFFAIIFAASALLVSATPVPRIDHHVEHAHHQAQADHHLQQAETHLGHAETHA
NHIHAAEQNGNQQLAAAHQVHYDHHMQQVDHHTNLHQEHQAQADYHARFIHH**RS
IDELD**

PIIN_04362 cDNA sequence showing signal peptide in bold

**ATGGCGCTGAGACTGCTAGTCGCATTATGGCTTGGGACTACAGTACTCGCAG
C**CGAAGGGTTTAATCCGAGAGCATACGAGAAAAAGCATGTTGAATGCCCCACTG
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CACGATCCCCCTCCCGGTTCTCGCCGTCTATCCAATAAACGACCCCGTGGCTGATT
GGCCCCTCGTGGCGCTCGTGGCCGGATCACATACATACCTGCCCTATCTTACAAA
GCGAGTACTGCAGACGGCACATTGGCCCCATTTGGAGAAGCCGAAAGAGTTTAA
CGCCATTCTGGAGGAATGGCTCGACGACCTTGATGTGAAATTGCTCGAAGCGCGT
ATCGGCGAACTGAAACGCGAGATGAGAGCCTTATCAAAAGAACTCTCGGCGGCG
AAAAAGAAGAAGAAAAGGCGAGGAGAGCTG

PIIN_04362 amino acid showing signal peptide bold and underlined, cysteine residues in red and nuclei localization signal in Orange bold

MALRLLVALWLGTTVLAAEGFNPRAYEKKHVECPTVHRKGGLGEPEETTINMSYV
DINPTANRTLLMVHGWPGLWSVWSNQIMHFSDKYHLLVPDLRGFGDSIHPGDVQAS
GTFSDIVGDLVCILQDAGVSKAVCIGHDWGSPICHEAARMRPDLFEGVVGASLPYLP
AAAPKMLSIEALIAALPKLTYNLYFMSETEKAIAELNKDVRRTIRAALRSLASPSPYRF
LRSKTSFLKAYNHLEEIPPVPFFTPDQEDYYVEQYQKQGFDHTLQFYTHENKYKNWE
FAHNQGNFTIPLPVLAVYPINDPVADWPLVALVAGSHTYLPYLTKRVLQTAHWPHLE
KPKEFNAILEEWLDDLDVKLLEARIGELKREMRALSKELSAA**KKKKKRR**GEL
GFP Sequence

ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAG
CTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGC
GATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGC
CCGTGCCCTGGCCCACCCTCGTGACCACCTTGACCTACGGCGTGCAGTGCTTCGC
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CTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCA
CATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAG
CTGTACAAGGGTTAA

D-lactate dehydrogenase (DLDH) promoter

TTCCGCAACACGACTGGCTATTTTTGATGGATATGCTGTAGATCCCAGTTCGACG
AGCTCTTCTCAACCTTCTCATCGTCTATATTGCCAACTTCTTTGGATGCGTCCTCAT
GTCGTACATTTTCTCGTACCAGACGGATATCTTTGAGGCAGAACCATGGCGGTCA
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GGAAATTTTGTCGGCGGTGCGATTGCCATCGGCACGGTGGAACACGCCATGAACC
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AACAGCAACTCCGGGAGCTGACACTGACGCGGACACGAAGCCGTGCGCCGAGTT
CGCCTGTGCAGATGCGGAATGGACACGCTTTGCCTGCATAATAGACCTGCTGCCT
CGGTCACACGTTTGCCGCTTTGAGGGGCCGCGTGTGGATAGATAGAGCCAGATAC
GATTTGTTCCTTTTTCTTCCGGGGAATCTCCCGAATGAACCCGTTCCGATGTTTCT
AGTGATCTATAGAAGTTACTGTTTCTGCAAAGCAATTCGCTACCAGGTCCGAGGA
GTCGAGACCAACAAGCGCTGGTCTGTGGCGAAATGTAGAAATACGCCGGCATCG
TCTGTCTGAACCCGTCATCACCTTTAGAGACAACAACAAATAGACAGAAAA

Hsp70 promoter

ACGACTCTGGTCTGTTGAGACCGTGGTGAGGCTGATAGGTGTTATTACGGTTCTG
GGTTGATCCAAGTTTTGAGAAATACTCTACTATTAGCGCCATTTTAGCGTCGTTTG
TCAAGTTGCACGACTTTCGACTCACAAACTGCAGCTCGCCGTTGTCTAGCTCGCA
GCATTCGCTGCCAATCCCATGAAGGTTTCCCGGTAGATGTTGATACATTCTTTTCT
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GCCAGCCGTTTTACTCCTCTAAAACACCTTAAAACTTGTTTCTTGTTTCTAGATTTC
TAAACAGAAC

Hygromycin B sequence

ATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGT
TCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTT
CAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGAT
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CAGGATCGCCGCGGCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTA
TCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGC
GACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCCCGC
AGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGA
AACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAA

Molecular ladder

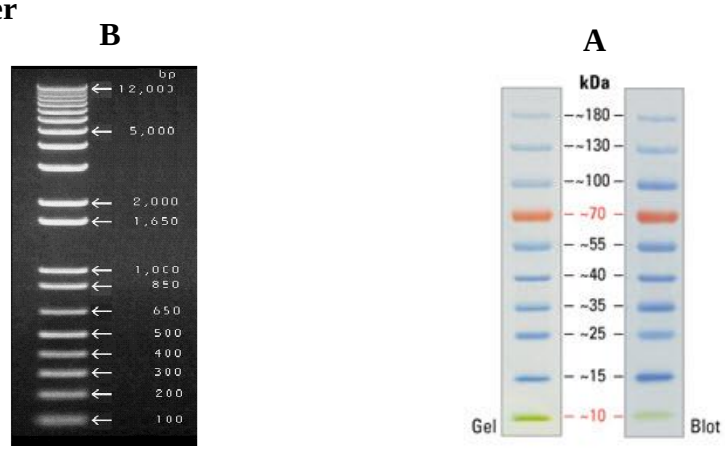

Figure S10) Molecular ladder band profile. A) PageRuler Prestained Protein Ladder showing band profile used during SDS-PAGE. B) DNA ladder (1 kb plus) showing band profil used during agarose gel electrophoresis

8. Declaration

I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation.

I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me.

At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the statutes of the Justus Liebig University Giessen for the Safe guarding of Good Scientific Practice.

Signature: Date: 29.06.2015

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