Molecular studies on compatibility in the mutualistic plant root-*Piriformospora indica* interaction

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Contents

List of Abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
ACO	ACC-oxidase
ACS	ACC synthase
Avr	Avirulence
BAK1	BRI1-associated kinase 1
Bgh	*Blumeria graminis* f.sp. *hordei*
BI-1	Bax Inhibitor -1
CIP	Calf intestine phosphatase
CSPs	Cold-shock proteins
CTR1	Constitutive triple reponse 1
DEPC	Diethylpyrocarbonate
E1	Ubiquitin activating enzymes
E2	Ubiquitin conjugating enzymes
E3	Ubiquitin ligase
EF-Tu	Elongation factor thermo-unstable
EIN2	Ethylene insensitive 2
ERS1	Ethylene response sensor 1
EST	Expressed sequence tag
ET	Ethylene
ETI	Effector-triggered immunity
ETR1	Ethylene resistant 1
ETS	Effector-triggered susceptibility
Flg22	A 22-amino-acid-long peptide derived from flagellin
FLS2	FLAGELLIN SENSING 2
HR	Hypersensitive Response
IAA	Indole-3-acetic acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
ISR	Induced systemic resistance
JA	Jasmonic acid
LPS	Lipopolysaccharides
LRR	Leucin-rich-repeat
MAMP	Microbe-associated molecular pattern
MAP	Mitogen activated protein
NF	Nod factor
NLP	Nep1-like protein
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PDB	Protein Data Bank
PR	Pathogenesis related
PRR	Pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP-triggered immunity
R-gene	Resistance gene
ROS	Reactive oxygen species

SA	Salicylic acid
SAR	Systemic acquired resistance
TAP	Tobacco acid pyrophosphatase
TTSS	Type III secretion system
Ub	Ubiquitin

1. Introduction

1.1 Plant-microbe interactions

Plants are being attacked by various microorganisms that aim to acquire adequate amount of nutrients from them. As a result of these interactions, the outcome can be neutral, harmful (as in the case of parasitism), or beneficial (in the form of mutualistic symbiosis) to the host (Shen et al., 2006; Thrall et al., 2007). In mutualistic associations, the interaction state between plant and microbes is thought to be well-balanced. However, even during these types of interactions, disease can be observed or host defense reactions are activated in unbalanced associations (Kogel et al., 2006). During evolution, plants have developed certain mechanisms to defend themselves against the attacking pathogens/herbivores. These responses to pathogen/herbivore invasion represent mostly targeted responses in which only those genes and pathways are activated that are required to arrest the invader, whereas others might be suppressed for conserving their resources (Glazebrook, 2005, Koornneef and Pieterse, 2008). Plant defense strategies were evolved to inhibit the growth of pathogens and mostly depend on the invading pathogen life style. For instance, biotrophic pathogens keep their host alive and feed on living tissues, such as viruses, some fungi (e.g. *Golovinomyces orontii*), oomycetes (e.g. *Peronospora parasitica*), and bacteria (e.g. *Pseudomonas syringae*). Necrotrophic pathogens such as the fungi *Botrytis cinerea* and *Alternaria brassicola* or the bacterium *Erwinia carotovora* often produce toxins or tissue-degrading enzymes to overwhelm the plant defense system and to promote the quick release of nutrients (Agrios, 2005; Rojo et al., 2003; Staples, 2001). Some pathogens even have an initial biotrophic phase of infection but become necrotrophic during later interaction stages and are so-called hemibiotrophic pathogens (Glazebrook, 2005; Perfect and Green, 2001). The ability of a plant to activate defense responses in order to minimize the damage or alleviate stresses partly relies on plant hormones especially salicylic acid (SA), jasmonates (JA) and ethylene (ET) as these hormones orchestrate a complex and interactive network of defense signaling pathways (Bostock, 2005; De Witt, 2007; Koornneef and Pieterse, 2008; Thomma et al., 2001). Other plant hormones, including abscisic acid (Asselbergh et al., 2008; De Torres-Zabala

et al., 2007), gibberellins (Navarro et al., 2008) and auxin (Navarro et al., 2006; Wang et al., 2007), have been reported to have an impact on defense mechanisms in different plant-microbe interactions (Robert-Seilaniantz et al., 2007). Generally, it was stated that pathogens with a biotrophic lifestyle are more sensitive to SA-mediated defenses, whereas necrotrophs and herbivorous insects are sensitive to JA/ET-dependent defense responses (Nimchuk et al., 2003, Glazebrook, 2005). As a result of the negative interaction between SA and JA signaling pathways, activation of the SA response should render a plant more susceptible to attackers that are resisted via JA-dependent defenses, and vice versa. The salicylate response was frequently deployed to protect plants against biotrophic pathogens and is often associated with the induction of a programmed cell death, the so-called hypersensitive response (HR) (McDowell and Dangl, 2000). The association between the salicylate response and the HR may limit the utility of this set of responses against necrotrophic pathogens, which draw nutrients from the host cells that they have killed prior to colonization (Cohn et al., 2001; Thomma et al., 2001).

1.2 Plant immune system

Plant survival depends on its ability to recognize, anticipate and respond to microbal challenges. There are several strategies used by plant to reduce the damage caused by pathogen attack including: structural, chemical, and protein-based defense mechanisms. Generally, plants exploit two types of defense responses to combat pathogens, constitutive (preformed) and induced (activated) responses. The preformed defense mechanisms include structural/physical barriers and biochemical reactions to delimit pathogen attachment, invasion and infection (Agrios, 2005; Buchanan et al., 2000). Mainly, the plant cell wall appears to be a major line of defense against fungal and bacterial pathogens (Hückelhoven, 2007a). During evolution, plants have developed active defense mechanisms by sensing pathogen attack and respond with inducible defense mechanisms, which provide more efficient ways to protect themselves (Agrios, 2005; Buchanan et al., 2000). This is achieved by a complex of signaling networks engaged in recognition, signal transduction and synthesis of antimicrobial proteins against invading pathogens (Agrios, 2005; Nürnberger et al., 2004).

During evolution, plants and microorganisms have established complex mechanisms of defense and attack, respectively, meeting in the development of the innate immune system of plants and virulence factors of pathogens. The most recent model of plant resistance, the zig-zag model, illustrates the process of co-evolution as shown in Fig. 1 (Jones and Dangl, 2006). Plants possess two highly effective layers of defense. The first layer of plant defense is activated after perception of pathogen-associated molecular patterns (PAMPs) by the plant, resulting in PAMP-triggered immunity (PTI). In contrast, well-adopted pathogens disrupt the first layer of defense by transferring virulence factors (also called effector proteins) into host cells to prevent activation of PTI and to establish plant susceptibility. A process defined as effector-triggered susceptibility (ETS). The second major layer of induced defense, known as effector triggered immunity (ETI), involves the specific recognition of pathogen effectors, directly or indirectly, by plant resistance proteins (*R*-gene mediated resistance) and results in a coordinated activation of defense reactions (Jones and Dangl, 2006). The elicitation of strong and rapid immune responses due to ETI is often associated with a HR (Jones and Dangl, 2006).

1.2.1 Plant basal defense

Plant basal defense (also called PTI) is immediately available to combat invading pathogens as it is activated after pathogen recognition (Ausubel, 2005; Nürnberger et al., 2004). As described before, pathogens as well as non-pathogens are triggering basal defense due to the widespread presence of molecular components, the so called microbe or pathogen-associated molecular patterns (MAMPs/PAMPs). MAMPs are structural, highly conserved microbial molecules, which are recognized by plant receptors and activate efficient innate immune responses by distinguishing between self and non-self molecules (Göhre and Robatzek, 2008; Schwessinger and Zipfel, 2008). MAMPs/PAMPs are including bacterial flagellin, cold-shock proteins (CSPs), lipopolysaccharide (LPS), bacterial elongation factor-Tu (EF-Tu), fungal glucans, chitin and oomycete elicitor INF1 (Chisholm et al., 2006; Kamoun et al., 1997; Nürnberger et al., 2004). In addition to MAMPs, plants also perceive breakdown products of their own cell wall that are released by hydrolytic activities of the invading plant microbes (Mackey and McFall, 2006).

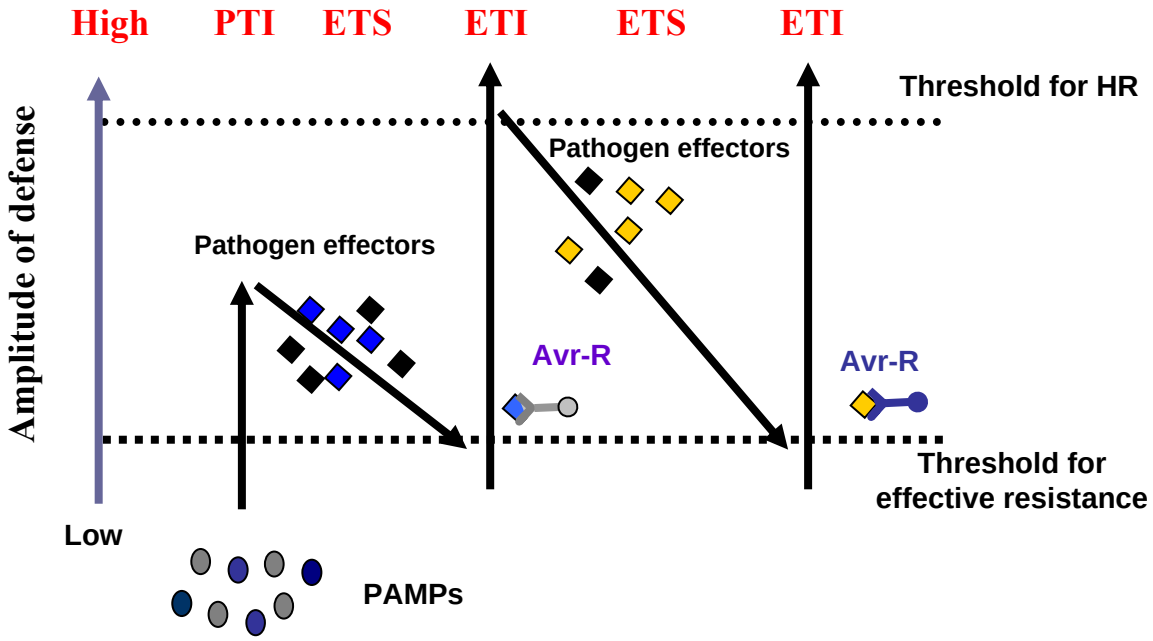

Figure 1: The proposed Zigzag model explains the processes of co-evolution meeting in the establishment of plant immunity (modified after Jones and Dangl, 2006) Firstly, plants recognize pathogen-associated molecular patterns (PAMPs) and in response to it PAMP-triggered immunity (PTI) is induced to stop further pathogen invasion. In a second step, well-adopted pathogens promote virulence by delivering effectors that interfere with PTI, resulting in effector-triggered susceptibility (ETS). In a third step, direct or indirect perception of pathogen effectors by R proteins leads to disease resistance, defined as effector-triggered immunity (ETI). In a fourth step, pathogens secrete another set of effector molecules to suppress ETI reestablishing ETS. Eventually, the plant surveillance system regenerates new *R*-gene that recognizes these effectors in order to regain ETI. The figure is taken from Jones and Dangl (2006).

The conserved plant plasma membrane localized receptor proteins, so called pattern recognition receptors (PRRs) are involved in MAMP recognition and activation of the plant innate immune system (Meyers et al., 2003; Nürnberger et al., 2004; Zipfel et al., 2004). MAMP recognition is characterised by the alteration of cytoplasmic calcium contents, generation of signaling molecules like nitric oxide (NO), reactive oxygen species (ROS) such as superoxide ions ($O_2^-\bullet$), hydrogen peroxide (H_2O_2), activation of MAP kinase cascades that eventually results in the induction of transcription factors leading to the synthesis of antimicrobial proteins and peptides (Asai et al., 2002; Bittel and Robatzek, 2007; Nürnberger et al., 2004; Torres and Dangl, 2005). One of the best characterized bacterial MAMP is flagellin. Recognition of flagellin in plant is achieved

by FLS2 (FLAGELLIN-SENSING2), a plasma membrane receptor-like kinase with extracellular leucine-rich repeat (LRR-RLK) (Gómez-Gómez and Boller, 2002). FLS2 directly binds to the 22 amino acid flagellin epitope flg22 (Zipfel and Felix, 2005). Flagellin was shown to induce plant defense responses, such as an oxidative burst, ethylene production, callose deposition, medium alkalinization in cell suspension cultures and induction of pathogenesis-related (PR) proteins (Felix et al., 1999; Gómez-Gómez and Boller, 2000). The impact of FLS2 on basal defense is highlighted in *fls2* mutant plants, which are more susceptible to the bacterium *P. syringae* (Zipfel et al., 2004). Another well-characterized MAMP is the bacterial elongation factor-Tu (EF-Tu), which is directly recognised by the EF-Tu plant receptor EFR (Zipfel et al., 2006). Interestingly, EF-Tu as well as flagellin could rapidly stimulate the association of FLS2 and EFR to another receptor like protein kinase, BAK1 (brassinosteroid-receptor-associated kinase 1). BAK1 was previously known as the co-receptor of Brassinosteroid insensitive 1 (BRI1), a receptor of the plant growth-promoting brassinosteroids (BRs) (Chinchilla et al., 2007). BAK1 silenced plants are compromised in responses to diverse MAMPs like flg22, CSPs and oomycetes INF1, suggesting that BAK1 also regulates the function of other PRRs (Heese et al., 2007; Shan et al., 2008). Importantly, microarray analyses revealed that the diverse MAMPs induce rapid but overlapping changes in gene expression (Zipfel et al., 2006). A significant overlap in gene expression has also been found between PTI and ETI indication that ETI includes magnified features of the PTI response (Zipfel et al., 2006).

1.2.2 *R*-gene mediated resistance

Many well-adopted pathogens can overcome plant basal defenses to promote the release of nutrients. Therefore, the second line of active defense known as *R*-gene mediated resistance or ETI is mounted by plants against those pathogens (Dangl and Jones, 2001; Jones and Dangl, 2006). This kind of resistance is typically detectable after the basal defense and is very restrictive to the growth of a pathogen (Fig. 1). As described in section 1.2, R proteins recognize the effector-virulence target complex, resulting in the activation of the HR (Nimchuk et al., 2003). R-mediated resistance can be activated through the recognition of effectors either by direct physical interaction (ligand-receptor

model) between R proteins and Avr proteins or via indirect perception of effectors by R proteins which has been described by the guard hypothesis (Dangl and Jones, 2001; Jia et al., 2000).

1.2.3 Non-host resistance

Non-host resistance (NHR) (general or basic resistance) is defined as resistance of all cultivars of a plant species against all genotypes of a pathogen species even under the most favorable conditions for disease development (Heath, 2001). It represents the most robust and durable plant resistance in nature and occurs at early interaction stages of pathogen infection and is characterised by failed penetration attempts or cell death (Heath, 1981). NHR may be due to preformed or inducible defense responses, but may also reflect lack of host compatibility or absence of pathogen virulence factors (Heath 2001; Li et al., 2005). Three *Arabidopsis* loci, designated *PEN1*, *PEN2* and *PEN3* were identified that are necessary for efficient cell wall penetration resistance against a non-host pathogen (*Blumeria graminis* f.sp. *hordei*) (Lipka et al., 2005; Nürnberger and Lipka, 2005). During evolution, an inappropriate or non-host pathogen must become insensitive to or must suppress or fail to elicit basal defenses in order to cause disease on a new host (Göhre and Robatzek, 2008).

1.2.4 Induced resistance

Induced resistance is thought to protect plants against the systemic spread of invading pathogens. As a result of systemic resistance, pathogenesis-related (PR) proteins with direct defensive roles often accumulate in both pest/pathogen-challenged and unchallenged (systemic) tissue of the same plant (Thomma et al., 2001). Systemic resistance can be divided into two main groups. Firstly, systemic acquired resistance (SAR), which builds up locally or systemically against a broad range of viral, bacterial and fungal pathogens via a SA-dependent pathway and is associated with local and systemic induction of PR proteins (Durrant and Dong, 2004; Maleck et al., 2000). The second type is induced systemic resistance (ISR), which is activated by root-associated non-pathogenic bacteria in the plant (Pieterse et al., 1998; van Loon, 1997). ISR depends on JA/ET pathways that function through a SA-independent manner and results in the

production of antimicrobial compounds (Pieterse et al., 1998; Van Loon et al., 1998). ISR is accompanied with accelerated, enhanced, or potentiated response to pathogens challenged host prior to its establishment (Conrath et al., 2002). ISR is as efficient as SAR. Nonexpressor-of-PR1 (NPR1) is an essential regulator for the successful establishment of both SAR and ISR. However NPR1 has a central impact on SAR but its impact on ISR is partly. Interestingly, plants expressing both types of induced resistance have not shown to elevate *NPR1* transcript levels, indicating the constitutive level of NPR1 is sufficient to facilitate simultaneous expression of SAR and ISR (Pieterse and van Loon, 2004).

1.3 Compatibility in plant-microbe interactions

To establish a compatible interaction, symbionts as well as pathogens must be able to overcome or manipulate hosts surveillance system for their own benefit (Pieterse and Dicke 2007; Robert-Seilaniantz et al., 2007). In different cases, different strategies have been used, like disturbing plants' defense signaling networks or even reprogramming host metabolism such as modifications on hormonal homoeostasis and antioxidant contents (Göhre and Robatzek, 2008; Robert-Seilaniantz et al., 2007). In general, plant hormones can quickly and potentially affect plant physiology. Therefore, it is not surprising that pathogens manipulate plant hormone signaling to promote disease. There is mounting evidence that plant pathogens either produce plant hormones or disturb host hormone signaling networks to mediate host susceptibility. SA, JA and ET are known to be involved in plant defense response but have also been reported to play a role in disease progression (Cohn and Martin, 2005). For instance, the effector proteins AvrPto and AvrPtoB have been shown to enhance the expression of the ET-forming enzyme ACC oxidase gene in susceptible tomato plants to induce late-onset cell death for a better access to nutrients or to improve dissemination in nature (Abramovitch *et al.,* 2003; Cohn and Martin, 2005; Jamir et al., 2004). Other specific strategies might be used by pathogens and symbionts. For instance, rhizobial Nod factors (NFs) are able to suppress salicylic acid accumulation and ROS production (Martínez-Abarca et al., 1998; Shaw and Long, 2003).

1.3.1 Microbial effector proteins

Since the plant innate immune system is very effective in stopping or at least restricting microbe invasion, successful pathogen relies on sophisticated mechanisms to overcome the plant surveillance system. As mentioned in sec. 1.2, microbes like bacteria, oomycetes and fungi secrete numerous effectors that presumably reprogram host cells in order to promote infection. Effectors (including *Avr* genes or AVR proteins) are defined as pathogen molecules that manipulate host cell structure and function, thereby facilitating infection and/or triggering defense responses (Hogenhout et al., 2009; Kamoun, 2006). Therefore, the term of effector is neutral and does not imply negative or positive consequences on the outcome of plant-pathogen interaction. Based on these ideas, effectors targets may contribute to host resistance or susceptibility. For instance, in susceptible plants, the interaction between effectors and targeted host protein results in molecular events that facilitate pathogen colonization, such as suppression of defense responses, and enhanced disease susceptibility. In resistant plants, the interaction between effectors and R-protein might lead to the activation of host defense and induction of HR, which finally eliminates the invading pathogens (Kamoun, 2006). To understand the function of secreted effector proteins it is necessary to identify their localisation. Generally two classes of effectors exists that target distinct sites in host plants. Apoplastic effectors are secreted into the plant extracellular space, while cytoplasmic effectors are characterized by their translocation inside the host cell (Kamoun, 2006; 2007). Gram negative bacteria (e.g *P. syringae*) deliver a battery of effector molecules inside living plant cells through type three secretion systems (TTSS) to subvert host metabolism and enhance pathogen fitness (Collmer et al., 2002; Grant et al., 2006). These effectors are actively suppressing the MAMP-mediated responses that are elicited either by TTSS-defective mutant bacteria or MAMPs (e.g. flg22) (Abramovitch et al., 2006; Chinchilla et al., 2006; He et al., 2007). *Arabidopsis* plants expressing the *P. syringae AvrPto* and *AvrPtoB* effector genes promote growth of non-pathogenic bacteria (TTSS deficient mutant bacteria) by suppressing callose deposition (Hauck et al., 2003). Deletion of AvrPto and AvrPtoB in bacteria reduces bacterial virulence in *Arabidopsis*. AvrPtoB exhibits ubiquitin ligase activity and targets host kinases for degradation by the proteasome, thereby suppressing ETI in addition to its PTI suppressing function

(Rosebrock et al., 2007). Hence, AvrPtoB suppresses two major layers of plant innate immunity, PTI and ETI (Alfano and Collmer, 2004; Mudgett et al., 2005; Rosebrock et al., 2007). No TTSS has yet been identified in eukaryotic plant pathogens, including hemibiotrophic or biotrophic fungi or oomycetes. Recently, remarkable advances have been made in the field of oomycete-associated effectors identification (Kamoun, 2006; 2007). Most of the oomycete effectors carry the RXLR (arginine, any amino acids, leucine and arginine) motif that is required for translocation into host cells (Birch et al., 2006; Whisson et al., 2007). Emerging findings indicate that several oomycete RXLR effectors suppress host immunity. For instance, *Phytophthora sojae* Avr1b, suppresses programmed cell death induced by the mouse protein BAX in yeast and plants (Dou et al., 2008). Gene clusters from *Ustilago maydis* encode secreted proteins and implicate the existence of over hundreds of small proteins with secretion signal in the *U. maydis* genome (Kämper *et al.,* 2006). However it is still unknown how these secreted effectors may shape the interaction after secretion into the host. Recently, Pep1 a secreted effector protein of *U. maydis* was characterised to be required for successful invasion of the fungus in the host cells (Doehlemann et al., 2009).

1.3.2 Plant compatibility factors

Mounting evidence indicates that the host plays an active role in the development of symbionts/pathogens. Apart from possible function of effectors in manipulating host metabolism, loss of a host factor may cause an altered physiological state that indirectly hampers microbial pathogenesis. The lack of host gene products might impact fungal development by altering cell signaling networks and nutrient exchange between the host and symbionts/pathogens (Toyoda et al., 2002). Thus, lack of essential host factors, could in principle lead to resistance against a given pathogen species without the constitutive activation of plant defence responses. The barley mildew resistance locus O (MLO) protein is well known as a plant compatibility factor for the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*). MLO is required for the successful entrance of *Bgh* spores into epidermal cells in barley. MLO-mediated defense suppression in barley seems rely on an interaction with calmodulin (Kim et al., 2002). Another example is BAX Inhibitor-1 (BI-1) proteins which belong to the small group of conserved

suppressors of PCD in mammals and plants (Hückelhoven, 2004). Overexpression of BI-1 in barley was sufficient to break both *mlo*-mediated resistances to *Bgh* and NHR to *B. graminis* f.sp. *tritici* (Eichmann et al., 2004; Hückelhoven et al., 2003). In addition, the small G-proteins from the RAC/ROP family are also influencing susceptibility of barley to *Bgh* (Schultheiss et al., 2003). In Arabidopsis, the loss-of-function mutation in the *Enhanced Disease Resistance1* (*EDR1*) gene confers, resistance to infection by powdery mildew fungi (e.g. *Golovinomyces cichoracearum*) (Vogel and Somerville, 2000).

1.4 Beneficial fungal microorganisms

Soil represents a favorable environment for a wide range of microorganisms including algae, bacteria, and fungi. Almost all the chemical changes that take place within the soil involve the active contribution of soil microflora. They are mainly participating in carbon and nitrogen cycling, nutrient acquisition and soil formation, processes which are necessary for plant growth and survival. In contrast, plants can have profound effects on soil microbial communities especially those colonizing the rhizosphere. This is because of the great carbon input in soils by plant root exudates. On the other side, plants are immobile organisms that are often confronted with unfavorable conditions (e.g. salinity, drought, pathogen attacks). Therefore, to evade abiotic and biotic stresses, one strategy is to establish associations with beneficial microbial organisms (Lum and Hirsch, 2003). However, one of the most complex tasks for a plant is to distinguish between mutualistic partners and parasites (Kogel et al., 2006; Schulz and Boyle, 2005), especially in view of the fact that symbiotic and parasitic interactions share many common signaling pathways (Paszkowski, 2006).

1.4.1 Mycorrhizal fungi

Mycorrhiza refers to symbiotic association between plant and fungi and approximately 80% of terrestrial plants have mycorrhizal relationship in their natural habitats (Harley, 1989). Symbiotic relationships are thought to exist since at least 400 million years (Rodriguez and Redman, 2008). The symbiotic association is characterized by the translocation of sugars and other compounds from the plant to the fungus, and in turn, the fungus facilitates the acquisition of mineral nutrients from the soil to plants. Improving

nutrient and water uptake, plus enhancing the level of resistance to a wide range of soil borne diseases and tolerance against extreme environmental conditions are the distinguishing characters of mycorrhization (Smith and Read, 1997). However, at situations the plant gets enough water and appropriate nutrients, symbiotic associations with plants are strongly reduced. The most common group of mycorrhizas are ectomycorrhiza (ECM) which are mostly Basidiomycetes that are colonizing the outer layer of root cells and grow through the root cortical cells of many tree species, forming a so-called Hartig's net (Kottke and Oberwinkler, 1987). Arbuscular mycorrhiza (AM) belong to the new fungal class Glomeromycota, which are forming highly branched intracellular structures within cortical root cells of many herbaceous and woody plant species called arbuscules (Hibbett et al., 2007). The fine crosstalk between fungi and host plants is necessary for mycorrhizal establishment and is indicated by a highly regulated exchange of compounds and signals between the two partners (Harrison, 2005; Paszkowski, 2006). Diffusible molecules released by mycorrhizal fungi are perceived by the plant even in the absence of physical contact as evidenced, for example, by a rapid and transient intracellular calcium increases (Navazio et al., 2007).

AM fungal spores can germinate and grow in the absence of a host; however, the hyphal growth is limited. In the presence of host roots, AM hyphae perform extensive branching to increase the chances to find host roots (Harrison, 2005). The strigolactone 5-deoxystrigol was recently identified in root exudates to induce hyphal branching in germinating mycorrhizal spores (Akiyama et al., 2005). Afterwards, the fungal hypha differentiates into a hyphopodium from which a penetration peg is formed at the root surface to enter the rhizodermis (Harrison, 2005). The initial AM infection is preceded by complex intracellular remodeling involving transcellular nuclear migration associated with the formation of a transient cytoplasmic assembly comprising cytoskeletal and endoplasmic reticulum (ER) components (Harrison, 2005; Genre et al., 2005; Parniske, 2004). This specialized cytoplasmic assembly, which has been termed the pre-penetration apparatus (PPA), defines the subsequent transcellular path of apoplastic AM infection and is thought to be responsible for the future intracellular path for fungal penetration (Genre et al., 2008; Parniske, 2008). The AM fungus progresses through the inner root cortex and

spreads intercellularly along the longitudinal axis of the root and develops arbuscules at the site for nutrient exchange (Genre et al., 2005; Parniske, 2008).

Several plant genes are required for AM development such as a *Symbiosis Leucine-Rich Repeat Receptor Kinase* (*SYMRK*), Does Not Make Infection genes (*DMI1, DMI2*, and *DMI3*); two predicted cation channels, *CASTOR* and *POLLUX*; two nucleoporins, *NUP85* and *NUP133*, which are required for calcium spiking (Kanamori et al., 2006; Kosuta et al., 2008; Parniske, 2008). *DMI1*, *DMI2*, and *DMI3* are involved in a common signaling pathway that implicated in the establishment of both mycorrhizal and bacterial endosymbioses (Catoira et al., 2000). The encoded proteins have been shown to participate in the initial transduction of the rhizobial Nod factors (NFs) signal in root hairs (Oldroyd and Downie, 2008).

1.4.2 Endophytic microorganisms

Endophytes are defined as microorganisms that accomplish parts of their life cycle within living host tissues without causing apparent damage to the plant (Schulz and Boyle, 2005). Endophytic bacteria and fungi have been isolated from different surface-sterilized plant sources like seeds, roots, stems, and leaves of various "symptomless" plant species (Brundrett, 2004; Carroll, 1998). Many endophytes are reported to be able to fix nitrogen or to enhance phosphorus uptake as well as plant growth and development (Bacon and Hill, 1996; Boddey et al., 2003). Endophytes may provide protection and enhance survival of their host resulting in enhanced stress-, insect-, and disease resistance as well as in improved yield (Schulz and Boyle 2005). The role of endophytes in terms of plant growth promotional effects has received increasing attention for providing a consistent and effective increase in the productivity of crops.

1.4.3 *Piriformospora indica*

P. indica was isolated from the Indian Thar Desert and soon after recognized as a plant root symbiont (Verma et al., 1998). *P. indica* colonizes a wide range of monocot and dicot plants. Even members of the *Brassicaceae* (e.g. *A. thaliana*) and *Chenopodiaceae*, known to be non-host plants of mycorrhiza, are interacting with *P. indica* (Pham et al., 2004b; Peškan-Berghöfer et al., 2004). However, *P. indica* transfers several benefits to

colonized plants like a better tolerance to various biotic and abiotic stresses, as well as improved plant fitness by increasing growth performance under normal and stress conditions (Schäfer et al., 2007; Waller et al., 2005). In this respect, the contribution of *P. indica* symbiosis to improve plant drought and salinity tolerance might point towards the natural habitat of its desert origin (Baltruschat et al., 2008; Sherameti et al., 2008; Waller et al., 2005). These data assigned *P. indica* as a novel mutualistic symbiont in comparison to known mycorrhizas and root-nodulating bacteria.

1.4.3.1 Phylogeny of *Sebacinales* ordo *nov.*

P. indica has been assigned as a member of the order Sebacinales within the Hymenomycetes. So far, all members of this order are involved in mycorrhizal associations encompassing ecto-, orchid-, ericoid-, cavendishioid- and jungermannioid mycorrhizas with plants (Setaro et al., 2006; Weiß et al., 2004). Based on the recent phylogenetic analyses, the beneficial fungal order *Sebacinales* are distributed over two distinct groups. Basidiomes and sebacinoid mycobionts are catagorised as group A, which harbors ectomycorrhizas and orchid mycorrhizas (Weiß et al., 2004). Group B represents a heterogeneous group concerning the type of mycorrhizal associations and contains ericoid-, cavendishioid-, and jungermannioid mycorrhizas. Phylogenetic analysis classified *P. indica* as well as a number of *Sebacina spp* in the latter group (Setaro et al., 2006; Weiß et al., 2004). *Sebacina vermifera* were isolated mostly from the Australian orchids and like *P. indica* transfer several beneficial characters to the colonized host. Morever, *P. indica* has got the ability to grow axenically (Deshmukh et al., 2006; Verma et al., 1998; Warcup, 1988,). *P. indica* is able to interact with orchids and forms hyphal coils within cortical cells in orchid roots similar in morphology to pelotons from orchid mycorrhizas (Blechert et al., 1999). As shown in Fig. 2, a similar structure to hyphal pelotons is observed in *Arabidopsis* plants colonised with *P. indica* (Schäfer and Kogel, 2009).

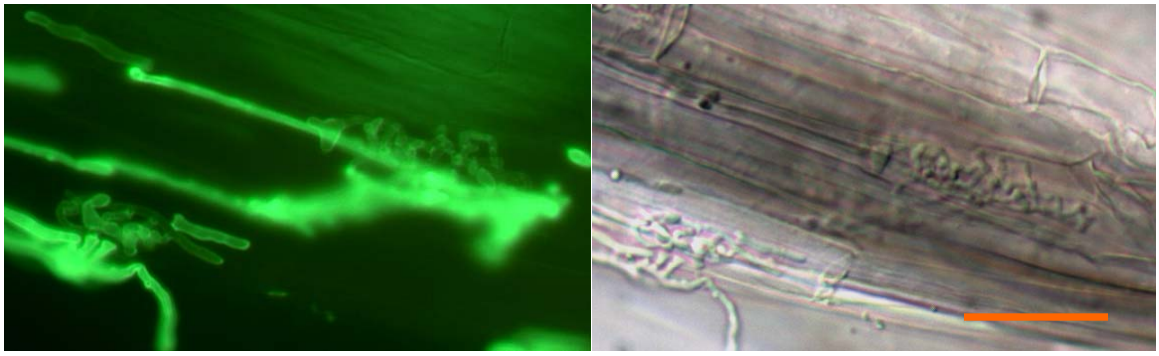

Figure 2: Coiled-like hyphae reminiscent of pelotons of orchid mycorrhizas are formed in *Arabidopsis* plants colonised with *P. indica* at 7 dai. The fungus was stained with wheat germ agglutinin-Alexa Fluor 488 (WGA-AF 488) and visualised by fluorescence microscopy. Bar = 20 µm (Schäfer and Kogel, 2009).

1.4.3.2 Bacteria associated with *P. indica*

P. indica forms intimate associations with the α-proteobacterium *Rhizobium radiobacter*, (Sharma et al., 2008). The isolated bacteria mediate growth promotion and systemic resistance against *Bgh* in barley similar to those conferred by *P. indica* (Sharma et al., 2008). However, quantitative analysis of bacteria associated with *P. indica* using QPCR and microscopic analyses revealed less number of bacteria associated with *P. indica* hyphae (Sharma et al., 2008). In this respect, a clear function of bacteria for the symbiotic interaction and the beneficial effects has yet to be defined.

1.4.3.3 Biological activities mediated by *P. indica*

P. indica is similar to AM fungi in terms of plant growth promotional effects. However, in contrast to AM fungi, *P. indica* has the potential to grow axenically without the requirement of living hosts (Verma et al., 1998). The ability of *P. indica* in improving the growth rate of various host plants is well documented (Pham et al., 2004b; Varma et al., 1999; Waller et al., 2005). It also has stimulatory effects on adventitious root formation in ornamental stem cuttings. However, the exact nature of plant growth promotional effects is still unclear (Druege et al., 2007; Pham et al., 2004a). *P. indica* was reported to activate nitrate reductase that plays a major role in nitrate acquisition and also a starch-degrading enzyme, glucan-water dikinase, involved in early events of starch degradation in the plants such as tobacco and *Arabidopsis* (Sherameti et al., 2005). On the other side,

improvement of plant performance in response to biotic and abiotic stresses have also been widely documented following colonisation by beneficial microorganisms and are considered as promising means to achieve sustainable agricultural production.

P. indica also conferred resistance against biotic stresses (Deshmukh and Kogel, 2007; Pham et al., 2004b; Waller et al., 2005). *P.* indica-colonised barley showed higher tolerance to the necrotrophic root pathogens *Fusarium culmorum*, *Cochliobolus sativus* (Waller et al., 2005), and *Fusarium graminearum* (Deshmukh and Kogel, 2007). Co-infection of barley plants with *P. indica* and *F. culmorum* showed reduced disease symptoms followed by increasing shoot and root biomass compared to non-inoculated plants (Waller et al., 2005). Data collected from both greenhouse and field experiments showed the reduction in symptom severity caused by stem rot (*Pseudocercosporella herpotrichoides*) and root rot (*Fusarium culmorum*) in wheat (Serfling et al., 2007). These evidences make *P. indica* a promising candidate for biological control of plant diseases. In addition to an enhanced local resistance in roots, *P. indica* has been reported to induce systemic resistance against the biotrophic leaf pathogen *Bgh* by reducing both frequency and size of powdery mildew pustules (Waller et al., 2005). However, the molecular mechanisms and the signaling pathways mediating such a protective effect in barley against *Bgh* is still unknown. In *Arabidopsis*, *P. indica* systemically protects leaves against powdery mildew caused by *Golovinomyces orontii* via the ISR pathway which is based on JA signaling and the cytoplasmic function of NPR1 (Nonexpressor-of-PR1) (Stein et al., 2008).

1.4.3.4 Plant root colonisation by *P. indica*

P. indica's lifestyle and the mechanisms underlying root colonisation have been partly uncovered from its associations with barley and *Arabidopsis* (Deshmukh et al., 2006; Schäfer and Kogel, 2009). In general, symbionts are colonizing rhizodermal and cortical cell layers of roots (Bolwerk et al., 2005; Parniske, 2000). Root colonisation by *P. indica* starts after chlamydospore germination followed by penetration of rhizodermal cells. In addition, the fungus inter- and intracellularly colonizes the root cortex, followed by fungal proliferation, and eventually, intracellular spore formation (Fig. 3). *P. indica* growth seems to be actively controlled by host plant (Deshmukh et al., 2006) and the

meristematic zone is protected from fungal colonisation (Deshmukh et al., 2006). *P. indica* penetration, colonization, proliferation and spore formation are fulfilled in the root elongation and maturation zones (Deshmukh et al., 2006).

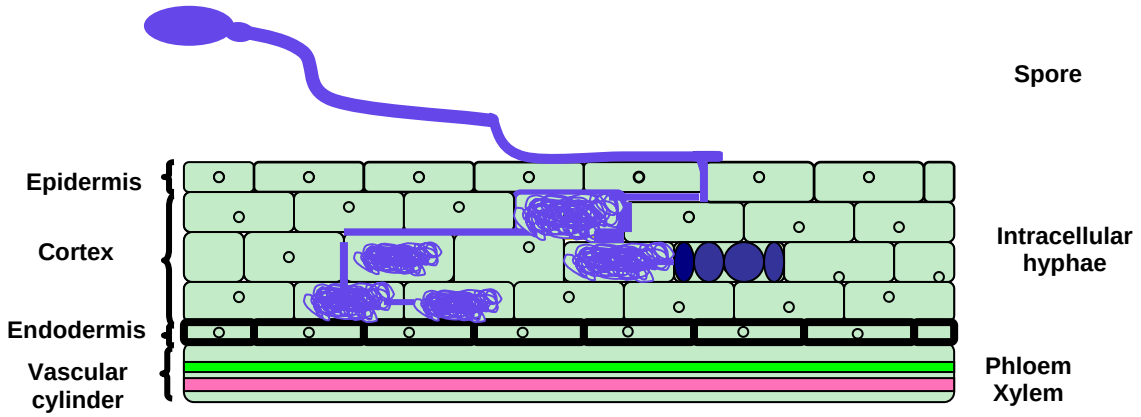

Figure 3: Schematic picture showing the processes of *P. indica* colonization of plant roots. After spore germination, the fungus penetrates epidermal cells and spreads quickly throughout the tissue and grows both inter- and intracellularly, follows by spore formation. The root endodermis and central cylinder is not colonised by the fungus.

Recently, *P. indica* has been found to colonize *Arabidopsis* roots by an initial biotrophic phase that is followed by cell death dependent phase. *P. indica*-colonised plant roots do not show disease symptoms like brown necrotic lesions (Deshmukh et al., 2006). Programmed cell death (PCD) is a mechanism by which multicellular organisms self-destruct superfluous or impaired cells e.g. as response to regular development or pathogen attack (Imlay and Linn, 1998). Some aspects of plant PCD are similar to apoptosis in animal and share common features, among these, chromatin condensation, cell shrinkage, DNA fragmentation followed by breakdown of the nucleus (Heath, 1998). Some of these PCD hallmarks (e.g. DNA fragmentation, cell shrinkage) have also been observed in roots during *P. indica* colonisation (Deshmukh et al., 2006). The requirement of host cell death for *P. indica* colonisation and proliferation was cytologically and genetically evidenced. Barley plants overexpressing the negative cell death regulator Bax Inhibitor -1 was shown to reduce invasion and proliferation of *P. indica* (Deshmukh et al., 2006).

1.5 Objectives

During fungal colonisation, numerous modifications at the cell wall or apoplast of host cells and tissue are happened. It is known that plant colonizing microbes secrete proteins during host colonisation and even translocation proteins (called effectors) into host cells in order to reprogram host physiology and modulate plant defense mechanisms and, hence, confer compatibility. However, physiological and molecular aspects of these alterations are mostly unknown. In order to understand theses physiological and molecular phenomena occurring during *P. indica* colonisation, a screening assay based on the yeast signal sequence trap (YSST) method was used for trapping genes carrying signal peptides and, therefore, putatively encode extracellular signaling molecules, transmembrane proteins and secreted proteins (e.g. effectors). Therefore, it was the aim to identify sets of secreted proteins of barley and *P. indica* during their association and to analyse their impact on the symbiotic interaction.

The second central interest of my project was to identify plant factors essential for *P. indica* colonization that might explain the wide host range of the fungus. Therefore, a novel subtractive hybridisation-based method, called Transcription Subtractive Hybridisation (TSH), was successfully established. As an outcome of procedure, gene redundancy was remarkably reduced and house-keeping genes were almost removed. Interestingly, the subtracted library was found to contain genes involved in ethylene metabolism (ET) and polyamine synthesis. Subsequent functional studies were performed to analyse the significance of ET and polyamine for plant root colonisation and to unravel to what extent these plant factors might explain *P. indica* compatibility to a multiplicity of host plants.

2 Materials and Methods

2.1 Plant materials and fungal inoculation

Barley seeds of cultivar Golden Promise were surface-sterilized with 70% ethanol for 5 min and with 6% sodium hypochloride (NaOCl) for 1.5 hours, and then rinsed thoroughly with sterile water. Two days after germination, barley seedlings were inoculated with *P. indica* spore suspension. Spore suspension was prepared by growing the fungus on complex medium (CM) plates for 3 weeks at 23°C. In order to remove the spores from the CM plates, 10-15 milliliters of sterilized tap water containing 0.05% Tween-20 was added on the surface. The surface was gently scratched with a spatula until the spores were released. Spore suspension was filtered through miracloth (Calbiochem, Bad Soden, Germany) to remove the mycelium, and the filtrate containing spores was transferred to 50 ml tubes. Spores were washed three times with dH_2O containing Tween-20 and repeatedly vortexed and sonicated in order to get rid off spore aggregations. After each washing step, spores were collected by centrifugation for 7 min at 4000 rpm and were finally resuspended in dH_2O. After determining the spore density using a hemacytometer and a microscope, the spore concentration was adjusted to $\sim 5 \times 10^5$ spores per milliliter and used as inoculum. The control seedlings of barley were immersed in sterile water, whereas, mock seedlings were inoculated with by immersion in *P. indica* spore suspension and subsequlely by gentle shaking for two hours. Eventually, the inoculated and mock-treated plants were transferred into glass jars containing $PNM_{(1/10)}$, followed by incubation in a climate chamber (16/8 h, day/night cycle, 22/18°C, 60% relative humidity). For the Transcription Subtractive Hybridisation (TSH) assay, barley roots were harvested at 60 hours after inoculation (hai), while root samples were harvested for the yeast signal sequence trap (YSST) experiment at 48, 60, 72 and 96 hai. For the latter experiments, harvested root samples were pooled, frozen in liquid nitrogen and stored at -80°C.

2.2 Cyto-histological techniques

P. indica was stained with wheat germ agglutinin-Alexa Fluor 488 (WGA-AF488, Molecular probes, Eugene, USA) and the root cell walls were stained with congo-red.

The roots were fixed in chloroform/ethanol/trichloracetic acid (TCA) (1/4/0.15% v/v/w), then rinsed with distilled H_2O three times, boiled for 1 min in 10% KOH, and washed with phosphate-buffered saline (PBS) pH 7.4. Thereafter, roots were transferred to the staining solution containing 10 µg/ml congo-red, 50 µg/ml WGA-AF488, and 0.2% Silwet L-77 dissolved in PBS. After immersing the roots into the staining solution, vacuum infiltration was done by applying vacuum (50 mm Hg) for 3 times (1 min each). Finally, the staining solution was removed and the roots transferred to PBS. If the microscopic analyses were not performed immediately, the samples were stored at 4ºC (dark). The roots were microscopically analysed using an Axioplan 2 fluorescent microscope (Carl Zeiss, Jena, Germany). WGA-AF488 was excited at 488 nm and analysed at 505-530 nm (emission) and congo-red at 530–585 nm (excitation) and at 600 nm (emission).

2.3 RNA isolation, biotin-labeled cDNA synthesis and subsequent hybridisation for TSH assay

Total RNA was extracted from barley roots (with and without *P. indica*). The mRNA was subsequently isolated from total RNA using oligo $(dT)_{25}$ magnetic beads (Invitrogen, Oslo, Norway). Therefore, 75 µg total RNA was adjusted in 100 µl distilled Diethylpyrocarbonate (DEPC) treated water and hybridised with 100 µl binding buffer (20 mM Tris-HCl pH 7.5, 1 M LiCl, 2 mM EDTA). The RNA was heated for 2 min at 65°C to disrupt RNA secondary structures and immediately placed on ice. In the meantime, 200 µl of resuspended oligo $(dT)_{25}$ beads were transferred into a 1.5 ml tube. The vial was placed on a magnetic stand and after 30 seconds, the supernatant was discarded and the beads were washed once again. This was done by removing the tube from the magnet before the beads were resuspended in 100 µl binding buffer and by placing the tube back on the magnet. After 30 seconds, the supernatant was removed from the tube and kept away from the magnet. The total RNA was mixed with beads thoroughly and allowed to anneal by inverting the tube for 3-5 min at room temperature. The tube was placed on the magnetic stand again until the solution was cleared; the supernatant was removed, and finally washed twice with 200 µl washing buffer B (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA). Thereafter, the supernatant was

removed and 5 µl of 10 mM Tris-HCl pH 7.5 was added. After heating at 85°C for 2 min, the tube was immediately placed on the magnet and the eluted mRNA was quickly transferred to a new RNase-free tube. The quantity and integrity of mRNA were spectrophotometrically checked and electrophoretically separated, respectively, on denaturing 1.5% agarose-gel containing 5% formaldehyde. For the synthesis of biotin-labeled cDNA, 300 ng mRNA from mock-treated roots was mixed with biotin-labeled oligo (dT) primers (MWG-Biotech, Ebersberg, Germany) and heat-denatured for 10 min at 70°C followed by immediately chilling on ice. In order to obtain cDNA, mRNA was reverse transcribed using a SuperScript II cDNA Synthesis Kit (Invitrogen, Karlsruhe, Germany). Therefore, mRNA was denatured and immediately chilled on ice in a 12 µl reaction mixture containing 500 ng biotin-labeled oligo (dT) primers and 1 µl 10 mM dNTPs mix. After the addition of 4 µl of 5 x first-strand buffer, 1 µl (40 units) ribonuclease inhibitor, and 2 µl 0.1 M DTT, the reaction was preheated for 2 min at 42ºC before adding of 1 µl (200 units) of SuperScript II (Invitrogen, Karlsruhe, Germany). The reaction mixture was incubated for 60 min at 42ºC, followed by heat inactivation for 15 min at 70ºC. Then, single-stranded RNA was hydrolysed by adding 2.5 units RNase H (MBI Fermentas, Vilnius, Lithuania) and incubated for 30 min at 37°C. Subsequently, RNase H was heat-inactivated for 5 min at 90°C. After the synthesis of biotin-labeled cDNAs, the excessive biotins as well as small biotinylated cDNA fragments (unincorporated biotin) were removed through Microcon YM-30 columns (Millipore, Schwalbach, Germany). Therefore, the single-stranded biotin-labeled cDNA was cleaned by adding 160 µl washing buffer (10 mM TE, 0.15 M LiCl, 1 mM EDTA) before transfer to an assembled Microcon filter (YM-30) unit. The labeled cDNA was centrifuged for 5 min at 12000 rpm and washed twice by adding 200 µl washing buffer and spinning for 5 min at 12000 rpm. The biotinylated cDNA was recovered by inserting the filter unit upside down into a new tube and by spinning at maximum speed for 4 min. For binding of biotinylated cDNAs to streptavidin-conjugated magnetic Dynabeads M-280 streptavidin (Invitrogen, Oslo, Norway)**,** 100 µl streptavidin beads were washed twice with 100 µl 2 x binding buffer (20 mM Tris-HCl pH 7. 5, 1 M LiCl, 2 mM EDTA). The solution was incubated by gentle shaking for 20 min at room temperature and finally stored overnight at 4°C. Eventually, biotinylated single-stranded cDNAs (sscDNAs)

probes obtained from mock-treated roots was hybridised with mRNA from *P. indica*-colonized barley roots. Therefore, the biotin-labeled cDNA coupled to streptavidin beads was washed twice with 100 µl hybridisation buffer [2 x binding buffer plus 0.2% **Lithium** dodecyl sulfate (LiDS)], and resuspened in 50 µl hybridisation buffer to which 10 units RNAsin (Invitrogen, Karlsruhe, Germany) was added. In order to avoid unspecific subtraction by binding of cDNA to poly-A tails of mRNAs, 1 µl oligo $(dT)_{17}$ (0.143 mM) in addition to 10 units of RNAsin was added to 300 ng mRNA. For hybridisation, the two solutions (mRNAs isolated from *P. indica*-colonized root material and biotinylated sscDNA that was labeled with streptavidin-labeled magnetic beads) were separately heated at 70°C and mixed. An equal volume (75 µl) of heated (70°C) hybridisation buffer (20 mM Tris-HCl pH 7.5, 1 M LiCl, 2 mM EDTA, 0.2% LiDS) was added and then incubated for 15 min at 65°C by gentle shaking. The mRNA/cDNA hybrids were collected at the tube wall after transfer to a magnetic stand while the supernatant solution got enriched with non-hybridised mRNAs. These mRNAs were transferred to a fresh tube while mRNA/cDNA hybrids were discarded. This procedure was repeated three times and a final fraction of subtracted mRNAs was used to construct a cDNA library.

2.4 Generation of a subtracted cDNA library, cloning and sequencing

A full-length cDNA library was generated using the GeneRacer Kit (Invitrogen Life Technologies, Karlsruhe, Germany). The subtracted mRNA was dephosphorylated using calf intestine phosphatase (CIP) (provided with the kit) and treated with tobacco acid pyrophosphatase (TAP) (provided with the kit) to remove the 5′-cap structure from intact full-length mRNA. Thereafter, the GeneRacer RNA oligo-nucleotide (containing 5' adaptor) was ligated to 5' decapped mRNA. Full-length mRNA was reverse transcribed to cDNA by SuperScript II reverse transcriptase kit (Invitrogen, Karlsruhe, Germany) using Gene Racer oligo dT primer (containing 3' adaptor) (Tab. S1). To obtain a full-length cDNA library, PCR was performed using the GeneRacer 5′- and 3′-primers followed by nested PCR using the GeneRacer 5′- and 3′-nested primers (Tab. S1). Subsequently, the amplicons were cloned into pGEM-T vector (Promega, Mannheim, Germany) and introduced into *E. coli* using the heat shock method. To assess the performance of the

TSH method, 15 colonies were randomly selected. Subsequently, plasmids were isolated and subjected to PCR using vector-specific primers (M13 forward, M13 reverse, Tab. S5). The amplicons were separated by electrophoresis and sequenced.

2.5 Evaluation of the efficiency and accuracy of the TSH assay and YSST method

Quantitative PCR was used to determine the efficiency of TSH using *ubiquitin* as the plant housekeeping gene*, PR10* and *SAM synthetase 2* as *P. indica*-responsive plant genes and *P. indica* elongation factor 1-α gene (*Pitef*) (Bŭtehorn et al., 2000). mRNA from root material harvested at 60 hai before and after several subtractive hybridisation steps (1, 2, 3, 6 and 9 times) was used as template. Comparative expression profiles of 10 randomly selected genes were determined at several time points (48, 60 and 72 hai) in independent experiments encompassing sterile and soil conditions. Similarly, QPCR was applied for quantification of plant isolated genes as the result of YSST screening during barley-*P. indica* interaction. Ten ng of total RNA served as a template for QPCR analyses. Amplifications were performed in 20 µl SYBR green JumpStart *Taq* ReadyMix (Sigma-Aldrich, München, Germany) with 350 nM oligonucleotides, using a Mx3000P thermal cycler (Stratagene, La Jolla, USA) with a standard amplification protocol as follows: 40 cycles consisting of three steps, one cycle at 95°C for 10 min ("hot start") followed by 95°C for 30 s, specified annealing temperature with 5 s at 60°C with 18 s at 72°C. Fluorescent product was detected at the last step of each cycle. Amplification was followed by melting curve analysis using the program run for one cycle at 95°C without hold, 60°C with 10 s hold, and 95°C with fluorescence collection at 0.2°C intervals. Melting curves were used to determine the specificity of the PCR. A negative control without cDNA template was run with every assay to assess the overall specificity. To determine the relative gene expression the $2^{-\Delta Ct}$ method was used (Livak and Schmittgen 2001). Cycle threshold (Ct) values were obtained by subtracting the raw Ct values of each gene of interested subtracted from the raw Ct values of *Arabidopsis*- or barley-specific *ubiquitin*. The list of primers used for transcript analysis and quantifications is presented in tab. S3 and 4.

2.6 Quantification of ACC in *Arabidopsis*/barley roots during *P. indica* association

For 1-aminocyclopropane 1-carboxylic acid (ACC) determinations, plant material (0.2 g) was homogenized in liquid N_2 and extracted with 1 ml 80% (v/v) ethanol. After centrifugation (10,000g; 5 min) the pellet was re-extracted two times (0.5 ml). The supernatants were combined and concentrated as above. The residue was dissolved in 1 ml of Milli-Q water and was extracted with 1 ml of $CHCl_3$. The water phase was concentrated to dryness *in vacuo* and dissolved in 1 ml of H_2O. The ACC content was assayed according to Lizada and Yang (1979). MACC (*N*-malonyl-ACC) was quantified after acid hydrolysis (2 N HCl, 3 h, 120°C) of the ethanol extracts by subtracting the ACC value of the non-hydrolyzed sample from that of the hydrolyzed.

2.7 Colonisation of *Arabidopsis* mutants by *P. indica*

Seeds of *A. thaliana* ecotype Columbia-0 (Col-0), *Arabidopsis* mutants *eto1-1* (N3072), *ein2-1* (N8844), *ctr1-1* (N8057), *etr1-3* (N3070), *eir1-1* (N8058) and *35S::ERF1* (N6142) as well as *adc1* (N9657) and *adc2* (N9659) were obtained from the Nottingham *Arabidopsis* Stock Center (NASC, Nottingham, UK). All *Arabidopsis* mutants have Col-0 background and were analyzed on 1/2 strength Murashige-Skoog (MS) medium (NH_4NO_3-free) without sucrose (Duchefa, Haarlem, Netherlands). Therefore, Petri dishes were filled with 100 ml medium and, after solidification, sterile seeds were placed on the medium and incubated for 48 hours at 4°C. Inoculation of *Arabidopsis* was performed on 3-week-old plants by spreading 1 ml spore suspension (500000 spores per ml) on each plate as described in section 2.1. Then, the control as well as colonised *Arabidopsis* plants was incubated at 22°C under short-day conditions in a climate chamber (8/12 h day/night cycle, 18/22°C, 60% relative humidity). *P. indica* biomass in *Arabidopsis* mutant as well as wild-type roots was quantified by QPCR at 3 and 14 dai.

2.8 Quantification of fungal colonisation by QPCR

The relative amount of *P. indica* in plant roots was determined by calculating the ratio of the amplified product of *P. indica* DNA to plant DNA in the same sample. Therefore, genomic DNA of wild-type and *Arabidopsis* mutant roots as well as barley roots was extracted from ~100 mg root material with the Plant DNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Ten ng of total DNA served as a

template for QPCR analyses. Cycle threshold (Ct) values were obtained by subtracting the raw Ct values of the *P. indica Tef* gene (Bütehorn *et al.,* 2000) from the raw Ct values of *Arabidopsis*- or barley-specific *ubiquitin* (Tab. S5).

2.9 Histochemical analyses of *Arabidopsis* ethylene- and polyamine reporter lines

Arabidopsis ethylene (ET) reporter plants expressing the β-glucuronidase (GUS) reporter gene under the control of the promoter of the respective *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS*) genes including: Theo-AT-ACS1-*GUS/GFP* (N31379), Theo-AT-ACS2-*GUS/GFP* (N31380), Theo-AT-ACS4-*GUS/GFP* (N31381), Theo-AT-ACS5-*GUS/GFP* (N31382), Theo-AT-ACS6-*GUS/GFP* (N31383), and Theo-AT-ACS9-*GUS/GFP* (N31386). ET reporter plants were harvested at early and advanced colonisation stages (3, 7 and 14 dai) and compared with mock-treated plants for GUS activity. GUS staining was performed as follows: the roots were immersed in staining solution (100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.1% Triton X-100, and 1 mM X-Gluc, stirred the solution until every component was dissolved and then filter sterilized) and incubated overnight at 37°C. The staining reaction was stopped by adding 70% ethanol. GUS stained roots were imaged by bright field using an Axioplan 2 microscope (Carl Zeiss, Jena, Germany).

2.10 Exogenous application of 1-aminocyclopropane 1-carboxylic acid (ACC) and 1-methylcyclopropene (MCP)

Barley seedling of cv Golden Promise were colonised with *P. indica* and cultured in $PNM_{(1/10)}$ supplemented with ACC (Sigma-Aldrich, St. Louis, USA) at two different concentrations (10- and 100 µM). ACC was dissolved in water and filter sterilized prior to its addition to autoclaved plant growth media $PNM_{(1/10)}$. On the other hand, barley seedlings colonised with *P. indica* were exposed to 1-methylcyclopropene (MCP) (Rohm and Haas Company, Philadelphia, USA). MCP was applied at two different concentrations (1.6- and 16 mg) and dissolved in distilled water (25- and 250 µl). MCP (0.14% active ingredient) is formulated as powder that releases 1-MCP when mixed in water. Mixing was carried out inside sealed glass jars. Due to the previous concentration calculation of MCP in the gas phase (Tamaoki et al., 2003); we expected to have about

500- and 5000 pl l^{-1} MCP, respectively, in each glass jar. Data was collected from three independent experiments at two different time points (3 and 7 dai).

2.11 cDNA library construction for YSST screening

Total RNA was extracted from barley roots colonised with *P. indica* (after 60 hours of infestaion) and mRNA was isolated using a magnetic approach (Invitrogen, Oslo, Norway) as described in section 2.3. Complete removal of DNA was achieved using RNase-free DNase (Qiagen, Hilden, Germany). Following RNA quantification and checking for RNA integrity, double-stranded cDNA was generated using SuperScript double-stranded cDNA synthesis kit (Invitrogen-life technologies, Karlsruhe, Germany). The first-strand cDNAs were synthesized from 5 µg purified mRNA as described above (section 2.3) with minor modifications, as we used degenerated primers instead of oligo dT primer for the first-strand cDNA synthesis. *Not*I and *Xho*I restriction sites (in bold or underlined, respectively) were incorporated into random nanomer as follows:

5′-CGATTA**CTCGAG**GCGGCCGCNNNNNNNNa-3′
5′-CGATTA**CTCGAG**GCGGCCGCNNNNNNNNc-3′
5′-CGATTA**CTCGAG**GCGGCCGCNNNNNNNNg-3′
5′-CGATTA**CTCGAG**GCGGCCGCNNNNNNNNt-3′

The second-strand cDNA was generated by adding the reagents for second-strand synthesis by adding the following component to a fresh 1.5 ml microcentrifuge tube: 30 µl reaction volume of the first-strand cDNAs, 91 µl DEPC-water, 30 µl 5 x second-strand buffer, 3 µl dNTP mix (10 mM), 1 µl DNA ligase (10 U/µl), 4 µl DNA polymerase I (10 U/µl), 1 µl RNase H (2 U/µl) in 150 µl final volume. The reaction was gently spun in a microcentrifuge and incubated for 120 min at 16°C. Thereafter, 2 µl T4 DNA polymerase (5 U/µl) was added and incubated for an additional 5 min at 16°C. The vial was placed on ice and 10 µl of 0.5 M EDTA was added to stop the reaction. After precipitation of dsDNA, the pellet was dissolved in 5 µl of DEPC-treated water. For ligation of the blunt-ended cDNAs to *Eco*RI adaptors (Promega, Madison, USA), the following reaction was performed. 3 µl 10 x T4 DNA ligase buffer, 3 µl acetylated BSA (1 mg/ml), 250 ng dsDNA, 25 pmol *Eco*RI adaptors, 2.5 units T4 DNA ligase (Promega Madison, USA)

were mixed and nuclease free water was added to a final volume of 30 µl. The *Eco*RI adaptor sequence was as follows:

5′-AATTCCGT TGCTGTCG
3′ -GGCAACGACAGC-5′-P

The reaction was incubated at 15°C overnight. The enzyme was heat-inactivated at 70°C for 10 min, before placing the reaction mixture on ice. Then, dsDNA was cleaved with *Not*I and cDNAs were size-selected using the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

2.12 Ligation of cDNA library into pSMASH, yeast transformation and screening

Size-selected cDNAs were cloned into pSMASH upstream of an invertase gene using *Not*I and *Eco*RI restriction sites of the pSMASH vector. The resulting constructs were electroporated into electrocompetent DH10B cells (Invitrogen, Karlsruhe, Germany) as described in the manual and the transformants were selected on LB plates containing ampicillin (100 mg/ml). Surviving clones were collected and subjected to plasmids isolation as indicated by the manufacturer of the Nucleobond PC 100 kit (Machery-Nagel, Düren, Germany). The invertase-deficient yeast mutant strain Y02321 (*MATa; his3; leu2; met15; ura3; suc2*) from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html) was grown on YPD medium (1% yeast extract, 2% peptone and 2% glucose). The yeast transformation was done by a high-efficiency lithium acetate-based method as follows: 5 ml 2 x YPDA (YPD supplemented with 50 mg adenine hemisulphate per liter) was inoculated with the yeast strain and incubated on a shaker (200 rpm) overnight at 30ºC. The second day, inoculated cells from the overnight culture were transferred to 50 ml 2 x YPDA (pre-warmed to 30ºC) and incubated at 30°C by vigorous shaking. At an OD of 0.6 at 600 nm, the medium was spin down at 5000 rpm for 10 min to harvest the yeast cells. The supernatant was discarded and the pellet was dissolved in 10 ml TE. Again the yeast cells were harvested by centrifugation at 5000 rpm for 10 min at room temperature. The supernatant was discarded and resuspended in a transformation mix [240 µl PEG 3500 (50% w/v), 36 µl 1 M LiAc, 50 µl (2 mg/ml) boiled salmon sperm carrier DNA (Sigma, Steinheim,

Germany), 0.1 µg pSMASH vector, water (adjusted to a final volume of 360 µl)], and well-mixed with a pipette. The reaction mix was incubated in a waterbath at 42°C for 40 min and mixed by inversion several times. The cells were centrifuged at maximum speed (13000 rpm) for 15 sec; the supernatant was removed completely and the pellet dissolved in 1 ml sterile water. 10 and 100 µl of the solution were then pipetted onto plates with appropriate selection medium as follows: Transformants were screened by auxotrophic marker and carbon source activity. Therefore, yeast cells were streaked on SC medium with complete amino acids mixture lacking leucine and containing 2% sucrose and 0.025% glucose as carbon sources. As true positive colonies getting bigger in size compared to others, they could be differentiated and got plated on SC lacking of leucine containing 2% sucrose and raffinose plates as the sole carbon source supplemented with 2 µg/ml antimycin A. The selective yeast plates containing sucrose were incubated for 4 to 5 days. However, the plates containing raffinose were stored 2 to 3 weeks at 30ºC. Basically, those yeast transformants, which were complemented by a cDNA harbouring a signal peptide and fused to the invertase gene in pSMASH (lacking the signal peptide), were rescued due to the reconstituted secretion of invertase and use of raffinose and sucrose as carbon source. Antimycin A mimics anaerobic growth condition and inhibit the growth of yeast cells that do not secrete invertase. Yeast colonies, which emerged on raffinose plates were selected and subjected to plasmid isolation by adding 0.025% SDS to disrupt the cell membrane. Afterward, PCR amplifications were performed using pSMASH-specific primers (ADH1 forward and Suc2 reverse, tab. S5). After separating the PCR products by electrophoresis on agarose gel, selected bands were sliced and forwarded to DNA gel purification using a gel extraction kit (Qiagen, Hilden, Germany). Finally, isolated fragments were sequenced.

2.13 Prediction strategy for putative proteins and domain delineation

The predicted amino acid sequences from selected genes were checked for the presence of a signal peptide using SignalP V2.0 (http://www.cbs.dtu.dk/ services/ SignalP-2.0) (Bendtsen et al., 2004), PsortII (http://psort.ims.u-tokyo.ac.jp) and SigCleave (http://bioweb.pasteur.fr/seqanal/interfaces/sigcleave.html). The proteins were further

checked for transmembrane domains by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html).

2.14 Methods for protein modelling of PIALH43 RING finger domain

The structural model for PIALH43 RING finger domain was predicted using SWISS-MODEL (Peitsch, 1995). The preparation of the homology project was carried out in the web-interface of the program SWISS-PDB VIEWER (http://SWISS-PDB VIEWER). At the beginning, the FASTA format of the PIALH43 sequence was loaded into the interface. Then suitable template structures based on their sequence similarity to this protein or rather to the special domain of this protein were identified. This was achieved by comparing the target sequence with all entries in the structure database used by SWISS-MODEL that is derived from the Protein Data Bank (PDB). Suitable templates were sorted by statistical significance and could be downloaded readily. For the whole sequence of PIALH43, no template was found which could be used as the model for constructing 3-D structure. Therefore, the structural model of PIALH43 was built based on the RING finger domain in homology with other known template structures. The backbone of the target protein was built up, based on the localisation of accordant atoms in the template structure. The entire project was submitted to SWISS-MODEL, where extensive optimisation and minimisation steps were carried out by the GROMOS96-force field (van Gunsteren, 1996). The model's coordinates and all intermediate analysis results were then returned to the user by e-mail. For more information about the used template structures, see the following link: http://www.rcsb.org/pdb/home/home.do.

2.15 Isolation of full-length *α- expansin* and *PIALH43* cDNAs

Total RNA was extracted from barley roots colonised with *P. indica* at early timepoints (60 hai) and used as the starting material for isolation of 5′- and 3′-ends of cDNAs using the GeneRacer Kit (Invitrogen Life Technologies, Carlsbad, USA) as described in section 2.4. The obtained 3'- and 5'-end cDNAs, PCR (touchdown PCR followed by nested PCR) were used with gene-specific primers designed from the known region of the sequences (Tab. S1). After electrophoresis, the 3'- and 5'-RACE products were purified from the gel and ligated into pGEM-T vector (Promega, Mannheim, Germany) and introduced into *E.*

coli. To avoid sequencing errors due to RACE artifacts, the sequence analyses were performed on four independent clones derived from each RACE product (5'- and 3'-RACE). The virtual full-length cDNA sequence of *α- expansin* and *PIALH43* genes were constructed by combining the overlapping regions. To determine the full-length cDNA sequences, primer pairs from both ends of the genes were designed to cover the whole region between the 5-' and 3'-RACE fragment. The final PCR was performed using Platinum *Pfu* DNA polymerase (Promega, Mannheim, Germany) under the following conditions: 95°C for 2 min, followed by 35 cycles 95°C for 40s, 56°C for 30s and 72°C for 4 min and 72°C for 10 min. The resulting PCR products were separated on a 2% agarose gel and the fragments were gel-purified (Qiagen, Hilden, Germany), After A-tailing, PCR products were cloned into pGEM-T vector and inserted cDNAs were sequenced in both directions to confirm the presence of unaltered sequences. The primer sequences used for amplification of full-length cDNA of *α- expansin* and *PIALH43* are listed in tab. S1.

2.16 Gene expression profile of *PIALH43 in planta* compared with axenic cultures

QPCR was used to reveal the gene expression profile of *PIALH43 in planta* at early and late stages of *P. indica* colonisation in barley plants compared to axenic cultures grown in liquid complete medium (CM). For the latter, *P. indica* was grown in liquid medium by gentle shaking at room temperature. Barley roots as well as *P. indica* material were harvested at 1, 2, 7, and 14 dai or after transfer to liquid CM respectively.

2.17 Construction of PIALH43-His_6, production of recombinant protein and purification

In order to produce recombinant PIALH43 protein and demonstrate the activity PIALH43, histidine-tagged protein related to the full-length coding sequence, corresponding to the predicted mature protein without putative signal peptide (1-22 amino acid residues) and without termination codon was generated by PCR using *PIALH43* forward and reverse primers (Tab. S2). The amplified fragment was cloned N-terminal to His_6-tags in the bacterial expression vector pEXP5-CT/TOPO (Invitrogen, Carlsbad, CA). Then, the bacterial expression vector harboring PIALH43-His_6 was

introduced into *E. coli* strain BL21 (DE3) (Stratagene, La Jolla, USA). The bacterial clones containing PIALH43-His_6 were immediately checked for the rate of protein induction. For large scale protein purification, a single bacterial clone, in which the protein was highly induced, firstly verified by a small scale protein induction procedure and was grown in Luria-Bertani (LB) medium overnight under shaking at 37°C. After inoculation of fresh medium with the overnight culture, bacteria were allowed to grow until mid log phase (OD600 of 0.4-0.6) before isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM and further incubation for 4-5 hours. Then, bacteria were harvested by centrifugation at 4000 rpm for 20 min at 4°C and the bacterial pellet was dissolved in 30 ml lysis buffer (50 mM sodium dihydrogen phosphate, 300 mM sodium chloride, 10% glycine, and 1 mg/ml lysozyme, adjusted to pH 8.0 using NaOH) and cell disruption by French press was performed two times at a pressure of 8000 lb/in^2. Subsequently, the lysate was kept on ice while adding 30 ml binding buffer (8 M urea, 100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, pH 8.0) and incubated under shaking for 3 hours at room temperature. Thereafter, the cell debris was precipitated from the lysate solution by centrifugation for 45 min in a Sorvall SS34 centrifuge (15000 rpm). The supernatant was collected and stored at 4°C. To prepare the column for purifying the 6× His-tagged fusion protein, 1 ml of Ni-NTA resin (Qiagen, Hilden, Germany) was pipetted into the column that was clamped onto a stand. The resin was allowed to settle down and once settled the valve was opened to drain off the residual liquids in the column. Thereafter, the supernatant containing soluble protein was loaded onto a Ni-NTA prepared column and washed three times with 4 ml washing buffer (8 M urea, 25 mM imidazole, 100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, pH 6.3) and thereafter, the column was eluted three times with elution buffer (8 M urea, 500 mM imidazole, 100 mM sodium dihydrogen phosphate, 10 mM Tris-HCl, pH 4.5). Finally, proteins were desalted and concentrated using an ultra-filtrate column (VIVASPIN 15 ml concentrator) with a cut-off at 50 kDa (Vivascience, Lincoln, UK) and stored at -20°C. Protein concentration was estimated by Bradford assay. Different concentrations of bovine serum albumin (BSA) were prepared and used to create a standard curve. Purity and integrity of PIALH43 recombinant protein was determined by separating protein aliquots using sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE). After electrophoresis, gel was fixed by fixation solution (one part Acetic Acid, 3 parts Isopropanol and 6 parts Water) for 30 min. Eventually, the gel was visualized with colloidal Coomassie blue (Roth, Karlsruhe, Germany). Staining solution was prepared (20 ml coomassie blue stock solution (Roth, Karlsruhe, Germany), 20 ml methanol and 60 ml water) and added to the gel till the bands were clearly seen. Destaining was performed with destaining solution (40% methanol, 10% glacial acid, 50% water) till background was faint.

2.18 *In vitro* ubiquitin ligase assay

An *in vitro* ubiquitination assay was performed to study the function of PIALH43 in protein degradation. The assay was performed by adding 0.5 µg of purified fractions of PIALH43 protein (without putative signal peptide), 0.5 µg rabbit ubiquitin activating enzyme E1 (Sigma-Aldrich, St. Louis, USA), 1 µg ubiquitin-conjugating enzyme E2 (Boston Biochem, Boston, USA), and 2 µg ubiquitin (Biomol, Exeter, United Kingdom). These components were added to 15 µl reaction buffer (50 mM Tris-HCl, pH 7.4, 2 mM DTT (Dithiothreitol), 5 mM $MgCl_2$). In addition, 2 mM ATP was added to the regenerating system consisting of 1 mM sodium creatine phosphate (Sigma, St Louis, USA), 3.5 units creatine phosphokinase from rabbit muscle (Sigma, St Louis, USA) and 0.3 unit inorganic pyrophosphatase from baker's yeast (Sigma, St Louis, USA). The reactions were incubated for 2 hours at 30ºC and then stopped by adding sample buffer (50 mM Tris-HCl pH 6.8, 10 % glycerol, 2% SDS, 12.5 mM EDTA, 0.02% bromophenol blue, and 1% β-mercaptoethanol) and heated at 95°C for 5 min. The reactions were analysed by SDS-PAGE and immunoblotting. Protein separation was done using 12% SDS-PAGE (100 mA for 1 hour) and the proteins were blotted onto a nitrocellulose membrane by semidry electro blotting (16 mA for 20 min). The membrane was blocked using phosphate buffered saline (PBS) containing 3% non-fat dry milk for 3 hours and washed three times in PBS containing 0.05% Tween 20 (PBS-T) for 5 min. The membrane was soaked with the respected antibodies diluted in PBS-T as follows: anti-ubiquitin at a dilution of 1:1000 (Sigma-Aldrich, St. Louis, USA); anti-His-tag (6-histidine) at a dilution of 1:2000 (Rockland, Gilbertsville, USA), and incubated overnight at 4°C. The membranes were washed three times with PBS-T for 5 min and then soaked

in PBS-T containing 1/10000 diluted goat anti-rabbit IgG (Sigma, Taufkirchen, Germany) for 3 hours at room temperature. Eventually, the membrane was washed three times in PBS-T for 5 min each. After the final wash, the membrane was completely covered with fresh NBT/BCIP solution (5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (25 mg/ml dissolved in dimethyl formamide) and 4-nitro blue tetrazolium chloride (NBT) (25 mg/ml dissolved in dimethyl formamide/warer: 70/30) was dissolved in revelation buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) and allowed the colour reaction to develop until optimal signal intensity was reached (usually 5-15 minutes). The reaction was stopped in 1 mM EDTA and the membrane was allowed to air dry and sealed membranes were stored in dark.

2.19 Statistical analysis

All the experiments were conducted at least twice and standard errors were calculated for all mean values and *t*-tests were performed. Data from single representative experiments were presented in cases repeated tests gave similar results.

3. Results

3.1 Establishment of the yeast signal sequence trap assay to identify proteins secreted during the interaction of barley roots with *P. indica*

Plant colonizing microbes release a plethora of apoplastically and cytoplasmically functioning effector proteins into their host cells in order to reprogram host physiology and modulate plant defense mechanisms, hence, it establishes compatibility within the host. However, physiological and molecular aspects of these alterations are mostly unknown. Understanding these processes might shed light onto the highly complex processes of symbiosis formation. Therefore, to gain insight into the physiological and molecular processes occurring during *P. indica* colonisation as well as identifying fungus effectors, the yeast signal sequence trap (YSST) was established. The YSST is used as a screening method for trapping genes carrying signal peptides, and therefore, putatively encoded extracellular signaling molecules, transmembrane and secreted proteins. Basically, the system consists of two components: (i) the yeast expression vector (pSMASH) that harbours an invertase gene, which lacks the signal sequence and the start codon methionine, and (ii) an invertase-deficient *Saccharomyces cerevisiae* strain (Fig. 4), which is unable to grow on sucrose or raffinose containing media. Invertase function can be reconstituted in the yeast vector in case a cDNA is introduced and provides the missing elements. By transforming the yeast mutant strain with such a reconstituted construct, it would regain a invertase protein function. Eventually, the complemented yeast clones were selected by their ability to grow on raffinose containing media. In the first step, the YSST method was successfully established to screen a cDNA library generated from *P. indica* colonised barley roots for *P. indica*-derived secreted proteins. The YSST procedure comprised the following steps barley roots (colonised with *P. indica*) were harvested at 48, 60, 72, and 96 hai and merged the samples together. These timepoint represent a critical interaction phase for the extracellular fungal development, its penetration-associated and early colonisation events (Schäfer *et al.*, in press). So, total RNA was extracted and subsequently, mRNA was isolated from *P. indica*-colonized barley root material at this time point. The first-strand cDNAs were generated from purified mRNA using degenerated primers (random nanomer) that *Not*I restriction sites

were incorporated into the 5′-end. Afterwards, the double -stranded cDNAs (ds cDNAs) was generated, then it was size selected and the *Eco*RI adaptors were ligated to the cDNA library consisted of both plant and *P. indica* transcripts. Afterwards, cDNA library was introduced into *Eco*RI and *Not*I-digested pSMASH yeast expression vector at upstream of the truncated invertase. The constructs were transformed into the invertase-deficient yeast mutant by a standard Lithium-Acetate transformation protocol (Gietz and Woods 2002). The screening of the transformants yeast were done initially by auxotrophic media lacking leucine to check the successful uptake of the yeast vectors and, thereafter, on raffinose-containing medium. The sequencing of the cDNA fragments in the emerged yeast colonies was the final step of the YSST screen.

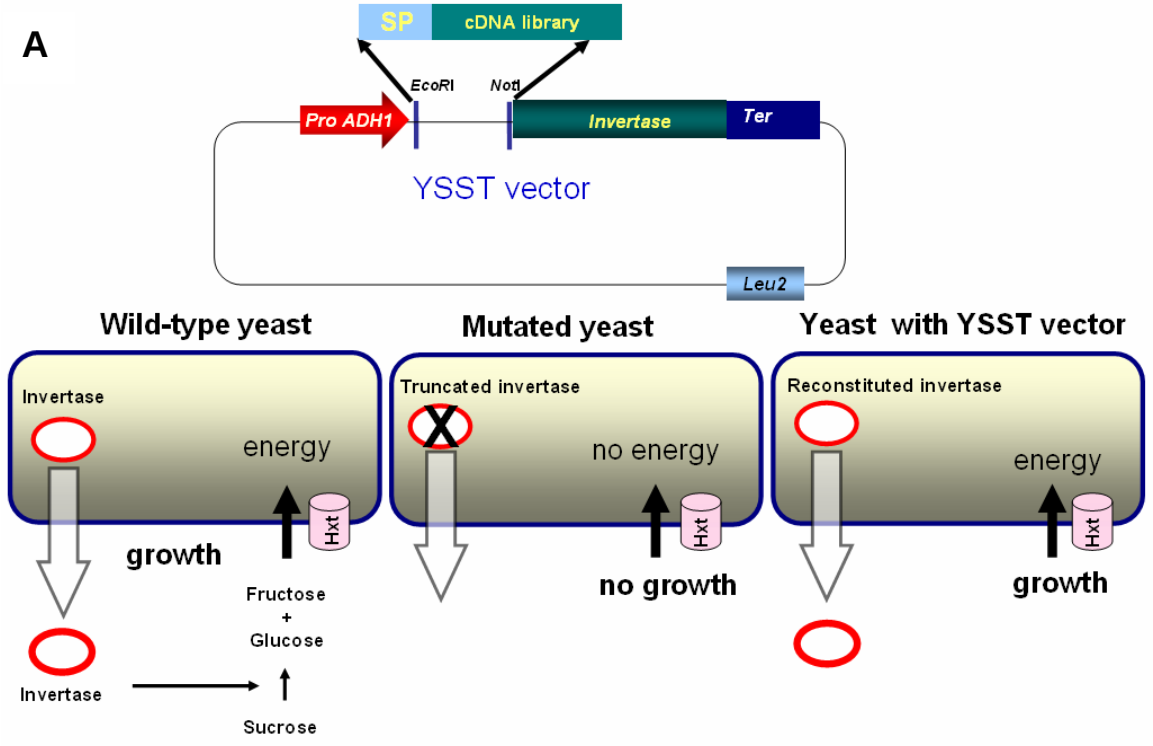

Figure 4: The basis of the yeast signal sequence trap (YSST) method for the isolation of genes encoding secreted proteins. Wild-type yeast is able to grow on sucrose and raffinose-containing medium by secreting invertase, while an invertase-deficient yeast strain was not able to grow on these media. Therefore, invertase-deficient yeast mutant requires the activity of the plasmid-derived invertase gene. The plasmid harbors selectable markers and origins of replication that allow the growth and selection in yeast and bacteria (A). The plasmid contains a truncated version of the invertase gene that lacks both an initiation codon and a functional signal sequence. Reconstitution of the invertase gene with a signal sequence and a start codon by introducing a competent

cDNA in frame with the truncated invertase and subsequent transformation in invertase-deficient yeast mutants restores their ability on sucrose media.

3.2 Isolation of secreted proteins using the yeast signal sequence trap approach

Initial screening was performed by plating the yeast transformants on Yeast Nitrogen Base (YNB) lacking leucine containing 2% (w/v) sucrose and 0.025% (w/v) D-glucose as carbon sources. The true positive colonies are appeared bigger in size when compared to invertase-deficient yeast colonies. The final screening process for verification of genes encoding secreted protein was done by re-growing the previously selected clones on YNB lacking leucine containing 2% (w/v) raffinose supplemented with antimycin A (Fig. 5).

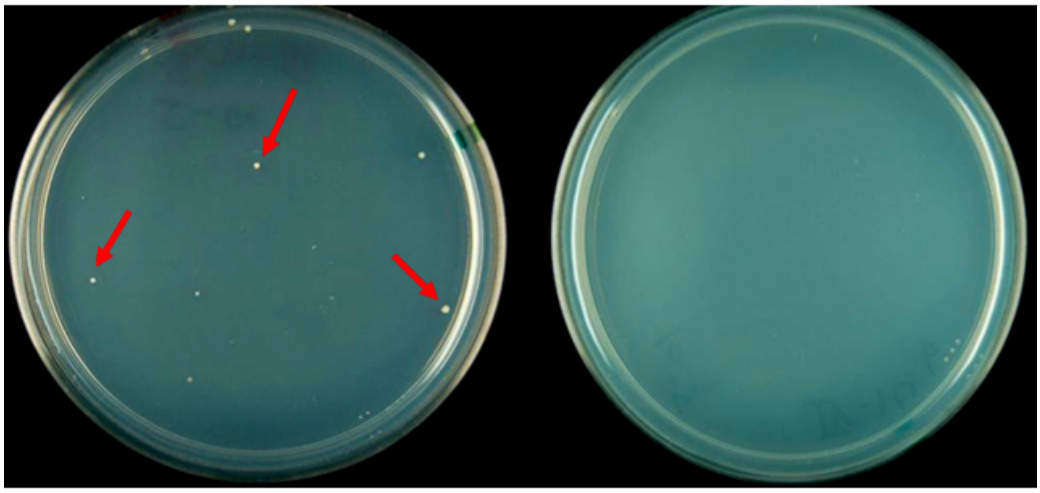

Figure 5: The final screening of genes encoding secreted proteins on YNB lacking leucine and containing 2% raffinose supplemented with antimycin A. The true positive clones (left) were able to grow in opposite to false positive clones (right).

In total, fifthteen sequences were obtained from the first screening representing five different sequences (Tab. 1). As shown in tab. 1, the annotations of the putative proteins revealed their function in different physiological processes like, stress- or defense responses that are mentioned as follows. The *Germin-like protein* and *secretory peroxidase* are categorized as stress- or defense related proteins (Baptista et al., 2007; Fester and Hause, 2005; Zimmermann et al., 2006). The α-expansin is involved in cell wall loosening (Balestrini et al., 2005; Cosgrove, 2005), while the heat shock protein 70

KDa (HSP70) is grouped as a chaperone involved in stress adaptation (Mambula et al., 2007).

Table 1: List of candidate genes, accession number, putative function with their origin isolated by yeast signal sequence trap.

Gene annotation	Accession number	Function	Organism
Germin-like protein	AK248686	Defence-related gene	Barley
Secretory peroxidase	AK249643	Defence-related gene	Barley
α-expansin	EU492895	Cell wall metabolism	Barley
HSP70	AK251839	Chaperone	Barley
PIALH43	----------	Unknown	*P. indica*

To examine whether the identified plant genes showed *P. indica*-responsive expression during barley root colonisation, the relative gene expression patterns were examined at different timepoints (3 and 7 dai) using QPCR (Fig. 6).

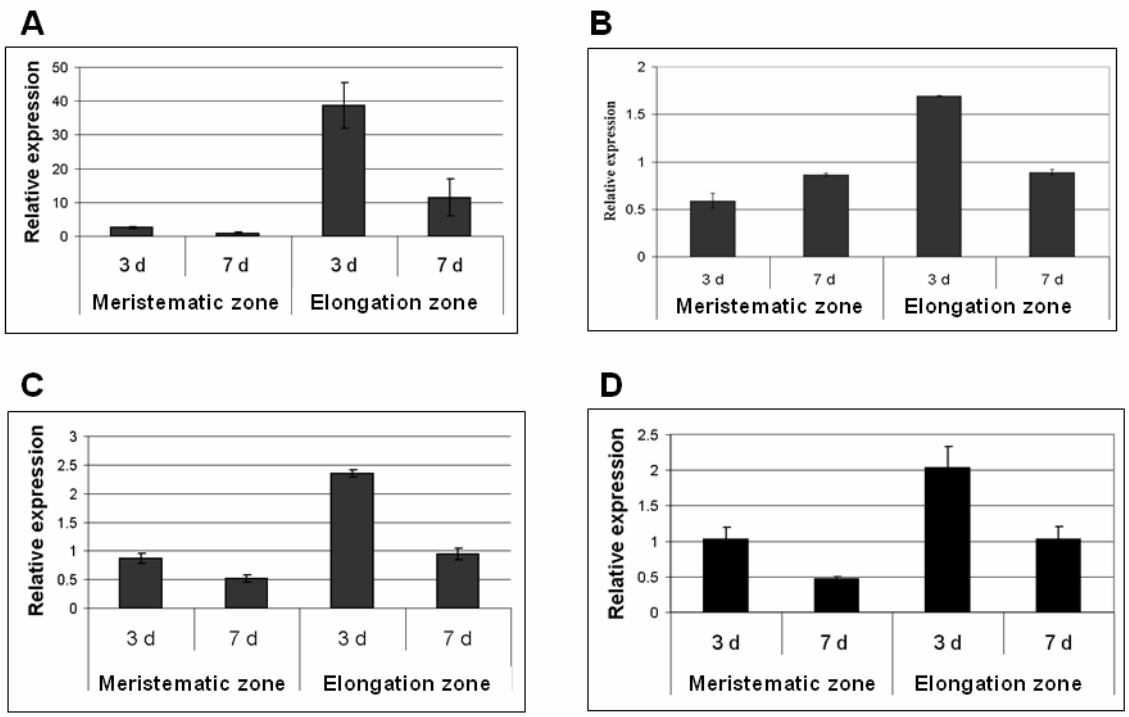

Figure 6: Gene expression patterns of plant genes isolated by YSST using QPCR. The gene expression analyses were performed on meristematc and elongation root zones. A) *Germin-like protein*, B) *α-expansin,* C) *Heat shock protein 70 KDa* (*HSP70*), and D) *Secretory peroxidase*, Ubiquitin was used as internal control to normalize data. Values are means and are based on three different independent experiments. The expression level

of *P. indica* non-inoculated plants was set to one due to enable the comparison of experiments.

P. indica growth depends on root development as the meristematic zone is protected from fungal colonisation while cell penetration, colonisation, fungal proliferation and spore formation are mostly observed in root elongation zone (Deshmukh et al., 2006). Therefore, the gene expression analysis was performed using segmented roots to monitor the local and systemic influence of *P. indica*. As shown in Fig. 6A, gene encoding *germin-like protein* was highly induced in elongation zones (approximately 38.6- and 11.4-fold) at 3 and 7 dai, respectively, but less induced in meristematic zones. The barley *α-expansin* showed approximately a 1.7-fold induction in elongation zones at 3 dai, but was twofold suppressed in meristematic zones at 7 dai (Fig. 6B). In case of HSP70 with higher expression levels in elongation zones (2.4-fold) at 3 dai, while the gene was suppressed in meristematic zones at 7 dai (Fig. 6C). The same expression patterns were observed for *Secretory peroxidase*, higher transcript levels were observed in elongation zones at 3 dai (2.6-fold), whereas the transcript levels in meristematic zone were unaltered or even twofold suppressed (Fig. 6D). The expression analysis of *P. indica* candidate gene *PIALH43* showed an 2,8-fold induction during *P. indica* interaction with barley at 5 dai in relation to its expression in axenically grown fungus (Fig. 7).

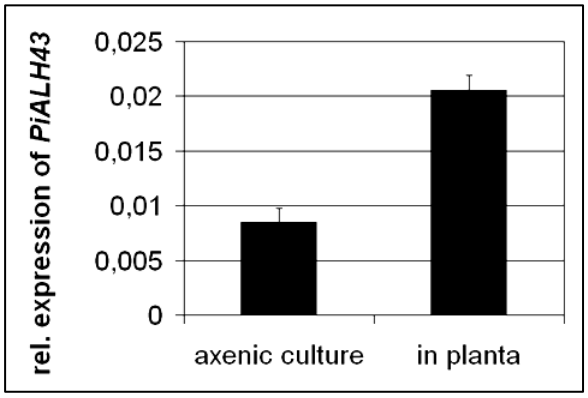

Figure 7: Gene expression analysis of the putatively secreted protein PIALH43 of *P. indica in planta* and in axenically grown *P. indica* at 5 day. *PiTef* was used as internal control to normalize the data.

3.3 *In silico*-based analysis of isolated genes and homology modeling of PIALH43

Firstly, comparisons of the predicted amino acid sequence of the isolated genes were done in the database (NCBI). The results from the sequence alignment revealed that the plant genes had homology with previously described genes with known functions as shown in (Tab. 1). The nucleotide sequence alignment of *α-expansin* cDNA showed the highest similarity (62%) with *α-expansin-22* from rice (TC298721) as well as 61-and 45% identical with two other barley *α-expansin* genes (TC170916 and TC160990). The other identified barley genes such as *Germin-like protein*, *Secretory peroxidase*, and *HSP70* were found identical in the barley EST database under the accession numbers AK248686, AK249643, and AK251839, respectively. Secondly, the predicted amino acid sequence that corresponds to selected genes was checked for the presence of a signal peptide using SignalP V2.0, PsortII and SigCleave. The proteins were further checked for transmembrane domains by TMpred. The predicted amino acid sequences of Germin-like protein, Secretory peroxidase and α-expansin have signal peptide at N-terminus, whereas HSP70 has no secretory domain (Fig. S1).

Thirdly, the full-length transcript of barley α-expansin was isolated. Therefore, barley roots colonised with *P. indica* were harvested at 60 hai and RNA was extracted. Then, RNA was subjected to the rapid amplification of cDNA ends (RACE) method. The results extracted from the assembly of different amplified fragments using 5′- and 3′-RACE (rapid amplification of cDNA ends) approaches of *α-expansin* revealed the full-length cDNA containing 986 nucleotides, which encodes a predicted protein containing 264 amino acids with a signal peptide at the N-terminus (Fig. S2).

Regarding the *P. indica* candidate gene *PIALH4*, sequence alignment using BLASTP for homology searches against the non-redundant protein database were performed and showed low similarity with other fungal proteins within the Basidiomycetes. It shares the highest homology (36, 31, and 29%, respectively) with hypothetical proteins from *Laccaria bicolor* (XP_001877371), *Coprinopsis cinerea okayama* (XP_001830579), and *Cryptococcus neoformans var. neoformans* (XP_571193), respectively. In addition, the *PIALH4* at protein level has 20% similarity with a putative RING finger protein from *Talaromyces stipitatus* (EED18541). Subsequently, the full-length cDNA clone corresponding to *PIALH43* was also determined through rapid amplification of cDNA ends (RACE). Lastly, *PIALH43* encodes a polypeptide of 529 amino acids (Fig. S3). The

deduced protein possesses a predicted molecular mass of 58.5 kDa and the isoelectric point (pI) value of 5.49. Prediction programs (e.g. SMART) were used to gain insights into the putative protein domains of PIALH43. As shown in Fig. 8, PIALH43 contained a signal peptide (residues 1-22), and E3-like ubiquitin ligase domain (residues 411-454) at the C-terminus of PIALH43.

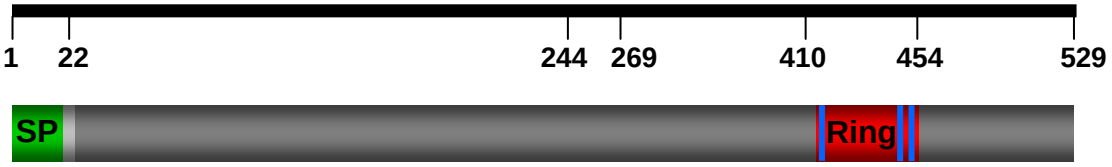

Figure 8: Different domains in *P. indica* candidate effector protein (PIALH43).
The position of putative signal peptide (residues 1-22), and RING finger-like domain (410-454) were predicted with SMART program.

In addition, I could identify other motifs with less significant prediction (Tab. 2). The function of these putative motifs might encompass the inhibition of plant proteases (SERPIN) or prevention chitin degradation (ChtBD3) as reported for AVR4 of *C. fulvum* (van den Burg et al., 2006). Interestingly, WWE motifs are apparently conserved motifs of E3 ligases and are discussed to mediate specificities for protein-protein interactions (Aravind, 2001). PIALH43 was also predicted to carry a transmembrane domain (248-270).

Table 2: Protein motifs predicted for PIALH43. SERPIN, serine proteinase inhibitor; B-lectin, bulb-type mannose-specific lectin; IFabd, interferon alpha, beta, gamma; WWE (named after three of its conserved residues); ChtBD3, chitin-binding domain type 3; FAS1, fasciclin I family of proteins; Cation_ATPase_N, Cation transporter/ATPase, N-terminus.

Name	Begin	End	E-value
SERPIN	9	268	2.25e+03
B_lectin	49	156	2.30e+03
IFabd	138	212	1.10e+03
WWE	146	204	4.02e+03
ChtBD3	175	215	1.44e+03
FAS1	176	264	1.74e+03
Cation_ATPase_N	210	272	1.02e+05

PIALH43 was subsequently forwarded to protein modelling studies. Since no template could be found for PIALH43, which would be usable for structural modelling, the analysis was focused on the conserved RING finger domain of PIALH43. This was achieved by comparing the predicted E3 ligase domain sequence of PIALH43 with the previously described models present in public databases (details on modeling are given in Materials and Methods). First, multiple amino acid sequence alignment was performed with the RING finger domain of PIALH43, human (Rbx1), *Arabidopsis* (AtPUB14), and the bacterium *Pseudomonas syringae* (AvrPtoB) using ClustalW (Fig. 9).

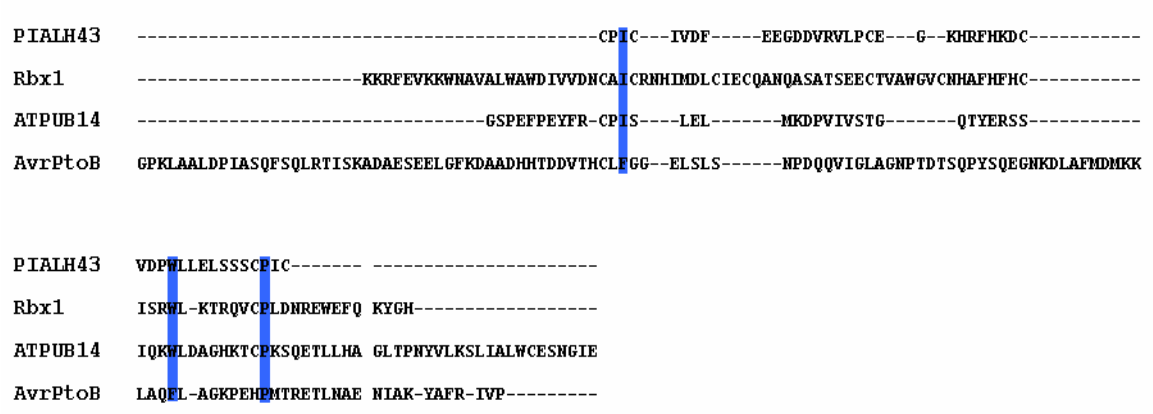

Figure 9: Multiple sequence alignment of the PIALH43 RING finger domain with human (Rbx1), *Arabidopsis* (AtPUB14), and the bacterium *Pseudomonas syringae* (AvrPtoB). The well-defined and functionally important E2 binding residues (blue marking) are conserved in PIALH43.

Ubiquitin conjugating E2 enzymes are interacting with specific amino acid residues of E3 ubiquitin ligases, so-called E2-binding sites, which are required for protein ubiquitination and subsequent degradation. Importantly, the three E2 binding sites encompassing tryptophane, isoleucine and proline were defined to be functionally relevant in known E3 ligases (e.g. AtPUB14, RBX1) were conserved in PIALH43 and implicates its function as E3 ligase. Furthermore, 3D structural analysis of PIALH43 revealed the accurate conformation and orientation of the E2 binding residues (Fig. 10).

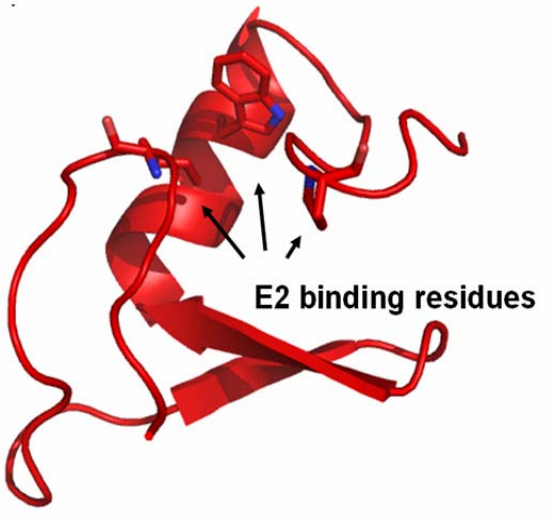

Figure 10: The tertiary structural model of PIALH43 based on the RING finger domain of *Homo sapiens* (RBX1). The three residues (tryptophane, isoleucine and proline) shown by arrows bind the E2-conjugating enzyme and are located in a conserved α-helix and two-loop structure.

A 3D alignment indicated a highly similar orientation of the E3 ligase domain and the E2 binding residues of PIALH43 with those of a human (RBX1) and plant (AtPUB14) RING finger domain (Fig. 11), which are known functional E3 ligases.

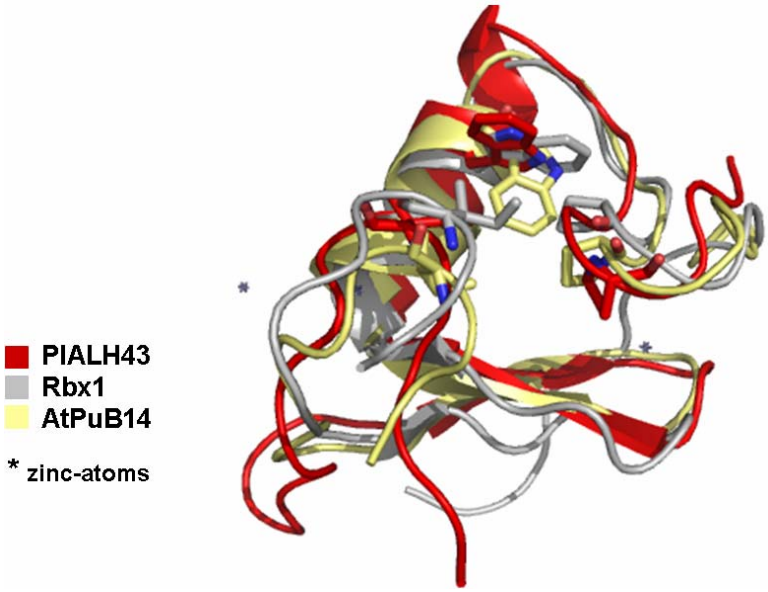

Figure 11: 3D alignment of the E3 ligase domain of PIALH43 with RING finger domains of RBX1 and AtPUB14.

3.4 *In vitro* E3 ubiquitin ligase assay showed the involvement of PIALH43 in protein degradation

Like other known E3 ligases, PIALH43 has a RING finger domain that might confer ubiquitin ligase activity which is crucial for its function as ubiquitination. Therefore, we used an *in vitro* E3 ubiquitin ligase assay to check the potential involvement of PIALH43 in protein degradation processes. Therefore, in order to generate a N-terminal His-tagged PIALH43 fusion protein, the corresponding full-length cDNA of *PIALH43*, without putative signal sequence (1-22 amino acid residues) and without termination codon, was amplified by PCR using specific PIALH43 primers and ligated in frame with a histidine-tag of the bacterial expression vector (pEXP5-CT/TOPO) as described in details in materials and methods. Thereafter, the recombinant protein was expressed in *E. coli* BL21 (DE3) and eventually purified by using Ni-NTA column. *In vitro* E3 ubiquitin ligase assay was performed using purified His-tagged PIALH43 in combination with ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzymes (E2). Indeed, there are several E2 enzymes existing, which exhibit high similarity in sequence and structure and carry out ubiquitination with E3 ubiquitin ligase. However, E2 needs first to be defined for a functional E3 ligase assay. Therefore, to identify the suitable E2 enzymes that can be recruited by PIALH43, several E2 enzymes were checked. The result experiment for screening different E2 enzymes in combination with E1 enzyme in the absence of E3 enzyme is presented in Fig. S4. As it shown, the differences among E2 enzymes were detectable. Subsequently, the promising and specific interactions among E1 and E2 were selected (UbcH5a, Boston Biochem) and was used in ultimate E3 ligase assay. Based on these pre-experiments, the final experiment was designed and conducted using E1, E2 (UbcH5a) and purified PIALH43 as presented in Fig. 12.

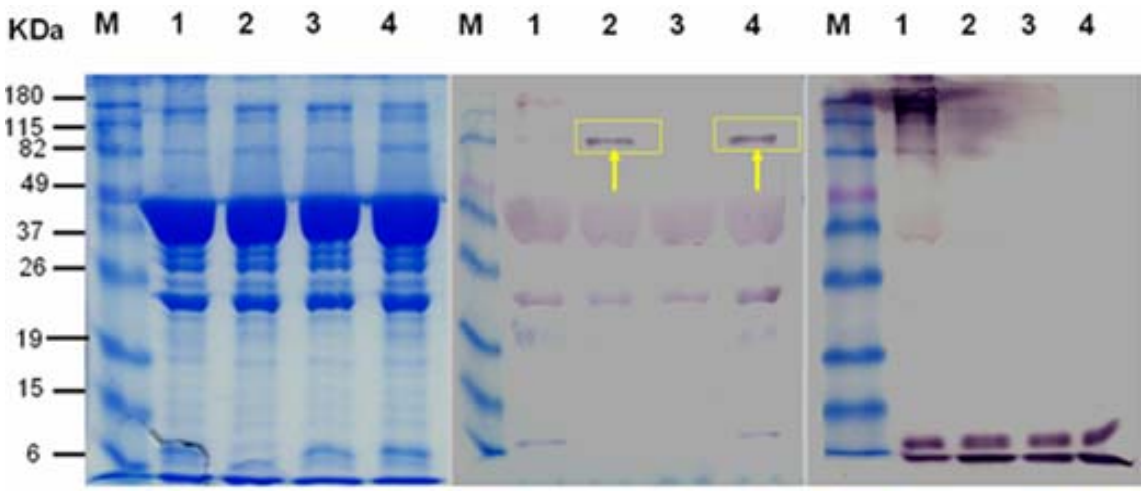

Figure 12: *In vitro* ubiquitination assay to test E3 ligase activity of PIALH43. First lane, combination of all components required for ubiquitination encompassing yeast E1, human E2 (UbcH5a), purified fraction of PIALH43, ubiquitin, and ATP. Second lane, combination of E1, PIALH43, ubiquitin and ATP. Third lane, combination of E1, E2, ubiquitin and ATP. Fourth lane, combination of E2, PIALH43, ubiquitin and ATP. The left photo is corresponding to the total protein stained with Coomassie blue. PIALH43 was detected by immunoblotting with anti-6xHis antibody in the middle image. Anti-ubiquitin blot from the ubiquitination assay is shown in the right image. Ubiquitin migrated at 5.5 kDa and has run off at the bottom of the gel, while ubiquitinated proteins (as shown in the first lane of right image) shifted to the top of the gel as it was detected by anti-ubiquitin antibodies. The arrows and boxes show the position of PIALH43 recombinant protein.

3.5 Establishment of TSH method for the identification *P. indica*-responsive genes

The identification and characterization of genes involved in specific physiological processes are important in order to understand the fundamentals of biological systems. Therefore, several reverse genetic approaches have been developed to specifically identify those genes that are differentially regulated in different organisms (e.g. plants, insects, mammals, fungi) in response to a given challenge ranging from developmental processes to abiotic or biotic stresses. In order to get deeper insights into the molecular mechanisms contributing to fungal establishment inside host tissue, plant compatibility factors influencing plant colonisation by microbes and nutrient exchange between plants and microbes should be considered. The second focus of my work was to identify putative plant compatibility factors that support root colonisation by the mutualist *P. indica* and that might help to explain the broad host range of the fungus. Therefore, we established a novel simplified subtraction method to identify differentially regulated

genes in barley roots during its interaction with the mutualistic basidiomycete *P. indica*. To identify those genes responsible for plant root colonisation by *P. indica*, root material was harvested at 60 hours after inoculation (hai) and subjected to mRNA isolation. This timepoint represents a critical interaction phase for root cell penetration and early colonization by the fungus (Fig. 13).

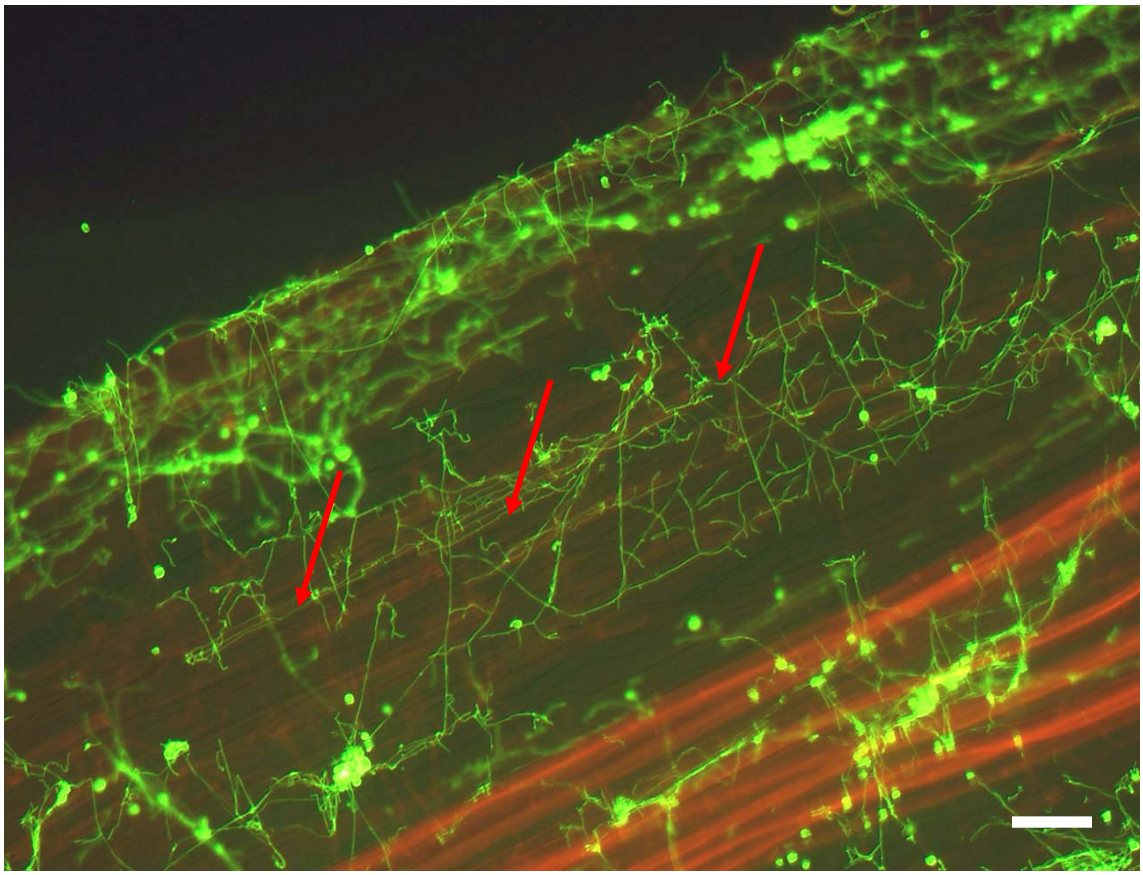

Figure 13: *P. indica* colonised barley root at 60 hai. The fungus was stained with wheat germ agglutinin-Alexa Fluor 488 (WGA-AF 488). WGA-AF488 was visualised by fluorescence microscopy (488 nm excitation and 505-530 nm emission). Congo-red that was used for barley root staining was excited at 530–585 nm and analysed at 600 nm (emission). Several rhizodermal cells have been intracellularly colonised by the fungus (arrows) (Bar = 30 µm).

The developed substration method so-called Transcription Subtractive Hybridisation (TSH), exploits subtraction at the mRNA level rather than at the cDNA level (Fig. 14), thereby circumventing the disadvantages of other methods like the subtractive suppressive hybridisation (SSH) technique (Ferreira et al., 1999; Sagerström et al., 1997).

Pivotal criteria for evaluating TSH were (1) high reliability in gene identification, (2) complete elimination of non-specific genes (e.g. housekeeping genes), (3) elimination of gene abundancy in subtracted cDNA libraries and, (4) the control on subtraction stringency. The subtraction procedure comprised the following steps (Fig. 14): Initially, mRNA was isolated from *P. indica*-colonized or mock-treated root material. The mRNA of mock-treated root material was used for the synthesis of biotinylated single-stranded cDNA (sscDNA). Following RNA hydrolysis and RNase heat inactivation, streptavidin-labeled magnetic beads were bound to the biotinylated sscDNA. Subsequently, mRNAs isolated from *P. indica*-colonized root material were added to the sscDNAs in order to obtain the complementary sscDNA/mRNA hybrids. By magnetic separation, the cDNA/mRNA hybrids were captured while uncoupled mRNAs remained in the solution and represented subtracted mRNA (Fig. 14). After a single or repeated subtraction cycles, this material was used for cDNA library construction and subsequently for *E. coli* transformation.

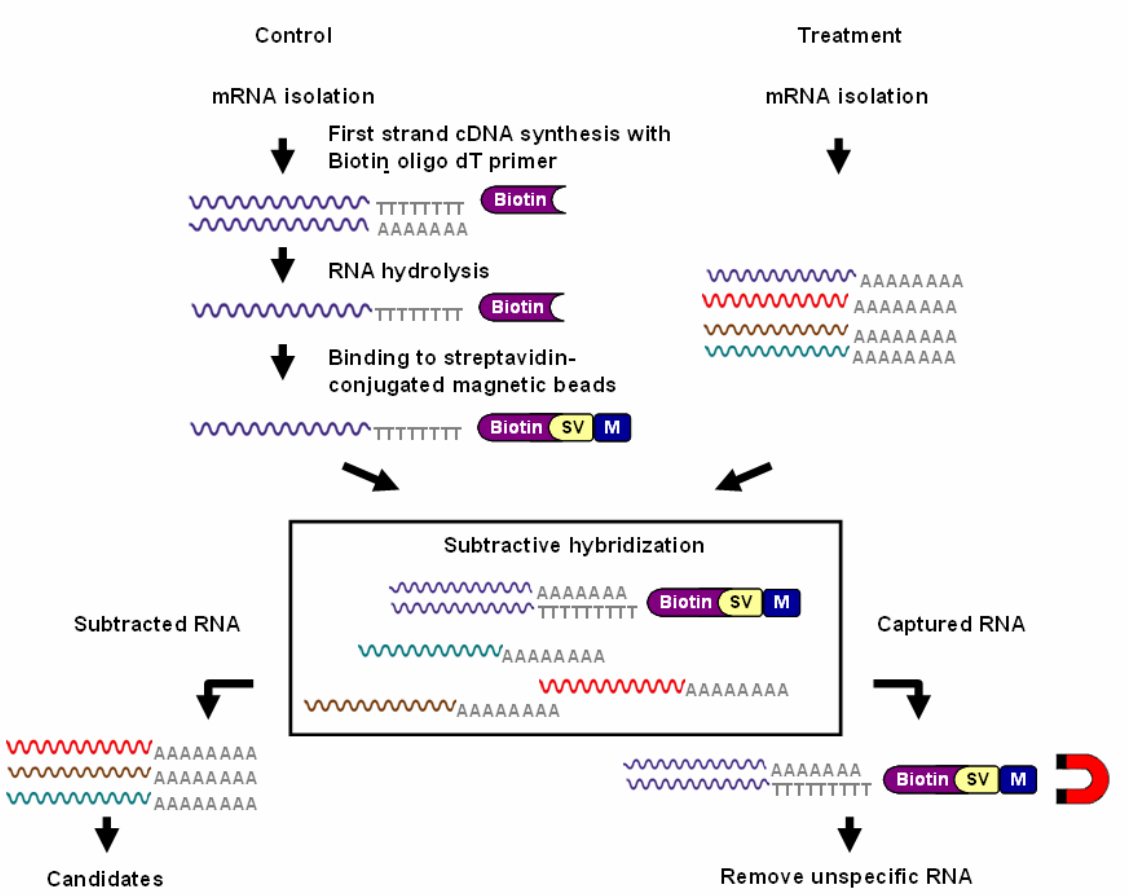

Figure 14: Schematic overview of Transcription Subtractive Hybridisation (TSH) assay to isolate differentially expressed genes. After first-strand cDNA synthesis using biotin-oligo (dT) primer, RNA was hydrolysed. The obtained biotinylated sscDNA was subsequently bound to streptavidin-labeled magnetic beads. For subtractive hybridisation, mRNA from *P. indica*-colonised roots was added to sscDNA from control roots. sscDNA/mRNA hybrids were subsequently removed by magnetic separation. Putative *P. indica*-responsive uncoupled mRNAs stayed in solution and were transferred to a fresh vial. This mRNA pool was used for cDNA library construction and isolation of *P. indica*-responsive genes.

In order to prove the functionality and stringency of the subtraction assay, a QPCR-based approach was performed to check the abundancy of the plant housekeeping gene *ubiquitin* and the *P. indica translation elongation factor* gene (*Pitef*) (Bütehorn et al., 2000), before and after several subtraction steps. As shown in Fig. 15, the barley housekeeping gene *ubiquitin* was almost completely removed from the mRNA pool after a single subtraction step leading to 95% reduction in relative mRNA abundancy. We used *Pitef* as additional control for subtraction specificity. As cDNA of mock-treated root

material did not contain fungal mRNA, the fungal housekeeping gene *Pitef* should be unaffected by the subtraction procedure. The quantitative PCR-based analysis revealed a constant *Pitef* transcript presence in the mRNA pool even after repeated subtractions. In turn, *P. indica*-responsive gene *PR10* was linearly reduced by consecutive subtractions with 20% left after 9 subtraction steps, while *SAM synthetase 2*, which displayed an about twofold induction in response to *P. indica* colonisation, was remarkably removed from the mRNA pool after one subtraction step (Fig. 15). The analyses confirmed the stringency and specificity of the subtraction procedure as indicated by the efficient elimination of housekeeping and abundant genes.

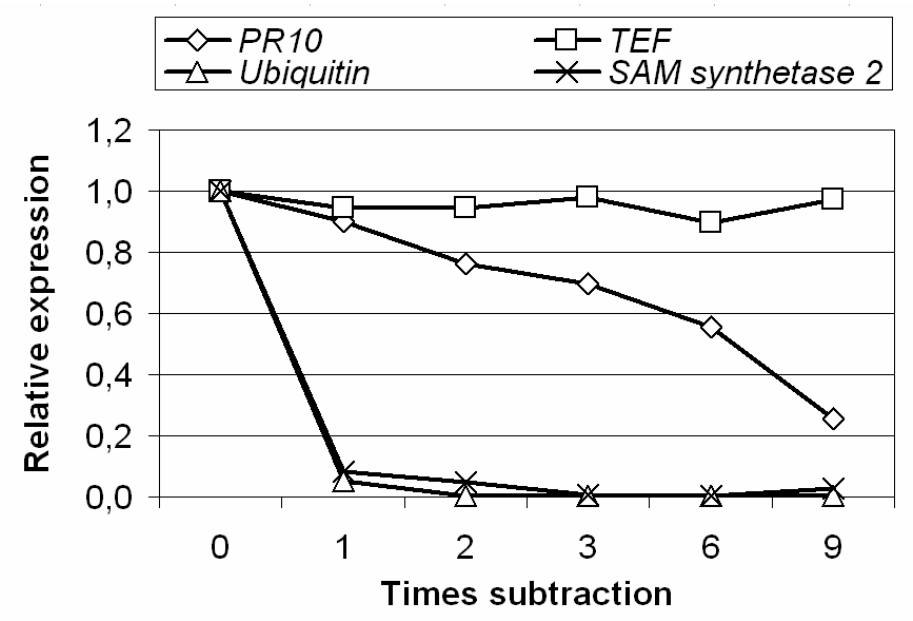

Figure 15: Determination of subtraction stringency of selected genes by subtractive hybridization. sscDNA of mock-treated plants was used to elucidate the effectivity of subtraction of *ubiquitin 5* (open triangle), *SAM synthetase 2* (cross), *PR10* (open diamond), and the fungal gene *Pitef* (open square) in barley roots at 60 hai. Amplification of transcripts was measured after each subtraction step using QPCR. Obtained values were related to transcript abundancy before subtraction. While slightly induced *SAM synthetase* 2 and non-responsive *ubiquitin 5* were almost completely removed after one subtraction step. Highly induced *PR10* was efficiently reduced after nine subtraction steps. *Pitef* was unaffected by the subtraction procedure.

3.6 Identification of genes involved in ethylene and polyamine biosynthesis by subtractive hybridisation

In order to identify the differentially regulated genes, mRNA isolated from respective root material was three times subtracted and used for cDNA library construction. After *E. coli* transformation, fiftheen colonies were randomly selected and sequenced. The approach resulted in a collection of genes encoding for proteins involved in nutrient and water uptake, plant defense, autophagy, phytohormone biosynthesis and signaling as well as polyamine synthesis (Tab. 3). None of these randomly selected clones encoded known housekeeping genes neither did we isolate candidates encoding identical proteins.

Table 3: List of barley genes isolated from subtracted cDNA library with corresponding accession number and putative function.

Gene annotation	**Accession number**	**Function**
Hordem vulgare, Germin F	AF250935	Defence-related gene
Secretory peroxidase	AK249643	Defence-related gene
Microtubule-associated protein	AK251678	Programmed cell death
RAV-like transcription factor	AK070487	Ethylene-responsive transcription factor
Drought inducible 22 kDa protein	AK249751	Stress-induced gene
NOD26-like membrane integral protein	AK112022	Aquaporin
S-adenosylmethionine synthetase 2	AK248590	Ethylene biosynthesis
S-adenosylmethionine decarboxylase proenzyme	AK250521	Polyamine biosynthesis
DnaJ-related proteins (Hsp40)	NM_001060953	Chaperone (protein folding)
Putative r40c1 protein	BM816173	Osmotic regulator
Delta tonoplast intrinsic protein	AY525640	Aquaporin
O-methyltransferase	EF189706	Secondary metabolism
Germin-like protein GLP3a	BQ740170	Stress response
Calcineurin B–like	EY965259	Protein processing

The reliability of TSH was confirmed by independent cDNA determinations through quantitative PCR at several timepoints (48, 60 and 72 hai) in different independent experiments encompassing sterile with or without soil conditions. As presented in Fig. 16A, the selected genes were shown to be induced by *P. indica* at 60 hai under sterile conditions. The same timepoint and conditions were used for the construction of the subtractive library. The expression profiles of plant genes in the soil experiment were a bit different compared to sterile conditions (Fig. 16A). However, most of the selected genes show a similar tendency of induction during the barley-*P. indica* interaction at 60 hai as seen in Fig. 16B. In TSH assay, I did not isolate fungal genes which might be explained by an under representation of fungal mRNAs in the total mRNA pool.

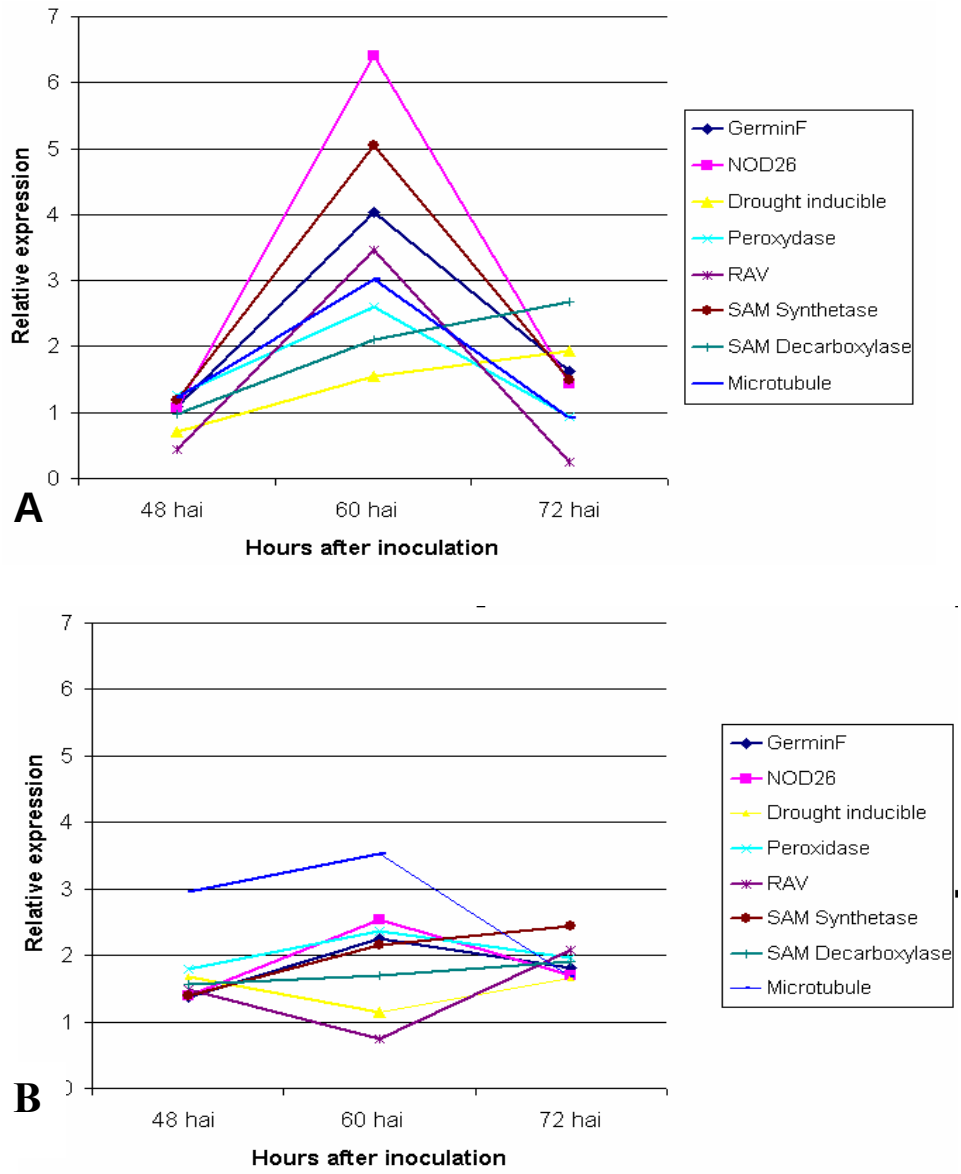

Figure 16: Relative expression patterns of barley genes isolated by Transcript Subtractive Hybridisation. Ten of the randomly selected genes were proven to be induced at 60 hai. Barley plants were inoculated with *P. indica* or mock-treated and grown under sterile conditions on $PNM_{(1/10)}$ (A) or on soil (B). Relative expression was determined by QPCR using gene-specific primers and *ubiquitin* as internal standard to normalize data.

3.7 Ethylene accumulation in barley roots colonized by *P. indica*

Among the identified cDNAs that are differentially expressed in *P. indica*-colonized roots was a gene encoding a *S-adenosylmethionine synthetase 2* (*SAM synthetase 2*). SAM serves as methyl donor for the synthesis of various physiologically important molecules including ethylene (ET). In an initial experiment, the amount of the precursor

of ET, 1-aminocyclopropane 1-carboxylic acid (ACC), was quantified in *P. indica*-colonised and mock-treated barley roots at 0, 6, 24, 36, 60, 120, and 168 hours after inoculation (hai) (Tab. S4). In addition, ACC quantities were determined in *Arabidopsis* colonised roots at 72 and 168 hai. The data analysis determined a higher ACC contents in *P. indica*-colonized barley roots at 60 and 120 hai encompassing early penetration to early colonisation stages (Fig. 17). Due to the limited sensitivity of the assay, no changes in ACC levels could be detected in *Arabidopsis* roots.

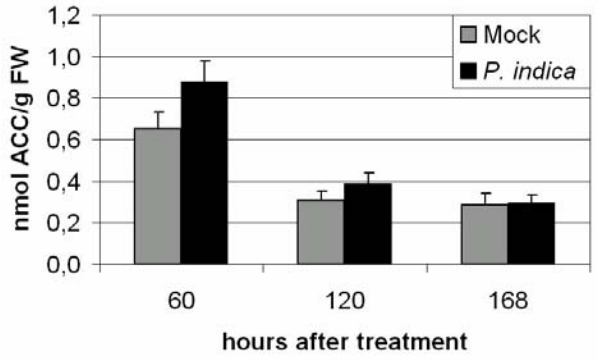

Figure 17: Free ACC contents in barley roots colonised by *P. indica*. Free contents in nmol g fresh weight-1 were determined at 60, 120, and 168 hours after treatment. Free ACC contents are elevated at 60 and 120 hai. Data shows the mean content of three biological experiments.

3.7.1 *De novo* ethylene synthesis during *P. indica* colonisation

An accurate approach to study ET signaling during *Arabidopsis* root colonisation by *P. indica* is to monitor the activity of respective reporter genes. The role of *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS*) in ET biosynthesis and turnover is well documented. The level of ACS closely correlates with the level of ethylene production in most plant tissues (Mattoo and Suttle, 1991). ACS is encoded by a multigene family. The different members are expressed differentially in response to various developmental, environmental, and hormonal factors. In *Arabidopsis*, 12 *ACS* genes (*ACS1* to *ACS12*) have been identified of which eight are functional ACS (Dugardeyn et al., 2008; Tsuchisaka and Theologis, 2004a). Therefore, we tested the transcription of *ACS* genes in *Arabidopsis* plants expressing of GUS gene (*uidA*) under the control of the promoters for *ACS* genes such as *ACS1, ACS2, ACS4, ACS5, ACS6,* and *ACS9* (Tsuchisaka and Theologis, 2004a,b). Ethylene reporter plants were harvested at

early biotrophic and advanced cell death-associated symbiosis stages (3 and 7 dai) and compared with mock-treated plants for GUS expression. Correlation between GUS activation and *P. indica* colonization was observed in the reporter lines ACS1::*GUS* and ACS8::*GUS* (Fig. 18 A, B, C, D). In the case of control roots of ACS1::*GUS*, weak GUS staining was detected at the quiescent center of root tips and at junctions of main to lateral roots (Fig. 18 E, F). In contrast, *P. indica*-colonised roots were more intensively stained at these sites and GUS expression co-localized with fungal penetration sites (Fig. 18 A, B).

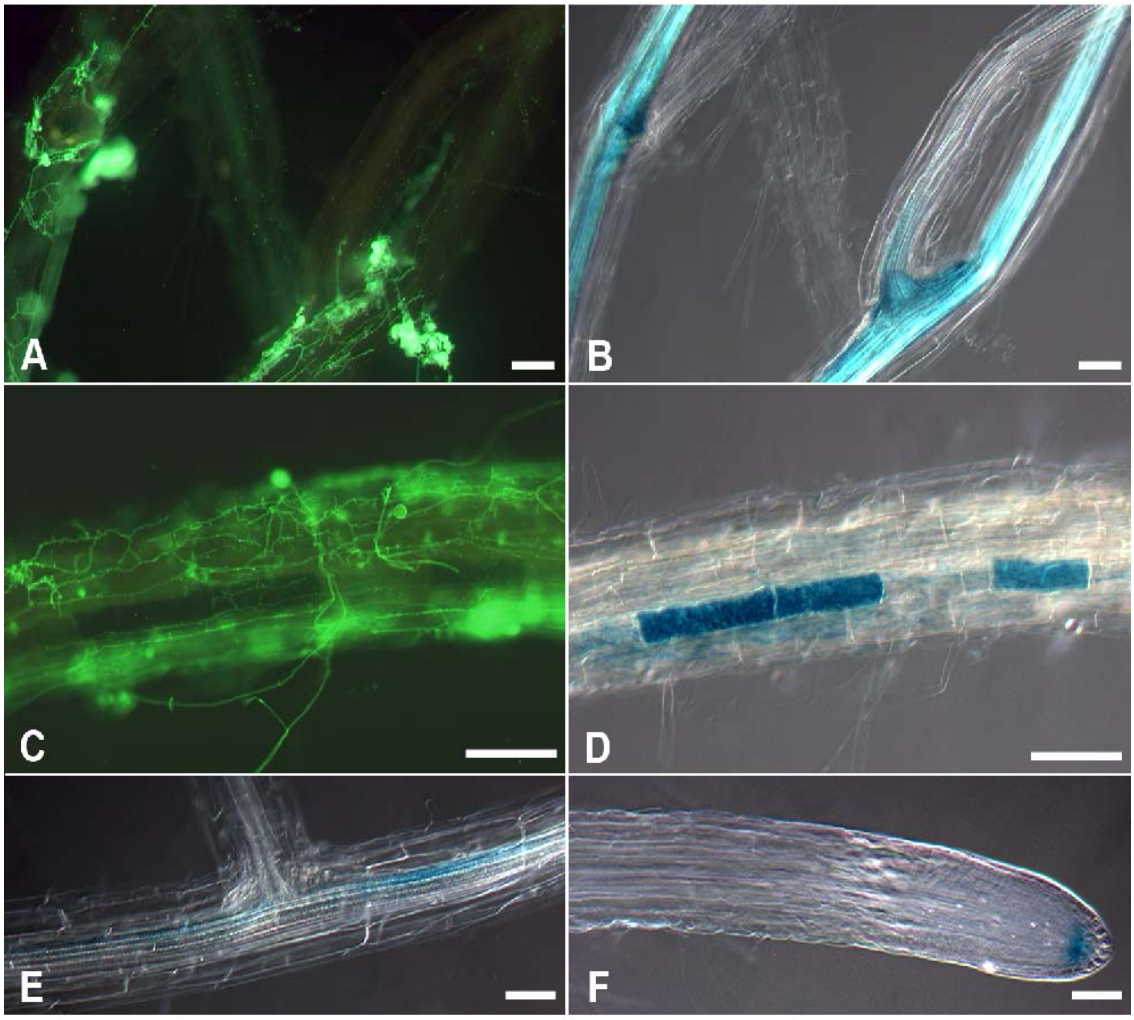

Figure 18: GUS expression in roots of ACS reporter plants colonised by *P. indica*. *Arabidopsis* lines ACS1::*GUS/GFP* and ACS8::*GUS/GFP* were harvested 3, 7, and 14 dai and, after GUS and WGA-AF488 staining, cytologically analysed. (A) In ACS1::*GUS/GFP* colonisation of the base of lateral roots or primordia was associated with enhanced GUS expression (B). (C) Occasionally, penetrated cells showed

pronounced GUS expression (D). In mock-treated ACS1::*GUS/GFP* GUS staining was weakly detectable at the lateral root base (E) and at the root tips (F). Staining pattern is reminiscent of GUS accumulation in this line after auxin treatment (see discussion for further description). (Bar = 60 µm).

In ACS8::*GUS/GFP*, GUS staining was more pronounced in root tip regions of colonised roots compared to mock-treated roots. GUS staining did not co-localise with infection attempts of *P. indica* or extracellular fungal growth. Interestingly, in ACS8::*GUS* roots, GUS expression was more strongly in root tips of *P. indica*-colonised roots (Fig. 19). Since the fungus was not detected at these sites, this accumulation pattern implicates a systemic induction by *P. indica*.

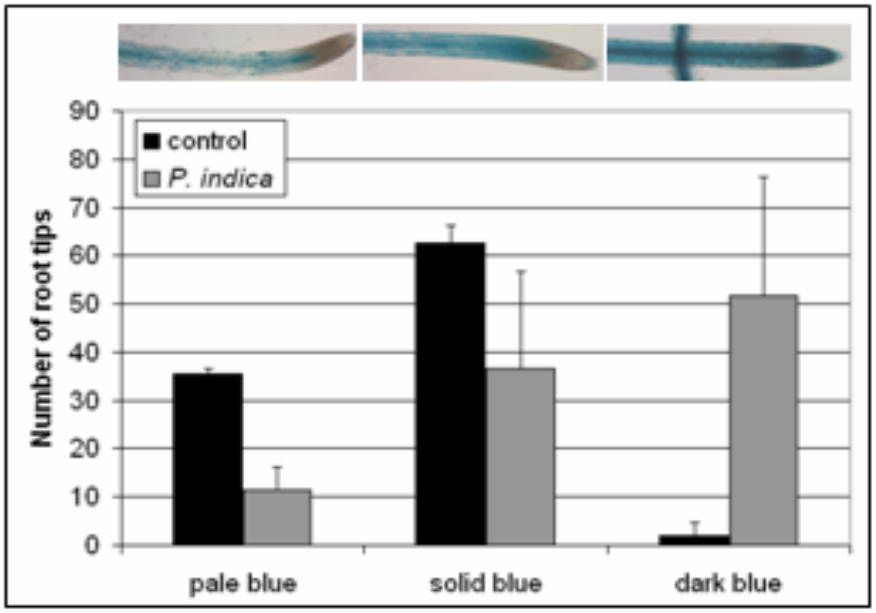

Figure 19: Number of pale, solid and dark blue tips in mock-treated and *P. indica*-colonised ACS8::*GUS* roots. The data depends on two independent biological experiments.

3.7.2 Ethylene – a compatibility factor during plant root colonisation by *P. indica*

Since no barley mutants altered in ET biosynthesis or signaling are available, a pharmacological approach was followed to elucidate the significance of ET for barley colonisation by *P. indica*. Barley roots were exposed to ET inhibitor 1-Methylcyclopropene (MCP) and ET precursor 1-aminocyclopropane 1-carboxylic acid

(ACC). MCP is very efficient and long-lasting in binding to ethylene receptors thereby blocking ethylene signaling (Sisler and Serek, 2003). This resulted in reduced and enhanced *P. indica* colonisation under MCP and ACC, respectively. The plants exhibited about 40% reduction in colonisation after application of the ET signaling blocker 1-methylcyclopropene (MCP), while treatment with ACC resulted in significant increase (~60%) in colonization at 7 dai. Minor changes were observed in *P. indica* colonisation for both treatments at 3 dai (Fig. 20).

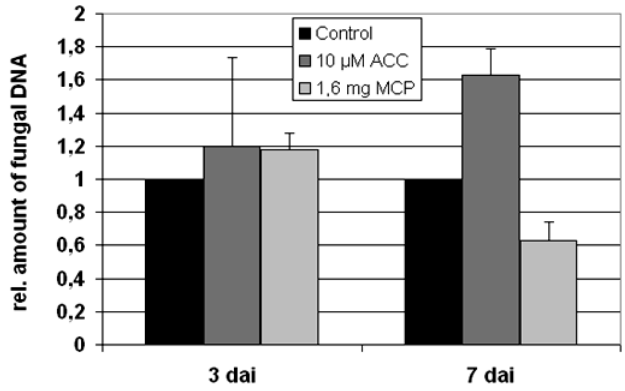

Figure 20: *P. indica* colonisation of barley cv Golden Promise in response to ACC and MCP treatment. Two day old plants were inoculated with *P. indica* and treated with 10 µM ACC and 1.6 mg MCP as described in experimental procedures. Colonisation was significantly inhibited by MCP and supported by ACC at 7 dai.

To further elucidate the impact of ET on plant root colonisation by *P. indica*, *Arabidopsis* mutants altered in ET metabolism were analysed. Ethylene perception and signaling was highly regulated at both transcriptional and post-transcriptional levels (Chen et al., 2005; Etheridge et al., 2006; Wang et al., 2002). In *Arabidopsis* ethylene is perceived by ER membrane-bound receptors (e.g. ETR1). In the absence of ethylene, the receptors activate a Raf-like kinase (CTR1) that in turn negatively regulates the downstream ethylene response pathway (Kieber et al., 1993). Binding of ethylene inactivates the receptors, resulting in deactivation of CTR1, which allows downstream effectors like EIN2 to function as a positive regulator of ethylene signaling (Guo and Ecker, 2004; Wang *et al.,* 2002). Hence, *ctr1* mutants display constutive ethylene signaling (Kieber et al., 1993). EIN2 activates nuclear EIN3 and EIN3-like 1 (EIL1), transcription factors that in turn

drive expression of primary ethylene transcriptional activators, e.g. ERF1. ERF1 is well-characterised to mediate transcription of ethylene responsive-genes including defense-associated genes (e.g. *PR3*, *PDF1.2*) (Adie et al., 2007). In addition, *ethylene overproducer 1* (*eto1*) mutant was included in the studies. ETO1 is a negative regulator of ethylene synthesis by inactivating and/or degrading ACS5 and probably other ACS isoforms (Chae and Kieber, 2005; Wang et al. 2004). Consequently, *eto1* displays an enhanced ethylene production in etiolated seedlings and ethylene synthesis is enhanced by external stimuli (e.g. hormones) (Chae and Kieber, 2005; Woeste *et al*., 1999). We investigated the colonization of the signaling (*ein2-1*, *etr1-3*, *ctr1-1*) and synthesis mutants (*eto1-1*) at 3 and 14 dai (Fig. 21). These timepoints were selected since *P. indica* is known to perform an initial biotrophic life style (~ 3 dai), which is followed by a cell death-associated colonization phase (> 5 dai). As shown in Fig. 21, *Arabidopsis* mutants that are enhanced in ET biosynthesis (*eto1*) or exhibiting constitutive ET signaling (*ctr1*) resulted in a 2-fold and 4-fold higher colonisation, respectively, at 14 dai. The higher colonisation in *ctr1* was also observed at 3 dai (55%), whereas the colonization rate in *eto1* was unaltered at 3 dai (Fig. 21). Mutants impaired in ET perception (*etr1*) showed almost no changes in colonisation at 3 and 14 dai. In contrast, *ein2-1*, a mutant that is impaired in ET signaling mutant was up to 36- and 29% less colonised after 3- and 14 dai, respectively (Fig. 21). *35S::ERF1*, the positive regulator of ET-related defense signaling showed a higher colonization rate (40%) at 14 dai.

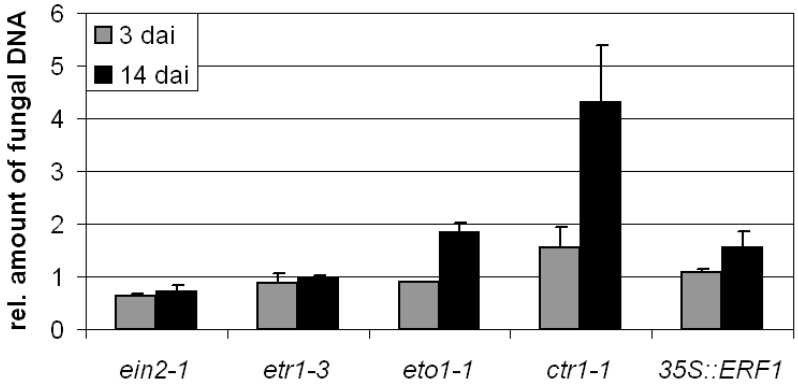

Figure 21: Colonisation of ET mutants by *P. indica*. Three weeks old *Arabidopsis* plants were inoculated with *P. indica* and fungal biomass in *ein2-1, etr1-3, eto1-1, ctr1-3*, and 35S::*ERF1* was determined by QPCR at 3 and 14 dai. The values are related to Col-0 (set

to one). While *ein2-1* showed reduced colonisation at 3 and 14 dai, more fungal biomass was detected in *eto1-1* and 35S::*ERF1* at 14 dai as well as in *ctr1-3* at 3 and 14 dai. The data base at least on two independent experiments.

3.8 Polyamines – compatibility factors for plant root colonisation by *P. indica*

Conspicuously, a cDNA clone was found by TSH, which is encoding a S-adenosylmethionine decarboxylase proenzyme (SAMDC), an enzyme specifically involved in the biosynthesis of the polyamines spermine and spermidine (Xiong et al., 1997). Accordingly, the experiments were conducted to determine the involvement of polyamine in *P. indica* colonisation. Arginine decarboxylase (ADC) is well characterized to participate in polyamine biosynthesis in plants. Subsequent functional studies using two *Arabidopsis* mutants *adc1* and *adc2* which are defective in encoding for arginin decarboxylases (ADC), displayed an 18% (*adc1*) and 27% (*adc2*) lower colonisation by *P. indica* (Fig. 22).

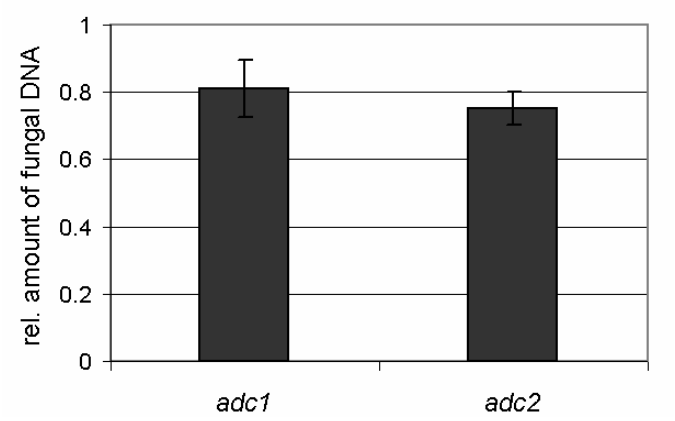

Figure 22: Amount of fungal colonisation of PA mutants. Three weeks old *Arabidopsis* plants were inoculated with *P. indica* and fungal biomass in *adc1*, and *adc2* was determined by QPCR at 14 dai. The values are related to Col-0 (set to one). Both *adc1* and *adc2* Showed lower colonisation by *P. indica* compared with Col-0. The data base on three independent experiments.

4. Discussion

4.1 The protein secretion system in eukaryotic cells

Plants have developed diverse strategies for protection against the threat of invading pathogens. In order to improve their performance as well as to evade abiotic and biotic stresses, one strategy of plants is to establish associations with beneficial microbial organisms. *Piriformospora indica* is a root interacting fungus, mimics the capability of a typical AM fungi. It was reported earlier that the interaction between the root colonizing fungus *P. indica* and the host plant, is a symbiotic association. Because of the root colonizing fungus, the host obtains several benefits including the abiotic stress tolerance, as well as improved plant fitness, biomass improvement, etc. *P. indica* colonizes a broad range of monocot and dicot plants. This broad host range indicates that *P. indica* has developed efficient strategies to overcome innate immune responses and manipulate metabolism in different plants. It has been widely accepted that plant colonisation by pathogenic or mutualistic aims to acquire adequate amounts of nutrients that are required for microbial reproduction. Irrespective of the microbial impact on the host organism, the innate immune system of plants is intended to avert any potential invader. However, microbes have co-evolved mechanisms to interrupt or circumvent potential defense responses and to redirect the plant metabolism on their own benefit. At least parts of these strategies are likely to be governed by the action of microbial metabolites and/or effector proteins. Therefore, understanding the molecular processes leading to the host compatibility and functions of symbiotic through the analysis of plant and fungus compatible factor was the main objectives. Hence, two different strategies were performed in parallel. In a first approach, the method so-called yeast signal sequence trap (YSST) was successfully established to identify *P. indica* effector proteins as well as plant secreted proteins that are involved in the manipulation of those processes required for successful fungal establishment *in planta*. In a second approach, a reliable, efficient, and simplified subtraction-based assay, designated *Transcript Subtractive Hybridization* (TSH), was successfully established to further study the plant compatibility factors in the host-*P. indica* interaction with the two model plants barley and *Arabidopsis*.

In general, secreted proteins play important roles in mediating cell-cell interactions, cell growth and differentiation, defense mechanisms and apoptosis (Diehn et al., 2000; Hückelhoven, 2007b; Jamet et al., 2006). In Fig. 23, the main features of the protein secretion system in eukaryotic cells are summarized. The first step in the transport of secreted proteins is translocation from the endoplasmic reticulum (ER) via their signal peptides to the Golgi apparatus. Signal peptides are usually 15-30 amino acids in length and consist of a highly basic N-terminus, a strong hydrophobic center, and a polar side at the C-terminus as well as a cleavage site (Nakai, 2000). Then, secreted proteins are transported through the cell membrane or cell exterior and are released from the secretory granules to the extracellular space (Matheson et al., 2006). Plasma membrane localised proteins are equally distributed via the protein secretion system. In this case, secreted proteins carry one or more transmembrane domain(s). It is well known that pathogens and microbes secrete a plethora of proteins inside the host cells to manipulate host physiology in order to promote their growth and dissemination (toxins, antimicrobial compounds and effector proteins) (Kwon et al., 2008; Hückelhoven, 2007b). Till now, several approaches have been developed for identification of secreted proteins or genes, encoding secreted proteins (Clark et al., 2003; Lee et al., 2004). Isolation of proteins from the apoplastic fluid using vacuum infiltration is one of the most straightforward ways (Lee et al., 2004). Another possibility is protein extraction from cell walls, gel-based separation followed by protein sequencing and identification (Jamet et al., 2006). Regarding the increasing numbers of full genome sequences from different microbes, new bioinformatic-based methods are provided as powerful tools for the prediction of secreted proteins (Dean et al., 2005; Kämper et al., 2006; Kleemann et al., 2008; Martin et al., 2008). However, bioinformatic analyses often result in quite divergent numbers of secreted proteins. This is mainly due to the lack or incompleteness of genomic data or expressed sequence tag (EST) databases (especially due to 5'-end truncated mRNA). However, based on the published fungal genomes, computational screenings have identified remarkable catalogs of secreted proteins expressed during plant-fungus interactions encompassing biotrophic fungi such as corn smut, is a disease of maize caused by the pathogenic plant fungus *Ustilago maydis* and in the flax rust fungus *Melampsora lini* as well as the ectomycorrhizal basidiomycete *Laccaria bicolor*

(Catanzariti et al., 2006; Kämper et al., 2006; Martin et al., 2008). Nevertheless, a reliable alternative way to identify secreted proteins is to use functional screening approaches. Both pro- and eukaryotic expression systems have been used successfully to screen for genes encoding secreted proteins (Klein et al., 1996; Jacobs et al., 1996).

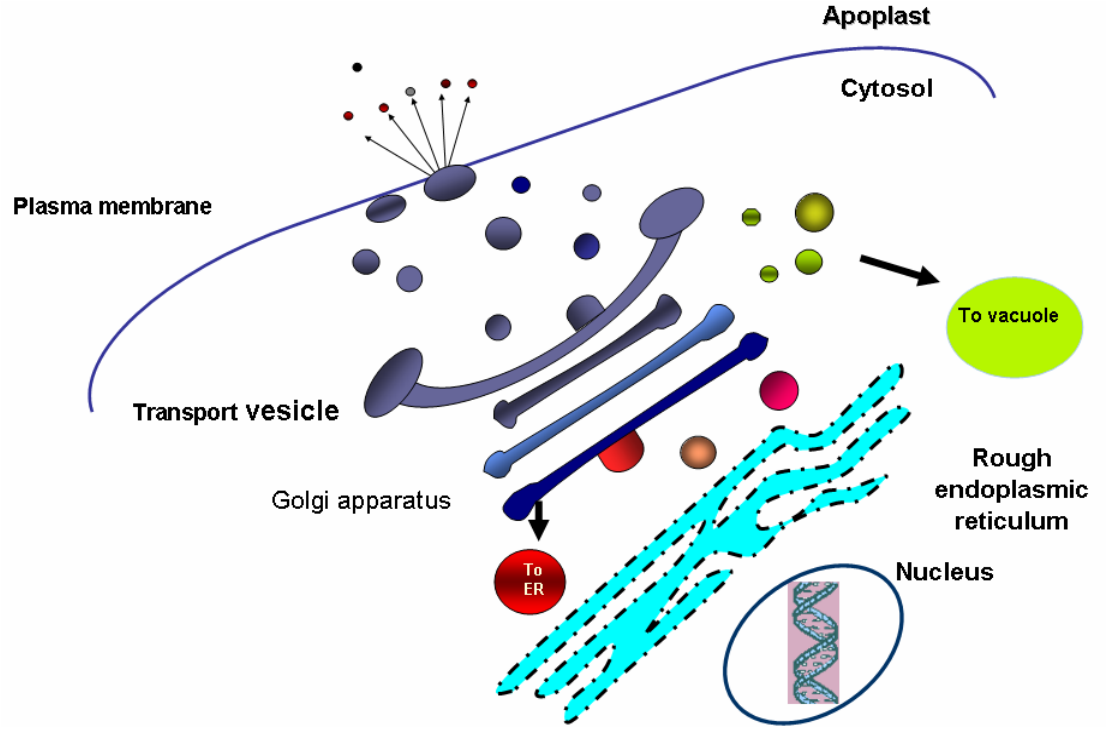

Figure 23: Simplified model for protein secretion. Proteins are synthesized via ribosomes associated with the endoplasmic reticulum (ER) and pass the ER membrane while their signal peptides are cleaved off. Thereafter, secreted proteins are transported from the ER through the Golgi apparatus to the plasma membrane, vacuole, chloroplast and mitochondrion. Protein sorting processes are occurring in order to direct secreted proteins to their definite destination. At the plasma membrane, secreted proteins that possess a extracellular destination are released from secretory granules to the extracellular space. The figure is adapted from Matheson et al., (2006) and modified.

4.2 The yeast signal sequence trap (YSST) assay identifies PIALH43 as a putative *P. indica* effector protein

In respect of the mutualistic interaction of plants and the basidiomycete *Piriformospora indica*, several molecular and biochemical processes are expected to be activated during host colonisation. *P. indica* colonizes a broad range of monocot and dicot plants. This broad host range indicates that *P. indica* has developed efficient strategies to overcome

innate immune responses and manipulate metabolism in different plants. This is even more intriguing as the fungus was shown to follow an initial biotrophic colonisation strategy at which penetrated cells are living (P. Schäfer, B. Zechmann, unpublished data). Therefore, it is interesting to know the factors responsible for successful colonization of fungus within the host. So I focused to determine and understand some of the fundamental mechanisms which are behind the compatible interaction between plant host and *P. indica* association in this thesis.

In order to study the secreted proteins from eukaryotic organisms like plants or fungi, baker's yeast (*Saccharomyces cerevisiae*) is commonly used since it has a similar protein processing system against the bacterial expression system. Many of the molecular mechanisms regulating the cellular processes including DNA metabolism, programmed cell death (PCD) and membrane traffic are conserved in eukaryotic cells (Valdivia, 2004). The so-called yeast signal sequence trap (YSST) has been successfully applied for screening cDNA libraries generated from different organisms like animals, plants and fungi (Belanger et al., 2003; Goo et al*.,* 1999; Lee et al., 2006b; Yamane et al., 2005). It represents a high-throughput method for the identification of genes encoding secreted proteins (Clark et al., 2003; Hugot et al., 2004; Jacobs et al., 1997; Klein et al., 1996). In case of plant-pathogen interactions, the YSST assay has been successfully used to identify proteins secreted by the hemibiotrophic corn pathogen *Colletotrichum graminicola* (Krijger et al., 2008), by the biotrophic rust fungus *Uromyces fabae* during colonisation of *Vicia faba* (Link and Voegele, 2008), and by the plant pathogenic oomycete *Phytophthora infestans* during infection of tomato (Lee et al., 2006a). In a present study, the YSST method was successfully used for trapping gene encoding secreted protein as well as fungal proteins encoding putative extracellularly acting proteins or fungal effectors during barley/*P. indica* interaction. As the result of YSST screening, several plant genes were identified that are known to be involved in stress responses and cell wall development (Tab. 1). The higher and specific level of gene expression of plant isolated gene as the result of YSST might reflect their impact on the symbiotic association (Fig 6A-D). For instance, α-expansin, one of the identified genes is closely belongs to expansin gene family that is known to induce cell wall relaxation and cell extension in acidic pH (Cosgrove, 2005). Loosening of cell wall is a key factor in

plant growth but may also make the plant vulnerable to biotic intruders. Recently, gene encoding α-expansin was localized at fungal penetration sites as well as those cells accommodated by AM fungi (Balestrini et al., 2005). Therefore, if our isolated gene has the same function, it might be speculated that α-expansin protein facilitates fungal symbiont entry into the host (Siciliano et al., 2007). Additionally, auxin induces the expression of expansin (Cosgrove, 2005). Hence, the induction of α-expansin during *P. indica* colonisation might be partly explained by the elevation of auxin in *P. indica* colonised roots. Therefore, the interpretation might be partly explained by the contribution of *α-expansin* in the reactions that promotes cell elongation. Among the isolated genes *Secretory peroxidase* and *Germin-like protein* are categorized as the stress- or defense-responsive genes. However, in barley *Germin-like protein* was expressed in response to biotic stresses, but not to abiotic stress (Zimmermann et al., 2006). It is well described as a defense-responsive protein in response to powdery mildew infection in barley (Zimmermann et al., 2006). *Germin-like protein* was strongly upregulated during *P. indica* colonisation mainly at early time points (3 dai). HSP70 is another class of functionally related proteins whose expression is increased when plants are exposed to stress, it is known as chaperone and has a function on protein folding as well as pathogen tolerance (Mambula et al., 2007). In addition to the identification of secreted plant proteins, a yet unknown fungal gene was identified via the YSST screen that did not show any similarities to other sequences deposited in public databases. However, the expression analysis of isolated plant and the fungal gene(s) showed a specific regulation in barley roots during *P. indica* colonisation (Fig. 6A-D and 7). The plant genes identified by the YSST screen were previously described to have an apoplastic localisation and function during symbiotic association (Balestrini et al., 2005; Waller et al., 2008). The additional root of evidence come from the presence of a predicted N-terminal signal peptide as suggested the functional capability of the YSST method. However, the screen also resulted in the identification of non-secreted proteins like ribosomal RNA (rRNA). The clones originated from 18S- and 28S-rRNA internal fragments were defined as false positives as previously reported for other biological systems (Belanger et al., 2003; Hugot et al., 2004; Lee et al., 2006a). This level of rRNA contamination is to be expected because only one round of polyA RNA enrichment was

performed prior to library construction by random priming. The other false positives in our analysis arose from incorrect ORF prediction. The signal peptide-mimicking transmembrane domains of truncated proteins have generated false positives in computational EST analyses (Klee and Ellis, 2005) and in functional screens for secreted proteins based on the yeast signal sequence trap (Klein et al., 1996). On the other hand, not all the clones recovered by signal sequence trapping have signal sequences. While no secretion signal was detected in the N-terminus of HSP70 protein. However, yeast harbouring HSP70 was able to grow on raffinose-containing media. In the past, it was believed that a signal peptide is strictly required for targeting a protein to the extracellular space. Recent studies have, however, shown that proteins can be exported to the apoplast without the requirement of a classical N-terminal signal peptide (Lee et al., 2006a; Krijger et al., 2008). The secretion of HSP70 was proven to occur by a non-classical protein secretion route in mammalian cells (Mambula et al., 2007). Nevertheless, no evidence concerning its ability to be secreted *in planta* is exciting.

4.2.1 The ubiquitin proteasome pathway and its function in plants

The identified *P. indica* protein (PIALH43) carries a signal peptide and was shown to be induced during barley root colonisation. Interestingly, PIALH43 harbours a highly conserved C-terminal RING finger motif. Protein modelling of PIALH43 confirmed a 3D structural overlap and verified the accurate conformation of the E2 binding residues compared to known human and plant ubiquitin ligases (Fig. 11). Accordingly, the role of PIALH43 in protein degradation was considered and its ligase activity determined *in vitro*. Therefore, recombinant PIALH43 protein was expressed in a bacterial expression system and then purified. The results from the *in vitro* E3 ligase assay implicate a role of PIALH43 in protein ubiquitination although this has to be confirmed in future experiments with PIALH43 versions mutagenised within the E2 binding residues. Taken together, the evidence presented here identifies PIALH43 as a novel *P. indica* protein that might function in ubiquitination. Regulation of protein function by post translational modifications such as glycosylation (the addition of oligosaccharides to the side chain amide of asparagine residues), phosphorylation (the addition of phosphate groups to serine, threonine or tyrosine residues) and protein ubiquitination (the covalent linkage of

the low-molecular-weight protein ubiquitin to substrate proteins) are highly effective method found within nature to fine tune protein activity in a highly sophisticated manner (Craig et al., 2009; Thrower et al., 2000). Ubiquitination is essential for protein turnover and degradation and it starts with the activation of ubiquitin by ubiquitin-activating enzymes (E1) follow by its transfer to ubiquitin-conjugating enzymes (E2). The E2 enzymes binds to E3 ubiquitin ligases thereby delivering activated ubiquitin, which is subsequently transferred to a substrate or target protein by an E3 ubiquitin ligase. In general, E3 ubiquitin ligases have a protein-protein interaction domain to appropriately capture substrates for the ubiquitin-mediated degradation of protein (Craig et al., 2009). One possible consequence of ubiquitination of a substrate protein is its degradation by the proteasome (Tanaka et al., 1998). Protein ubiquitination plays important roles in plant signaling pathways linked to hormone biosynthsis and signaling, growth, development and plant defense mechanisms (Devoto et al., 2003; Trujillo et al., 2008). Plant responses to biotic stresses or defence-associated signaling molecules have been shown to include transcriptional up-regulation of a number of E3 ligases indicating that E3 ubiquitin ligases have an impact in signal transduction pathways that lead to disease resistance during both PTI and ETI (Delauŕ et al., 2008; Dreher and Callis, 2007). For instance, the RING-finger type E3 ligases *ATL2* and *ATL6* genes encode are induced rapidly in *Arabidopsis* after chitin elicitor (Serrano and Guzman, 2004).

In contrast to the positive function of E3 ligases that are required for activation of defence, some of the E3 ligases negatively regulate the defense system (Trujillo *et al.*, 2008). *Arabidopsis* U-box proteins PUB22, PUB23, PUB24 show increased expression following flg22 treatment as well as after infection with *Pseudomonas syringae* and *Hyaloperonospora parasitica* (Trujillo *et al.*, 2008). Study the *pub22 pub23 pub24* triple mutant showed increased resistance to bacterial and oomycete pathogens and impaired down-regulation of responses triggered by PAMPs, suggesting the roles of these E3 ligases as a negative regulator of basal resistance (Trujillo *et al.*, 2008).

On the other hand, ubiquitination has been shown to contribute to the transcriptional regulation of hormone-responsive genes in the auxin, gibberellin, abscisic acid, ethylene and jasmonate signaling pathways and influence disease resistance and even

susceptibility, most notably through the regulation of defences orchestrated by signaling of plant hormones (Dreher and Callis, 2007).

Beyond the role of plant ubiquitination in defense, plant pathogens often follow a strategy to mimicry of host proteins to establish compatibility. It is well known that the plant pathogenic bacteria secrete effector proteins that attack the host signaling machinery to suppress immunity. *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) is a pathogen of tomato and *Arabidopsis*, and it injects ~30 effectors into host cells. One of these effectors is AvrPtoB that is widely conserved among diverse bacterial pathogens, including *Xanthomonas*, *Erwinia*, and many strains of *Pseudomonas*. AvrPtoB bacterial mutants with loss-of-function in ubiquitin ligase activity elicit a host immune response, leading to defense gene induction and localized cell death known as the hypersensitive response. The C-terminal of AvrPtoB portion contains a domain with the activity and structural similarity of an E3 ligase (Jansusevic et al., 2006). This activity has been reported to cause the ubiquitin-dependent degradation of Fen kinase, a host protein that activates the plant innate immune response (Abramovitch et al., 2006; Janjusevis et al., 2006). Therefore, AvrPtoB degrades host protein kinases through ubiquitination by a C-terminal E3 ubiquitin ligase domain (Rosebrock et al., 2007) to mediate the suppression of the plant immune response by evading the plant's cell death and basal defence responses (Abramovitch and Martin, 2005; De Torres et al., 2006). On the other hand, the PAMP receptor FLS can be ubiqtinatated through the action of the bacterial effector AvrPtoB. Therefore, AvrPtoB could interfere with the plant innate immune system and suppresses PCD associated with plant immunity in order to colonize the host during both PTI and ETI. Thus, AvrPtoB is an example of a pathogen evolving molecular mimicry of host proteins by modification of the host ubiquitin machinery (Abramovitch et al., 2006; Janjusevis et al., 2006). Interestingly, the identification of E3 ligase at the *P. indica* effector protein (PIALH43) that is involved in ubiquitination may represent a novel mechanism for fungal establishment by modification of the host ubiquitin machinery. Annotation of plant eukaryotic pathogens such as *Phytophthora infestans* genome has revealed members of the E3 ubiquitin ligase family that may be secreted, and could thus interact with host ubiquitin enzymes to trigger, suppress, or otherwise modify ubiquitination (Birch er al., 2009**)** .

In conclusion, the same model like the bacterial effectors can be designed for PIALH43 as a novel *P. indica* protein. Based on this model, PIALH43 might act in substrate recognition thereby supporting rapid intracellular degradation of target proteins as exemplified in bacterial infection through its activity of E3 ligase domain. Ultimately, *P. indica* targeted the host ubiquitin proteasome system for theirs own benefit.

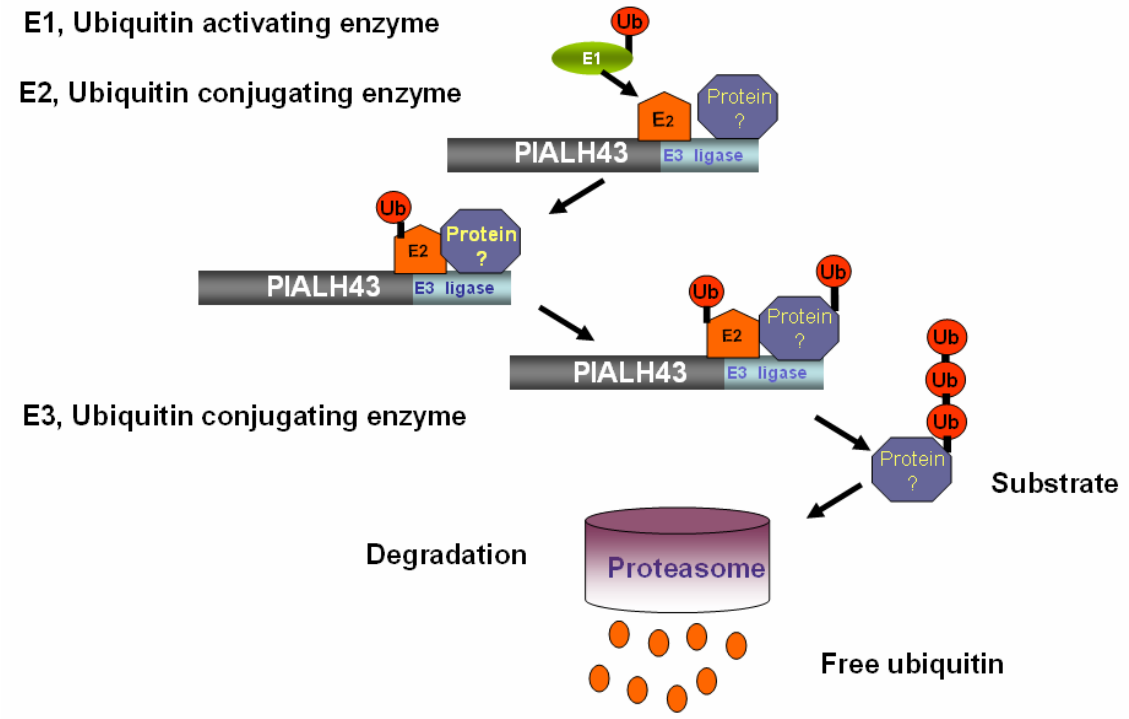

Figure 24: Putative function(s) of PIALH43 in protein ubiquitination. Ubiquitination has been associated with protein degradation of target proteins by the 26S proteosome. The components of ubiquitination are ubiquitin activating enzymes E1, ubiquitin conjugating enzymes E2 and ubiquitin protein ligases E3. Proteins tagged with poly-ubiquitin chains are targeted to 26S proteasome. Eventually, after disassembly of tagged proteins in proteasomes, free ubiquitin are released. PIALH43 might function as E3 ubiquitin ligase that is translocated into plant cells thereby targeting an unknown plant protein for degradation. The figure is adapted from Abramovitch et al., (2006) and modified.

4.2.2 PIALH43 as a putative *P. indica* effector protein and symbiotic reprogramming

Hypersensitive Response (HR) as a type of programmed cell death (PCD) in plants is an important plant resistance mechanism against biotrophic pathogens and suppression of HR induced by biotrophic pathogens could facilitate plant colonisation. In contrast, cell

death or necrosis helps necrotrophic pathogens to colonize plant hosts. It is still a matter of debate whether this type of necrotic cell death is always programmed and, hence, genetically determined as compared to HR. Hemibiotrophic pathogens might manipulate cell death depending on their infection phase since these fungi have an initial biotrophic followed by a necrotrophic life style. The mechanism of effectors in plant defense suppression might be performed by inactivation, modification or suppression of host defense proteins. For instance, many pathogen effectors have been demonstrated to suppress HR. AvrPtoB suppresses cell death in yeast cells in response to proapoptotic conditions such as oxidative stress or extreme heat shock (Abramovitch et al., 2003). Another example is the *Avr* gene, *Avr1b*, from *Phytophthora sojae*, which was characterized as suppressor of HR (Dou et al., 2008). On the other hand, some effector protein can cause necrosis in hosts when it is secreted during the interaction. For instance, Nep1-like protein (PiNPP1.1) is an extracellular fungal protein from *P. sojae* that causes necrosis and its expression is associated with the necrotrophic phase of colonisation (Qutob et al., 2002). Therefore, to determine the function of PIALH43, the PIALH43 should be expressed in yeast to see if the putative *P. indica* effector protein PIALH43 has any inhibitory effect on yeast growth or not. In future, the expression pattern of PIALH43 in different *P. indica* life style should be investigated

Taken together, the used YSST screening method provides an effective tool to identify proteins secreted by either interacting partner and represents a first step in elucidating those biological processes that are involved in the establishment of mutualistic symbioses.

4.3 Plant factors influencing root colonisation by *P. indica*

The molecular and biochemical processes associated with *P. indica* colonisation of plant roots are only partly understood. In *Arabidopsis* by transmission electron microscopy (but which most probably represents a general infection strategy in other plants), root colonization is initiated with a biotrophic followed by a later cell death-dependent colonization phase (Schäfer and Kogel, 2009 and unpublished data). So far, overexpression of the negative cell death regulator, Bax Inhibitor -1, was found to restrict barley root colonisation by *P. indica*, which is in agreement with the proposed cell death-

dependent colonisation at later interaction stages (Deshmukh et al., 2006). In *Arabidopsis* mutant with the loss-of-function PYK10, *P. indica* root colonization was increased (Sherameti et al., 2008). PYK10 is an ER-localised leucine-rich repeat protein with high sequence similarity to At*PEN2* that is required for Non-host resistance (Sherameti et al., 2008). Interestingly, *pyk10* mutant showed enhanced root colonization, but were inhibited in the transfer of beneficial effects (e.g. growth promotion and seed production) by the endophyte (Peškan-Berghöfer et al., 2005). Owing to its broad host range, *P. indica* must have evolved molecular and biochemical strategies, which most probably possess a plant kingdom-wide efficiency. Recently, microarray analysis was performed to understand signaling pathway which are targeted by *P. indica* for compatibility (Schäfer et al., in press). In this respect, the phytohormone gibberellic acid apparently takes an essential role in barley as the barely mutants impaired in GA synthesis and perception exhibited a reduced colonisation (Schäfer et al., in press). However, the mechanisms underlying and determining host compatibility in the plant root-*P. indica* association are mostly unknown. The present study aimed to identify host compatibility factors and to define their universal function in the respective monocot and dicot model plants barley and *Arabidopsis*.

4.4 Plant genes identified by Transcript Subtractive Hybridization (TSH) are induced by *P. indica* during root colonisation

Several reverse genetics approaches have been developed to identify genes differentially regulated in organisms in response to abiotic or biotic stresses. However, the available methods have certain drawbacks in that they are costly, technically demanding or have a reduced sensitivity. Microarray-based approaches are commonly employed when extensive genome sequences are available (Busch and Lohmann, 2007). Other routinely applied analytical methods like RNA arbitrarily primed PCR (RAP-PCR), representational difference analysis (RDA), differential display (DD) often deliver a high percentage of false positives and might loss some differentially expressed genes due to PCR bias (Donson et al., 2002; Huang et al., 2007; Liang and Pardee, 1992; Stein and Liang, 2002). Furthermore, RDA uses multiple high-stringency hybridizations, which could result in a low recovery frequency of moderately upregulated genes (Huang et al.,

2007; Hubank and Schatz, 1994). The increasing tendency to use subtractive hybridization reveals its attraction for discovering novel genes (Akopyants et al., 1998; Ferreira et al., 1999; Mishra et al., 2005; Thomas and Pauls, 2001). However, an intrinsic limitation of subtraction can often be attributed to technical difficulties faced during performance of the procedure. The latest advancement, the subtractive suppressive hybridization (SSH) is more efficient and provides a better solution than the other subtraction techniques for different applications (Diatchenko et al., 1996; Ferreira et al., 1999; Huang et al., 2007; Sagerström et al., 1997). The drawbacks of SSH is a one step subtraction, that increases the incidence of false positive signals and the dependence of SSH on the efficiency of adaptor ligation (Huang et al., 2007). The key solution for these difficulties would be the performance of subtraction at the mRNA level, which circumvents PCR-based bias.

For the identification of differentially regulated barley genes in response to *P. indica* colonisation, we used subtraction at the mRNA level and established so-called Transcript Subtractive Hybridisation (TSH) assay (Fig. 14). The convenience of this method results (1) from its flexibility in terms of subtractive stringency, (2) from the ability to simultaneously isolate rare and more abundant (or weakly and highly induced) transcripts in one step, (3) from the mRNA-defined specificity of the subtraction process and (4) from the independent of sequence information. The efficiency and accuracy of this method was proven by monitoring the elimination of transcripts of the housekeeping gene *ubiquitin* and the *P. indica*-induced gene *PR10* while the transcript abundance of the *P. indica* gene *Pitef* was unaffected (Fig. 15). In contrast to other assays, the present method would offer the unique ability for the identification of suppressed genes with a similar efficiency. Therefore, challenged samples might be used for sscDNA synthesis and hybridisation with mRNA of mock-treated material. Further than proving the high sensing of TSH performance. The transcript level of identified genes in the subtracted cDNA library was also checked. It contained the genes that have been described before and are involved in mutualistic interactions (Tab. 2). The function of barley genes stimulated in response to *P. indica* colonisation can be divided in different categorizes including stress- or defense response genes, gene encoding proteins involved in water and nutrient uptake and hormone synthesis. The Secretory peroxidase and Germin F are

categorized as stress-related proteins. Secretory peroxidase has an impact on the oxidative burst especially at early responses to pathogen attacks (Foyer and Noctor, 2005). Oxidative burst is a part of the response to pathogen attack in plants and plays direct as well as indirect roles in plant defense. The production of Reactive oxygen species (ROS) can act as a toxic compound for pathogen as well as reinforce the cell wall by catalyzing cross-linking of cell wall proteins through a peroxidase-dependent reaction (Apel and Hirt, 2004; Torres and Dangl, 2005). ROS are also known as a second messenger that is critical for activation of defense reactions like pathogenesis-related (PR) proteins synthesis as well as phytoalexin accumulation (Apel and Hirt, 2004; Mittler, 2002; Torres and Dangl, 2005). The regulatory functions of ROS in symbiotic performance like mycorrhiza and endophyte establishment are well studied (Baptista et al., 2007; Hause and Fester, 2005). Among the genes, the expression level of a *Germin-Like Protein* (*GLP*) was highly induced at early stages of the barley-*P. indica* interaction. GLPs are involved in plant development and defense (Brechenmacher et al., 2004; Manthey et al., 2004). Germins are known to restrict powdery mildew infection of barley leaves (Manthey et al., 2004; Zimmermann et al., 2006) as well as mycorrizal progression. In analogy, germin induction in roots might aim at restricting *P. indica* invasion (Schäfer et al., in press). The induction of stress- or defense response genes is in agreement with recent results showing that *P. indica* transiently induced stress and defense-related genes in barley roots during penetration/early colonization (Schäfer et al., in press). The other cluster covers gene encoding proteins that regulate and facilitate water and nutrient uptakes including *NOD26-like membrane integral protein* involved in active transport of solutes across the perisymbiotic membrane during the formation of nodules by *Rhizobium* sp (Uehlein et al., 2007). The upregulation of Nodulin-26 has been previously reported during nodulation and similarly in AM mycorrhiza association also (Brechenmacher et al., 2004; Manthey et al., 2004). It was currently accepted that the symbiosis protects hosts against the detrimental effects of water deficiency due to the combination of physical, nutritional and cellular effects. The other gene in this group is a gene encoding a Drought-inducible 22 kDa protein was more pronounced in barley roots during *P. indica* colonisation. Drought-inducible 22 kDa protein plays a role in drought stress tolerance by facilitating water and nutrition uptake (Uehlein et al., 2007). The

contribution of *P. indica* in plant drought tolerance might be a link to its natural habitat, the desert (Waller et al., 2005). The other members of this group are Delta tonoplast intrinsic proteins and putative r40c1 protein that are documented as aquaporins and osmotic regulator respectively (Luo et al., 2005). In addition, an *S-adenosylmethionine (SAM) synthetase 2* and a *SAM decarboxylase proenzyme* were identified genes associated with ethylene and polyamine biosynthesis respectively. *SAM synthetase 2* was more expressed in *P. indica* colonised roots compared to non-inoculated barley plants at 60 hai. ET acts as a pivotal mediator and coordinator of several functions in plant like growth and development as well as defense response. ET is a negative regulator of *Rhizobium* infection and nodule formation as well as a negative regulator of mycorrhiza symbiotic establishment. Further, a *RAV-like* transcription factor was identified, which might participate in ethylene signaling (Kim et al., 2005; Sohn et al., 2006). *S-adenosylmethionine decarboxylase* takes part in polyamine (PA) biosynthesis. PA is known as a factor affecting growth, development and survival including regulation of gene expression, translation, and modulation of cell signaling and membrane stabilization (Kumar et al., 1997). The accumulation of PA in host plant is one of the first indicators for the establishment of ectomycorrhizal interactions. PA can also regulate cell death, particularly apoptosis (Seiler and Raul, 2005). Interestingly, none of the isolated genes identified as being differentially regulated by *P. indica* in barley roots by microarrays (Schäfer et al., in press).

4.5 Ethylene acts as a compatibility factor in plant root colonisation by *P. indica*

As result of present studies, ET was identifed as a compatibility factor in the mutualistic plant-*P. indica* interaction. Moreover, ethylene (ET) was effectively supporting colonisation in the monocot barley and dicot *Arabidopsis*. As, an elevated content of free 1-aminocyclopropane-1-carboxylic acid (ACC) during early colonisation stages (60 and 120 hai) was monitored (Tab. S4). The studies revealed an improved endophytic colonisation of barley roots treated with the ET precursor ACC while barley roots exposed to the ET inhibitor 1-methylcyclopropene (MCP) displayed a reduced colonisation (Fig. 20). These results are in accordance with recent microarray-based studies, in which we monitored the induction of two ACC oxidases at 72 hai (Schäfer et

al., in press). As *P. indica* is able to colonize *Arabidopsis*, we investigated mutants to determine whether ET signaling also is required in a dicot. In general, ET perception and signaling is highly regulated at both transcriptional and post-transcriptional levels (Chen et al., 2005; Etheridge et al., 2006; Wang et al., 2002). In *Arabidopsis*, ET is perceived by membrane-bound receptors (e.g. ETR1) but in the absence of ET, the receptors activate a Raf-like kinase (CTR1) that in turn negatively regulates the downstream ET response pathway (Kieber et al., 1993).Therefore, binding of ET inactivates the receptors, resulting in deactivation of CTR1, which allows downstream effectors like EIN2 to function as a positive regulator of ET signaling (Guo and Ecker, 2004; Wang et al., 2002). The two *Arabidopsis* mutants *constitutive triple response 1* (*ctr1-1*) and *ET overexpresser 1* (*eto1-1*) characterised for a constitutive ET signaling and enhanced ET biosynthesis, respectively, exhibited a significant increase in fungal biomass, while less colonization (about 20%) was detected in the ET-insensitive mutant *ein2-1* compared to parent lines in response to *P. indica.* These data are in accordance of other *Arabidopsis* mutants *ctr1-1* and *eto1-1* exhibited a significant increase in fungal biomass, while fungal colonisation was reduced in *ein2-1* (Fig. 21).

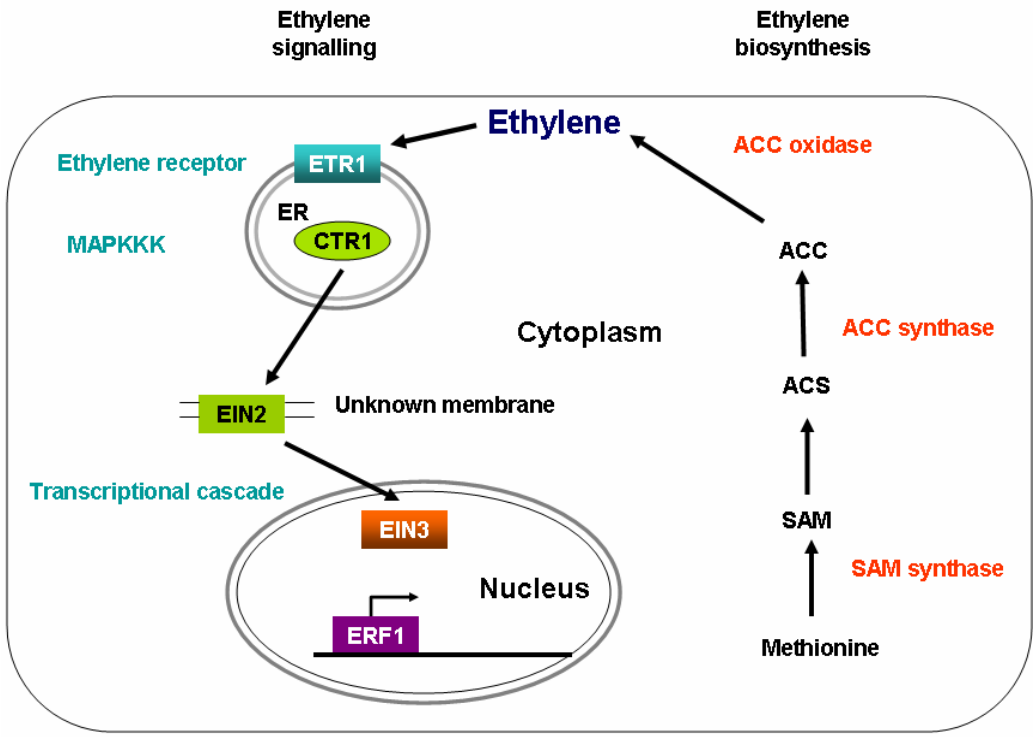

Fig. 25: The ethylene biosynthesis and signaling pathway in plants. ET is formed from methionine via the action of *S*-adenosyl-L-methionine synthetase (SAM synthetase) which converts methionine to SAM. ACC synthases convert S-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is converted to ET by the enzyme ACC oxidase (ACO). ET is sensed by endoplasmic reticulum (ER) localized receptors. ER-localised CTR1 is known to negative regulate ET responses. EIN2 is a positive regulator of ET and located downstream of CTR. EIN3 is a transcriptional regulator important for ET signaling. ERF1 as a transcriptional regulator activate genes in response to ET.

As *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS*) proteins mediate the rate limiting step in ethylene synthesis. In Arabidopsis, nine ACS gene familiymembers are described so far (Yamagami et al., 2003). As the various *ACS* proteins have different enzymatic properties, it has been proposed that the various *ACS* genes may respond to different stimuli and show a divergent spatial regulation to perform specific functions (Yamagami et al., 2003). Broekaert et al. (2006) have recently offered a brief overview of the ACS transcriptional regulation following biotic stress by analysis of the Genevestigator database (Zimmermann et al., 2004). This analysis shows that ACS2, ACS5, and ACS6 represent a major focus of transcriptional regulation after pathogen challenge.

Therefore, ACS transcription of various ACS::*GUS* fusion lines was cyto-histochemically analysed. Finally, a elevated GUS accumulation in ACS1::*GUS/GFP* and ACS8::*GUS/GFP* reporter plants colonized by *P. indica* was monitored. The induction of *ACS1* was unexpected since it was previously reported not to be expressed in roots (Tsuchisaka and Theologis, 2004a). ACS1 itself is enzymatically inactive as homodimer but attains activity after heterodimerization with other ACS isozymes (Tsuchisaka and Theologis, 2004b; Yamagami et al., 2003). ET synthesis is known to be induced by auxin, which stimulates the activities of several ACS (Stepanova et al., 2007; Ruzicka et al., 2007). Intriguingly, the pronounced GUS expression in ACS8::*GUS/GFP* roots after *P. indica* inoculation (Fig. 18 H-I) is reminiscent of the previously reported expression pattern of *ACS8* in *Arabidopsis* roots after auxin treatment (Fig. 18 I) (Tsuchisaka and Theologis, 2004). Auxin might be synthesised by the plant as several genes participating in auxin biosynthesis were induced by *P. indica* (Schäfer et al., in press) and/or by the

fungus as was recently reported (Sirrenberg et al., 2007). Hence, *GUS* expression in *ACS8* might be a result of auxin activity. Interestingly, neither defense-associated ACS2 nor ACS6 was found to be *P. indica*-responsive, which is in line with the defense-suppressive activity of the fungus.

In plant-microbe interactions, ET is generally regarded as a compound that confers resistance against necrotrophic pathogens, while biotrophic pathogens are rather unaffected (van Loon et al., 2006). For instance, ET application protects plants against *Botrytis cinerea* while *ein2* mutants are more susceptible (Broekart et al., 2006). ET suppresses the formation of nodules in legumes by rhizobium (Guinel and Geil 2002; Penmetsa and Cook, 1997; Riedel et al., 2008). The ERF/AP2 transcription factor (ERN) is essential for NOD factor signal transduction (Middelton et al., 2007). In addition, decreased mycorrhization of tomato *sitiens* mutants (abscisic acid deficient) was partly explained by enhanced ET contents in mutant roots (Herrera-Medina et al., 2005). Plant-growth-promoting rhizobacteria are able to modulate ET responses by altering the levels of ACC produced by plants. A reaction, catalyzed by the microbial-encoded enzyme ACC deaminase, hydrolyses ACC to α-ketobutyrate and ammonia thereby decreasing the levels of ACC that are available for ET production (Glick, 2005). We included 35S::*ERF1* plants in our studies in which jasmonate and ethylene-induced defense genes (e.g. *PDF1.2*, *b-CHI*) are constitutively expressed and which are more resistant against necrotrophic leaf and root pathogens (Berrocal-Lobo et al., 2002, Berrocal-Lobo and Molina, 2004). Interestingly, 35S::*ERF1* plants were stronger colonized by *P. indica* at 14 dai. By analysing the colonisation phenotypes observed within this study, ethylene signaling has a complete contrary meaning for *P. indica* in terms of compatibility as compared to necrotrophic pathogens especially during cell death-dependent interaction stages (14 dai). The data might also indicate that *P. indica*-induced cell death is molecularly and biochemically different to necrotrophy-associated cell death as already implicated by the absence of root necrotization in *P. indica*-colonized roots (Schäfer and Kogel, 2009). Nevertheless, ethylene is apparently also a basal component of compatibility during biotrophic *P. indica* colonisation as imposed by reduced compatibility in *ein2-1* and enhanced susceptibility in *ctr1-1* at 3 dai.

Recently, auxin was found to be strongly elevated in *P. indica*-colonised *Arabidopsis* roots at early interaction stages (Vadassery et al., 2008). Auxin has been shown to be negatively regulating plant defence processes and the induction of auxin signaling represents an active strategy of pathogens to establish compatibility (Navarro et al., 2006; Wang et al., 2007). Interestingly, barley root colonisation by *P. indica* is associated with a broad suppression of the barley innate immune system during colonisation as indicated by microarray studies (Schäfer et al., in press). The possible involvement of elevated auxin during *P. indica* colonisation in suppression of plant defense needs to be investigated. A possible explanation for the support of *P. indica* colonisation by ET might be found in its ability to induce auxin synthesis. ET also participates in developmental (e.g. tracheary element differentiation, adventitious root development) as well as defence-related cell death processes (Bouchez et al., 2007; Kuriyama and Fukuda, 2000; Mergemann and Sauter, 2000; Nasir et al., 2005). However, *P. indica* might alternatively use the proapoptotic activities of ET in favour of its cell death-dependent life style. In conclusion, ET signaling (in crosstalk with auxin and/or other hormones) might be activated by the fungus in various monocot and dicot hosts.

The hormone takes a crucial part in abiotic and biotic stress responses (Finkelstein and Rock, 2002) and often shows antagonistic activity for other hormones (Asselbergh et al. 2008).The supporting activity of ethylene in terms of root colonization by *P. indica* was surprising in consideration of the recent identification of GA as compatibility factor (Schäfer et al., in press). Ethylene is reported to have rather antagonistic activity towards GA responses by stabilizing GA signaling repressing DELLA proteins (Achard et al., 2003, 2006) and by generally down regulating GA synthesis genes (Dugardeyn et al., 2008). However, both hormones synergistically support hypocotyl elongation in light-grown *Arabidopsis* (De Grauwe et al., 2007), root cell elongation, and maintainance of the apical hook (Achard et al., 2003; Vriezen et al., 2004). Microarray-based data even indicate genes whose GA-responsiveness was dependent on a functional ethylene signaling pathway (De Grauwe et al., 2007) and exogenous GA induces several *ACS* genes (including *ACS8*) (Dugardeyn et al., 2008). De Grauwe *et al.* (2008) identified three to fourfold higher contents of bioactive GA_4 in *eto2-1* mutants. Taken together,

ethylene-GA crosstalk is obviously multilayered and cannot be reduced to a simple antagonistic interaction level.

As it was mentioned, the phytohormone might be regarded as one master key in the initiation of signal cascades that might at least partly explain *P. indica*'s extraordinary ability to colonize the multiplicity of hosts. Interestingly, ET biosynthesis is also required for virulence function of individual TTSS effectors. Interestingly, ET is elevated by the *P. syringae* pv. *tomato* effectors, AvrPto and AvrPtoB as virulence factors to enhance symptom development on tomato leaves during a compatible interaction (Lin and Martin, 2005). Therefore, it might be of future interest to investigate to which extent ET elevation in *P. indica*-colonized root rely on effectors activities.

4.6 Diamine and polyamine recruitment to support the *Arabidopsis* root-*P. indica* symbiosis

Our analyses resulted in the identification of a gene encoding a S-adenosylmethionine decarboxylase proenzyme (SAMDC) and is induced in barley roots during *P. indica* colonisation. *SAMDC* is a key enzyme in polyamine synthesis as it provides decarboxylated SAM, which is added to putrescine by spermine- and spermidine synthases (Alcazar et al., 2006; Franceschetti et al., 2001; Urano et al., 2005). Plants neither have any *ornithine decarboxylase* (*ODC*) gene nor do they possess ODC activities meaning that putrescine synthesis is exclusively performed via arginine decarboxylation by arginine decarboxylase (ADC) (Hanfrey et al., 2001). *Arabidopsis* contains two *ADC* genes (Urano et al., 2005). Our genetic studies revealed the significance of diamine/polyamine in the *Arabidopsis*-*P. indica* interaction. As the Arabidopsis mutants impaired in PA biosynthesis (*adc1* and *adc2*) displayed reduction in colonisation by *P. indica*. Accumulation of diamine/polyamine has been reported at the early stages of endo- and ectomycorrhizal establishment (Efrose et al., 2008; Niemi et al., 2006; Sannazzaro et al., 2007). Polyamines also accumulate during the Hypersensitive Response (HR) in plant-microbe interactions (reviewed in Walters, 2003). This accumulation is discussed to be associated with the activities of diamine/polyamine oxidases that lead to the generation of H_2O_2, a postulated inducer of cell death (Apel and Hirt, 2004). HR has not been observed to occur in plant-*P. indica* interaction even at later

stages of cell death-dependent interactions. For instance, the whole-cell autofluorescence or H_2O_2 accumulation as indicator for HR has not been detected so far (Deshmukh et al., 2006; Schäfer, unpublished data). However, spermine was shown to cause caspase activation and cytochrome *c* release, hallmarks of apoptosis (Steffanelli et al., 1998; Steffanelli et al., 1999; Steffanelli et al., 2002).

Future perspectives

●The knowledge about the function of the putative *P. indica* effector protein (PIALH43) is still limited. Neither a general signaling pathway is resolved, nor functions in suppression of defense. Therefore, to identify PIALH43 interacting plant protein(s) that helps to elucidate its biological function, a yeast two-hybrid screen should be applied.

●To fully understand the ubiquitination in concern of PIALH43 protein, future efforts should be focused on the specificity of interfaces between the RING domains with E2.

● using the *P. indica* transformation techniques can provide us with the functional analysis of overexpression/knock-down genes and study the phenotype of transformants during plant/P. indica interaction.

●To which extent, *P. indica* rely on effector proteins to manipulate their host should be investigated.

5. Summary

Plants have developed diverse strategies for protection against the threat of invading pathogens. In order to improve their performance as well as to evade abiotic and biotic stresses, one strategy of plants is to establish associations with beneficial microbial organisms. *Piriformospora indica* is a root interacting fungus, which transfers several benefits to colonized plants like a better tolerance to various biotic and abiotic stresses, as well as an improved plant growth and yield. *P. indica* colonizes a broad range of monocot and dicot plants. This broad host range indicates that *P. indica* has developed efficient strategies to overcome innate immune responses and to manipulate the metabolism in different plants. This is even more intriguing as the fungus was shown to follow an initial biotrophic colonisation strategy at which penetrated cells are living. Plant colonizing microbes are known to secrete proteins (also called effectors) in order to modify host physiology and modulate plant defense mechanisms and, hence, confer compatibility. The aim of this study was to identify *P. indica* effector proteins as well as plant compatibility factors that are involved in the manipulation of those processes required for successful fungal establishment *in planta.* Therefore, two different strategies were followed. In the first approach, the so-called yeast signal sequence trap (YSST) assay was established. As the result of YSST, several plant genes were identified that are known to be involved in stress responses and cell wall development. These genes were shown to have a specific expression in barley roots during *P. indica* colonisation. In addition, a fungal gene was identified that does not show any similarities to other sequences deposited in public databases. The identified *P. indica* protein (PIALH43) carries a signal peptide and was shown to be induced during barley root colonisation. Interestingly, PIALH43 harbours a highly conserved C-terminal RING finger motif. *In silico* protein modelling of PIALH43 confirmed a 3D structural overlap and verified the accurate conformation of the E2 binding residues when compared with known human and plant ubiquitin ligases. Moreover, E3 ligase activity of PIALH43 was confirmed *in vitro*. Currently, PIALH43 is overexpressed *in planta* and in *P. indica* in order to study its function in mutualistic root colonisation. In a second approach, a simplified subtraction-

based assay, designated *Transcript Subtractive Hybridization* (TSH), was established to identify and study plant compatibility factors in the barley-*P. indica* interaction. The subtraction assay delivered various differentially regulated genes. These genes are known to be involved in stress responses, phytohormone- and secondary metabolism, autophagy, and protein processing. Among the up-regulated candidates was a gene encoding S-adenosylmethionine synthetase 2, which is thought to be involved in the synthesis of ethylene. *De novo* synthesis of ethylene during root colonization was verified by quantifying the ethylene precursor 1-aminocyclopropane 1-carboxylic acid (ACC) in barley and by cytologically monitoring GUS accumulation in ACC synthase reporter plants of *Arabidopsis*. In addition, the effects of ethylene precursor ACC or ethylene antagonist 1-methylcyclopropene (MCP) was determined. In these pharmacological experiments, barley plants were about 40% less colonised by *P. indica* after application of MCP while treatment with ACC resulted in significant increase (~ 60%) in colonization. To further elucidate the impact of ethylene on plant root colonization by *P. indica*, genetic analyses were performed with *Arabidopsis* mutants altered in ethylene synthesis and signaling at early biotrophic (~ 3 dai) and later cell death-associated colonization phases (~ 14 dai). In accordance with the studies in barley, *Arabidopsis* mutants *ctr1-1* (constitutive ethylene signaling) and *eto1-1* (ethylene overproducer) exhibited a significant increase in fungal colonization (especially at later interaction stages), while a reduced colonization was observed in *ein2-1* (ethylene insensitive). In summary, ethylene might function as general plant compatibility factor in the plant-*P. indica* system.

Zusammenfassung

Pflanzen haben verschieden Strategien entwickelt, um sich vor den Bedrohungen durch eindringende Pathogene zu schützen. Um ihre Leistungsfähigkeit zu verbessern und abiotischem und biotischem Stress zu entgehen, besteht eine Strategie der Pflanzen darin, Verbindungen mit nützlichen Mikroorganismen einzugehen. *Piriformospora indica* ist ein Pilz, der mit Pflanzenwurzeln interagiert und der besiedelten Pflanze verschiedene Vorteile verschafft, zum Beispiel eine bessere Toleranz gegenüber verschiedenen biotischen und abiotischen Stressfaktoren, verbessertes Wachstum und höhere Ernteerträge. *P. indica* ist in der Lage, ein breites Spektrum an monokotylen und dikotylen Pflanzen zu besiedeln. Dieses breite Wirtsspektrum deutet darauf hin, dass *P. indica* effiziente Mechanismen entwickelt hat, um pflanzliche Immunantworten zu überwinden und den Metabolismus verschiedener Pflanzen zu manipulieren. Dies ist umso mehr erstaunlich, da gezeigt werden konnte, dass der Pilz zu Beginn der Besiedlung in einer biotrophen Phase lebende Zellen penetriert. Pflanzen besiedelnde Mikroorganismen sekretieren bekanntermaßen Proteine (so genannte Effektoren), um die Wirtsphysiologie zu verändern, die Abwehrmechanismen der Pflanze zu modulieren und schließlich Kompatibilität zu erreichen. Das Ziel dieser Untersuchungen war es, Effektorproteine von *P. indica* und Kompatibilitäts-faktoren der Pflanze zu identifizieren, die an manipulativen Prozessen beteiligt sind und die erfolgreiche Etablierung des Pilzes in der Pflanze ermöglichen. Hierfür wurden zwei verschiedene Strategien verfolgt. Zunächst wurde der so genannte *yeast signal sequence trap* (YSST) etabliert. Als Ergebnis des YSST konnten verschiedene pflanzliche Gene identifiziert werden, die an Stressantworten und Zellwandbildung beteiligt sind. Es wurde gezeigt, dass diese Gene spezifisch in Gerstenwurzeln exprimiert wurden, wenn diese mit *P. indica* besiedelt waren. Zusätzlich konnte ein pilzliches Gen identifiziert werden, das keinerlei Ähnlichkeiten mit bisher bekannten Sequenzen aus öffentlich zugänglichen Datenbanken aufweist. Das entdeckte *P. indica*-Protein (PIALH43) trägt ein Signalpeptid, es wurde gezeigt, dass es während der Besiedlung von Gerstenwurzeln exprimiert wird. Interessanterweise beinhaltet PIALH43 ein stark konserviertes, C-terminales RING-

Finger Motiv. *In silico* Proteinmodellierung von PIALH43 bestätigte eine dreidimensinole Überlappung und wies die genaue Konformation der der E2-Bindestellen im Vergleich mit bekannten menschlichen und pflanzlichen Ubiquitin-Ligasen nach. Die E3-Ligase-Aktivität konnte *in vitro* bekräftigt werden. Im Moment wird PIALH43 in der Pflanze (*Arabidopsis thaliana*) und in *P. indica* überexprimiert, um seine Funktion in der mutualistischen Wurzelkolonisierung zu studieren. In einer weiteren Annäherung wurde eine vereinfachte, auf Subtraktion basierende Methode, bezeichnet als *Transcript Substractive Hybridization* (TSH), etabliert. Mit Hilfe dieser Methode wurden pflanzliche Kompatibilitätsfaktoren der Gerste-*P. indica* Interaktion identifiziert und studiert. Die Subtraktionsmethode lieferte viele verschieden regulierte Gene. Die gefundenen Gene sind bekanntermaßen in Stressantworten, Phytohormon- und Sekundärmetabolismus, Autophagie und Proteinprozessierung beteiligt. Unter den hochregulierten Kandidaten war eine S-Adenosylmethionin-Synthetase 2, bei der vermutet wird, dass sie in der Synthese von Ethylen eine Rolle spielt. Die *de novo* Synthese von Ethylen während der Wurzelbesiedlung wurde durch die Quantifizierung des Ethylen-Vorläufers 1-Aminocyclopropan-1-carboxylsäure (ACC) in Gerste und durch zytologische Kontrolle der Akkumulation von GUS in ACC-Synthase-Reporterpflanzen (*Arabidopsis*) bestätigt. Zusätzlich wurden die Effekte des Ethylen Vorläufers ACC und des Ethylen-Antagonists 1-Methylcyclopropene (MCP) auf die Besiedlung bestimmt. In diesen pharmakologischen Untersuchungen waren Gerstenpflanzen nach der Zugabe von MCP um etwa 40 % weniger mit *P. indica* besiedelt, während die Behandlung mit ACC zu einem signifikanten Anstieg (~ 60 %) in der Besiedlung führte. Um den Einfluss von Ethylen auf die Besiedlung von Pflanzenwurzeln durch *P. indica* weiter zu beleuchten, wurden genetische Untersuchungen mit *Arabidopsis*-Mutanten, deren Ethylensynthese und Ethylen-Signalwege verändert waren, in der frühen biotrophen (~ 3 *days after inoculation*, dai) und späteren Zelltod-assoziierten Phase (~ 14 dai) durchgeführt. In Übereinstimmung mit Studien in Gerste waren die *Arabidopsis*-Mutanten *ctr1-1* (Ethylen-Signalweg konstitutiv aktiv) und *eto1-1* (Ethylen-Überproduzierer) signifikant stärker besiedelt, vor allem in späteren Interaktionsstadien. Bei der Ethylen-insensitiven Mutante *ein2-1* wurde hingegen eine reduzierte Besiedlung beobachtet. Zusammenfassend scheint Ethylen ein genereller Kompatibilitätsfaktor zu sein, der durch

den Pilz rekrutiert wird, um verschiedene Wirtspflanzen zu besiedeln, wie hier beispielhaft an den Modellpflanzen Gerste und *Arabidopsis* gezeigt werden konnte.

6. References

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7. Supplement

Table S1: List of primers used in rapid amplification of cDNA ends RACE-PCR in order to determine full-length *PIALH43* and α-expansin cDNAs. Primers are in 5'-3' direction.

Primer name	**Sequence**
*PIALH4*3 Racer 5′	ATCCAGGGCGTGGTTGTGGTATCAT
PIALH43 Racer 3′	CACAAGTACACCGTATGGGATCCTG
PIALH43 Racer 5′ Nested primer	CCATCTGCTGATAGGAAACCACAG
PIALH43 Racer 3′ Nested primer	AATGCGACTGGAGAAGAGAGTGCAG
α-expansin Racer 5′	GGGGATTGTGTGGTCGGGTTGTC
α-expansin Racer 3′	GGCAGTGCTACAAGATCGCATGT
α-expansin Racer 5′ Nested primer	ACAAGTCGAGCTGGAGCCATATCT
α-expansin Racer 3′ Nested primer	GCAGACCCGTTGTTTTGCAAGC
α-expansin full-length-for	CAACCATCAGCAACAAATCAA
α-expansin full-length-rev	TGACAAATCCATAGCTCGGTA
*PI*ALH43 full-length-for	ATGGGCAGATATTCATTGG
*PI*ALH43 full-length-rev	TGTACACAGCACGTCCACA
GeneRacer 5′ Primer	CGACTGGAGCACGAGGACACTGA
GeneRacer 3′ Primer	GCTGTCAACGATACGCTACGTAACG
GeneRacer 5′ Nested Primer	GGACACTGACATGGACTGAAGGAGTA
GeneRacer 3′ Nested Primer	CGCTACGTAACGGCATGACAGTG
GeneRacer Oligo dT Primer	GCTGTCAACGATACGCTACGTAACGGCATGACAGTG(T)18
GeneRacer RNA Oligo Sequence	CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA

Table S2: Primers used for cloning of *PIALH43* gene in different intermediate and destination vectors (primers are in 5'-3' direction). Incorporated restriction enzyme site is shown in bold at the 5'-end of primer.

Primer name	**Sequence (restriction enzyme site)**
PIALH43, full length-for	ATGGGCAGATATTCATTGG
PIALH43, full length-rev	TGTACACAGCACGTCCACA
PIALH43, without SP-for	ATGATTCCAGCTAGGGCT
PIALH43, mut stop codon-rev	GAATGTCATCTTCGCCGG
PIALH43, middle-for	CGAGGGCGATGATGTC
PIALH43, with SP-for	**CCCGGG**ATGGGCAGATATTCATTGG (*Xma*I site)
PIALH43, without SP-for	**CCCGGG**ATGATTCCAGCTAGGGCT (*Xma*I site)
PIALH43, with SP-for	**ACTAGTA**TGGGCAGATATTCATTGG (*Spe*I site)
PIALH43, without SP-for	**ACTAGTA**TGATTCCAGCTAGGGCT (*Spe*I site)
PIALH43, with stop-rev	**CTGCAGT**GTACACAGCAACGTCCACA (*Pst*I site)
PIALH43, mut stop codon-rev	**CCATGGA**ATGTCATCTTCGCCG (*Nco*I site)
PIALH43, with stop codon-rev	**CTGCAGT**GTACACAGCACGTCCACA (*Pst*I site)
pEX-5, ter-rev	**CTGCAGT**GCTCAGCTAAGGTTTCTTC (*Pst*I site)

Table S3: List of oligonucleotides designed for QPCR analysis of the identified genes as the outcome of the yeast signal sequence trap screening. Primers are in 5'-3' direction.

Gene annotation	**Forward primer**	**Reverse primer**
Germin-like protein	GGTCCAACGTGACCTTGATT	CACCCTTCTTGAGCTCCTTG
Secretory peroxidase	CACGAACAGCAGCCAGT	CGAGGGCGATGATGTC
α-expansin	CATACACAGCTAGCGGCAGA	CGTCCCGTACCCTTCAGAG
HSP70	ACCAAGGACAACAACCTGCT	CTTCTCGATGTCCTCCTTGC
PIALH43	CTGGATTGCGCTTATTGGAT	CGAAACTGGGACTGGATGAT

Table S4: Primer sequences of genes investigated as the results of Transcript Subtractive Hybridisation (TSH) assay. Primers are in 5'-3' direction.

Primer name	**Sequence**
Hordem vulgare, Germin F-for	ATTCGGCACGAGGCTAC
Hordem vulgare, Germin F-rev	GCATGGTTGTGAGCTGTG
Secretory peroxidase-for	CACGAACAGCAGCCAGT
Secretory peroxidase-rev	CGAGGGCGATGATGTC
Microtubule-associated protein-for	CAGTTGATTGGCACGCCTTA
Microtubule-associated protein-rev	TCCGACCAAGGAACCCAAGG
RAV-like transcription factor-for	TTCCTCCAGAGCAGCAATC
RAV-like transcription factor-rev	ATCCTCGAAAGACAGGAGCA
Drought inducible 22 kDa protein-for	AGTACGAGCGGATCACCAAG
Drought inducible 22 kDa protein-rev	GTGGTCCTGCTTCTTCTCGT
NOD26-like membrane integral protein-for	CCGAGCTGTATGTAGCGAGAG
NOD26-like membrane integral protein-rev	ACAGGTCGTGGATCTCGTTC
S-adenosylmethionine synthetase 2-for	ACACTCATGCACGACCTGAC
S-adenosylmethionine synthetase 2-rev	CTCGGAAGTGAAGAGGAACG
S-adenosylmethionine decarboxylase proenzyme-for	GAGGTTGTGTTCGTCCGTTT
S-adenosylmethionine decarboxylase proenzyme-rev	GGGAGGAACGACTACTGCTG

Table S5. The gene-specific primers and the universal primers used in this study. Primers are in 5'-3' direction.

Primer name	**Sequence**
ADH1-for	CTCGTTCCCTTTCTTCCTTGTTTC
suc2-rev	GGACCAAAGGTCTATCGCTAGTTTC
M13-for	GTAAAACGACGGCCAG
M13-rev	CAGGAAACAGCTATGAC
AtUBQ5-for	CCAAGCCGAAGAAGATCAAG
AtUBQ5- rev	ACTCCTTCCTCAAACGCTGA
HvUBQ5-for	ACCCTCGCCGACTACAACAT
HvUBQ5- rev	CAGTAGTGGCGGTCGAAGTG
Pitef-for	TCGTCGCTGTCAACAAGATG
Pitef-rev	ACCGTCTTGGGGTTGTATCC
At ITS-for	CAACACATGTGCACGTCGAT
At ITS- rev	CCAATGTGCATTCAGAACGA3

Table S5: ACC contents in barley roots colonised by *P. indica*. (A) Free and malonyl-ACC contents in nmol g fresh weight^{-1} were determined at 60, 120, and 168 hai. While contents of malonyl-ACC are slightly reduced at 60 and 168 hai, free ACC contents are elevated at 60 and 120 hai. Data shows the mean content of three biological experiments. (B) Free and malonyl-ACC contents in percent of control at 60, 120, and 168 hai. Displayed are the relative values of three biological experiments. While contents of malonyl-ACC are slightly reduced at 60 and 168 hai, free ACC contents are elevated at 60 and 120 hai.

A

		0 hours	60 hours	120 hours	168 hours
ACC (f)	Control	4,02 ± 0,95	0,52 ± 0,06	0,28 ± 0,07	0,24 ± 0,06
	P. indica		0,75 ± 0,27	0,36 ± 0,09	0,25 ± 0,08
ACC (f+M)	Control	8,13 ± 6,25	10,96 ± 1,95	8,22 ± 1,94	7,19 ± 3,01
	P. indica		9,1 ± 4,56	8,99 ± 3,32	6,03 ± 2,98

B

		60 hours	120 hours	168 hours
ACC (f)	Exp. 1	171,31	177,72	92,80
	Exp. 2	168,33	140,81	111,30
	Exp. 3	92,76	85,32	98,43
	Sum	144,13	134,62	100,84
	STD	44,52	46,51	9,48
ACC (f+M)	Exp. 1	56,80	144,60	85,35
	Exp. 2	122,78	103,55	112,14
	Exp. 3	67,37	77,62	55,19
	Mean	82,32	108,59	84,22
	STD	35,44	33,77	28,49

>Barley, Germin-like protein

```
MASSPTFLLLVAPFALISWQAVASDPGPLQDFCVADMHSPVRVNGFVCKN 50
PMDVNADDFFKAAALDKPRVTNKVGSNVTLINVMQIAGLNTLGISIARID 100
YAPLGQNPPHTHPRATEILTVLEGTLYVGFVTSNLPAPNRNKFLSKVLNK 150
GDVFVFPVGLIHFQFNPNPHQPAVAIAALSSQNPGAITIANAVFGSDPTI 200
SDDVLAKAFQVEKNTIDWLQAQFWENNQN
```

>Barley, Secretory peroxidase

```
MASSSYTSLLVLVALVTAASAQLSPTFYDTSCPRALATIKSGVMAAVTSD 50
PRMGASLLRLHFHDCFVQGCDASVLLSGMEQNAIPNAGSLRGFGVIDSIK 100
TQIEAICKQTVSCADILTVAARDSVVALGGPSWTVPLGRRDSIDANENEA 150
NTDLPGFNSSRAELEAAFLKKGGLNTVDMVALSGAHTIGQAQCSTFRARI 200
YGGDTNINAAYAASLRANCPQTVGSGDGSLANLDTTTANTFDNAYYTNLM 250
SQKGLLHSDQVLFNNDTTDNTVRNFASNPAAFSSSFTTAMIKMGNIAPKT 300
GTQGQIRLSCSRVNS
```

>Barley, Heat Shock Protein 70 kDa (HSP70)

```
MAGKGDGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVAFTDT 50
ERLIGDAAKNQVAMNPINTVFDAKRLIGRRFSDASVQSDAKLWPFKVIPG 100
PADKPMIGVQYRGEDKQFSAEEISSMVLNKMKETAEAYLGTTIKNAVVTV 150
PAYFNDSQRQATKDAGVISGLNVMRIINEPTAAAIAYGLDKKSTSVGEKN 200
VLIFDLGGGTFDVSLLTIEEGIFEVKATAGDTHLGGEDFDNRMVNHFVQE 250
FKRKNKKDISGNPRALRRLRTACERAKRTLSSTAQTTIEIDSLFEGIDFY 300
TTITRARFEELNMDLFRKCMEPVEKCLRDAKMDKSTVHDVVLVGGSTRIP 350
RVQQLLQDFFNGKELCKSINPDEAVAYGAAVQAAILTGEGNEKVQDLLLL 400
DVTPLSQGLETAGGVMTVLIPRNTTIPTKKEQVFSTYSDNQPGVLIQVYE 450
GERARTKDNNLLGKFELSGIPPAPRGVPQITVCFDIDANGILNVSAEDKT 500
TGQKNKITITNDKGRLSKEDIEKMVQEAEKYKAEDEEHKKKVDSKNALEN 550
YAYNMRNTIKDDKIASKLPEADKKKIEDAIEGAITWLDNNQLAEADEFDD 600
KMKELEGICNPIIAKMYQGAGAEMPGGMDEDAPASAAGGSSGPGPKIEEV 650
D
```

Figure S1: The predicted amino acid sequences of genes as the result of YSST screening. Plant genes (*Germin-like protein, α-expansin and Secretary peroxidase*) contain putative signal peptide at N-terminus of their predicted proteins, except HSP70 with a lack of secreted domain.

```
  1 CACAACCATC AGCAACAAAT CAATATAGAC ACACGAGCAA ACGCATCCAT
 51 ACAGGTCACA AGTTCATCAA ACTTCAACGG CATACACAGC TAGCGGCAGA

101 TATGGCTCCA GCTCGACTTG TTGCAGGGAT GCTGCTGGCG GCGATCGGCT
 17   M  A  P   A  R  L  V   A  G  M   L  L  A   A  I  G  C
151 GTGTGCTCTC TGTGGCCGCG GACAACCCGA CCACACAATC CCCCCAACCG
 33    V  L  S   V  A  A    D  N  P  T   T  Q  S   P  Q  P
201 TTCGTCTGGC AGAAGGCGCA TGCGACGTTC TATGGCGGCG CGGACGCCTC
 50 F  V  W  Q   K  A  H   A  T  F   Y  G  G  A   D  A  S
251 TGACACAATG GGTGGCGCGT GCGGGTACGG CAACCTCTAC TCTGAAGGGT
 67  D  T  M   G  G  A  C   G  Y  G   N  L  Y   S  E  G  Y
301 ACGGGACGCG AACCGCAGCT CTAAGCACAG TGTTGTTCAA TGACGGTGCC
 83   G  T  R   T  A  A   L  S  T  V   L  F  N   D  G  A
351 GCTTGCGGGC AGTGCTACAA GATCGCATGT GATCGCAAGC GCGCAGACCC
100 A  C  G  Q   C  Y  K   I  A  C   D  R  K  R   A  D  P
401 GTTGTTTTGC AAGCCCGGCG TGACGGTCAC CGTCACGGCC ACAAACTTCT
117  L  F  C   K  P  G  V   T  V  T   V  T  A   T  N  F  C
```

```
451 GTCCACCCAA CGACGCCCTC CCCAATGACA ACGGCGGTTG GTGCAACCCG
133   P  P  N   D  A  L   P  N  D  N   G  G  W   C  N  P
501 CCACGGCCAC ACTTCGACAT GGCACAACCG GCCTGGGAGA AGATCGGTGT
150  P  R  P  H   F  D  M   A  Q  P   A  W  E  K   I  G  V
551 TTATAGAGGT GGCATCATCC CCGTCATGTA CCAGAGAGTT CCGTGCGTGA
167  Y  R  G   G  I  I  P   V  M  Y   Q  R  V   P  C  V  K
601 AGCGGGGTGG CGTGAGGTTC AAAATTAATG GTCACGATTA CTTCATTCTT
183  R  G  G   V  R  F   K  I  N  G   H  D  Y   F  I  L
651 GTGCTTGTGA GCAACGTTGC TGCAACAGGC TCGATCCAGT CCATGGATGT
200 V  L  V  S   N  V  A   A  T  G   S  I  Q  S   M  D  V
701 GAAGTGCTCT GATTCCGAGG ACTGGACGCC TATGGCACGC AACTGGGGCG
217  K  C  S   D  S  E  D   W  T  P   M  A  R   N  W  G  A
751 CTAACTGGCA CTCGCTGGCT AACCTCACCG GCAAGATGCT CTCCTTCAGG
233   N  W  H   S  L  A   N  L  T  G   K  M  L   S  F  R
801 CTAACCAACA CTGATGGACA CACGCTTGTA TTCAACAACA TTGTGCCAAA
250  L  T  N  T   D  G  H   T  L  V   F  N  N  I   V  P  K
851 GGGGTGGAAC TTTGGGCAAT CATTTGCTAG CAAATTGCAA TTCTAGTGAA
264   G  W  N   F  G  Q  S   F  A  S   K  L  Q   F  *
901 CAATGTCTAC CGAGCTATGG ATTTGTCATA ATTATGTTGT GTAAAATACT
951 AGTTATGGTG CTTAAAAAAA AAAAAAAAAA AAAAAA
```

Figure S2: The full-length cDNA and deduced amino acid of barley α-expansin. The full-length cDNA contains 986-nucleotide which encodes a putative protein with 264 amino acids. The open reading frame starts with a signal peptide of 23 hydrophobic amino acids (boxed in yellow). Nucleotides are numbered from 5'- to 3'-end, and the amino acid residues are numbered starting with the first ATG codon in the open reading frame.

```
1 ATGGGCAGAT ATTCATTGGC AGCGCAGGCC ATCTGCCTCT TATCGAGCAT
 17  M  G  R  Y   S  L  A   A  Q  A   I  C  L  L   S  S  I
 51 ACACTCTGCT CTCGCATATA TTCCAGCTAG GGCTGCAAAC ATTAGCCAGG
 34  H  S  A   L  A  Y  I   P  A  R   A  A  N   I  S  Q  G
101 GTCTCGGCCT CGACGTCCAC GACAATTCCA AGGTCACCCT GACATGGAAC
 50   L  G  L   D  V  H   D  N  S  K   V  T  L   T  W  N
151 CCCAGCGGAA CCTATGAGAC TGTGGTTTCC TATCAGCAGA TGGGCAATAA
 67 P  S  G  T   Y  E  T   V  V  S   Y  Q  Q  M   G  N  N
201 TTCTCAAGGT ATCTCAAAGG GCGCGCTCAT TCCAATCCGC GAAGAAGATT
 84  S  Q  G   I  S  K  G   A  L  I   P  I  R   E  E  D  F
251 TTACAAATAA TGATACCACA ACCACGCCCT GGATTGCGCT TATTGGATGC
100   T  N  N   D  T  T   T  T  P  W   I  A  L   I  G  C
301 GACTACAATG CCACCAACGC CTCTATGGAG CTCGACATAT TCACTATGGT
117 D  Y  N  A   T  N  A   S  M  E   L  D  I  F   T  M  V
351 TCGAGACCGA GGCGCTAGAG CCGCTCTCTT GTATTCAAAC ACCTCGGACG
134  R  D  R   G  A  R  A   A  L  L   Y  S  N   T  S  D  G
401 GCTGTTTACT CAATGAAGGA TATCGAACGG GAGATTTTGA ACAAATCTTT
150   C  L  L   N  E  G   Y  R  T  G   D  F  E   Q  I  F
451 GATATTTTCA CATCCAAAAC GGCCGCTAAT TCTATCATCA TCCAGTCCCA
167 D  I  F  T   S  K  T   A  A  N   S  I  I  I   Q  S  Q
501 GTTTCGCATA CTCGAACACA AGTACACCGT ATGGGATCCT GCTCTTCTCA
184  F  R  I   L  E  H  K   Y  T  V   W  D  P   A  L  L  T
551 CAGCCAACAA CCAAAGCGTT ACCTCTGCCC TTTATCGTAA TGCACTCAAT
200   A  N  N   Q  S  V   T  S  A  L   Y  R  N   A  L  N
601 ACCTCGCCCT ATTTGGTCGC GGCGCTAAGA GCATGGAATG CGACTGGAGA
217 T  S  P  Y   L  V  A   A  L  R   A  W  N  A   T  G  E
651 AGAGAGTGCA GATGATCCGA GCGCTGTTCC GACGACGGTC TATAATCCTT
234  E  S  A   D  D  P  S   A  V  P   T  T  V   Y  N  P  S
701 CAACTACGCA CGACAGCGAG CCAAGCCAGA GTTTGGCTAT GATCATTCTC
250   T  T  H   D  S  E   P  S  Q  S   L  A  M   I  I  L
751 TACGTGATCA TCAGCTTGGT ATCGGCGCTT TTTATCATTG TCATTGTCTC
267 Y  V  I  I   S  L  V   S  A  L   F  I  I  V   I  V  S
```

```
 801 GGGGGCTGTC CGCGCTTTCC GCCACCCGGA ACGCTATGGA CCAAGACTGT
 284  G  A  V  R  A  F  R   H  P  E   R  Y  G   P  R  L  Y
 851 ATGATCCGAC ACTGGAAGGA GATGGAGGTC AGCCGCAAAC AAGGGCAGCT
 300   D  P  T   L  E  G   D  G  G  Q   P  Q  T   R  A  A
 901 GGACTTACCC GCGCGATTCT CGAAACGTTC CCTGTCATCA AGTTTGGCCG
 317 G  L  T  R   A  I  L   E  T  F   P  V  I  K   F  G  R
 951 CACCAACGAC CAGATGCAGA ACCAATCTAC ACGCACTTAT CGTCAAGAAA
 334  T  N  D   Q  M  Q  N   Q  S  T   R  T  Y   R  Q  E  M
1001 TGAAGAAGTG GAGTCTGGAG AACGGCGAGC AGCCATCACG AGACCTCTTG
 350   K  K  W   S  L  E   N  G  E  Q   P  S  R   D  L  L
1051 CAGCCAGCGC ACGGGCAACC AAACTCGGTC TTTGATGCCT CTCGACAAGC
 367 Q  P  A  H   G  Q  P   N  S  V   F  D  A  S   R  Q  A
1101 TTCGCCCATT AGACATAGTT CCGAGGTTGC GAATCGTGCT ATGAGACCTC
 384  S  P  I   R  H  S  S   E  V  A   N  R  A   M  R  P  H
1151 ATTCTACAGA GATGGCACCG TCCACATCGG ATGCTTCTGA TACGCAGCAG
 400   S  T  E   M  A  P   S  T  S  D   A  S  D   T  Q  Q
1201 CTCGATCCCG CCGCCATCGG AAACCAAACC TGTCCTATCT GCATCGTCGA
 417  L  D  P  A   A  I  G   N  Q  T   C  P  I  C   I  V  D
1251 TTTCGAAGAA GGCGATGACG TTCGCGTACT ACCGTGCGAG GGGAAGCATC
 437  F  E  E   G  D  D  V   R  V  L   P  C  E   G  K  H  R
1301 GTTTCCACAA GGATTGTGTG GACCCATGGC TGTTGGAGCT TTCGAGTTCT
 450   F  H  K   D  C  V   D  P  W  L   L  E  L   S  S  S
1351 TGTCCCATAT GCCGTGAAGA TTTTCATGTG TTGGAAGAAA TGGCCGTCGC
 467 C  P  I  C   R  E  D   F  H  V   L  E  E  M   A  V  A
1401 TGCGGATGGT CGTGACCGTG AGCGTTCAGA ATCTGGTCAC AGAGAAGAGG
 484  A  D  G   R  D  R  E   R  S  E   S  G  H   R  E  E  E
1451 AAGACCATGT CCCGCCGGCA GAACACCATA CCTCGTCCCG CTTCACGCGC
 500   D  H  V   P  P  A   E  H  H  T   S  S  R   F  T  R
1501 TATCTTCGGT TCGCGAACAA GAGGAGACGA AGTCAGCGCT CTAGCCAGCA
 517 Y  L  R  F   A  N  K   R  R  R   S  Q  R  S   S  Q  Q
1551 ACCGCCAGAC AACGCAGCGG TGTCGCCGAC CCCGGCGTAG ATGACATTCG
 529  P  P  D   N  A  A  V   S  P  T   P  A  *
1601 CCTTTATTTT CTGTATTCCC ATCCCACCGT TTAGTATGGA CTTTGATCTC
1651 AAAGAATCCT TCGTATCCAT AATTCACCCT CATTTCCTAT CTACTGTGGA
1701 CGTGCTGTGT ACAATGCTCT TTTTCAGCCG TTAAAAAAAA AAAAAAAAAA
1751 AAAAAAAAAA
```

Figure S3: The full-length cDNA and deduced amino acid sequence of PIALH43. It consists of 1714 nucleotides which encodes 1519-bp (open reading frame) corresponds to 529-amino acid. The putative signal peptide, potential transmembrane spanning domains and RING finger-like domain are shaded yellow, red and green respectively. Nucleotides are numbered from 5'- to 3'-end, and the amino acid residues are numbered starting with the first ATG codon in the open reading frame.

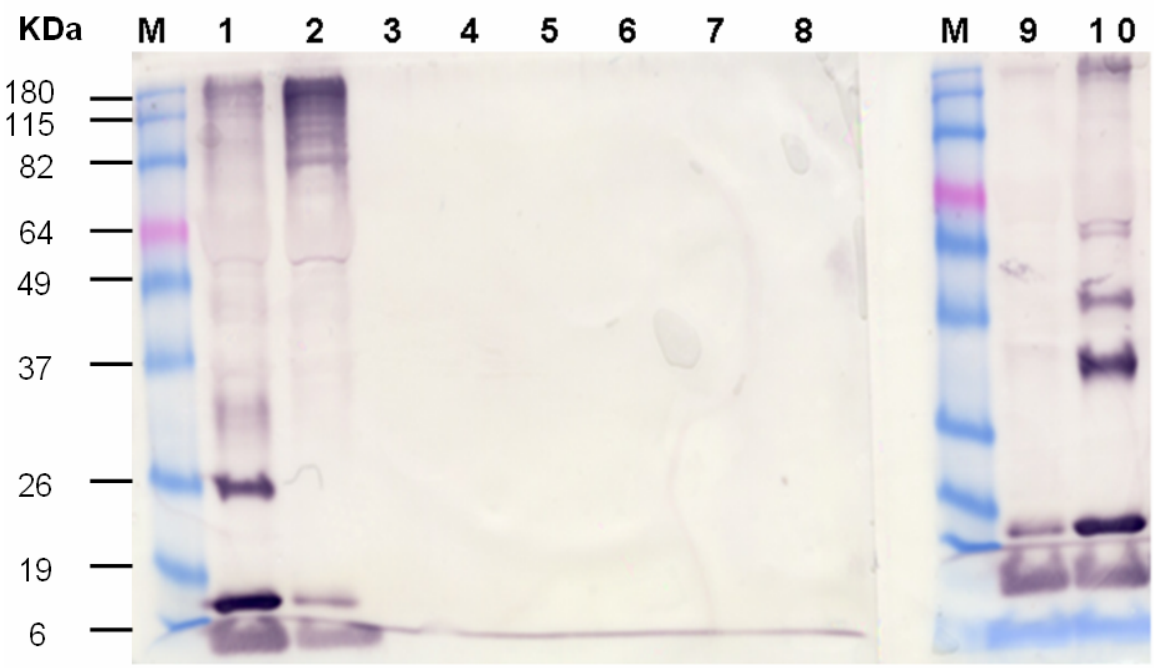

Figure S4: *In vitro* ubiquitination assays with a number of different E2s. Lane 1-10 (UbcH2, His_6-UbcH3, UbcH5a, UbcH5b, UbcH5c, His_6-UbcH6, UbcH7, UbcH8 His_6-UbcH10 and His_6-$UbcH13_{complex}$) from Boston Biochem, Boston, USA. E2 shows polyubiquitination activity independent of E3 ubiquitin ligase. The reactions including all the necessary components (E1, different E2 enzymes, ubiqutin and ATP but except E3) were performed. Immunobloting with anti-ubiquitin antibody showed polyubiquitination activity of some of E2s. Lane M**:** molecular weight marker.

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10 July, 2009

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.