Investigating function of effector candidates in the interaction between *Piriformospora indica* and plants

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Contents

Introduction 1

1.1 Plant immunity system 1

1.1.1 Pattern-trigged immunity (PTI) 2

1.1.2 Effector trigged immunity (ETI) 6

1.2 The mutualistic fungus *Piriformospora indica* 7

1.2.1 *P. indica*'s beneficial symbioses with host plants 7

1.2.2 *P. indica* confers tolerance and resistance to abiotic and biotic stresses 8

1.2.3 Cellular colonization pattern of *P. indica* 9

1.2.4 Effector candidates secreted by *P. indica* 10

1.3. E3 ubiquitin ligases and plant innate immunity 11

1.3.1 ubiquitin-26S proteasome system 11

1.3.2 Interaction of E3 ubiquitin ligase effectors with plant defense 12

1.4 Objective 14

2. Materials and Methods 15

2.1 Plant, fungal material and plant inoculation 15

2.1.1 Arabidopsis thaliana 15

2.1.2 *Piriformospora indica* 16

2.1.3 Inoculation of plant roots with *Piriformospora indica* 16

2.2 Molecular biological standard methods 16

2.2.1 DNA-Extraction 16

2.2.2 RNA-Extraction 17

2.2.3 Reverse transcriptional polymerase chain reaction (RT-PCR) 18

2.2.4 Quantitative Real-Time PCR (qPCR) 18

2.2.5 Polymerase chain reaction (PCR) 18

2.2.6. Agarose gel electrophoresis 19

2.2.7 Production of chemically competent *E. coli* DH5*α* cells 20

2.2.8 Transformation of chemically competent *E. coli* cells 20

2.2.9 Western blotting 21

2.3 Candidate gene expression studies with quantitative real-time PCR 22

2.3.1. PIIN29 expression kinetics 22

2.3.2 Quantification of fungal colonization by qPCR 22

2.4 Analysis of flagellin- or chitin-induced Pattern-Trigged Immunity (PTI) 23

2.4.1 Analysis of flg22-induced oxidative burst of Arabidopsis roots 23

2.4.2 Analysis of flg22- or chitin-induced oxidative burst of leaves 23

2.4.3 Analysis flg22-induced callose deposition in Arabidopsis leaves 23

2.4.4 Analysis flg22-induced MAP kinase activity 24

2.5 Transient expression DELD candidates in *Nicotiana tabacum* and *Nicotiana benthamiana* . 24

2.5.1 Growth of *N. tabacum* and *N. benthamiana* 24

2.5.2 Agrobacterium mediated infiltration 24

2.5.3 Type III secret system mediated infiltration 26

2.6 *Botrytis cinerea* inoculation and quantification 27

2.7 Preparation of TEM samples 28

2.8 Methods for 3D protein modeling of PIIN29 RING finger domain 28

3. Result 30

3.1 Functional analysis of effector candidate PIIN_00029 (PIIN29) 30

3.1.1 *In silico* analysis of PIIN29 30

3.1.2 Gene expression of *PIIN29* during colonization of Arabidopsis root with *P. indica* .. 32

3.1.3 Overexpression of PIIN29 in plant 34

3.1.4 Colonization of transgenic Arabidopsis PIIN29OE 37

3.1.5 Suppression of basal defense by PIIN29 39

3.1.6 PIIN29 is not involved in the MAP kinase pathway trigged by flg22 44

3.1.7 PIIN29 failed to abolish growth inhibition trigged by flg22 45

3.2 Characterization of DELD effector candidates of *P. indica* 46

3.2.1 Subcellular localization of DELD effector candidates of *P. indica* using agroinfiltraion 47

3.2.2 Functional analysis of DELD effector candidates of *P. indica* using *P. fluorescens* EtHAn mediated infiltration 49

3.3 Cytology of *P. indica*'s infection structure using transmission electron microscopy (TEM) . 52

4. Discussion 53

4.1 Functional analysis of effector candidate PIIN29 53

4.2 Functional analysis of one group of effector candidates belonging to DELD family 58

4.3 New insights into the cytology of *P. indica*'s infection structure 59

4.4 Future perspectives 59

5. Summary/Zusammenfassung 60

5.1 Summary 60

5.2 Zusammenfassung 61

6. References 64

7. Supplement 74

7.1 Sequence information of PIIN29 74

7.2 plasmid map 75

7.3 List of primers mentioned in this thesis 78

7.4 Sequence of DELD effector candidates 80

8. Declaration 85

9. Acknowledgements 86

List of Abbreviations

ABA	Abscisic acid
Avr	Avirulence
BSA	Bovine serum albumin
cDNA	Complementary DNA
CEBiP	Chitin elicitor binding protein
CTAB	Cetyltrimethylammonium bromide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleosidtriphosphate
dpi	day post inoculation
DTT	Dithiothreitol
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
EFR	EF-Tu receptor
EF-Tu	Elongation factor TU receptor
ER	Endoplasmic reticulum
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
flg22	A 22-amino-acid-long peptide derived from flagellin
FLS2	FLAGELLIN SENSING 2
HR	Hypersensitive Response
JA	Jasmonic acid
kDa	Kilodalton
LRR	Leucin-rich-repeat
LysM	Lysine motif
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen activated protein kinase
MEKK	MAP kinase kinase kinase
min	Minutes
MKK/MEK	MAP kinase kinase

MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger RNA
NB-LRR	Nucleotide-binding and a leucine-rich repeat domain
NR	Nitrate reductase
NTR	N-terminal region
PAMP	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PR	Pathogenesis related
PRR	Pattern recognition receptor
PTI	PAMP-trigged immunity
qRT-PCR	Quantitative real-time PCR
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
R-protein	Resistance protein
RT	Room temperature
SA	Salicylic acid
SDS	Sodium dodecyl sulfate
SSPs	Small secreted proteins
T3SS	Type III secretion system
TBE	Tris-Boric acid-EDTA
TE	Tris-HCl+ EDTA
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
Ub	Ubiquitin

Introduction

1.1 Plant immunity system

Unlike animals, plants can't move to avoid attack of living organisms, particular microbes. During evolution, plants develop to a special immunity system-two layers innate immunity system (Figure 1.1). The first layer is mediated by pattern recognition receptors (PRRs) at the plasma membrane that recognize pathogen-associated molecular patterns or microbe-associated molecular patterns (PAMPs or MAMPs) and establish a relatively weak immune response as pattern-triggered immunity (PTI) (Boller and Felix 2009; Dodds and Rathjen 2010). The second layer of plant innate immunity is based on highly polymorphic resistance (R) proteins that are activated to recognize highly variable molecules called as effectors, to initiate a rapid and robust response: effector-triggered immunity (ETI) (Chisholm *et al.* 2006; Jones and Dangl 2006; Dodds and Rathjen 2010). These two layers of defense constitute the plant immune system which protects plants from pathogen attack.

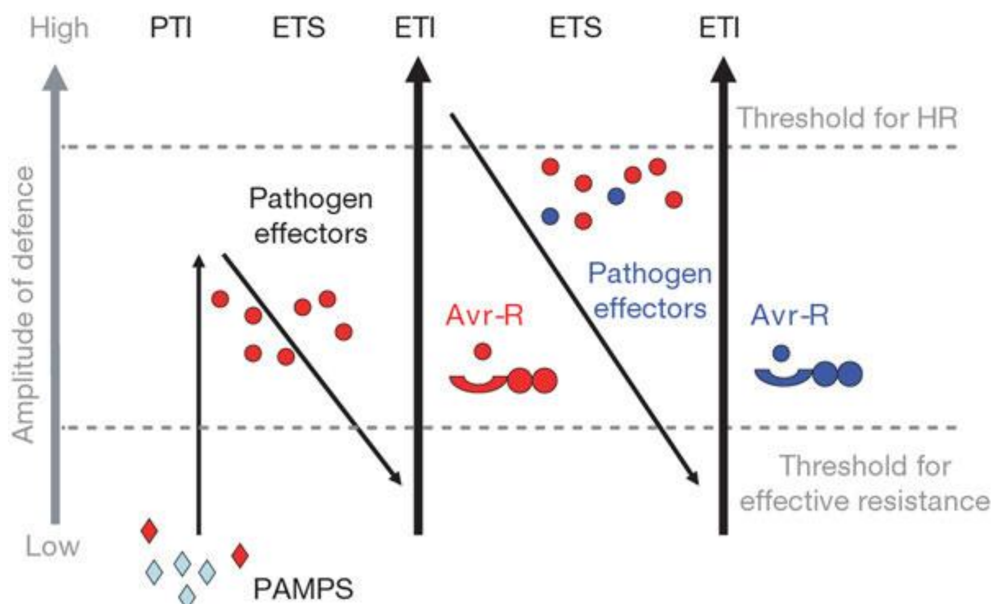


Figure 1.1 Zigzag model of plant immunity system (taken from Jones and Dangl 2006). In phase 1, plants detect MAMPs/PAMPs (red diamonds) by PRRs to trigger PTI. In phase 2, successful microbes deliver effectors that block PTI for effector-triggered susceptibility (ETS). In phase 3, resistance (R)

protein recognizes one effector (red), and then actives ETI. In phase 4, microbes secret new effectors (in blue)-these can help pathogens to suppress ETI.

1.1.1 Pattern-trigged immunity (PTI)

As the first inducible response of microbial perception, molecular patterns are conserved microbes structure components (Boller and Felix 2009). PTI responses are weak and slow, otherwise plants would spend more 'costs' for immunity without distinguishing pathogens from begin microbes. Recognition of molecular patterns by plant pattern-recognition receptors (PRRs), which are typical localized in extracellular and in plant cell membranes. In the major case, PTI inhabits microbe spreading at an early stage, such as induction of pathogen-responsive genes, production of reactive oxygen specials (ROS), and deposition of callose (Schwessinger and Zipfel 2008) (Figure 1.2).

The best studied elicitor of PTI is bacterial flagellin, which triggers defense responses in various plants (Gomez-Gomez and Boller 2002). Flg22, 22-amino-acid peptide from a conserved N-terminus of bacterial flagellin domain, is sufficient to induce many cellular responses (Felix *et al.* 1999; Zipfel and Felix 2005). Arabidopsis FLS2 (FLAGELLIN-SENSEING2) is a transmembrane leucine-rich repeat receptor (LRR-receptor) kinase which mediates plant basal defenses by directly binding flg22 (Chinchilla *et al.* 2006) and fls2 mutants exhibit enhanced susceptibility to bacterial infection (Zipfel *et al.* 2004; Dunning *et al.* 2007; Sun *et al.* 2012). Characterization of other flg22-insensitive mutants uncovers the function downstream of flagellin perception as mitogen-activated protein kinase (MAPK-Group 2002) cascade and WRKY signaling pathway (Asai *et al.* 2002; Suarez-Rodriguez *et al.* 2007).

Additionally, bacterial elongation factor Tu (EF-Tu) activates as a PAMP to trigger plant defense responses. The first 18 amino acids of EF-Tu, elf18, induce an oxidative burst and biosynthesis of ethylene, and trigger resistance to subsequent infection with pathogenic bacteria (Kunze *et al.* 2004; Zipfel *et al.* 2006). EFR (EF-Tu receptor), a LRR-kinase, recognizes and percepts EF-Tu in Arabidopsis. *Nicotiana benthamiana* plants fail to percept EF-Tu but trigger response by this PAMP when transformed with EFR. Arabidopsis efr mutants, lacking EF-Tu perception, are more susceptible to transient transformation by

Agrobacterium tumefaciens, supporting that EF-Tu induces similar plant defense as flg22 (Kunze *et al.* 2004; Zipfel *et al.* 2006).

Chitin (polymer of N-acetyl-D-glucosamine), an important component of the fungal cell wall, activates various defense responses in a wide range of plant cells including both monocots and dicots plant (Shibuya and Minami 2001). The plasma membrane glycoprotein CEBiP (chitin elicitor binding protein) recognizes chitin and plays a critical role in chitin signaling in rice (Kaku *et al.* 2006; Wan *et al.* 2008b). Since there is not an obvious intracellular domain of CEBiP protein, which uses partner, such as a receptor-like kinase, to translate the perceived chitin signal into intracellular events. The rice receptor-like kinase (RLK) chitin elicitor receptor kinase 1 (OsCERK1) forms chitin-induced hetero-oligomers with CEBiP, both contain extracellular lysine motif (LysM) domains for binding chitin (Miya *et al.* 2007; Wan *et al.* 2008a). Additionally, LYK4 protein (LysM-containing receptor-like kinase), localized to the plasma membrane, is involved in the chitin recognition receptor complex in plant innate immunity (Wan *et al.* 2012).

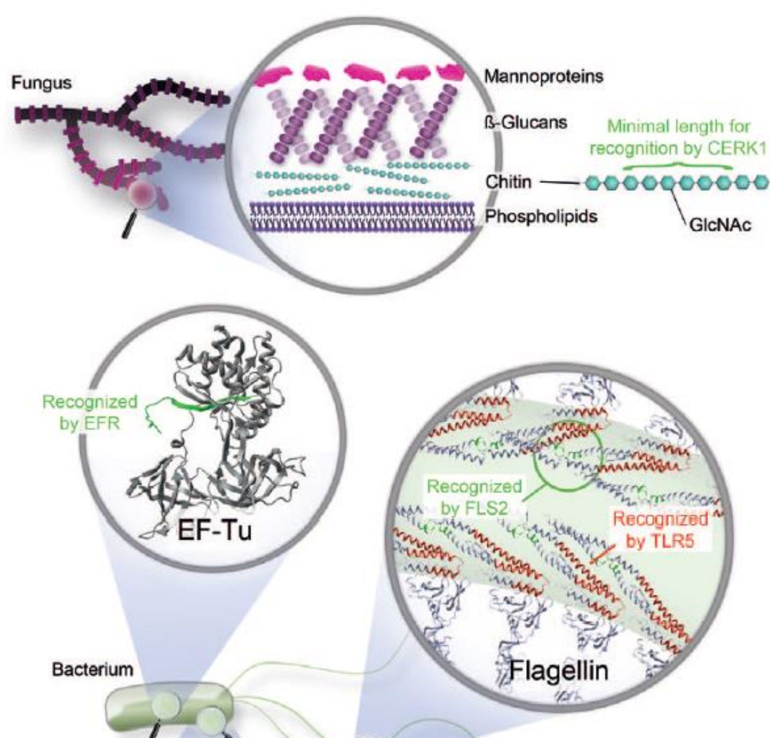


Figure 1.2 Schematic representation of the structure, location and recognized domains of the described PAMPs (taken from Pel and Pieterse 2013). Chitin: The chitin (blue) polymer chitin is an important component of the fungal cell wall. EF-Tu: Structure of *E. coli* EF-Tu (Song *et al.* 1999). EF-Tu is present in the bacterial cytoplasm and the acetylated N terminus of the protein (green) is recognized by the plant receptor EFR. Flagellin: Structure of *Salmonella typhimurium* flagellin molecules (Maki-Yonekura *et al.* 2010). The bacterial flagella are formed by flagellin monomers. These monomers have an exposed part that forms the outside of the flagellum and a non-exposed part that is on the inside. Both the site recognized by FLS2 (green) and the site recognized by TLR5 (red) are in the conserved non-exposed part of the flagellin protein.

1.1.1.1 The oxidative burst

One of the most characteristic PAMP responses studies is the oxidative burst: the rapid and transient accumulation of reactive oxygen species within a few minutes of PAMP perception (Chisholm *et al.* 2006; Jones and Dangl 2006; Boller and Felix 2009). Plant cells produce ROS at the cell surface well known as the “oxidative burst”, such as hydrogen peroxide (H_2O_2), superoxide anions and hydroxyl radicals. However, reactive oxygen species are also involved at the later levels of the hypersensitive response and systemic acquired resistance (Torres *et al.* 2005, 2006). ROS production regulates hormonal signaling pathways such as salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA) and ethylene (ET), and play key roles in the crosstalk between biotic and abiotic stress signaling (Torres *et al.* 2002; Torres and Dangl 2005; Torres *et al.* 2005, 2006; Miya *et al.* 2007).

1.1.1.2 Mitogen-activated protein kinase signaling cascade

Mitogen-activated protein (MAPK-Group 2002) kinase cascades are conserved signaling modules in plant immunity. A MAP kinase cascade consists of a MAP kinase kinase kinase (MAP3K, also called MEKK), a MAP kinase kinase (MAP2K, also called MKK or MEK), and a MAP kinase (MAPK-Group 2002). More over 60 apparent MAPKKKs, 10 MAPKKs, and 20 MAPKs are encoded in *Arabidopsis thaliana* (MAPK-Group 2002). Signals from upstream receptors are transduced and amplified through the MAP kinase cascade by phosphorylation (Figure 1.3). There are a few PRRs that are demonstrated to activate MAPK signaling upon perception of PAMPs, such as the flagellin receptor FLS2 (Felix *et al.* 1999;

Gomez-Gomez and Boller 2002), the bacterial elongation factor EF-Tu receptor EFR (Zipfel *et al.* 2006), and the chitin receptor CERK1 (Miya *et al.* 2007). MPK3, MPK4, and MPK6 are all activated by flg22 (Felix *et al.* 1999). In Arabidopsis, there are two MAP kinase cascades activated by PAMP receptors. One is activation of MPK3 and MPK6 (Asai *et al.* 2002), the other is activation of MPK4 as a negative regulator of plant immunity (Gao *et al.* 2008; Qiu *et al.* 2008).

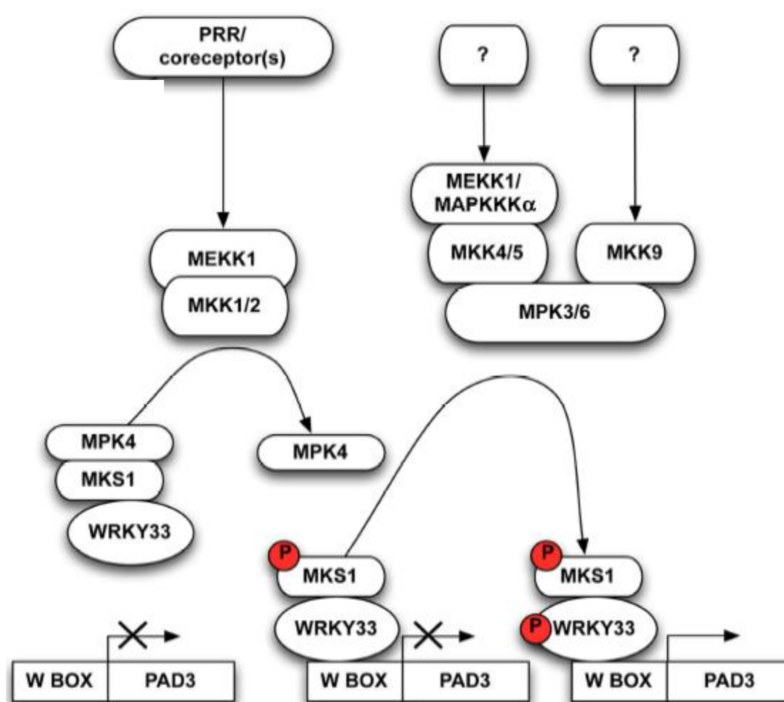


Figure 1.3 PAMP perception by PRRs triggers a signaling cascade (taken from Rasmussen *et al.* 2012). The co-receptors, PAMPs cause activation of MAP3K MEKK1and two MAP2Ks MKK1 and MKK2. These phosphorylate and activate MPK4 which then phosphorylates its substrate MKS1, releasing MKS1 in complex with WRKY33. MPK3/MPK6 sequentially phosphorylate WRKY33, allowing it to promote PAD3 transcription, thus activating plant defense.

1.1.1.3 Callose deposition

Later responses to PAMP treatment include cell wall reinforcement through callose deposition. Callose (a β-1,3-glucan polymer), on the inner side of epidermal cell walls, is produced at different locations in response to biotic and abiotic stress (Humphrey *et al.* 2007; Clay *et al.* 2009). Callose synthase mutants display low levels of callose, and enhance

pathogen resistance as a result of activation of the salicylic acid (SA) defense signaling pathway. Thus, callose or callose synthase negatively regulates the SA pathway (Jacobs *et al.* 2003; Nishimura *et al.* 2003).

1.1.2 Effector trigged immunity (ETI)

Successful microbes secret effector molecules to modify host plant defense response to enhance microbial fitness. During these decades, effectors have emerged as a central class of molecules of plant-microbe interactions. Functions of effector are as diverse as suppressing immune responses or enhancing access to nutrients through altering host-cell structure and function (Hogenhout *et al.* 2009). In the major case, ETI follow gene-to-gene resistance, resistance (R) proteins are proposed to monitor the integrity of host effector targets (Dangl and Jones 2001). R proteins recognize effectors directly or indirectly to induce Effector-Trigged-Immunity (ETI), which accompany localized programmed cell death (PCD) called hypersensitive cell death response (HR). Most plant R proteins contain well characteristic nucleotide-binding and a leucine-rich repeat domain (NB-LRR) class (Ausubel 2005; Caplan *et al.* 2008). Plant NB-LRR R proteins, similar as mammalian NOD like immune receptors, contain either a Toll-interleukin 1-like receptor (TIR) domain or a coiled-coil (CC) domain at their N termini (Meyers *et al.* 2003) (Figure 1.4).

Effectors can be involved in many key plant processes as plant hormones, plant development, plant receptors, signal transduction pathways, and epigenetics. There are some remarkable effectors. Transcription activator-like (TAL) effectors of Xanthomonas bacteria which directly bind specific plant promoter elements in the plant cell nucleus by central domain of tandem repeats to activate plant gene transcription (Boch *et al.* 2009). The bacterial effectors AvrPtoB effector, expressed by the plant pathogen *Pseudomonas syringae pv. Tomato* via type III secretion system (T3SS), has E3 ubiquitin ligases activity to inhibit immunity-associated programmed cell death (Janjusevic *et al.* 2006; Rosebrock *et al.* 2007). SAP11 of phytoplasma bacteria, binding host TCP transcription factors, destabilizes CIN-TCPs to modulate plant development and inhibits jasmonate (JA) synthesis for the advantage of the AY-WB insect vector *Macrosteles quadrilineatus* (Sugio *et al.* 2011). The viral protein P19 of *Cymbidium ringspot virus* (CymRSV) that suppresses RNA interference

(RNAi) in host plant cells by binding double-stranded RNA with high affinity (Lakatos *et al.* 2004). The knowledge of effectors mostly is discovered in plant pathogens and immunosuppression processes. However there is little known about identity and function of effectors from fungi as powdery mildews, rust fungi, and mycorrhizal fungi.

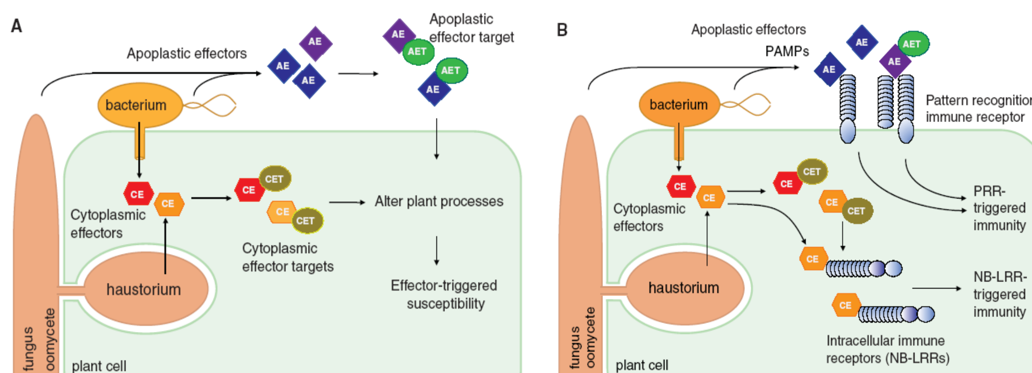


Figure 1.4 The concept of effectors in plant immunity (taken from Win *et al.* 2012). Infectious pathogens deliver effectors at the interface of the host plant (apoplastic effectors, AE) or inside the cell (cytoplasmic effectors, CE). Cytoplasmic effectors are delivered into the host cytoplasm through a type III secretion system or specialized infectious structures called haustoria that form within the cell. Depending on their localization in the cells, these targets are designated as apoplastic effector target (AET) and cytoplasmic effector target (CET). In susceptible genotypes (A), these molecular interactions can alter plant cell processes and suppress immune responses, leading to effector-triggered susceptibility (ETS) and host colonization. However, in resistant genotypes (B), these interactions are perceived by key sensing receptors of the immune system to stop pathogen growth.

1.2 The mutualistic fungus *Piriformospora indica*

Microbes adopt different strategies for gaining nutrients from hosts. Necrotrophs kill plant cell for nutrients, contrastingly the other fungi establish biotrophic relationship with their hosts transiently as hemibiotrophs or for all eternity (Kamper *et al.* 2006; Tyler *et al.* 2006; Stuttmann *et al.* 2011).

1.2.1 *P. indica*'s beneficial symbioses with host plants

P. indica (Hymenomycetes, Basidiomycota) was first described as a cultivable endophyte that colonizes roots in the 1990s in the sandy soils of the Thar region in India (Varma *et al.* 1999).

P. indica was classified as a member of Sebacinales family. As a mutualistic root endophyte, *P. indica* colonizes a broad spectrum of plant hosts as agriculturally important monocot barley and the dicot model plant Arabidopsis thaliana (Waller *et al.* 2005; Peškan-Berghöfer *et al.* 2004). This root-endophytic fungus induces resistance to fungal diseases, tolerance to salt stress in barley and yield in crop plants (Waller *et al*. 2005). Recently, it is reported that important medicinal plant *Coleus forskohlii* interacted with *P. indica* obtains higher biomass and early flowering by regulating development genes (Das *et al.* 2012). Given the capability of *P. indica* to colonize a broad range of hosts, the fungus must evolve efficient strategies to overcome plant immunity and to establish a proper environment for nutrient acquisition and reproduction. Arabidopsis colonized with *P. indica* exhibits constitutive ethylene signaling (Khatabi *et al.* 2012), growth promotion (Peškan-Berghöfer et al. 2004), and suppression of innate immunity (Jacobs *et al.* 2011). Therefore *P. indica* is a powerful model system to study mutualistic fungi interaction with plant host at the molecular level.

It is recently reported that *P. indica* stimulates nitrate uptake via inducing the expression of nitrate reductase (NR) genes in transgenic tobacco seedlings. *P. indica* induced growth-promotion is accompanied by a co-regulated stimulation of enzymes involved in nitrate and starch metabolisms (Sherameti *et al.* 2005). Moreover, *P. indica* promotes maize growth depending on a phosphate transporter of *P. indica* (PiPT), which is thought to mediate phosphate transport to the host plant (Yadav *et al.* 2010). Considering the various beneficial effects, it is worthy to study the mechanism of interaction of *P. indica* and host plants for significant agronomic and high ecological relevance.

1.2.2 *P. indica* confers tolerance and resistance to abiotic and biotic stresses

P. indica has developed efficient colonization strategies to suppress host MTI (Jacobs *et al.* 2011). The microarray-based studies suggested that *P. indica* suppresses plant defense and significantly changes gibberellic acid (GA) metabolism. Barley plants impaired in GA synthesis and perception shows a significant reduction in mutualistic colonization, which is associated with an elevated expression of defense-related genes (Schafer *et al.* 2009).

1.2.3 Cellular colonization pattern of *P. indica*

Cytological studies in Arabidopsis and barley have shown that *P. indica* has a biphasic colonization strategy (Deshmukh *et al.* 2006; Zuccaro *et al.* 2011; Lahrmann and Zuccaro 2012; Qiang *et al.* 2012; Lahrmann *et al.* 2013) (Figure 1.5). The initial root cell invasions using a biotrophic strategy that colonized host cells maintain membrane integrity and invasive hyphae of *P. indica* remain enveloped by the host plasma membrane, therefore it is failed to detect hyphae in cell wall staining with wheat germ agglutinin–Alexa Fluor 488 (WGA-AF488) conjugate (Jacobs *et al*. 2011; Zuccaro *et al*. 2011; Lahrmann and Zuccaro 2012). At later stage, *P. indica* is found more often in dead or dying host cells, especially in the root cortex of barley. Moreover it is exhibited *P. indica* colonization was reduced by overexpression of the negative cell death regulator BAX inhibitor 1 (HvBI-1) in barley and mediated by an endoplasmic reticulum stress-triggered caspase-dependent cell death in Arabidopsis (Deshmukh *et al*. 2006; Qiang *et al*. 2012).

Figure 1.5 Schematic representation of *P. indica* colonization strategies at different symbiotic stages in barley and in Arabidopsis (taken from Lahrmann *et al.* 2013). (A) Invasive hyphae (IH) and secondary thin hyphae (SH) of *P. indica* in barley dead cells (10 days post inoculation). (B) *P. indica* biotrophic broad invasive hyphae in an Arabidopsis epidermal cell (10 days post inoculation). Fungal structures were stained with WGA-AF488 (green); membranes were stained with FM4-64 (red).

1.2.4 Effector candidates secreted by *P. indica*

Current knowledge on fungal effectors derives mainly from biotrophic and hemibiotrophic plant fungal pathogens, and are predicted relying on up-regulated genes during in colonization and coding for predicted small secreted proteins (SSPs) (Martin *et al.* 2008; Zuccaro *et al*. 2011; Hacquard *et al.* 2012). Complete genome sequencing provides an opportunity to investigate the role of effectors during the interaction of *P. indica* with plant hosts (Zuccaro *et al*. 2011). *P. indica* effector protein candidates are established through *in silico* pipeline independent of protein size and Pfam domain-containing (Rafiqi *et al*. 2013). 976 genes are predicted to code for proteins with signal peptide using SignalP, 543 secreted proteins are selected with Pfam domains suggesting possible intracellular functions, and 389 proteins are characterize as predicted fungal effectors with unknown functions (Rafiqi *et al.* 2013) (Figure 1.6). Among SSPs rich in small repeats, 17 effector candidates have the conserved C-terminal motif RSIDELD related sequences enriched for alanine and histidine residues and may have expanded from a single ancestral sequence (Zuccaro *et al.* 2011). The majority of *P. indica* SSPs are not showing significant homology to known sequences in other organisms except DELD proteins and 14 other SSPs showing similarity to predicted secreted proteins of *Laccaria bicolor* (Basiewicz *et al.* 2012).

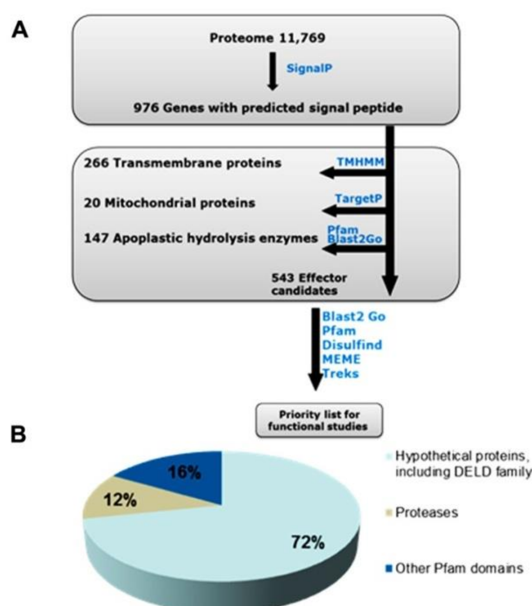


Figure 1.6 Overview of the computational pipeline used to mine the list of effector candidates in the secretome of *P. indica* (taken from Rafiqi *et al.* 2013). (A) *P. indica* secretome, consisting of 972 proteins, was predicted using SignalP. Proteins containing transmembrane domains and proteins with mitochondrial signals were removed using TMHMM and TargetP, respectively. Apoplastic hydrolysis enzymes, such as chitinases and glucanases, were removed based on their function and not on their size, using Pfam and Blast2Go. The 543 proteins are considered effector candidates. (B) 72% of effector candidates are novel sequences of unknown function using Markov-Cluster-Algorithm (MCL http://micans.org/mcl/) analysis.

1.3. E3 ubiquitin ligases and plant innate immunity

1.3.1 ubiquitin-26S proteasome system

Ubiquitination pathway, removing or modifying most abnormal peptides and short-live cellular regulator, is an important mechanism in regulating hormone biosynthesis and signaling. Ubiquitin is a 76 amino acid globular protein and contains seven lysines (K6, K11, K27, K29, K31, K48 and K63) (Callis *et al.* 1995). There is an ATP-dependent E1-E2-E3 enzyme conjugation cascade for free ubiquitin to attach substrates (Weissman 2001) (Figure 1.7). The cascade starts with E1 (or ubiquitin-activating enzyme), which catalyzes the formation of an acyl phosphoanhydride bond between the adenosine monophosphate (AMP) of ATP and the C-terminal glycine carboxyl group of ubiquitin. The activated ubiquitin is transferred from E1 to E2 (or ubiquitin-conjugating enzyme). Finally, ubiquitin-protein conjugate combines target substrate under catalysis of E3 (or ubiquitin ligase) enzymes. After attachment of an initial ubiquitin moiety to a substrate, additional ubiquitins form poly-ubiquitin chains. Polyubiquitinated proteins with K48 ubiquitin chains are degraded by the 26S proteasome, a 2.5 MDa ATP dependent protease complex that is present in both the

cytoplasm and the nucleus (Yang *et al.* 2004). Comparing with E1 and E2, E3 enzymes are more abundant. It is found about 1415 E3 enzyme in Arabidopsis (Mazzucotelli *et al.* 2006). Based on the protein structure and mechanistic properties, E3 has been classified as HECT (homologous to E6-associated protein C-terminus), RING/U-box (really interesting new gene) (Pavletich 2002; Ardley and Robinson 2005). RING domains are defined by the consensus sequence Cx2Cx9-39Cx1-3Hx2-3C/Hx2Cx4-48Cx2C (Deshaies and Joazeiro 2009). RING domain E3 ubiquitin ligase act independently and determine substrate specificity allowing between the E2 and the target protein by tethering them in close proximity (Vierstra 2009).

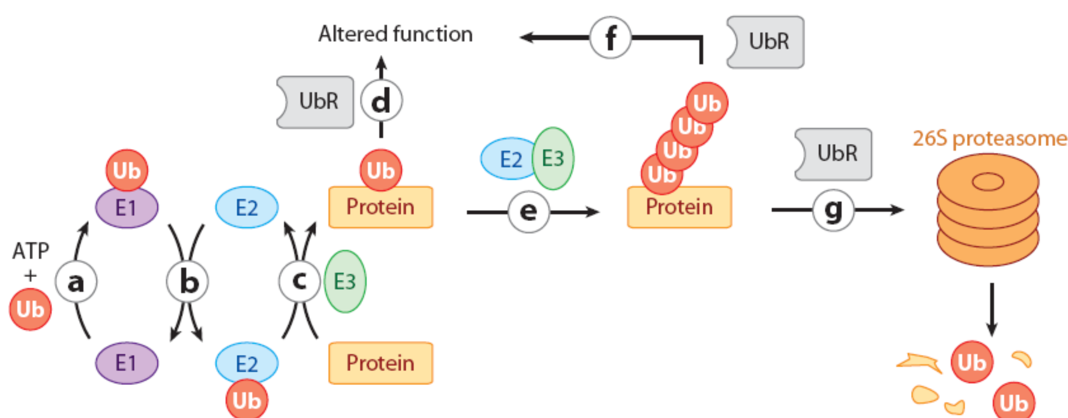


Figure 1.7 Scheme of ubiquitin-26S system (taken from Vierstra 2009). (a) Ubiquitin (Ub) and ubiquitin-like proteins are activated for transfer by E1. (b) Activated ubiquitin is transferred in thioester linkage from the active-site cysteine of E1 to the active-site cysteine of an E2. (c) The E2~Ub thioester next interacts with an E3 ubiquitin ligase, which effects transfer of Ub from E2~Ub to a lysine residue of a substrate. Monoubiquitinated substrate can either dissociate from E3 (d) or an ubiquitin chain (e). Whereas monoubiquitin and some types of chains serve mainly to alter the function of the modified protein (f), and polyubiquitin chains assembled via the K48 residue of ubiquitin typically direct the appended substrate to the proteasome for degradation (g).

1.3.2 Interaction of E3 ubiquitin ligase effectors with plant defense

Some bacterial plant pathogens manipulate of host ubiquitination signaling to suppress defense and promote their own survival (Dreher and Callis 2007). The AvrPiz-t effector from the rice blast fungus *Magnaporthe oryzae* suppresses the ubiquitin ligase activity of the rice

RING E3 ubiquitin ligase APIP6 (AvrPiz-t Interacting Protein 6) to mediate suppression of PAMP-induced ROS production, induces susceptibility to *M. oryzea* (Park *et al.* 2012). The RXLR (R is arginine, X is any amino acid, and L is leucine) effector AVR3a from oomycete *Phytophthora infestans* is an essential virulence factor that targets and stabilizes the plant host U-box E3 ligase CMPG1 to prevent host cell death during the biotrophic phase of infection (Bos *et al.* 2010).

The most famous example is AvrPtoB, a 59-kDa protein secreted by *Pseudomonas syringae pv. tomato DC3000* using type III secret system (T3SS), which manipulates the host ubiquitin machinery and employs intrinsic E3 ubiquitin ligase activity to suppresses plant immunity by inhibiting hypersensitive response-based programmed cell death (Abramovitch *et al.* 2003; Abramovitch *et al.* 2006). Since AvrPtoB also suppresses PCD in yeast, AvrPtoB is considered as a general eukaryotic cell death suppressor (Abramovitch *et al*. 2003; Rosebrock *et al*. 2007; Gohre *et al.* 2008). AvrPtoB N-terminal region (NTR) (1–387aa) is recognized by resistance protein Pto to trigger HR and resistance in tomato (Pedley and Martin 2003), and interacts with the kinase domain of BAK1 and FLS2 to suppress signaling following flagellin perception (Gohre *et al.* 2008; Shan *et al.* 2008). Moreover, the Fen protein is ubiquitinated by the E3 ligase domain of AvrPtoB C-terminal region (CTR) (308–553aa) to suppress ETI triggered by the interactions between the N-terminal domain and Fen (Abramovitch *et al.* 2003; Abramovitch *et al.* 2006; Rosebrock *et al.* 2007).

1.4 Objective

Plant recognize microbes via perception of conserved pathogen-associated molecular patterns (PAMPs) to induced Pattern-trigged immunity (PTI), which is sufficient to restrict microbes growth and eventually leads to cell death. Since some successful microbes can avoid the induction of PTI for promoting their living. For this aim, plant-associated microbes secrete a suite of proteins called effectors that often act to suppress the plant immune system and promote pathogen spread. Effectors use diverse strategies to alter host immunity. *Piriformospora indica,* as a mutualistic endophyte, is a root-colonizing basidiomycete that confers a wide range of beneficial traits to its host. The agricultural potential of *P. indica* is deduced from the wide range of mutualistic symbioses. *P. indica* thus represents a genetically accessible model to study the molecular basis of processes associated with fungal accommodation and the establishment of root symbioses. Genome sequencing of *P. indica* has thrown up many effector candidates, but the challenge now is to investigate functions of these effector candidates. The aim of this study is to reveal the biological function of selected effector proteins and get new insights into the cytology of *P. indica*.

A putative effector, named candidate-PIIN29, has been discovered by B. Khatabi (Khatabi, 2009) and was shown to possess E3 ubiquitin ligase activity. The aim of my work was to further characterize this effector candidate. In addition, I focused on one group of SSPs containing conserved DELD domain to investigate function of these effector candidates.

2. Materials and Methods

2.1 Plant, fungal material and plant inoculation

2.1.1 Arabidopsis thaliana

Arabidopsis Col-0 (*Arabidopsis thaliana* L. Columbia-0) was obtained from the Nottingham Arabidopsis Stock Center. The Arabidopsis seeds were sterilized with 3% sodium hypochlorite for 15 minutes and rinsed in autoclaved water for 3 to 4 times before drying. Next, the sterilized seeds were put on ½ MS medium or ATS medium in squared petri-dishes. The culture conditions were 8 h light (fluorescent cool white, Toshiba FL40SSW/37, 180 $\mu mol\ m^{-2}\ s^{-1}$ photon flux density) / 16 h night, 22 ℃ / 18 ℃, and 60% relative humidity. The petri-dishes with sterilized seeds were vertically arranged and wrapped with aluminium foil to guarantee all plant roots grew on the surface of the medium. For propagation, Arabidopsis seeds were put on the top of a 1: 3 (v/v) sand : soil mixture (type P), and then incubated at 4 ℃ for 48 hours before being transferred to a greenhouse and their growth under long day conditions.

½MS medium (1L)

4% gelrite

4.2 g MS salts

1% sucrose

Adjust pH to 5.4 with KOH

ATS medium

Stock solution	Vol. of stock to add for 1L	Micronutrients
1 M KNO_3	5 ml	0.01 mM $CoCl_2$
1 M KPO_4	2.5 ml	10 mM NaCl
1 M $MgSO_4$	2 ml	0.2 mM Na_2MoO_4
1 M $Ca(NO_3)_2$	2 ml	1 mM $ZnSO_4$
Micronutritients	1 ml	0.5 mM $CuSO_4$
		14 mM $MnCl_2$
		70 mM H_3BO_3

2.1.2 *Piriformospora indica*

The isolate of *P. indica* DSM11827 (German collection of microorganisms and cell cultures in Braunschweig, Germany) was applied in this work. The glycerol stock of chlamydospores of the applied *P. indica* isolate was stored at -80 ℃, which could be used to prepare master plates. The *P. indica* was cultured in CM medium at 25°C for 6 to 8 weeks.

CM medium (Modified Aspergillus minimal medium)

	20 x Salt solution Microelements (1L)
50 ml 20 x Salt solution	
20 g Glucose	120 g $NaNO_3$
2 g Peptone	6 g $MnCl_2x4H_2O$
1 g Yeast extract	10.4 g KCl
1 g Casamino-acid	1.5 g H_3BO_3
1 ml Microelements	10.4 g $MgSO_4x7H_2O$
Add 950 ml H_2O dest	2.65 g $ZnSO_4x7H_2O$
Optional: 15 g Agar	30.4 g KH_2PO_4
	750 mg KI
	2.4 mg $Na_2MO_4x2H_2O$
	130mg CuSO4x5H2O

2.1.3 Inoculation of plant roots with *Piriformospora indica*

Sterilized water containing 0.05% Tween-20 was added on CM agar plates, and then the surfaces of plates were gently scratched with a spatula. The washed spores were filtered using miracloth (Calbiochem, Bad Soden, Germany) to remove mycelium. Spores were collected by centrifuging suspension solution at 4,000 rpm for 10 minutes, and were washed pellet at least 3 times with sterilized Tween-H_2O. To determine the spore concentration was 500,000 spores/ml, a hemacytometer in combination with a microscope was used. For inoculation, 1 ml spore suspension was pipetted on top of plant roots in one squared petri-dish.

2.2 Molecular biological standard methods

2.2.1 DNA-Extraction

Plant material was grinded to fine powder with liquid nitrogen and was added 700 µl warm Doyle&Doyle extraction buffer (65 ℃). This mixture was incubated for 30-60 minutes at

65 ℃ with continuous gentle shaking. After that, 700 µl of chloroform / isoamylalcohol (24:1) was added to the samples and mixed gently for 5-10 min. Then the samples were spun for 15 min with a speed of 10,000 rpm at 4 ℃. The supernatant was collected and added 500 µl isopropanol, 50 µl 10M NH_4OAc and 60 µl 3M NaOAc (pH 5.5) to precipitate DNA. The DNA pellet was centrifuged at 13,000 rpm at 4 ℃ for 22 min, and was washed with 70% ethanol for 10 min at room temperature after centrifuging. Finally, the dry DNA pellet was dissolved in 50 µl H_2O. 1 µl 10 mg/ml RNase was added in for digestion over 30 min at 37 ℃. The concentration was measured using a NanoDrop ND-1000 (peqLab Biotechnology GmbH, Erlangen, Germany).

Doyle&Doyle extraction buffer

100 mM Tris-HCl (pH 8.0)	2% CTAB
20 mM EDTA (pH 8.0)	1% $Na_2S_2O_5$
1.4 M NaCl	0.2% β-mercaptoethanol

2.2.2 RNA-Extraction

Before RNA extraction, samples were grinded to fine powder with liquid nitrogen and stored at -80 ℃. Grinded powder was added to 1 ml of TRIzol (Invitrogen, Karlsruhe, Germany) and incubated for 5 minutes at room temperature. 200 µl of chloroform was added and vigorously mixed with vortex for 10 minutes at room temperature before centrifugation (12,000 rpm, 15 min, 4 ℃). Subsequently, the supernatant was transformed into a new tube and precipitated by 700 µl isopropanol for 30 min at room temperature. The RNA pellet was obtained through centrifugation (12,000 rpm, 20 min, 4 ℃) and washed with 70% ethanol (H_2O_{DEPC}). The dry RNA pellet was dissolved in 40 µl of H_2O_{DEPC}. Finally, the RNA concentration was determined by NanoDrop ND-1000 (peqLab Biotechnology GmbH, Erlangen, Germany).

In order to remove DNA contamination in RNA, DNase-I digestion system was used. The mixture was incubated at 37 ℃ for 30 min, and then the reaction was inactivated by adding 2 µl EDTA and incubated at 70 ℃ for 10 minutes.

DNase-I digestion system

2 µg RNA

1 µl 10x DNase-I buffer (Fermentas, Germany)

1 µl DNase-I (Fermentas, Germany)

0.25 µl RNase inhibitor (Fermentas, Germany)

Added H_2O_{EDPC} up to 10 µl

2.2.3 Reverse transcriptional polymerase chain reaction (RT-PCR)

Different from standard PCR, the template of RT-PCR is RNA. For one-step RT-PCR, Bio-Rad iScript™ cDNA synthesis kit was applied. According to the protocol, a master mix was prepared with 4 µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase, 1 µg RNA template and added with nuclease-free water up to 20 µl. Subsequently, incubate complete reaction mix with this scheme. When the reaction was completed, 80 µl H_2O_{DEPC} was added for final concentration is 10 ng/µl.

Reverse transcription scheme

25 ℃ 5 min

42 ℃ 30 min

85 ℃ 5 min

2.2.4 Quantitative Real-Time PCR (qPCR)

The quantitative real-time PCR was applied either to quantify the relative amount of *P. indica* genomic DNA and plant genomic DNA in colonized Arabidopsis roots or to analyze the relative expression level of candidate transcripts in cDNA samples. In each reaction (20 µl), 40 ng of genomic DNA or cDNA was used as template using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) according to manufacturer’s instructions. In order to minimize the operating errors, each sample was triplicatedly pipetted. The amplification was performed in cyclers of Applied Biosystems (Applied Biosystems 7500 Fast Real Time PCR, Applied Biosystems Inc., CA, USA). The cycle threshold (Ct) value of specific genes was determined by the cycler software, from which the relative expression value $2^{-\Delta Ct}$ was calculated (Livak and Schmittgen 2001).

2.2.5 Polymerase chain reaction (PCR)

A standard PCR mixture with DNA polymerase (DCS Pol, DNA cloning service) was used for amplification of DNA from different template types (cDNA, plasmids, genomic DNA). A standard PCR program is shown below. Annealing temperatures usually depend on the

sequence of the primers, and the elongation time on the size of the amplified DNA fragment (1kb/min).

PCR mixture (1x)

2 µl 10×Buffer BD

1 µl 10 µM Primer fwd

1 µl 10 µM Primer rev

2 µl 2 mM dNTPs

2 µl 25 mM MgCl2

0.2 µl DCS Pol

0.5 - 2 µl Template DNA

Added H_2O up to 20 µl

Cycler program for PCR

Step	Temperature	Time	
Denaturation	94 °C	3 min	
Denaturation	94 °C	30 sec	30-35 cycles
Annealing	x °C	30 sec	
Elongation	72 °C	x min	
Final elongation	72 °C	10 min	

2.2.6. Agarose gel electrophoresis

2.2.6.1. DNA samples

1 x TBE buffer with 1-2% agarose containing 1 µg/ml ethidium bromide (EB) was used to separate DNA samples. The samples were mixed with 10 x DNA loading buffer and separated at 80 -120 V for about 1 hour. The 1kb plus DNA ladder from Invitrogen (Darmstadt, Germany) was used as standard. Subsequently, the gel was detected with a UV-Transluminator (Fröber Larbortechnik, Lindau, Germany) at a wavelength of 312 nm. All results were documented by video documentation equipment (digitStore, INTAS, Göttingen, Germany).

2.2.6.2. RNA samples

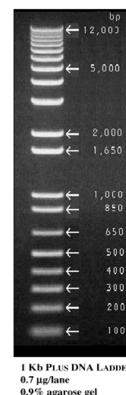
The quality of RNA isolations was checked on 1.2% agarose gels made of 1 x MOPS buffer including 5% (v/v) formaldehyde. RNA (0.25 - 1.0 µg) was mixed 1:1 (v/v) with 2 x RNA loading dye (Fermentas, St. Leon-Roth, Germany) and denatured at 95 °C for 5 min. Subsequently, the gel was detected with a UV-Transluminator (Fröber Larbortechnik, Lindau, Germany) at a wavelength of 312 nm. All results were documented by video documentation equipment (digitStore, INTAS, Göttingen, Germany).

10 x MOPS buffer pH 7.0

20 mM MOPS

5 mM sodiumacetate

1 mM EDTA



1 Kb PLUS DNA LADDER
0.7 µg/lane
0.9% agarose gel

10 x TBE (Tris-Borate-EDTA)

0.9 M Tris

0.9 M Boric acid

0.025 M $Na_2EDTAx2H_2O$

10 x DNA loading buffer

50% Glycerol

0.1 M EDTA

1% SDS

0.04% Bromphenol blue

2.2.7 Production of chemically competent *E. coli* DH5*α* cells

3 ml liquid LB medium was inoculated with *E. coli* DH5*α* cells from a glycerol stock and grown over night at 37 ℃ with 220 rpm. The next day, 1 ml overnight culture was transformed into 100 ml LB medium and incubated at 37 ℃ with 220 rpm for 4-6 hours until OD_{600} ~0.4-0.6. The culture was harvested and centrifuged at 3,000 rpm at 4 ℃ for 10 min. The supernatant was discarded and the pellet was resuspended carefully with 20 ml cold 0.1M $MgCl_2$. After centrifugation, the pellet was dissolved in 20 ml cold 0.1M $CaCl_2$, and inoculated on ice for 30 min. Centrifuge the culture again with 3,000 rpm at 4 ℃ for 10 min, and resuspend the pellet in 4 ml 0.1M $CaCl_2$ containing 20% glycerol. 1.5 ml tubes were filled with aliquots of 50 µl and then frozen immediately in liquid nitrogen.

2.2.8 Transformation of chemically competent *E. coli* cells

Competent cells of *E. coli* DH 5α were thawed on ice for several minutes, and then were added with interest plasmids or ligation products. After incubation ice for 20 min, cells were incubated for 90 seconds at 42 ℃ for hot shock and cooled down on ice for 2 min. Next, 500 µl SOC or LB medium was added and the cells were incubated at 37 ℃ for 60 minutes. Finally the cells were spread on solid LB medium containing respective antibiotics for selection and incubated upside down at 37 ℃ overnight.

SOC medium	10 mM NaCl
2% Tryptone	10 mM $MgSO_4$
0.5% Yeast Extract	10 mM $MgCl_2$

LB medium

1% Tryptone

0.5% Yeast Extract

1% NaCl

2.2.9 Western blotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a PVDF membrane (Roti®-PVDF, pore size 0.4um, ROTH, Germany) with semi-dry electrophoretic transfer cell (Bio-Rad) at 30 mA for 1 h. The PVDF membrane was balanced with 1×Towbin buffer for 15 min. After protein transfer, the membrane was washed by TBS-T buffer for 10 min three times. Non-specific binding sites were blocked using 5% (w/v) milk powder (ROTH, Germany) in TBS-T buffer at room temperature for 1 hour. After three times of washing with TBS-T buffer, primary antibody was applied for overnight incubation at 4 ℃ on a shaker. The membrane was washed for 5 min in TBS-T buffer three times and incubated with second antibody at the dilution 1: 5,000 in TBS-T buffer for 2 h at room temperature. After three times of washing by TBS-T, the blot was developed using Chemiluminescent substrate (SuperSignal® WestPico, Germany).

Polyacrylamide gel (for 2 mini-gels, 12%)

Separating gel	**Stacking gel**
2.5 ml 1.5 M Tris-HCl (pH 8.8)	600 µl 1 M Tris-HCl (pH 6.8)
3.4 ml H_2O	3.6 ml H_2O
4 ml 30% acrylamide/bisacrylamide (ROTH)	1 ml 30% acrylamide/bisacrylamide (ROTH)
100 µl 10% SDS	50 µl 10% SDS
100 µl 10% ammonium persulfate	50 µl 10% ammonium persulfate
10 µl TEMED (Merck)	10 µl TEMED (Merck)

Note: Ammonium persulfate is always freshly prepared.

10x SDS running buffer (pH 8.3)	**1×TBS-T buffer (pH 7.5)**

250 mM Tris-HCl	100 mM Tris-HCl
1.92 M Glycine	150 mM NaCl
1% SDS	0.1% Tween-20
1 X Towbin buffer	**Staining buffer**
25 mM Tris-HCl	0.1% Coomassie-blue
192 mM Glycine	40 % Ethanol
20% Methanol	10 % Acetic acid

Destaining buffer

10% Ethanol

5% Acetic acid

2.3 Candidate gene expression studies with quantitative real-time PCR

2.3.1. PIIN29 expression kinetics

The expression levels of *PIIN29* were analyzed with *in vivo* Arabidopsis Col-0 roots colonized by *P. indica*. For the analysis, Arabidopsis Col-0 plants were grown on ATS in squared petri-dishes for one week. Then roots were inoculated with *P. indica* (~ 500,000 spores/ml) and immediately frozen in liquid nitrogen at 3, 7, 14, and 21 days post inoculation (dpi). RNA was extracted for cDNA by reversed transcription. 40ng cDNA was as template for qPCR analysis. PIIN29-E primers were used to amplify *PIIN29* expression, while the Pi*ITS* (Intragenic transcribed spacer) specific primers were used to amplify *P. indica* DNA.

The sequences of Pi*ITS*-specific primers were 5'-CAACACATGTGCACGTCGAT-3' and 5'-CCAATGTGCATTCAGAACGA-3'. The sequences of *PIIN29*-E specific primers were 5'-CTGGATTGCGCTTATTGGAT-3' and 5'-CTCCCGTTCGATATCCTTCA-3'.

2.3.2 Quantification of fungal colonization by qPCR

The sterilized seeds were put on ATS medium in squared petri-dishes. Three-week-old Arabidopsis seedlings were cultured, and 1 ml of *P. indica* chlamydospores solution (500,000 spores/ml) was well spread on the roots in each plate. At 3 and 7 dpi, roots were harvested (3-4 plates/time point) and DNA was extracted by using the described CTAB method (see 2.2.1). Subsequently, the colonization level of plant roots by *P. indica* was analyzed by qPCR (see 2.2.4). For each sample, 40 ng of genomic DNA was served as template.

At*UBQ4*-specific primers were used to amplify plant genomic DNA, while the Pi*ITS* specific primers were used to amplify *P. indica* DNA. The sequences of At*UBQ4*-specific primers were 5'- CGCAGTTAAGAGGACTGTCCGGC -3' and 5'-GCTTGGAGTCCTGCTTGGACG-3'.

2.4 Analysis of flagellin- or chitin-induced Pattern-Trigged Immunity (PTI)

2.4.1 Analysis of flg22-induced oxidative burst of Arabidopsis roots

Roots of three-week-old Arabidopsis seedlings were harvest and cut to 1 cm length. Root fragments were incubated overnight in glass tubes containing water to avoid wound affection. By removing the water, 195 µl luminol solution and 5 µl phosphate buffer was added. After running 5 cycles as blank, samples were treated with 1.5 µM flg22. Oxidative burst was measured using Berthold Lumat LB 9501(Berthold, Bad Widbach) for 40 min.

2.4.2 Analysis of flg22- or chitin-induced oxidative burst of leaves

Arabidopsis were grown on a sand/soil (V/V=1:3) for around six weeks at short day conditions. Leaves of 6-week-old plants were cut to obtain 25 mm^2 leaf discs. Leaves discs were carefully moved into white 96-well plates filled with 180 µl luminol solution for incubation in water overnight. After running 10 cycles as blank, samples were treated with 100 nM flg22 or 10 mg/ml chitin. Oxidative burst was measured using TECAN Infinite® F200 microplate reader (TECAN, Switzerland).

Luminol solution

30 µg/ml Luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione, stock 15 mg/ml in DMSO)

20 µg/ml Horseradish peroxidase (HRP, stock 10 mg/ml in H_2O)

Wrap the tube with aluminium foil and keep solution avoid light

Phosphate buffer

200 mM Na_2HPO_4

200 mM NaH_2PO_4

Adjust solution to pH 8.0.

2.4.3 Analysis flg22-induced callose deposition in Arabidopsis leaves

Two-week-old seedlings used in this assay were grown in a ½ MS, and treated with 1 µM flg22 for 24 hours. After the elicitor treatment, the seedlings were fixed in an ethanol/acetic

acid (V/V=3:1) solution overnight on a shaker. Seedlings were subsequently rehydrated in 70% ethanol for 2 hours, then in 50% ethanol for another 2 hours. After washed by water twice, seedlings were suspended in 150 mM K_2HPO_4 (pH 9.5) buffer containing 0.01% aniline blue for at least 2 hours on a shaker. The amount of callose deposition was viewed by microscopy (Axioplan 2 Imaging, Zeiss, Germany).

2.4.4 Analysis flg22-induced MAP kinase activity

Arabidopsis seedlings were grown on ½ MA medium containing 1% sucrose for 14 days. The seedlings were incubated with 100 nM flg22 and harvested after different time points as 0, 15, 30, 60 and 120 min. Proteins were extracted from seedlings in LUCAS buffer. 20 μg protein was separated by 12% SDS-PAGE gel immunoblotting with anti-αpMPK antibody (1:1,000 dilution, Cell Signaling) was used to detect Phosphorylated MPK proteins.

LUCAS buffer

25mM Tri-HCl (pH 7.8)	75mM NaCl
10mM $MgCl_2$	1mM DTT
15mM EDTA	

10 ml LUCAS buffer was added 1x protease inhibitor cocktail tablet (Roche) and 1x phosphatase inhibitor cocktail tablets (Roche).

2.5 Transient expression DELD candidates in *Nicotiana tabacum* and *Nicotiana benthamiana*

2.5.1 Growth of *N. tabacum* and *N. benthamiana*

N. tabacum and *N. benthamiana* were grown in soil (Fruhstorfer Erde Typ T), and kept in a chamber under 16 h light/8 h dark intervals, at a light intensity of 160 μmol m^{-2} s^{-1} at 20 ℃. Seven to eight-week-old plants were transferred to the laboratory at the day of infiltration.

2.5.2 Agrobacterium mediated infiltration

2.5.2.1 Preparation of effector delivery plasmids by golden gate

After purification of effector candidates PCR product (Phusion® High-Fidelity DNA Polymerase, NEB), add pICH51277 (CaMV 35S promoter), pICH 41421 (Nos terminal), pICH 47732 (destination vector) and GFP PCR product using the following Protocol. And

then transform 5 µl of the assembly reaction into chemical competent *E. coli* DH 5α. All the primers and vector detail are listed in supplement.

100ng effector candidates PCR product	
100 ng GFP PCR product	Thermocycler program:
100 ng pICH51277	37 ℃, 1 hour
100 ng pICH 41421	50 ℃, 5 min
100 ng pICH 47732	80 ℃, 5 min
0.75 µl T4 DNA ligase (Promega)	
1 µl 10x Buffer	
0.5 µl BsaI (NEB)	
Add H_2O up to 10 µl	

2.5.2.2 Transformation and growth of *A. tumefaciens* strains

The Agrobacterium strains GV310::pMP90 were transformed with the candidate constructions by electroporation. Electro-competent agrobacteria cells were thawed on ice for several minutes and added 100 ng plasmids. After inoculation on ice for 10 minutes, the cells were transferred to precooled electroporation cuvettes (0.2 cm gap) and exposed to one pulse of 2.4 kV using Gene Pulser MXcell Electroporation System (BioRad). Thereafter, the cells were supplied with 600 µl SOC medium and cultured in the cuvettes at 28 ℃ for 1.5 hours. Finally, bacteria were spread on LB medium with the appropriate antibiotics and incubated at 28 ℃ for about two days. Positive colonies were cultured in LB medium at 28 ℃ and 140 rpm for two days.

2.5.2.3 Agroinfiltration of *N. tabacum* and *N. benthamiana*

Positive agrobacteria were grown at 28 ℃ and 140 rpm for two days, and the cultures were harvested by centrifugation at 3,500 rpm for 20 minutes at room temperature. Discard supernatant and resuspend pellet in induction medium. Adjust the OD_{600} ~1.0 and store cell in dark for 1-2 hours. Infiltrate the agrobacterium into the pre-marked circles on the leaves using 1 ml needleless syringe. After 48 hours, the infiltrated leaves were harvested for microscopy or western blotting.

Infiltration buffer (pH 5.6)

10 mM $MgCl_2$

10 mM MES

150 μM Acetosyringone (stock 15 mM in ethanol)

2.5.3 Type III secret system mediated infiltration

2.5.3.1 Preparation of effector delivery plasmids

All GATEWAY entry vectors of candidate effectors and yellow fluorescent protein (YFP venus) were constructed in pENTR™/D-TOPO® according to manufacturer's (Invitrogen) instructions. A high-fidelity DNA polymerase such as Phusion (Thermo Scientific) was used for initial PCR amplification from cDNA of Arabidopsis roots colonized by *P. indica* (all the primers were listed in supplement information). The candidate effector entry clones included the coding sequence of the predicted mature protein without the signal peptide region. 25 mM PCR product, 0.5 μl salt solution and 0.5 μl TOPO vector were combined in a 3 μl reaction, and inoculated at room temperature for 30 min. After that, the reaction was transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen™). Expression vectors were then generated using Gateway® LR Clonase® II Enzyme mix (Invitrogen™) with candidate effector entry clones and the AvrRPM1 T3SS destination vector pNR526G2AC3A. Positive expression clones were analyzed by gene/destination vector specific PCR and sequencing to confirm correct integration.

2.5.3.2 Production of electroporation competent *Pseudomonas fluorescens* EtHAn cells

3 ml liquid LB medium was inoculated with *P. fluorescens* EtHAn cells from a glycerol stock and grown over night at 30 ℃ and 220 rpm. The next day, 1 ml overnight culture was transferred into 100 ml LB medium and incubated at 30 ℃ and 220 rpm for 18 hours to an OD_{600} ~ 0.8-1.2. The culture was cooled on ice for 20min and centrifuged at 3,000 rpm at 4 ℃ for 10 min. The supernatant was discarded and the pellet was resuspended carefully in 20 ml cold 10% glycerol. This washing step was repeated for 5 times and the pellet was resuspended in 4 ml 10% glycerol. Aliquots of 50 μl were placed in tubes (1.5 ml) and then frozen immediately in liquid nitrogen.

2.5.3.3 Transformation of electroporation competent *P. fluorescens* EtHAn cells

Electro-competent cells of *P. fluorescens* EtHAn were thawed on ice for several minutes, and then 100ng interest plasmids were added. After incubated on ice for 20 min, cells were transferred into precooled electroporation cuvettes (0.2 cm gap) and exposed to one pulse of 2.0 kV using the Gene Pulser MXcell Electroporation System (BioRad). Next, 500 μl SOC or LB medium was added and the cells were incubated at 30 ℃ for 3 hours. Finally the cells were spread on solid LB medium containing respective antibiotics for selection and incubated upside-down at 30 ℃ for two days.

2.5.3.4 Preparation of *P. fluorescens* EtHAn cells under T3SS inducing conditions for infiltration

Glycerol stock cultures were placed onto LB media with antibiotic selection (30 μg/ml chloramphenicol and 20 μg/ml gentamycin) and grown at 29°C for 36 hours. A loopful of the cultures was inoculated into 5 ml LB (with antibiotics) and grown at 29°C with shaking overnight (O/N). 50 ml LB (with antibiotics) containing 1 ml this O/N culture was incubated to OD_{600} ~0.8 at 29°C for 4-6 h with shacking. These cultures were cooled on ice, transferred to 50 ml Falcon tubes and cells harvested by centrifugation (4,000 rpm, 4°C, 10 min). Culture pellets were resuspended in 25 ml of pre-chilled 10 mM $MgSO_4$ and centrifuged to harvest cells. Pellets were resuspended in a minimal medium with antibiotic (15 μg/ml gentamycin) to OD_{600}=0.8, and incubate with shaking at 20°C O/N. After harvesting cells by centrifugation (4,000 rpm, 4°C, 10 min), pellets were resuspended in 10 mM $MgCl_2$ to the OD_{600} ~ 1.8-2.0 and used for infiltration.

Minimal T3SS induction medium (pH 5.8)

50 mM potassium phosphate buffer	1.7 mM NaCl
7.6 mM $(NH_4)_2SO_4$	10 mM fructose
1.7 mM $MgCl_2$	

Sterilize with 0.22 μm filter.

2.6 *Botrytis cinerea* inoculation and quantification

Botrytis cinerea strain B 05.10 was grown on HA medium as described previously (Doehlemann *et al.* 2006). Six-week-old Arabidopsis plants were detached and placed in

petri-dishes containing 1% agar. To infect plants, 14-day-old *B. conidia* was washed by 12 g/L potato dextrose broth (PDB, Duchefa Biochemie, Haarlem, The Netherlands) and adjusted spore concentration to 50,000 conidiospore/ml. 5 μl spore suspension was inoculated in the middle of leaf vein. Depending on the symptom development, two to six days after infection, measure the area of lesion using ImageJ.

HA medium (pH 5.5)

1% malt extract

0.4 % yeast extract

0.4 % glucose

1.5% agar

2.7 Preparation of TEM samples

The roots of barley colonized by *P.indica* were fixed in 1.5 % paraformaldehyde and 1.5% glutaraldehyde in 0.05 M cacodylate (pH 7.2) buffer for 24 hours at 4°C, and washed three times using 0.05 M cacodylate buffer. And then the small portions were refixed in 4% paraformaldehyde in 0.05 M cacodylate buffer for 1 hour at 4°C. After washed three times by 0.05 M cacodylate buffer, the portions were post-fixed in 1% osmium tetroxide for 1 hour in room temperature. Following two times rinses in 0.05 M cacodylate buffer, the samples were dehydrated in a graded ethanol series. The pieces were embedded in Epon resin (propylene oxide: resin 2:1, 1:1, 1:2 for 60min each) in blocks. After hardening at 68°C for 2 days, ultra-thin sections were cut using a diamond knife, mounted on microscope slides in grid.

2.8 Methods for 3D protein modeling of PIIN29 RING finger domain

The structural model for PIIN29 RING finger domain was predicted using SWISS-MODEL (Schwede *et al.* 2003). 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 PIIN29 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). For the whole sequence of PIIN29, no template was found

which could be used as the model for constructing 3-D structure. Therefore, the structural model of PIIN29 was built based on the RING finger domain in homology with other known template structures (these sequences were listed in supplement). The backbone of the target protein was built up, based on the localization of accordant atoms in the template structure. For more information about the used template structures, see the following link: http://www.rcsb.org/pdb/home/home.do.

3. Result

3.1 Functional analysis of effector candidate PIIN_00029 (PIIN29)

3.1.1 *In silico* analysis of PIIN29

Since genomic sequence of *P. indica* is known, there was a good chance for us to select effector candidates. We found an interest candidate effector, named PIIN29, which had high homology with E3 ubiquitin ligase.

We got the nucleotide and amino acid (aa) sequences of PIIN29 (CCA66343) of *Piriformospora indica* DSM 11827. The full length of cDNA is 1590 bp (the sequences detail can be found in supplement 7.1).

PIIN29 shares 41% identity on the aa level with a hypothetical protein of *Serpula lacrymans var. lacrymans* (XP_007317404), 42% with *Fomitiporia mediterranea MF3/22* (XP_007266647), 42% with *Gloeophyllum trabeum ATCC 11539* (XP_007864356), 41% with *Agaricus bisporus var. bisporus H97* (XP_006461112), 43% with *Coniophora puteana RWD-64-598 SS2* (XP_007766531), and 40% with *Laccaria bicolor S238N-H82* (XP_001877371) using BLASTP alignment against the non-redundant protein database. In addition, PIIN29 has 41% similarity with a putative RING-7 (really interesting new gene) domain from *Rhizoctonia solani AG-1 IA* (ELU42672) and 31% similarity with E3 ubiquitin-protein ligase RING1 from *Pyronema omphalodes CBS 100304* (CCX31590). Since effectors always have signal peptide to guide target in host cell, we used SignalP 4.0 to check PIIN29 aa sequence, and there is a putative signal peptide (residues 1-22). A predicted E3 ubiquitin ligase domain (residues 410-454) was contained at C-terminus of PIIN29 using SMART (Figure 3.1).

Figure 3.1 Sequence and domain structure of the effector PIIN29. Domain of 1-21 aa was signal peptide (green) and domain of 411-454 aa was RING domain E3 ubiquitin ligase (red).

Subsequently we predicted protein modeling of PIIN29 to search activity sites of catalysis. Because there was low homologous with the other functional proteins, the analysis was focused on conserved RING finger domain. We used 2KizA (SWISS-MODEL template library) as template (predicted second structure alignment in Figure 3.2) for PIIN29 RING domain E3 ubiquitin ligase modeling (406-547aa).

```
TARGET     1                      GNQTCP ICIVDFEEGD DVRVLPCEGK HRFHKDCVDI
2kizA      1     mkqdgeegte ed--teekct iclsileege dvrrlpcm-- hlfhqvcvdc

TARGET                                                sssss ss sss hhhhhh
2kizA                                                 sss      sss hhhhhh


TARGET     37    WLLELSSSCP ICREDFHVLE  ---
2kizA      47    wl-itnkkcp icrvdieaql pses

TARGET           hhhh
2kizA            hh hh
```

Figure 3.2 Predicted second structure alignment of PIIN29 (target) and 2kizA. H: α-helix; s: β-sheet.

There were three well studied protein models of RING finger, human (Rbx1), *Arabidopsis* (AtPUB14), and the bacterium *Pseudomonas syringae* (AvrPtoB). It is possible to predict the activity site of RING figure domain through modeling study. Figure 3.3 shows the alignment result of PIIN29 RING domain with the others.

```
PIIN29          ----------------------GN--------------------QTCPIC---IVDF--- 12
Rbx1            -------GSMDVDTPSGTNSGAGKKRFEVKKWNAVALWAWDIVVDNCAICRNHIMDLCIE 53
AtPUB14         ----------------------GSPEFP--------------EYFRCPIS----LEL--- 17
AvrPtoB         GPKLAALDPIASQFSQLRTISKADAESEELG-FKDAADHHTDDVTHCLFGGELSLSNPDQ 59
                                      ..                    * :     :.

PIIN29          --EEGDDVRVLPCE-----G--KHRFHKDC-VDPWLLELSSSCPICRE---------DFH 53
Rbx1            CQANQASATSEECTVAW--GVCNHAFHFHC-ISRWL-KTRQVCPLDNR---------EWE 100
AtPUB14         -----MKDPVIVST-----G---QTYERSS-IQKWLDAGHKTCPKSQETLLHAGLTPNYV 63
AvrPtoB         QVIGLAGNPTDTSQPYSQEGNKDLAFMDMKKLAQFL-AGKPEHPMTRETLNAEN-IAKYA 117
                            .      *     :     :  :*       *  ..         .:

PIIN29          VLE------------ 56
Rbx1            FQKYGH--------- 106
AtPUB14         LKSLIALWCESNGIE 78
AvrPtoB         FRIVP---------- 122
                .
```

Figure 3.3 Multiple sequence alignment of the PIIN29 RING figure domain with human (Rbx1), Arabidopsis (AtPUB14) and the bacterium *Pseudomonas syringae* (AvrPtoB). The well-defined and functionally important E2 binding residues (red arrow) were conserved in PIIN29.

Ubiquitin conjugating E2 enzymes were interacting with specific amino acid residues of E3 ubiquitin ligase, so-called E2-binding sites, which are required for protein ubiquitination and subsequent degradation. The three E2 binding sites encompassing tryptophane, isoleucine and proline were defined to be functionally relevant in known E3 ligase were conserved in PIIN29 and implicates its function as E3 ligases. Furthermore, three dimensional structural deduced analysis of PIIN29 revealed the accurate conformation and orientation of the E2 binding residues.

A 3D alignment indicated a highly similar orientation of E3 ligase domain and the E2 binding residues of PIIN29 with those bacterial (AvrPtoB) and plant (AtPUB14) RING finger domain (Figure 3.4), which were known functional E3 ligases.

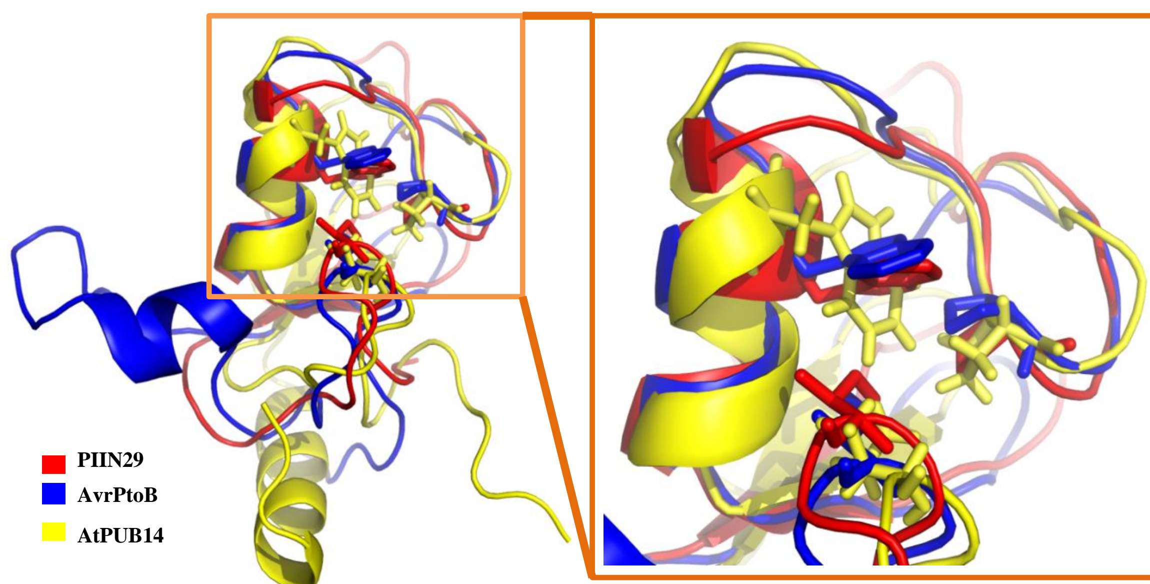


Figure 3.4 3D alignment of the ubiquitin E3 ligase of PIIN29 with RING figure domains of AvrPtoB and AtPUB14. Conformation and orientation of the E2 binding residues had shown in small frame.

3.1.2 Gene expression of *PIIN29* during colonization of Arabidopsis root with *P. indica*

Since redundant effector candidates, we need to determine the expression of *PIIN29* during *P. indica* colonization. To this aim, transcription level of *PIIN29* was quantified of Arabidopsis Col-0 roots, which were inoculated with *P. indica* at different stage as 3, 7, 14 and 21 days post inoculation (dpi). It was shown that *PIIN29* expression level was increased gradually

after inoculation (Figure 3.5A). To reduce the background, we only cultured *P. indica* spore in CM liquid medium. There was no different expression of *PIIN29* between 7 and 10 days (Figure 3.5 B). In addition, expression of *PIIN29* was 2.6-fold and 5.2-fold induction during *P. indica* interaction with Arabidopsis root comparing with its expression in liquid CM medium at 7 and 10 dpi respectively (Figure 3.5 B). Therefore, as an effector secreted by *P. indica*, PIIN29 increased expression level during colonization completely irrelevant to growth in liquid culture. The data presented here demonstrated that PIIN29 was induced in presence of plant root.

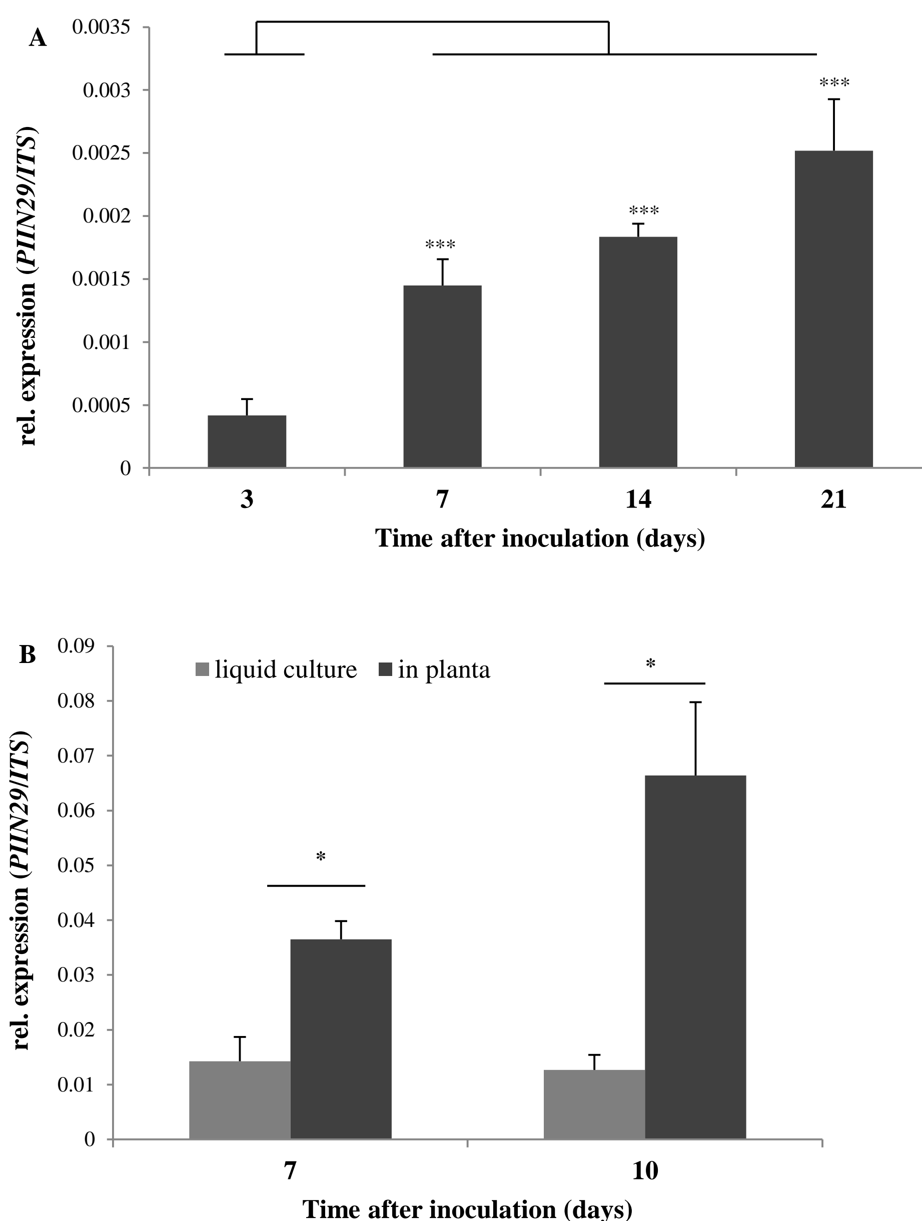


Figure 3.5 Expression of *PIIN29* in Arabidopsis root colonized by *P. indica*. A. One-week-old Arabidopsis Col-0 were inoculated with 500,000 chlamydospores of *P. indica* and harvested at 3, 7, 14 and 21 dpi with qRT-PCR for transcription of *PIIN29*. B. *P. indica* spore was cultured in liquid CM medium and three-week-old Arabidopsis Col-0 roots. Harvest samples at 7 and 10 dpi for *PIIN29* expression using qRT-PCR. Expression values were calculated by the $2^{-\Delta Ct}$ method using relating Ct values of *PIIN29* to the *P. indica* specific primer PiITS. Asterisks indicate significant differences expression level of *PIIN29* at P<0.001(**) and P<0.05(*) as analyzed by Student's test.

3.1.3 Overexpression of PIIN29 in plant

During *P. indica* colonizing Arabidopsis root, expression of *PIIN29* increased significantly compared with its expression in liquid CM medium. Therefore, there was a question for us to answer: what is the function of PIIN29 as effector secreted by *P. indica*. To investigate effects of PIIN29 in plant immunity, we constructed stably transgenic plants which consistent expression PIIN29 (PIIN29OE).

3.1.3.1 Construct binary vector for overexpression PIIN29 in plant

We got fragment of PIIN29 without native signal peptide fusion with mCherry using overlapping PCR (Figure 3.6 A). To avoid function loss, six aa (GSGSGS) were added as linker to enhance flexibility. And then we cloned this fragment *piin29*:*mCherry* into binary vector pLH6000 for expression in Arabidopsis and barley constitutive controlling under CaMV35S promoter (Figure 3.6 B). All the primers and plasmid map were listed in supplement information.

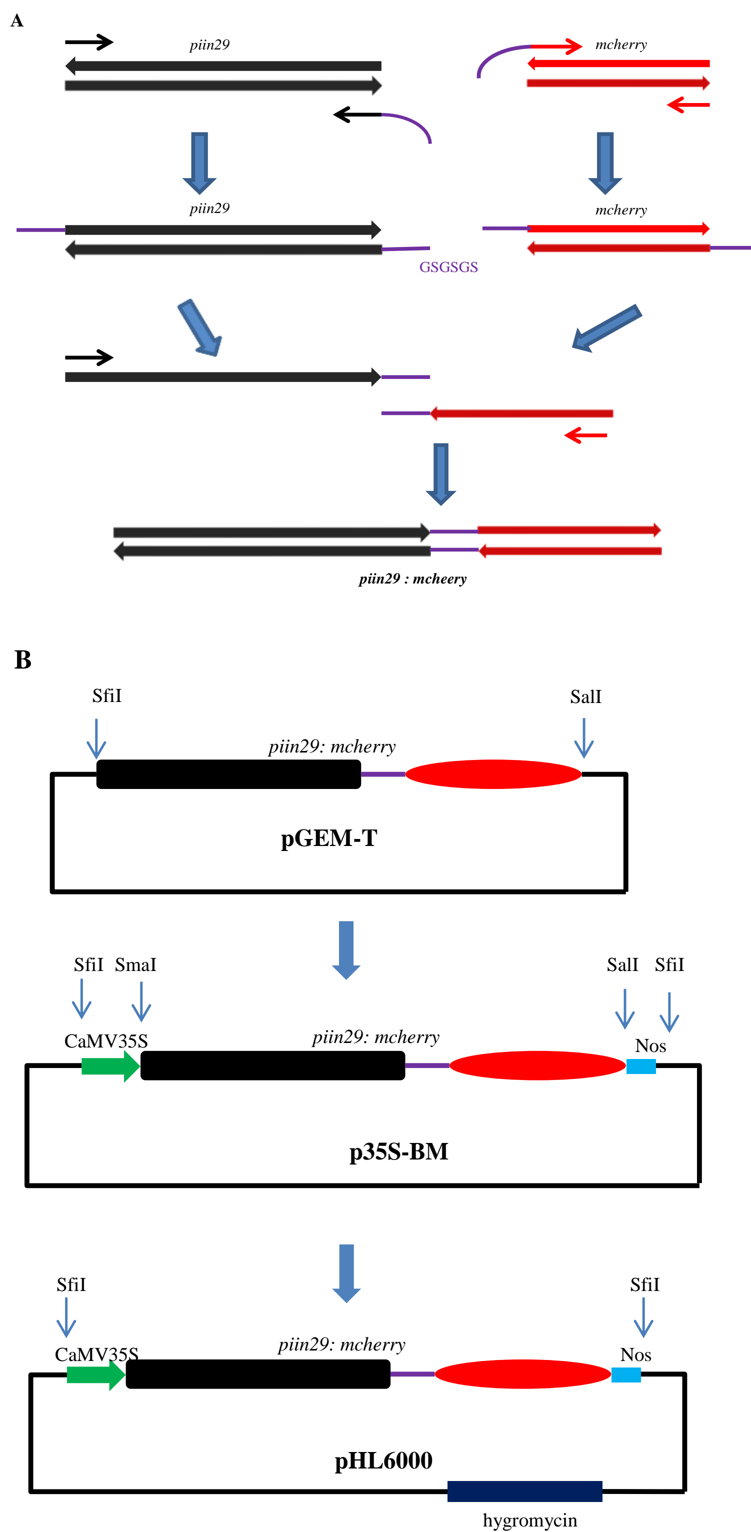


Figure 3.6 Schematic diagram of the subcloning strategy for PIIN29 expression in plants. A. Strategy of *piin29:mcherry* by overlapping PCR. B. Scheme of construction of pHL6000:*35S:piin29:mcherry*. Cut *piin29: mcherry* from pGEM-T using SfiI and SalI, and then fill the

fragment to blunt end for ligation with vector p35S-BM to form cassette with CaMV 35S promoter and Nos terminal. The last step was that whole cassette was cloned into binary vector pHL6000 for transformation into plants.

3.1.3.2 Identify positive transgenic plants

3.1.3.2.1 Identify T2 generation of positive transgenic barley

The deleted signal peptide of PIIN29 was cloned in the binary vector pLH6000 in order to consistent expression the effector under control of CaVM35S promoter. We got transgenic barley using stable root transformation system (Imani *et al.* 2011, Fatima 2013).

To confirm the T2 generation of positive transgenic barley, we harvested one-month-old different transgenic barley lines and extracted genomic DNA with Doyle&Doyle buffer. Genomic DNA was serviced as template to check expression of PIIN29 in positive transgenic barleys. The positive transgenic barley can be detected the expected band as 1854bp using PIIN29 specific primer (Figure 3.7). The sequence of PIIN29 specific primer is PIIN29-F: 5'-TATGAATTCATGTATATTCCAGCTAGGGCTGC-3' and PIIN29-R: 5'-AATCTCGAGCTACGCCGGGGTCGGCGACAC-3'.

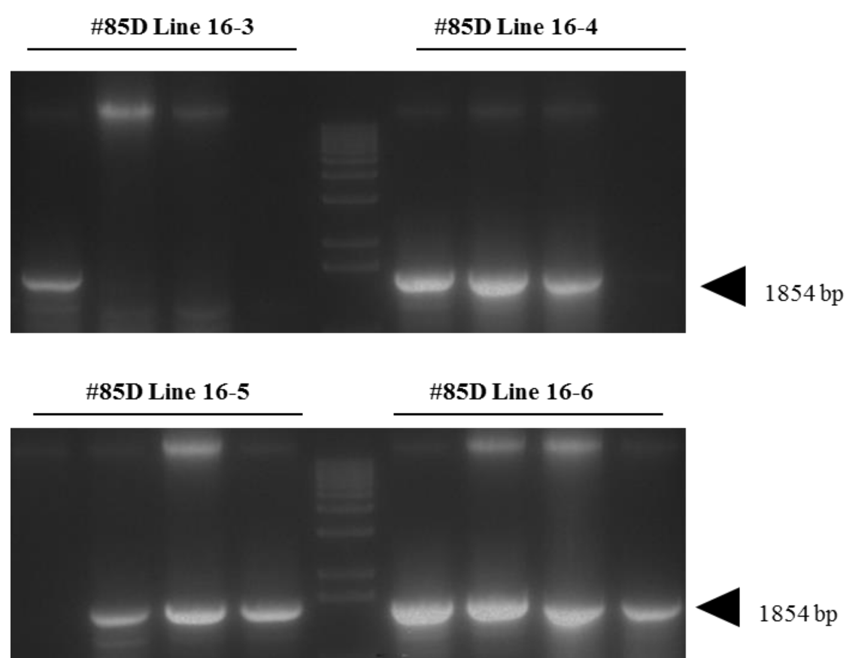


Figure 3.7 Screening of positive transgenic barleys of PIIN29 construct. Different T2 generation plants as #85D lines 16-3, 4, 5 and 6 were used in this experiment. Genomic DNA of positive transgenic barleys got the expected size as 1854bp using gene specific primers.

3.1.3.2.2 Identification T3 generation of positive transgenic Arabidopsis

We cultured seeds on ½ MS agar medium (1% sucrose) containing 30 μg/μl hygromycin as selection marker. After three generations, two-week-old seedlings were tested expression of PIIN29 using RT-PCR, and wild type Col-0 as control. Transgenic Arabidopsis (PIIN29OE) exhibited expression level of PIIN29 as expected size, however there was no band shown in Col-0 (Figure 3.8). Arabidopsis housing keeping gene *AtUBQ4*-specific primers were used as control. The sequence of PIIN29 specific primer is PIIN29E-F: 5'-AAGTGGAGTCTGGAGAACGGCG-3' and PIIN29E-R: 5'-AGGTTTGGTTTCCGATGGCG-3'.

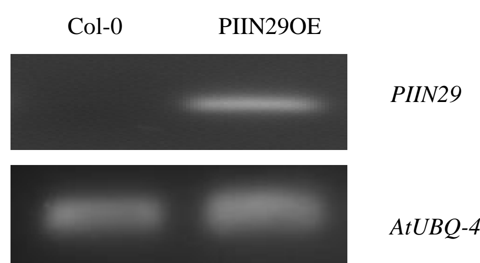


Figure 3.8 Confirmation of positive transgenic Arabidopsis by RT-PCR. Two-week-old Arabidopsis seedlings were extracted RNA with TRIzol and reserved transcription as cDNA. Arabidopsis housing keeping gene *AtUBQ4*-specific primers were used to amplify plant cDNA as control, but only PIIN29OE had band using PIIN29E gene specific primer.

3.1.4 Colonization of transgenic Arabidopsis PIIN29OE

3.1.4.1 Colonization of transgenic Arabidopsis PIIN29OE with *P. indica*

Given present data, the expression of PIIN29 increased gradually during *P. indica* colonization of Arabidopsis roots. Because effector can induce effector-trigged susceptibility (ETS), we subsequently elucidated this effector affecting the colonization of *P. indica*. Therefore transgenic Arabidopsis PIIN29OE and wild type Col-0 were forward to colonization studies. Roots of three-week-old Arabidopsis seedlings were inoculated with *P. indica* spore, and harvested at 3, 7 and 10 dpi for DNA extraction. The 40ng genomic DNA was used to quantify the fungal biomass in the roots by qPCR. There was no difference at the early stage between Col-0 and PIIN29OE, but at the late stage as 10 dpi, the *P. indica* biomass was almost 2 times higher in PIIN29OE than Col-0 (Figure 3.9). This result indicated

that effector PIIN29 was able to significantly increase *P. indica* colonization in Arabidopsis root at late stage.

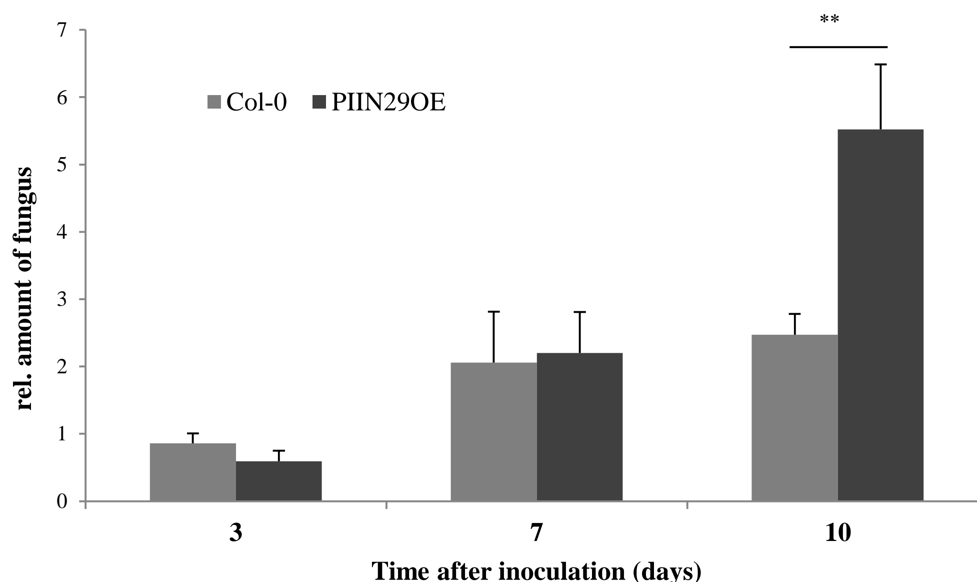


Figure 3.9 PIIN29 promoted colonization of Arabidopsis root by *P. indica.* Three-week-old Arabidopsis Col-0 and PIIN29OE roots were inoculated with *P. indica*. The fungal biomass was determined at different stages as 3, 7, and 10 dpi by qPCR. Arabidopsis house keeping gene *AtUBQ-4* was used as internal control to normalize the data. The values represent means ±SE of three independent experiments. For each experiment, around 100 plants were analyzed per line at each time point. Asterisks indicate significant differences in the colonization of transgenic plant compared with Col-0 at 10 dpi at $P<0.01$(**) as analyzed by Student's test.

3.1.4.2 Colonization of transgenic Arabidopsis PIIN29OE with *Botrytis cinerea*

PIIN29 can enhance *P. indica* colonization of Arabidopsis roots at later stage as 10 dpi. In contrast to mutualistic fungus *P. indica*, *B. cinerea* is a necrotrophic fungus. To verify whether PIIN29-induced ETS is based on host or not, we design experiment to inoculate Arabidopsis with *B. cinerea.* The six-week-old Arabidopsis of Col-0 and PIIN29OE detached leaves were speared on 1% agar plates and inoculated with necrotrophic fungus *B. cinerea* spore. After 3 days inoculation, disease symptoms of PIIN29OE Arabidopsis were significantly stronger than those on wild type Col-0 (Figure 3.10 A, B). The PIIN29OE were more susceptible to *B. cinerea* as evidenced by the complete rot and overgrowth with fungal

mycelium than Col-0. It is suggested that PIIN29 enhanced susceptibility of *B. cinerea* and colonization of *P. indica* independent on tissues.

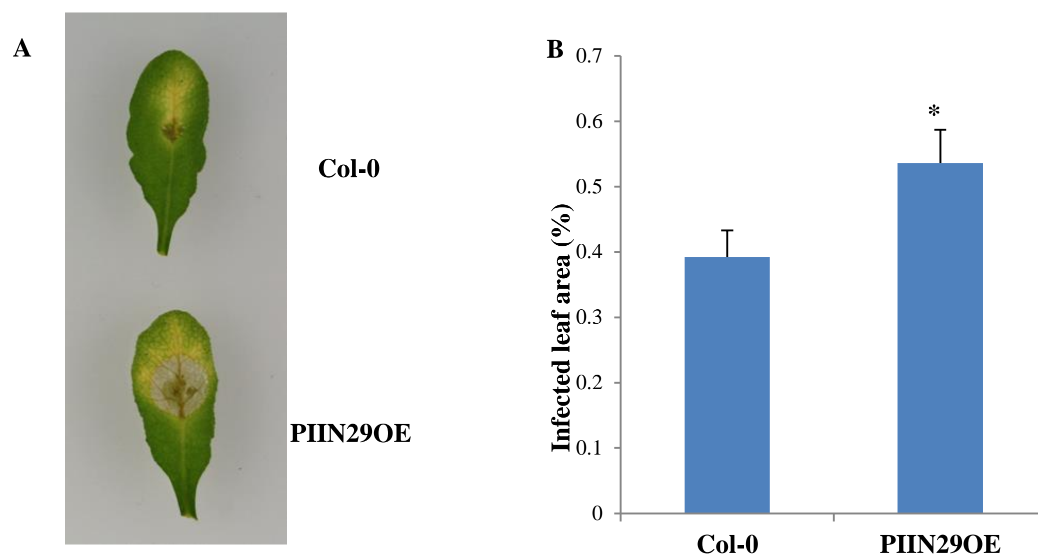


Figure 3.10 PIIN29 enhanced susceptibility to the necrotrophic pathogen *B. cinerea*. Infection symptoms on Arabidopsis leaves were followed inoculation with *B. cinerea*. A. Leaves were detached from six-week-old Arabidopsis and inoculated with 5 µl spore suspension (50,000 conidiospores/ml) on the middle vein for 3 days. B. Quantification of infected leaf area at 3 dpi, and the lesion size was measured by ImageJ. Data represent average ±SE of at least 30 leaves collected from 15 different plants of transgenic plant and wild-type. Three independent experiments showed similar result. The asterisks indicate significant differences in infection symptoms on transgenic plant compared with Col-0 at 3 dpi at $P<0.05$ (*) as analyzed by Student's test.

3.1.5 Suppression of basal defense by PIIN29

Pattern trigged immunity (PTI) summarized a strong immunity response against microbes that is triggered by different PAMPs (Jones and Dangl 2006). Successful microbes are able to suppress PTI by effectors for their survival. Since PIIN29 enhanced fungal colonization in Arabidopsis, mechanism of PIIN29 overcoming plant immunity system was an essential challenge for us.

3.1.5.1 PIIN29 suppresses flg22- and chitin-induced reactive oxygen species (ROS)

Transient generation of reactive oxygen species induced by PAMPs is a rapid signaling response, which depends on Rboh enzymes (respiratory burst oxidase homologs) (Torres and

Dangl 2005; Torres *et al*. 2005). Flg22, 22 aa peptide derived from bacterial flagellin, is one of best studied PAMPs.

3.1.5.1.1 PIIN29 suppresses flg22-trigged ROS in Arabidopsis roots

P. indica-colonized roots were almost completely suppressed ROS trigged by flg22 (Jacobs *et al*. 2011). Considering redundant *P. indica* effectors, it is necessary to verify the function of PIIN29 in basal defense such as flg22-trigged ROS. Three-week-old Arabidopsis roots were cut to 1cm fragment and inoculated in water overnight to reduce wound influence. After treated with 1.5 μM flg22, samples were monitored based on lumino assay by TECAN. Transgenic Arabidopsis PIIN29OE roots also completely abolished the oxidative burst trigged by flg22 comparing with Col-0 (Figure 3.11).

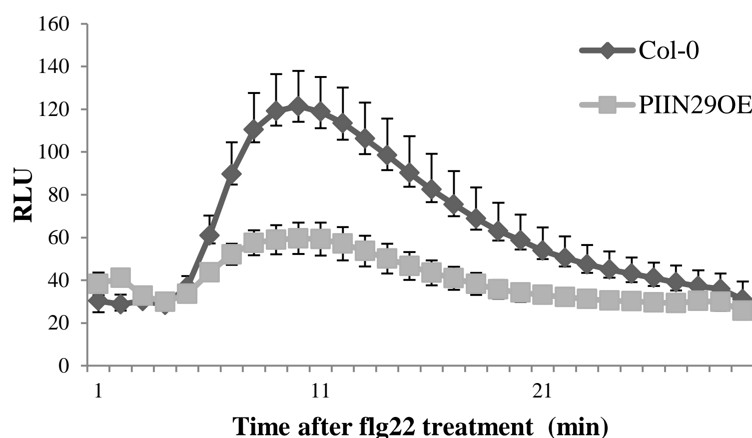


Figure 3.11 Suppression of flg22-trigged oxidative burst in root by PIIN29. Three-week-old Arabidopsis roots were treated with 1.5 μM flg22. Oxidative burst were measured in 10 mg root segments (1 cm each segment) by a luminol-based assay. Value were given as relative light unites (RLU) over time. Experiments were repeated three times with similar results. ±SE values are from four independent measurements per treatment one experiment.

3.1.5.1.2 PIIN29 suppresses flg22- and chitin-trigged ROS in Arabidopsis leaves

The benefit of stable transgenic plants is consistent expression PIIN29 in whole plant. Whether PIIN29 has similar function in other tissues beside roots, we need to investigate function of PIIN29 in leaves of transgenic Arabidopsis.

Five-week-old Arabidopsis, which grew in short time condition, were applied in this experiment. Leaves were cut to 25 mm^2 discs using punch and cultured in water overnight to

reduce wound influence. Leaf discs were treated with 100 nM flg22 or 10 mg/ml chitin, and monitored by lumino-based assay. It is clearly shown that PIIN29OE strongly suppressed chitin-induced ROS comparing with Col-0 (Figure 3.12 A), additionally completely abolished flg22 trigged ROS (Figure 3.12 B). It is represented that PIIN29 was able to suppress PAMP-trigged ROS in different tissues.

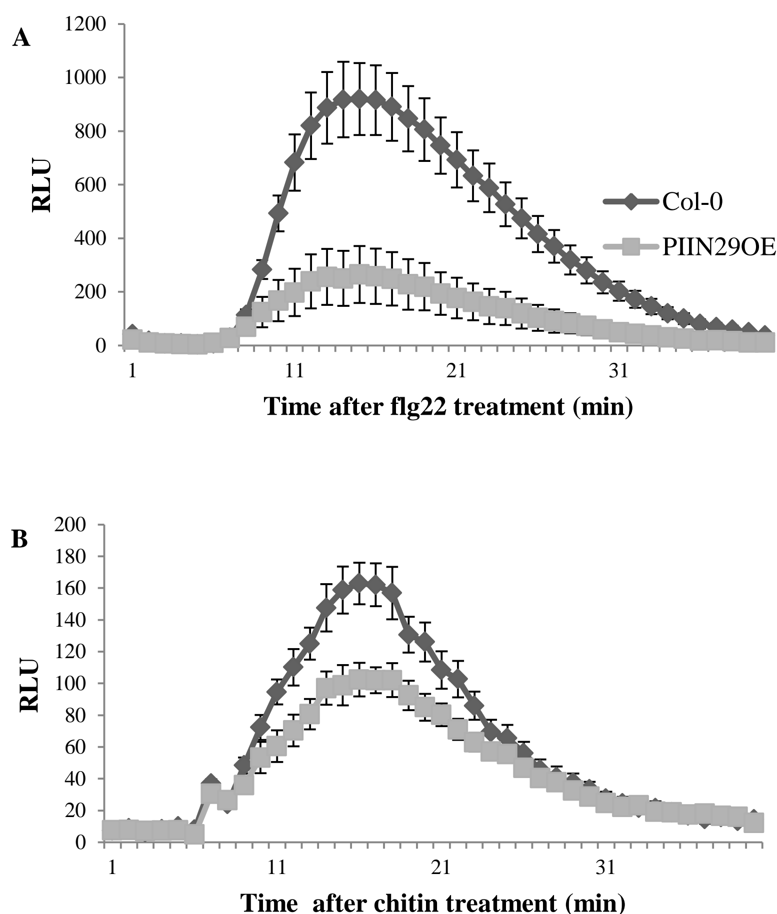


Figure 3.12 Suppression of flg22- and chitin-trigged oxidative burst in leaves by PIIN29. Five-week-old Arabidopsis leaves were treated with A. 10mg/ml chitin or B. 100 nM flg22, oxidative burst were given as relative light unites (RLU) over time. ±SE values are from 10 independent measurements per treatment one experiment. Experiments were repeated three times with similar results.

3.1.5.1.3 PIIN29 suppresses flg22-trigged ROS in barley leaves

The presented data showed that PIIN29 efficiently suppressed ROS trigged by flg22 or chitin in Arabidopsis leaves and roots. We subsequently wanted to elucidate PIIN29 had the same

function in monocot plant as barley. Five-week-old transgenic barleys were treated with 100 nM flg22 and measured relative light unites based lumino assay by TECAN. Interestingly, transgenic barley with PIIN29 also strongly suppressed flg22-trigged ROS (Figure 3.13) coincided with the transgenic Arabidopsis. PIIN29 was capable of efficient suppression of chitin or flg22-induced ROS in not only Arabidopsis roots and leaves but also barley leaves.

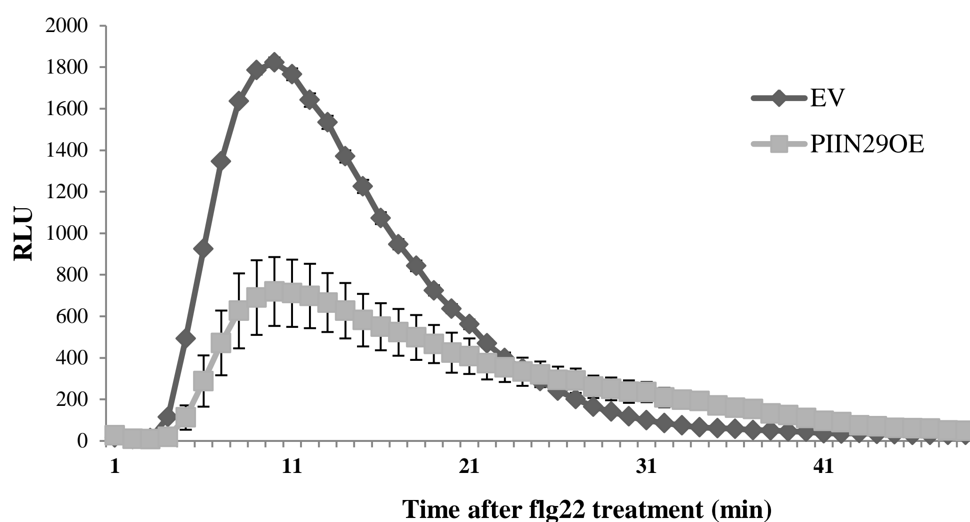


Figure 3.13 Suppression of flg22-trigged oxidative burst in barley leaves by PIIN29. Five-week-old transgenic barley leaves were treated with 100 nM flg22, oxidative burst were given as relative light unites (RLU) over time. ±SE values were from 10 independent measurements per treatment one experiment. Experiments were repeated three times with similar results. EV, transgenic barley with empty vector; PIIN29OE, transgenic barley expression of PIIN29.

3.1.5.2 PIIN29 suppresses expression of resistant marker gene

Facing microbes attack, plants are capable of extensive reprogramming of their network of various transcription factors (TFs). WRKY TFs are a large family of regulatory proteins forming such network in plant immunity (Eulgem and Somssich 2007). Arabidopsis WRKY22 (*AtWRKY22*) expression was markedly induced by H_2O_2, therefore *AtWRKY22* was involved in signal pathways in response to plant innate immunity.

Arabidopsis seedling were cultured in ½ MS containing 1% sucrose under short light for two weeks, and then treated with 100 nM flg22 for 0, 2, 6, 12 and 24 hours. We checked the expression level of *AtWRKY22* using 40ng cDNA by qPCR. Expression of PTI marker gene

as *WRKY22* reduced significantly after 2 hours treatment of 100 nM flg22 in PIIN29OE comparing with wild type Col-0 (Figure 3.14).

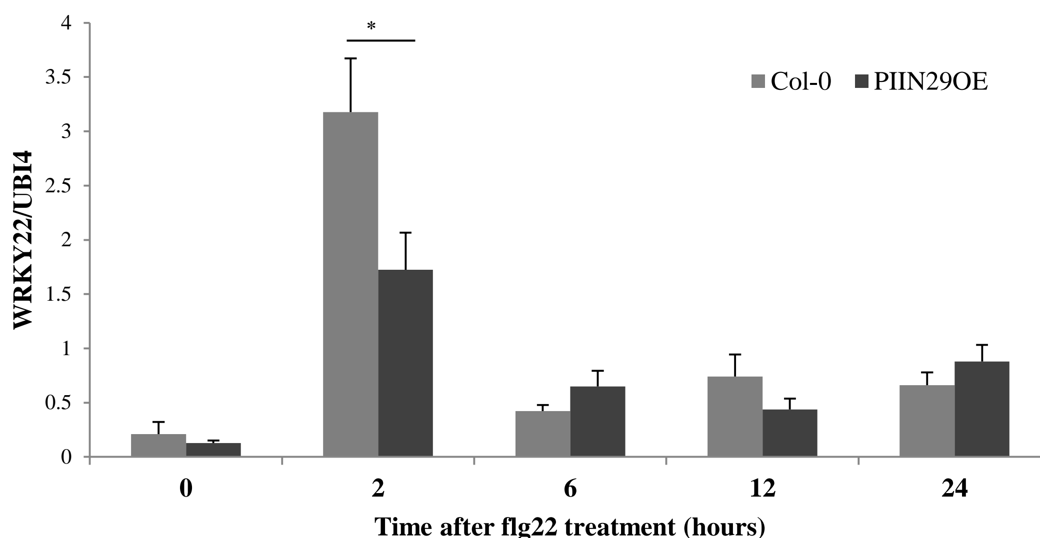


Figure 3.14 PIIN29 reduced *WRKY22* transcription trigged by flg22. Two-week-old Arabidopsis seedling were treated with 100 nM flg22 and harvested after 0, 2, 6, 12 and 24 hours for transcription of *WRKY22* using qRT-PCR. Expression values were calculated by the $2^{-\Delta Ct}$ method by relating Ct values of candidates to those of the housekeeping gene *AtUBQ-4*. Data presented show means of three independent experiments ±SE. Asterisks indicate significant differences of *AtWRYK22* expression of transgenic plant compared with Col-0 at $P<0.05$(*) as analyzed by Student's test.

3.1.5.3 PIIN29 suppressed flg22-induced callose deposition

Another flg22-induced late response of PTI in Arabidopsis is the callose deposition which is regulated by indole glucosinolates (IGs) (Clay *et al*. 2009). Two-week-old Arabidopsis seedlings were involved for checking callose deposition. PIIN29OE exhibited less callose accumulation than wild type staining with Aniline blue after treatment of 1 μM flg22 for 24 hours (Figure 3.15). In summary, as an effector, PIIN29 suppressed plant host a conserved set of PTI responses to enhance fungus colonization into plant host.

A

Col-0 PIIN29OE

B

Callose intensity

100 90 80 70 60 50 40 30 20 10 0

*

Col-0 PIIN29OE

Figure 3.15 PIIN29 suppresses callose deposition trigged by flg22 in Arabidopsis seedlings. Arabidopsis seedlings were cultured in ½ MS containing with 1% sucrose and treated with 1 µM flg22 for 24h, after which these seedlings were staining with Aniline blue. A. Callose deposition was detected by microscopy, Bar = 200 µm. B. Relative callose intensities were counted as the numbers of same area. ±SE values were from 8 independent measurements per treatment one experiment. Experiments were repeated three times with similar results. Asterisks indicate significant differences in the colonization of transgenic plant compared with Col-0 at P<0.01(**) as analyzed by Student's test.

3.1.6 PIIN29 is not involved in the MAP kinase pathway trigged by flg22

An important pathway of plant innate immunity is MAPK signal cascades which play an essential role in plant defense (Asai *et al*. 2002). Therefore we needed to investigate PIIN29

interacting with plant immunity involved in which pathway. Arabidopsis seedling were cultured in ½ MS medium containing 1% sucrose and treated with 100 nM flg22. And then harvest samples after 0, 15, 30, 60 and 120 min. Proteins of different time points were extracted with LUCAS buffer (containing proteinase inhibitor and phosphorylase inhibitor), and then 20 μg proteins were separated by 12% SDS-PAGE gel for western blotting. We applied immunoblot assays using the p44/42 antibody, raised against phosphorylated MAP kinases, for assessing the impact of PIIN29 on the MAPK activation by flg22. There is not clear difference of MAPK activity between PINN29OE and Col-0 (Figure 3.16). This data strongly supports that PIIN29 regulated PTI independent MAP kinase pathway.

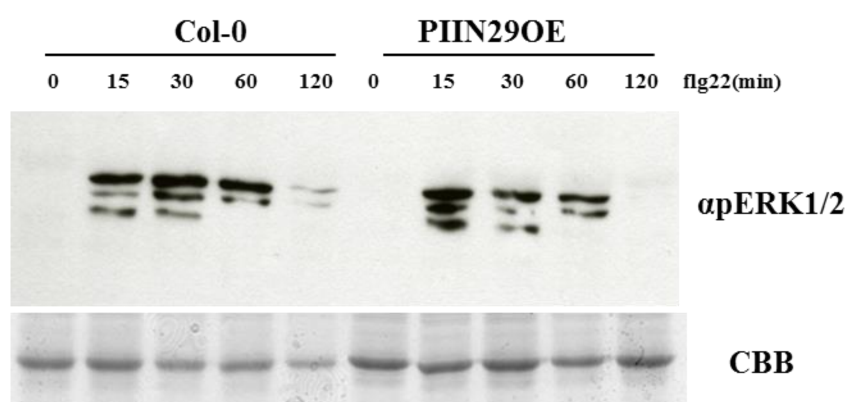


Figure 3.16 MAP kinase activation upon flg22 treatment in Arabidopsis seedlings of PIIN29OE and Col-0. Immunoblotting of phosphorylated MAP kinase was used two-week-old Arabidopsis seedlings which were collected at 0, 15 and 30 min after 100 nM flg22 treatment. Antibody raised against activated MAP kinase p44/p42 (αpERK1/2) was used for detection. Experiments were repeated three times with similar results. Coomassie brilliant blue (CBB) staining served as a loading control.

3.1.7 PIIN29 failed to abolish growth inhibition trigged by flg22

P. indica can abolish flg22-trigged growth inhibition and PTI in Arabidopsis root (Jacobs *et al.* 2011). Therefore, it is necessary for us to elucidate the function of PIIN29 in flg22-induced growth inhibition, since PIIN29 suppressed flg22-trigged PTI as *P. indica*. Five-day-old Arabidopsis seedlings were cultured in ½ MS medium in square petri-dishes and treated with 1 μM flg22 for 10 days. The root length of different treatments was measured every two days. PIIN29OE exhibited the similar inhibition trigged by flg22 as Col-0 (Figure

3.17). Based on this result, PIIN29 fails to abolish flg22-trigged growth inhibition in Arabidopsis as *P. indica*.

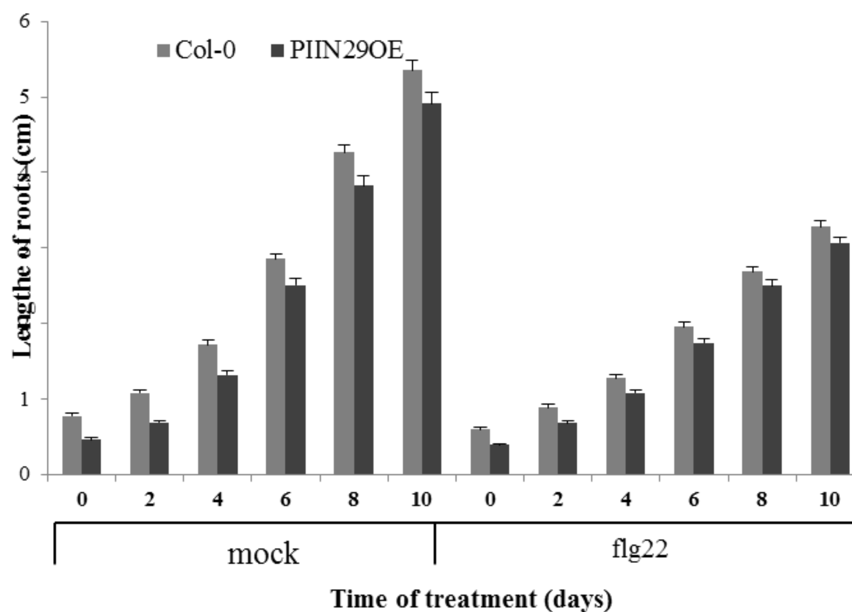


Figure 3.17 PIIN29 fails to abolish growth inhibition trigged by flg22. Five-day-old seedlings were treated by 1 µM flg22 for 10 days. ±SE values are from 25 independent measurements per treatment one experiment. Experiments were repeated two times with similar results.

3.2 Characterization of DELD effector candidates of *P. indica*

P. indica genomic sequence has support a wonderful opportunity for us to characterize the mechanism of suppression of innate immunity by *P. indica* (Zuccaro *et al.* 2011). About 10% of the genes induce during *P. indica* colonization of living barley root encoded putative small secreted proteins (SSPs). There are 543 secreted proteins that are considered effector candidates with predicated apoplastic function as cell wall hydrolysis (Rafiqi *et al.* 2013). A search for motifs in the amino acid sequences identified a group of 17 proteins with a highly conserved pattern of seven amino acids “RSIDELD” at the C-terminus (named as DELD) (Zuccaro *et al.* 2011). All DELD proteins have a similar size ranging between 101 and 135 aa with no known functional protein domain. To analyze the function and subcellular localization of DELDs of *P. indica*, we transiently expressed these effector candidates into plant leaves by agroinfiltration or type III secret system (T3SS) -mediated infiltration.

3.2.1 Subcellular localization of DELD effector candidates of *P. indica* using agroinfiltraion

Golden gate cloning is based on the use of type IIs restriction endonucleases (REases), which cut outside of their recognition sequence. Therefore it is allowed fusion of different fragments without leaving site-specific recombination sequences in the final construct genes of interest (Engler *et al.* 2008). Generated constructs were able to elucidate subcellular localization and function of different DELD candidates using agroinfiltration (Figure 3.18). Golden gate cloning strategy allows the cloning of 35S promoter, GFP, effectors (without native signal peptide) and Nos terminal into the plant expression vector in one reaction. Because the kanamycin resistance is more stable in agrobacterium GV3101, we need clone whole cassette into expression vector pICH86966.

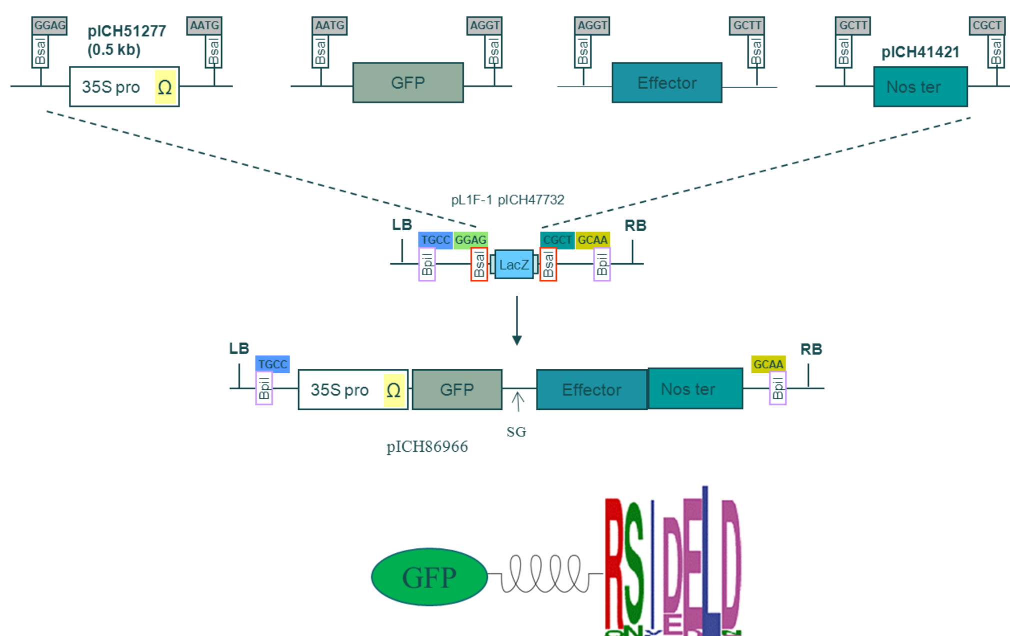


Figure 3.18 Cloning strategy of golden gate construction. PCR Fragment of GFP and effector candidates with BsaI enzyme site plus 4 bp overhang sequences in a bin were used throughout a combinatorial bin. The fragment of CaMV35S promoter and Nos terminal were offered from plasmid pICH51277 and pICH41421, respectively. All these fragments were combined together with destination vector pICH47732 through golden gate. There was two amino acid (S and G) adding

between GFP and effectors as linker for flexibility. And then get the whole cassette containing promote and terminal using PCR for ligation with expression pICH86966 by enzyme XmaI.

Based on *in silico* analysis, cDNA sequence without signal peptide of the candidates PIIN_10147, PIIN_09226, PIIN_06837, PIIN_05865 and PIIN_05851 were cloned in expression vector pICH86966. These candidates fusion with GFP at N-terminal were controlled by CaMV35s promoter, and transformed into agrobacterium GV3101 competent cell. For subcellular localization study, five-week-old *N. benthamiana* leaves were used for transient expression of these candidates by agroinfiltration, and then checked GFP fluorescence signal of these candidates by confocal microscopy after 48-72 hours treatment. The candidate -PIIN_06837 showed the GFP fluorescence signal associated with plasma membrane (Figure 3.19). Unfortunately, we did not get clear GFP fluorescence signal of the other effector candidates.

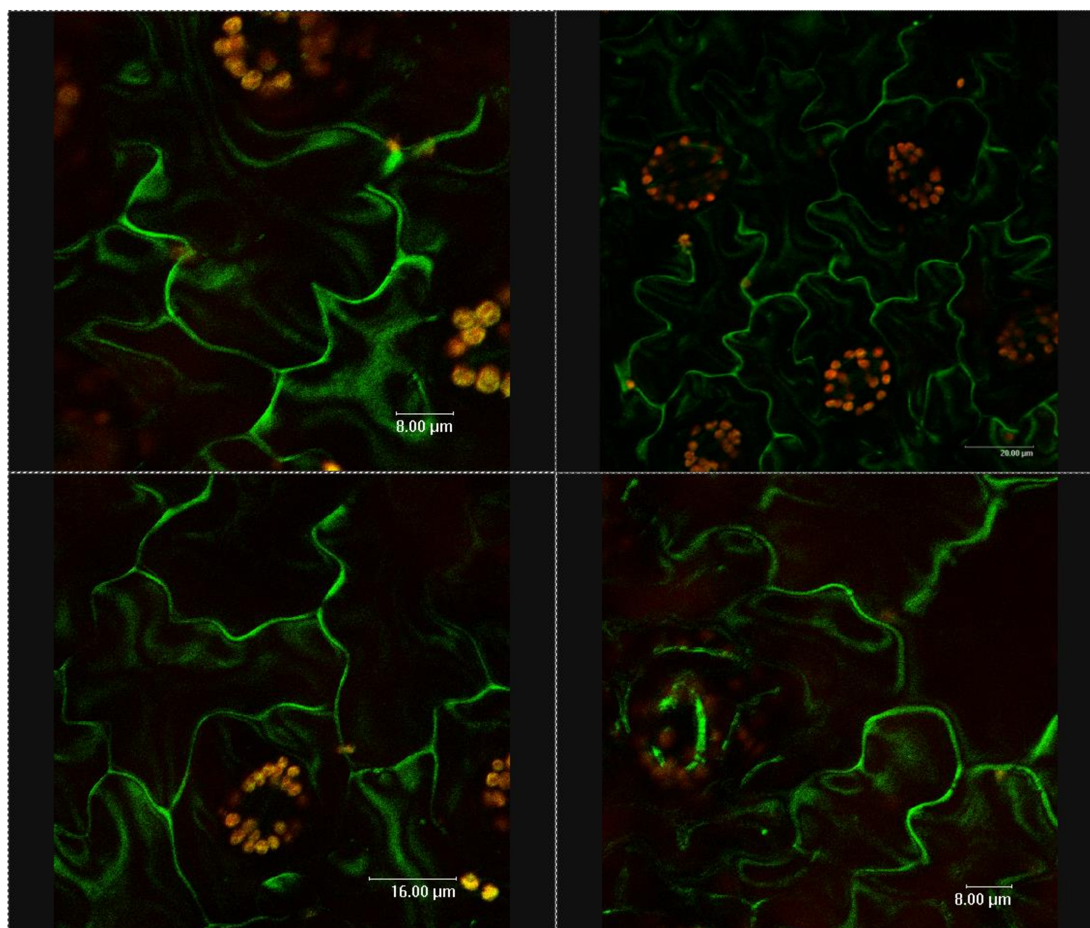


Figure 3.19 Subcellular localization of PIIN_06837 with plasma membrane. Five-week-old *N. benthamiana* leaves were infiltrated with PIIN_06837 using a needleless syringe. Confocal laser scanning microscopy image of *N. benthamiana* leaves epidermal cells 48 hours after infiltration.

3.2.2 Functional analysis of DELD effector candidates of *P. indica* using *P. fluorescens* EtHAn mediated infiltration

The DELD candidates of GFP fusion constructions were unstable in *N. benthamiana* leaves by *Agrobacterium* assay. We therefore have adapted an alternative assay based on effector protein delivery using the T3SS of a non-pathogenic *Pseudomonas fluorescens* strain Effector-to-Host Analyzer (EtHAn) (Upadhyaya *et al.* 2014). The pENTR™ Directional TOPO® Cloning Kit (Invitrogen) was utilized to quickly and directionally clone a blunt-end PCR product into a vector pENTR™/D-TOPO for entry into the Gateway® System. I designed primers to amplify different DELD candidates without their native signal peptides. The primers included the additional sequence CACCATG at the 5' terminal to allow directional cloning. The 3' primers deleted the native stop codon to allow for fusion with HA tag. The destination vector pNR526-G2AC3A contains the promoter (189 nt) plus first 267 nt of coding region (1 to 89 aa) of the *Pseudomonas syringae AvrRPM1* gene which was mutated at the N-terminal of AvrRPM1 (Gly at position 2 and Cys at position 3 to Ala) to abolish plasma membrane targeting of the delivered protein, and an HA tag flanking the reading frame *attR1-ccdB-cm^R-attR2* cassette (information of vector and primer listed in supplement). Expression vectors were generated by performing LR Clonase reactions (Invitrogen, Gateway® LR Clonase® II enzyme mix) with candidate effector entry clones and the AvrRPM1 T3SS destination vector (Figure 3.20). Expression vectors were transformed into engineered *P. fluorescens* strain EtHAn by electroporation. The positive transformants were confirmed by colony PCR, and then infiltrated into *N. tabacum* leaves with needleless syringe.

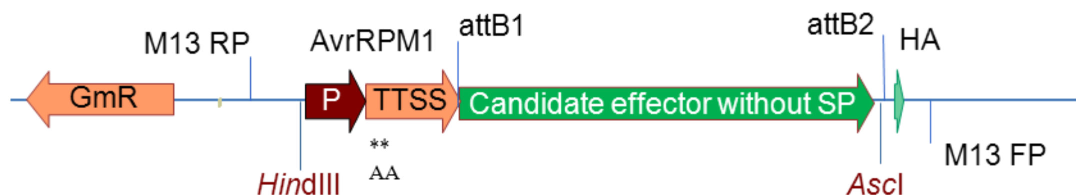


Figure 3.20 Schematic representation of destination vectors pNR526-G2AC3A (taken from Upadhyaya *et al.* 2014). The destination vector have the broad host-rang vector pBBR 1MCS-5 (Kovach *et al.* 1995; Sohn *et al.* 2007) as the backbone having gentamycin resistance gene as the selectable marker. The *attR1-ccdB-cmR-attR2* cassette was performing LR Clonase reactions. There were two site mutants at the N-terminal of AvrRpm1 (G2 to A and C3 to A) to abolish plasma membrane targeting of the delivered protein.

As a positive control to confirm effector delivery by this bacterial system, I used the flax rust AvrM protein which is recognized by the corresponding flax M resistance protein in a gene-for-gene manner (Catanzariti *et al.* 2006). To confirm that AvrM delivery could induce a HR, *P. fluorescens* EtHAn containing the AvrM construct was infiltrated into transgenic tobacco expression the M gene and wild type tobacco. No HR was observed when *P. fluorescens* EtHAn containing the AvrM was infiltrated into wild type tobacco. However, HR response was strongly induced by *P. fluorescens* EtHAn-mediated transient expression of AvrM by T3SS (Figure 3.21). The construct of YFP venus did not induce any HR in M expression tobacco nor in wild type tobacco. Importantly, a HR also was induced by DELD effector PIIN_05098. The HR induced by this effector candidate was seen in both M tobacco and in wild type tobacco.

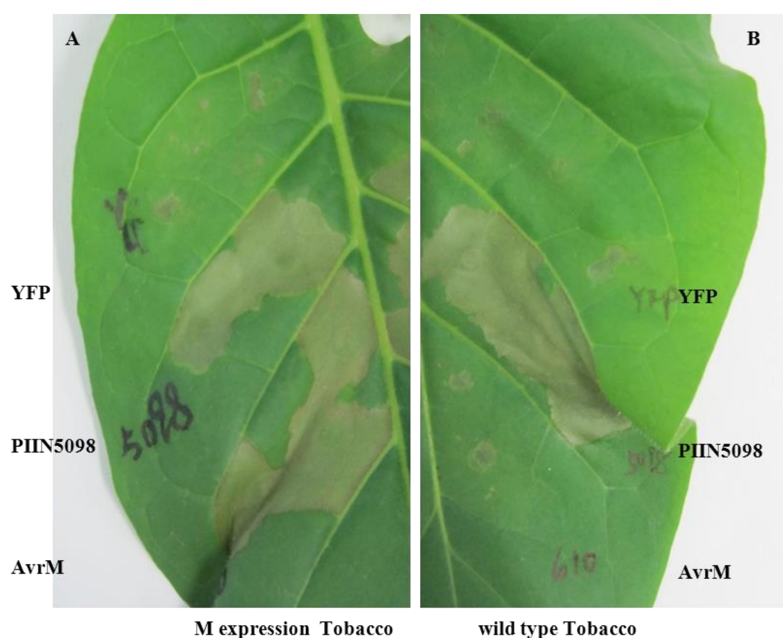


Figure 3.21 Efficient delivery effector to tobacco leaves by *P. fluorescens* EtHAn. Delivery of AvrM, PIIN_05098 and YFP venus constructions into M transgenic tobacco (A) and wild type tobacco (B) leaves by T3SS system after 2 dpi. Tobacco (*N. tabacum*) leaves were infiltrated with *P. fluorescens* EtHAn containing different constructions.

The other three DELDs candidates- PIIN_05851, PIIN_09689 and PIIN_07579 also induced significant cell death in wild type *N. tabacum* leaves after 24 hours (Figure 3.22).

Figure 3.22 HR induced by three DELD effector candidates. Wild type tobacco was infiltrated with *P. fluorescens* EtHAn carrying DELD effector candidates PIIN_05851, PIIN_09689, and PIIN_07579 after 2dpi.

3.3 Cytology of *P. indica*'s infection structure using transmission electron microscopy (TEM)

To reveal specialized biotrophic fungal structure formed by *P. indica* inside infected plant root cells, wild type barley *Golden Promise* roots were inoculated with spores of *P. indica* for 3 days, and prepared as ultra-thin sections for TEM. Our preliminary results show that *P. indica* differentiates biotrophic hyphal bodies that penetrate into barley root cytoplasm (Figure 3.23). It is the first time to character the infection structure of barley's roots colonized by *P.indica* using TEM.

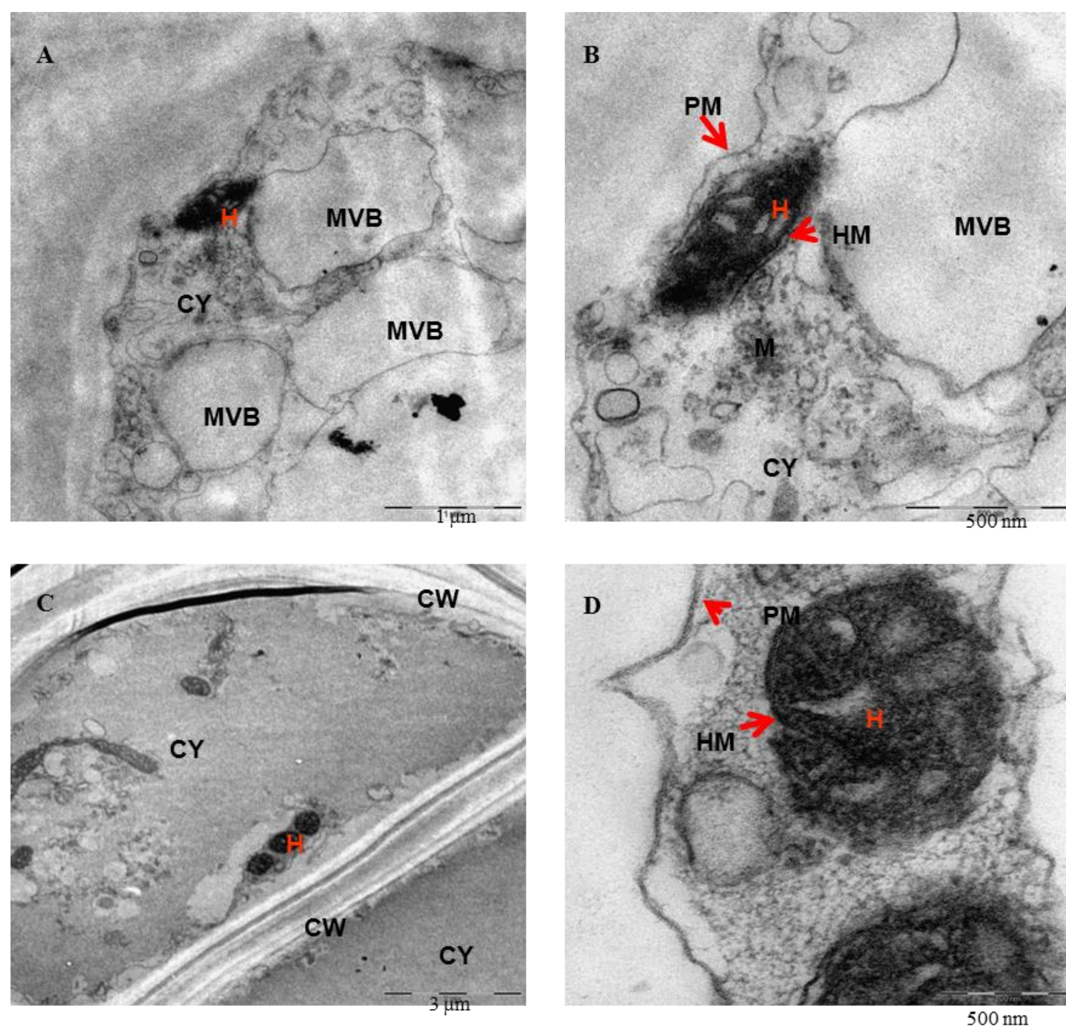


Figure 3.23 Transmission electron micrographs of barley root colonized by *P. indica* at 3 dpi. (A-D) *P. indica* hypha penetrated into host cytoplasm. CW-root cell wall, CY-cytosol, H-hypha, V-vacuole, PM-plasma membrane, HM-hyphal membrane, PM-plasma membrane, MVB-multivesicular bodies.

4. Discussion

As development of molecular biology, more and more genomic DNA of fungi is finished sequence. It is possible for us to characterize interaction of fungi with plant hosts on molecular level. The broad-spectrum root-colonized endophytic *P. indica* confers various beneficial effects to host plants, such as growth promotion, seed yield increase, abiotic stress tolerance, and biotic stress resistance (Peškan-Berghöfer *et al.* 2004; Sherameti *et al.* 2005; Waller *et al.* 2005; Jacobs *et al.* 2011; Das *et al.* 2012). Plants have developed a complex defensive response system to protect themselves against invasion by detrimental organism. In contrast, invading organisms have adopted various methods to circumvent the plant's defense or control plant cell function for their benefit. Like pathogenic bacterial, mutualistic fungi affect plant immune response to achieve colonization (Liu *et al.* 2007; Jacobs *et al.* 2011), although there is little knowledge of the effectors in mutualistic fungi (Kloppholz *et al.* 2011; Plett *et al.* 2011; Plett *et al.* 2014a; Plett *et al.* 2014b).

4.1 Functional analysis of effector candidate PIIN29

Reversible protein conjugation with ubiquitin, or ubiquitination, is a key regulatory mechanism that controls a variety of cellular processes in eukaryotic cells, including DNA repair, gene transcription, protein activation or receptor trafficking, although the best characterized function of ubiquitin involves selective protein degradation through the 26S proteasome (Vierstra 2009). Therefore, it has become increasingly evident that E3 ubiquitin-ligase proteins play important roles in the regulation of immune signaling such as microbial effectors that either target host E3 ubiquitin ligases or act as ubiquitin ligase inside plant cells. Recently, there are some microbial effectors exhibiting E3 ubiquitin ligase activity involved in establishment of plant immune responses to pathogen attack to promote disease. For example, the AvrPiz-t from the rice blast fungus *Magnaporthe oryzae* is translocated into rice cells, where it is able to inhibit the ubiquitin ligase activity of APIP6 for suppression of PAMP-induced ROS production, inducing susceptibility to *M. oryzae* (Park *et al.* 2012). Moreover, the effector AVR3a from the oomycete *Phytophthora infestans*, targets and stabilizes the U-box-type ubiquitin ligase CMPG1, prevents development t of cell death induced by *P. infestans* elicitin NF1 (Bos *et al.* 2010). In addition to microbial effectors that

are able to target host E3 ubiquitin ligase proteins, effectors that present E3 ubiquitin ligase-related domains have also been reported in various pathogenic microbes (Marino *et al.* 2012). However, the best characterized microbial E3 ubiquitin ligase is the AvrPtoB from *Pseudomonas syringae* that presents a C-terminal domain with remarkable structural homology with RING-and U-box-type ubiquitin ligases (Janjusevic *et al.* 2006), which is an effector able to inhibit PCD. One effector candidate, PIIN29, contains a signal peptide and high conserved C-terminal RING figure domain (Figure 3.1). 3D protein modeling analysis confirms the structure overlap and exhibits accurate conformation of the E2 binding residues compared to known plant and bacterial ubiquitin ligase (Figure 3.4). Combining with *in vitro* assay, PIIN29 possesses E3 ubiquitin ligase activity (Khatabi 2009). It is the first characterized mutualistic fungus effector with E3 ubiquitin ligase activity.

The transcription level of *PIIN29* is qualified by real time PCR in different conditions. When colonized with living Arabidopsis roots, transcription level of *PIIN29* was gradually increased and significantly high compared with cultured in liquid culture medium (Figure 3.5). This result suggests that PIIN29, as an effect candidate, is induced during symbiosis.

As a mutualistic fungus, *P. indica* actives several molecular and biochemical processes during host colonization. *P. indica* efficiently suppresses the immune response trigged by various PAMP as flg22, chitin and elf18 (Jacobs *et al.* 2011) (Figure 4.1). The function of effector is that suppressed host immune response for microbe survival. Therefore, we are focus on the interaction of PIIN29 which exhibited E3 ubiquitin ligase activity with host plant.

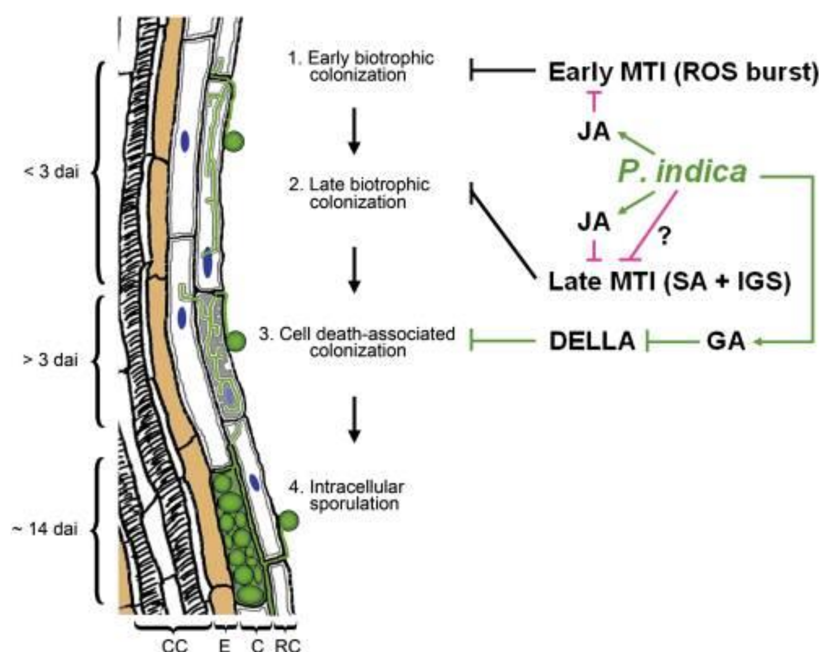


Figure 4.1 Model of the spatiotemporal colonization pattern of Arabidopsis roots (taken from Jacobs *et al.* 2011). Root colonization by *P. indica* can be divided into four stages. After germination of the spores and extracellular growth, hyphae penetrate epidermal or cortical cells and establish an early biotrophic colonization phase. Biotrophic stages can be preceded by intercellular colonization (nucleus, blue; plasma membrane, dark gray lines inside cells). Biotrophically colonized cells die (light gray filling of cells) during subsequent cell death-associated colonization. Intracellular sporulation takes place in epidermal and cortical cells at about 14 dpi (CC, Central cylinder; E, endodermis; C, cortex; RC, rhizodermal cells). PTI is restricting root colonization by *P. indica* from early through late interaction stages. The fungus achieves biotrophic root colonization by the suppression of early PTI. SA-mediated defense and antimicrobial indole glucosinolates (IGS) participate in PTI. *P. indica* recruits JA to suppress ROS. *P. indica* might further induce GA signaling to achieve DELLA protein degradation, thereby elevating the proapoptotic threshold in root cells and initiating cell death-associated colonization.

Stable transgenic barley and Arabidopsis which expresses of PIIN29 drived under consistent CaMV35S promoter are constructed to clarify function of PIIN29. As a model plant, Arabidopsis has clear background for studying function of PIIN29. In classic plant immunity system, success microbes overcome host PTI and induce ETS by secreting effectors (Jones

and Dangl 2006). We demonstrated that transgenic Arabidopsis expressing PIIN29 illuminated susceptibility of necrotrophic fungus in leaves and increased colonization of *P. indica* in roots (Figure 3.9, 3.10). This implies that PIIN29 alters Arabidopsis process to induce ETS independent of host. It is speculated that PIIN29 function reflects an evolutionary adaptation to protein substrates of unspecific hosts.

Pseudomonas type III effector AvrPtoB, as model effector with function of E3 ubiquitin ligase, induces plant disease susceptibility by inhibition of host programmed cell death (Abramovitch *et al.* 2003; Abramovitch *et al.* 2006). PIIN29 exhibits suppression flg22 or chitin-induced ROS in roots and leaves (Figure 3.11, 3.12). This result represents that PIIN29 depresses ROS similar as *P. indica*. We choose flg22 for our research as it represents the best-studied PAMP and thus allows more detailed comparison of *P. indica*. PIIN29 effectively counteracts immune signaling, as seen by the abolishment of conserved PTI response as oxidative burst (Figure 3.11, 3.12), callose deposition (Figure 3.14) and response related gene expression (Figure 3.13). The suppression of flg22-induced *AtWRYK22* transcription in transgenic Arabidopsis seedlings independent of MAPK-mediated signal pathway (Figure 3.16) suggests that this effector targets transcription of defense related genes in nucleus. It is consistent with subcellular localization of nucleus by agroinfiltration (Pena, 2013). In addition, PIIN29 can't abolish growth inhibition trigged by flg22 (Figure 3.17). Considering Arabidopsis seedlings of PIIN29OE are smaller than wild type Col-0 (Figure 4.2), PIIN29 with function of E3 ubiquitin ligase can degrade some related defense proteins to overcome host PTI. All these data represent that suppression of PTI response by *P. indica* is different with the activity showed by PIIN29.

Col-0

PIIN29OE

Figure 4.2 Transgenic Arabidopsis seedlings PIIN29OE are smaller than wild type. Four-week-old Arabidopsis were cultured in short light condition.

Based on presented data, we propose an interaction model of PIIN29 with plant host (Figure 4.3). *P. indica* penetrates plant cell to delivery cytoplasmic effectors. As one of this kind effectors, PIIN29 with function of E3 ubiquitin ligase is able to suppress host PTI response as ROS, callose deposition through down-regulating some defense genes as *AtWRYK22* transcription in nucleus and degrading some related immune proteins. Unlike well characterized effector AvrPtoB, PIIN29 fails to abolish MAPK activity.

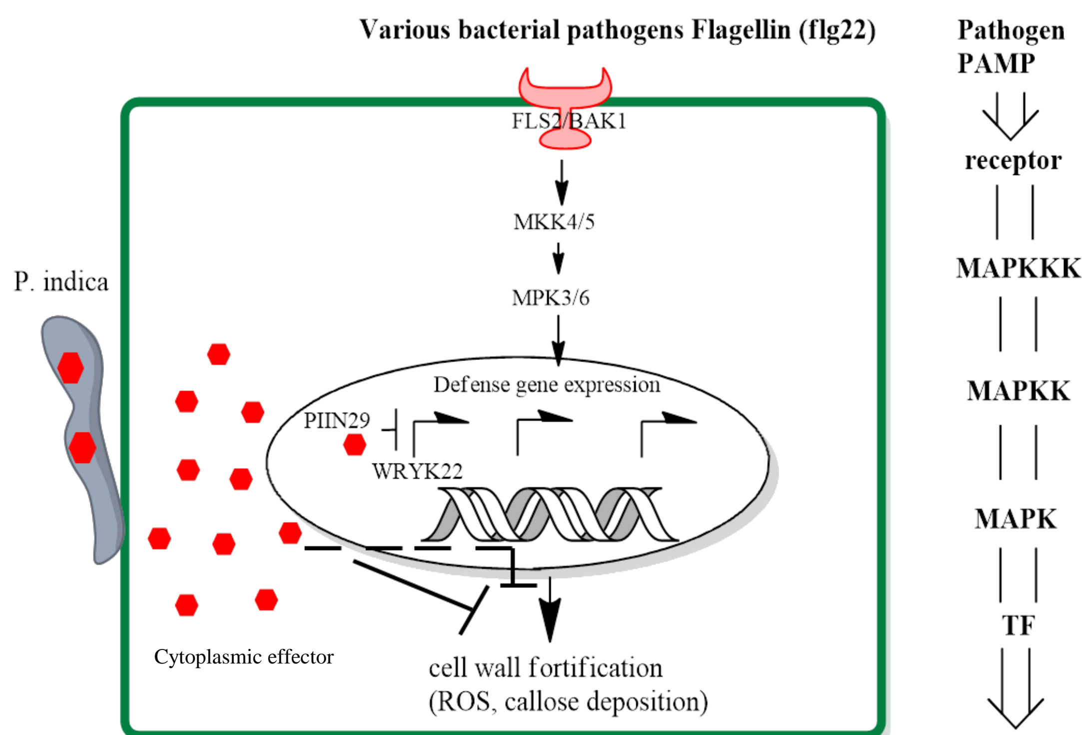


Figure 4.3 Putative mechanism of suppression of PTI by PIIN29. *P. indica* (brown outside of plant cell) colonized plant host and secreted some cytoplasmic effectors into plant cell (small red particle). One effector is PIIN29 which be localized into nucleus to suppress transcription of defense genes as *WRKY22* trigged by flg22. Therefore the later stage of immune response, ROS and callose deposition, are suppressed by this effector. However, PIIN29 failed to suppress the upstream MAPK-mediate signal pathway.

Taken together, our results give the first mechanistic insight into how an effector of *P. indica* "negotiates" a symbiosis relationship by altering host cell process.

4.2 Functional analysis of one group of effector candidates belonging to DELD family

Almost the knowledge of effector function is obtained from phytopathogenic microbes such as reprogramming plant defense and cell metabolism (Dodds *et al.* 2009; Hogenhout *et al.* 2009; Stergiopoulos and de Wit 2009). Recently it has been shown that effector proteins exist also in mutualistic fungi (Kloppholz *et al.* 2011; Plett *et al.* 2011; Plett *et al.* 2014a; Plett *et al.* 2014b). About 10% of the genes induced during *P. indica* colonization of living barley roots encoded putative small secreted proteins (SSPs <300 aa) (Zuccaro *et al.* 2011), in which 17 proteins with a highly conserved pattern of seven amino acids "RSIDELD" at the C-terminus (named DELD). All DELDs are less than 135 aa in size and with no known functional protein domain. Interestingly, the other mutualistic fungi, *L. bicolor* possess a similar DELD motif at the C-terminus but lack a high content of histidines. Secondary structural prediction shows that the DELD proteins most probably form a two-helix bundle interrupted by a central conserved glycine residue. For investigating the function of these special proteins, we transiently express DELDs using agrobacterium and Pseudomonas mediated infiltration. Only one effector-PIIN_06837 showed subcellular localization of plasma membrane, however it failed to confirm the other effector candidates through agroinfiltration (Figure 3.19). It is represented that *P. indica* secreted abound effectors during symbiosis, only litter effectors were stable and functional in host cell. One more efficient method for transient expression protein in plant is T3SS of bacterial infiltration (Upadhyaya *et al*. 2014). Four of DELD candidates as PIIN_05098, PIIN_07519, PIIN_09689, PIIN_05851, fusion with HA using T3SS-media infiltration, are able to induce cell death in tobacco leaves (Figure 3.21, 3.22). However, it failed to obtain immunoblotting signal of HA in these infiltrations, maybe there is too less expression of HA-tag based on only one HA fusion. Since the mutualistic fungus *P. indica* adopts two stages of colonization, biotrophic colonization followed by cell death-associated colonization phage through ER stress (Qiang *et al.* 2012; Lahrmann *et al.* 2013). We proposed that some DELD effector candidates are involved in that latter stage to induce cell death for *P. indica* living.

4.3 New insights into the cytology of *P. indica*'s infection structure

Membranes are a central feature of life, which allow the interior of cell to establish controlled conditions separated from the environment to provide optimal conditions for biochemical processes. In endosymbiosis, two organisms cooperated closely through a membrane and cell wall for initial recognition to the establishment of the symbiotic interface and nutrients exchange. Our preliminary results show that *P. indica* differentiates biotrophic hyphal bodies that penetrate into barley cytoplasm to investigate using TEM (Figure 3.23). These structures are similar to specialized fungal structures formed in other fungal systems (Islam *et al.* 2009; Kottke *et al.* 2010).

4.4 Future perspectives

A). Confirm secretion of PIIN29 during colonization. We have constructed plasmid containing full length sequence including intron and exon of PIIN29 (1920 bp) and mcherry tag. This cassette is controlled by the promoter pHSP70 and transformed into wild type *P. indica*. Therefore, it is possible for us to investigate the localization of PIIN29 during symbiosis.

B). Construction of knockout PIIN29 mutant through *P. indica* transformation characterizes the function PIIN29. Because silencing PIIN29 in *P. indica* can only reduce expression of this gene, it is unclear to show the function of PIIN29.

C). Screening target protein of PIIN29 in plant by yeast two-hybrid (Y2H), and then revealing interaction of this gene and target by Co-Immunoprecipitation (Co-IP).

5. Summary/Zusammenfassung

5.1 Summary

Although plants are exposed to a wide range of microorganisms in nature, they adapt their immune systems to allow infection only by limited numbers of adapted pathogens. However, ‘smart’ microbes determine the outcome of plant-microbe interactions to overcome plant immunity. During this co-evolution, there is one group of special proteins, effectors secreted by microorganisms, playing important roles in immune response during infection. *Piriformospora indica,* a mutualistic root-colonizing basidiomycete, promotes biomass formation and plant health in a wide range of host plants. The fungus colonizes plant roots without causing any visible disease symptoms and thus represents a genetically accessible model to study the molecular basis of processes associated with fungal accommodation and the establishment of root symbioses. It is recently reported that *P. indica* has developed efficient colonization strategies to suppress host PTI. Genome sequencing of *P. indica* has identified many effector candidates, so the challenge is to characterize “mutualistic” effector functions.

Structural and functional analysis of PIIN29 showed that it fullfils the definition of a fungal effector candidate, and possesses E3 ubiquitin ligase activity *in vitro*. In order to establish PIIN29 as a mutualistic effector that controls the establishment and/or maintenance of the symbiotic relationship, it is necessary to prove these three points: First, it is induced by the presence of a plant root. Second, it is necessary for *P. indica* symbiosis. Third, it alters functioning of the plant cell.

The transcription level of *PIIN29* is induced during Arabidopsis root colonized by *P. indica* as shown by real time PCR in different conditions. Heterologous expression of mature version of PIIN29 (without its native signal peptide) driven by CaMV35S promoter in Arabidopsis results in higher colonization rates of *P. indica* and pathogen of *B. cinerea* compared with wild type Col-0. Moreover, PIIN29OE inhibits the production of ROS in response to both flg22 and chitin, decreased transcription of defense genes such as *AtWRKY22*, and abolished callose deposition elicited by flg22. In summary, PIIN29 is one of the first characterized mutualistic effector, which suppresses a conserved set of PTI responses in plant tissues.

Cytological studies in Arabidopsis and barley have shown that *P. indica* has a biphasic colonization strategy. To investigate the cytology of *P. indica* during symbiosis, the result of TEM represented that *P. indica* differentiates biotrophic hyphal bodies penetrated into barley root cytoplasm.

Based on *in silico* analysis, 17 SSPs containing the conserved C-terminal motif RSIDELD are involved in interested effector candidates. Using agroinfiltration, we identify the subcellular localization of PIIN_06837 as plasma membrane. In addition, we reveal that four effectors, PIIN_05098/05851/09689/07519, induce cell death in *N. tabacum* leaves through T3SS media infiltration.

The results of this work can be used as base for the characterization of further *P. indica* secreted proteins.

5.2 Zusammenfassung

Obwohl Pflanzen in der Natur einer Vielzahl von Mikroorganismen ausgesetzt sind, passen sie ihr Immunsystem an, dass nur eine begrenzte Anzahl von adaptierten Krankheitserregern Pflanzen infizieren. Nur die „smarten“ Mikroorganismen sind dazu fähig das Ergebnis der Mikroben-Pflanzen Interaktion zu ihren Gunsten zu bestimmen und die pflanzliche Immunität zu überwinden. Während dieser Co-Evolution, gibt es eine Gruppe von speziellen Proteinen, Effektor die von Mikroorganismen sekretiert werden, die eine wichtige Rolle bei der Unterdrückung der Immunanwort spielen.

Piriformospora indica, ein mutualistischer Wurzeln besiedelnder Basidiomycet, fördert die Bildung von Biomasse und das pflanzliche Immunsystem in einer Vielzahl von Wirtspflanzen. Der Pilz besiedelt Pflanzenwurzeln ohne das irgendwelche Krankheitssymptome sichtbar. Daher stellt diese Pflanzen-Pilz Interaktion ein Modell dar, welches für die molekulare Analyse für Wurzel etablierter Symbiosen genutzt werden kann. Vor kurzem wurde berichtet, dass *P. indica* effiziente Besiedelungsstrategien entwickelt hat, um die Wirtsabwehr (MTI) zu unterdrücken. Eine Mikroarray basierte Studie hat gezeigt, dass *P. Indica* bei der Unterdrückung der pflanzlichen Abwehr, den GA Stoffwechsel wesentlich beeinträchtigt. Dennoch zeigen dir Transkriptionsdaten eine erhöhte Expression von Verteidigungsgenen. Die Sequenzierung von *P. indica* hat viele Effektor-Kandidaten identifiziert. Nun liegt die

Herausforderung darin, Effektor-Proteine zu charakterisieren, die für die mutualistische Interaktion verantwortlich sind. Struktur- und Funktionsanalysen zeigen, dass PIIN29 die Definition eines pilzlichen Effektors erfüllt und eine E3-Ubiqutin-Ligase-Aktivität in vitro besitzt. Um PIIN29 als mutualistischen Effektor, der die Herstellung und Aufrichterhaltung der Symbiose kontrolliert, zu etablieren, mussten folgende Punkte überprüft werden: Erstens, ist dieses Protein induziert in Anwesenheit einer Pflanzenwurzel. Zweitens, wird es gebraucht bei der *P. indica*-Pflanzen Interaktion. Drittens, hat es einen Einfluss auf die zellulären Pflanzenfunktionen. Das PIIN29 Effektor-Protein ist exprimiert, wenn Arabidopsis Wurzeln von *P. indica* besiedelt sind. Dies konnte durch quantitative-PCR Analyse Methoden in unterschiedlichen Bedingungen gezeigt werden. Die heterologe Expression von PIIN29 (ohne Signalpeptid) mit dem starken Promotor CaMV35s in Arabidopsis resultiert in erhöhte Kolonisationsraten von *P. indica* und B. cinerea verglichen mit wildtyp Pflanzen. Außerdem induziert die Überproduktion von PIIN29 eine Hemmung von ROS nach Zugabe von flg22 und Chitin, eine Verminderung der Expression des Transkriptionsfaktors *AtWRKY22* und verhindert Callose Depositon nach flg22 Behandlung. Zusammenfassend, PIIN29 ist der erste mutualistische Effektor, der konservierte PTI Reaktionen in pflanzlichen Gewebe unterdrückt. Zytologische Untersuchung in Arabidopsis und Gerste haben gezeigt, dass *P. indica* eine zweiphasige Besiedelungsstrategie hat. Um die Zytologie von *P. indica* in Symbiose zu untersuchen, konnten TEM Ergebnisse zeigen, dass *P. indica* mit biotrophischen Hyphen in Zytoplasma von Gerstenwurzeln penetriert. In silico Analysen von 17 Effektorkandidaten beinhalten das konservierte C-terminale Motiv RSIDELD von SSPs. Mit Agroinflitration konnten wir die subzelluläre Lokalisation von PIIN_06837 als Plasmamembran zeigen. Zusätzlich, konnten wir mit T3SS media Infiltration zeigen, dass vier weiter Effektorproteine PIIN_05098/05851/09689/07519 Zelltod in N. tobacum induzieren.

Die Ergebnisse dieser Arbeit können als Basis genutzt werden, für die weitere Charakterisierung von *P. indica* sekretierten Proteinen.

6. References

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7. Supplement

7.1 Sequence information of PIIN29

7.1 The full length cDNA and amino acid sequence of PIIN29. It consists of 1590 nucleotides which encodes 529 amino acids. The putative signal peptide and RING domain E3 ubiquitin ligase were shown as Italic and underline respectively.

ATGGGCAGATATTCATTGGCAGCGCAGGCCATCTGCCTCTTATCGAGCATACACTC
TGCTCTCGCATATATTCCAGCTAGGGCTGCAAACATTAGCCAGGGTCTCGGCCTC
GACGTCCACGACAATTCCAAGGTCACCCTGACATGGAACCCCAGCGGAACCTAT
GAGACTGTGGTTTCCTATCAGCAGATGGGCAATAATTCTCAAGGTATCTCAAAGG
GCGCGCTCATTCCAATCCGCGAAGAAGATTTTACAAATAATGATACCACAACCAC
GCCCTGGATTGCGCTTATTGGATGCGACTACAATGCCACCAACGCCTCTATGGAG
CTCGACATATTCACTATGGTTCGAGACCGAGGCGCTAGAGCCGCTCTCTTGTATT
CAAACACCTCGGACGGCTGTTTACTCAATGAAGGATATCGAACGGGAGATTTTGA
ACAAATCTTTGATATTTTCACATCCAAAACGGCCGCTAATTCTATCATCATCCAGT
CCCAGTTTCGCATACTCGAACACAAGTACACCGTATGGGATCCTGCTCTTCTCAC
AGCCAACAACCAAAGCGTTACCTCTGCCCTTTATCGTAATGCACTCAATACCTCG
CCCTATTTGGTCGCGGCGCTAAGAGCATGGAATGCGACTGGAGAAGAGAGTGCA
GATGATCCGAGCGCTGTTCCGACGACGGTCTATAATCCTTCAACTACGCACGACA
GCGAGCCAAGCCAGAGTTTGGCTATGATCATTCTCTACGTGATCATCAGCTTGGT
ATCGGCGCTTTTTATCATTGTCATTGTCTCGGGGGCTGTCCGCGCTTTCCGCCACC
CGGAACGCTATGGACCAAGACTGTACGATCCGACACTGGAAGGAGATGAAGGTC
AGCCGCAAACAAGGGCAGCTGGACTTACCCGCGCGATTCTCGAAACGTTCCCTGT
CATCAAGTTTGGCCGCACCAACGACCAGATGCAGAACCAATCTACACGCACTTAT
CGTCAAGAAATGAAGAAGTGGAGTCTGGAGAACGGCGAGCAGCCATCACGAGAC
CTCTTGCAGCCAGCGCACGGGCAACCAAACTCGGTCTTTGATGCCTCTCGACAAG
CTTCGCCCATTAGACATAGTTCCGAGGTTGCGAATCGTGCTATGAGACCTCATTCT
ACAGAGATGGCACCGTCCACATCGGATGCTTCTGATACGCAGCAGCTCGATCCCG
CCGCCATCGGAAACCAAACCTGTCCTATCTGCATCGTCGATTTCGAAGAAGGCGA
TGACGTTCGCGTACTACCGTGCGAGGGGAAGCATCGTTTCCACAAGGATTGTGTG
GACCCATGGCTGTTGGAGCTTTCGAGTTCTTGTCCCATATGCCGTGAAGATTTTCA
TGTGTTGGAAGAAATGGCCGTCGCTGCGGATGGTCGTGACCGTGAGCGTTCAGAA
TCTGGTCACAGAGAAGAGGAAGACCATGTCCCGCCGGCAGAACACCATACCTCG
TCCCGCTTCACGCGCTATCTTCGGTTCGCGAACAAGAGGAGACGAAGTCAGCGCT
CTAGCCAGCAACCGCCAGACAACGCAGCGGTGTCGCCGACCCCGGCGTAG
MGRYSLAAQAICLLSSIHSALAYIPARAANISQGLGLDVHDNSKVTLTWNPSGTYETV
VSYQQMGNNSQGISKGALIPIREEDFTNNDTTTTPWIALIGCDYNATNASMELDIFTM
VRDRGARAALLYSNTSDGCLLNEGYRTGDFEQIFDIFTSKTAANSIIIQSQFRILEHKYT
VWDPALLTANNQSVTSALYRNALNTSPYLVAALRAWNATGEESADDPSAVPTTVYN
PSTTHDSEPSQSLAMIILYVIISLVSALFIIVIVSGAVRAFRHPERYGPRLYDPTLEGDEG
QPQTRAAGLTRAILETFPVIKFGRTNDQMQNQSTRTYRQEMKKWSLENGEQPSRDLL
QPAHGQPNSVFDASRQASPIRHSSEVANRAMRPHSTEMAPSTSDASDTQQLDPAAIG

NQTCPICIVDFEEGDDVRVLPCEGKHRFHKDCVDPWLLELSSSCPICREDFHVLEEMA VAADGRDRERSESGHREEEDHVPPAEHHTSSRFTRYLRFANKRRRSQRSSQQPPDNA AVSPTPA

7.2 The sequences were involved in the 3D protein model analysis.

>PIIN29

GNQTCP ICIVDFEEGD DVRVLPCEGK HRFHKDCVDP WLLELSSSCP ICREDFHVLE

>AvrPtoB

GPKLAALDPIASQFSQLRTISKADAESEELGFKDAADHHTDDVTHCLFGGELSLSNPD QQVIGLAGNPTDTSQPYSQEGNKDLAFMDMKKLAQFLAGKPEHPMTRETLNAENIA KYAFRIVP

>Rbx1

GSMDVDTPSGTNSGAGKKRFEVKKWNAVALWAWDIVVDNCAICRNHIMDLCIECQ A NQASATSEECTVAWGVCNHAFHFHCISRWLKTRQVCPLDNREWEFQKYGH

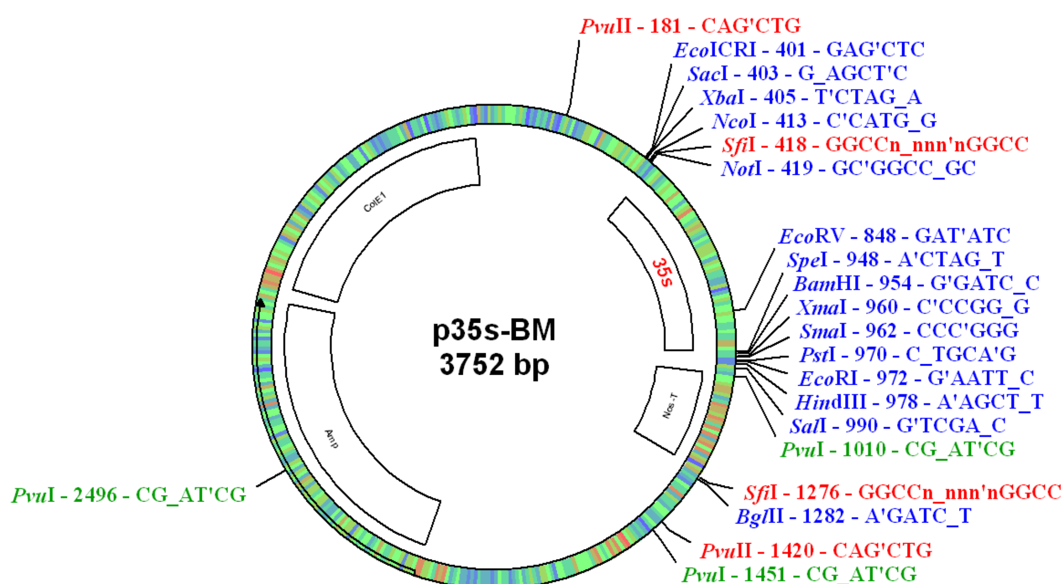
>2KIZ

MKQDGEEGTEEDTEEKCTICLSILEEGEDVRRLPCMHLFHQVCVDQWLITNKKCPICR VD IEAQLPSES

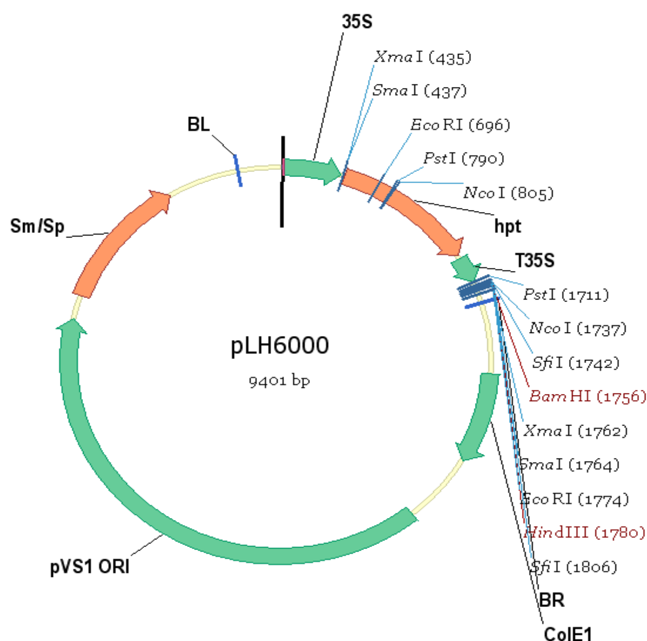
>AtPUB14

GSPEFPEYFRCPISLELMKDPVIVSTGQTYERSSIQKWLDAGHKTCPKSQETLLHAGLT PNYVLKSLIALWCESNGIE

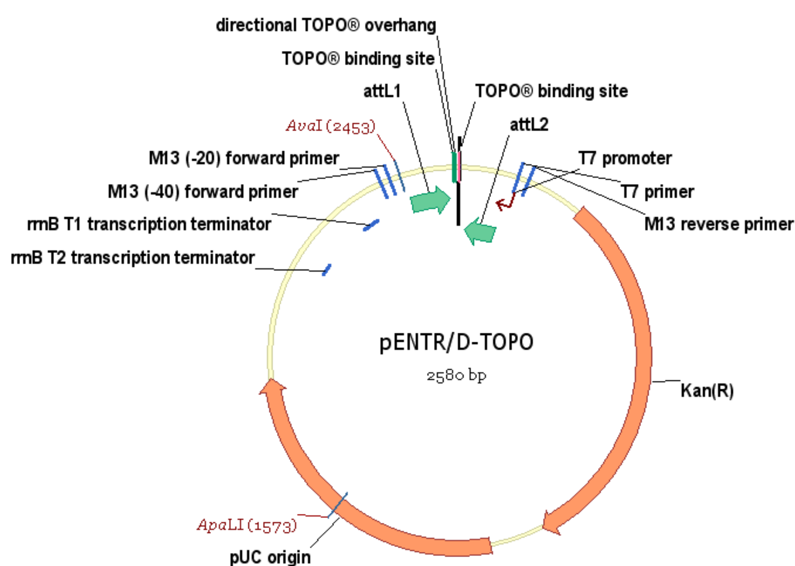
7.2 plasmid map



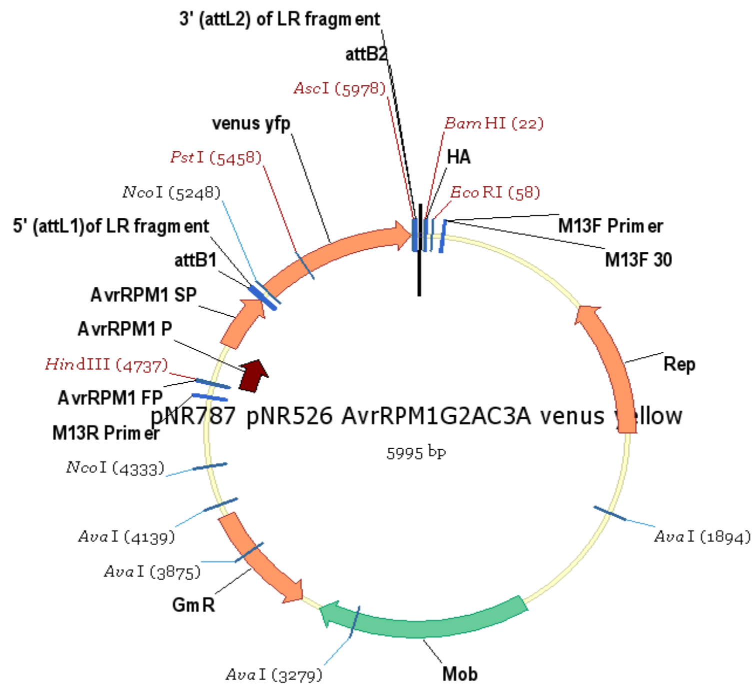
7.2.1 Vector map of the vector p35S-BM. The vector was applied to add CaMV35s promoter and Nos terminal to fragment of *piin29:mcherry* .



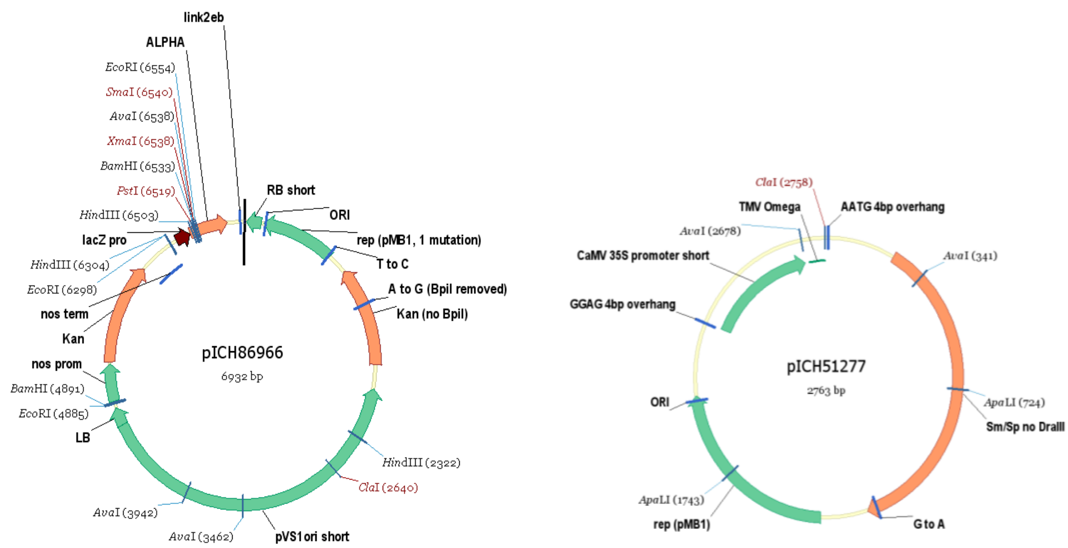
7.2.2 Vector map of the binary expression vector pLH6000. The vector was applied for the generation of the pHL6000:CaMV35S:*piin29:mcherry* construct used in transgenic plants.

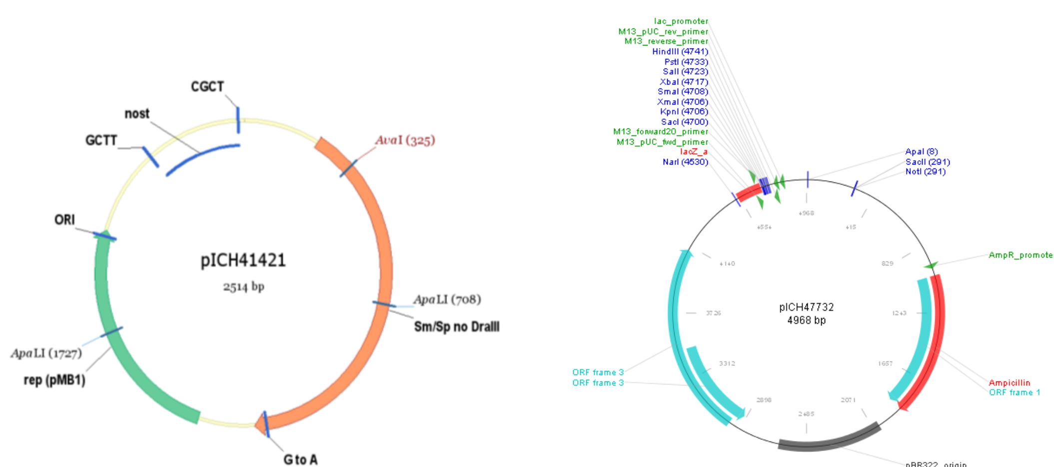


7.2.3 Vector map of pENTR/D-TOPO. The vector was applied for the entry clone for GATEWAY constructions.



7.2.4 Vector map of the destination vector pNR526 G2AC3A. The vector was applied for the generation of the DELD effector candidate constructs used for *P. fluorescens* EtHAn infiltration.





7.2.4 Vectors were used in Golden gate. pICH51277 and pICH41421 offered CaMV35S and Nos terminal respectively. The destination vector was pICH47732. pICH86966 was expression vector and applied for the generation of the DELDs constructs used for agroinfiltration.

7.3 List of primers mentioned in this thesis

7.3.1 Primer sequences for overlapping PCR of *piin29:mcherry* without signal peptide.

Primer name	Sequence (5'-3')
PIN29 DSP-F	ATCACTAGTATGTATATTCCAGCTAGGGCTGC
mcherry-R	ATCTCTAGATTACTTGTACAGCTCGTCCATGC
SGSG-PIN29-R	TGAACCTGATCC CGCCGGGGTCGGCGAC
GSGS-cherry-F	GGTTCAGGTTCTATGGTGAGCAAGGGCG

7.3.2 List of primer sequence of DELDs constructions for T3SS.

Primer name	Sequence (5'-3')
5872-F	CACCATGGCTCCGCTTCCGAACCCC
5872 dsc-R	TATCCAGCTCGTCTATGCTCCGCTTG
5932-F	CACCATGGCTCCAATCCCTAGTCCTGAAG
5932 dsc-R	TATCCAACTCGGCTATGCTCCGACG
6837-F	CACCATGACTCCGGTCCCTAGAATCGATCAC
6837 dsc-R	TATCCAACTCGTCTATGCTCCGATGG

7595-F	CACCATGGCTCCCCTCCCTAGGCC
7595 dsc-R	TATCCAATTCGTCTATGCTCCGAAATTTCAGG
9226-F	CACCATGACACCACTCCCCAGGGATCG
9226 dsc-R	TATCCAACTCGTCTATGCTCCTACGG
9643-F	CACCATGGCTCCAATTCCAGACCCTAGC
9643 dsc-R	TATCCAACTCGTCTATGCTTTGAACACTAC
9644-F	CACCATGGCCCCAGTTCCAGAACCTAGC
9644 dsc-R	TATCCAACTCGTCTATGCTTTGAACACTAC
9687-F	CACCATGACTCCGCTGCCCAGCCC
9687 dsc-R	TATCCAAGTCTTCTATGCTCCTACTGAAAATTC
9689-F	CACCATGGCTCCGCTTCCCAAGGACG
9689 dsc-R	TATCCAACTCGTCTATGCTCCGACG
9859-F	CACCATGACTCCATCCCCCGCATACGAC
9859 dsc-R	TATCCAACTCGTCTATGCTTCGGCG
10147-F	CACCATGGCTCCGCTCCCCAAGCC
10147 dsc-R	TTTCCAACTCGTCTATGCTCCGACG
11595-F	CACCATGGCTCCACTCCCCGTTCTTGATATC
11595 dsc-R	TATCCAACTCCTCTATGCTCCGAGTC

7.3.3 List of primer used in Golden gate construction

Primer name	Sequence (5'-3')
N-GFP BbsI-F	ATTGAAGACTTGTCGGGTCTCAAATGATGGTTTCCAAGG GTGAGG
N-GFP BbsI -R	ATTGAAGACTTTACCGGTCTCAACCTGATTTGTAAAGTTC ATCCATTC
C-10147dsp-F	ATTGAAGACTTGTCGGGTCTCCAGGTAGGCTCCGCTCCC CAAGC
C-10147-R	ATTGAAGACTTTACCGGTCTCAAAGCTTATTTCCAACTCG

	TCTATGCTCCG
C-09266dsp-F	ATTGAAGACTTGTCGGGTCTCCAGGTATGATGACACCACTCCCCAGGGATCG
C-09266-R	ATTGAAGACTTTACCGGTCTCAAAGCTTAATCCAACTCGTCTATGCT
C-06837dsp-F	ATTGAAGACTTGTCGGGTCTCCAGGTATGACTCCGGTCCCTAGAATCG
C-06837-R	ATTGAAGACTTTACCGGTCTCAAAGCTTAATCCAACTCGTCTATGCTCC
C-05865dsp-F	ATTGAAGACTTGTCGGGTCTCCAGGTATGATGGCTCCCTTCCCTTTTTTGC
C-05865-R	ATTGAAGACTTTACCGGTCTCAAAGCTTAATCCAACTCATCTATGTTCCG
C-05851dsp-F	ATTGAAGACTTGTCGGGTCTCCAGGTATGGTTCCGCTCCCCAGTCC
C-05851-R	ATTGAAGACTTTACCGGTCTCAAAGCTTATATCCAACTCGTGTATGCTTCG

7.4 Sequence of DELD effector candidates

The cDNA and amino acid sequences of the DELD effector candidates were taken from the database of *P. indica*. All the candidates have signal peptide which was marked by Bold and Italic.

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8. Declaration

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

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

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

Signature:

Giessen, July 2014

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