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Detection and characterization of secreted proteins from *Piriformospora indica* during *Arabidopsis* roots colonization

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Für meinen Vater

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ERKLÄRUNG **132**

DANKSAGUNG **133**

List of abbreviations

aa	Amino acid(s)
A. bidest.	Aqua bidistilled from Millipore filter unit
A. dest.	Aqua distilled
ADH	Alcohol dehydrogenase
AM	Arbuscular mycorrhiza
APS	Ammonium persulfate
bp	Base pairs
BLAST	Basic local alignment search tool
cDNA	Complementary DNA
CLSM	Confocal laser scanning microscopy
CM	Complex medium
CTAB	Cetriumonium bromide
dai	Day(s) after inoculation
DB	Database
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DNase	Desoxyribonuclease
dNTP	Desoxyribonucleoside triphosphate
ds	Double-stranded
dscDNA	Double-stranded complementary DNA
ECM	Ectomycorrhyza
EDTA	Ethylene diamine tetraacetic acid
ER	Endoplasmic reticulum
EST	Expressed sequence tag
ET	Ethylene
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility
EtOH	Ethanol
Fwd	Forward
GFP	Green fluorescent protein
HR	Hypersensitive response
IGS	indole glycosinolate
ISR	Induced systemic resistance
IAA	Indole acetic acid
LiAc	Lithium acetate
JA	Jasmonic acid
LB	Lysogenic broth
LRR	Leucin-rich repeat
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	Messenger RNA
MS medium	Murashige & Skoog medium
MTI	MAMP-triggered immunity
NaOCl	Sodium hypochlorite
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

PR	Pathogenesis-related
PRR	Pathogen recognition receptor
qPCR	Quantitative real-time PCR
rev	Reverse
RLK	Receptor-like kinase
RNA	Ribonucleic acid
RNase A	Ribonuclease A
ROS	Reactive oxygen species
rcf	Relative centrifugal force
rpm	Rounds per minute
RT	Room temperature
RT reaction	Reverse transcriptase reaction
SA	Salicylic acid
SAR	Systemic aquired resistance
SD medium	Synthetic complete drop out medium
SDS	Sodium dodecyl sulfate
ss	single-stranded
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylendiamine
Tris	Tris-(hydroxymethyl)-aminomethane
UV	Ultraviolet
YNB	Yeast nitrogen base
YEPD	Yeast complete medium
YSST	Yeast signal sequence trap

1. INTRODUCTION

1.1. Plant-microbe interactions

In their natural environment, plants are surrounded by many microbial organisms such as fungi, bacteria and protozoa. The vast majority of all these life forms does not disturb the plant, only very few of them are able to overcome the plants' resistance mechanisms and evoke disease symptoms. In addition, viruses and herbivores like insects and nematodes are potential threats because they use the plants for their proliferation and/or feed on them. Plant pathogenic microbes have different life styles. Biotrophic organisms are dependent on their ability to colonize living host cells, which need a living host plant for survival (e. g. powdery mildew fungi like *Blumeria graminis*). Other pathogens rely on dead tissue for plant colonization and follow a necrotrophic life style (e. g. *Botrytis cinerea*). Hemi-biotrophic pathogens such as the oomycete *Phytophthora infestans* change their life style during host plant colonization. Here, living host cells are initially colonized but are subsequently killed by the fungus in order to pursue a necrotrophic colonization strategy (Pieterse *et al*., 2009).

1.2. Role of plant hormones in stress signaling

Plants synthesize hormones for the coordination of developmental and growth processes. The hormones ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) have important relevance in plant development. Ethylene acts for example in seed germination, cell elongation and fertilization. JA regulates the formation of anthers and pollen and SA modulates plant growth (Taiz & Zeiger, 2006). Hormones also play a role in signaling and in the reaction to biotic or abiotic stress. Especially ET, JA and SA have been studied regarding their function in defense response after pathogen infection or abiotic stresses. The signaling pathways induced by these three hormones during stress response show an extensive interaction. Necrotrophic pathogens and herbivores are more hindered by JA and ET defense signaling. JA and ET signaling is also induced after wounding (O'Donnell *et al.*, 1996; Wasternack 2007). Biotrophic and hemi-biotrophic pathogens are more hampered by SA-associated defense signaling (Bari & Jones, 2009; Glazebrook, 2005). Several studies show the antagonistic interaction of JA and SA signaling. The activation of the SA pathway after infection with a biotrophic pathogen, for example, simultaneously suppresses JA signaling activated upon attack by a necrotrophic pathogen (Spoel *et al*., 2007). In *Arabidopsis*, the rise in SA levels is accompanied by an increase in the level of glutathione and the JA signaling pathway is attenuated.

Thus the influence of SA signaling on the redox status of the cells influences the tradeoffs between JA and SA pathway (Koornneef *et al*., 2008). JA, ET as well as SA signaling also participate in the establishment of systemically induced defense responses.

1.3. Systemic acquired resistance (SAR), induced systemic resistance (ISR) and priming

Plants which were infected by (necrotrophic) pathogens and were able to repel the attack are afterwards in a primed state so that they can react faster and more effective to biotic or abiotic stress situations. The priming effect can also be achieved by treatment with chemicals or beneficial microbes (Goellner & Conrath, 2008). This SA-dependent phenomenon, so called systemic acquired resistance (SAR), is mainly caused by an increased induction of pathogenesis-related genes (*PR* genes) which have antimicrobial capacities. SAR can last for a long period of time (Durrant & Dong, 2004). The mobile signal responsible for the activation of SAR is still not clearly identified. Recently it was published that methyl salicylate (MeSA) and one or several lipid-derived signals could induce SAR (Liu *et al.*, 2011). Liu and coworkers suggested a model for long distance SAR signaling in *Arabidopsis*. SA levels increase after pathogen challenge at the site of infection and the enzyme AtBSMT1 (benzoic acid/SA carboxyl methyltransferase 1) partly converts SA into MeSA. The accumulating, biologically inactive, MeSA is also distributed in uninfected parts of the plant. At the site of infection, additionally a yet not clearly identified lipid-derived complex is formed which is also delivered into systemic tissue. There, the enzyme MSE (MeSA esterase) converts the inactive MeSA into active SA and the SA accumulation as well as the inhibition of AtBSMT1 by the lipid-derived complex allows the establishment of SAR (Liu *et al*., 2011).

NPR1 (NONEXPRESSER OF PR GENES 1) is a key regulator of SA-induced *PR* genes and thus of SAR (Dong, 2004). The accumulation of SA due to pathogen challenge causes the transfer of NPR1 into the nucleus where it interacts with different transcription factors and activates SA defense signaling. For the specific activation of target genes, a balanced turnover of nuclear NPR1 protein is important. Without pathogen attack, NPR1 is located in the cytoplasm and is removed from the nucleus by the proteasome (Spoel *et al*., 2009). In the cytoplasm, NPR1 is also regulating the suppression of JA-responsive genes elicited by SA accumulation (Spoel *et al*., 2003). An *Arabidopsis npr1* mutant does not express *PR* genes because it is non-responsive to altered SA levels and thus SAR development is not possible (Cao *et al.*, 1994).

Induced systemic resistance (ISR), a disease resistance against a wide variety of pathogens, is initiated by non-pathogenic rhizobacteria which colonize plant roots.

Hence the colonized plants are in a primed state and JA responsive genes are induced for the establishment of ISR (Pozo *et al*., 2008). In contrast to SAR, ISR is not dependent on SA accumulation and *PR* gene induction. Instead, regulators of JA and ET signaling, JAR1 (JASMONATE RESISTANT 1) and ETR1 (ETHYLENE RECEPTOR 1), and the regulator NPR1 are necessary for ISR. Therefore, pathogens sensitive to JA and ET-dependent defense responses are repelled by ISR (Pieterse *et al*, 1998; Ton *et al*., 2002).
NPR1 is acting downstream of pathogen induced SA and JA/ET signaling and is thus functioning in the regulation of SAR and ISR (Pieterse *et al*., 1998).

1.4. Plant innate immunity

1.4.1. MAMP-triggered immunity (MTI)

If a microorganism gets in contact with a plant it is confronted with permanent structural and biochemical barriers, such as the wax cuticle of leaves, the plant cell wall and antimicrobial components in the apoplastic space, which protect the plant from invaders (Gimenez-Ibanez & Rathjen, 2010). Among the antimicrobial secondary metabolites phenols, unsaturated lactones, sulfur compounds, and saponins have been identified (Osbourn, 1996). Those pathogens that are able to overcome these barriers are confronted with induced immune responses. Each plant cell is able to recognize microbes in a cell autonomous manner (Zipfel, 2008). Plants recognize pathogens initially by highly conserved molecules defined as pathogen-associated molecular patterns (PAMPs) or, more generally, so called microbe-associated molecular patterns (MAMPs).
Among the best studied MAMPs are bacterial flagellin, the bacterial elongation factor Tu (EF-Tu), and lipopolysaccharides as well as fungal chitin (oligomers) (Bent & Mackey, 2007). Bacterial flagellin represents an important component of the flagellum in eubacteria. Most plant species react to a 22 amino acids epitope at the N-terminus of the protein, defined as flg22 (Felix *et al.*, 1999). MAMPs are recognized by plant cells through plasma membrane-localized pattern recognition receptors (PRRs) (Boller & He, 2009). For instance, in *Arabidopsis* flg22 is specifically recognized by the receptor-like kinase (RLK) FLS2 (FLAGELLIN SENSITIVE 2) (Gómez-Gómez *et al*., 1999). Orthologous proteins for the perception of flagellin in *Nicotiana benthamiana*, NbFLS2, and in tomato, LeFLS2, were recently identified (Hann & Rathjen, 2007; Robatzek *et al.*, 2007). FLS2 consists of an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and a cytoplasmic serine-threonine kinase domain (Gómez-Gómez & Boller, 2000). Upon binding of flg22, FLS2 changes its conformation and forms a complex with the LRR receptor-like kinase BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) in order to activate defense processes like for example oxidative burst, MAPK (MITOGEN ACTIVATED

PROTEIN KINASE) signaling and ethylene biosynthesis (Chinchilla *et al*., 2007; Boller & Felix, 2009). The elongation factor Tu (EF-Tu) is another bacterial MAMP activating very similar signaling pathways and defense responses in plants like flagellin (Zipfel *et al.*, 2006). EF-Tu is only perceived in *Arabidopsis* and other Brassicaceae. In *Arabidopsis*, especially the N-terminus and an N-acetylated peptide (elf18) are sensed by the receptor kinase EFR (EF-Tu receptor); the initiated defense responses enable resistance against further bacterial invasion (Kunze *et al.*, 2004).

The polysaccharide chitin of fungal cell walls is the most prominent MAMP found in fungi. Also this MAMP is, like flg22 and elf18, recognized by a RLK; in *Arabidopsis* it is named CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1). This RLK consists of three extracellular LysM motifs, a transmembrane domain, a juxtamembrane domain and an intracellular serine/ threonine kinase domain and is necessary for the resistance of plants against nonhost pathogens (Miya *et al*., 2007). The three LysM domains are required for the binding of chitin and chitinoligomers. Upon chitin binding, CERK1 is phosphorylated, probably for transmission of defense signaling processes (Petutschnig *et al.*, 2010). The recognition of chitin by CERK1 seems to be independent from flagellin recognition, but similar downstream elements like MAPK signaling and activation of defense related WRKY transcription factors are initiated during both perception processes. Thus different MAMPs elicit similar defense associated pathways in the plant (Wan *et al*., 2008). The downstream responses triggered by MAMP perception finally lead to MTI. MTI is considered as the first instance of the plant immune system (Jones & Dangl, 2006). The paragraphs 1.4.1.1 to 1.4.1.4 below describe the major defense mechanisms elicited by MAMPs in more detail.

1.4.1.1. Elevation of intracellular calcium levels

A very early response in MAMP signaling is the elevation of intracellular calcium levels. In plants, there are mainly three forms of calcium sensors, the CAM (= calmodulin) and CAM-like proteins, the calcineurin B-like proteins and the calcium-dependent protein kinases (CDPKs). *Arabidopsis* CDPKs perceive the calcium levels in the cytosol. Upon challenge with flg22, several CDPKs are transiently activated; they act independently and also synergistically with MAPKs in the regulation of early target genes in MAMP defense signaling downstream of PRRs. CDPKs are also involved in the regulation of the oxidative burst reaction (Boudsocq *et al*., 2010).

1.4.1.2. The oxidative burst

The fast production of reactive oxygen species (ROS), defined as oxidative burst, is an immediate defense response in plants which occurs within minutes after challenge with MAMPs such as flagellin or chitin. The oxidative burst is the result of the fast production of apoplastic superoxide (O_2^-) and hydrogen peroxide (H_2O_2) at the site of MAMP recognition (Felix *et al.* 1999). Enzymes responsible for this ROS production are plasma membrane-localized NADPH oxidases (Torres *et al.*, 2006; Zhang *et al*., 2007; Mersmann *et al*., 2010). It was discovered, that there are differences between ROS synthesis in compatible and incompatible host-microbe interactions. In incompatible interactions, a biphasic ROS production was observed. The first short and unspecific phase is immediately observed after pathogen contact and can be seen in compatible as well as in incompatible interactions.

The second phase is only found in incompatible contacts and is characterized by a higher, long-lasting ROS level, which is associated with a programmed cell death reactions termed hypersensitive response (HR) (Baker & Orlandi, 1995, Dangl *et al.*, 1996). This HR is found in incompatible host-pathogen interactions at the site of pathogen attack. It is thought that the HR is only effective against (hemi-) biotrophic pathogens but might be less effective against the cell death-dependent life style of necrotrophic microbes. Importantly, signals transmitted by dying cells and the adjacent living cells activate further defense processes which stop the spreading of pathogens (Heath, 2000)

Interestingly, the synthesis of ROS is also involved in the establishment of symbioses between legumes and rhizobial bacteria. The crosstalk between the two partners starts with the synthesis of flavonoids by the legume roots. Subsequently, specific lipochitooligo-saccharides, the Nod factors (NFs), are secreted by the bacteria whose recognition by the plant roots initiates a short but reversible synthesis of ROS. Finally different physiological changes in the plant roots lead to nodule formation (Cárdenas *et al.*, 2008).

1.4.1.3. Mitogen-activated protein kinase (MAPK) signaling cascade

Protein kinases are responsible for the phosphorylation of proteins and are normally arranged in signaling cascades. The composition of a MAPK cascade often starts with a MAPKKK (or MAP3K) which phosphorylates and thus activates a MAPKK (or MAP2K) which in turn phosphorylates and activates a MAPK. The regulation of the MAPK cascades is done by MAPK phosphatases (MKPs). Protein kinases have various functions. They are involved in developmental processes as well as pathogen defense (Colcombet & Hirt, 2008).

When the MAMP flg22 is bound to the FLS2 receptor in *Arabidopsis*, the kinase cascade MEKK1 (MAPK/ERK kinase kinase 1; (ERK = EXTRACELLULAR SIGNAL-REGULATED KINASE)), MKK4/MKK5 and MPK3/MPK6 and thus the targets FRK1 (FLS2 INDUCED RECEPTOR KINASE1) as well as the transcription factors WRKY22 (WRKY DNA-BINDING PROTEIN 22) and WRKY29 are activated and confer resistance to bacterial and fungal pathogens (Asai *et al.*, 2002). A second signaling cascade which is activated by flg22 perception consists of MEKK1, MKK1/MKK2 and MPK4. The targets of MPK4 are MKS1 (MPK4 SUBSTRATE 1) as well as the transcription factors WRKY25 and WRKY33. The *Arabidopsis* loss-of-function mutants *mekk1* and *mpk4* have a dwarf phenotype due to the accumulation of SA and H_2O_2; furthermore *PR* genes like *PR1* are constitutively activated making the mutants more resistant to pathogen attack (Colcombet & Hirt, 2008; Suarez-Rodriguez *et al*., 2010). Normally, upon activation, the PRs accumulate at the site of pathogen infection. They are also induced systemically as a part of SAR for the protection against further pathogen attack (see Par. 1.3). 17 families of *PRs* have been identified so far of which several have glucanase or chitinase activity and thus could functioning in the defense against fungal pathogens. Other groups comprise proteins with lysozyme activity against bacteria or proteins with broad-spectrum antimicrobial capacity (e. g. defensins and thionins) (Van Loon *et al.*, 1999; 2006). In addition, MAPK signaling controls the synthesis of phytoalexins, which are secondary metabolites with antimicrobial properties like some of the PR proteins. This was recently discovered for the major phytoalexin in *Arabidopsis*, camalexin, after infection with *B. cinerea* (Ren *et al.*, 2008). Phytoalexins are produced after pathogen challenge at the site of attack. For example several sesquiterpenes in potato, tobacco and tomato are classified as phytoalexins (Taiz & Zeiger, 2006).

1.4.1.4. Callose deposition

Cell wall appositions composed of callose are synthesized at the site of microbe invasion after recognition of MAMPs like flg22 (Gómez-Gómez *et al.*, 1999) several hours after MAMP challenge. Callose is a β-(1,3)-glucan polymer and is deposited between the plasma membrane and the cell wall. It can be produced also in response to wounding and other stress stimulators (Taiz & Zeiger, 2006). Callose can additionally serve as a matrix for the accumulation of antimicrobial compounds (Brown *et al*., 1998).

Recently it was published that flg22-triggered callose deposition requires the synthesis of indole glucosinolate (IGS) as well as ET defense signaling in *Arabidopsis*. IGSs are secondary metabolites deduced from the amino acid tryptophan (Clay *et al.*, 2009, Bednarek *et al*., 2009).

1.5. Effector-triggered immunity (ETI)

Pathogens have developed strategies to overcome MTI by the secretion of effector/Avr (avirulence) proteins. As a result of effector action, pathogens increase their virulence. However, effector proteins can be recognized by plant R (resistance) proteins, which results in the fast and strong activation of defense responses. Hence in such scenarios, effectors are not longer virulence but avirulence factors. In most cases, R proteins belong to the group of NB-LRR proteins. They consist of a nucleotide binding and a C-terminal leucine-rich repeat domain and contain a variable amount of LRRs. The nucleotide binding domain is responsible for ATP binding and hydrolysis. Two sub-classes of NB LRR proteins are known in plants which contain either a Coiled-coil (CC) or a Toll and human interleukin receptor (TIR) at the N-terminus (Belkhadir *et al.*, 2004; Eitas & Dangl, 2010). The interaction of these plant R proteins with the corresponding effector/avr (avirulence) protein of the pathogen is considered as the second branch of the plant immune system. If both gene loci are co-existing, plant resistance is observed. If either of both gene loci is missing or not active disease occurs (Dangl & Jones, 2001). This so-called gene-for-gene concept was developed by H. H. Flor.

He hypothesized that for each gene that confers resistance in the host plant a corresponding gene exists that causes pathogenicity of the pathogen (Flor 1955; 1971). R proteins have been found to guard certain plant proteins that are potential targets of effectors. Any change in the guardee is recognized by the R protein resulting in an immediate defense reponse. This model is summarized as the ‘guard hypothesis’. If R proteins recognize effector proteins, different defense signaling pathways are activated which leads to ETI (effector-triggered immunity) (Jones & Dangl, 2006). Resistance is achieved by R protein signaling and is often associated with a HR. In addition, SA signaling pathways and expression of *PR* genes participate in the establishment of ETI. ETI has only been reported for (hemi-) biotrophic pathogens (Glazebrook, 2005). The evolutionary concept of plant immunity is described in the “zigzag model” (Jones & Dangl, 2006) (Fig 1.1).

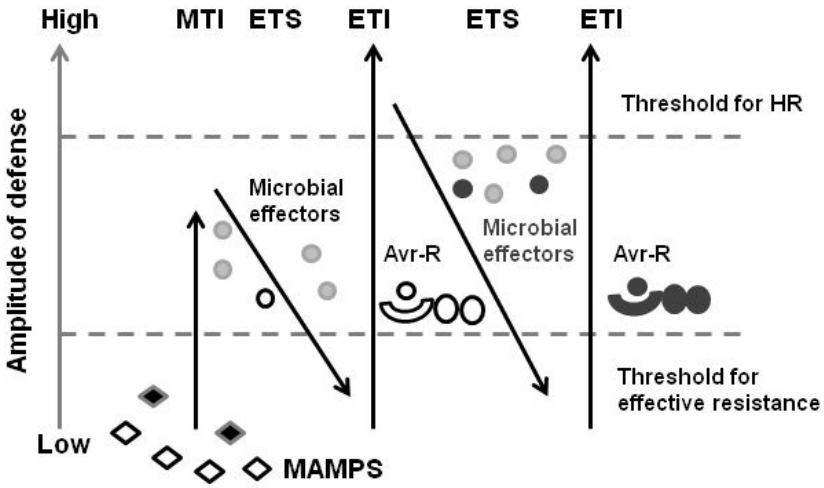


Fig. 1.1 The plant immune system illustrated as zigzag model (modified Jones & Dangl, 2006). At the beginning of the interaction, MAMPs are perceived by PRRs. The spreading of the invaders is blocked and this leads to MTI. As a result of selection pressure as evolutionary force, microbes developed effector proteins as strategy to overcome MTI and to establish effector-triggered susceptibility (ETS). Thereafter, host plants regained the ability to detect effector proteins by the development of R proteins. R protein-mediated/effector-triggered immunity (ETI) represents a faster and stronger immune response as compared to MTI as indicated by the amplitude of defense at the y-axis. During evolution, microbes have developed other or modified versions of existing effectors in order to prevent R protein-mediated recognition and to reestablish ETS. Consequently, plants established adequate R proteins to obtain a high immune status. In summary, the zigzag model defines an evolution-driven arms race between microbes and plants.

1.6. Nonhost resistance (NHR)

The immunity of a plant species against non-adapted pathogens is called nonhost resistance (NHR). This form of disease resistance is the most durable and efficient in plants. In general, pathogens are only able to infect a very limited number of plant species. Most plants are nonhosts for this pathogen. The invading microbes are confronted with different defense strategies of the plant. Permanent barriers like the wax cuticle, MTI and ETI protect the plant from disease development (Thordal-Christensen, 2003; Nürnberger & Lipka, 2005). NHR in *Arabidopsis* in response to incompatible powdery mildew species consists of a pre-invasive entry control where the genes *PEN1*, *PEN2* and *PEN3* (PEN = penetration) play an important role in restriction of these pathogens (Lipka *et al*., 2005; Stein *et al*., 2006). The second part of NHR is the post-invasive resistance, which is associated with HR-like cell death elicitation and callose deposition. The lipase-like proteins EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1), PAD4 (PHYTOALEXIN DEFICIENT 4) and SAG101 (SENESCENCE ASSOCIATED GENE 101) are involved in post-invasive resistance signaling against the powdery mildew species. These studies clearly indicate that NHR is genetically determined.

It is currently assumed that NHR is the result of the inability of non-adapted pathogens to overcome MAMP or effector-triggered immunity (Lipka *et al.*, 2010; Lipka *et al.*, 2005). Examples for microbial effector proteins will be given in the following paragraphs.

1.7. Plant Resistance proteins and microbial effectors

1.7.1. Bacterial effector proteins

The interaction of microbial effector/Avr proteins and plant resistance (R) proteins was especially studied on the basis of the interaction of different *Pseudomonas syringae* strains with model plants like *Arabidopsis* and tomato. *Pseudomonas syringae* pv*. tomato* causes the speck disease in tomato. The bacteria enter the host cell through stomata or wounds and proliferate in the apoplastic space (Gimenez-Ibanez & Rathjen, 2009). Depending on the strain, 15-30 bacterial effector proteins are delivered into the host cells by the type III secretion system (TTSS) (Jones & Dangl, 2006). The TTSS is found in Gram-negative pathogens of plants and animals and is an important factor for their virulence. In *P. syringae*, it is encoded by the hypersensitive response and pathogenicity (*hrp*) genes. Upon expression of the *hrp* genes a so-called needle complex is formed which consists of an envelope-embedded base and the protruding needle (Galan & Collmer, 1999; Jin *et al.*, 2003).

Two *P. syringae* effector proteins which have been extensively investigated are AvrPto and AvrPtoB. AvrPto and AvrPtoB are excellent examples for the confirmation of the zigzag model suggested by Jones and Dangl (2006). These two effectors influence the plant defense responses triggered during MTI through interference with different plant PRRs and PRR defense signaling. FLS2 and CERK1, the receptors for the detection of bacterial flagellin and fungal chitin, respectively, are ubiquitinated for degradation (Gimenez-Ibanez *et al.*, 2009; Göhre *et al.*, 2008; Xiang *et al.*, 2011). In resistant plants, AvrPto and AvrPtoB are recognized by the plant R protein Pto, a serine/threonine protein kinase, by direct physical interaction. Pto acts together with the LRR protein Prf to induce ETI (Pedley & Martin, 2003). AvrPtoB has ubiquitin ligase activity and ubiquitinates the plant kinase Fen, which is involved in AvrPtoB defense signaling, for degradation. Thus the effector AvrPtoB prevents HR by interference with the plant's ubiquitination machinery (Abramovitch *et al.*, 2006; Rosebrock *et al.*, 2007).

1.7.2. Oomycete effector proteins

Oomycetes belong to a separate group of microorganisms and are taxonomically related to algae. Among this group, several species cause economically important losses in yield.

For instance, *Phytophthora infestans* is responsible for the late blight disease in potato and tomato; *Plasmopara viticola* is a downy powdery mildew species which infests grapevine.

P. infestans is able to infect different *Nicotiana* species which results in a defense response including HR (Kamoun *et al.*, 1998). In the interaction of *P. infestans* and *Nicotiana benthamiana* several *Avr*/*R* gene interactions have been identified. The *P. infestans* effector protein Avr3a is recognized in the host cytoplasm by the R protein R3a, this perception triggers host cell death (Armstrong *et al.*, 2005). Avr3a contains a characteristic amino acid sequence, RXLR, present in many secreted proteins of *P. infestans* and other oomycetes. The motif is located next to the C-terminal site of the signal peptide and is involved in the translocation of effector proteins into the host cell (Whisson *et al*., 2007). In total, *P. infestans* possesses 563 predicted RXLR proteins (Haas *et al*., 2009). A protein motif similar to the RXLR sequence was found in the malaria parasite *Plasmodium* where it participates in the delivery of proteins in human erythrocytes (Birch *et al.*, 2006). The N-terminal RXLR motif and a dEER motif (including the surrounding amino acids) of an oomycete effector protein are even sufficient to guide the protein into the host cell in the absence of the pathogen. This was shown for a RXLR-dEER-GFP fusion protein (Dou *et al.*, 2008). Two different protein versions of Avr3a have been identified: $Avr3a^{EM}$ and $Avr3a^{KI}$. Especially $Avr3a^{KI}$ is a strong suppressor of plant cell death (HR) triggered by the MAMP-like secreted *P. infestans* effector protein INF1. In resistant plants, $Avr3a^{KI}$ is recognized by the plant R protein R3a and a HR is triggered. $Avr3a^{EM}$ is not perceived by R3a and suppresses HR only weakly. Especially $Avr3a^{KI}$ but also $Avr3a^{EM}$ interact with and stabilize the plant U-Box E3 ligase CMPG1 which is necessary for the cell death response. Thus, the RXLR protein manipulates the plants' ubiquitin proteasome system to disturb plant immunity (Bos *et al.*, 2006, 2009, 2010; Gilroy *et al*., 2011).

1.7.3. Basidiomycete effector proteins

Melampsora lini: The interaction of the flax rust *Melampsora lini* with flax, *Linum usitatissimum*, was investigated by Flor and served for the establishment of the gene-for-gene concept (Flor, 1971, see Par. 1.5). *M. lini* is an obligate biotrophic and pathogenic basidiomycete which forms haustoria during the infection of plant cells. In a screen of a flax rust haustorium specific cDNA library, 21 haustorially expressed secreted proteins (HESPs) have been identified; some of these HESPs have been shown to possess avirulence activity (Catanzariti *et al*., 2006). Several R proteins in flax recognize secreted Avr effector proteins. For example AvrM is perceived by the M resistance protein. Upon recognition, M-dependent host cell death occurs (Catanzariti *et al.* 2010). It was also demonstrated that the AvrM effector is delivered from the haustoria in the host cells. Even in the absence of the fungus the effector is internalized into the plant cell (Rafiqi *et al.*, 2010).

Ustilago maydis: *Ustilago maydis* is a biotrophic maize pathogen, which is growing in two different forms, a unicellular haploid and a filamentous dicaryotic form. The dicaryotic pathogenic form grows intracellularly in the host plant and induces the formation of tumors (Banuett, 1995). The genome of *U. maydis* was sequenced and 426 sequences were found coding for secreted proteins. Interestingly, 12 gene clusters coding for small secreted proteins were identified. The majority of these genes are induced upon plant infection and the gene clusters are regulated together. Furthermore, the deletion of individual clusters impaired the virulence of the fungus (Kämper *et al.*, 2006). The secreted protein Pep1 was identified as an apoplastic effector, which is important for the entrance of the hyphae into the host cell. Mutants deficient in this protein trigger an intensive defense response in the plant (Doehlemann *et al*., 2009).

Laccaria bicolor: The basidiomycete *Lacaria bicolor* belongs to the group of ectomycorrhiza and grows in association with different trees, for example *Pinus sylvestris*. The genome was sequenced and it contains sequences for 278 cysteine-rich, small secreted proteins with a size less than 300 amino acids (aa). Some of them are expressed exclusively in symbiotic interactions; thus these proteins could serve as effectors in the establishment of the symbiosis of *L. bicolor* with plant roots (Martin *et al.*, 2008). Further studies are necessary to collect more information about the function of these secreted proteins. The association of *L. bicolor* with roots of forest trees can be seen as an example for mutualism, which is further described in the following paragraph.

1.8. Mutualistic plant-microbe interactions

Disease is an exception in plant–microbe interactions and can be regarded as an imbalance in symbiosis. Balanced stages like commensalistic or neutral interactions imply a beneficial effect for one partner while the other one is not disturbed. By contrast, mutualism describes a situation in which the host plant and the colonizing microbe gain some benefits (Kogel *et al.*, 2006; Newton *et al.*, 2010; Redman *et al.*, 2001).

The term ‘fungal endophyte’ is used for fungi that inhabit plants without causing visible disease symptoms (Schulz & Boyle, 2005). Relationships of endophytic or mycorrhizal fungi with plant roots are important examples for beneficial interactions. A distinction is drawn between different types of mycorrhiza. Arbuscular mycorrhiza (AM) or glomeromycotan mycorrhiza form the most frequent connection between plant roots and fungi.

About 70- 90% of all land plants are associated with AM. Orchidaceae and Ericales harbor special types of mycorrhiza and are colonized by AM, ericoid and orchid mycorrhiza.

Ectomycorrhizal associations (ECM) are characteristic for certain families of gymnosperms, dicotyledons and few monocotyledonous plants. They extracellularly colonize their hosts (Brundrett 2002; Parniske 2008).

Arbuscular mycorrhiza (AM): AM belong to the order Glomales (Glomeromycota) and are obligate biotrophs. They reproduce asexually by forming multinucleate spores. So far, no sexual form has been identified (Hause & Fester, 2005). Short time after germination of AM spores on plant roots the formation of appressoria can be observed. The hyphae penetrate root cell walls and intracellular tree-like structures, the arbuscules, are formed by dichotomous branching (Strack *et al.*, 2003). Arbuscules are supposed to be the predominant regions for nutrient exchange. The hyphae provide water and especially phosphate for the plant, the fungus receives carbohydrates in return (Parniske, 2008).

Ectomycorrhiza (ECM): Ectomycorrhiza can be found in the phyla ascomycota and basidiomycota; their hyphae cover plant roots, form a mantle and grow within the apoplastic space of the root. In the apoplastic space the Hartig net is formed, which consists of interweaved hyphae between root cells. The Hartig net is the site of nutrient exchange in the interaction (Brundrett, 2004; Plett & Martin, 2011).

1.9. *Piriformospora indica*

1.9.1. Taxonomic classification

The fungus *Piriformospora indica* was firstly described by Verma *et al*. in 1998. It was isolated as a contaminant of collected AM spores from the rizhospere of two wood species growing in the Indian Thar desert. *P. indica* can be cultivated in different axenic cultures. Due to its morphology and the analysis of the 18S rRNA, *P. indica* was classified as a basidiomycete. The fungus asexually produces pear-shaped chlamydospores with eight to 25 nuclei (Verma *et al.*, 1998). It belongs to the Sebacinales, an order containing ectomycorrhizae as well as ericoid and orchid mycorrhizae, which are associated with roots of a huge variety of plant species. The Sebacinales can be found in different ecosystems all over the world and are divided in two clades: Clade A harbors ectomycorrhizal fungi; in clade B, a more heterologous group, endomycorrhiza and endophytes, including *P. indica*, are sorted (Selosse *et al*., 2007; 2009; Weiß *et al.*, 2004). Fungi of the order Sebacinales are sometimes intimately associated with bacteria. For *P. indica* an intimate association with *Rhizobium radiobacter*, a Gram-negative α-proteobacterium, was detected (Sharma *et al.*, 2008).

1.9.2. Beneficial effects on host plants

Like several species of the order Sebacinales, *P. indica* is able to colonize a broad range of host plants, including model organisms like *Arabidopsis*, tobacco and barley (Deshmukh *et al.*, 2006; Peškan-Berghöfer *et al.*, 2004; Varma *et al.*, 1999). Until now, no plant species has been identified whose roots cannot be colonized by the fungus (Schäfer & Kogel, 2009). In symbiotic interactions with host plants, *P. indica* has growth promoting effects. For barley, an increase in plant biomass and grain yield was demonstrated upon fungal inoculation under greenhouse as well as open top conditions (Achatz *et al.*, 2010; Waller *et al.*, 2005). Also tomato plants, which were grown in hydroponic culture and inoculated with *P. indica* showed an increase in fruit biomass and dry weight per plant (Fakhro *et al.*, 2010). In Chinese cabbage, *P. indica* promotes shoot and root growth and lateral root development. Additionally the plants show a higher tolerance to drought stress. The enhanced drought tolerance could be due to the activation of antioxidant enzymes, drought related genes, and the plastid-localized Ca^{2+}-sensing regulator (CAS) protein (Sun *et al.*, 2010). Antioxidants are also involved in the establishment of increased salt tolerance of barley plants, which are inoculated with *P. indica* (Baltruschat *et al.*, 2008; Waller *et al.*, 2005). The fungus also confers increased resistance to different plant pathogens.

Barley plants develop higher resistance to the necrotrophic fungal pathogens *Fusarium graminearum* and *Fusarium culmorum* (Deshmukh & Kogel, 2007; Waller *et al.*, 2005), the hemibiotrophic root pathogen *Cochliobolus sativus* and also enhanced systemic resistance to the biotrophic leaf pathogen *Blumeria graminis* f. sp. *hordei* (Waller *et al*., 2005). In wheat, *P. indica*-colonized plants are more resistant to the leaf pathogen *B. graminis* f. sp *tritici*, the stem base pathogen *Pseudocercosporella herpotrichoides* and the root pathogen *F. culmorum* under greenhouse conditions. Under field conditions, wheat plants infected with *P. herpotrichoides* developed significantly attenuated disease symptoms (Serfling *et al.*, 2007). For *Arabidopsis*, a higher resistance to the biotrophic leaf pathogen *Golovinomyces orontii* was reported, which might base on ISR (Stein *et al.*, 2008).

1.9.3. Colonization strategy of *P. indica*

The colonization procedure of barley by *P. indica* starts with the germination of chlamydospores on the root surface. The growing hyphae later form an extracellular net and also inter- and intracellular growth can be observed. During further development, hyphae grow extensively in cortical and rhizodermal cells, this phase seems to be associated with host cell death. In contrast, no fungal hyphae were found in the plant meristematic zone or in the aerial part of the plant. Sometimes the synthesis of new chlamydospores can be seen (Deshmukh *et al*., 2006, Schäfer *et al.*, 2009).

For *Arabidopsis* a similar colonization pattern was documented recently. Jacobs *et al.* (2011) divided the growth of *P. indica* with *Arabidopsis* roots in four phases. At the beginning of the interaction, the fungus grows extracellularly (~ one day after inoculation (dai)). Thereafter, a biotrophic colonization phase (< three dai) is established, which is followed by a cell death-associated colonization phase. Finally extra- and intracellular chlamydospore production occurs. Although a cell-death associated phase was discovered (Deshmukh *et al*., 2006, Jacobs *et al*., 2011), roots colonized by *P. indica* look as healthy as not inoculated control plants. Pathogenic changes like necrosis have not been observed (Schäfer & Kogel, 2009). These studies indicate that the colonization of plant roots by *P. indica* seems to depend on an elaborated communication between the host plant and the fungus.

1.9.3.1. Role of plant hormones in the *P. indica*-plant association

Several plant hormones revealed to be important for the growth promoting effects conferred by the fungus and also for the colonization success of *P. indica*. JA signaling is intimately involved in the establishment of the beneficial effects as well as in the colonization success of *Arabidopsis* roots. The resistance against powdery mildew in *Arabidopsis*, which is built up after inoculation with *P. indica*, was found to rely on JA signaling (Stein *et al.*, 2008). Further, *P. indica* seems to be able to actively suppress MTI by using JA signaling and requires the JA signaling pathway for *Arabidopsis* root colonization (Jacobs *et al.*, 2011). Also gibberellin signaling is involved in the establishment of the plant *P. indica* association. Barley mutant plants impaired in gibberellin synthesis and perception were less colonized by *P. indica* than wild type plants (Schäfer *et al.*, 2009). In addition, ethylene (ET) signaling is relevant for the maintenance of the growth promoting effects conferred by *P. indica* (Camehl *et al.*, 2010). The *Arabidopsis* mutant line *ctr1* with constitutive activation of ET responsive genes was stronger colonized by *P. indica*. So ET signaling seems to be affected in *P. indica*-colonized plant roots (Schäfer & Kogel, 2009). Furthermore, *P. indica* is able to produce the phytohormones auxin (Sirrenberg *et al.*, 2007; Vadassery *et al.*, 2008) and cytokinin (Vadassery *et al*., 2008). Cytokinins are discussed to be important for the growth promoting effects of *P. indica* on host plants (Vadassery *et al.*, 2008). Thus plant hormone levels as well as their signaling pathways are modulated by the fungus or the plant allowing a mutualistic connection between the two partners.

1.9.3.2. Further factors influencing the crosstalk of *P. indica* and the host plant

Several factors involved either in growth promotion or stress tolerance have been identified. The intracellular calcium (Ca^{2+}) level of the host plant seems to be important in early signaling processes at the beginning of the symbiosis between *P. indica* and the host plant. *Arabidopsis* and tobacco plants, which were treated with *P. indica* cell wall extract, exhibited a transiently increased cytosolic calcium level (Vadassery *et al.*, 2009b). Furthermore, *P. indica* confers long-term tolerance to salt and drought stress in barley (Baltruschat *et al.*, 2008; Waller *et al.*, 2005) as well as drought stress in *Arabidopsis* (Vadassery *et al.,* 2009a; see also Par. 1.9.2). This is mediated by an enhanced antioxidative capacity through the activation of the gluthatione-ascorbate cycle in the host plant. Enzymes of the gluthatione-ascorbate cycle like monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) are more active in plants colonized by *P. indica* upon confrontation with salt or drought stress (Baltruschat *et al.*, 2008; Vadassery *et al.*, 2009a). Thus a balanced level of ascorbate in the host plant is important for the mutualistic interaction with the fungus (Vadassery *et al*., 2009a).

The plant gene *PYK10* is coding for a root- and hypocotyl-specific β-glucosidase/myrosinase, which is known to play a role in defense reactions against herbivores and pathogens. Growth promoting effects are absent in *Arabidopsis* plants deficient in the *PYK10* gene, when their roots are colonized by *P. indica* (Sherameti *et al.*, 2008). Sherameti and coworkers (2008) assumed that *PYK10* is involved in the restriction of root colonization by *P. indica* and thus reduction of defense responses by the plant improving establishment of a mutualistic interaction.

1.10. Objectives

Only little is known about effector proteins of mycorrhiza or endophytic fungi. These effector proteins could be secreted during the interaction of the symbionts with their respective host plants. They are thought to influence the plant innate immune system and to modulate host metabolism to establish compatibility. *P. indica* is an interesting candidate to study these compatibility factors due to its broad host range and its cultivability in axenic culture. For the isolation of such secreted effector proteins of *P. indica*, a method called yeast signal sequence trap (YSST) was adopted. This yeast-based technique allows the trapping of proteins harboring a signal peptide (SP) and thus is suitable to isolate extracellular proteins which could be functioning as effectors in the *P. indica*-plant association. The secretion of putative effector proteins was triggered by growing *P. indica* in liquid culture including cell walls and root extracts of *Arabidopsis* plants. This experimental design should mimic plant roots and enrich the amount of secreted proteins originating from *P. indica*. Isolated candidate genes should be characterized further by *in silico* studies and functional analyses.

In a second project, the candidate Pi*ALH43* (*PIIN_00029*) should be characterized further concerning its function in root colonization. PiALH43 was already described as a secreted protein of *P. indica*, which is induced *in planta* and contains a RING domain with E3 ubiquitin ligase activity *in vitro* (Khatabi, 2009).

2. MATERIALS AND METHODS

All chemicals and laboratory equipment used for these studies were delivered by the following companies: Sigma-Aldrich (Munich, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Sarstedt (Nümbrecht-Rommelsdorf, Germany), Greiner (Frickenhausen, Germany), BD (Heidelberg, Germany), Duchefa (Haarlem, the Netherlands). The suppliers of the electrical equipment and kits are listed in the corresponding description of the methods.

2.1. Basic molecular biological methods

2.1.1. RNA extraction (modified according to Logemann *et al.*, 1987)

For the extraction of RNA, 1 ml of RNA extraction buffer was added to the powdered tissue sample. Then samples were vortexed thoroughly until all powder was dissolved. Immediately 200 µl chloroform was added and vortexed again. All samples were incubated for ten minutes on a shaker at room temperature (RT). Then they were centrifuged for 15 min. at 4°C and 14000 rpm. The upper phase was transferred to a new tube containing 850 µl chloroform. This tube was vortexed for 15 seconds at high speed and incubated for about 2 min. at RT prior to another centrifugation step (15 min., 4°C, 14000 rpm). The upper phase was transferred in a new tube, mixed with 1 ml 5 M LiCl and incubated at -20°C over night.

The next day, all samples were centrifuged for 20 min. at 4°C and 14000 rpm. After removal of the supernatant, pellets were washed twice with 900 µl 70% $Ethanol_{DEPC}$ and centrifuged for 10 min. at 4°C and 14000 rpm. Pellets were air dried and dissolved in 53 µl H_2O_{DEPC}. This solution was finally centrifuged for 10 min. at 4°C and 14000 rpm. 50 µl of the supernatant were transferred to a new tube. RNA concentrations were determined using Nanodrop (Peqlab, Erlangen, Germany).

RNA extraction buffer (100 ml)

Phenol saturated	38%	30 ml
Guanidine thiocyanate	0,8 M	11,82 g
Ammonium thiocyanate	0,4 M	7,5 g
3 M Sodium acetate (pH 5)	0,1 M	3,33 ml
Glycerol	5%	5 ml

ad 100 ml A. bidest. treated with Diethylpyrocarbonate (DEPC)

DEPC-treatment of A. bidest.

DEPC was added at a concentration of 0,1% to water. The mixture was stirred for about 2 hours at RT. Thereafter, the water was incubated at 37°C over night and finally autoclaved.

2.1.2. Polymerase chain reaction (PCR)

For the amplification of DNA from different template types (cDNA, plasmid, genomic DNA) a standard PCR mixture with DNA polymerase (DCS Pol, DNA cloning service) was used. A standard PCR program is given below. Annealing temperatures depend on the sequence of the primers, the elongation time on the size of the amplified DNA fragment.

PCR mixture (1x)	
Buffer BD	2 µl
25 mM $MgCl_2$	2 µl
2 mM dNTPs	2 µl
Primer fwd 10 µM	1 µl
Primer rev 10 µM	1 µl
DCS Pol	0,2 µl
Template DNA	0,5 – 2 µl
A. bidest	ad 20 µl

Cycler program for PCR		
94℃	5 min.	
94℃	30 sec.	} 28 -37 x
x℃	30 sec.	
72℃	x sec.	
72℃	5 min.	

2.1.3. Agarose gel electrophoresis

2.1.3.1. DNA samples

The analysis of DNA and RNA samples was performed with agarose gel electrophoresis. For the separation of DNA samples 1 x TAE or 1 x TBE buffer with 0,8 – 1,5% agarose, supplied with ~0,75 -1 µg/ml ethidium bromide, was used. The samples, for example PCR products, were mixed 1:10 with 10 x DNA loading buffer and separated at 80 -120 V for about 1 hour. The 1kb plus DNA ladder from Invitrogen (Darmstadt, Germany) was used as standard.

10 x TBE (Tris-Borate-EDTA) (1 l)	
Tris	0,9 M (109,03 g)
Boric acid	0,9 M (55,65 g)
$Na_2EDTA \times 2\ H_2O$	0,025 M (9,31 g)

50 x TAE (Tris-Acetate-EDTA) (1 l)	
Tris	242 g
Glacial acetic acid	57,1 ml
$Na_2EDTA \times 2\ H_2O$	37,2 g

10 x DNA loading buffer	
Glycerol	50%
$Na_2EDTA \times 2\ H_2O$	0,1 M
SDS	1%
Bromphenol blue	0,04%

2.1.3.2. RNA samples

The quality of RNA isolations was checked on 1,2% agarose gels made of 1 x MOPS buffer including 5% (v/v) formaldehyde. Prior to gel loading an adequate amount of RNA (0,25 – 1,0 µg) was mixed 1:1 (v/v) with 2 x RNA loading dye (Fermentas, St. Leon-Roth, Germany) and denatured at 95℃ for 5 min.

10 x MOPS (3-(N-morpholino)propanesulfonic acid) buffer (1 l)

MOPS	0,2 M	41,86 g
Sodium acetate	0,05 M	4,102 g
EDTA	0,01 M	3,722 g

pH 7,0 with 10 M NaOH, DEPC-treatment over night, finally autoclaved

2.1.4. Transformation of chemically competent *E. coli*

Glycerol stocks of *E. coli* DH5α were thawed on ice for several minutes. Then the plasmid of interest was added to the cells (usually between 5 - 10 ng plasmid) and the mixture was incubated on ice for 20 min. Thereafter, the cells were incubated for 45 seconds at 42°C in a water bath or a heating block and cooled down on ice for 2 min. Then 500 µl SOC or LB medium was added and the cells were incubated at 37°C and 220 rpm for 90 minutes. Finally the cells were plated in adequate dilutions on LB medium containing the respective antibiotics for selection and incubated upside down at 37°C over ni ght.

LB medium, 1 l (lysogeny broth, modified Bertani, 1951)

Peptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar-Agar	15 g (for plates only)

pH adjusted to 7,0 with NaOH, if necessary

After autoclaving filter-sterilized antibiotic (e. g. 100 µg/ml ampicillin or 50 µg/ml kanamycin) was added to the LB medium.

SOC medium (100 ml)

Tryptone	2 g
Yeast extract	0,5 g
NaCl	10 mM
KCl	2,5 mM
$MgCl_2$	20 mM, from 2 M stock, filter-sterilized, add after autoclaving
Glucose	20 mM, from 2 M stock, filter-sterilized, add after autoclaving

2.1.5. Colony-PCR with *E. coli* or *Agrobacterium tumefaciens* transformants

Single colonies from the transformation plate were collected with a toothpick and transferred to a new LB plate for further applications. Then the toothpick was directly put in a PCR reaction tube containing the colony PCR mixture.

Colony PCR mixture (1 x; total volume 10 µl)

10 x buffer BD	1 µl
2 mM dNTPs	1 µl
25 mM $MgCl_2$	1 µl
Primer fwd 10 µM	0,2 µl
Primer rev 10 µM	0,2 µl
DCS Pol	0,1 µl
H_2O bidest.	6,5 µl

2.1.6. Production of chemically competent *E. coli* DH5α cells

Liquid LB medium (3 ml) was inoculated with DH5α cells from a glycerol stock and grown over night at 37℃ and 220 rpm. The next day, 200 ml LB medium was inoculated with the overnight culture and incubated at 37℃ and 220 rpm for 4 – 6 hours until an OD_{600} of 0,6 was reached. The culture was harvested and centrifuged for 10 min. at 4℃ and 3000 rpm. The supernatant was discarded and the pellet was resuspended carefully in 20 ml cold transformation buffer 1 (TFB1). The cells were incubated on ice for 10 min. and centrifuged again. The supernatant was removed and the pellet was dissolved carefully in 8 ml cold transformation buffer 2 (TFB2). After 10 min. incubation on ice aliquots of 200 µl were filled in 1,5 ml tubes and immediately frozen in liquid nitrogen.

Transformation buffer 1 (TFB1)

RbCl	100 mM
$MnCl_2$	45 mM
Potassium acetate	35 mM
$CaCl_2$	19 mM
LiCl	0,5 mM
Glycerol	15%

Transformation buffer 2 (TFB2)

MOPS	10 mM
$CaCl_2$	75 mM
RbCl	10 mM
Glycerol	15%

2.2. Cultivation of *Piriformospora indica*

2.2.1. Maintenance of *P. indica* cultures

For all experiments, *P. indica* DSMZ 11827 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was used. Glycerol stocks of *P. indica* with a concentration of 500.000 spores/ml were stored at -80℃ and used for the preparation of master plates. These plates served for the preparation of working plates containing *Aspergillus* complex medium (CM) by transferring agar blocks with mycelium to the working plates. Alternatively, master plates were covered with tween water (0,002% Tween 20) and scratched with a rubber spreader. The obtained spore and mycelium suspension was spread on fresh plates. After four to six weeks incubation at 24℃ in the dark, spores were collected from working plates and resulting spore suspension was used for the experiments.

Aspergillus complex medium (CM, 1 l)	
20 x salt solution	50 ml
Glucose	20 g
Peptone	2 g
Yeast extract	1 g
Casamino acids	1 g
1000 x Microelements	1 ml
Agar-Agar (for plates only)	15 g

20 x salt solution (1 l)	
$NaNO_3$	120 g
KCl	10,4 g
$MgSO_4$ x 7 H_2O	10,4 g
KH_2PO_4	30,4 g

1000 x microelements (1 l)	
$MnCl_2$ x $4H_2O$	6 g
H_3BO_3	1,5 g
$ZnSO_4$ x $7H_2O$	2,65 g
KI	750 mg
Na_2MO_4 x $2H_2O$	2,4 mg
$CuSO_4$ x $5H_2O$	130 mg

2.2.2. Preparation of *P. indica* chlamydospore suspension

Plates with *P. indica* were covered with distilled water including 0,002% Tween 20 and scratched with a rubber spreader. Thereafter, the supernatant was filtered through a sheet of Miracloth (Calbiochem, Merck, Darmstadt, Germany) using a funnel and collected in an Erlenmeyer flask. The spores were sonicated for three minutes in an ultrasonic bath, washed three times with Tween water and collected after centrifugation for seven minutes at 3500 rpm at room temperature (RT). Finally the spores were counted using a Fuchs-Rosenthal counting chamber. The spore concentration was adjusted to 500.000 spores/ml.

2.3. Yeast signal sequence trap (YSST)

2.3.1. Growth and treatment of plants and fungal inoculum for YSST

2.3.1.1. *Arabidopsis thaliana*

A. thaliana ecotype Columbia-0 (Col-0, NASC N1092) was used to receive root cell walls and root extract. Therefore, *Arabidopsis* seeds were surface-sterilized applying 70% ethanol for less than one minute followed by 3% NaOCl for ten minutes and several washing steps with sterile distilled water. Finally, seeds were dried under sterile conditions on filter paper. About 50 seeds were germinated in petri dishes containing ½ Murashige & Skoog (MS) medium including vitamins (Duchefa), 1% sucrose and 0,22% gelrite and stored at 4°C for two days in the dark. Thereafter, seedlings were grown at 22-25°C (16 hou rs light/ 8 hours dark, light intensity approx. 47 µmol m^{-2} s^{-1}) for one to two weeks. Adult plants or alternatively only roots were transferred to liquid MS-medium including vitamins, 2% sucrose and 2 ppm indole-3-acetic acid (IAA). IAA was added in order to intensify root growth.

These liquid cultures were grown at 22-25℃ (16 hou rs light/ 8 hours dark, light intensity approx. 47 µmol m^{-2} s^{-1}) by shaking at 90 rpm. Roots were harvested after two to three weeks and after determining the fresh weight, the material was frozen in liquid nitrogen and stored at –70℃.

2.3.1.2. Preparation of root cell walls and root extract

Root cell walls: The root cell walls were prepared with slight modifications according to Sposato *et al.* (1995). The roots were crushed in liquid nitrogen and washed in cold distilled water in a glass beaker (ratio of 1:2 in relation to the fresh weight of the roots). The material was filtrated using a small meshed gaze (mull) and squeezed.

This washing and filtration step was repeated several times (four to five washes). Then fibers were extracted with a mixture of methanol/chloroform (v/v 1:1). To remove the methanol/chloroform mixture the roots were filtrated again. This extraction procedure was repeated 5 - 7 times. Finally the fibers were washed with acetone and dried under sterile conditions in glass petri dishes. The fibers were crushed with a mortar and pistil using liquid nitrogen under sterile conditions to receive cell wall powder for further experiments. The cell walls were stored at RT.

Root extract: The roots were homogenized using liquid nitrogen. To receive a final concentration of 50% root extract, 110% of the measured fresh weight was added in form of cold 10 mM potassium phosphate buffer pH 7,0 and mixed properly. For example, if the measured fresh weight of the roots was 20 g, the root material was mixed with 22 ml potassium phosphate buffer. This homogenate of roots and potassium phosphate buffer was centrifuged at 25.000 x g for 30 minutes at 4℃. The supernatant was vacuum f iltrated with a suction filter and filter paper. At the end the root extract was filter-sterilized (0,2 µm filter) and stored at -70℃.

2.3.1.3. Liquid culture of *P. indica* for YSST

P. indica spore suspension was prepared as described above (Par. 2.2.2). 20 ml spore suspension was poured in 1 l CM and mixed well. Then, 50 ml aliquots of this solution were given in 100 ml Erlenmeyer flasks. The flasks were incubated on a shaker at ~115 rpm and at 27-29℃ in the dark.

After one week, *P. indica* cultures were washed individually with A. dest. using a sieve and a glass beaker and thereafter incubated in 50 ml A. dest. over night. The next day, the mycelium was again washed with A. dest. and transferred to modified SNA minimal medium.

SNA medium, 1 l (modified according to Nirenberg, 1976)

KH_2PO_4	1 g
KNO_3	1 g
$MgSO_4 \times 7H_2O$ (autoclaved separately)	0,5 g
KCl	0,5 g
1 M NaOH	6 ml

Some flasks were supplied with 1% *Arabidopsis* cell walls and 1% *Arabidopsis* root extract. The other flasks were kept in pure SNA medium as a control. The mycelium was harvested one, two and three days after transfer in the new (cell wall) medium. Therefore it was washed with A. dest. and dried shortly on filter paper and immediately frozen in liquid nitrogen. Fungal material was crushed under liquid nitrogen; the powder was kept at -70°C until further processing.

2.3.2. Isolation and concentration of *P. indica* mRNA

After extraction of total RNA from the *P. indica* samples as described in Par. 2.3.1.3, isolation of mRNA was performed according to the protocol of Invitrogen Dynabeads® Oligo $(dT)_{25}$ (Invitrogen). For each sample different batches of Dynabeads were used. To increase the amount of isolated mRNA, the beads were used two times on the same sample as described in the manufacturer's protocol. The mRNA concentration was determined using Nanodrop (Peqlab, Erlangen, Germany).

About 1,5 µg *P. indica* mRNA from each sample (one, two and three days after transfer to inducing conditions) was collected in one tube (totally about 4,5 µg mRNA). To decrease the volume of mRNA for the following cDNA synthesis, Microcon 30 filter units (Millipore, Billerica, USA) were applied. The membrane of the filter units was initially washed three times with RNase free water (30 µl, 11000 rpm, 4°C). Thereafter, mRNA samples were loaded and centrifuged at 11000 rpm and 4°C for several minutes. The amount of residual liquid in the collection tube was checked and this procedure was repeated until the volume on the membrane was applicable for the synthesis of double stranded cDNA (dscDNA). Finally the filter unit was turned upside down and centrifuged for 3 minutes at 3000 rpm to regain mRNAs.

2.3.3. First and second-strand cDNA synthesis

The mRNA (Par. 2.3.2) was forwarded to double-stranded cDNA (dscDNA) synthesis. First and second-strand cDNA synthesis was performed according to SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). For the synthesis, random primers with *Not*I and *Xho*I restriction sites were used.

Primer sequence:
Random nonamers with *Not*I and *Xho*I restriction site
5'-CGATTACTCGAGGCGGCCGCNNNNNNNNa-3'
5'-CGATTACTCGAGGCGGCCGCNNNNNNNNc-3'
5'-CGATTACTCGAGGCGGCCGCNNNNNNNNg-3'
5'-CGATTACTCGAGGCGGCCGCNNNNNNNNt-3'

2.3.3.1. Clean-up of dscDNA and integrity check

In order to remove salts and nucleic acids smaller than 100 bp (primers, nucleotides etc.), the dscDNA was purified with a Qiaquick PCR Purification Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The dscDNA was eluted in 30 µl nuclease-free water and the concentration was determined using Nanodrop (Peqlab). The dscDNA was separated by agarose gel electrophoresis. About 25 ng of dscDNA containing 1 x DNA loading buffer was given on a 1 x TAE gel with 1,5% agarose. The gel was run over a distance of 1-2 cm (30 minutes, 50 V) and stained afterwards in an ethidium bromide bath (~ 0,75 -1 µg/ml ethidium bromide).

To determine the quality of synthesized dscDNA, self-ligation of a small amount of dscDNA was performed. A 10 µl reaction mix with one unit T4 DNA-Ligase (Fermentas) and PEG 4000 for blunt end ligation was prepared. The mixture was incubated for one hour at RT; prior to heat inactivation of the enzyme. The result of the self-ligation was analyzed on an agarose gel (1%). A small amount of the original dscDNA was loaded as reference and the samples were separated by gel electrophoresis.

2.3.4. Synthesis and integrity check of *Eco*RI adapters

For the synthesis of *Eco*RI adapters, primers with the following sequences were ordered:

Multilinker-low: 5'- CTC GAC GTC CAG ATC TTC AG -3',
Multilinker-up: 5'- AAT TCT GAA GAT CTG GAC GTC GAG -3'

The multilinker-low primer was phosphorylated at the 5' end. Both primers were dissolved in nuclease-free water to a concentration of 1 mM and kept on ice until further processing. 5 µl of each primer and 10 µl nuclease-free water was put in a PCR tube. The concentration was adjusted to 250 µM for each primer.

For the synthesis, a cycler from Biometra (Biometra, Göttingen, Germany) was employed. The sample was put into the machine when the PCR block reached a temperature of 80°C. The synthesized adapters were always kept on ice and stored at -20°C.

Tab. 2.1 Program for adapter synthesis with Biometra PCR cyclers

temperature [℃]	**time**	**ramp**
80	∞	4℃/sec
after 1 minute press "start/stop" and then "continue"		
70	∞	4℃/sec
after a few seconds press "start/stop" and then "continue"		
50	∞	0,01℃/sec
after a few seconds press "start/stop" and then "continue"		

The synthesized adapters were checked on a DNA acrylamide gel. To remove un-polymerized acrylamide, the empty gel was run for 30 min. at 35 V. An aliquot of the double-stranded (ds) ds adapters and aliquots of each single-stranded (ss) primer were mixed with loading buffer, loaded on the gel and run for 30 min at 50 V. As DNA marker a low range marker (Fermentas) was applied. Thereafter, the gel was stained in an ethidium bromide bath and analyzed under UV light.

Recipe for 2 small DNA acrylamide gels

50 x TAE	240 µl
Acrylamide (30%)	8 ml
Ammonium persulfate (APS)	50 µl
TEMED	7 µl
H_2O dest.	3,7 ml
Total	12 ml

2.3.5. Ligation of *Eco*RI adapters and dscDNA and subsequent purification

The ligation of dscDNA and adapters was performed with a T4 DNA ligase including PEG 4000 for blunt end ligation according to the manufacturer's protocol (Fermentas). The dscDNA and the adapters were added in a molarity of 1:100 (part dscDNA: part adaptors) and incubated for four hours at RT. Then the enzyme was heat inactivated for 10 minutes at 65℃. At the beginning and at the end of the ligation reaction a small amount of the reaction mix was taken to analyze the success of the reaction on an acrylamide gel as described for the synthesis of adapters (Par. 2.3.4). The purification of the ligation mixture was performed with SureClean kit from Bioline (Bioline, Luckenwalde, Germany) according to the manufacturer's protocol with some modifications. An equal volume of SureClean solution was added to the dscDNA and mixed thoroughly. The mixture was incubated at RT for at least 20 minutes.

Then, the sample was centrifuged at 14000 rpm for 20 min. and the supernatant was removed until 2-3 µl were left in the tube. Afterwards a mixture of SureClean solution and water (v/v 1:1) was added, mixed and incubated for up to 10 minutes at RT.

The mixture was centrifuged for 15 min. at 14000 rpm. The supernatant was removed as described above. One volume of 70% ethanol, equal to the double amount of the original sample volume was added to the pellet and mixed thoroughly for ten seconds. The sample was incubated for up to 10 min. at RT and centrifuged again (10 min, 14000 rpm). After removal of the supernatant, the pellet was washed again using 70% ethanol and air-dried. Finally the pellet was dissolved in 50 µl nuclease-free water. To analyze the success of the purification, a small amount of the purified dscDNA and the supernatants containing SureClean solution were loaded on a 1% agarose gel.

2.3.6. *Not*I digestion of cDNA library

The dscDNA with Eco*R*I adapters was digested with *Not*I according to the manufacturer's protocol (Fermentas) and incubated at 37°C over night. The purification of the *Not*I digested library was again performed using the SureClean kit from Bioline as described in Par. 2.3.5. The success of the clean-up was again verified on an agarose gel. Additionally, a PCR was performed in order to amplify dscDNA fragments and to determine the average size of the fragments.

Sequences of primers (annealing temperature 60°C):

Primer fwd (from Multilinker-up): 5'- AAT TCT GAA GAT CTG GAC GTC GAG-3'

Primer rev (from *Not*I random primers): 5'-ATT ACT CGA GGC GGC CGC-3'

2.3.7. Preparation of the YSST vector (pSMASH)

The binary vector used for the YSST screen, pSMASH (Goo *et. al.*, 1999), includes a truncated version of an extracellular invertase. The invertase is lacking a signal peptide and a starting methionine and is flanked by a strong ADH promoter. The dscDNA fragments were cloned in pSMASH upstream of the invertase gene.

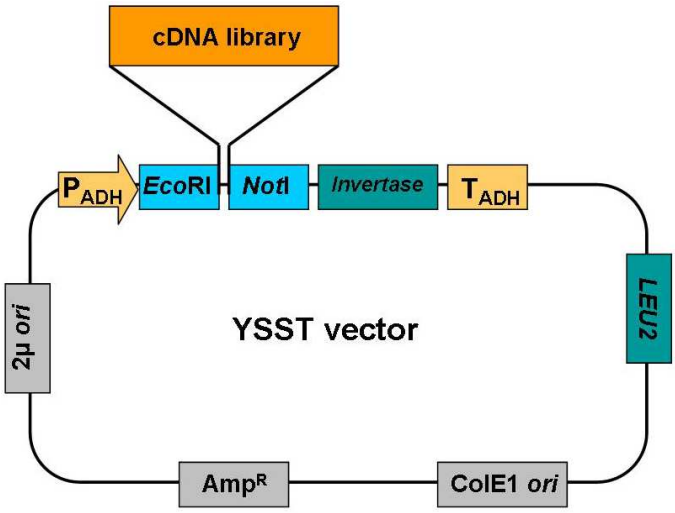


Fig. 2.1 Map of the YSST vector pSMASH. In addition to the truncated invertase complex the vector contains an ampicillin resistance gene and a leucine synthesis gene for the selection in bacteria and yeast, respectively.

E. coli DH5-α competent cells were transformed with the pSMASH vector by heat shock using a standard transformation protocol (see Par. 2.1.4). The transformed cells were plated on LB medium including ampicillin and incubated at 37°C over night. One single colony was used for overnight culture in liquid medium. The next day, a midi-preparation (midiprep) was performed using the Plasmid Midi Kit (Qiagen) according to the manufacturer's manual. The plasmid DNA was dissolved in 1 x TE buffer and the concentration was measured with a photometer. The quality of the plasmid isolation was checked on an agarose gel (0,8% agarose).

1 x TE buffer (Tris-EDTA buffer)
10 mM Tris HCl pH 7,4
1 mM EDTA pH 8,0

2.3.7.1. *Not*I and *Eco*RI digestion of pSMASH and re-ligation test

20 µg of pSMASH was digested with *Not*I according to Fermentas protocol at 37°C over night. The digested plasmid was directly purified by phenol-chloroform extraction and ethanol precipitation as described by Moore & Dowhan (2007). The DNA pellet was finally dissolved in nuclease-free water. Agarose gel electrophoresis was applied to check the integrity of the enzymatic digestion. The purified *Not*I digested plasmid was digested with *EcoR*I according to Fermentas protocol and the mixture was incubated at 37°C over night. Additionally, uncut pSMASH vector was digested with *EcoR*I as a control.

The digested plasmid was again purified by phenol-chloroform extraction followed by ethanol precipitation for the removal of low-molecular-weight oligonucleotides and triphosphates according to Moore & Dowhan (2007). The DNA pellet was finally dissolved in nuclease-free water. Agarose gel electrophoresis was again used to control the integrity of the enzymatic digestion. The potential of re-ligation of the vector was investigated with different ligation and transformation arrangements.

Tab. 2.2 Approaches for re-ligation tests of the pSMASH vector.

component	**pSMASH *Not*I digestion**	**pSMASH Eco*R*I digestion**	**pSMASH Eco*R*I & *Not*I digestion**
digested vector	x µl (~30 ng)	x µl (~30 ng)	x µl (~30 ng)
10 x Buffer	1 µl	1 µl	1 µl
T4-DNA ligase (5U/µl)	0,2 µl	0,2 µl	0,2 µl
H_2O nuclease-free	x µl	x µl	x µl
total	10 µl	10 µl	10 µl

The mixtures were incubated for one hour at room temperature and the enzyme was heat inactivated for ten minutes at 65°C.

2.3.7.2. Test-Transformation of *E. coli* DH5α

E. coli DH5α (NEB 5-alpha competent *E. coli* (High Efficiency) from New England Biolabs) (NEB, Ipswich, USA) were transformed as follows:

- ➔ pSMASH, *Not*I digested, re-ligated and linear
- ➔ pSMASH, Eco*R*I digested, re-ligated and linear
- ➔ pSMASH, *Not*I and Eco*R*I digested, re-ligated and linear
- ➔ pSMASH non-digested
- ➔ H_2O (to verify the integrity of the competent cells and the medium)

The transformation was performed according to the manufacturer's protocol, for each transformation 10 µl of cells were used.

The following dilutions were plated on petri dishes containing LB medium with Ampicillin:

- ➔ digested samples: undiluted, 1:10, 1:100
- ➔ re-ligated samples: 1:10, 1:100, 1:1000
- ➔ uncut vector: 1:10, 1:100
- ➔ H_2O: undiluted

The plates were incubated at 37℃ over night and the number of colonies was counted to determine the transformation efficiency (cfu/ µg DNA) with the following formula:

$$\frac{\text{CFU for control plasmid}}{\text{pg control plasmid}} \times \frac{1 \times 10^6 \text{ pg}}{\mu\text{g}} \times \frac{\text{volume of transformants}}{\text{volume plated}} \times \text{dilution factor}$$

The values for the control plasmid were taken from the manual of the competent cells (NEB). The formula was adapted from Promega Corporation (Madison, USA).

2.3.8. Ligation of dscDNA inserts into pSMASH

To improve the transformation efficiency, a ratio of 3:1 of insert to vector was chosen. The ligation procedure was performed with a T4 DNA ligase from Fermentas according to the company's protocol. The mixture was incubated at 18℃ in a PCR cycler over night. Simultaneously, a second control ligation reaction was run including all components except the insert cDNA. The transformation efficiency of the cDNA library was tested in small scale using again NEB5-alpha cells as described above (Par. 2.3.7.2). For this transformation, samples were used as follows: The dscDNA pSMASH ligation, the pSMASH re-ligation, undigested pSMASH and H_2O. The transformation efficiency was determined as described above in order to estimate the fraction of colonies with pSMASH plus cDNA insert. To remove all salts and enzymes from the ligation mixture, a phenol extraction followed by an ethanol precipitation was performed according to Moore & Dowhan (2007). The DNA pellet was finally dissolved in A. bidest..

2.3.9. Electroporation of *E. coli*

The electroporation of the Stratagene ElectroTen-Blue competent *E. coli* cells was conducted according to the manufacturer's protocol (Stratagene, La Jolla, USA). A small amount of the purified cDNA library (~ 7,5 ng) was applied. As a control, plasmid pUC18 was transformed. The whole transformation mixture was plated on one 15 cm plate with LB ampicillin and incubated at 37℃ over night. After determination of the transformation efficiency, some colonies were picked for analysis by colony PCR. Then the colonies were harvested from the plate by adding 10 ml LB medium and scratching with a glass applicator. Half of the supernatant was used for the preparation of glycerol stocks containing 18% glycerol and frozen at -80℃. The other part of the bacteria was pelleted in 500 µl aliquots and used for plasmid isolation. This plasmid mixture was forwarded to YSST yeast transformation.

2.3.10. Growth and maintenance of yeast strain

For the YSST screen the *Saccharomyces cerevisiae* strain Y02321 (Mat a; *his3*Δ*1*; *leu2*Δ*0*; *met15*Δ*0*; *ura3*Δ*0*; YIL162w::kanMX4) was received from Euroscarf (Frankfurt, Germany) and cultured on YEPD medium. All yeast media and solutions were prepared with A. bidest..

Yeast complete medium (YEPD)	
Yeast extract	1%
Peptone	2%
Dextrose/glucose	2%
Difco-Agar (BD, Heidelberg, Germany)	1,7%

2.3.11. Transformation of yeast for YSST

The transformation was performed according to Gietz & Woods (2002). On the first day, the yeast strain was inoculated into 2 ml of liquid medium (2 x YPAD) and incubated over night on a rotary shaker at 200 rpm and 30°C. If necessary, kanamycin and streptomycin (working concentration of 250 µg/ml each) were added to the medium to avoid bacterial contaminations.

2 x YPAD	
Yeast extract	2%
Peptone	4%
Glucose	4%
Adenine hemisulphate	0,01%
Difco-Agar (BD, Heidelberg, Germany)	1,7%, for plates only

On the second day, the titer of the yeast culture was determined by counting an adequate dilution of overnight culture with a Fuchs-Rosenthal counting chamber. The overnight culture was grown until an amount of about 2,5 x 10^8 cells could be transferred to pre-warmed 50 ml 2 x YPAD resulting in a cell concentration of approximately 5 x 10^6 cells/ml. The culture was incubated on a rotary shaker at 30°C and ~200 rpm for three to five hours until a cell titer of at least 2 x 10^7 cells/ml was reached. Cell concentrations were again determined by counting. Cells were harvested by centrifugation at 3000 x g for five minutes at RT. Thereafter, cells were washed in 25 ml A. bidest., centrifuged and finally resuspended in 1 ml of sterile water. After transfer to a 1,5 ml microcentrifuge tube the yeast cells were centrifuged for 30 sec at maximum speed. Then the supernatant was removed and A. bidest. was added to a final volume of exactly 1 ml, the pellet was dissolved by pipetting. For each transformation, 100 µl cell suspension (corresponding to ~ 10^8 cells) was put in 1,5 ml tubes and centrifuged for 30 seconds at top speed to remove any liquid.

Water and plasmid DNA (100 ng) was added to the freshly prepared transformation mix and added to the yeast cells. The cells were completely resuspended by pipetting. Then, the cells were incubated in a water bath at 42℃ for 33 minut es.

After centrifugation for 30 seconds at top speed, the transformation mix was removed. The cell pellet was dissolved in 1 ml sterile A. bidest. by pipetting. Cells from one tube were plated on a 15 cm plate with synthetic complete drop out (SD) medium including 2% sucrose and 0,025% glucose. Glucose was added to reduce the growth of false positive colonies (Krijger *et al.*, 2008). The yeast cells were spread on the plates with a glass spatula and petri dishes were kept open until all liquid was absorbed by the medium. The plates were sealed with Parafilm and incubated at 30℃ upside down for 3 to 5 days.

Tab. 2.3 Composition of the transformation mixture for up to ten transformations. The mix was always kept on ice.

	number of transformations		
reagents	1x	5 (6x)	10 (11x)
PEG 3350 50% w/v	266,66 µl	1440 µl	2640 µl
LiAc 1 M	40 µl	216 µl	396 µl
boiled SS-carrier DNA	55,55 µl	300 µl	550 µl
plasmid (100 ng) plus water	37,77 µl	-	-
total	400 µl	2160 µl	3960 µl

Synthetic complete drop out medium

For this yeast selection medium several sterile stock solutions were added to autoclaved water agar (finally 1,7% Difco Agar).

Synthetic complete drop out medium (1l)

100 ml	10 x yeast nitrogen base without amino acids (YNB), pH 5,4 (NaOH)
50 ml	20 x synthetic complete drop out mix (amino acids, aa)
50 ml	40% sucrose
50 ml	0,5% glucose

Tab. 2.4 Composition of the 20 x stock of the synthetic complete drop out mix (amino acids), prepared without leucin.

compound	Final concentration [µg/ml]	20 x stock [mg/l]
Adeninsulfate	40	800
L-Arginine	20	400
L-Aspartate	100	2000
L-Glutamate	100	2000
L-Histidin HCl	20	400
L-Lysine	30	600
L-Methionine	20	400
L-Phenylalanine	50	1000
L-Serine	375	7500
L-Threonine	200	4000
L-Tryptophan	40	800
L-Tyrosine	30	600
L-Valine	150	3000
Uracil	20	400

Positive colonies were transferred to 150 µl liquid synthetic complete drop out medium with 2% glucose instead of sucrose in a sterile 96 well plate. The plate was incubated over night at 30℃ and 210 rpm. To achieve better ventilation each well was provided with a sterile 2 mm glass bead. The next day, the cell density was determined by counting the cells of some wells with a Fuchs-Rosenthal chamber. The cells were diluted with sterile distilled water to a final concentration of about 1000 cells/ml. 50 µl of this dilution was plated on 6 cm selection plates with SD medium with 2% sucrose and 2 µg/ml antimycin A. Antimycin A blocks oxidative respiration, yeast cells are forced to live on fermentation which supports the elimination of false positive colonies. As an additional control, cells were plated on SD medium plates supplied with raffinose instead of sucrose and antimycin A. Raffinose is hardly metabolized by false positives compared to real positive colonies. The plates were incubated for 3 to 5 days at 30℃.

Antimycin A (stock: 2 mg/ml)

Antimycin A (Sigma-Aldrich) was dissolved in ethanol (p. a.) and diluted 1:10 in ethanol p. a. before it was added to the autoclaved medium (avoid precipitation). The final concentration of ethanol in the medium was 1%.

Single-stranded carrier DNA (2 mg/ml)

High molecular weight DNA (deoxyribonucleic acid sodium salt type III from salmon testes, Sigma-Aldrich) was dissolved in TE buffer (10 mM Tris-HCl pH 8,0, 1 mM EDTA) and dispersed by pipetting the solution up and down and shaking on a shaker at low speed in an ice water

bath. Aliquots of the DNA were stored at -20°C. Prior to further use, an aliquot of DNA was boiled in a water bath for 5 min and, thereafter, always kept on ice.

Lithium acetate stock solution (1 M)

The lithium acetate solution was prepared as a 1 M stock in A. bidest. and autoclaved. The pH should be between 8,4 -8,9.

Polyethylene glycol (PEG 50% w/v)

Polyethylene glycol (PEG), MW 3350 (Sigma-Aldrich) was dissolved in A. bidest. to an exact concentration of 50% (w/v) and sterilized by autoclaving. An accurate concentration of PEG is important for optimal transformation efficiency.

2.3.12. SDS lysates for yeast colony PCR

For further analysis of cDNA inserts, yeast colony PCR was performed according to Krijger *et al.* (2008). Cells from a single yeast colony were resuspended in 40 µl 0,25% sodium dodecyl sulfate (SDS) with a toothpick. Then, lysates were mixed for 5 minutes at RT and incubated at 90°C for 5 minutes. After chilling to RT, 160 µl A. bidest. was added and the mixture was centrifuged at 3200 rpm for 5 minutes. An average amount of 1 µl of the supernatant was used for the PCR reaction. To reduce the disturbing influence of SDS on the DNA Polymerase (DNA Cloning Service, Hamburg, Germany), the provided buffer B was employed which contains Tween-20.

Primer sequences (Krijger *et al.*, 2008)

ScADH.F1	5'- CTC GTT CCC TTT CTT CCT TGT TTC-3'
ScSUC2.R2	5'- TTT GGG TCA TTC ATC CAG CCC TTG-3'

PCR mixture		PCR Cycler Setup		
10 x Buffer B	2,5 µl	94°C	1 min	
25 mM $MgCl_2$	2,5 µl	94°C	30 sec	37x
2 mM dNTPs	2,5 µl	61°C	30 sec	
Primer ADH fwd (10 µM)	1,25 µl	72°C	2 min	
Primer Suc2 rev (10 µM)	1,25 µl	72°C	5 min	
DCS Pol DNA Polymerase	0,2 µl	12°C	∞	
Template	0,5-2 µl			
A. bidest.	ad 25 µl			

The result of the PCR was analyzed on a 1% agarose TBE gel using 4 µl PCR reaction and loading buffer. The main part of the PCR reaction was purified using Wizard® SV Gel and PCR Clean-Up System from Promega according to the manufacturer's protocol.

The concentration of the PCR product was measured with Nanodrop. Then PCR fragments with a similar size (bp) were test digested with the restriction enzymes *Hae*III and *BsaR*I (Fermentas) to identify those which have the identical sequence.
The PCR products which appeared to be unique were sent for sequencing to Agowa Genomics, now LGC Genomics (Berlin, Germany) and sequenced using ADH.F1 forward primers. The sequences were labeled with "K" (for "Kandidat") and provided with the colony number, for example "K1". Later candidate labelling was changed in accordance to the corresponding protein number in the *P. indica* genome database ("PIIN...."). The assignments can be found in Tab. 2.5.

2.4. *In silico* analysis

All obtained sequences of the cDNA fragments were analyzed *in silico* by alignment with the *P. indica* genome and transcriptome database to achieve the full length sequence of the putative genes. Thereafter, all sequences were put in the NCBI blastx database to gather information about homologies. SignalP 3.0 and TMHMM were applied to look for putative secretory signal peptides and transmembran helices. The SMART database was used to identify putative protein domains. Furthermore, homologous proteins were identified with the help of the *P. indica* GBrowse database. For the respective internet addresses of the databases, see references.

2.5. Candidate gene expression studies with quantitative real-time PCR

The expression levels of identified candidate genes harboring a signal peptide were analyzed *in vivo* with *P. indica* growing on *Arabidopsis* Col-0 roots and *in vitro* with *P. indica* growing in liquid CM or on dead *Arabidopsis* Col-0 roots.

2.5.1. Kinetics with living *Arabidopsis* Col-0 roots and *P. indica* for qPCR analysis

For the *in vivo* analysis, *Arabidopsis* Col-0 plants were grown on ½ Murashige & Skoog medium (½ MS, mod. IV (without ammonium), Duchefa, plus 0,4% Gelrite) in squared petri dishes for three weeks at 8 hours light (22℃)/16 hours dark (18℃). Then they were inoculated with *P. indica*; isolation of spores was performed as described in Par. 2.2.2. Each petri dish was inoculated with 1 ml spore suspension containing ~ 500.000 spores. The roots were harvested and immediately frozen in liquid nitrogen at two and seven days after inoculation and grinded to fine powder using liquid nitrogen. RNA extraction was performed with Trizol reagent (Invitrogen) according to the company's manual. The RNA concentration was determined with Nanodrop. The integrity of the RNA was analyzed with gel electrophoresis (see Par. 2.1.3).
2 µg RNA of each sample were digested with DNase I using the DNase I kit from Fermentas as described by the manufacturer.

Synthesis of cDNA was performed with 1 µg total RNA applying the qScript cDNA synthesis kit (Quanta, Gaithersburg, USA). The cDNA was diluted with A. bidest. to a final concentration of 5 ng/µl. 10 ng cDNA served as template in the qPCR reaction.

2.5.2. Kinetics with dead *Arabidopsis* Col-0 roots and *P. indica* for qPCR analysis

Arabidopsis ecotype Col-0 (N1092) was grown on ½ MS medium in squared petri dishes for 3 weeks under short day conditions (8 hours light (22℃), 16 hours dark, 18℃)). Then, the roots were harvested and autoclaved. The dead roots were again put on fresh ½ MS plates and inoculated with *P. indica* spore suspension (1 ml per plate) at a concentration of 500.000 spores/ml. The plates were put back for two or seven days to short day conditions (eight hours light (22℃)/16 hours dark (18℃)). Then the roots were harvested and frozen in liquid nitrogen. RNA extraction and all following steps for cDNA synthesis were performed as described in Par. 2.5.1

2.5.3. Kinetic with *P. indica* in axenic culture for qPCR analysis

Liquid culture of *P. indica* was grown in 100 ml Erlenmeyer flasks containing 50 ml *Aspergillus* complex medium (CM). The flasks were inoculated with 1 ml *P. indica* spore suspension containing about 500.000 spores, incubated on a horizontal shaker at RT and harvested at two and seven days after inoculation. The harvesting was performed by centrifugation at 3500 rpm for seven minutes. After decantation of the medium, the fungal material was immediately frozen in liquid nitrogen and stored at -80℃. For RNA extraction, the mycelium was crushed using liquid nitrogen. RNA extraction was performed as described for the YSST screen (Par 2.1.1). The integrity of the RNA was analyzed with gel electrophoresis (Par. 2.1.3). DNase I digestion was also done with the enzyme from Fermentas and for cDNA synthesis the qScript cDNA synthesis kit (Quanta) was used. The cDNA was diluted to a final concentration of 5 ng/µl and 10 ng cDNA served as template in the qPCR reaction.

2.5.4. Quantitative real-time PCR

Quantitative real-time PCR was performed using the 7500 Fast Real-Time PCR System from Applied Biosystems (Foster City, CA, USA). The expression level of candidate genes identified by the YSST screen was compared to the expression level of the *P. indica* housekeeping gene *transcription elongation factor 1α* (*tef 1α*) (Bütehorn *et al.*, 2000).

For every primer pair a standard curve was run using 10 ng, 1 ng, 0,1 ng and 0,01 ng of template cDNA to determine amplification efficiency of the primer pairs and the tendency of the primer pairs to form primer dimers.

If the standard curve gave acceptable results for the different cDNA concentrations, primer pairs were applied. 10 ng cDNA served as template. Every time point of the kinetics was measured in triplicates.

PCR mixture (1x)

Green JumpStart Taq ReadyMix (Sigma-Aldrich)	10 µl
Primer fwd 10 µM	0,7 µl
Primer rev 10 µM	0,7 µl
Template cDNA	2,0 µl
A. bidest.	6,6 µl
total	20 µl

The program used for the PCR reaction was always identical for all measurements and just annealing temperatures were adjusted for the different primer pairs. The first part of the measurement comprised a standard PCR amplification phase with 40 cycles. The reading point for data collection was set at 72℃ elongation time . Additionally a melting curve was recorded from 95℃ to 66℃ (every 0,5℃), the reading point was set at the ramp between 95℃ and 66℃.

PCR program

2 min	95℃ sample denaturation	
30 sec	95℃ denaturation	40 x
30 sec	x℃ annealing	
30 sec	72℃ elongation	
15 sec	95℃	melting curve record
30 sec	66℃	
15 sec	95℃	
15 sec	24℃	

The threshold cycle (C_T) values were determined by the Applied Biosystems software. Further analysis was done using the comparative C_T method according to Schmittgen and Livak (2008).

Tab. 2.5 Sequences of qPCR primers, their annealing temperature and the resulting product sizes of candidate genes and housekeeping gene *TEF1α*. In addition, the gene ID of the *P. indica* database (DB) is listed.

Name	**Sequence**	**AT [℃] / size [bp]**	**Gene ID *P. indica* DB**
tef150f	TCGTCGCTGTCAACAAGATG	60 / 110	PIIN_03008
tef 150r	ACCGTCTTGGGGTTGTATCC		
K3_qPCR_fwd	CGCTGTAAACCTCCAAATCC	57 / 176	PIIN_07104
K3_qPCR_ rev	CGACATTGCTGCCTGTATTG		
K12.1 fwd	AATCACAATCGTTCAACCAG	53 / 131	PIIN_03806
K12.1 rev	CTCAACTTTCCATTGTCCGT		
K16_qPCR_fwd	TACGAGCCCAAGAAGGAAGA	57 / 227	PIIN_08513
K16_qPCR_rev	CAGCTGGTTGTGTTGTGCTT		
K19.2 fwd	CTCACATACACAATCTGAAACC	55 / 193	PIIN_10643
K19.2 fwd	ATCGTTTCTGCTGTTCTTGAC		
K37_qPCR_ fwd	GTCGCCCTTATCACACTCGT	59 / 178	PIIN_02519
K37_ qPCR_rev	AAGTCGTCACCGTCTGGAAC		
K38.1 fwd	GAGAATACTGCGTTTACCGT	55 / 119	PIIN_00308
K38.1 rev	GATCAGGATACCGTTACCGT		
K40_qPCR_ fwd	TCCGTCAGTCATTTGAGCAG	57 / 166	PIIN_02169
K40_qPCR_rev	CCATAAGGTGGCCTCAAGAA		
K43.2 fwd	ACCAGTACGGCTACAACATT	55 / 137	PIIN_04526
K43.2 rev	CTTCAGTGTCACCGATCAAG		
K46_qPCR_ fwd	TCAACGGGAAGAAGGTTGAC	57 / 235	PIIN_09796
K46_qPCR_ rev	GACGTCAGGATTGGTGGATT		
PIALH43 fwd	TCTTATCGAGCATACACTCTG	56 / 175	PIIN_00029
PIALH43 rev	GATACCTTGAGAATTATTGCCC		

2.6. Subcellular localization of candidate genes

2.6.1. Cloning strategy for candidate GFP fusions

Four candidate genes, *K12* (*PIIN_03806*), *K16* (*PIIN_08513*), *K43* (*PIIN_04526*) and Pi*ALH43 (PIIN_00029)*, were chosen for further functional analysis. The full length cDNA sequence of these selected candidate genes including the endogenous signal peptide and lacking a stop codon was cloned into the expression vector (GFP+0fwd in pGY-1) (vector provided by R. Eichmann, TU Munich). In this vector, the fusion protein consisting of the candidate gene and a C-terminal GFP, was set under the control of a CaMV 35S promoter. The full length cDNA of the candidates without stop codons was amplified from *P. indica* cDNA with primers including *Sac*II and *Nco*I restriction sites (*K16*, *K43*) or only *Sac*II restriction sites (*K12*, Pi*ALH43*) using Pfu DNA Polymerase (Promega) or Phusion DNA Polymerase (Finnzymes, Vantaa, Finland). Then the PCR products were gel purified (SV Wizard PCR Clean-up Kit, Promega), the concentration was determined with Nanodrop. Afterwards 3' end adenine overhangs were added to the PCR products (A-tailing) using DCS Pol (DNA cloning service) and dATP.

Finally, the fragments were cloned in the pGEM-T vector using the pGEM-T system (Promega). The ligation reaction was set up as described in the manual (ratio 3:1 insert:vector) and incubated over night at 4℃. The ligation reaction was used for transformation of *E. coli* DH5α cells as described in Par. 2.1.4 and the bacteria were plated on LB medium containing ampicillin. Colony PCR was performed with M13 fwd/rev primers (M13 fwd 5'-GTTTTCCCAGTCACGAC -3'; M13 rev 5'-AACAGCTATGACCATGA -3') and positive colonies were used for minipreparation (Pure Yield Miniprep Kit, Promega).

Plasmids were sent for sequencing to verify the correctness of the sequences. Then the pGEM-T vectors were digested with *Sac*II and *Nco*I or only with *Sac*II (Fermentas). The released candidate fragments were gel purified (SV Wizard Gel Clean-up Kit, Promega) and ligated into the *Sac*II/*Nco*I digested pGY-1 GFP vector with T4 DNA ligase (Fermentas) or in the dephosphorylated *Sac*II digested pGY-1 GFP vector using the Rapid DNA Dephos & Ligation Kit (Roche Diagnostics, Mannheim, Germany). The constructs were used to transform *E. coli* DH5α and transformed colonies were identified by colony PCR. Positive clones were used for minipreparation (Pure Yield Plasmid Miniprep System, Promega) and preparation of glycerol stocks (bacterial culture including 15% glycerol). The isolated plasmids were sent for sequencing to assure the success of the cloning process. Suitable glycerol stocks were applied for midipreparation (Plasmid DNA Purification NucleoBond PC100, Macherey-Nagel, Düren, Germany). Sequences of primers used for cloning and colony PCR can be found in Tab. 2.6 and 2.7. The vector map can be found in Suppl 7.1.

Addition of 3' end adenine overhangs to PCR products (A-tailing)		Cycling program	
Purified PCR product	15 µl	70℃	20 min.
10x Buffer DB	2 µl	12℃	∞
10 mM dATP	1 µl		
DCS Pol	1 µl		
25 mM $MgCl_2$	1 µl		

Tab. 2.6 Sequences of primers used for cloning of candidate genes into (GFP+0fwd-pGY-1).

name	sequence (5'-3')	AT [℃]	size [bp]
K12 *Sac*II fwd	CCGCGGATGATCTTCACACCCTAC	60	2000
K12 *Sac*II rev	CCGCGGTTTGCATGCCTTTCAGCC		
K16 *Nco*I fwd	CCATGGATGGAGAAAGCCTACACC	53	750
K16 *Sac*II rev	CCGCGGATATACGTGAAGGAGAGAACT		
K43 *Nco*I fwd	CCATGGATGCTCACGCTCCTCGC	62	1060
K43 *Sac*II rev	CCGCGGTGGAGCGCGACGGCAAAG		
PIALH43 *Sac*II fwd	CCGCGGATGGGCAGATATTCATTGGC	67	1600
PIALH43 *Sac*II rev	CCGCGGTTCGCCGGGGTCGGCG		

The table contains information on the annealing temperature (AT) of the primer pairs and the size of the PCR product in base pairs (bp).

Tab. 2.7 Sequences of primers used for colony PCR with transformants containing candidate genes into (GFP+0fwd-pGY-1).

name	sequence (5'-3')	AT [℃] / size [bp]	comments
K12 *Sac*II fwd	CCGCGGATGATCTTCACACCCTAC	60 / 2200	K12 insert
GFP5'rev	GTGGTGCAGATGAACTTCAG		
K16 *Nco*I fwd	CCATGGATGGAGAAAGCCTACACC	60 / 900	K16 insert
GFP5'rev	GTGGTGCAGATGAACTTCAG		
K43 *Nco*I fwd	CCATGGATGCTCACGCTCCTCGC	60 / 1250	K43 insert
GFP5'rev	GTGGTGCAGATGAACTTCAG		
PIALH43 FLN fwd	ATGGGCAGATATTCATTGGCAGCG	60 / 1860	PIALH43 insert
GFP5'rev	GTGGTGCAGATGAACTTCAG		
pGY-1 fwd2	CGTTCCAACCACGTCTTCAA	60 / 270	empty vector
GFP5'rev	GTGGTGCAGATGAACTTCAG		

The table contains information on the annealing temperature (AT) of the primer pairs and the size of the PCR product in base pairs (bp). Additionally, comments inform about the insert which can be tested with the given primer combinations.

2.6.2. Transient expression of candidate genes by particle bombardment

The midipreparations of pGY-1 candidate-GFP vectors were employed for transient expression in leaves of *Arabidopsis* ecotype Col-0 (N1092) or onion epidermal cells. The *Arabidopsis* plants were grown in a soil (Fruhstorfer Erde Typ P) sand mixture (2:1) for 4 to 6 weeks under short day conditions (22℃, 7 hours light, 17 hours dark) .

Arabidopsis leaves were placed in 6 cm petri dishes with 1% water agar. Onion scale leaves were cut into pieces of about 4 cm² and put in 6 cm petri dishes with wet filter paper. The transient transformation was performed according to Schweizer *et al.* (1999). Per shot 1 µg of the pGY-1-candidate-GFP vector and as a control 0,5 µg of pGY-1-mCherry with 312 µg tungsten particles (1,1 µm, M-17, BioRad, Hercules, USA) were released. After the particle bombardment petri dishes with *Arabidopsis* leaves were placed on the lab bench.

Petri dishes with onions were sealed with Parafilm and incubated in the dark. 16 to 24 hours later subcellular localization was monitored. For microscopy, the onion epidermis was peeled of and put on microscope slides embedded in distilled water with 0,002% Tween 20. *Arabidopsis* leaves were also mounted on microscope slides and covered with Tween water. The analysis was done with an epifluorescence microscope (Axioplan 2, Carl Zeiss MicroImaging, Jena, Germany) and a confocal laser-scanning microscope (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany). Using the confocal laser scanning microscope, GFP was excited using a 488 nm laser line and emission was detected at 505-530 nm; for mCherry the excitation was at 543 nm laser line and the detection at 580-650 nm. The excitation used for epifluorescence microscopy was 485/20 nm for GFP and 546/12 nm for mCherry, the emission was 515 nm for GFP and 590 nm for mCherry.

2.6.3. Proteasome inhibitor MG-132

To improve GFP signal detection the proteasome inhibitor MG-132 (Calbiochem, Merck, Darmstadt, Germany) was infiltrated into transiently transformed *Arabidopsis* leaves about 16 hours after particle bombardment. MG-132 prevents destruction of proteins. MG-132 was dissolved in DMSO and diluted with distilled water to a final concentration of 100 µM. For the infiltration a syringe without needle was applied. Four to six hours after infiltration the leaves were analyzed by microscopy as described in Par. 2.6.2.

2.7. Transient expression of candidate genes in *Nicotiana benthamiana*

2.7.1. Cloning strategy for candidates

The candidate genes *K12* (*PIIN_03806*), *K16* (*PIIN_08513*), *K43* (*PIIN_04526*) and Pi*ALH43 (PIIN_00029)* were cloned in the expression vector pCXSN (Chen *et al.*, 2009), which can be used for *Agrobacterium*-mediated transient transformation. For all candidates, constructs with and without signal peptide were created. The sequences of the genes were amplified from the pGEM-T vectors mentioned in Par. 2.6.1 with Phusion DNA polymerase (Finnzymes) according to the company's protocol. Then the PCR products were gel purified (Wizard® SV Gel and PCR Clean-Up System, Promega) and the DNA concentration was measured with Nanodrop. Afterwards addition of 3' end adenine overhangs (A-tailing) was performed with DCS Pol (DNA Cloning Service) and dATP (Par. 2.6.1). The vector pCXSN was isolated from overnight cultures of *E. coli* strain DB3.1 containing the antibiotic kanamycin (50 µg/ml) with Pure Yield Miniprep Kit (Promega). Then the plasmid was digested with *Xcm*I (New England Biolabs) according to the manual to produce 5' thymine overhangs and to remove the killing *ccDB* gene.

The linearized pCXSN was gel purified and the concentration was determined with Nanodrop. The ligation reaction was performed with the pGEM-T kit (Promega) but with pCXSN instead of pGEM-T (ratio 6:1, insert:vector). Transformation of *E. coli* DH5α (with kanamycin) as selection marker (50 µg/ml), colony PCR, minipreparation and sequencing were performed as described above (Par. 2.1.5/2.6.1). Primers used for the cloning procedure and colony PCRs are listed in Tab. 2.8 and 2.9. The vector map can be found in Suppl. 7.1)

Tab. 2.8 Primer combinations used for cloning of pCXSN candidate constructs.

name	**sequence (5'-3')**	**AT [℃]/ size [bp]**	**comments**
K12 ATOME fwd	GGAGATAGAACCATGGCTCCGATCACCGTA	51 / 1940	w/o SP, with stop
K12 ATOME rev	AAGGCATGCATAGKGATCCGGAGGTGGA		
K12 ATOME fwd	GGAGATAGAACCATGATCTTCACACCC	51 / 2000	with SP, with stop
K12 ATOME rev	AAGGCATGCATAGKGATCCGGAGGTGGA		
K16 ATOME fwd	GGAGATAGAACCATGGTGCTTTTCCTTGCA	51 / 690	w/o SP, with + w/o stop
K16 ATOME rev	TCTCTCCTTCACGTATKGATCCGGAGGTGGA		
K16 ATOME fwd	GGAGATAGAACCATGGAGAAAGCCTACA	51 / 750	with SP, with and w/o stop
K16 ATOME rev	TCTCTCCTTCACGTATKGATCCGGAGGTGGA		
K43 ATOME fwd	GGAGATAGAACCATGCAAGGCGGCGCGTTTA	57 / 990	w/o SP, with and w/o stop
K43 ATOME rev	TTGCCGTCGCGCTCKGATCCGGAGGTGGA		
K43 ATOME fwd	GGAGATAGAACCATGCTCACGCTCCTC	57 / 1050	with SP, with and w/o stop
K43 ATOME rev	TTGCCGTCGCGCTCKGATCCGGAGGTGGA		
PIALH43 ATOME fwd	GGAGATAGAACCATGTATATTCCAGCTAGGGCTG	63 / 1530	w/o SP, with Stop
PIALH43 FLN rev	CTACGCCGGGGTCGGCGA		
PIALH43 FLN fwd	ATGGGCAGATATTCATTGGCAGCG	63 / 1590	with SP, with Stop
PIALH43 FLN rev	CTACGCCGGGGTCGGCGA		

The table lists the sequences, the annealing temperature (AT) and the size of the PCR product in base pairs (bp). The comments provide information whether the PCR product contains a signal peptide (with SP) or not (w/o SP). The comments give information about the stop codon of the reverse primers because some reverse primers contain a mixture of primers with or without stop codon for different cloning strategies (with and w/o stop).

Tab. 2.9 Primer combinations used for colony PCR with pCXSN candidate constructs.

name	sequence (5'-3')	AT [℃] / size [bp]
NosT #516	ATTGCCAAATGTTTGAACGA	55/ 2000
K12 ATOME fwd	GGAGATAGAACCATGGCTCCGATCACCGTA	
NosT #516	ATTGCCAAATGTTTGAACGA	53/ 720
K16 ATOME fwd	GGAGATAGAACCATGGTGCTTTTCCTTGCA	
NosT #516	ATTGCCAAATGTTTGAACGA	55/ 1050
K43 ATOME fwd	GGAGATAGAACCATGCAAGGCGGCGCGTTTA	
NosT #516	ATTGCCAAATGTTTGAACGA	53/ 1540
PIALH43ATOME fwd	GGAGATAGAACCATGTATATTCCAGCTAGGGCTG	

The table lists the sequences, the annealing temperature (AT) and the size of the PCR product in base pairs (bp).

2.7.2. Growth of *N. benthamiana*

N. benthamiana was grown in soil (Fruhstorfer Erde Typ T) supplied with *Steinernema feltiae* (Katz Biotech AG, Baruth, Germany), nematodes which feed on plant impairing larvae of flies. The tobacco was kept in a chamber under 16 h light/8 h dark intervals, at a light intensity of 160 µmol m^{-2} s^{-1} at 20℃. The plants were fertilized every 2 -3 weeks with Wuxal (1ml/l). About four weeks old plants were transferred in new soil (Fruhstorfer Erde Typ T), again supplied with nematodes. For the experiments, seven to eight weeks old plants were transferred to the laboratory at the day of infiltration.

2.7.3. Transformation and growth of *A. tumefaciens* strains

The *Agrobacterium* strains GV3101 pMP90 (*K12*, *K16*, *K43*) or AGL1 (Pi*ALH43*) were transformed with the pCXSN candidate constructs by electroporation. Therefore, glycerol stocks of electrocompetent agrobacteria were thawed on ice for several minutes. The cells were mixed with 100 ng of the plasmid. The mixture was kept on ice for 10 minutes. Then the cells were transferred to precooled electroporation cuvettes (0,2 cm gap) and exposed to one pulse of 2,4 kV using Gene Pulser MXcell Electroporation System (BioRad). Thereafter, the cells were supplied with 600 µl SOC medium and kept in the cuvettes at 28℃ for 1,5 hours. Finally, bacteria were plated on YEB medium with the appropriate antibiotics (1 x 25 µl cells, 1 x 50 µl cells) and incubated at 28℃ for about two days. Colony PCR was performed with the same primers as described in Par. 2.7.1. Positive colonies were used for liquid cultures (LB medium with appropriate antibiotics), which were grown at 28℃ and 140 rpm for two days. Glycerol stocks (15% glycerol) of these bacterial cultures were applied for the infiltration experiments.

YEB medium (1l)

Yeast extract	1 g
Beef extract	5 g
Sucrose	5 g
Casein hydrolyzate	5 g
$MgCl_2$ x 6 H_2O	2 mM → autoclave stock (1 M) separately, add after autoclaving
Agar-Agar	15 g
Antibiotics	strain and construct specific, add after autoclaving

Antibiotics for GV3101 pMP90

Rifampicin (stock in methanol)	100 µg/ml
Gentamycin (stock in H_2O)	25 µg/ml
Kanamycin (stock in H_2O)	50 µg/ml (pCXSN selection)

Antibiotics for AGL1

Rifampicin (stock in methanol)	25 µg/ml
Carbenicillin (stock in H_2O)	25 µg/ml
Kanamycin (stock in H_2O)	50 µg/ml (pCXSN selection)

2.7.4. *Agrobacterium* infiltration of *N. benthamiana*

Overnight cultures (10 ml LB medium with respective antibiotics and 30 µl of Agrobacteria from glycerol stocks) of the transformed agrobacteria were grown at 28℃ and 140 rpm. In addition to transformed Agrobacteria, untransformed competent cells (GV3101 pMP90 or AGL1) were grown in LB medium with antibiotics except kanamycin. The next day, the cultures were harvested by centrifugation at 3500 rpm for 20 minutes at RT and resuspended in induction medium. The OD_{600} was adjusted to 0,1 and the bacteria were incubated at RT for 2 hours. Then the bacteria or buffer (control) were pressure-infiltrated with a syringe on the lower side of the leaves; 4-5 leaves per plant were infiltrated. The infiltrated area was labeled with a red marker. The plants were kept in the laboratory for 24 hours. Then infiltrated leaves (3 per construct) were harvested. Leaf discs of the infiltrated area were cut using a 5 mm biopsy punch (8 leaf discs from each construct or treatment). The leaf discs were transferred to a white 96 well plate which was supplied with 200 µl water per well (1 leaf disc per well). The leaf discs were incubated over night in the water to avoid any interference with wound responses. The plate was sealed with Parafilm.

Induction medium

MES (4-Morpholineethanesulfonic acid) pH 5,6	10 mM
$MgCl_2$ x 6 H_2O	10 mM
Acetosyringone (stock 15 mM in ethanol (p. a.)) in H_2O dest.	150 µM

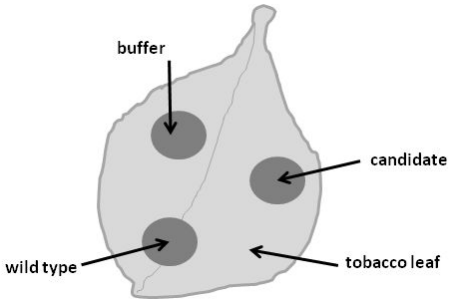


Fig. 2.2 Schematic drawing of an infiltrated tobacco leaf: The areas which were pressure-infiltrated are shown in dark grey. buffer = buffer infiltrated; wild type = untransformed Agrobacteria infiltrated; candidate = Agrobacteria with the construct of interest infiltrated.

2.8. Measurement of oxidative burst and callose detection

The next day, the water was removed from the wells containing the tobacco leaf discs and replaced by aqueous luminol solution (200 µl per well). Light emission was monitored as relative light units (RLU) in a 96-well luminometer (Tecan Infinite 200, Tecan, Männedorf, Switzerland) for 80 minutes (40 cycles, 1 cycle = 2 min.). To trigger ROS production, 20 µl flg22 was added after 20 minutes to a final concentration of 1 µM using the injector of the Tecan reader.

Flg22 is an oligopeptide derived from the N-terminus of bacterial flagellin, which is an elicitor of defense response in many plants (Felix *et al.*, 1999). At the end of the measurement the luminol solution was removed and replaced by a mixture of water and flg22 (1 µM). 24 hours after flg22 treatment, leaf discs were put in 96% ethanol for callose detection. The callose staining was performed as described by Nguyen *et al.* (2010). Therefore, leaf discs were incubated in 96% ethanol for at least six hours at 37°C to remove chlorophyll. Then the leaf discs were washed twice with 70% ethanol and three times with A. dest. Finally, leaf discs were stained with 1% aniline blue in 150 mM K_2HPO_4, pH 9,5, for one hour in the dark and put in 50% glycerol until they were analyzed by epifluorescence microscopy.

Luminol solution for ROS detection

Luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione; stock 15 mg/ml in DMSO)	30 µg/ml
Horseradish peroxidase (HRP, stock 10 mg/ml in H_2O)	20 µg/ml
in H_2O dest	

2.9. Functional analysis of Pi*ALH43* (PIIN_00029)

2.9.1. Yeast cell viability assay

From a former YSST screen dealing with the interaction of *P. indica* with barley roots (Khatabi, 2009) the putative effector candidate Pi*ALH43* from *P. indica* was discovered. Because PiALH43 contains an E3 ligase motif as was reproted for the bacterial effector protein AvrPtoB, which is involved in manipulation of programmed cell death (PCD), a yeast cell viability assay was conducted.

For the assay, the *S. cerevisiae* wild type strain WCG4a (MATa *his3-11,15 leu2-3,112 ura3*) was used. The yeast transformation was done applying the small scale LiAc (= lithium acetate) yeast transformation procedure as described in the yeast protocols handbook (Clontech, Mountain View, CA USA). The plasmids pSD10.a and pL009 were used containing the *Mus musculus* genes *Bax* with proapoptotic activity or its antagonist *Bcl-X_L*, respectively (Ligr *et al.*, 1998). Pi*ALH43* hypothetical ORF (*PIIN 00029*) was cloned in the yeast expression vector pGADT7 (Clontech) where it was set under the control of a constitutive ADH promoter. The cloning procedure is described in more detail in Par. 2.9.2. The plasmid p416-PiALH43 (clone No 61) with a galactose inducible promoter was created by Behnam Khatabi (Khatabi, 2009). As control plasmids pRS315, pRS316 (Sikorski & Hieter, 1989), p416-Gal1 and pGADT7 (empty) were employed. The plasmids used in this assay contained either *LEU2* or *URA3* as selection markers. The transformation combinations of these plasmids, their respective selection marker and the purpose are listed in Tab. 2.10. The pGADT7 vector map can be found in Suppl. 7.1.

Tab. 2.10 List of plasmids co-transformed in the yeast viability assay.

plasmid 1	**plasmid 2**	**selection marker**	**purpose**
pRS315	pRS316	*LEU*/*URA*	control
pRS315	pSD10.a-Bax	*LEU*/*URA*	control
pRS316	pL009-Bcl-X_L	*URA/LEU*	control
pL009-Bcl-X_L	pSD10.a-Bax	*LEU*/*URA*	control
pL009-Bcl-X_L	p416-GAL1	*LEU*/*URA*	control
pRS315	p416-GAL1	*LEU*/*URA*	control
pSD10.a-Bax	pGADT7	*URA/LEU*	control
pRS316	pGADT7	*URA/LEU*	control
pSD10.a-Bax	pGADT7-PIALH43	*URA/LEU*	antiapoptotic
pRS316	pGADT7-PIALH43	*URA/LEU*	antiapoptotic
pRS315	p416-PIALH43 c61	*LEU*/*URA*	proapoptotic
pL009-Bcl-X_L	p416-PIALH43 c61	*LEU*/*URA*	proapoptotic

2.9.2. Cloning strategy for pGADT7-Pi*ALH43*

The vector pGADT7 (Clontech) was digested by *Hind*III (Fermentas), which allows the removal of the activation domain from the vector. The vector without activation domain was purified via gel extraction applying Wizard® SV Gel and PCR Clean-up System from Promega as described in the manual. The sticky ends of the vector fragment were filled in with the Klenow-fragment (Fermentas). Afterwards a dephosporylation step with Calf Intestine Alcaline Phospatase (CIAP, Fermentas) was performed to avoid re-ligation of the vector in the following ligation step. The Pi*ALH43* hypothetical ORF was amplified from a pGEM-T vector (Promega) containing the Pi*ALH43* gene with gene specific primers.

Then it was purified with the PCR clean-up kit from Promega. The ligation reaction was performed using T4 DNA ligase with PEG4000 for blunt end ligation (Fermentas). Chemically competent *E. coli* DH5α cells were transformed with the ligation reaction applying a standard protocol. The transformed cells were plated on LB medium with ampicillin. Resulting colonies were analyzed with colony PCR; positive colonies were used for overnight culture. The plasmid was isolated from the bacterial culture with the PureYield™ Plasmid Miniprep System (Promega) and confirmed via sequencing (Agowa Sequencing Service, now LGC Genomics, Berlin). The vector map can be found in Suppl. 7.1.

Primers for pGADT7-Pi*ALH43* construction (annealing temperature 63°C)
PIALH43 FLN fwd 5'- ATGGGCAGATATTCATTGGCAGCG - 3'
PIALH43 FLN rev 5' - CTACGCCGGGGTCGGCGA - 3'

2.9.2.1. Yeast transformation procedure for yeast viability assay

S. cerevisiae strain WCG4 was grown over night at 30°C in complete medium (YPDA, 1% yeast extract, 2% peptone, 2% glucose and 30mg/l adenine) to a cell density of OD_{600} > 1,5. About 10-15 ml of this culture was transferred to 300 ml YPDA to an OD_{600} of 0,2-0,3. Then this culture was grown at 30°C and 240 rpm for about three hours until an OD_{600} of 0,4-0,6 was reached. After centrifugation (5 min., 1000 x g, RT), the cells were washed with sterile water, centrifuged and resuspended in 1 x TE/1 x LiAc.

For simultaneous co-transformation, 100 ng of each plasmid (in the respective combinations) and 100 µg of salmon testes carrier DNA (Sigma-Aldrich) were added to 100 µl of yeast competent cells, finally 600 µl of PEG/LiAc solution (40% polyethylene glycol in 1x TE/1x LiAc) was added to the transformation mixture. This mixture was incubated at 30°C for 30 min. Every 10 minutes cells were mixed by inversion of the tubes. After addition of 70 µl DMSO, cells were transferred to a 42°C water bath for 15 minutes and subsequently kept on ice for two minutes.

Then the cells were centrifuged, the supernatant was removed and the cells were resuspended in sterile A. bidest.. Yeast cells were spread onto agar plates containing selective medium (synthetic dropout (SD) medium containing 0,17% nitrogen base (w/o amino acids), the defined amino acid mixture and 2% glucose). The plates were incubated at 30℃ for about five days.
For the drop assay, single colonies of each co-transformation were transferred to master plates containing selective medium and grown at 30℃ for s everal days.
Then the colonies were picked for yeast colony PCR (see Par. 2.3.12). The primers used for colony PCR are listed in Tab. 2.11. Positive colonies were diluted in sterile water to obtain a concentration of 400 cells/µl. Therefore, cells were counted using a Fuchs-Rosenthal chamber.
Serial 10-fold dilutions (5 µl) of each approach were dropped onto squared petri dishes with selective media including glucose (SD-Leu$^-$/Ura$^-$/Glu) or galactose (SD-Leu$^-$/Ura$^-$/Gal). The plates were incubated at 30℃ for four to five days and photographed (Chae *et al.*, 2003).

Tab. 2.11 Names and sequences of primers used for colony PCR in the cell viability assay.

name	**sequence (5'-3')**	**vectors**	**AT[℃]/ size [bp]**
pSD10.a Bax fwd	GGCCAGGCAACTTTAGTG	pSD10.a-Bax	51 / 250
BAX-rev	TGATCTGTTCAGAGCTGGTG		
pSD10.a fwd	GGCCAGGCAACTTTAGTG	pL009-Bcl-XL	51 / 250
Bcl-X_L rev	TCTGGGAAAGCTTGTAGGA		
p416 Gal1 fwd	ACTTTCAACATTTTCGGTTTGT	p416-Gal1	54 / 240
p416 Gal1 rev	CTTTTCGGTTAGAGCGGATG		
pGADT7-seq fwd	CGTTCCCTTTCTTCCTTG	pGADT7	54 / 860
pGADT7-seq rev	TGGCGAAGAAGTCCAAA		
43 fwd	CTGGATTGCGCTTATTGGAT	p416-Gal1-PIALH43	54 / 190
43 rev	CGAAACTGGGACTGGATGAT		
43 fwd	CTGGATTGCGCTTATTGGAT	pGADT7-PIALH43	54 / 190
43 rev	CGAAACTGGGACTGGATGAT		

Additionally the corresponding vectors, the annealing temperature (AT) and the size of the PCR products are listed.

YPDA medium

Peptone	2%
Yeast extract	1%
Adenine sulfate	0.003%
Glucose	2%
Agar (for plates only)	2%

in A. bidest.
pH 6,5 (4M HCl); autoclave: 12 min. 120℃

Synthetic dropout (SD) medium

Yeast nitrogen base w/o amino acids	0,17%
$(NH4)_2SO_4$	0,5%
Amino acids from 10 x dropout stock	0,059%
Glucose or galactose	2%
Agar (for plates only)	2%

in A. bidest.
pH 5,8 (HCl); autoclave: 12 min at 120°C

10 x Dropout stock (without leucine and uracil, powder form)

L-Adenine hemisulfate salt	200 mg
L-Arginine HCl	200 mg
L-Histidine HCl monohydrate	200 mg
L-Isoleucine	300 mg
L-Lysine HCl	300 mg
L-Methionine	200 mg
L-Phenylalanine	500 mg
L-Threonine	2000 mg
L-Tryptophan	200 mg
L-Tyrosine	300 mg
L-Valine	1500 mg

10x TE buffer

Tris-HCl	0.1 M
EDTA	10 mM

in A. bidest.
pH 7.5 (HCl)

10x LiAc solution

Lithium acetate	1 M

in A. bidest.
pH 7,5 (acetic acid)

2.9.3. *Arabidopsis* plants overexpressing Pi*ALH43*

2.9.3.1. Identification of homozygous lines expressing Pi*ALH43* constructs

Arabidopsis ecotype Col-0 (N1092) plants were transformed with two different constructs of Pi*ALH43*, one with Pi*ALH43* full length cDNA with signal peptide (#24) and the other one without signal peptide (#23) under the control of a dexamethasone inducible promoter. The vectors were created by B. Khatabi (Khatabi, 2009). Plant transformation was performed by J. Imani

For the analysis of Pi*ALH43* expression, different lines of the T2 generation of stable transformed plants were grown on MS medium including vitamins (Duchefa), 0,5% sucrose, 0,4% gelrite as well as 150 mg/l ticarcillin and 30 mg/l hygromycin as selection markers. The same medium without antibiotics was used for control plants Col-0 (N1092). The plants were grown under 16 hours light/8 hours dark intervals at 24°C and ~ 47 µMol m^{-2} s^{-1}.

Tab. 2.12 Tested Pi*ALH43*-expressing *Arabidopsis* lines (T2 generation).

construct	**line**	**construct**	**line**
#23 PiALH43 w/o SP	1	#24 PiALH43 w SP	1
#23 PiALH43 w/o SP	3	#24 PiALH43 w SP	2
#23 PiALH43 w/o SP	4	#24 PiALH43 w SP	3
#23 PiALH43 w/o SP	6	#24 PiALH43 w SP	4
#23 PiALH43 w/o SP	7	#24 PiALH43 w SP	5
#23 PiALH43 w/o SP	8	#24 PiALH43 w SP	10
#23 PiALH43 w/o SP	9	#24 PiALH43 w SP	11
#23 PiALH43 w/o SP	10	#24 PiALH43 w SP	12
#23 PiALH43 w/o SP	14		

After two weeks, half of the plants were sprayed twice with 100 µM dexamethasone (Sigma-Aldrich, stock dissolved in ethanol p. a.) within 24 hours to induce Pi*ALH43* expression, the other half of the plants served as untreated control. 24 hours after the second treatment, whole plants were harvested and ground using liquid nitrogen. RNA isolation and further processing was performed as described in Par. 2.1.1 and Par. 2.1.3.

After DNase I treatment (Fermentas), RNA samples were forwarded to semi-quantitative onestep RT PCR (Qiagen OneStep RT PCR kit) using *Ubiquitin5* (fwd 5'-CCAAGCCGAAGAAGATCAAG -3'; rev 5'- ACTCCTTCCTCAAACGCTGA -3'; annealing 60°C) as housekeeping gene and *43* fwd/rev primers (43 fwd 5'-CTGGATTGCGCTTATTGGAT-3'; 43 rev 5'-CGAAACTGGGACTGGATGAT-3'; annealing 54°C) to monitor Pi*ALH43* expression. The PCR was performed according to the manufacturer's manual with 50 ng template per reaction. On the basis of this analysis, several lines were chosen for propagation.

2.9.3.2. Propagation of T2 and analysis of T3 Pi*ALH43* plants

Plants of the T2 generation were grown on MS medium including vitamins (Duchefa), 0,5% sucrose, 0,4% gelrite as well as 150 mg/l ticarcillin and 30 mg/l hygromycin as selection markers. The plants were grown under 16 hours light/8 hours dark intervals at 24°C and ~ 47 µMol m^{-2} s^{-1}. After ten days, viable plants of those lines listed in Tab. 2.13 were transferred to glass jars with the same medium except antibiotics. Finally, some of these plants were transferred to soil (Fruhstorfer Erde Typ P / sand mixture, v/v 2:1) and grown in the green house for propagation.

Tab. 2.13 *Arabidopsis* T2 lines selected for propagation

construct	line	comments
#23	1	
#23	3	
#23	9	
#24	1	
#24	4	
#24	10	
#23	10	non-transformed control line

T3 seeds of each plant were harvested separately. From some of these plants seeds were grown on soil under short day conditions (7 hours light, 17 hours dark) at 22℃. Leaves from five plantlets were harvested separately and frozen in liquid nitrogen and forwarded to the extraction of genomic DNA.

2.9.3.3. Quick genomic DNA isolation for the identification of homozygous plants

One leaf per plant was harvested in a 1,5 ml tube and crushed with a small pestle. After the addition of 500 µl DNA extraction buffer the samples were vortexed and incubated at room temperature for 5-10 minutes. Then 500 µl chloroform was added and the sample was vortexed. After centrifugation (13000 rpm, 10 min.), the upper phase was transferred to a fresh tube with 500 µl isopropanol.

The mix was incubated at room temperature for two minutes and centrifuged again (13000 rpm, 10 min.). The supernatant was discarded and the pellet was washed once with 500 µl 70% ethanol. After the removal of the supernatant the pellet was dried and finally dissolved in A. bidest. The concentration of the genomic DNA was determined with Nanodrop (Peqlab). 1-3 µl genomic DNA served as template for the PCR reaction and those primers were used as described for onestep RT PCR (chapter 2.9.3.1). The PCR results indicated homozygous plants, which could be utilized for colonization kinetics with *P. indica*. The selected T3 lines are listed in Tab. 2.14.

DNA extraction buffer

Tris-HCl pH 7,5	200 mM
NaCl	250 mM
EDTA	25 mM
SDS	0,5%

2.9.3.4. Colonization of Pi*ALH43 Arabidopsis* plants by *P. indica*

Homozygous lines selected for colonization assays are listed in Tab. 2.14. As control, Col-0 (N1092) and the non-transgenic line #23T3L10 were included.

Tab. 2.14 *Arabidopsis* T3 plants selected for colonization assays. The construct #23 contains the Pi*ALH43* sequence without signal peptide; the construct #24 possesses a signal peptide.

construct	generation	line	plant number (T2)
#23	T3	3	5
#23	T3	9	2
#23	T3	10	6
#24	T3	1	5
#24	T3	10	9

For the colonization assay, *Arabidopsis* seeds were surface sterilized as described in Par. 2.3.1.1. Plants were grown on ATS medium in squared petri dishes for three weeks under short day conditions (8 hours light (22°C), at 16 hours dark (18°C)). Half of the plates were treated with 100 µM dexamethasone (stock in ethanol p. a.) or tween water with the same amount of ethanol p. a. (mock treatment). Therefore, 1 ml solution was pipetted on the roots. 24 hours after the dexamethasone treatment all plates were inoculated with *P. indica*. Spore suspension was prepared as written in Par. 2.2.2. Per plate 1 ml spore suspension was pipetted on the roots. Three and seven days after inoculation with *P. indica* the roots were harvested and frozen in liquid nitrogen.

The root material was ground using liquid nitrogen and the powder was used for the extraction of genomic DNA and subsequent qPCR analyses. qPCR analyses were performed with *Ubiquitin5* as housekeeping gene (Par. 2.9.3.1) and *P. indica ITS* primers (fwd 5'-CAACACATGTGCACGTCGAT-3' and rev 5'-CCAATGTGCATTCAGAACGA-3').

ATS medium (1l) (Estelle & Somerville 1987)

KNO_3	5 mM
KPO_4	2,5 mM
$MgSO_4$	2 mM
$Ca(NO_3)_2$	2 mM
Fe-EDTA	50 µM
Micronutrients	1 ml

Micronutrients

H_3BO_3	70 mM
$MnCl_2$	14 mM
$CuSO_4$	0,5 mM
$ZnSO_4$	1,0 mM
Na_2MoO_4	0,2 mM
NaCl	10 mM
$CoCl_2$	0,01mM

2.9.3.5. Genomic DNA extraction with CTAB (cetrimonium bromide) method

The powder of root material from Pi*ALH43*-expressing plants was mixed with 900 µl prewarmed (65°C) CTAB extraction buffer by inverting the tube. Then the samples were incubated for 60 - 90 minutes in a water bath at 65°C and mixed several times by inversion. Thereafter, the samples were cooled to RT, mixed with 450 µl chloroform/isoamylalcohol (24:1) and were carefully shaken for 5 – 10 minutes. The samples were centrifuged (15 min, 10000 rpm, RT) and the upper phase was transferred to a new tube with 450 µl chloroform/isoamylalcohol and shaken again carefully for 5 – 10 minutes followed by another centrifugation step. The upper phase was now transferred to a tube with 3 µl RNase A (10 mg/ml) and incubated for 30 min. at RT. Then the samples were mixed with 600 µl isopropanol by inversion and incubated again for 20 minutes. Samples were centrifuged for 30 minutes at 13000 rpm at RT. The supernatant was discarded and the pellet was washed with 350 µl washing buffer 1. After 20 min. incubation and centrifugation (15 min., 10000 rpm, RT), the supernatant was removed and the pellet was washed with 200 µl washing buffer 2. Thereafter, the samples were centrifuged (15 min., 10000 rpm, RT) and the pellet was air dried.

Finally the genomic DNA was dissolved in 30 – 50 µl 1 x TE buffer at 4°C over night. The concentration of DNA was determined using Nanodrop and adjusted to 20 ng/µl. 40 ng DNA served as template for the qPCR reaction. The qPCR was performed as described in Par. 2.5.

CTAB extraction buffer (prepare always fresh)

Tris-HCl pH 7,5	100 mM	
NaCl	700 mM	
EDTA pH 8,0	50 mM	
$CTAB_2$	1%	add after heating to 65°C
β-mercaptoethanol	140 mM	

Washing buffer 1 (WASH1)

Ethanol	76%
NaOAc	0,2 M
in A. bidest.	

Washing buffer 2 (WASH2)

Ethanol	76%
NH_4OAc	10 mM
in A. bidest.	

3. RESULTS

3.1. Yeast signal sequence trap (YSST)

The YSST approach was applied to identify secreted proteins of *P. indica*, which could play an important role in the interaction of the fungus with plant roots. For the YSST screen, an invertase deficient *S. cerevisiae* mutant was transformed with a binary vector (pSMASH, Goo *et al.*, 1999) containing a truncated extracellular invertase gene without signal peptide and start codon. Extracellular invertase hydrolyses sucrose in fructose and glucose. If a dscDNA fragment with a signal peptide sequence is inserted in front of this truncated invertase gene and the invertase can be synthesized correctly, the yeast mutant transformed with this plasmid is able to grow on minimal medium with sucrose as only carbon source.

P. indica mRNA was isolated from fungal inoculum grown in liquid minimal medium including plant cell walls and root extract as only carbon source. These stimulating conditions should trigger the secretion of proteins necessary for the colonization of plant roots. The fungal mycelium was harvested at one, two and three days after transfer to minimal medium and mRNA was isolated. Then the mRNA was transcribed in dscDNA and, after the addition of *Eco*RI adapters, *Not*I digestion and several purification steps, cloned in the linearized and purified pSMASH YSST vector. Finally, yeast colonies transformed with this cDNA library, which were growing on sucrose minimal medium, were tested for their incorporated pSMASH construct. The yeast colonies were additionally transferred to minimal medium with raffinose or sucrose as carbon source and antimycin A to receive single cell colonies. Furthermore, this step should eliminate false positive yeast clones harboring a construct without signal peptide. The antibiotic antimycin A blocks oxidative respiration so that the yeast cells have to rely on anaerobic fermentation where a functional extracellular invertase is essential (Krijger *et al.*, 2008). The single colonies growing on minimal medium with sucrose and antimycin A were analysed with colony-PCR, the PCR products were purified and subsequently sequenced.

Through successful application of the root cell wall and root extract approach, the proportion of sequenced cDNA fragments originating from *P. indica* mRNA was 100%. Thus, plant genes were not found in the assay. Fifty cDNA fragments were analyzed by sequencing and could be clearly related to 14 different ORFs listed in the meanwhile available *P. indica* GBrowse database (Tab. 3.1). Especially the candidate genes *K1* (*PIIN_03211*) and *K29* (*PIIN_04018*) were highly expressed in the *P. indica* mRNA so that cDNA fragments of these genes were sequenced ten and 16 times, respectively. Also *K3* (PIIN_07104), *K16* (PIIN_08513), *K19* (PIIN_10643), *K40* (PIIN_02169) and *K46* (PIIN_09796) were retrieved several times.

The other candidates were found once. Yeast clones assigned to seven of the 14 ORFs were able to grow on raffinose/ antimycin A minimal medium in contrast to the other ones that did not grow (Tab. 3.1). The established YSST screening retrieved several interesting *P. indica* genes whose putative function in the interaction with a host plant should be investigated further.
Thus, the *P. indica* ORFs identified by the YSST approach were firstly subjected to different *in silico* studies. In addition, the candidate gene *PIIN_00029* (Pi*ALH43*), which was identified by B. Khatabi (2009), was included in the *in silico* analysis so that in total 15 sequences were studied.

3.2. *In silico* analysis of *P. indica* candidate genes

3.2.1. Signal peptide, protein and transmembrane domain identification

The full length nucleotide and amino acid sequences of the YSST candidates were retrieved from the *P. indica* GBrowse genome and transcriptome database (DB). Their protein length ranged from 117 to 1054 amino acids (aa). These sequences were inserted in several internet databases for structural and functional protein analysis.
The SignalP internet platform can be used for the identification of signal peptides in protein sequences. It predicted a signal peptide for eleven of the *P. indica* sequences; three proteins did not carry a signal peptide (Tab. 3.1). Among the candidates which turned out to have no signal peptide, the ORF *PIIN_04018* (*K29*) was retrieved very often in the YSST screen (16 times). A special case was found for *PIIN_03211* (*K1*). Comparing the YSST sequencing results of *PIIN_03211* with the predicted ORF in the *P. indica* GBrowse database, it was discovered that two possible ORF sequences could exist. The shorter version of 104 amino acids, which was deduced from the YSST sequencing results, was predicted to include a signal peptide. The longer version (126 aa), deduced from the *P. indica* database, did not contain a signal peptide but a transmembrane domain was predicted. *PIIN_03211* was retrieved ten times during the screen. For the five candidate genes *PIIN_03211* (GBrowse DB version, *K1*), *PIIN_08513* (*K16*), *PIIN_02519* (*K37*), *PIIN_04526* (*K43*) and *PIIN_00029* (Pi*ALH43*) one transmembrane domain was found using the TMHMM internet database although almost all of them were predicted to be secreted according to SignalP (except *PIIN_03211*, GBrowse DB version). For *PIIN_08513*, the detected transmembrane domain could be due to the prediction of a signal peptide or a signal anchor by SignalP. For one sequence (*PIIN_04018*, *K29*) two transmembrane (TM) domains were identified.
The SMART platform was applied for the identification of protein domains. For nine of the 15 protein sequences no convincing match to known protein domains could be found. Both versions of PIIN_03211 belong to this group.

Candidate PIIN_07104 (K3) was reported to include an inhibitor_I9 and a peptidase_I8 protein domain and thus could function as a serine endopeptidase in proteolytic processes (e. g. Hoffman & Breuil, 2002). PIIN_03806 (K12) was identified to possess a collagen domain, which could play a role in the formation of connective tissue (e. g. Celerin *et al.*, 1996). PIIN_05415 (K36) was considered to have an ELFV dehydrogenase domain and might be involved in metabolic mechanisms like amino acid transport. Additionally, PIIN_02169 (K40) was predicted to contain a deacetylase domain and four LysM domains, so that a putative function in carbohydrate metabolism and cell wall catabolic processes might be assumed (e. g. Buist *et al.*, 2008). PIIN_09796 (K46) was analyzed to comprise two thioredoxin domains, as reported for protein disulphide isomerases, which are involved in the formation and rearrangement of disulphide bounds (e. g. Freedman *et al.*, 1994). Finally, a RING domain was identified in PIIN_00029 (PiALH43) which is known to be associated with E3 ubiquitin-protein ligase activity and functions in protein and zinc ion binding (e. g. Deshaies & Joazeiro, 2009). The findings obtained with the SMART, TMHMM and SignalP databases are additionally depicted and summarized in Fig. 3.1A for the candidate proteins with signal peptide and in Fig. 3.1B for the identified proteins without signal peptide. The vast majority of the isolated YSST candidates was predicted to carry a SP for the secretory pathway and thus could have a function in the interaction with a host plant.

Tab. 3.1 *In silico* analysis of the *P. indica* candidates using the SignalP, TMHMM and SMART internet databases (retrieved on Feb 21th, 2011).

YSST			*P. indica* GBrowse		SignalP	TMHMM	SMART		
No YSST[a]	times sequenced	growth on raffinose	PIIN N[b]	length (aa)[b]	signal peptide	trans-membran regions	protein domains	begin-end (aa)	possible function of domain
K1[1]	10	7x yes/ 3x no	PIIN_03211	104	yes	0	-	-	-
K1[2]	10	7 x yes/ 3 x no	PIIN_03211	126	no	1	-	-	-
K3	5	4 x yes/ 1 x no	PIIN_07104	425	yes	0	Inhibitor_I9; peptidase_S8	43-124; 150-420	serine-type endopeptidase, proteolysis
K12	1	no	PIIN_03806	666	yes	0	collagen	275-334	formation of connective tissue structure
K16	3	3 x no	PIIN_08513	250	yes	1	-	-	-
K19	2	1x yes/ 1 x no	PIIN_10643	198	yes	0	-	-	-
K29	16	1 x yes/ 15 x no	PIIN_04018	383	no	2	-	-	-
K35	1	no	PIIN_06847	117	yes	0	-	-	-
K36	1	no	PIIN_05415	1054	no	0	ELFV_dehydrog (Glutamate/Leucine/ Phenylalanine/Valine dehydrogenase)	693-955	amino acid transport, metabolism
K37	1	yes	PIIN_02519	314	yes	1	-	-	-
K38	1	yes	PIIN_00308	259	yes	0	-	-	-
K40	4	1 x yes/ 3 x no	PIIN_02169	513	yes	0	Polysacc_deac_1; 4x LysM	30-158; 264-308, 340-384, 412-457, 466-512	carbohydrate metabolism; cell wall catabolic processes
K43	1	no	PIIN_04526	353	yes	1	-	-	-
K46	3	3 x no	PIIN_09796	628	yes	0	2x Thioredoxin	33-140; 168-268	protein disulphide isomerases (PDI), formation and rearrangement of disulphide bonds during protein folding
K48	1	no	PIIN_10506	324	no	0	-	-	-
PiALH43	Khatabi (2009)	isee Khatabi (2009)	PIIN_00029	529	yes	1	Ring finger	411-454	E3 ubiquitin-protein ligase activity, protein binding, zinc ion binding

[a] = classification of the numbers chosen for the YSST screen
[b] = ORF numbers and protein length (aa) from the *P. indica* GBrowse database
[1] = YSST version of PIIN_03211; [2] = *P. indica* GBrowse database version of PIIN_03211
aa = amino acid(s)

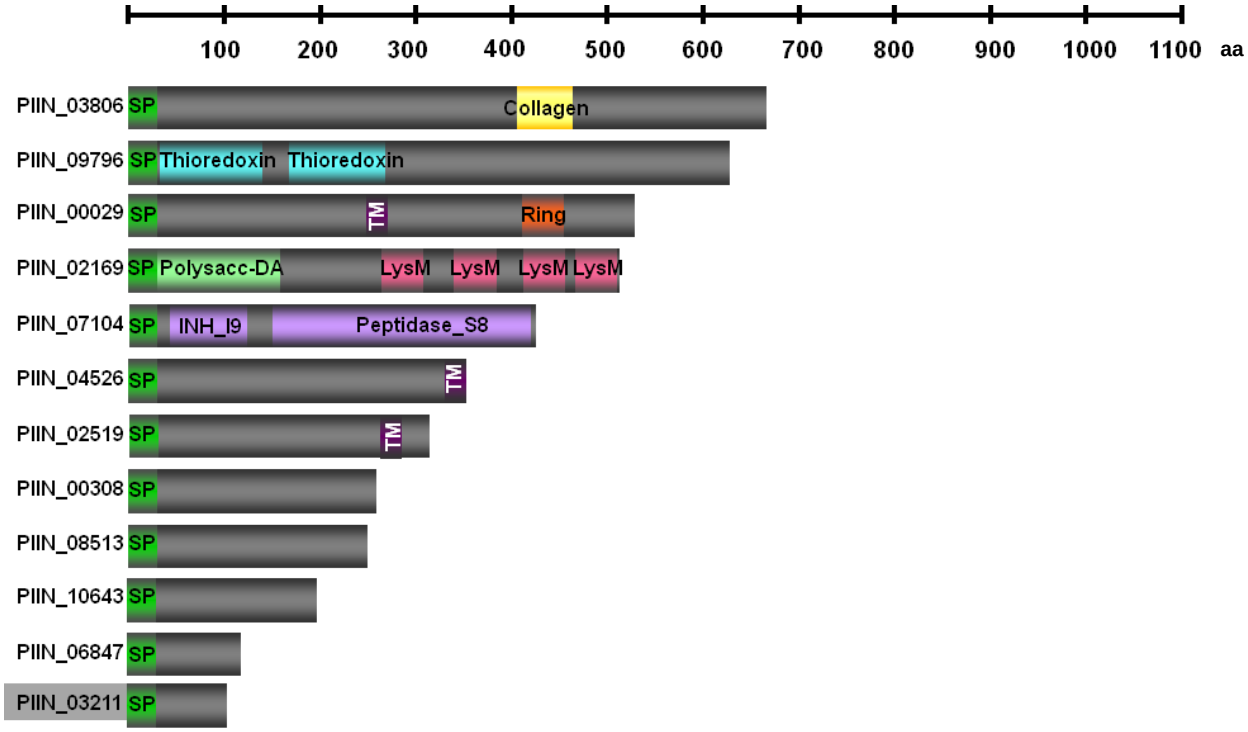


Fig. 3.1A Schematic diagram of the *P. indica* proteins with signal peptide identified by YSST. *In silico* studies were performed by using the full length ORFs from the *P. indica* GBrowse DB. These sequences were blasted against the SignalP, TMHMM and SMART database. The positions and length of the identified protein domains, as indicated by the scale bar, are given as suggested by SMART. The alternative version of PIIN_03211 as it was found in the YSST screen is highlighted with grey color. aa = amino acids; SP = signal peptide; TM = transmembrane domain; Polysacc-DA = polysaccharide-deacetylase domain; Inh I9 = inhibitor I9 domain.

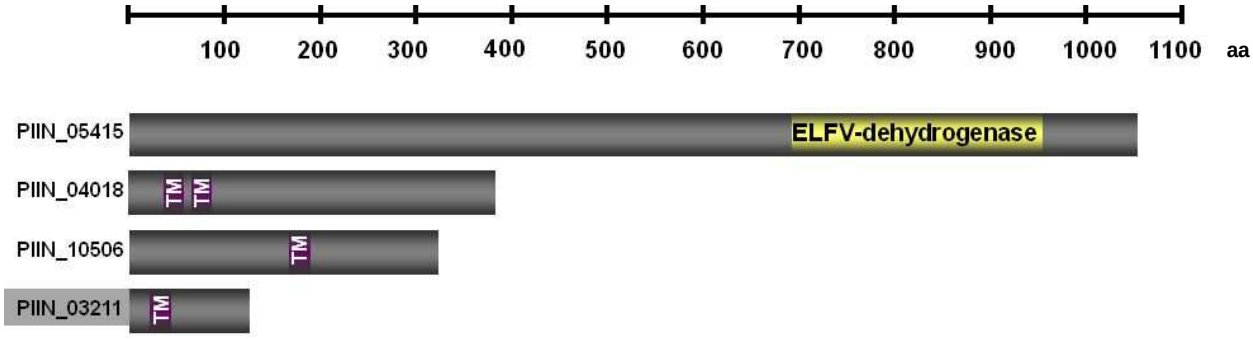


Fig. 3.1B Schematic diagram of the *P. indica* proteins without signal peptide identified in the YSST screen. *In silico* studies were performed by using the full length ORFs from the *P. indica* GBrowse database. These sequences were blasted against the SignalP, TMHMM and SMART database. The positions and length of the identified protein domains is based on SMART-based analyses. The alternative version of PIIN_03211 as it is retained in the GBrowse DB is highlighted with grey color. aa = amino acids; TM = transmembrane domain.

3.2.2. Annotations of the YSST candidates in the *P. indica* GBrowse and NCBI DB

In silico analyses were further performed by using the *P. indica* GBrowse database (DB) (Tab. 3.2). The scaffold comprises the sequence of *P. indica* genomic DNA with exons and introns. It can be used to collect information about the position of the respective gene in the *P. indica* genome. Similar to the results of the SMART protein domain analysis given in Tab. 3.1, eleven of the 15 investigated genes were annotated as hypothetical ORFs in the *P. indica* GBrowse database. For PIIN_07104, PIIN_05415, PIIN_02169, and PIIN_09796 the annotations in the *P. indica* database were almost identical to those retrieved from the SMART database (compare Tab. 3.1/ Tab. 3.2) The *P. indica* candidate genes were additionally analyzed using the blastx algorithm (for internet address see references). Therefore, the nucleotide sequences of the candidates were translated and blasted against the protein database. The results are presented in Tab. 3.2. Here the best hit, retrieved from the BLAST search, and the best annotated hit - if the first hit was specified as a hypothetical protein - is listed. The best hit in NCBI blastx resulted in “hypothetical ORF/ hypothetical protein” for eight of the candidates; for one candidate (PIIN_04018) nothing was found. Similar to the SMART and *P. indica* GBrowse findings, PIIN_07104 was referred to as a serine protease, PIIN_05415 as a NAD-specific glutamate dehydrogenase and PIIN_02169 as a carbohydrate esterase family 4 protein. PIIN_09796 was, consistent with the other sources, retrieved as protein disulfide isomerase. Finally PIIN_00029 was also identified as RING 7 protein and PIIN_04526 was assigned as a mannoprotein. Almost all annotations were obtained due to sequence similarities to proteins of other fungal species. Proteins of the species *Laccaria bicolor* (2x), *Schizophyllum commune* (3x) and *Coprinopsis cinerea okayama* (3x) provided the main part of basidiomycota hits. Three of the best hits belonged to the phylum of ascomycota. For two candidates, a protein from a *Drosophila* species or a protein of a *Caenorhabditis* species, respectively, showed highest similarities.

In order to obtain more information about the 15 candidate genes, the best annotated hit was determined (Tab. 3.2). For most of the candidates, no annotated database entries could be found. However, both PIIN_03211 versions showed similarities to a Pep2 protein *of Fusarium oxysporum* while PIIN_03806 was similar to a protein from the ysirk family (*Gemella haemolysans*, firmicutes). PIIN_10506 displayed a similarity to a LipA and NB-ARC domain protein of *Aspergillus fumigatus*. The use of different internet resources retrieved consistent results; this increases the plausibility of the findings.

Tab. 3.2 *In silico* analysis of the *P. indica* candidates using the *P. indica* GBrowse database and the blastx search tool from the NCBI platform (retrieved on Feb 21th, 2011).

The NCBI blastx tool compares the inserted nucleotide sequence to a protein database.

classification *P. indica* GBrowse				NCBI blastx				
No YSST[a]	**PIIN**	**scaffold**	**annotation**	**best hit**	**phylum best hit (NCBI)**	**Accession/ E-value**	**best annotated hit**	**Accession/ E-value**
K1[1]	PIIN_03211	PIRI_contig_0056	-	hypothetical orf (*Arthroderma gypseum*)	ascomycota	XP003176477.1 /9e^{-7}	Pep2 (*Fusarium oxysporum*)	ADO60277.1/ 2e^{-5}
K1[2]	PIIN_03211	PIRI_contig_0056	hypothetical orf	hypothetical orf (*Arthroderma gypseum*)	ascomycota	XP003176477.1 /9e^{-7}	Pep2 (*Fusarium oxysporum*)	ADO60277.1/ 2e^{-5}
K3	PIIN_07104	PIRI_contig_0211	probable endopep-tidase K	serine protease (*Hypsizygus marmoreus)*	basidiomycota	ABL98208.2 /4e^{-70}	see best hit	see best hit
K12	PIIN_03806	PIRI_contig_0071	hypothetical orf	GI21630 (*Drosophila majovensis*)	arthropoda	XP_002010590.1 /6e^{-22}	gram-positive signal peptide, ysirk family (*G. haemolysans*)	ZP_04776810.1 /3e^{-15}
K16	PIIN_08513	PIRI_contig_0333	hypothetical orf	hypothetical protein (*Schizophyllum commune*)	basidiomycota	XP_003037150.1 /3e^{-16}	yd repeat protein (*Riemerella anatipestifer*)	YP 004046258.1 /0,9
K19	PIIN_10643	PIRI_contig_0907	hypothetical orf	hypothetical protein MGG (*Magnaporthe oryzae*)	ascomycota	XP_001414338.1 /3,5	pchromodomain-helicase-DNA-binding protein 8 isoform 2 (*Callithrix jacchus*)	XP_002753817.1 /7,8
K29	PIIN_04018	PIRI_contig_0076	hypothetical orf	no hit	no hit	no hit	no hit	no hit
K35	PIIN_06847	PIRI_contig_0195	hypothetical orf	hypothetical protein CRE_27851 (*Caenorhabditis remanei*)	nematoda	XP_003093512.1 /0,002	PFG377 protein (*Plasmodium falciparum* 3D7)	XP_001350885.1 /0,004
K36	PIIN_05415	PIRI_contig_0126	probable glutamate dehydrogenase, NAD(+)-specific	NAD-specific glutamate dehydrogenase (*Laccaria bicolor*)	basidiomycota	XP_001876152.1 /0	see best hit	see best hit
K37	PIIN_02519	PIRI_contig_0040	hypothetical orf	hypothetical protein (*Schizophyllum commune* H4-8)	basidiomycota	XP_003030519.1 /2e^{-14}	IS30 transposase (*E. coli*)	NP_289191.1 /3
K38	PIIN_00308	PIRI_contig_0003	hypothetical orf	hypothetical protein (*Coprinopsis cinerea okayama*)	basidiomycota	XP_001828926.1 /2e^{-52}	no annotated hit	no annotated hit
K40	PIIN_02169	PIRI_contig_0033	related to deacetylase	carbohydrate esterase f amily 4 protein (*Schizophyllum commune*)	basidiomycota	XP_003027593.1 /2e^{-37}	see best hit	see best hit
K43	PIIN_04526	PIRI_contig_0092	hypothetical orf	mannoprotein (*Coprinopsis cinerea okayama*)	basidiomycota	XP_001838460.1 / 4e^{-111}	see best hit	see best hit
K46	PIIN_09796	PIRI_contig_0536	related to protein disulfide isomerase (*Laccaria bicolor*)	protein disulfide isomerase *(Laccaria bicolor)*	basidiomycota	XP_001884326.1 / 3e^{-82}	see best hit	see best hit
K48	PIIN_10506	PIRI_contig_0813	hypothetical orf	hypothetical protein (*Pyrenophora teres f. teres*)	ascomycota	EFQ89369.1 /3e^{-17}	LipA and NB-ARC domain protein (*Aspergillus fumigatus*)	EDP52601.1 /6e^{-15}
PiALH43	PIIN_00029	PIRI_contig_0001	hypothetical orf	RING-7 protein (*Coprinopsis cinerea okayama*)	basidiomycota	XP_001830579.2 / 6e^{-86}	see best hit	see best hit

[1] = YSST version of PIIN_03211
[2] = *P. indica* GBrowse database version of PIIN_03211

3.2.3. Homology search within the *P. indica* genome

Next, the *P. indica* genome was checked for genes which can be regarded as homologs or more precisely paralogs to the YSST candidates (Tab. 3.3). Homologous sequences originate from a common primary sequence. The homology analysis distinguishes between paralogous and orthologous sequences (proteins or genes). Orthologous genes occur through a speciation event, paralogous genes originate from gene duplications (Lee *et al*., 2007). The *P. indica* GBrowse database provides also a platform to search for paralogs within the *P. indica* genome ("best self match"). This tool uses a blastp algorithm; the blastp approach compares a protein sequence to a protein database. In the *P. indica* DB, proteins are partially sorted in clusters of paralogs within the genome. Because the clustering only focuses on one linkage ("single linkage cluster") and a lot of proteins contain several protein domains, it is possible to have sequences grouped in one cluster which are not related to each other (see *P. indica* GBrowse DB for further explanations). The best self match and protein cluster information was collected from the GBrowse database in February 2011.

PIIN_03211 (K1) had two similar paralogs in the *P. indica* genome; the three proteins were grouped together in cluster 620 "predicted protein". The version of PIIN_03211 identified by YSST could not be included in the analysis because it was not listed in the database. For PIIN_07104 (K3), the putative endopeptidase, ten additional similar proteins were identified in the *P. indica* genome. PIIN_07104 and its paralogs were put in cluster 29, which was defined as "related to ABC1 - ubiquinol- cytochrome-c reductase complex assembly protein". For PIIN_03806 (K12), the candidate with the collagen domain, one paralog (PIIN_03801) could be found in the *P. indica* genome. This ORF was located on the same scaffold (contig00071) and thus probably in physical proximity to candidate PIIN_03806. The cluster comprising these two proteins was named "cluster679, predicted protein [(*Coprinopsis cinerea*)/ (*Hormographiella aspergillata*)]". PIIN_08513 (K16) had three paralogs and was grouped in cluster 863, "hypothetical orf". Two of these paralogs were located on the same scaffold than PIIN_08513. Their PIIN numbers are PIIN_08511 and PIIN_08512. PIIN_10643 (K19) possessed one paralogous protein in the *P. indica* genome. Both were grouped in a cluster entitled "hypothetical orf". For PIIN_04018 (K29), no matching proteins could be found. PIIN_06847 (K35) showed similarities to at least 30 other proteins.

Like PIIN_06847, all of them were small secreted proteins; a screenshot of those proteins with the highest similarity to PIIN_06847 is shown in Fig. 3.2. These proteins had a length of 110 – 130 amino acids.

Noticeable was the huge number of histidines in the protein sequence and the characteristic amino acid motif “RSIDELD” (or similar) at the C-terminus of many of these proteins. PIIN_06847 did not show this “RSIDELD” amino acid sequence. PIIN_05415 (K36), the probable glutamate dehydrogenase, and PIIN_02519 (K37) were lacking homologs in the genome of the fungus. PIIN_00308 (K38) was grouped together with four other similar proteins in cluster 114, “predicted protein”, in total there were six proteins showing similarities to PIIN_00308. For PIIN_02169 (K40), the protein with the deacetylase and LysM domains, 25 homologous proteins were listed of which one was declared as “invalid protein model” (PIIN_02171). PIIN_02169 was part of the large cluster 107: “related to glycoside hydrolase family 71 protein –*Laccaria bicolor*”, which comprised 78 proteins. PIIN_04526 (K43) had similarities to eight proteins in the genome. All of them were sorted in cluster 329 “predicted protein [*Laccaria bicolor* (strain S238N-H82)/(*Laccaria laccata var. bicolor*)]”. PIIN_09796 (K46, with two thioredoxin domains) showed paralogous sequence passages to seven other protein sequences. Six of these proteins, among them PIIN_09796, were put in the large cluster 2 “related to RSA4 - WD-repeat protein (maturation, intra-nuclear transport), pre-60S ribosomal subunits”. The candidate protein PIIN_10506 (K48) had sequence similarities to at least 30 other proteins in the *P. indica* genome. Finally, PIIN_00029 (PiALH43) had similarities (especially the RING domain) to eleven other proteins in the genome. PIIN_00029 was grouped in cluster 29 (“related to ABC1 - ubiquinol- cytochrome-c reductase complex assembly protein”), to which also candidate PIIN_07104 belonged to. Cluster 29 was a quite large group of proteins with 35 entries and not all proteins of this group were related to each other. For instance, PIIN_00029 and PIIN_07104 showed no similarities. Thus, some of the YSST candidates seem to be part of larger groups of homologous proteins, for example PIIN_06847 (K35) or PIIN_02169 (K40) (Tab. 3.3). Their importance for the fungus, for example in the interaction with a host plant, should be investigated further.

Tab. 3.3 Homology (paralogy) search (best self match, blastp) with the YSST candidates within the *P. indica* GBrowse database (retrieved in February 2011).

		best self-match (blastp) *P. indica* GBrowse					
No YSST	**PIIN**	**PIIN best self-match**	**score**	**p-value**	**region[a] start stop (aa)**	**total[b] amount similar proteins**	**protein cluster[c]**
K1[1]	PIIN_03211	PIIN_10087	111	6,40E-26	27 - 102	2	620: predicted protein
		PIIN_06855	83	1,20E-17	28 - 126		
K3	PIIN_07104	PIIN_04006	406	6,30E-114	4 - 422	10	cluster29: related to ABC1 – ubiquinol-cytochrome-c reductase complex assembly protein
		PIIN_07492	320	5,40E-88	41 - 422		
		PIIN_07598	268	1,80E-72	4 - 419		
		PIIN_04374	251	3,40E-67	92 - 422		
K12	PIIN_03806	PIIN_03801	671	0	1 - 659	1	cluster679: predicted protein [(*Coprinopsis cinerea*)/ (*Hormographiella aspergillata*)]
K16	PIIN_08513	PIIN_05901	179	6,20E-46	68 - 249	3	cluster863: hypothetical orf
		PIIN_08512	150	3,90E-37	78 - 248		
		PIIN_08511	109	8,00E-25	74 - 234		
K19	PIIN_10643	PIIN_08618	214, 46	1,40E-56, 3,70E-06	1 - 140, 178 - 197	1	cluster1048: hypothetical orf
K29	PIIN_04018	-	-	-	-	-	-
K35	PIIN_06847	PIIN_06837	110	8,70E-26	1 - 111	at least 30	-
		PIIN_05848	104	5,70E-24	1 - 111		
		PIIN_09689	101	4,30E-23	1 - 111		
		PIIN_09643	100	5,60E-23	1 - 100		
K36	PIIN_05415	-	-	-	-	-	-
K37	PIIN_02519	-	-	-	-	-	-
K38	PIIN_00308	PIIN_09540	197	1,80E-51	47 - 227	6	cluster114: predicted protein
		PIIN_06796	157	1,80E-39	31 - 201		
		PIIN_04114	149	9,30E-37	29 - 215		
		PIIN_05452	142	9,00E-35	36 - 200		
K40	PIIN_02169	PIIN_02172	614	1,50E-176	1 - 456	25	cluster107: related to glycoside hydrolase family 71 protein – *Laccaria bicolor*
		PIIN_02171	592	7,60E-170	26 - 455		
		PIIN_02170	390	4,60E-109	261 - 513		
		PIIN_03655	380	4,40E-106	6 - 511		
K43	PIIN_04526	PIIN_06613	471	1,80E-133	3 - 353	8	cluster329: predicted protein [*Laccaria bicolor* (strain S238N-H82)/ (*Laccaria laccata var. bicolor*)]
		PIIN_04527	453	3,70E-128	21 - 338		
		PIIN_01237	195	1,40E-50	23 - 276		
		PIIN_01243	184	2,50E-47	39 - 241		
K46	PIIN_09796	PIIN_02932	80	1,10E-15	166 - 395	7	cluster2: related to RSA4 - WD-repeat protein (maturation, intra-nuclear transport), pre-60S ribosomal subunits
		PIIN_02623	73	2,10E-13	163 - 315		
		PIIN_09795	69	2,30E-12	30 - 141		
		PIIN_09630	68	4,50E-12	36 - 244		
K48	PIIN_10506	PIIN_08587	228	1,70E-60	63 - 324	at least 30	-
		PIIN_10963	219	6,50E-58	70 - 324		
		PIIN_02090	218	1,70E-57	19 - 324		
		PIIN_09058	212	1,30E-55	40 - 324		
PiALH43	PIIN_00029	PIIN_07106	68	3,80E-12	394 - 458	11	cluster29: related to ABC1 - ubiquinol- cytochrome-c reductase complex assembly protein

[a] = start and stop of the protein regions with similarities to the YSST candidate.
[b] = total amount of similar proteins in the genome, only those with the highest similarity are listed in this table.
[c] = protein cluster of paralogous proteins.
[1] = PIIN_03211 version from the *P. indica* GBrowse DB; aa = amino acid(s)

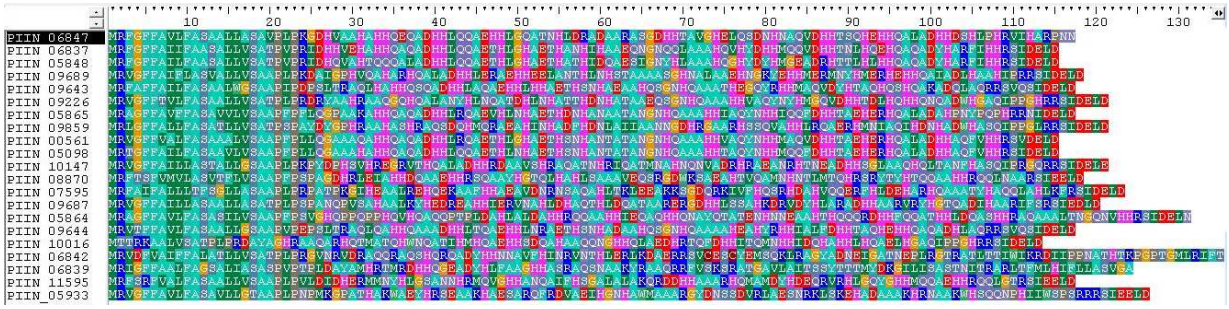


Fig. 3.2 Screenshot displaying sequences of small secreted proteins in the *P. indica* genome with highest similarity to PIIN_06847 (K35). The score was at least 50. The sequences are sorted according to the grade of similarity to PIIN_06847. The PIIN number of the YSST candidate gene is highlighted in black. The number of amino acids is given as scale on top of the screenshot.

3.3. Quantitative real-time PCR with YSST candidate genes

The expression levels of the candidate genes with signal peptide were determined under different culturing conditions using quantitative RT-PCR. All candidate genes were analyzed except *PIIN_06847* (*K35*) for which it was not possible to design specific primers because of the high number of homologous genes in the genome (see Fig 3.2). Three different growth conditions were investigated. Firstly, *P. indica* was grown in liquid axenic culture (complex medium, CM). Therefore, the medium was inoculated with *P. indica* chlamydospores. The cultures were harvested at 2 and 7 days after inoculation and used for RNA isolation and cDNA synthesis. Secondly, *P. indica* was grown on living *Arabidopsis* wild type (Col-0) roots. The roots of three weeks old plants were inoculated with *P. indica* spore suspension, harvested at 2 and 7 days after inoculation (dai), and forwarded to RNA isolation and cDNA synthesis. Thirdly, *P. indica* was grown on dead autoclaved *Arabidopsis* wild type roots. The inoculation and processing of the dead roots was identical to the second approach with living roots.

3.3.1. Relative expression of secreted candidates under different growth conditions

Generally, the analyzed *P. indica* genes could be sorted in three different groups according to their expression pattern. The sorting basically focused on the tendencies and not on the absolute values. At 2 dai, the expression levels of the putative endopeptidase K, *PIIN_07104* (*K3*), the collagen domain protein *PIIN_03806* (*K12)*, *PIIN_08513* (*K16*), *PIIN_10643* (*K19*) and *PIIN_02519* (*K37*) were highest in the interaction with living plant roots and lowest in dead roots, the expression in axenic culture was intermediate.

At 7 dai, the expression levels were highest in CM for these five genes and again lowest in dead roots (Fig. 3.3A-E).

A very similar expression pattern was also measured for the putative RING E3 ligase *PIIN_00029* (Pi*ALH43*). At the early time point (2 dai), the highest *PIIN_00029* expression was found in living plant roots and the lowest in axenic culture (CM); at the late time point the expression was highest in axenic culture and lowest in dead roots (Fig. 3.3J).Thus this first group consisted of six genes which showed the highest expression in living plant roots at 2 dai and in CM at 7 dai. The second group comprises the genes *PIIN_00308* (*K38*), the putative protein disulphide isomerase *PIIN_09796* (*K46*) and the putative mannoprotein *PIIN_04526* (*K43*). Here, the expression was highest in living plant roots and lowest in dead plant roots at 2 dai, the CM values lay intermediate. At 7 dai, the expression was also highest in living plant roots, especially for PIIN_04526. The lowest expression was again seen in dead roots at this late time point (Fig. 3.3F, H, I). Thus, these three genes showed the highest expression in living plant roots at both time points. *PIIN_02169* (*K40*), the putative deacetylase with four LysM domains, forms the third group. The expression of *PIIN_02169* was highest in CM at 2 dai. The expression in living and dead plant roots was lower; there was almost no difference between these two approaches at this early time point (Fig. 3.3G). At the late time point (7 dai), the expression was by far the highest in axenic culture, the lowest value was measured in dead plant roots. The expression patterns of all *P. indica* genes are schematically summarized in Tab. 3.4. This table considers the tendencies of the three approaches at the respective time point; the absolute levels are not taken into account. The highest expression level is illustrated with dark grey and three plus signs; the lowest level is marked with one plus sign and a light grey color. The intermediate values are labeled with two plus signs and a medium grey color (Tab. 3.4). In general, the expression level of the investigated *P. indica* genes was lowest in dead *Arabidopsis* roots at both investigated time points. For seven genes the expression level in CM increased very strongly at 7 dai. At 2 dai, the expression levels of nine of the tested genes were higher in living plant roots than in CM or dead roots. Only for *PIIN_02169* (*K40*) the highest expression level was detected in CM at this early time point. Thus, these qPCR results indicate that the majority of the candidate genes are induced at 2 dai in living plant roots and could play a role in the interaction of *P. indica* with plant roots.

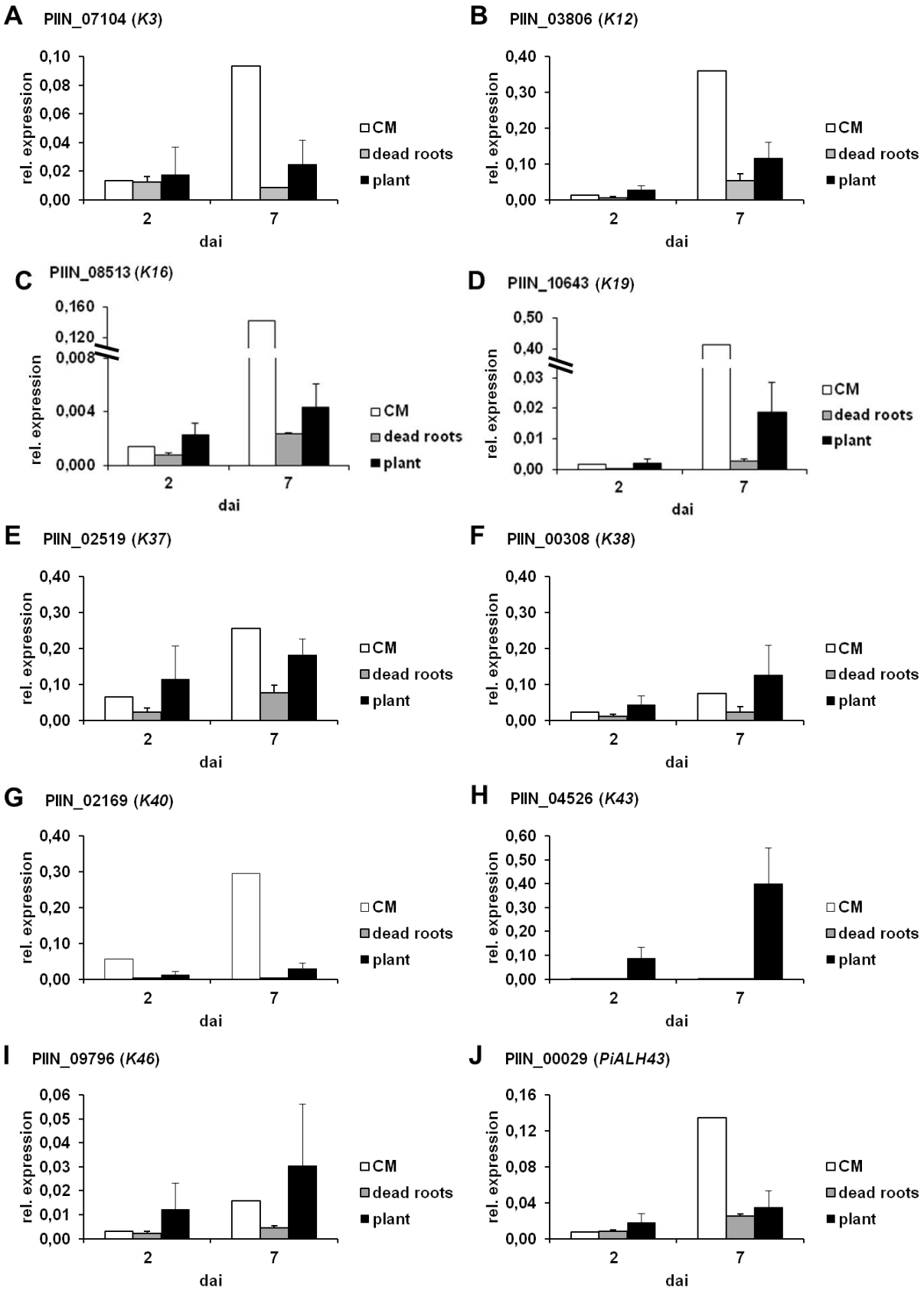

A PIIN_07104 (K3)
rel. expression
0,10
0,08
0,06
0,04
0,02
0,00
2
7
dai
CM
dead roots
plant
B PIIN_03806 (K12)
rel. expression
0,40
0,30
0,20
0,10
0,00
2
7
dai
CM
dead roots
plant
C PIIN_08513 (K16)
rel. expression
0,160
0,120
0,008
0,004
0,000
2
7
dai
CM
dead roots
plant
D PIIN_10643 (K19)
rel. expression
0,50
0,40
0,03
0,02
0,01
0,00
2
7
dai
CM
dead roots
plant
E PIIN_02519 (K37)
rel. expression
0,40
0,30
0,20
0,10
0,00
2
7
dai
CM
dead roots
plant
F PIIN_00308 (K38)
rel. expression
0,40
0,30
0,20
0,10
0,00
2
7
dai
CM
dead roots
plant
G PIIN_02169 (K40)
rel. expression
0,40
0,30
0,20
0,10
0,00
2
7
dai
CM
dead roots
plant
H PIIN_04526 (K43)
rel. expression
0,60
0,50
0,40
0,30
0,20
0,10
0,00
2
7
dai
CM
dead roots
plant
I PIIN_09796 (K46)
rel. expression
0,06
0,05
0,04
0,03
0,02
0,01
0,00
2
7
dai
CM
dead roots
plant
J PIIN_00029 (PiALH43)
rel. expression
0,16
0,12
0,08
0,04
0,00
2
7
dai
CM
dead roots
plant

Fig. 3.3 Expression of *P. indica* genes encoding for secreted proteins identified by YSST. **A-J** *P. indica* was grown in axenic culture (CM, white bars), on dead *Arabidopsis* roots (dead roots, grey bars) or on living *Arabidopsis* roots (plant, black bars) and harvested at 2 and 7 days after inoculation (dai). The relative expression (2^-ΔCt) of the *P. indica* genes was calculated in relation to the housekeeping gene Pi*Tef1α*. The expression study with axenic culture was performed once and standard deviations were not calculated. For all other experiments, standard deviations derive from two (dead roots) and three (living roots, plant) independent biological experiments.

Tab. 3.4 Expression of *P. indica* genes encoding for secreted proteins identified by YSST. The tendencies of the expression levels are illustrated with the help of colors and plus signs.

		2 dai			**7 dai**		
No YSST	**PIIN**	**CM**	**dead roots**	**plant**	**CM**	**dead roots**	**plant**
K3	*PIIN_07104*	++	+	+++	+++	+	++
K12	*PIIN_03806*	++	+	+++	+++	+	++
K16	*PIIN_08513*	++	+	+++	+++	+	++
K19	*PIIN_10643*	++	+	+++	+++	+	++
K37	*PIIN_02519*	++	+	+++	+++	+	++
Pi*ALH43*	*PIIN_00029*	+	++	+++	+++	+	++
K38	*PIIN_00308*	++	+	+++	++	+	+++
K43	*PIIN_04526*	+	++	+++	++	+	+++
K46	*PIIN_09796*	++	+	+++	++	+	+++
K40	*PIIN_02169*	+++	+	++	+++	+	++

The candidate genes are sorted according to their expression similarities.
Highest expression level: "+++" and dark grey
Intermediate expression level: "++" and medium grey
Lowest expression level: "+" and light grey
CM = complex medium
dai = days after inoculation

3.4. Functional analysis

For further functional analysis the four genes *PIIN_03806* (*K12*), *PIIN_08513* (*K16*), *PIIN_04526* (*K43*), and *PIIN_00029* (Pi*ALH43*) were chosen on the basis of their qPCR expression levels and the results of the *in silico* studies. All of these four candidate proteins were predicted to carry a signal peptide. Please see Tab. 3.1 and Tab. 3.2 for further details (e.g. predicted domains) on these candidates.

3.4.1. Subcellular localization of YSST candidates

To analyze the subcellular localization of the *P. indica* proteins and to collect information about putative plant targets, *P. indica* candidate genes were overexpressed in leaf or onion epidermal cells. Therefore, the full length cDNA sequences of the candidate genes *PIIN_03806* (*K12*), *PIIN_08513* (*K16*), *PIIN_04526* (*K43*), and *PIIN_00029* (Pi*ALH43*) including their endogenous signal peptides were cloned in the expression vector pGY-1-*GFP* (for the vector map see Suppl. 7.2) in order to obtain candidates that were fused to *GFP* at their C-terminus. These candidate GFP fusion proteins were set under the control of a 35S CaMV promoter. For the subcellular localization studies, *Arabidopsis* Col-0 leaf or onion epidermal cells were transformed using a biolistic transformation approach. The pGY-1 candidate-GFP constructs were always co-expressed with the pGY-1-*mCherry* plasmid as a technical control. As additional control, the empty plasmid pGY-1-*GFP* was transiently expressed in onion epidermal cells. Transformed cells were monitored 24 – 48 hours after particle bombardment by confocal laser-scanning microscopy (CLSM) or epifluorescence microscopy. Fig. 3.4A-C display CLSM images of an onion epidermal cell transformed with pGY-1-*GFP* (Fig 3.4A) and pGY-1-*mCherry* (Fig. 3.4B). The localization patterns of the constructs are also presented as an overlay (Fig. 3.4C). Both fluorescent marker proteins (GFP and mCherry) showed the same cytoplasmic localization pattern. In Fig. 3.5A-C, CLSM images of an *Arabidopsis* leaf epidermal cell transformed with pGY-1-*K16-GFP* (Fig. 3.5A) and pGY-1-*mCherry* (Fig. 3.5B) are shown. The localization patterns of the fusion proteins are also presented as an overlay (Fig. 3.5C). Both proteins, K16-GFP and mCherry, exhibited the same localization pattern and were expressed in the cytoplasm. On the right side of Fig. 3.5D-F, CLSM images of an onion epidermal cell are displayed which was co-transformed with pGY-1-*K16-GFP* (Fig 3.5D) and pGY-1-*mCherry* (Fig 3.5E). Finally, in Fig. 3.5F, an overlay of the two images is presented. The localization pattern in the onion epidermal cell was identical to that observed in *Arabidopsis* leaf epidermal cells for both proteins. In summary, GFP alone and K16-GFP showed cytoplasmic localization in onion and *Arabidopsis* leaf epidermal cells. The K16-GFP signal could not be assigned to cell organelles, cell membranes or the apoplast.

The transient expression of K12-GFP, K43-GFP and PiALH43-GFP fusion proteins did not lead to GFP fluorescence in transformed cells. The particle bombardment for these three constructs was performed with *Arabidopsis* Col-0 leaf epidermal cells and onion epidermal cells. The constructs were always co-expressed with pGY-1-*mCherry*. Only the expression of the cytoplasmic control plasmid pGY-1-*mCherry* was visible nine to 48 hours after biolistic transformation. In order to enhance fusion protein stability, the proteasome inhibitor MG-132 was applied at a concentration of 100 µM.

MG-132 inhibits the protein degradation machinery in eukaryotic cells (Rock *et al.*, 1994). About 16 hours after particle bombardment, the onion epidermis was peeled of and put on microscope slides with MG-132. The *Arabidopsis* leaves used for particle bombardment were infiltrated with 100 µM MG-132 with a needleless syringe at 16 hours after transformation. Then onion epidermis or *Arabidopsis* leaves were incubated for four or five hours after MG-132 treatment before they were analyzed by CLSM.

The addition of MG-132 did not result in detectable fluorescence of K12-GFP, K43-GFP and PiALH43-GFP fusion proteins in onion or *Arabidopsis* leaf epidermal cells. So it could be assumed that the stability of the candidate GFP fusion proteins is not affected by the plant's proteasome machinery.

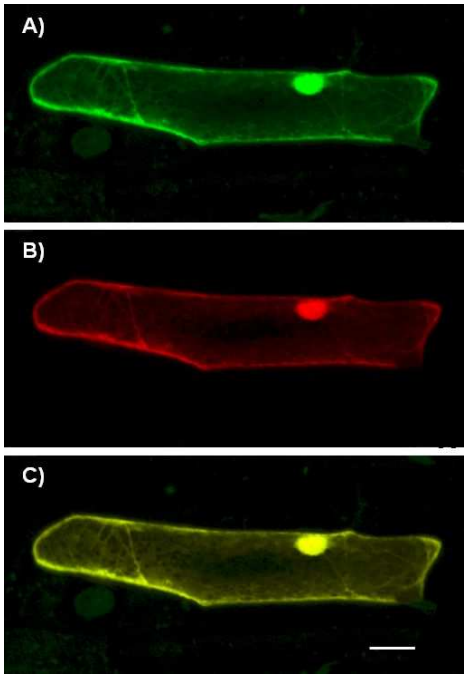


Fig. 3.4 Confocal laser scanning microscopy image of onion epidermal cells overexpressing pGY-1-*GFP* and pGY-1-*mCherry* (**A-C**) The overlay (merge) of the *GFP* and the *mCherry* expression is displayed on the bottom of the figure. Scale bar = 10 µm.

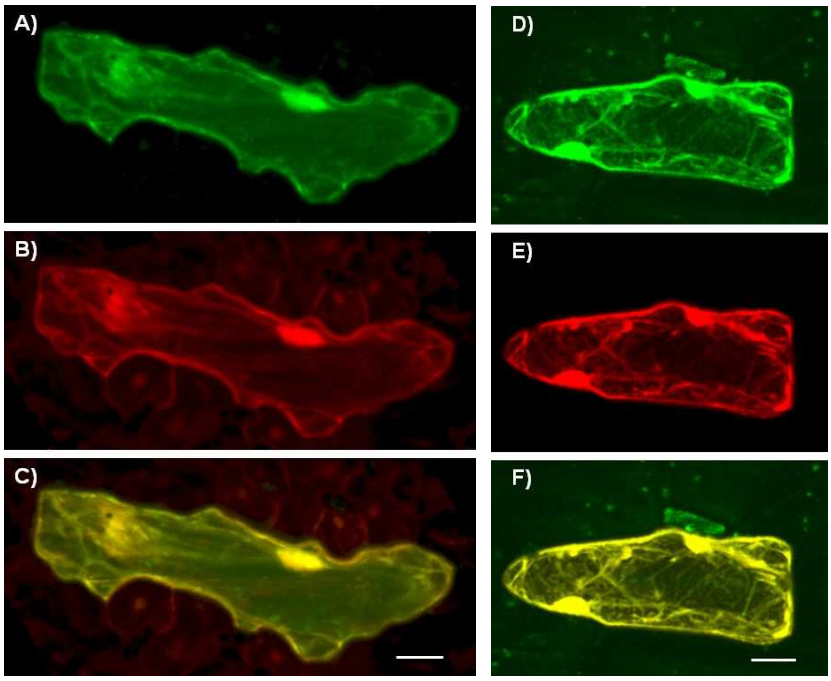


Fig. 3.5 Confocal laser scanning microscopy image of *Arabidopsis* Col-0 leaf epidermal cells (**A-C**) and onion epidermal cells (**D-F**) overexpressing pGY-1-*GFP-K16*. Cell were co-transformed with pGY-1-*mCherry* (red fluorescence). The overlay (merge) of the *GFP* and the *mCherry* expression is displayed on the bottom (**C, F**). Scale bar = 10 µm

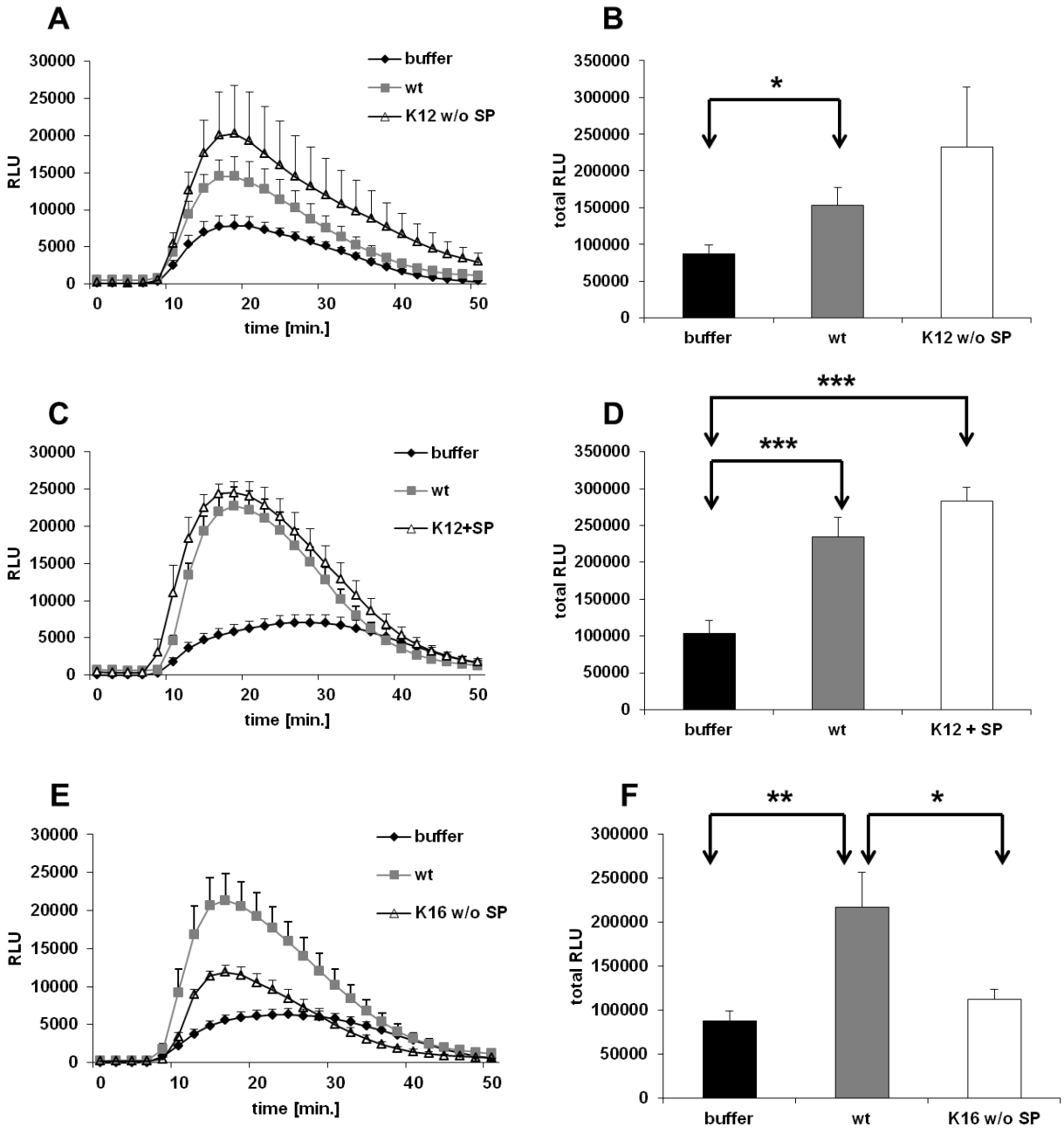
3.4.2. Suppression of basal defense by *P. indica* secreted proteins

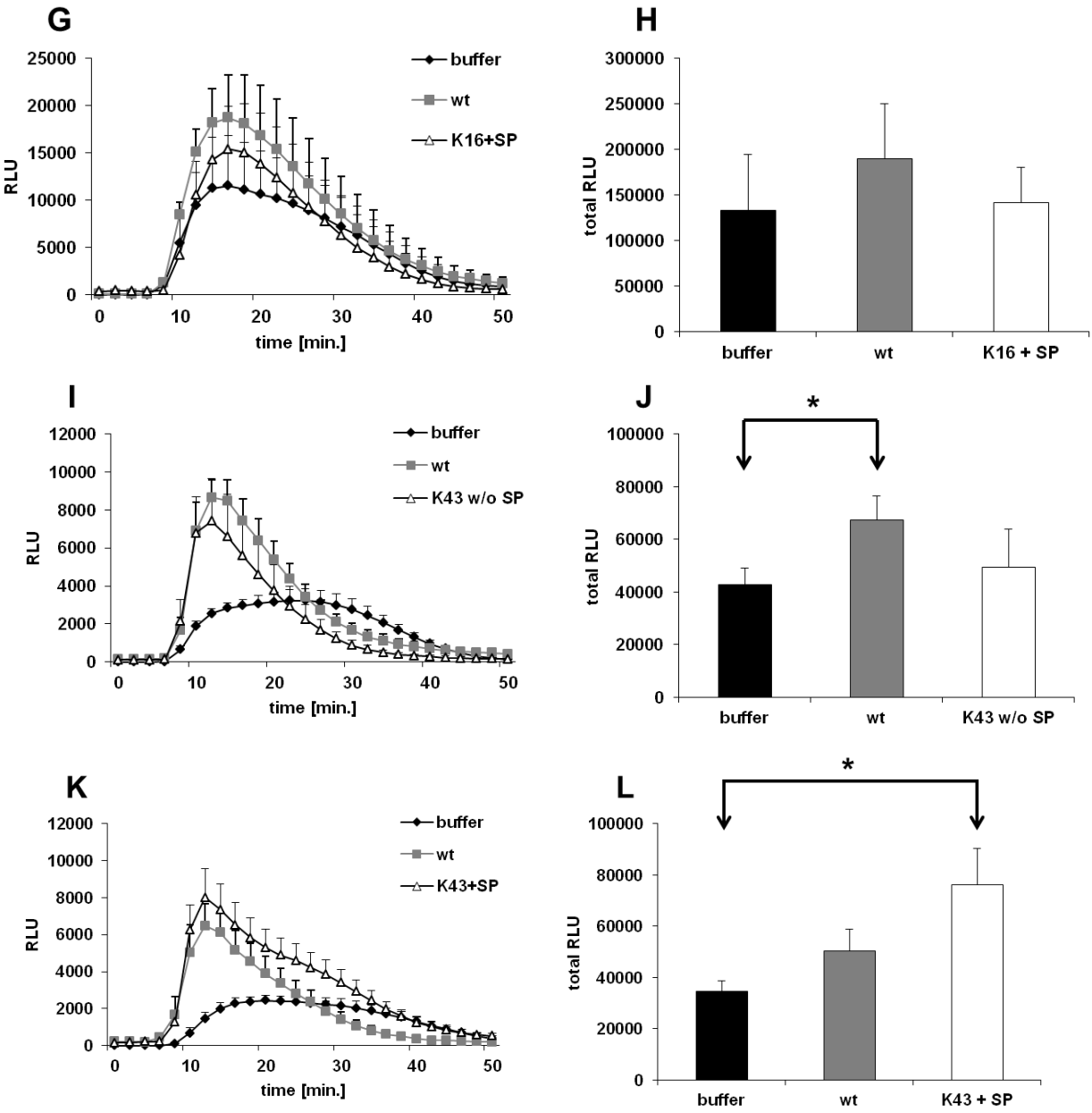
MAMP triggered immunity (MTI) summarizes a strong immune response against microbes that is triggered by different MAMPs (e.g. flagellin, chitin) (Jones & Dangl, 2006). One of the best studied MAMPs is the bacterial flagellin (Felix *et al.*, 1999). Its active epitope flg22 is perceived by the plant receptor-like kinase FLS2 (Gómez-Gómez & Boller, 2000). As one of the fastest reactions to microbial attack, plants react with the rapid production of reactive oxygen species (ROS). This oxidative burst reaction can induce programmed cell death (PCD) (Apel & Hirt, 2004). Successful microbes are able to overcome MTI by the secretion of effector proteins. Because *P: indica* is able to colonize a broad range of host plants (Verma *et al.*, 1999) it is thought to posses a sophisticated spectrum of manipulative tools like the secretion of effector proteins, which influence the metabolism of the host plant. Thus the aim of this study was to elucidate if the identified *P. indica* secreted proteins play a role in the suppression of MTI.

The four *P. indica* genes *PIIN_03806* (*K12*), *PIIN_08513* (*K16*), *PIIN_04526* (*K43*) and *PIIN_00029* (Pi*ALH43*) were amplified from *P. indica* cDNA and cloned in the plant expression vector pCXSN (Chen *et al*., 2009) with and without their endogenous signal peptides.

These constructs were used to transform *A. tumefaciens;* the transgenic Agrobacteria were pressure-infiltrated in *N. benthamiana* leaves. Additionally to the transgenic Agrobacteria,

untransformed (“wild type”) Agrobacteria and infiltration buffer were pressure infiltrated as controls in the same tobacco leaves. 24 hours after infiltration, leaf discs were cut from the infiltrated areas and incubated in water over night to reduce wounding effects that might affect subsequent oxidative burst measurements. The oxidative burst elicited by the addition of flg22 was monitored by a luminol-based assay.

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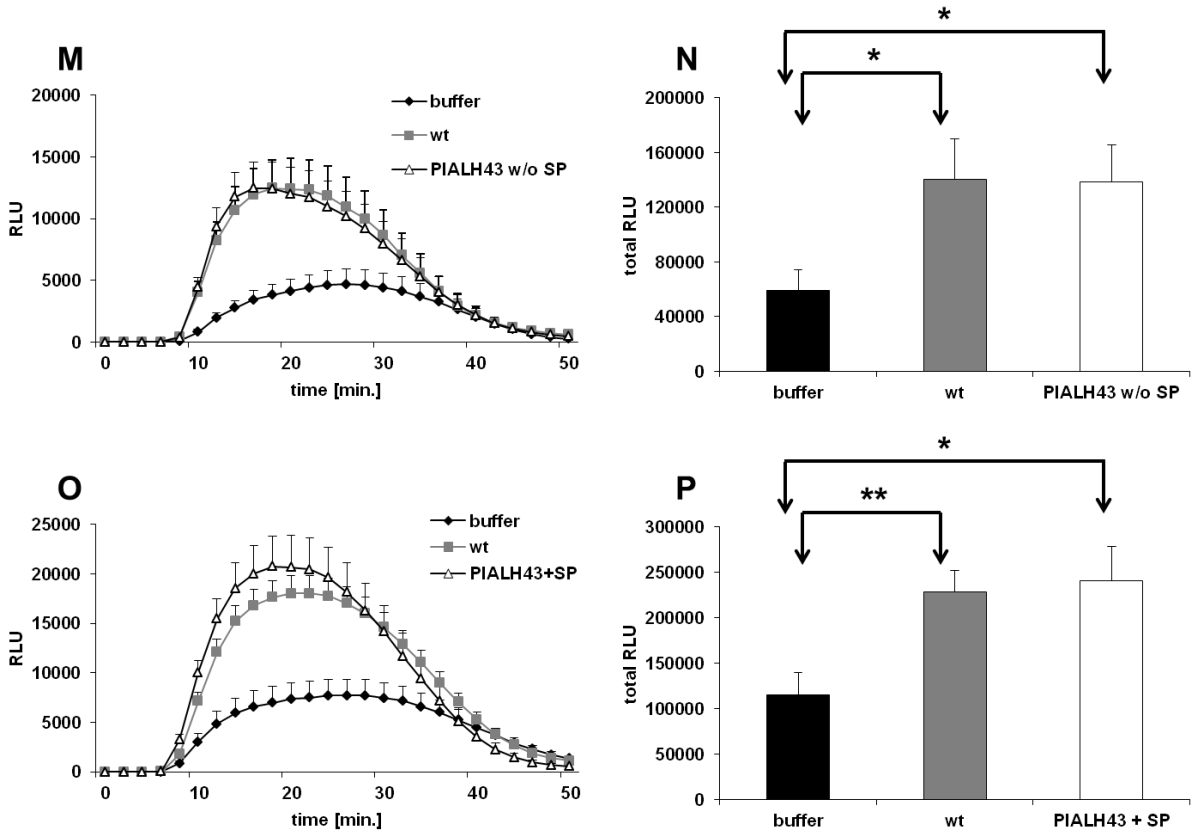

Fig. 3.6 Impact of candidates on flg22-triggered oxidative burst. **A-P** Oxidative burst in *N. benthamiana* leaf discs infiltrated with buffer, untransformed Agrobacteria (wt) or Agrobacteria transformed with the respective construct. The candidate genes *K12* (*PIIN_03806*), *K16* (*PIIN_08513*), *K43* (*PIIN_04526*) and *PiALH43* (*PIIN_00029*) were cloned in the pCXSN vector with or without the endogenous signal peptide (+ SP or w/o SP). At time point zero, flg22 was added to the leaf discs to a final concentration of 1 µM. Each diagram displays one representative measurement of three independent biological repetitions. The error bars represent standard errors of eight independent samples. The total amount of RLU was calculated and analyzed with Student's t-test (* = p<0,05; ** = p<0,01; ***= p<0,001). The error bars represent the standard errors of eight independent samples.

The ROS levels of leaf discs infiltrated with agrobacteria harboring pCXSN-*K12* without signal peptide (w/o SP) (Fig. 3.6A+B) were similar to the ROS levels of those infiltrated with wild type bacteria after challenge with flg22. But the reaction of leaf discs infiltrated with infiltration buffer was lower and also delayed compared to the reactions of those with Agrobacteria. The results for leaves infiltrated with pCXSN-*K12* with signal peptide (+SP) (Fig. 3.6C, D) were similar. The two approaches including Agrobacteria (wild type or transgenic) showed almost identical and significantly higher ROS levels compared to the treatment with buffer. The reaction of leaf discs infiltrated with buffer was delayed and the maximum was only about 20% of the maximum of the other treatments.

So the expression of the constructs seemed not to influence the level of ROS production upon flg22 trigger. There was no clear difference between transformed and untransformed Agrobacteria.

The magnitude of ROS generated by leaf discs infiltrated with wild type bacteria was higher than the level in leaf discs infiltrated with pCXSN-*K16* w/o SP (Fig. 3.6E). The peak of leaf discs with transgenic bacteria was only half of that monitored after the application of wild type bacteria. The lowest level was again measured in leaf discs infiltrated with buffer.

Its maximum was about 25% of the maximum found in leaf discs treated with wild type Agrobacteria (Fig. 3.6E). Fig. 3.6F illustrates that the total amount of ROS produced was significantly reduced in leaf discs infiltrated with buffer ($p<0,01$) as well as in leaf discs infiltrated with transgenic bacteria (with pCXSN-*K16* w/o SP, $p<0,05$) compared to leaf discs treated with wild type bacteria. Similar results were obtained for *K16* (= *PIIN_08513*) with signal peptide (Fig. 3.6G+H). The smallest ROS level was detected in leaf discs infiltrated with buffer and the most intensive reaction took place in leaf discs treated with wild type agrobacteria upon challenge with bacterial flg22. An intermediate ROS production was observed in leaf discs infiltrated with transgenic bacteria. But the differences between pCXSN-*K16* + SP and the two controls were not so pronounced as compared to the treatment with pCXSN-*K16* w/o SP. The level of reactive oxygen species in leaf discs infiltrated with Agrobacteria harboring pCXSN-*K43* w/o SP (Fig. 3.6I+J) was only slightly reduced in comparison to the ROS levels in leaves infiltrated with wild type bacteria after challenge with flg22. Again the reaction of leaf discs infiltrated with infiltration buffer was lower and delayed compared to the reactions of the other two treatments with Agrobacteria. The total amount of ROS production in leaf discs treated with wild type bacteria was significantly higher ($p<0,05$) as compared to leaf discs treated with buffer (Fig. 3.6J).

Opposite results were obtained for leaves infiltrated with pCXSN-*K43*+SP (Fig 3.6K). Here the leaf discs treated with wild type bacteria showed only minor differences in ROS production as compared to those challenged with the transgenic bacteria harboring the construct. In line with all other measurements, the reaction of leaf discs infiltrated with buffer was delayed and the maximum was only about 25 -30 % of the maximum of the other treatments. The total amount of ROS produced in leaf discs infiltrated with transgenic agrobacteria was significantly higher as compared to the control with buffer (Fig 3.6L). Taken together, the expression of the construct appeared to have only a slight influence on the ROS level upon flg22 trigger. There was no convincing difference between transformed and untransformed Agrobacteria. The level of reactive oxygen species of leaf discs infiltrated with Agrobacteria harboring pCXSN-Pi*ALH43* (= *PIIN_00029*) w/o SP (Fig 3.6M) was nearly identical to the ROS levels of those leaves infiltrated with wild type bacteria after challenge with flg22.

Again the total amount of ROS in leaf discs infiltrated with infiltration buffer was significantly lower ($p < 0,05$) in comparison to the other two treatments (Fig 3.6N). Similar results were obtained for leaves infiltrated with pCXSN-Pi*ALH43*+SP (Fig 3.6O). Those leaf discs treated with wild type bacteria showed only minor differences in ROS production as compared to those challenged with the transgenic bacteria carrying the construct. The reaction of leaf discs infiltrated with buffer was delayed and the maximum was only about 30% of the maximum of the other treatments.

The total amount of ROS produced was significantly lower in the control treatment with buffer as compared to the other treatments with bacteria (Fig 6P). The construct appeared to have no clear influence on the ROS level upon flg22 trigger. No significant differences between the ROS levels of leaf discs with transformed and untransformed Agrobacteria were quantified. The ROS production in the control leaf discs infiltrated with buffer was always lower and delayed in comparison to the approaches with Agrobacteria after challenge with flg22. The transient expression of the candidate genes *PIIN_00308* (*K12*), *PIIN_04526* (*K43*) and *PIIN_00029* (Pi*ALH43*) in tobacco leaves by agroinfiltration did furthermore not influence the ROS production in these infiltrated leaves after treatment with flg22 when compared to leaves infiltrated with non-transgenic Agrobacteria. For the constructs harboring *PIIN_08513* (*K16*), especially the construct without SP, a reduction in the ROS production was detectable in comparison to untransformed Agrobacteria. Thus, *PIIN_08513* seems to influence the basal defense in tobacco leaves.

3.4.3. Morphology of infiltrated *N. benthamiana* leaves

It was investigated whether the transient expression of these secreted *P. indica* proteins caused any morphological changes in the infiltrated leaf areas. Therefore in every experiment one additional leaf with all three treatments was infiltrated and analysed for morphological alteration at least up to three or four days after infiltration. All constructs did not evoke morphological changes such as necrotic lesions in the infiltrated areas. Such morphological changes are often seen in host plants during colonization by pathogenic hemibiotrophic fungi and bacteria (Kelley *et al.*, 2010).

3.4.4. Callose deposition in infiltrated leaf discs

The leaf discs used for oxidative burst measurements were also analyzed for the deposition of callose upon flg22 challenge. Therefore, the luminol mixture was removed after the luminescence measurement and substituted by an aqueous solution with 1 µM flg22.

The leaf discs were incubated in this solution for additional 24 hours and subsequently decolorized and stained with aniline blue to detect callose depositions. The fluorescence microscopic investigation did not reveal any differences between the leaf discs infiltrated with bacteria harboring the construct of interest as compared to the corresponding control treatments. There were also no significant differences between the eight constructs tested. Callose was mainly seen at the cutting edge of the leaf discs and at positions where dead cells were located (e. g. because of the damage caused by the infiltration process).

3.5. Functional analysis of Pi*ALH43* (*PIIN_00029*)

3.5.1. Yeast cell viability assay with *PIIN_00029* (*PiALH43*) constructs

The candidate gene *PIIN_00029* (Pi*ALH43*) was previously identified and characterized (Khatabi, 2009) as a *P: indica* secreted protein with E3 ligase activity and homologies to the bacterial effector protein AvrPtoB. AvrPtoB is secreted in plant cells by phytopathogenic bacteria, suppresses programmed cell death and inhibits plant immunity (Abramovitch *et al.*, 2006).

Therefore, *PIIN_00029* was assumed to play a role in the interference with host cell death processes. This hypothesis was investigated by an approach based on a *S. cerevisiae* viability assay. The *PIIN_00029* full length cDNA sequence including signal peptide was cloned in different yeast expression vectors and co-transformed with vectors harboring the mammalian agonist of apoptosis *Bax* or its antagonist *Bcl-X_L*. Bax and Bcl-X_L are functional in metazoans and in yeast (Ligr *et al.*, 1998). The *Bax* gene was set under the control of a galactose-inducible promoter. Thus, yeast cells transformed with the *Bax* plasmid could grow on media without galactose. In Fig 3.7 the photos with the drop assay are shown. The experiment was performed twice with similar results. On the left side of Fig. 3.7, the control plate with glucose-containing selection medium is shown. On the right side the plate with galactose-containing medium is presented. The yeast cultures transformed with the different plasmid combinations were dropped on selection medium with glucose or galactose as carbon source in dilutions starting with 400 cells/µl. The plasmid combinations one to eight were control set-ups to check *Bax* or *Bcl-X_L* constructs as well as empty vectors for their influence on yeast growth. Additionally the control combinations should assure the functionality of the *Bax* and *Bcl-X_L* plasmids. Plasmid combinations nine and ten were used to analyze the anti-apoptotic activity of the PiALH43 (PIIN_00029) protein. Here Pi*ALH43* was co-expressed with *Bax*. In order to analyze the anti-apoptotic activity of the candidate gene, it was cloned in the pGADT7 vector where it was set under a constitutive ADH promoter.

Finally the combinations eleven and twelve studied the pro-apoptotic activity of the candidate gene; Pi*ALH43* was co-expressed with *Bcl-X_L*. Here the candidate gene was cloned in the p416-Gal1 plasmid where it was set under the control of a galactose inducible promoter.

On glucose medium all co-transformed yeast cells were able to grow, but yeast cells transformed with the *Bax* vector (combinations 2, 7 and 9) displayed a more poor growth than yeast with the other plasmid combinations tested except in combination with Bcl-X_L (combination 4, Fig. 3.7, left side). Yeast cells transformed with the *Bax* plasmid were not able to grow on galactose medium (combinations 2, 7 and 9), except when co-transformed with the *Bax* antagonist *Bcl-X_L* (combination 4, Fig. 3.7, right side).

The co-transformation with the Pi*ALH43* construct did not rescue the *Bax* phenotype (combination 9). The yeast cells expressing Pi*ALH43* and the empty vector pRS316 (combination 10) were able to grow on glucose and on galactose. The analysis performed to examine the pro-apoptotic activity of the candidate revealed that yeast cells transformed with the Pi*ALH43* construct were able to grow on galactose and glucose containing medium in a similar manner (combinations 11 and 12). Here the expression of Pi*ALH43* cloned in pGADT7 did not depend on the induction by the presence of galactose. It was constitutively expressed. These findings illustrate that the PiALH43 protein did not elicit cell death processes in yeast cells. Although the expression of Pi*ALH43* in combination with the strong pro-apoptotic Bax protein did not rescue the Bax phenotype, a weak anti-apoptotic activity of PiALH43 cannot be excluded.

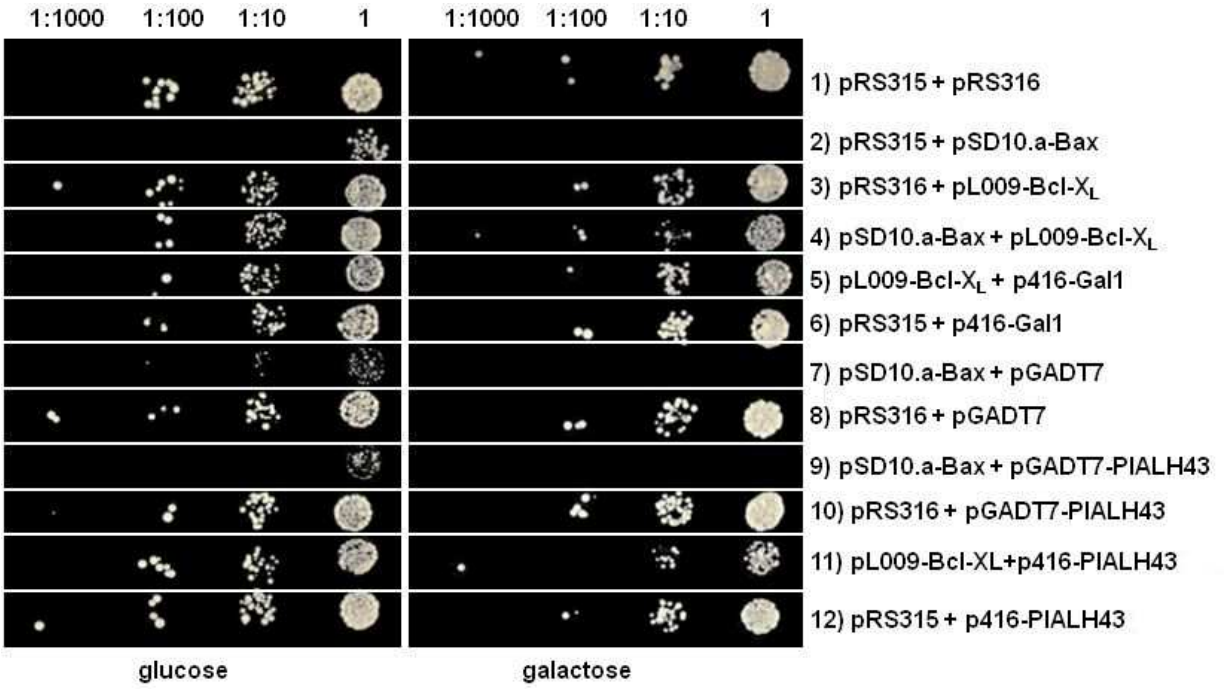

Fig. 3.7 Cell viability assay with *PIIN-00029* (Pi*ALH43*). The image was taken five days after yeast cells were dropped on the media. Yeast cells were either grown on SD medium with glucose (left) or with galactose (right). The plasmid combinations are listed on the right hand. The upper eight plasmid combinations display control combinations. The combinations 9 and 10 were applied to analyze the anti-apoptotic activity of the candidate. The 11th and 12th combination were performed to analyze the pro-apoptotic activity of the candidate.

3.5.2. Identification of homozygous Pi*ALH43* (*PIIN_00029*) *Arabidopsis* plants

3.5.2.1. Analysis of the T2 generation of Pi*ALH43 Arabidopsis* plants

The full length cDNA sequence of Pi*ALH43* (*PIIN_00029*), without and with signal peptide (#23 and #24, respectively), was cloned in the binary vector pINDEX3 in order to give the candidate under control of a dexamethasone inducible promoter. These constructs were used for the transformation of *Arabidopsis* Col-0 plants. Different lines of the T2 generation of these transgenic plants were analyzed for their expression of the Pi*ALH43* insert after dexamethasone treatment. Therefore, plants were grown on MS-medium including hygromycin as selection marker (without hygromycin for non-transgenic Col-0). Two-week-old plants were sprayed twice within 24 hours with 100 µM dexamethasone; untreated plants were used as control. The whole plants were harvested and used for RNA isolation. The results of the one-step RT-PCR are presented in Fig 3.8. All transgenic lines tested expressed the Pi*ALH43* gene. Unexpectedly, the expression of the gene could also be detected in the transgenic plants, which were not treated with dexamethasone. Almost all lines exhibited a higher expression level of Pi*ALH43* after treatment with dexamethasone.

Only in lines #23L3 and #24L10 a strong constitutive expression was detected. As expected, no Pi*ALH43* expression could be detected in *Arabidopsis* Col-0 control plants. For further experiments, the lines #23L1, #23L9, #24L1, and #24L4 were chosen. In addition, the lines #23L3 and #24L10 were selected because they had a quite high constitutive expression level of Pi*ALH43*. As an additional control, #23L10 was included as azygous line that was not expressing the transgene.

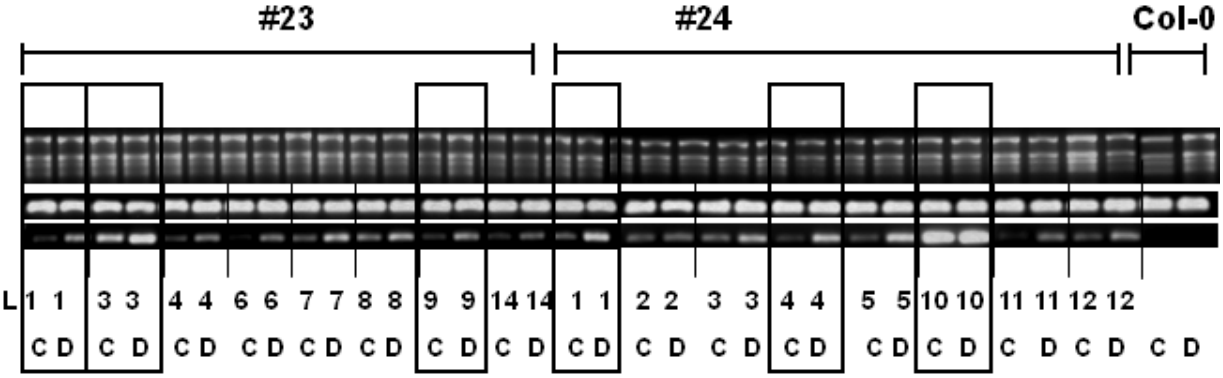

Fig. 3.8 Screening of *Arabidopsis* plants overexpressing *PiALH43* (*PIIN 00029*) constructs. From the #23 construct (Pi*ALH43* w/o SP), the lines 1, 2, 4, 6, 7, 8, 9 and 12 were analyzed. From the #24 construct (Pi*ALH43* with SP), the lines 1, 2, 3, 4, 5, 10 and 12 were investigated. In addition, parental line Col-0 N1092 served as control. The constructs were set under the control of a dexamethasone-inducible promoter. The transgenic plants were treated twice with 100 µM dexamethasone (D); non-treated plants served as control (C). The upper row displays the RNA extracted from total plants. The middle row shows the amplicons obtained by onestep PCR using primers specific for the housekeeping gene At*Ubiquitin5*.
The row at the bottom illustrates amplicons obtained by onestep PCR using Pi*ALH43* specific primers. The highlighted lines were chosen for further experiments.

3.5.2.2. Analysis of the T3 generation of *Arabidopsis* Pi*ALH43* (*PIIN_00029*) plants

The selected lines of the T2 generation (except #23L10) were pre-grown on MS-medium including hygromycin and ticarcillin to distinguish the transgenic from wild type plants. After one week, plants were transferred to jars with MS-medium without antibiotics. Thereafter, the plants were transferred to soil and grown in the greenhouse for the production of seeds. Line #23T10 seeds were directly sowed in soil and transferred to the greenhouse. The plants did not show morphological differences in comparison to wild type plants. Finally the seeds from each plant were harvested separately. Plants growing from these T3 seeds were analyzed with PCR for the presence of the Pi*ALH43* (*PIIN_00029*) gene to identify homozygous plants. Therefore, seeds from several plants were grown in soil and emerging leaves were harvested for the isolation of genomic DNA that was used for semi-quantitative PCR analysis.

Homozygous plants were identified from the following lines and used for colonization assays with *P. indica*: #23T3L3, #23T3L9, #24T3L1, #24T3L10. As a control, plants from line #23L10 were selected, which did not carry the Pi*ALH43* gene (Tab. 3.5). Thus, homozygous plants from both constructs were successfully identified; their seeds could be used for further functional analysis.

Tab. 3.5 *Arabidopsis* T3 seeds selected for colonization assays. The construct #23 contains the Pi*ALH43* sequence without signal peptide; the construct #24 possesses a signal peptide.

construct	generation	line	plant number (T2)
#23	T3	3	5
#23	T3	9	2
#23	T3	10	6 (non-transgenic)
#24	T3	1	5
#24	T3	10	9

3.5.3. Colonization of *PiALH43* (*PIIN_00029*) *Arabidopsis* plants by *P. indica*

The chosen *Arabidopsis* plants expressing the Pi*ALH43* gene were subjected to colonization studies with *P. indica*. In a pre-experiment, *P. indica* was grown on CM medium including 100 µM dexamethasone to check whether the glucocorticoid hormone would disturb the growth pattern of the fungus. The mycelium was analyzed microscopically and no obvious morphological changes were seen.

Transgenic T3 seeds of the Pi*ALH43* lines #23L3, #23L9, #24L1 and #24L10 were forwarded to colonization studies with *P. indica*. Lines #23L10 (azygous) and Col-0 served as controls.

Roots of three-week-old plants were treated once with 100 µM dexamethasone or mock-treated with tween water. One day after these treatments, roots were inoculated with *P. indica*, harvested at three and seven days after inoculation and forwarded to DNA extraction. The DNA was used to quantify the amount of fungal DNA in the roots by qRT-PCR using primers specific for At*Ubiquitin*5 and Pi*ITS*. At 3 dai, the relative amount of fungal DNA in transgenic plants, which were pretreated with dexamethasone (Dex), were similar to the amount found in wild type (Col-0) plants and the non-transgenic control line #23L10 (Fig. 3.9A). In the mock treated plants, the relative amount of fungal DNA was slightly elevated in #23L10 (azygous) and #23L3, a line with quite high level of constitutively expressed Pi*ALH43* without endogenous signal peptide (Fig. 3.9A). In lines #23L9 and #24L1, no difference between dexamethasone treated and control plants was detected. However, in line #24L10, which displayed a high constitutive expression of Pi*ALH43* + SP, the relative amount of fungal DNA was significantly increased in the mock-treated plants (Fig. 3.8/ 3.9A).

At 7 dai with *P. indica*, the relative amount of fungal DNA was significantly increased in the line #23L3 (Pi*ALH43* w/o SP) upon treatment with dexamethasone. The amount of fungal biomass was almost 3,5 times higher in comparison to the wild type plants (Fig. 3.9B). Also in mock treated #23L3 plants an insignificant elevation of fungal DNA was detected. In all other lines, the amount of fungal DNA was similar to wild type plants.

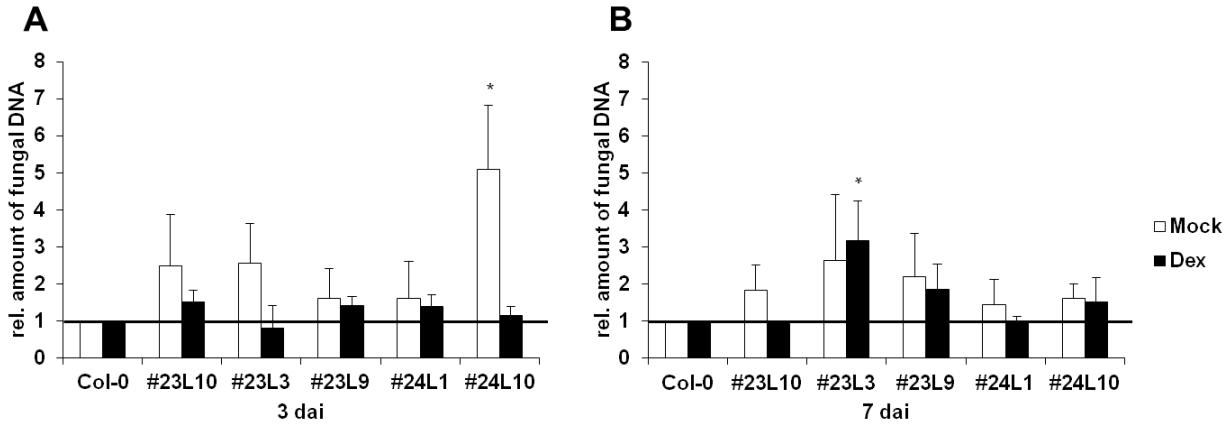

Fig. 3.9 *P. indica* colonization of *Arabidopsis* lines expressing Pi*ALH43* (*PIIN_00029*). **A-B** Transgenic *Arabidopsis* plants expressing Pi*ALH43* (*PIIN_00029*) with signal peptide (#24) or without signal peptide (#23) were analyzed for *P. indica* colonization by quantitative RT-PCR. The wild type Col-0 and the non-transgenic line #23L10 served as control. Three–week-old plants were treated once with dexamethasone (Dex) or tween water (Mock) and were inoculated with *P. indica* one day later. Roots were harvested at three (left side) and seven (right side) dai. The relative amount of fungal DNA in the transgenic lines was related to the amount of DNA in wild type plants (Col-0). The data were analyzed using a Student's t-test (*= p< 0,05). Displayed are the results of three independent biological repetitions with standard deviations. dai = days after inoculation

Taken together, the treatment with dexamethasone and thus the elevation of Pi*ALH43* transcript in the transgenic plants seemed to reduce the fungal growth in comparison to mock treated plants at 3 dai. However, in line #24L10, which constitutively overexpressed Pi*ALH43* + SP at high levels, colonization was significantly increased in mock-treated plants (Fig. 3.9A). Thus, at the early time point, higher expression of Pi*ALH43* seems to have a negative influence on the colonization, at least in line #24L10 carrying full-length *PiALH43*. At 7 dai, only the line #23L3 (Pi*ALH43* w/o SP) showed a significantly higher colonisation with *P. indica* upon treatment with dexamethasone. #23L3 was a line with elevated constitutive expression of Pi*ALH43* w/o SP (see Fig. 3.8/ Fig. 3.9B). At the late time point, a higher expression of Pi*ALH43* w/o SP seems to improve the colonization by *P. indica*, at least in #23L3.

4. DISCUSSION

The root colonizing basidiomycete *P. indica* is has beneficial impact on a broad multitude of host plants. It belongs to the order Sebacinales, which harbors various mycorrhizal and endophytic fungi. Like *P. indica*, many Sebacinales have similar advantageous influence on their host plants (Weiß *et al.*, 2011). The colonization of their roots by *P. indica* improves stress resistance of the host plant against pathogens as well as abiotic stress tolerance like drought or salt stress. Furthermore, growth promoting effects have been described for plants colonized by the fungus (Waller *et al*., 2005).

For successful colonization, *P. indica* actively suppresses the immune system of the plant and manipulates different hormone signaling pathways (Jacobs *et al*., 2011; Schäfer *et al*., 2009). Until now, no fungal compounds or effector proteins have been identified which could be involved in the crosstalk of the plant and *P. indica*. From other, mainly pathogenic plant-microbe associations several microbial secreted effector proteins have been identified. These effector proteins support the microbe in circumventing or suppressing defense reactions of the plant.

This work aimed at the identification and characterization of putative *P. indica* effector proteins functioning in the establishment of a compatible interaction with the host plant. Therefore the yeast signal sequence trap (YSST) was applied. Isolated *P. indica* genes were analyzed *in silico* as well as in functional studies to shed light on their putative relevance for the fungus. In a second project, the putative *P. indica* effector *PiALH43* (*PIIN_00029*), which was previously identified (Khatabi 2009), was investigated concerning its role in the *P. indica*- plant association.

4.1. The yeast signal sequence trap (YSST)

4.1.1. Secretion of proteins in eukaryotes

The synthesis of proteins in eukaryotes starts at the ribosomes in the cytosol of the cell. There, the mRNA is translated in a protein sequence. If the protein has to be delivered to a special cell compartment, its amino acid sequence contains a sorting signal. Because of this sorting signal the protein is subsequently delivered for example to the nucleus, the ER or the mitochondria. Proteins which are destined for the secretory pathway enter the ER where the folding and glycosylation of the proteins takes place (Alberts *et al.*, 2002). The sorting signal of secreted proteins is called signal peptide (SP). It is located at the N-terminus of the protein sequence and consists of 15-30 amino acids. The SP is cleaved off during the passage of the protein across the membrane. It is usually composed of three regions: The *n*-region at the amino-terminus, which often contains positively charged amino acids, the hydrophobic *h*-region, and the C-terminal *c*-region with polar uncharged residues.

The *c*-region comprises the signal peptidase cleavage site (Emanuelsson *et al.*, 2007; von Heijne, 1990). Not all proteins, which enter the secretory pathway, are thereafter transmitted to the cell membrane or the cell surface. Some of them remain in the ER due to their retention amino acid sequences like “HDEL” and “KDEL”. Additionally, some proteins are guided to other cell organelles (e.g. vacuoles). Secretory proteins are delivered from the ER to the Golgi apparatus and the *trans*-Golgi network (TGN) via vesicle trafficking. From the TGN the proteins are transported in vesicles to their ultimate destination (van Vliet *et al*., 2003).

For the identification of secreted proteins harboring a SP, the YSST was developed. The YSST makes use of a vector system with a truncated extracellular invertase gene, which is complemented by an inserted cDNA fragment carrying a SP. Transformed with this construct, an invertase lacking yeast mutant is able to grow on sucrose containing medium. This system can be applied for cDNA libraries of animal, plant or fungal material (Goo *et al*., 1999; Jacobs *et al.*, 1997; Klein *et al*., 1996; Lee *et al*., 2006).

4.1.2. Identification of putative *P. indica* effector proteins

In this work, the YSST was used to identify secreted proteins of the beneficial basidiomycete *P. indica* growing on *Arabidopsis* root cell wall medium. This cell wall approach was for example already successfully deployed to discover secreted proteins of the maize-pathogenic ascomycete *Colletotrichum graminicula* (Krijger *et al*., 2008). The proportion of identified secreted proteins from the fungus, which participate in the interaction of the fungus and the host plant is supposed to be enriched by providing plant cell walls and root or leaf extract instead of a living plant. In total, 14 different cDNA fragments which were found with the YSST could be clearly assigned to 14 different ORFs in the *P. indica* genome and transcriptome database. Thus the proportion of *P. indica*-derived genes was 100%. If one had chosen an approach using *P. indica* grown on living plant roots, the vast majority of isolated genes would be of plant origin. Therefore the chosen technique is very useful to enrich fungal secreted proteins.

All yeast clones transformed with the cDNA fragments of these 14 ORFs were able to grow on sucrose medium with antimycin A, but not all of them grew on raffinose medium and antimycin A. Antimycin A blocks mitochondria and thus inhibits respiration. The application of raffinose medium and antimycin A forms a stringent environment where only yeast clones with a functional extracellular invertase are able to generate their energy by fermentation (Schmidt *et al*., 1999). Due to unknown reasons, several of the yeast clones which were not growing on raffinose antimycin A medium were convincingly predicted to carry a SP (Tab. 3.1).

The YSST is a useful tool to identify promising secreted proteins which could have a role as effectors in the interaction of the fungus and the host plant.

4.2. *In silico* studies of YSST candidates - features and functions

4.2.1. Non-secreted candidates

From the 14 identified *P. indica* ORFs, only a minor part of three ORFs (PIIN_04018, PIIN_05415 and PIIN_10506) emerged to carry no SP. PIIN_04018 was predicted to have two transmembrane domains (TMs), and the other two proteins did not have a TM (Tab.3.1). Nonetheless, these proteins could still be secreted by an alternative secretion pathway. In the obligate biotrophic powdery mildew fungus *B. graminis* f. sp. *hordei,* for example, several avirulence proteins have been identified which are active inside the host plant cell but lack an N-terminal SP for the secretory pathway. Thus there seems to be an alternative route for the delivery of avirulence proteins into host cells (Ridout *et al*., 2006).

4.2.1.1. PIIN_04018 (K29)

PIIN_04018 was 16 times sequenced during the YSST screen. Furthermore, it has neither any annotated protein domains nor homologies to known proteins of microbes including *P. indica* (Tab. 3.1-3.3). The presence of *PIIN_04018* in the sequenced YSST clones is probably due to the second ATG in its nucleotide sequence, which is localized in the second of the two predicted transmembrane domains. Thus this transmembrane sequence is functioning as signal peptide. In the YSST screen, only truncated versions of *PIIN_04018* have been found where the second ATG was seen as the first start codon in the sequence. *S. cerevisiae* clones harboring such construct with an inserted truncated cDNA fragment of *PIIN_04018* were able to grow on the selection medium. To complement extracellular invertase, a minimal amount of protein sequence conservation is enough to allow protein secretion in *S. cerevisiae* (Kaiser *et al.*, 1987). The further analysis of the truncated version of *PIIN_04018*, using the second ATG in the sequence as start codon, confirmed the assumption. This truncated protein was clearly predicted to be secreted by SignalP. In addition it is known that transmembrane domains can sometimes mimic SPs if they are located at the beginning or close to the N-terminus of a protein. Transmembrane domains, like SPs, consist of hydrophobic amino acid sequences resulting in inaccurate predictions by SignalP (Krogh *et al.*, 2001). The putative function of this protein for *P. indica* itself or for the interaction of the fungus with the host plant remains unclear because PIIN_04018 did not show any similarities to known proteins in the databases.

4.2.1.2. PIIN_05415 (K36, NAD specific glutamate dehydrogenase)

PIIN_05415 was consistently denominated as NAD-specific glutamate dehydrogenase in all analyzed databases (Tab. 3.1/3.2). The *P. indica* genome did not contain any similar proteins (Tab. 3.3).

Despite a putative alternative secretion pathway for PIIN_05415, the isolation of non-secreted candidates by YSST could arise from frameshifts in the nucleotide sequence. This allows the formation of false SPs. In addition, truncated versions of a protein might result in alternative start codons in the sequence or the 3'-untranslated regions (Jacobs *et al.*, 1997). In ectomycorrhizal fungi, the glutamate dehydrogenase is involved in the assimilation of ammonia and the synthesis of amino acids (Martin *et al.*, 1987).

4.2.1.3. PIIN_10506 (K48)

The best annotated hit for PIIN_10506 revealed a LipA and NB-ARC domain protein of *Aspergillus fumigatus* (Tab. 3.2). Furthermore, there were at least 30 ORFs with similarities to PIIN_10506 in the *P. indica* genome (Tab. 3.3). The group of proteins with NB-ARC domain was expanded in the *P. indica* genome compared to other fungal genomes (Zuccaro A., Kogel K.-H., unpublished). LipA and NB-ARC domains can be found in putative serine esterases. The NB-ARC domain is a NTP binding domain while the LipA domain contains the serine esterase function. Such LipA-NB-ARC fusion proteins are assumed to have a switch function in signaling cascades, for example in developmental processes, bistable morphological modifications and stress responses in fungi (Fedorova *et al*., 2005; Graziani *et al.*, 2004). Whether PIIN_10506 has such functions in *P. indica* is currently unknown.

4.2.2. Secreted candidates without transmembrane domain

4.2.2.1. PIIN_03211 (K1)

For the candidate protein PIIN_03211 (K1) two sequence versions were identified. The first version, listed in the *P. indica* database, was predicted to have no SP. The second version emerged from the sequencing of the YSST constructs; it was predicted to carry a SP by SignalP. The finding of two possible protein sequences for PIIN_03211 probably arose from incorrect ORF prediction. The longer version of PIIN_03211 from the *P. indica* database was predicted to have a transmembrane domain. The YSST version of PIIN_03211 did not own a transmembrane domain. Protein domains were not identified, but the similarity search within the NCBI protein database found similarities to the Pep2 protein of *Fusarium oxysporum* (Tab. 3.1/3.2).

The Pep1 and Pep2 proteins (Pep = putative effector proteins) of *F.* oxysporum f. sp *vasinfectum* are expressed only in pathogenic isolates of this species and harbor a SP and several cysteines (Chakrabarti *et al*., 2011).

Also both versions of PIIN_03211 contain eight cysteine residues. Cysteine-rich proteins have been described as effector proteins in different host-microbe interactions.

In the tomato pathogen *Cladosporium fulvum*, for example, cysteine-rich small secreted proteins play an important role as apoplastic effectors. The disulphide bonds formed by the cysteine residues in these effectors are assumed to stabilize the proteins in the protease-rich environment of the plant apoplast (Thomma *et al*., 2005). These results support the assumption that the version of PIIN_03211 with SP could have a similar role in the *P. indica*- plant association and the protein could be secreted into the host apoplast.

4.2.2.2. PIIN_07104 (K3), endopeptidase

The protein PIIN_07104 (K3) was annotated as endopeptidase K, more generally as serine protease and on the protein domain level as a protein with a peptidase S8 (S = serine) and an inhibitor I9 domain (Tab. 3.1/3.2). Serine proteases are functioning in many metabolic and physiologic processes in animals, humans, plants and fungi, for example digestion, development and fertilization in humans or as regulators of other proteolytic enzymes in fungi (Avanzo *et al.*, 2009). Serine proteases are classified in two superfamilies: The trypsins and chymotrypsins are found in fungi, actinomycete bacteria and animals; the subtilase (or subtilisin-like) superfamily is found in archaea, bacteria, fungi and higher eukaryotes. Of the six families of subtilases, three were also discovered in the fungal kingdom: the proteinase K, the subtilisin and the kexin family (Hu & St. Leger, 2004; Siezen & Leunissen, 1997). The proteinase K type proteases are composed of a subtilisin N-terminal propeptide domain and a catalytic peptidase S8 domain (Bryant *et al.*, 2009). The subtilisin family S8 harbors peptidases with an asparagine, a histidine and a serine as catalytic-site residues (Rawlings & Barret, 1993). The putative endopeptidase PIIN_07104 was also found to include an inhibitor I9 domain. Such inhibitor domains are seen as part of the propeptide. They can function as intramolecular chaperones in protein folding or as inhibitors of the peptidase activity (Bryant *et al*., 2009). It might be possible that the inhibitor is blocking other protease target proteins and not its own peptidase activity, perhaps even targets in the host plant. There is only little information about the role of subtilisin-like proteases in the interaction of fungi with their host plants. In the plant pathogen *Magnaporthe poae*, for example, a subtilisin-like protease was described which was highly expressed in infected roots (Sreedhar *et al.*, 1999).

A secreted subtilisin-like protease was also identified in the plant pathogen *C. graminicola*, which showed a biphasic expression pattern *in planta* at early (0-16h) and late (48-120h) interaction time points (Krijger *et al.*, 2008).

Two secreted plant subtilases *of Lotus japonicus* revealed to be induced during the symbiosis development with arbuscular mycorrhiza and apparently play a role in the formation of arbuscules (Takeda *et al*., 2009).

In the *P. indica* genome, there were ten proteases listed with similarities to PIIN_07104 (Tab. 3.3). PIIN_07104 and its homologs could be involved in metabolic processes, which are important for the interaction with a host plant.

4.2.2.3. PIIN_03806 (K12), collagen domain protein

PIIN_03806 (K12) contains a collagen domain (Tab. 3.1); furthermore, the best annotated hit in the NCBI database was a so-called YSIRK family protein from a Gram-positive bacterium (Tab. 3.2). The YSIRK cell surface proteins of Gram-positive bacteria carry the sequence YSIRK in their SP, it is important for the correct secretion of the protein (Bae & Schneewind, 2003). The YSIRK sequence motif is not present in PIIN_03806, thus it seems unlikely that PIIN_03806 has similar functions. The analysis of paralogs within the *P. indica* genome revealed one very similar protein on the same scaffold (Tab. 3.3). The high similarity could be seen as a hint for a recent duplication event (Li *et al*., 2003).

Collagen molecules are structural parts of the extracellular matrix especially in animals and humans. They are normally formed of three polypeptide chains, two identical α1 chains and one α2 chain, connected in a triple helix and coiled coil conformation (van der Rest & Garrone, 1991). Collagen or collagen-like proteins have occasionally been described in fungi. Celerin *et al*. (1996) reported that fimbriae of the basidiomycete *Microbotryum violaceum* consisted of fungal collagen. Fimbriae are cell surface appendages found in all phyla of fungi. They have functions in mating and pathogenic adhesion. Another collagen-like protein was identified in the insect pathogenic fungus *Metarhizium anisopliae.* At the beginning of the *M. anisopliae* infection, fungal conidia can be eliminated by the insect's immune system. In an advanced state, the fungus spreads in hyphal bodies, which are short hyphae with yeast-like blastospores. These hyphal bodies are covered with the collagen-like protein MCL1, which seems to function like a protective mask. MCL1 has an N-terminal SP and a C-terminal GPI (glycosyl-phosphatidylinositol) anchor site and hides antigenic structures like β-glucans from the insect's immune response. Furthermore, proteins with collagenous domains are rather rarely distributed among fungi and not linked to a special lifestyle (Wang & St. Leger, 2006). Recently, the analysis of the cell wall proteome of *P. infestans* isolated a collagen protein without SP from a cell wall fraction of *P. infestans* appressoria.

The importance of this collagen protein for the structure and function of appressoria has to be investigated further (Grenville-Briggs *et al*., 2010). These examples of fungal collagens or collagen-like proteins reveal several putative functions for the YSST candidate PIIN_03806.

Until now, it has not been described whether *P. indica* is having fimbriae on its cell surface, but PIIN_03806 could also be associated with the cell surface, either as part of fungal fimbriae or as a kind of “mask” to hide MAMPs like fungal chitin from the immune system of the host plant.
As mentioned above, fungal fimbriae are also important for the adhesion of fungi to their hosts or for mating processes. Thus the candidate protein could have a similar function for *P. indica* although only asexual reproduction of *P. indica* has been described until now (Verma *et al.*, 1998). The growth environment of *P. indica* chosen for the YSST approach was very poor in nutrient and the given plant cell wall fragments mimicked plant roots. So it seems rather convincing that the fungus is trying to attach its growing hyphae to the assumed plant roots with special surface associated effector proteins.

4.2.2.4. PIIN_06847 (K35)

The next candidate, PIIN_06847, was identified as a small secreted protein with a quite high proportion of histidine residues. Protein domains or transmembrane domains could not be discovered (Tab. 3.1). The best annotated hit revealed a PFG377 protein of the protozoon *Plasmodium falciparum* (Tab. 3.2). *P. falciparum* is a protozoon which is responsible for the malaria disease in humans and transmitted by sanguivorous mosquitos. The PFG377 protein of *P. falciparum* is associated with the sexual reproduction process of the parasite. It is expressed in special organelles of female gametocytes. The sexual reproduction process is important for the transmission of the parasite from the red blood cells to the mosquito alternate host (de Koning-Ward *et al*., 2008). Another interesting connection of *P. falciparum* and plant pathogens was the discovery that translocation signals of secreted *P. falciparum* proteins are functional in the oomycete potato pathogen *P. infestans*. This implies that there are conserved protein recognition sequences even in unrelated species (Grouffaud *et al*, 2008). Another amazing discovery was the huge number of paralogous proteins in the *P. indica* genome (Tab. 3.3/ Fig. 3.2). All of these paralogs were small secreted proteins with a high number of histidine residues. Histidine-rich proteins are for example found in human saliva; these histidine-rich proteins are called histatins. Histatins are small, cationic and secreted proteins with antimicrobial capacities against fungi and bacteria (De Smet & Contreras, 2005). The production of antimicrobial compounds by *P. indica* has not been described so far but might help *P. indica* to control competitors.
Furthermore, many of the PIIN_06847 paralogs consistently carried a special amino acid sequence at their C-terminus, “RSIDELD”. It is tempting to speculate whether it is a special sorting signal, like the RXLR motif in secreted effector proteins of oomycete fungi.

Contrary to the “RSIDELD” sequence, the RXLR motif is located near the N-terminal SP of such effector proteins (Rehmany *et al.*, 2005) and not at the C-terminus. Investigations of the possible function of PIIN_06847 and its paralogs for *P. indica* are hindered by their high level of similarity.

4.2.2.5. PIIN_02169 (K40), deacetylase LysM protein

Five protein domains were found in this secreted effector candidate, one deacetylase domain and four LysM (= Lysine Motif) domains (Tab. 3.1). Hence, the best hit in the NCBI database revealed a carbohydrate esterase family 4 protein of the basidiomycete *Schizophyllum commune* (Tab. 3.2). Furthermore, 25 paralogs of this protein were found in the *P. indica* database (Tab. 3.3). LysM domains can be found in prokaryotes and eukaryotes; they usually consist of 44–65 amino acids and bind different derivatives of peptidoglycans or chitin (Buist *et al.*, 2008). The group of the carbohydrate esterase family 4 proteins contains chitin deacetylases, rhizobial NodB chitooligosaccharide deacetylases, peptidoglycan-N-acetylglucosamine deacetylases, acetyl xylan esterases and xylanases. Chitin deacetylases hydrolyze N-linked acetyl-groups of N-acetylglucosamine residues of their respective substrates, such as chitin (Caufrier *et al.,* 2003). In plants, LysM domains can be found in LRR receptor-like kinases, for example in the chitin receptor CERK1 of *Arabidopsis*. CERK1 is functioning in the perception of the microbe-derived MAMP chitin and thus in plant innate immunity (Miya *et al*., 2007; see introduction, Par. 1.4.1, for further description).

The ascomycete *Cladosporium fulvum* is the causal agent of leaf mold in tomato. The fungus secretes the effector protein Ecp6 during the infection of its host plant. Ecp6 contains three LysM domains which specifically bind to chitin oligosaccharides. The effector *Ecp6* is expressed *in planta* and is important for the virulence of the fungus. A plant immune response upon treatment with the MAMP chitin, for example the oxidative burst reaction and medium alkalization in cell cultures, is actively suppressed by Ecp6. Another effector protein of *C. fulvum*, Avr4, also carries a chitin binding domain and is responsible for the protection of the mycelium from plant chitinases (Bolton *et al*., 2008; de Jonge *et al*., 2010).

The plant pathogenic fungus *Colletotrichum lindemuthianum* secretes a chitin deacetylase to alter its hyphal chitin during the infection of the host plant. The enzyme changes chitin to chitosan during the extracellular infection process. This can prevent the lysis of the hyphae by secreted plant derived chitinases (Blair *et al.*, 2006).

Chitosan is less active in plant defense induction than chitin. The treatment of tomato cell culture with chitosan, for example, did not result in an alkalization of the medium as it was observed upon treatment with chitin (de Jonge *et al.,* 2010). A protein which contains a combination of a deacetylase and several LysM domains like PIIN_02169 has not been characterized so far.

Perhaps the four LysM domains bind the MAMP chitin, which is then modified by the deacetylase enzyme to the less immuno-active chitosan.
This could be seen as a strategy to evade the activation of plant immune responses during root colonization. The huge amount of homologous proteins in the *P. indica* genome could be interpreted as an evidence for the importance of this protein group for the fungus. It has to be determined whether they are involved in the interaction of the fungus and its hosts or in other metabolic processes.

4.2.2.6. PIIN_09796 (K46), protein disulphide isomerase (PDI)

The next candidate, PIIN_09796, had a SP and two thioredoxin domains (Tab. 3.1); the alignment to the NCBI database revealed a protein disulphide isomerase of *L. bicolor* as best hit (Tab. 3.2). Finally, there were seven paralogs with similarities to PIIN_09796 listed in the *P. indica* database (Tab. 3.3).
In many bacterial species, for example in the phytopathogen *Erwinia carotorova*, a thiol disulphide oxidoreductase, DsbA, is responsible for the introduction of disulphide bonds in secreted proteins; among them important virulence factors. Thus, an inactivation of *DsbA* has a huge impact on the virulence of the bacterium (Coulthurst *et al*., 2008).
Protein disulphide isomerases (PDIs) in mammals and yeasts are part of the thioredoxin superfamily. Their classical location is the lumen of the ER. Thus they are carrying the amino acid ER retention signals “HDEL” or “KDEL” at their C-terminus. PDIs are responsible for the formation, isomerization or reduction of disulphide bridges in the ER. In addition, chaperone activity has been described for PDIs. Thioredoxins catalyze redox reactions and PDIs in mammals contain four thioredoxin domains (Ferrari & Söling, 1999). In contrast to these findings, PIIN_09796 did not have a “HDEL” or “KDEL” ER retention signal but it contained a SP for the secretory pathway. Instead of four, it had only two predicted thioredoxin domains. Other non-classical locations for PDIs have been discussed in the literature. PDIs have been reported to be secreted to the extracellular space and have also been found on the cell surface in different mammalian cell types. There they could have similar functions as in the ER lumen, namely the reduction of disulphide bonds of interaction partners or isomerase activity.
Thiol groups of cell surface or extracellular proteins can furthermore work in cell adhesion processes, for example in platelets of animals (Turano *et al*., 2002). Aichinger *et al.* (2003) found a PDI (*pig2*, *pig* = plant induced gene) in *U. maydis*, which was induced upon contact with the host plant. This protein had, like PIIN_09796, two thioredoxin domains and there was no ER anchor or transmembrane domain indicative of ER retention detectable. It was rather predicted to have an extracellular localization.

The PDI of *U. maydis* showed the highest expression in the late biotrophic developmental interaction stage. It was assumed that the up-regulation of the PDI at this stage could be associated with a stress response of the fungus due to nutrient limitation and plant defense responses. The poor growth conditions chosen for the YSST screen with *P. indica* could also evoke such stress might induce the secretion of PIIN_09796.

4.2.3. Secreted candidates without any known function

4.2.3.1. PIIN_08513 (K16)

The YSST candidate protein PIIN_08513 (K16) was predicted to carry a SP or a signal anchor by SignalP (Tab. 3.1). A signal anchor, in contrast to a SP, is not cleaved of and the protein is retained in the membrane in an N-in/C-out orientation. Such a protein is named type II membrane protein (Emanuelsson *et al*., 2007; von Heijne, 1988). The TMHMM analysis revealed an N-terminal transmembrane domain, but the TMHMM output additionally indicated that this N-terminal transmembrane domain could also be the SP (Tab. 3.1). The hydrophobic domains of SPs can sometimes be misinterpreted as transmembrane domains (up to 20% of the investigated sequences; Krogh *et al*., 2001). The search for protein domains and the BLAST similarity search did not reveal further information on the protein function. The best alignment with the BLAST search was seen for a yd repeat protein of the bacterium *Riemerella anatipestifer* (Tab. 3.2). The yd repeat proteins are found in several bacteria and phage species. They have been described as putative toxins. Furthermore, orthologs of the yd repeat protein family have been described as membrane-bound adhesins (Degnan & Moran, 2008). The term adhesins is used for surface structures in bacteria, which help to establish an association with surfaces, for example host cells (Danhorn & Fuqua, 2007). In the *P. indica* DB, three proteins with similarities to PIIN_08513 were found, two of which were in direct proximity to the candidate on the same scaffold (Tab. 3.3). This constitution could be interpreted as a recently occurred duplication event. Taken together, the *in silico* studies with PIIN_08513 did not retrieve much information about its structure but an association with the cell surface could be assumed.

4.2.3.2. PIIN_10643 (K19)

The *P. indica* candidate protein PIIN_10643 harbors no protein or transmembrane domains. PIIN_10643 is a quite small (about 200 aa) secreted protein and has no similarities to any annotated protein listed in a database (Tab. 3.1., Tab. 3.2). In the *P. indica* genome, one paralogous protein was assigned (Tab. 3.3). Thus the function of this protein for the fungus remains unclear.

4.2.3.3. PIIN_02519 (K37)

PIIN_02519 was identified as a secreted protein with one transmembrane domain but no confidentially predicted protein domains (Tab. 3.1). The homology search in different databases did not return much information. The best annotated hit in the NCBI database was an IS30 transposase of *E. coli* with a questionable E-value (Tab. 3.2). Other homologous proteins could not be found in the *P. indica* genome (Tab. 3.3). Because of the transmembrane domain in the protein sequence, it can be assumed that the protein is located at the cell surface. Perhaps it is involved in the attachment of the fungal mycelium to the surface of the host plant. Further investigations are necessary to collect more information on the function of this secreted *P. indica* protein.

4.2.3.4. PIIN_00308 (K38)

The *in silico* analysis with the YSST candidate PIIN_00308 revealed a secreted protein according to SignalP. An additional evaluation with the TargetP database retrieved an ambiguous result. The protein could harbor a SP for the secretory pathway or a mitochondrial signal sequence for the guidance into the mitochondria. The alignment with the NCBI database retrieved no annotated proteins. In addition, protein domains could not be identified (Tab. 3.1/3.2). Finally, there were six proteins with similarities to PIIN_00308 present in the *P. indica* genome (Tab. 3.3). Therefore, the features and putative functions of PIIN_00308 for *P. indica* remain elusive.

4.2.4. Secreted candidate proteins with transmembrane domain

4.2.4.1. PIIN_04526 (K43), mannoprotein

The putatively secreted *P. indica* protein PIIN_04526 harbors a transmembrane domain at the C-terminus; thus it is likely a cell surface protein. The alignment with the NCBI database identified a mannoprotein of the basidiomycete *C. cinerea* as best hit (Tab. 3.1/3.2).

Eight other proteins were found in the *P. indica* database, among which certain members showed high similarity to PIIN_04526 (Tab. 3.3). One of the proteins with the highest similarity was PIIN_04527, a hypothetical protein that is located next to PIIN_04526. Their high similarity could be seen as a hint for a recently occurred duplication event (Li *et al*., 2003).

Cell wall mannoproteins of the ectomycorrhizal basidiomycete *Pisolithus tinctorius* are glycoproteins with mannose residues. They are the most prominent part of the cell wall of free-living *P. tinctorius* mycelium. In the association with plant roots, the biosynthesis of the mannoprotein gp95 is reduced, a characteristic step during the establishment of the symbiosis (Martin *et al*., 1999).

Mannoproteins are prominent activators of an immune response in mammals, for example during the perception of the human pathogenic basidiomycete *Cryptococcus neoformans*. Their characteristic mannosylations are recognized by special mannose receptors which are involved in the transmission of the immune response. The mannoproteins of *C. neoformans* contain an N-terminal SP, a serine threonine-rich region and a hydrophobic region at the C-terminus for the addition of a GPI (= glycosylphosphatidylinositol) anchor motif, which serves for the attachment to the cell membrane or to β-1,6 glucans in the cell wall (Levitz & Specht, 2006). In the facultative human pathogenic ascomycete *Candida albicans*, mannoproteins are also known to be an important target for the human immune system. The fungus can switch between yeast-like and filamentous hyphal growth. The gene *Camp65p* codes for a putative β-glucanase mannoprotein. It can be seen as a so called adhesin. Mutants deficient in this protein show severe growth defects during the hyphal growth phase and are impaired in their adherence to surfaces, for example host cells. Furthermore, *Camp65p* is an important virulence factor in *C. albicans*; knock-outs of this gene are less pathogenic than wild type *C. albicans* (Sandini *et al.*, 2007).

Because the *P. indica* mannoprotein PIIN_04526 seems to have a similar structure, it may be possible, that a GPI anchor attaches this protein to the cell membrane or the cell wall. It can be speculated whether PIIN_04526 has an important function as adhesin during the colonization of plant roots; this should be investigated further.

4.2.4.2. PIIN_00029 (PiALH43), RING E3 ubiquitin ligase

This possibly secreted *P. indica* protein was identified in a former YSST screen. The material used for the construction of the YSST cDNA library, which retrieved PIIN_00029, originated from living barley roots colonized by *P. indica* (Khatabi, 2009). The protein was additionally annotated to have a RING 7 (RING = really interesting new gene) protein domain (Tab. 3.2).

Furthermore, PIIN_00029 was shown to have E3 ubiquitin ligase activity *in vitro.* The RING domain of PIIN_00029 had in addition homologies to the *P. syringae* effector AvrPtoB; the binding domains for the E2 interacting enzyme were conserved, which was shown by 3D modeling (Khatabi, 2009). Finally, there were eleven *P: indica* proteins with similarities mainly to the RING domain identified in the *P. indica* DB (Tab. 3.3).

E3 ligases are part of an enzyme complex where they are involved in the ubiquitination of proteins for many cellular processes. In a primary step, which needs ATP, the protein ubiquitin is activated by the E1 enzyme. After the transfer of the activated ubiquitin to the E2 conjugation enzyme, the E3 ubiquitin ligase binds the E2 ubiquitin complex and mediates the ligation of ubiquitin to a target protein.

Thus the target protein is marked for degradation by the proteasome or for other cellular purposes (Vierstra, 2009). Investigations concerning the role of E3 ubiquitin ligases in the crosstalk between plants and microbes have mainly focused on the plant side. Additionally, secreted effectors of bacterial plant pathogens, the most prominent is the *P. syringae* effector AvrPtoB, have been shown to possess E3 ubiquitin ligase activity. These effectors interfere with the host plants immune system (e. g. Abramovitch *et al*., 2006; see introduction, Par. 1.7.1, for further description).

Plant E3 ubiquitin ligases have important impact in plant immune response. Ubiquitination is involved in different hormonal signaling cascades, which are modulated upon infection of plants with (pathogenic) microorganisms (Trujillo & Shirazu, 2010). Some plant RING E3 ligases participate in the establishment of resistance against pathogens. For instance, overexpression of the RING ubiquitin ligase StRFP1 enhanced resistance in potato plants against the oomycete pathogen *P. infestans* (Ni *et al.*, 2010). Until now, it is not known whether E3 ligases of phytopathogenic, endophytic or mycorrhizal fungi have functions in the interaction with their host plants. If further studies of PIIN_00029 reveal an interaction with targets in the host plant, it would be one of the first fungal E3 ubiquitin ligases assigned to function in plant-microbe interactions.

4.2.5. YSST *in silico* studies - conclusions

The YSST screen, aiming at the isolation of putative secreted proteins of *P. indica*, resulted in the successful identification of several interesting secreted fungal proteins for further functional studies. Only a minor part of the identified proteins revealed to carry no SP. Such false positives have been reported to be isolated by YSST screens (Jacobs *et al.*, 1997). It is still possible, that these at the first glance non-secreted proteins are delivered out of the fungal cells by an alternative secretion pathway.

Four of the identified secreted proteins did not show similarities to any other annotated proteins. This makes it difficult to assign their putative function for the fungus. According to the features found in *in silico* studies, eight of the 14 identified proteins might function as effector proteins in the interaction with the host plant because they have similarities to known or putative effector proteins. Furthermore, six of the secreted proteins found in this YSST screen seem to be associated with the cell membrane, either by transmembrane domains or putative signal / GPI anchors. The cDNA library for the YSST screen was obtained from *P. indica* mycelium grown on minimal medium with plant cell walls mimicking a host plant. Thus it is plausible that the fungus is trying to attach its hyphae to the surface of the assumed plant roots and secretes the appropriate proteins for this purpose.

Two of the YSST candidates, the putative mannoprotein PIIN_04526 and the putative RING E3 ligase PIIN_00029, might function as virulence factors, because proteins with similar features have been described as virulence factors in other biological systems. The YSST candidates PIIN_06847 (small secreted histidine-rich protein) and its paralogs as well as PIIN_02169 (deacetylase with LysM motifs) and its paralogs have been also identified in the *P. indica* GBrowse genome and transcriptome database as putative *P. indica* effectors (A. Zuccaro, K.-H. Kogel, unpublished). Thus the experimental approach with the YSST and the *in silico P. indica* genome and transcriptome studies led to consistent results.

P. indica revealed to be able to actively suppress plant immunity and to achieve root colonization by influencing JA and gibberellin signaling (Jacobs *et al.*, 2011; Schäfer *et al*., 2009). Secreted proteins obviously linked to plant hormone signaling were not identified in the YSST screening. This might be either explained by the experimental arrangement, which might not be adequate to mimick host hormone responses, or by poor sequence similarities of fungal proteins to respective plant homologs. Therefore, the modulation of plant hormone signaling by putative effectors identified within this study has to be determined in further functional studies. On the basis of the YSST findings, *P. indica* might need special cell surface proteins for root surface adhesion and to evade the activation of the plant immune system in order to establish a compatible interaction. Thus one could hypothesize, that in addition to the active suppression of MTI as described by Jacobs *et al.* (2011), the fungus avoids exposure of MAMPs like chitin oligomers to the plant immune system by secreting proteins such as PIIN_02169 (deacetylase-LysM-protein) or PIIN_03806 (collagen domain protein). A graphical summary of the categories of the identified *P. indica* proteins is given in Fig. 4.1.

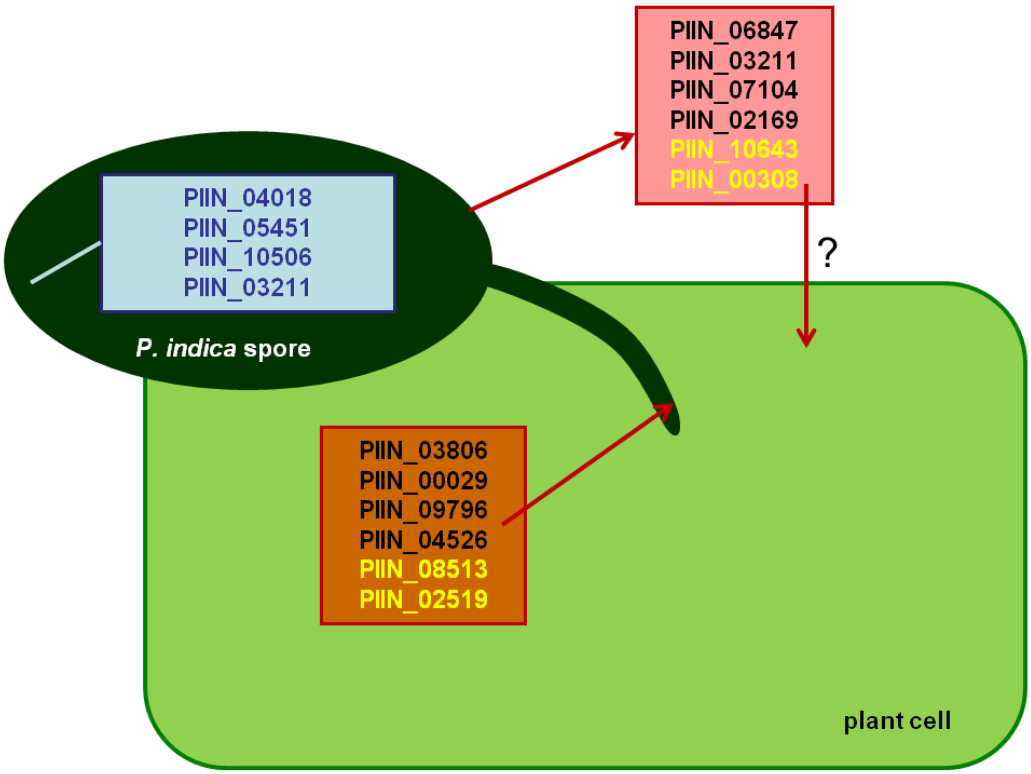

Fig. 4.1 Graphical summary of the *in silico* studies showing a plant cell and a germinating *P. indica* spore. Candidates harboring a SP are listed outside the *P. indica* spore. Those without a SP are put inside the spore.
Depending on the presence of a transmembrane domain or examples found in literature, the candidates with SP were arranged outside the *P. indica* spore (rose box) or on the cell surface / in the cell membrane of the penetrating hypha (orange box). The candidates PIIN_03806 (collagen domain protein), PIIN_09796 (PDI), PIIN_04526 (mannoprotein), PIIN_00029 (RING E3 ligase), PIIN_03211 (YSST version, Pep2), PIIN_071104 (endopeptidase), PIIN_02169 (deacetylase, LysM) and PIIN_06847 were declared as putatively effector associated proteins and thus their PIIN-numbers were written in black letters. The PIIN-numbers of candidates without known effector-association were written with yellow letters. The question mark is set to point to the putative delivery of the secreted *P. indica* candidates in the host cell The two possible versions of PIIN_03211 were put either to the secreted effector associated proteins (YSST version) or inside the fungal spore to the non-secreted candidates (*P. indica* GBrowse database).

4.3. Relative expression of secreted candidates under different growth conditions

The relative expression of putative *P. indica* effectors was analyzed with quantitative real-time PCR (qPCR). Therefore, the fungus was grown in axenic liquid culture (CM), with dead and with living *Arabidopsis* Col-0 roots (mimicking saprophytic and mutualistic fungal growth, respectively). The expression levels were measured in 2 and 7 day–old-mycelium. At 2 dai, the fungus colonizes living host cells. This biotrophic phase is followed by a cell death-associated colonization phase at 7 dai (Deshmukh *et al.*, 2006; Jacobs *et al*., 2011). Except for PIIN_02169 (putative deacetylase-LysM protein), nine of the investigated genes, showed highest expression level at 2 dai in living plant roots (Fig. 3.3A-J, Tab. 3.4). The *P. indica* cDNA library used for YSST was constructed with fungal material harvested at early time points after transfer to inducing conditions (one, two and three days). Therefore, it is not surprising that the isolated genes were mostly induced at early interaction time points. The relative expression of all genes increased at 7 dai in comparison to their corresponding values measured at 2 dai. This was especially true for the expression levels of those genes originating from fungal hyphae grown in CM and harvested at 7 dai. Seven out of ten analyzed candidates displayed a massive increase in their expression levels (Fig 3.3A-J). This might be explained by a suboptimal nutrient supply in CM after seven days of growth, which forces the fungus to secrete (putative) effectors in order to improve its living conditions by the colonization of a host plant. The expression levels of the three genes *PIIN_00308*, *PIIN_04526* (putative mannoprotein) and *PIIN_09796* (putative PDI) were highest in the samples with living roots at both time points (Fig 3.3F, H, I; Tab. 3.4). Especially the putative mannoprotein *PIIN_04526* was highly induced *in planta* (Fig 3.3H). This might be related to their described function in cell adhesion in other biological systems, for example in the attachment to the host cell surface (Sandini *et al.*, 2007; Lahav *et al.*, 2000). Thus it is convincing that genes, which are needed for adhesion, are upregulated during the colonization process (Fig 3.3F, H, I). The relative gene expression of all investigated genes was lower in dead *Arabidopsis* roots in comparison to living *Arabidopsis* at both time points. It is tempting to speculate that the fungus perceives other signals from living as compared to dead plant roots and accordingly adapts its transcriptome or secretome (Fig. 3.3A-J).

4.4. Subcellular localization of candidate GFP fusion proteins

The subcellular localization of the *P. indica* candidate proteins were investigated by the overexpression of candidate-GFP fusion proteins in *Arabidopsis* or onion epidermal cells. Therefore, the full length cDNA sequences of the candidate genes *PIIN_03806* (*K12*, collagen domain), *PIIN_08513* (*K16*), *PIIN_04526* (*K43*, mannoprotein) and *PIIN_00029* (Pi*ALH43*, RING E3 ligase) including their endogenous SPs were cloned in the expression vector pGY-1-GFP.

No fluorescence could be observed after transient expression of Pi*ALH43-GFP* (= *PIIN_00029-GFP*), *K43-GFP* (= *PIIN_04526-GFP*) and *K12-GFP* (= *PIIN_03806-GFP*). Even the addition of the proteasome inhibitor MG132 did not lead to a detectable GFP fluorescence. Thus the absent GFP fluorescence is presumably not due to rapid degradation of the fusion proteins by the proteasome machinery of the cell. Another reason might be very low and thus undetectable expression levels of the fusion proteins by CLSM. It is furthermore known that GFP fluorescence is reduced or impaired under unfavorable pH conditions, like the acidic environment of the apoplast (Schweizer *et al*., 1999; Zheng *et al*., 2004). Thus a shifting pH in the apoplast could impair the GFP fluorescence so that a fusion protein, which is secreted to the apoplast, cannot be detected. To exclude such problems, one might create fusion proteins with a pH stable visual marker. Alternatively, the heterologous expression of a fungal gene in a plant might not result in the synthesis of a functional protein. Therefore, GFP fusion proteins should be expressed in *P. indica*. The transformation of *P. indica* was recently shown by Zuccaro *et al.* (2009) and Yadav *et al.* (2010). However, the technique has not been adapted to perform protein localization studies.

GFP protein alone (Fig. 3.4A-C) showed a cytoplasmic localization pattern in onion epidermal cells. Also the K16-GFP fusion protein (K16 = PIIN_08513; Fig. 3.5A-F) revealed a cytoplasmic expression pattern. Thus the synthesized protein was not guided to a specific cellular compartment or membrane. PIIN_08513 (= K16) was predicted to carry a signal peptide or a signal anchor. Furthermore, it showed homologies to a group of bacterial adhesins, the yd repeat proteins (see Par. 4.2.3.1). The cytoplasmic localization of PIIN_08513 might be explained by the secretion of the protein and its subsequent re-uptake due to an inherent translocation signal. Future studies might use GFP fusions with truncated versions of PIIN_08513 to study their localization in more detail *in planta*. If a yet unidentified translocation signal permits the re-uptake of the protein, this should be abolished by the removal of this sequence by truncations.

The targeting of secreted effector proteins into the cells of host plants has been intensively studied in plant pathogens of the genus *Phytophthora.* Here, the RXLR and dEER motifs in effectors serve as translocation signals and are required for effector uptake by host cells.

In basidiomycetes such a translocation sequence has not been identified until now. In transgenic *P. infestans*, expressing a fusion protein of the effector protein Avr3a and mRFP (red fluorescent protein), the RXLR and dEER motif in Avr3a guided the fusion protein into the plant cell when the transgenic oomycete was grown on a host plant.
It was additionally discovered, that the RXLR and dEER translocation motif is not necessary for the secretion of the effector into the apoplast but required for the translocation of the effector via the plasma membrane into the host cell (Whisson *et al*., 2007). The RXLR and dEER translocation signal even guided the *Phytophthora sojae* effector Avr1b into the host cell in the absence of the fungus (Dou *et al*. 2008).
In the obligate biotrophic basidiomycete *Uromyces fabae*, which infects *Vicia faba*, a secreted protein (Uf-RTP1p, rust transferred protein 1p), was detected in the host-parasite interface defined as the extrahaustorial matrix at early interaction stages. At later infection stages, the protein was also detected in the cytoplasm and the nucleoplasm of the host cells. Furthermore, a nuclear localization signal could be identified. It has been speculated, that the protein is involved in the establishment and maintenance of the intimate biotrophic association of the fungus with the host plant (Kemen *et al*., 2005).

4.5. Suppression of MAMP triggered immunity by putative *P. indica* effectors

In plants, MAMPs like flg22 are recognized by pattern recognition receptors (PRRs), which activate signaling cascades resulting in various defense responses. Among them the ROS production is an immediate answer to pathogen perception (see also introduction, Par. 1.4.1.2). *P. indica* is able to suppress MAMP-induced immune responses in *Arabidopsis* roots, such as the root oxidative burst, the induction of defense genes, and callose deposition. For immune suppression and thus root colonization, *P. indica* relies on the manipulation of plant hormone signaling. In this respect, JA signaling plays an important role in the crosstalk between *P. indica* and the host plant (Jacobs *et al*., 2011; Schäfer *et al.*, 2009).
Although *P. indica*-colonized roots have an increased antioxidative capacity as indicated by an activated glutathione-ascorbate cycle (Waller *et al.*, 2005; Baltruschat *et al.,* 2008), it has not been demonstrated whether it is associated with immune suppression. However, ROS are cytotoxic compounds and thus the enhanced antioxidative capacity of the host plant might be associated with ROS detoxification.
It is assumed, that *P. indica* secretes effector proteins to suppress MTI in multiple ways. Many plant colonizing microbes are able to manipulate and suppress the defense responses of their hosts by the secretion of effector proteins.

For instance, the effector proteins AvrPto and AvrPtoB of *P. syringae* are secreted into host cells in order to suppress MTI. Moreover, they inhibit cell death in *N. benthamiana*, which occurs after treatment with bacterial flagellin or the oomycete MAMP INF1 elicitin. Their transient expression in *N. benthamiana* suppresses the oxidative burst reaction after flagellin perception.

Callose deposition after MAMP application is also reduced in *N: benthamiana* plants expressing AvrPto (Hann & Rathjen, 2007). Insects which are feeding on plants are also assumed to secrete effector proteins into the plant cells to manipulate host metabolism. With the help of transient gene expression in *N. benthamiana*, a putative effector of the aphid *Myzus persicae* was identified, which suppressed flg22-triggered oxidative burst (Bos *et al.*, 2010). Thus, the transient expression of putative effector proteins in *N. benthamiana* leaves can be used to analyze their impact on plant defense reactions like oxidative burst and callose deposition.

The effector candidates *PIIN_03806* (*K12*, collagen domain), *PIIN_08513* (*K16*), *PIIN_04526* (*K43*, mannoprotein) and *PIIN_00029* (Pi*ALH43*, RING E3 ligase) were transiently expressed in *N. benthamiana* leaves. The infiltrated leaf areas were used for oxidative burst measurements and callose deposition analysis after flg22 treatment. Therefore, the candidates (with and without SP) were overexpressed in *N. benthamiana* leaves by agroinfiltration. Generally, the oxidative burst in control leaf discs infiltrated with induction buffer was (often significantly) reduced in comparison to leaf discs infiltrated with wild type or transgenic Agrobacteria (Fig. 3.6A-P). Leaf discs infiltrated with Agrobacteria carrying one of the *PIIN_03806* (= *K12*) constructs showed no significant difference in RLU levels compared to leaf discs infiltrated with non-transgenic Agrobacteria, although the RLU levels were slightly increased in the transgenic approach (Fig. 3.6A-D). Thus, the overexpression of this *P. indica* gene seemed not to influence ROS production after flg22 treatment. Similar results were obtained with both *PIIN_00029* (= Pi*ALH43*) and *PIIN_04526* (= *K43*) constructs (Fig. 3.6I-P). There was no significant difference between leaf discs infiltrated with transgenic or non-transgenic Agrobacteria. *PIIN_03806*, *PIIN_04526* and *PIIN_00029* might have other roles during the establishment of a compatible interaction of the host plant and *P. indica*, such as cell adhesion, as implicated by the *in silico* data (Par. 4.2). By contrast, *PIIN_08513* (= *K16*) overexpression resulted in reduced ROS levels in transgenic leaf discs (Fig. 3.6E-H). PIIN_08513 did not carry a special protein domain and was predicted to carry a SP or a signal anchor (Par. 4.2.3.1). Because the reduction in ROS production was more intense in leaf discs expressing PIIN_08513 without SP, it could act inside the plant cell as suppressor of MAMP-triggered oxidative burst.

4.5.1. No induction of leaf cell death and callose deposition by PIINs

All infiltrated areas of the *N. benthamiana* leaves did not exhibit necrosis or PCD after infiltration. Thus, the overexpression of the candidates and also the respective controls seemed not to trigger cell death processes in plant cells.

Callose deposition at the plant cell wall is an immune response and commonly used to characterize the degree of defense response in different plant species. It is often analyzed by infiltration of MAMPs or bacteria eliciting callose deposition in plant leaves. Alternatively, *Arabidopsis* seedlings are often incubated in solutions containing MAMPs like flg22 to study immune responses such as callose depositions (Brown *et al.*, 1998; Luna *et al.*, 2011). It was recently shown that at 24 hours after infiltration with flg22, the callose deposition in *N. benthamiana* leaves reaches a maximum (Nguyen *et al.*, 2010). The analysis of callose depositions in the infiltrated *N. benthamiana* leaf discs 48 hours after infiltration and 24 hours after incubation in flg22 did not reveal differences between leaf discs expressing effector candidates or the control approaches.

4.6. Characterization of the putative RING E3 ligase *PIIN_00029* (Pi*ALH43*)

4.6.1. Involvement of *PIIN_00029* into host cell death processes

PIIN_00029 was described as a secreted RING E3 ligase. It was furthermore demonstrated to have E3 ligase activity *in vitro*. 3D modeling revealed conserved E2 binding residues in PIIN_00029 in comparison to other E3 ligases from humans, bacteria and plants; among them the *P. syringae* effector protein AvrPtoB (Khatabi, 2009). The secreted E3 ubiquitin ligase AvrPtoB is a suppressor of PCD and MTI in plants (Janjusevic *et al*., 2006). If one of the effectors AvrPto or AvrPtoB is recognized by the plant resistance (R) protein Pto in tomato, PCD and other defense responses are elicited, which effectively stop bacterial invasion (Pedley *et al.*, 2003). Thus a yeast viability assay was performed to investigate the potential role of PIIN_00029 in pro- or antiapoptotic activities involved in the interaction of *P: indica* with its host plant.

The model organism *S. cerevisiae* can be used to study apoptotic processes of plants, animals and humans because apoptosis occurs in *S. cerevisiae* with mechanisms similar to those in metazoans. This hints to an evolutionary conserved origin of these cell death processes (Madeo *et al.*, 2004). Also bacterial effectors can be analyzed with a yeast viability assay. Such effector proteins from the plant pathogens *P. syringae* and *Xanthomonas campestris* sp. *vesicatoria* have been shown to cause attenuation or death of yeast cells upon heterologous expression (Munkvold *et al*., 2008; Salomon *et al*., 2011).For the yeast viability studies with *PIIN_00029*, a system using the mammalian proapoptotic protein Bax and its antagonist Bcl-X_L was applied.

The single overexpression of *Bax* in *S. cerevisiae* results in various cellular destructions, e.g. asymmetries and blebbing of the plasma membrane, chromatin condensation and DNA fragmentation, due to its proapoptotic capacities. These phenotypes can be rescued by simultaneous overexpression of an antagonist of apoptosis, *Bcl-X_L*.

Because of the proapoptotic nature of the Bax protein, it was set under the control of a galactose-inducible promoter (Ligr *et al.*, 1998). The full length ORF of *PIIN_00029* (Pi*ALH43*) was co-expressed with the Bax protein or with the Bcl-X_L protein to study its anti- or proapoptotic capacities, respectively. Additionally, several control approaches analyzed the functionality of the system and putative site-effects arising from the empty vectors. All yeast cells transformed with one of the *PIIN_00029* combinations or control approaches were able to grow on glucose containing medium, although all yeast clones transformed with the Bax plasmid grew slower as compared to the other combinations (Fig 3.7, left side). Thus, the galactose-inducible promoter of the pSD10.a-*Bax* vector might be leaky resulting in reduced protein synthesis even in the absence of galactose. This phenomenon has been described for several other vectors with galactose-inducible promoters (e. g. Munkvold *et al*., 2008). Another reason for this phenomenon could be that the glucose or other components used for the SD medium contained traces of galactose. However, I obtained similar results with glucose from different distributors. Despite these unexpected findings, conclusions can be drawn from the study. The growth of yeast colonies harboring the *Bax* construct was totally abolished on the plate with drop out medium containing galactose. Yeast growth was rescued by co-transformation with *Bcl-X_L* (Fig 3.7, right side). The simultaneous expression of *PIIN_00029* did not alter this phenotype (Fig. 3.7, combination 9). Because the Bax protein is a quite strong inducer of apoptosis, it is still possible that PIIN_00029 has anti-apoptotic capacities which were not able to compensate the Bax effect. Furthermore, yeast cells expressing *PIIN_00029* were able to grow and did not show an apoptotic or inhibited phenotype. This is consistent with the findings gained with its transient expression in *N. benthamiana* leaves. The transient expression of *PIIN_00029* in tobacco leaves did not result in necrotic lesions or cell death (Par. 4.5.1). According to these findings, *PIIN_00029* has probably not a key role in the establishment of the cell death observed at later colonization phase of *P. indica*.

4.6.2. Transgenic *PIIN_00029* (Pi*ALH43*)-expressing *Arabidopsis* plants

The full length cDNA sequence of PIIN_00029 without (#23) or with (#24) endogenous SP was cloned in a binary vector with a dexamethasone-inducible promoter and used for the stable transformation of *Arabidopsis*. Dexamethasone is a synthetic glucocorticoid hormone, which is not present in plants. The dexamethasone-inducible system used in the *PIIN_00029* studies consists of a chimeric transcription factor.

This transcription factor comprises a DNA binding domain from the yeast transcription factor GAL4, a transactivation domain of the V16 protein originating from a herpes virus and a receptor domain from the rat glucocorticoid receptor GR. Thus the name chosen for this artificial gene is GVG (Aoyama & Chua, 1997).

The expression of *PIIN_00029* could be detected in the T2 generation of transgenic lines after treatment with dexamethasone using onestep PCR. The expression of *PIIN_00029* was also detectable in non-treated *PIIN_00029* plants although to a lesser extent (Fig. 3.8). Contrary to these findings, the GVG dexamethasone-inducible promoter system has been described to be tightly regulated (Ouwerkerk *et al*., 2001). The transgenic *PIIN_00029* plants had the same appearance like wild type plants and their growth was not impaired. These findings are again in line with the results from the transient expression studies in *N. benthamiana* plants and the viability assay in *S. cerevisiae*. The heterologous expression of *PIIN_00029* did not cause morphological changes or PCD reactions. Several dexamethasone-inducible homozygous transgenic lines and an azygous control line were used for further analysis.

4.6.3. Characterization of *PIIN_00029* (Pi*ALH43*) expressing *Arabidopsis* plants

4.6.3.1. Colonization by *P. indica*

Homozygous lines from the T3 generation of *PIIN_00029*-transformed *Arabidopsis* plants were treated with dexamethasone or mock-treated and thereafter inoculated with *P. indica*. Genomic DNA of this root material was harvested at 3 and 7 dai and forwarded to qPCR-based analysis. At 3 dai (biotrophic phase), the colonization in mock-treated roots was increased compared to the dexamethasone treated roots in line #24L10 (*PIIN_00029* with SP; Fig 3.9A). The overexpression of *PIIN_00029*, after dexamethasone treatment, apparently restricted fungal growth. By contrast, at 7 dai (cell-death associated phase), #23L3 plants (PIIN_00029 without SP) were significantly more colonized by *P. indica* after dexamethasone treatment in comparison to the control (Fig. 3.9B).

Hence, intracellular overexpression of *PIIN_00029* seems to improve *P. indica* colonization at later interaction stages. The E3 ligase activity might improve colonization by supproting the degradation of plant target proteins controlling root colonization.

4.7. Conclusion

The present work aimed at the isolation and functional characterization of secreted proteins of the beneficial basidiomycete *P.indica* which could be involved in the establishment of a compatible interaction with plant roots. The experimental YSST approach successfully retrieved several secreted *P. indica* proteins. The majority of these candidates could be linked to effector proteins as indicated by *in silico* studies and the comparison with published protein sequences. The identified candidates could be assigned, for example, to the group of surface associated adhesins. In addition, several candidates could be involved in the hiding of MAMPs like fungal chitin in order to avoid the activation of the plant immune system. Thus in addition to the active suppression of plant innate immunity, as published by Jacobs *et al.* (2011), the fungus seems to possess such additional facilities to prevent defense responses of the host plant. Furthermore, the candidate proteins identified by the YSST approach are in line with *in silico* studies of the *P. indica* genome and transcriptome (Zuccaro A., Kogel K.-H., unpublished). Both investigations resulted in the identification of putative effector proteins from the group of the LysM domain proteins and a special *P. indica* group of small secreted, histidine-rich proteins harboring the putative sorting sequence “RSIDELD”.

A direct participation of the four investigated candidates *PIIN_03806*, *PIIN_08513*, *PIIN_04526* and *PIIN_00029* in the suppression of MTI, perhaps except for *PIIN_08513*, could not be observed. Thus further studies would be necessary to identify their function for the fungus, for example in cell adhesion or in the evasion of MAMP recognition. Nontheless, the transient overexpression of *P. indica* proteins in tobacco leaves for the measurement of oxidative burst or callose deposition provides a useful tool for the characterization of further effector candidates. Thus this work might serve as base for future functional studies.

An important research field for future investigations will be the overexpression or knock-out/knock-down of the secreted candidates in *P. indica* itself to study their impact on colonization success and the morphology or development of the fungal mycelium. It would be advantageous if the *P. indica* transformation system could be developed further to perform for example localization studies with GFP fusion proteins inside the fungal cells.

5. SUMMARY/ZUSAMMENFASSUNG

5.1. Summary

The basidiomycete *P. indica* is a root endophyte, which has a beneficial impact on host plants. *P. indica* promotes growth in colonized host plants and mediates biotic stress resistance as well as abiotic stress tolerance such as drought or salt stress (Waller *et al.*, 2005; Baltruschat *et al*., 2008). The fungus has a broad host spectrum and non-host plants have not been identified so far. During the establishment of a compatible interaction, the fungus is able to actively suppress root immune responses of the host plant including the root oxidative burst, induction of defense genes and callose deposition. Especially JA signaling seems to be influenced by *P. indica* during the active suppression of host basal defense. The colonization pattern of *P. indica* can be mainly divided in an initial biotrophic and a later cell death-associated phase (Schäfer *et al.*, 2009; Jacobs *et al.*, 2011). To reach compatibility with the host plant, *P. indica* is assumed to secrete a battery of effector proteins, especially during the biotrophic interaction phase. Effector proteins are secreted by pathogenic as well as beneficial microbes during plant colonization in order to suppress the plant innate immune system and to manipulate the host metabolism. Due to the broad host range which includes model plants like *Arabidopsis*, barley and tobacco, *P. indica* is an ideal model organism for the investigation of fungal derived effector proteins and their impact on plant innate immunity.

The aim of this study was the identification and subsequent characterization of *P. indica* effector proteins. Therefore, the yeast signal sequence trap (YSST) was successfully applied. Eleven different secreted *P. indica* proteins could be isolated. Additionally, the *P. indica* effector candidate *PIIN_00029* (Pi*ALH43*), which was previously identified (Khatabi, 2009), was included in this study. The *in silico* analyses revealed similarities of eight candidates to known or putative effector proteins identified in other biological systems. Furthermore, six of the candidates are probably associated with the cell membrane. The isolated effector candidates could be functioning in the adhesion of the fungus to the host surface or even in “masking” *P. indica*-derived MAMPs. In qPCR-based analyses, nine candidates showed the highest expression during biotrophic colonization (2 dai) of *Arabidopsis* roots in comparison to axenically grown *P. indica* or *P. indica* colonizing dead roots. Localization studies with GFP fusion proteins revealed a cytoplasmic expression pattern of the candidate PIIN_08513. This candidate was furthermore demonstrated to suppress MAMP-triggered oxidative burst upon transient overexpression in *N. benthamiana* leaves. The present work is a base for future studies on *P. indica* effectors and their impact on plant innate immunity and host metabolism.

Functional studies on the RING E3 ligase PIIN_00029 (PiALH43) revealed that this enzyme had neither a proapoptotic nor antiapoptotic activity in yeast viability assays. Further, plant basal defense was not influenced in *N. benthamiana* leaves transiently expressing *PIIN_00029*. *Arabidopsis* plants stably expressing *PIIN_00029* showed partly enhanced colonization by *P. indica*. PIIN_00029 might manipulate plant metabolism by the targeted labeling of proteins for degradation in order to enhance root colonization.
The results of this work can be used as base for the characterization of further *P. indica* secreted proteins.

5.2. Zusammenfassung

Der Basidiomyzet *P. indica* ist ein Wurzelendophyt, der verschiedene nützliche Auswirkungen auf seine Wirtspflanzen hat. *P. indica* hat wachstumsfördernde Effekte auf die Wirtspflanze, zusätzlich werden besiedelte Pflanzen resistenter gegen Pathogenbefall und abiotischen Stress wie Trocken- oder Salzstress (Waller *et al.*, 2005; Baltruschat *et al*., 2008). Der Pilz verfügt über ein breites Wirtsspektrum. Bisher wurden keine Pflanzenarten gefunden, die nicht von *P. indica* besiedelt werden können. Während der Ausbildung einer kompatiblen Interaktion mit der Wirtspflanze ist der Pilz in der Lage, die Abwehrreaktionen der Pflanze aktiv zu unterdrücken. Der Pilz unterdrückt in Wurzeln die Bildung reaktiver Sauerstoffspezies, die Aktivierung von Abwehrgenen oder die Auflagerung von Callose. Insbesondere der Jasmonsäure-Signalweg wird vermutlich zur Unterdrückung der basalen Abwehr durch *P. indica* beeinflusst. Die Interaktion von Pflanze und Pilz kann im Wesentlichen in eine anfängliche biotrophe und eine spätere Zelltod-assoziierte Phase unterteilt werden (Schäfer *et al*., 2009; Jacobs *et al.*, 2011). Um die Kompatibilität mit der Wirtspflanze zu erreichen wird vermutet, dass *P. indica* zahlreiche Effektorproteine, vor allem in der biotrophen Interaktionsphase, sekretiert. Effektorproteine werden von Pathogenen wie auch nützlichen Mikroorganismen sekretiert, um die Abwehr zu unterdrücken und den Metabolismus der Wirtspflanze zu manipulieren. Aufgrund seines breiten Wirtsspektrums ist *P. indica* ein idealer Modellorganismus zur Studie pilzlicher Effektorproteine, da auch Modellpflanzen wie *Arabidopsis*, Gerste und Tabak von dem Pilz besiedelt werden können.
Das Ziel dieser Studie war die Identifizierung und anschließende Charakterisierung von *P. indica*-Effektorproteinen. Hierfür wurde der *yeast signal sequence trap* (YSST) erfolgreich durchgeführt. Es konnten insgesamt elf sekretierte, putative Effektorproteine isoliert werden. Zusätzlich wurde der Effektor-Kandidat *PIIN_00029* (Pi*ALH43*), welcher kürzlich identifiziert wurde (Khatabi, 2009), in die Studien einbezogen.

Die *in silico* Analyse ergab Ähnlichkeiten von acht Kandidaten zu bekannten oder putativen Effektorproteinen aus anderen biologischen Systemen. Sechs der Kandidaten könnten außerdem mit der Zellmembran assoziiert sein.
Somit könnten die isolierten Kandidaten eine Funktion bei der Anheftung des Pilzes an die Wurzeloberfläche oder auch in der „Maskierung“ von MAMPs vor dem pflanzlichen Immunsystem haben.
In qPCR-basierten Analysen konnte für neun Kandidaten das höchste Expressionsniveau während der biotrophen Besiedlung (2 dai) lebender Pflanzenwurzeln nachgewiesen werden. Im Vergleich dazu waren die Expressionsniveaus während der Besiedlung toten Wurzelmaterials oder von in Flüssigkultur wachsenden Hyphen weitaus geringer. Lokalisationsstudien mit GFP-Fusionsproteinen ergaben ein zytoplasmatisches Expressionsmuster des Kandidaten PIIN_08513. Dieser Kandidat scheint außerdem die basale Pflanzenabwehr supprimieren zu können, wie in transient mit *PIIN_08513* transformierten *N. benthamiana* Blättern gezeigt werden konnte. Zum Nachweis wurde hier der *oxidative burst* nach flg22 Behandlung gemessen.
Funktionale Studien mit der putativen RING E3 Ligase *PIIN_00029* (Pi*ALH43*) ergab keine proapoptotische oder antiapoptotische Wirkung des Enzyms in Hefe-basierten Zelltodstudien. Eine Beeinflussung der basalen Pflanzenabwehr nach transienter Überexpression in *N. benthamiana* konnte nicht festgestellt werden. Die stabile Überexpression von *PIIN_00029* in *Arabidopsis* ergab eine transient höhere *P. indica* Besiedlung von Arabidopsiswurzeln, welche *PIIN_00029* überexprimieren. *PIIN_00029* könnte durch die gezielte Degradierung von Proteinen den Pflanzenmetabolismus und somit die Besiedlung beeinflussen.
Die Ergebnisse dieser Arbeit können als Basis für die Charakterisierung weiterer sekretierter *P. indica* Proteine verwendet werden.

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Database internet addresses for DNA and protein sequence analysis (29.03.2011)

http://mips.helmholtz-muenchen.de/gbrowse-1.69/piri/cgi-bin/gbrowse/piri/
(Password protected)

http://www.cbs.dtu.dk/services/SignalP/

http://www.cbs.dtu.dk/services/TargetP/

http://www.cbs.dtu.dk/services/TMHMM/

http://smart.embl-heidelberg.de/

http://blast.ncbi.nlm.nih.gov/Blast.cgi

7. SUPPLEMENT

7.1. Vector maps

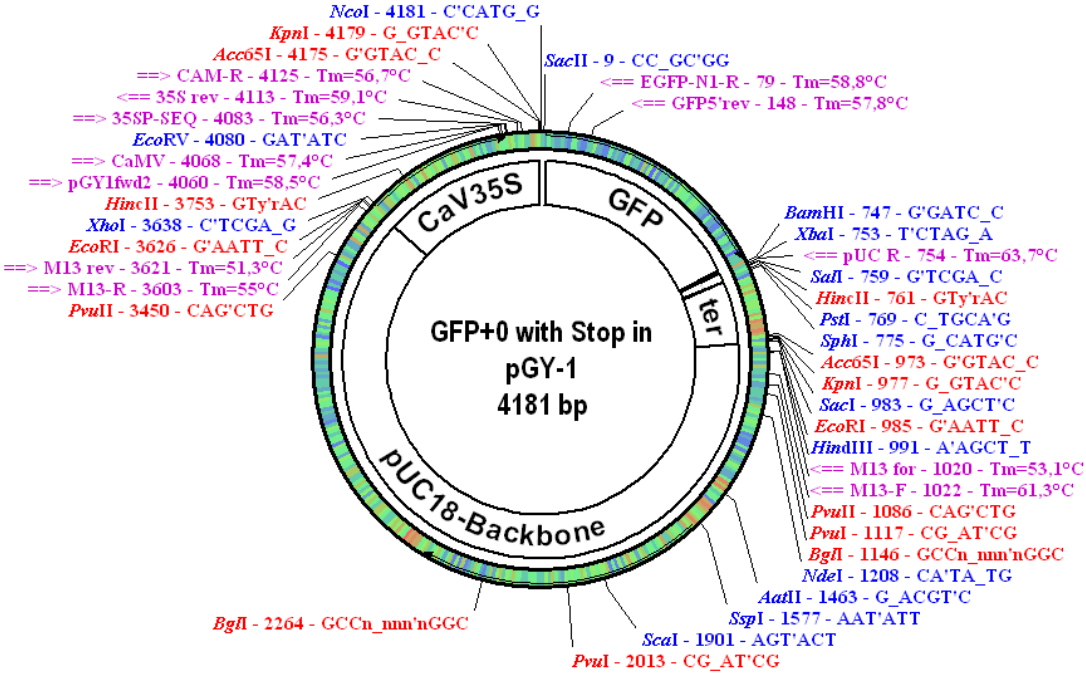

Fig. 7.1 Vector map of the expression vector (GFP+0fwd in pGY-1). The vector was kindly provided by Ruth Eichmann (TU Munich, Germany). It was used for the synthesis of the candidate-GFP fusion construct for subcellular protein localization studies.

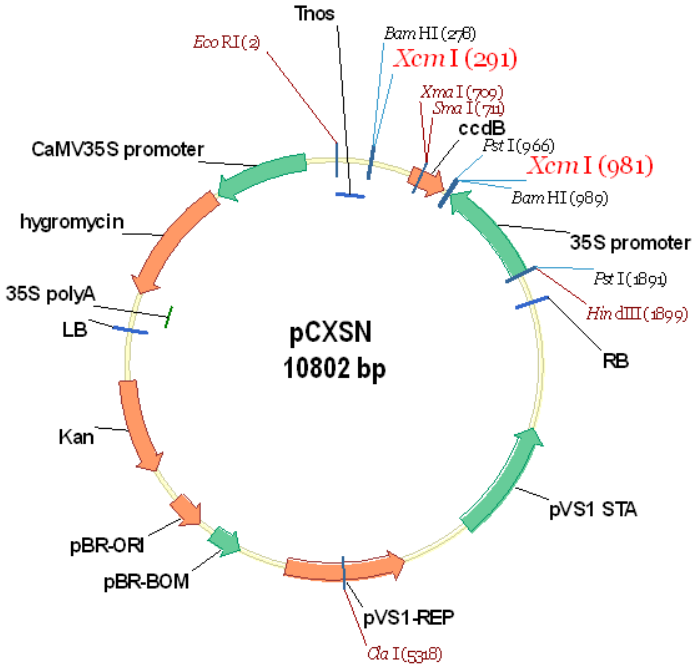

Fig. 7.2 Vector map of the binary expression vector pCXSN (Chen *et al*., 2009). YSST candidates were cloned in pCXSN for the transformation of Agrobacteria and transient expression *in N. benthamiana* leaves.

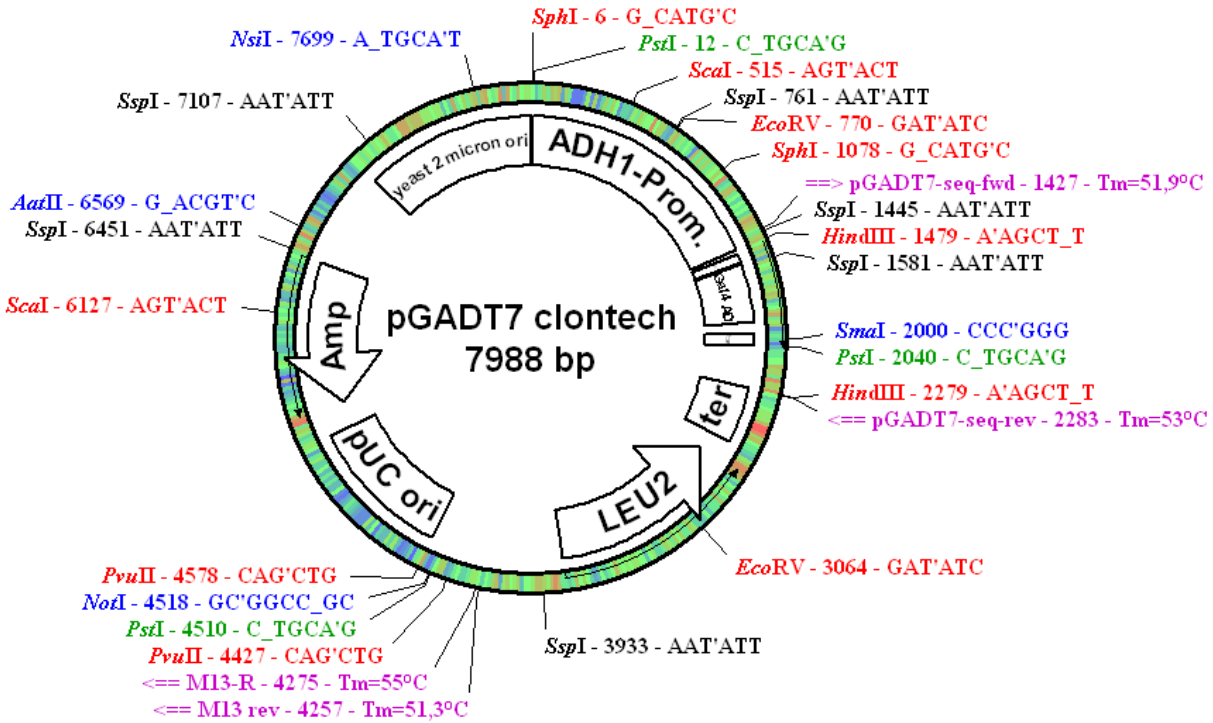

Fig. 7.3 Vector map of the binary expression vector pGADT7 (Clontech Mountain View CA USA). The vector was applied for the synthesis of the pGADT7-Pi*ALH43* construct used in the yeast viability assay.

7.2. ORF sequences of the YSST candidates

The sequences of the hypothetical ORFs of the YSST candidates were taken from the *P. indica* GBrowse Database in February 2011. Both versions of PIIN_03211 are listed. Due to the identification of SNPs (single nucleotide polymorphisms) in the sequences of *PIIN_04526* (*K43*) and *PIIN_03806* (*K12*), the sequences listed here were those which were used for the cloning procedure for the functional studies which were slightly different in comparison to the versions listed in the *P. indica* DB.

***PIIN_03211* (*K1*) *P. indica* GBrowse database version**

ATGCCCGATACTGCAGCCGTCATCAAACAAGTCCAAACAGAGGCCCAGGAGCGTATAAAAGAGGCTT
TACAGTTCACTACCGTCTTCGTCACCCTCGCCATCTTCGCCTCCTCTGCTTTCGCAGCAGCTGTCGC
CGATGACGAAACAGCGGTCATTGCTCGTGCTCGCAGCGAGGATGCTGCTGGAAGTGTCGATCTTCT
CAAGCGCGCTTGCAACTATAACACGCCTTGCAAGGGATACTACTGGTCCAAAGGGCTCCACTGTGGC
GATGGCAACTATGGATGCGTCTACGGCCACGTCTACCAGGTTGGTTCTGACACCAACGTCTGTGACT
ATGGTGTTCGGACCTCTTGCCAGAAGTGCGGGAAGCTCTCGTGCTAG

***PIIN_03211* (*K1*) YSST version**

ATGAAGTTCACTACCGTCTTCGTCACCCTCGCCATCTTCGCCTCCTCTGCTTTCGCAGCAGCTGTCG
CCGATGACGAAACAGCGGTCATTGCTCGTGCTCGCAGCGAGGATGCTGCTGGAAGTGTCGATCTTC
TCAAGCGCGCTTGCAACTATAACACGCCTTGCAAGGGATACTACTGGTCCAAAGGGCTCCACTGTGG
CGATGGCAACTATGGATGCGTCTACGGCCACGTCTACCAGGTTGGTTCTGACACCAACGTCTGTGAC
TATGGTGTTCGGACCTCTTGCCAGAAGTGCGGGAAGCTCTCGTGCTAG

***PIIN_07104* (*K3*)**

ATGATCAAAATTTACGCCCTCGTGCTTTCAATTGCATCACTTGCATACTCTCTTCCAACACCAGATGAC
CTCCTTGCGCCCATCAAGCGGGCAGTCGATATTCCTGGAGCACTTGCGCCAAACCCACAGGCCTAT
GTTGTCGCTTTCAAGCCCAACACCGTAGATCCTACTGGACGTGGAGAATGGCTCAATAATGTGCTCT
CGACCAGAGGTCACCGCCGACGTGACCTCTCCTCGTCCGACCTCAAGCTCAACTGGAACGAAACTG
TGTACAATGGCTTGGCTGGAACATTTAGTGACGCCGAGATACGTACGCTACGAAGACAAAACGAAGT
CAAGTATATCCAGCACGATACATTACTCCATACCAATGAACTCGTCGTACAGACCAATGCTGTATGGG
GAATCGCTCGCCTTTCGTCTGGCGGGAACCCTATCCCTTTTGGCTCGGACCCCTCGACGTTTAGTTT
CTCCTACACTTTCGACAGCTCTGGTGGCAACGGCGTCGATATCTACGTCTTGGATACAGGAGTCCGT
GCTACACACCAGGACTTTGGCGGTCGAGTCAACTTTCTCCAAACCTTTGGATCAGGAACGCCGGGCG
TTGATGTAAACGGACATGGCAGCCATGTCTCTGGAACTGCAATCGGGTCGGTATTCGGCGTCGCAAA
GAGCGCCACGGTGCAAATGATCAAGTGCATGGCAGACGACGGGTCCGGATTCACTTCAGACATTGT
GTCTGCCATCAATCTTGCAGCCAACACTGCCGCAACCTCTGGTCGGCCCTCTGTCGTGTCCATGTCG
CTTGGTGGTCCAGCCAACGATGCCATTGACGACGCTGTGATCAACGCTGTAAACCTCCAAATCCCCT
TTGTCGTCGCGGCTGGCAATAACGGAGCGGATGCGTCAACGAGCTCGCCTGCGAGACTCGGTGGGT
CTGCTGGAAATCCGGGGGTGATTACGGTTGGCGCCACCACAATCGACGATACCTTTGCCACCTTTAG
CAATACAGGCAGCAATGTCGACGTCCTTGCACCTGGCCAAGATATTCTCAGCTGTGGTATTACAGAC
GACGCGGCGGTCAAGAACCTCTCAGGGACGAGCATGTCAACGCCTCATGTCGCTGGTCTGGCTGCG
CTCATCATGGGATTGGAAGGCAAGATCAGCCCGACCGCGCTCAAGCAGAAAATTATCGCTCAAAGCG
CACTCGGTGTCATAAGTGGACTCCCAGTGACTACTGTGAACACGTTGGCGAACAACGGAGCCACCGT
GGTCTAG

***PIIN_03806* (*K12*) (ORF version which was inserted in cloning procedure)**

ATGATCTTCACACCCTACCTACTCGCGTTGCTGAGCGCGTCCACGTTGGCCGTAGAGGCCGCTCCG
ATCACCGTAGGCGGAGCTGCTCAATTATGCTCAGTTGCACCCTGCCGAATGCGACGAGTGAGACGC
ACGCCTGAGATTATGCGCAACTGGAAGCGACAAGAAGAAGGAGACGACGATGGCGAGGGAGATGGA
GAGAGTGCACCTCCTCCATCTGAAGATGGATCTGGCGCCCCGCTGACTTCGACTCCGCCCGCTAAC
GCACCTGCTGTCGTCGTAGTCCCGGTCACATCAACCACGACGATTCCAGTCAGTCAGGAGCTTCCAC
CGGGTCCTGTTGGAGGTAGTACGACCACGACACCTGAAACTGGTACGAACACAACGACAACACCGC
CCGCGACGTCCTCGACGGCAGTTGGCACCATTCCGGCAACACCAGCGACCCCCTCTACTGGCACAC
TACCTGGAACTGTACCTGGAGCGGCTGTTCCTCCGACCGGCCAGACACCTCCGTTCTCACCATTTGG
CCAGCCGACGACGGGATTCAATCCGTTTGTTCAGCCACCGACCACGGGCTTCACACCATTTGGACAG
CCACCGGTCGGTGCGGTTCCACCATTTGGCCAACCACCGTTTGGCACTGTACCGCCGTTTGGCGCT
GTGCCACCGTTTGGTCAACCGCCTATTGGATCGGTGCCACCATTTGGCGTCCCTCCAACTGGGTTCG
AACAGACTGGCCAATTTGGTAACAATCCGTTTGCCAACGGTCTGCCACCTACGACTGGGTTTGGAAC
AGAGTTTGGTGCAAATGGACAATTTGGGTCAACTGGACAATTAGGATCAGCTGGACAGTTTGGATCG
ACGGAACAGCCTGGATCAACTGGACAGTTTGGATCTGCTGGACAATTTGGATCGACGGAGCAGCCT
GGATCAACTGGCCAATTCGGTTCGACTGGCCAATCTGGATCCACCGGACAATTTGGTTCCACTGGAC
AACCCGAGACATGTGGTCCGACTGGATGCACTCCTACTTCGGCAACGAACACGCAGTGTGGCGCAT
CTGGATGTGGAACATTTGG~CCGTGATCACGAATTCGGCGTGCCACCCAACTTTAGTCCGTTCCCGA
GCTACATGCAAAACTATGCGATGTACTACCTGCCGCATATGATGTACTCGGTGGACGCGTCGATGAG
CTACGTGGGACAACTGCTTGGAGCAGCGATGCAGTCTCTGCAGTCGGCTTTGATGCACGTGCAGCA
GAATATGGCGGCGTACGGGATCATGATGACGCCACACCTGAACTCGACCTTTGGCCATAACAATATG
CAATTTGGACACAATATGCACTTTGAGAATCAACAATCCGGAGGACATCAATTCTCACATAACCAGGA
GCAGTTTTCATCTCATGGTCAATCCTCCAACGGTCAATTTGAATCCTCAAACAATCACTTTTCGTCGTC
GAATGGCCACTCTGGATCCTTCAACCACAATAATAACGACGTTGGATCCAATAACCAGTTCTCTTCGT
CGAATAACAACGTCGGATCGTCCAATAATCAGTTTTCATCGTCGAACAACAACTTTGGCTCGTCTGGT
TCATCACAGTTTAGCTCTTCTGCCTCGTTCCCGCAGTCGACTCCTCTCGGTGGCGCCAGCACCACAT
CGTCGTCCTCGTTCCCTCAGGAGACGCAGTCGTTTAACAATCACGGGTCACAGTCGTTTAACAGTCA
CGAATCACAATCGTTCAACCAGCAGCAGCCACAATCCTTTGACCAGACCAACCAACAGCCCCAATGC
AGCCATGGCGAAAAGACATGTATGGGACAGAGCTTTGCCGTTTGCGTGCACGGACAATGGAAAGTTG
AGCAATGCTCACACGGCACCCAATGCTATGCTCTCCCGTACAGTGGTCAAGCCACGACATTTGCGTG
CGCGACGCCGCAGTACTTTGGAAAATGGATGCAAATGGCCAAGCAGGCTGAAAGGCATGCATAG

***PIIN_08513* (*K16*)**
ATGGAGAAAGCCTACACCCCAGTTGATGCACGCAAACATCGAAGGATCATCCTCTCTTCCGTGCTTTT
CCTTGCAGCACTCGTGACGGGAACACTCCTCTGTGTATCCGCAGCGTGCCTTTCAGCGATGCTCCAC
GGATTCACACAAAGCCACAACACCCAGAACAATCCCATCACCGCCGAGCATCGACTTCTCAATGCAT
TCGGTCTCAACCGACGAGCAGACGTGTCGGCAATTGGGCCTTCGAACCCTGCTGTCGGCTGGCACG
CGCGCGCCGTTCAGCGTGCTCTGGTCTACAAGGGCCCACTATACGAGCCCAAGAAGGAAGACCTCA
CTCTCACGGTTCTCTTCAGCACGCCTGCCGAGCCAAATGGCTTCACGGTCGCACTCTTCAAGCCCTC
CTACGCGGTCGATGCGAACGGACGGATCCTAACGATGACGACAACTGAGTACGATGTCTTCGCTGAT
TTGATCAAAAAGGTCGGCGCTCTGCCGACAGTCGACCAATGGCGCGTCAAGCACAACACAACCAGC
TGGCCCATCGACAAGTTGATCATACCGGGAGACGACGAGGTTTCAGTGTATGGCTGGAGCACGGAA
ACGGACGTCCTCGTGAAGCCGACAAAGGGATATAAGAGACTCCCCCCATCGCTGAACACCCTCATTG
GCTTGGCAAGGGAGGGCAGAGAAGACTACTCGCCTGACTTGCGCATGGATGAGAACATTAAGAAAG
TTCTCTCCTTCACGTATTGA

***PIIN_10643* (*K19*)**
ATGCAGATCGTCAATCTCTCTCTCCTCGTCCTCGCGATTCGTCTCGCGTCCGCTGCTCCCATTCCAG
CACCAAACCCAAGTGGACACCTCGTCAAATCTCTTGCACATGGTGCAGCTGCCGTAGCGGATTCCGG
TTCCGTCACGGACGGTATTGCCGCAGCAGTCTCTCATCATCACCACCACTCAAACCCGCATTATGAA
CACTCACATACACAATCTGAAACCCGCCGTAGCATCGAGCGTCGGGGACCCAAGGGCAAAGGCAAA
GGCGGTGGAGGCGTCAACCTCAATGTAAACGTCGACGCGAGTGTAATCCACCATCATGACAAGCGC
TCACCAGAGCCAGAGCCGCATCACATTCACTTCCACGCTGGAGGTCAAGAACAGCAGAAACGATCAT
TGAACGCACGCTCCCCGACTCACCCTCACTCTCAACACCAGCATCATCCTCATCCACACCACATGCA
CGCTTCTCACTCCGTCGACGAGAGTCAGTCCGGGGGGTGGAGATGCAAGCCAAGGAGACGTCCGCAA
GCGCTCACCAGAGCCAGAGCCGCATCATATTCACTTCCACGCTGGAGGTCAAGAACAGCAGTAG

***PIIN_04018* (*K29*)**
ATGCTTCTTTCGCTAGACGCGGTGAAACATCTGGCCGTCAACGATTCCTCACCACCTCTTCTTTCGTG
GACACCCAGCTCTTTCATCTTTTCTTCGCAGCGGCCAGGCGCCCCTTTCTTTTTTCACACTTTCAACT
ACTTTTTTTTCAACAAGTTCTTCTTTTTCTTTATCTTTCAACACCCAACAATCAACATGTTTGTCAAGAA
CACCCTCGTTGCAGTCCTCGCCGTTGCCCCCGCCATCTTGGCTGCCCCTCTCCCGAGCACCACTCC
TACGCACACTCAAGCTGTTGCTACTGCATCGCATACCTCGGCTGCTCCTCACGAGACCACGACTGCG
GTGCACCACCATCACCAACACGCTCACCAGCACCACGACCACCCTCACAAGCACCACGTCCCTGCTA
GTGCAGAGGCTCGCAAGGAAGAGGAGCACAAGCTCAAGGAGAAGGAGCACAAGCTCGAAGCCAAG
GACAAAGAGCTCGAGAAAGAGGTGCACGATCTCAAGGAACACATCAAGAAGGATGAGAAGGCTGAG
CACCAACACCATGCTGCGACGACGCCTGCCCCAACTGCGTCGTCGACTTCTGCCAAGCTCAGTCGC
CGTAGTGAGACCGCAGCAGAGCTTCAGAAGGAGAAGCAGCGCCTCAAGGCGCTCGAGGAGAAGAA
GGACAAGGTTGAGGCACAGGAAAAGGAGCTCAAGGCTGAAGAAAAGGCCGATGAAAACGCTGGCAA
GGCTCCTACCCACCACAACAAGCAGCACCACAACAAGCACCACAAGCATGCCTCCTCGACGCACAC
CTCTACGCACACCTCCTCTACGCACACCTCCACTGCGACCCCGCATCCTACTGCTACCGAAGCAGCA
AAGGTCCAGCGCCGTGCCGAGTCGTCCTCGTCCGCTGCCGCCGCTACGTCCACGAGTGGAACGCA
CCACGCTCACCACGAGTACAAGCAAGCTCTCCAGCGCCTCATTGAGCTCGCCAAGATTATCGAAACG
GGTGCTCGCAAGCTCGAGGCCGATCCCTCTGGCAAGCACGCCCTCAAGGCCATCGACAGCCTCGAG
AAGAAGTTTGAAAAGATTCACGCAAAGCACGCTAAGCACCACTCTTCGTCTGCGAGCGCTCCTGCTG
CCACGTCCACCAAGGTTTAG

***PIIN_06847* (*K35*)**
ATGCGCTTCGGTTTCTTTGCCGTACTTTTTGCCTCGGCTGCTCTCCTGGCGTCCGCCGTTCCGCTCC
CCAAGGGAGATCACGTTGCGGCGCACGCACACCACCAAGAACAAGCAGACCACCATTTACAACAGG
CAGAACATCACCTTGGTCAGGCAACGAATCATTTAGATCGCGCCGACGCTGCACGAGCGAGTGGCG
ATCATCACACTGCTGTGGGGCACGAACTTCAATCCGATAACCATAACGCGCAAGTTGATCATCATACG
TCTCAACACGAACATCACCAAGCTCTGGCTGACCATCATGATTCACACCTTCCCCACCGGGTCATACA
TGCCCGCCCCAACAACTAA

***PIIN_05415* (*K36*)**

ATGAGCACACCAACAAGCTCGACCCCAGACGCTCAGGCAAGTTTGCGCGTTCCGGGCATCTACACT
GCAGCTGCGAACAAAACGGGCAGCGATTCTTCTCTTCATGTGATCAAAAATGTCCCCGGCTACAAAA
CTCCAGTCTTCCCCGCAAAGGAAGAGCAACGCCTCAAGGTATATGAAAACGTCACTCAAAAGGGATT
CCTCCCAAAGGAGTTTGTGCGTCCAGAAGTAGATTGGTTCTATGACGGCCTCGGCATCGAGGAAAAT
TACTTTGCAAACGAGTCCATTGACGTCATTACGGACCATATCATTGCACTCTTTGCCGCAAAGACACT
TGCGTACACCAAACATTCCAACCAGCTCTTAATCGATCTCGAAAAAGTGACCGAAAATGGTGCCCTCT
TCATCCACACAAGCCCCCCTGGTGTCACGAGTGAAGAGGGACCCGGCGCAACCTGCGAAACTCGAA
TCGATGACCTCTTCCTCGATATTTCGACGCCTCAAAAGGCCTTCCGCCTCGAAACGTATCGCTCTGCT
GGTGTGACAGCCGGCAACTCGCAGTCCCTGCGCTGCTACTTTGTCTCGCAATGCAACTTCCCCTCTA
TTCCTCCAGACTCGTTTCAGCCCGAGAATACGTCCACGAGCACCGTCGACATCCGCACCGTATCCGA
CCCTCACTTCCTCGAAAAGGCGTCAGAAAACACCCTCGAGATTTATCAGTCGATCATGAACCACGTTC
TTCAGCGACGTGGCTTTGGCCCTGTCCTCGAGTTCTACGAAGTCGAAGGTACGAGGGAGAGGCGAG
TCGTCATTGGCTACAAGATGGGCTCGACCAAGCACTTCTTCAGCGCGCTTAGTCAGCTTTACCACTG
GTATGGGCTCTACTCAAAGAGGAAATACGTCGAGCAATTTGCCAATGGCGTTACGATCATCTCTCTCT
ATCTCAACCCTCTTCCTGGAGCAAACGCACCTCCGATTGAGCACTCAATTCATCAGGTCACCAAGGA
AGCATCTCTCCTTCATTGCCTACCAGACAACCCCTTCTTCCGCACCACTACTTCCGGTGATCGTCCAG
GCGGAATTCCGACTACTCATGCCGTACAAGAGGCGACATATGCTTACGCCGGATGGATTTTCGCGCA
GCACTTCTGTAACCGACTCGGTCCCGCATACTCTGCTCTCCGCAATGTTCTTGACGAAGCCAACCCT
GGTCACGCAGAGGTTCTCAATCAGATCAAGAAGCGTTTCAGAGAAGAGACGTTTACGCGTCAAGGCA
TTTCCGAGGCTATTCTCGCGTACCCGGAGCTCATTCGTATGCTCTACGTCAACTTTGCAATGACGCAC
TACCCGAATGATTCGGAGGCGACCAAACTCATGCCCACACTGTCGTACCAGCGACTCAAGACTGAGC
AACCTCTCTCTGTCCCCGAGTTGCAGGAGAAGATTCGTAAGACTGTCGCGAACAAGCACGACTTGCA
AGTCTTCGAGGCGCTCCTCACATTCAACCAGCACGTTCTCAAGACCAATTTCTACCAGCCCACCAAG
GTTGCCATCTCGTTCCGCTTGGATGGAAACTTTTTGCCAGACATCGAGTACCCAACGAAGCCTTTTGG
TGTCTTCATTGTCGTCGGCTCCGACTTCCGTGGATTCCATGTCCGCTTCAGGGACGTCGCTCGTGGT
GGCATCCGTATCGTTCGCTCGAGGGGACGAGAGAACTATTCCATCAATCAGCGTACGCTCTTTGATG
AAAACTATGCGCTCGCAAGCACACAGGCACTCAAGAACAAGGACATTCCCGAAGGAGGTGCGAAAG
GAACAATTCTTCCATCTCTGGGTGCCGATCCTCGTGCATGCTTTGAGCGATACGTCGATGCCATCATA
GATCTTCTCCTCCCAGGAACAACCCCCGGTATTAAAGAGCTGCTGGTCGACAAGTACGGCAAGCCG
GAGATTCTCTTCTTCGGCCCAGACGAGGGCACTGCAGACATGATGGACTGGGCGGCTTTGCACGCT
CGCTCACGTGGAGCACCTTGGTGGAAGTCATTCACCACTGGCAAGAGCGCAGAGAAACTGGGTGGT
GTCCCGCACGATACATATGGCATGACCTCGCTCTCCGTCCGCCAATACGTCGTTGGGTCGTATCAGA
AGCTTAGCCTTACCGAGTCGGAGATTACTAAAGTACAAACCGGAGGACCCGACGGTGATTTGGGCTC
GAATGAGATTCTGCTCAGCTCGGAGAAGTACGTCGCCATCATCGACGGTTCAGGTGTTCTCGCGGAT
CCCAATGGCCTTGATCGCGACGAACTCGTTCGCTTGGCAAAGGGCAGGAAGATGATTGTCAACTTTG
ATCGCTCCAAGCTCAGCAAAGATGGCTATGTTGTACTCGTCGAGGACAAGGACCTCAAACTCCCCTC
TGGTGAGATCGTAGCAGATGGTACCGACTTCCGCAACACGGCACACTTCCGCTACCATGCAGACCTC
TTCGTCCCATGCGGTGGCCGTCCAGAGGCCGTCAATATCTCCAATGTTGCCGAGTTGTTCGACGAGG
ACGGCAAATGTCACTACAAGTATATCATCGAGGGTGCAAATCTGTTCCTTACGCAACAAGCGAGACT
GTATCTCGAGAAGAGGGGTATCGTCGTCTTCAAGGATGCCAGTGCCAACAAGGGTGGCGTGACTTC
GTCATCCCTCGAAGTGCTTGCTGGTCTCGGTCTCAGCGACCAAGAGTATCTCGACCTCATGGTGTTC
AAGAACGGAAAGCCTTCCTCTTTCTATAGGGATTACGTCAAGGAGATCCAGGGCAAGATTGTCGAAA
ACGCCGCGATGGAGTTTTCGTGCATCTGGCAAGAGTATACTCGAGCAGGAGGTGCCAAGCCACGCA
CGCTCATCTCGGACGAACTCTCGAGCACGCTTAACAACTTGCAAGCCGAGTTGGAAAACTCTGACTT
GTTTGACGACGTCGCGAGTCGTCGAGGTGTGCTTAACCGCGCTATTCCCCAGACACTCGTCAAGCAA
GTTGGGTTGGACACGCTAATGAAGCGTCTGCCGGAGCCGTATCAGCGCGCCTTGTTCTCGAGCTGG
GTGGCTGCTCATTTCATTTACCAATATGGCGTCGCCGGAAGCAACGTCGATTTCTTCAACTTTGCACG
CACGCTCGCTCAGTAG

***PIIN_02519* (*K37*)**

ATGGTCGCCCTTATCACACTCGTTTTTATCGCCAGCCTTCTCTCGACCTCGACGGCGTCGCCCATCTA
TCAGATTGGCGAGGTAATTGGGGAAATTCGAGATCTCCGCGCAGAGCCGCGACAAGAATCTCGCTC
AGGAAATCTCCTGACAGGCCCAATAACGTTCCAGACGGTGACGACTTCTCAAACTCAAGCGGGCACG
ATTACACAAACTTGCGAGGTCACGCTCACCCCGGTGACCGCCAGGGATGGCACCCCAGCCGTTCTG
CACTCCAAGCAATGTAAATTCACAGTAGGAAACGGTGGAGGTGGACAACAATCGTCCTCGTCTTCTG
CCGAGAGCAGCACCGCAGTAGAAACATCGACGAGCAGCCAAGCCTCGGCCACTGAGACGTCAACGA
GCTCTCAGGAGACTCTAACGAGTAGCTCAACAGCTTCTTCATCCACTGTTTCGGGAACCGCGCCACC
AGCCATTTCTGAAATTGGCACAAACATTATTCCCGCATCCGGCTCGACAACTCTAGAGTCATCATCCG
CCACTCAATCGACCTCATCTGTCACCGGTAGTCAATCTGCTGCGCAGCCAGTCGTTTCCGTCGTGGG
CCCATCTTCGATCTCGAATACCGGTAATCCAGTCATAACCACTGGCGGATCGAGCACGGCGACAGGA
ACCGAACCCATCACCGTAGTAACGACCTTGTCGAATGGGCAAGTCTCGACGGCCGTCTCGACCCCA
ACACCCGCAGCTGCGGAAGCTGCAGCACAAACTCCTGGCAGAAAACTTGAAGTTTTGCCCATTGGTC
TTGGCGTCTTTGCAGGTGTTTCTGTGATTGCCTTGATCGTCGTTGGCTTGGTCACCTACGAACGAACC
AAGTACAGAAAGGCTTTCCGAAAGAGAAAGCTCGCCGAAGCCGGCGCACAGATGGGCTATGGAGGA
ATGGCGTAA

***PIIN_00308* (*K38*)**

ATGTTCAACAAGGCATCCTTGTTTTCTGCTCTCGTCCTCACTGCCTCTTTCGTCCACGCCCGTCCAGG
TCCCACTTGGGTTCGTCGTCAGGAGAATGCTCAGTCATCATTGACGCTTGACCCTGCCGTCATCTCT
CAAGGTCTGGCTCAGGACGGACAAGGCAATACTCCAGACCCCGGACAAGTTCGTTCCGAAACATCG
ACCAACAATTTCATCAACCACTGTCTCAAGTTCCTCGACCAAGGCGTCCCCCTCACCGATGGTAAACA
AATCACTGGTGGCTCCTGCAACCAGACGCCGATGGGCCGCATCATCTCCCTTGACAAGGCTCCTCG
CTGCAAGTTTGTCTTCCCAAAGAACGGCGACGACTCTATCGTCGAGAATACTGCGTTTACCGTCCAAA
TGCGTATGGTAAACATGGTCGCTGGCAATTTCGTCAACGCCCAGGCCAACTACTACGCCGCGCCACA
ACAAGTCGACGGTAACGGTATCCTGATCGGCCATTCGCATATTGTCATCGAACCTCTTCCATCTCTCG
ACTCTACAGAGCCACTTGATCCTCTCGCGTTCAAATTTTTCAAGGGCCTCAATGACCCCGCGGACGG
ACAAGGCATTCTCGAAGACAGAGGTGACTGCTGGTCTCCTCAAAGGTGTCTACCGCATGTGTTCCAT
CAATACTGCGGCCAACCATCAACCTGCATTGGTTTCAAATGCCCAACATGGAGCACTGGACGACTGT
TCCTACTTTACTGTAAAAGGAAGAGGCGAGCTGGGAAATAA

***PIIN_02169* (*K40*)**

ATGTTCCCCGCCTCGTTTGTCGCCTTTGTCGCTGCTGCCCTCGTTGTACGCGCTGCTCCGCTCGAGA
AAAAGGGTCTCGCACAGGTCATAAGCAACTGTAATCACGACTTTGCGTATACTTGGGACGATGGACC
ATACACCGCCCATCGTGACATTAACAGTGCCTTTAACCGAAACAACGGAAAGACTACCTTTTTTATGA
ATGGCGACAACTGGTCGTGCATCTACAACGAAGATAATGTCCAAGCCATCCGTCAGTCATTTGAGCA
GGGACACATGCTCGCCTCGCACACCTGGTCACATGCCCACCTCACCCAACTCAGTGATGCCCAAATT
GACTACGAGATCCAGCGCCTCGACGAAGCCTTTGTCAAGATTGTGGGTGTCATTCCAAAGTTCTTGA
GGCCACCTTATGGTGACATCGACGAGCGGACCGCGTCGTACATCCAGCAAAAGCACGGCAAGACCA
TTGTCCTCTGGTCAGACGATTCTGGAGACTCGACCGGCGGCTCAACCCAGCAGTCAATCAATTTCTA
TAATACATTTGCGAATGAGAACCCCAAGCGTCCTCATATGGCGCTCTCACATGAGACTGAGCCATCG
GGTTACAACGCCATGTACTCGGGTACCGTCGACAACTTGGCCAGCCACGGCGTCCAACTCGTTACG
GTAGCTCAATGCCTCGATACTGATGCGTATATCTATGTTGGCGGATACCAGCAGCGTGACTCGTCCT
GGTACTGCGGTGGTTCATGGACACCGTCACCTGGTGGAGGAACGTGTGTGCAAACATACACTTCCAA
GGTCGGTGATACATGCGCAAGCATTGAAAGCCAGTTTGGCCTTCCATCCGGCTCGATCTTAGCAGCC
AACTCTTTCTTGAACTGCAACGATATCTGGGTGAACACGCCAATCTGCATTCCTCGAGGAGGCACGA
CGACAACTCGAACTTCGACCACGAGCCCCTCAACTACTCGAACTAGCACGAGCTCGACGCCTGCGC
CGACCTGCGTTCAGACGTACACCTCCAAGGCCGGTGACACATGTGCGAGCATCGAGGGTCAGTTCG
GCCTCCAGTCTGGCTCAATCTTGGCTGCCAACTCGTTCTTGAACTGCCAAGATATTTGGGTGAACAC
GCCAATTTGTATCCCTCCCGGAGGCTCAACGACGACCCGTACTACTACGACGCCTACAAGCACCAGC
ACCAGTGGACCTGCACCGACCTGCGTCTCTACGTACACTGCCGTCGCCGGTGATACCTGCGCGAAG
ATTGAGTACAACTATGAGCTACCGGCAGGCTCGATCCAAGCTGCTAACCCATCTTGGCTCAATTGTG
CGGATATCTGGGCTTACACGCCAATCTGCATTCCTCCTGGCGGATCGGGATGCACCCTCAAGATCTC
TACGGCTTCGACTGACAGGACTTGTGCAGCTGTGGCTTCTCGCTATGGAACCACGGCCGCTCTGATT
CAAGCCTGGAATCCAAGCTGGCTCAACTGCAATGATGTGTGGCCCAACACCCCGCTTTGTGTGAGAC
ACTAA

***PIIN_04526* (*K43*) (ORF version which was inserted in cloning procedure)**

ATGCTCACGCTCCTCGCGACCGCCCTCGTCTCCTCTACCGCCGTCCTTGCCCAAGGCGGCGCGTTT
ACCCCGCTCGCATCTCAGAGCTTTACGTACACTGCACTCCCTTACCAGGCCGACCCAAACACGGGTG
AGCGTGGCCACCAGTACGGCTACAACATTTGCAACTCGACGACTCAGGGCCAAGAATCACTCTGCCA
GACGGCCATTATCAACAGCATTGACGACTTTTGCCTTTGGGGTCCCCCCGAGCCCAACAGCTTGATC
GGTGACACTGAAGGTGAAGCCGTTGCTTGGTGCACAAAAGGAGGTCACGGAACTCGTGTCATTCCC
GAGGGTGCCCTCACCGGTGTCCAGTTTATGCGCACACCAGATTACATCCAAGTCACTGGTCATATTA
GACAAGAGCGTATCAACATTGCTGCCGACGACTCTGGAGGAGAGATGGATCCCCACGGTGCCGACC
AACGTGGCAACCCCCTCGGTGGCTTGCTCTTCTCCAACGCCTGGGGTGGAGGATACGTCCAAGCCA
TCGAATGGCACAATTTCATGGGTGGTGGCGTCTTCTGTCTCAAGGCCTGCGACCCCAGGGGCCCCA
ACGCTGCTCGCTTCTGCGAACATATCTTTGATAGAATTGGATGCGCATACAACGCCCCAGCTGCGTA
CGAGGACGGTGTGTTCGAATCCTGCCTCGGCGAGAACCAAGACTTCCCGGGTATCTATACCTCGAAT
GGCGTCGTCATGACATACACTCAACCCCCCGAGTCGCTTGGTCCCATTAGCGTCCGCCCATATGAGC
CACGCATTCCGGCAAGCTCTTCGTGCACACCCTACACGAGCTCTGCACTCTACACAGACGCACCACC
AGCCACTGGTGCCGCCGCCTCGTCTTCGTCCACGACGAGCCGTGCCTCGACCTCGACTCCACGCAG
CACCGCGACGCGTTCGACGACGGGTACTAGCGCAAGTGCTACGTCGACTCCAGGTGCTGGATTCGT
TGGTGCATCTGTCAGCCTTTCTGCTCTCCTCGCTGGCTTCTTTGCCTTTGCCGTCGCGCTCTGA

***PIIN_09796* (*K46*)**

ATGTGGACGACACTCCGACCAGCTAGCATCTTTCTCTCCTCTCTTCTCCTCCTTGTTGCATCCACGCC
GCTCCCTGTACAAAGCAAGGAGACGCCAGAGCTGCGCCAACTCACCGGCCAAAACTTTGAGGAATC
GATAAAGACAAACCATTGGTTTGTCGAGTTCTTCTCACCATATTGCCCGCACTGTACTGCGTTCAAAC
CTGATTGGACCAAGATTGTTGCCGCGCGGGATGACCCACCACGCCTGAACCTCGCTCAAGTGGACT
GCGTTGCCAACGGCGACCTTTGCCGAGCGCAAAATGTACCTTATTATCCGTATCTACGGCTCTACCG
CAACGAAGTCAATGGCACGCAGACTCAAGACGTCTTCGGCGGGTCGCGGACTATCGAAAACATTGAA
AAATGGCTTGATGAGCGTTCACCAAAGCCAAAATCCAATTCTATACCCCAGCATAACGACCAAGTATC
TATACAGCAGAAGAAGTATAATGTGGATGGAAAGGTCCTGGTGTTGGGAGCGAACACGTTCCGCGAA
CAAGTCGCCAAGGAGCCTACATTTGTCAAATTCTTCGCTCCTTGGTGTGGACATTGCCAGAAGTTGG
CACCGAAATGGGTCGAGCTTGCCGAAAAGCTAAAAGGCGTCATCAACGTGGCCGAAGTCAACTGCG
ATGCTCACGGTACCTTGTGTCGCGACCAGGAAATCGAAGGCTATCCTACTGTCACGCTGTATCTCAA
CGGGAAGAAGGTTGACTACACCGGTAGCAAATCTGTTCCCGCGATGGAGGATTTCGCCCGCAAGGT
CATGTCGCCTCGCGTCGAGGCTGTCGACGAGGAGCAATTTGAGAAGAAGCTCAAGGAGCAAGAAGT
GATCTTCCTCCTCTTACATTCTGGAAAGGACGAGCGACTAGTTTCCGTCGTAGCCTCTGCCGCTCAA
CCACTTCTTGGAAATCCACCAATCCTGACGTCGACATCCAAGGCACTCTACGCGCAATATAACCTGCA
GTCTGCATCTTCTTCGTCTGTGCCAGTCTTATTGGCGATCAAATCGCACTCGCAGAAAAAGTATGCGG
CCAAGATTGAACTCGATGGACTCGTGACGGGTTCTGGTAATGCCGAGAGATTGGAGACGTGGTTGCA
ACGCCATCGGTTCCCTGTCGTGAGCAAGTTGGGGATGGACAATTTTTATAGCGTCATGAAGAACCCG
ACCAAGCCGTTTGTCGTTCTCGCTGCCATTGACTCTGGGCACGACGTCGAAGCTGGTGGCGACGAA
GAAGCGGTACTTCCTAATGCGCACCCCGAACAGCAGCTTTCGTCGGCTGCTCAAGACCATATCCAAC
GGTTGAGCAAGATTTCGGAAAAGTGGGCAGTCGAAAGTGAGAAAAAGGAGCGCACGCGCGAGGTTG
TGTTTGTGTGGATGGATGGGAGACGGTGGGGACGGTGGTTGAAGAATATGTATGGTATCAAGGATGT
GACGATGTCTGGTGGCGTTGGGCTCGTCGTTGTTGATCATGGAAGCTTGCTTTACTATGACTCTGAC
GATCATGGAAAGCCTCTTGTGCTTAGCGAAGAGTCGGTGTGGTCGACGTTGCGTGCGATTTATGATG
GCAAGGCGAAGCCACGCCATAGCGAAAACTTGTTTGAGCGGATAGCAAGGAGTGTGAATAATCGTCT
CATGGGACTGGAGACGTTTGTGAGAGAGAATGTCAAGACGACAGTCTTCCTTGGTGTCATGTTCTTT
GCTTTTTGTTCATGTCATTCCGATGGTGCATCAACTCTGACTTGCCAAACGACTATTATGATGGCGAA
AAGCGCGAGGACCGACGGTTGGACTAGTCTGTTGTGTACTGTATTGGATTTTGTTTACCACTGTCGG
TGTGAGTGGTGA

***PIIN_10506* (*K48*)**

ATGAAGGAATTCTTCAAATGGACTCGGAAAGGAGAGGAGAAAAAGAAACAATATTCAGATTTACACAG
TGCCTCGTTGGGTTCAACCCCGACTACCGTAACCACCGTCGCTGGTGAATCTAACAGCGCCGCTGCT
TCAAGCATCACCGATACAAACTCTGCAAGTGAGCCGACAAGGGTGCACAAGCGCATTCGTGGGTTGA
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ERKLÄRUNG

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Gießen, im August 2011

Anna Klute

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