Investigation of programmed cell death mechanisms in *Arabidopsis* roots during colonization with *Piriformospora indica*

Dissertation zur Erlangung des Doktorgrades
(Dr. rer. nat.)
der Naturwissenschaftlichen Fachbereiche
der Justus-Liebig-Universität Gießen

durchgeführt am
Institut für Phytopathologie
und Angewandte Zoologie

vorgelegt von
M.Sc. Xiaoyu Qiang
aus China

Gießen, November 2010

Dekan: Prof. Dr. Volkmar Wolters

1. Gutachter: Prof. Dr. Karl-Heinz Kogel

2. Gutachter: Prof. Dr. Volker Wissemann

Contents

List of Abbreviations

AM	Arbuscular mycorrhiza
Avr	Avirulence
Bgh	*Blumeria graminis* f.sp. *hordei*
BI-1	Bax Inhibitor -1
BiP	Luminal binding protein
bZIP	Basic domain/leucine zipper factor
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDPK	Ca^{2+}-dependent protein kinase
CNX / CRT	Calnexin / Calreticulin
CTAB	Cetyltrimethylammonium bromide
cv.	Cultivar
dai	day after inoculation
DAMPs	Damage-associated molecular patterns
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleosidtriphosphate
DTT	Dithiothreitol
EF-Tu	Elongation factor TU receptor
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ER-QC	Endoplasmic reticulum-quality control
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
flg22	A 22-amino-acid-long peptide derived from flagellin
FLS2	FLAGELLIN SENSING 2
f. sp.	forma specialis
h	Hour
HR	Hypersensitive Response
ISR	Induced systemic resistance
JA q	Jasmonic acid
kDa	Kilodalton
LRR	Leucin-rich-repeat
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen activated protein kinase
min	Minutes
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger RNA
NaCl	Sodium chloride
OST	Oligosaccharide transferase

PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PR	Pathogenesis related
PRR	Pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
qRT-PCR	Quantitative real-time PCR
R-gene	Resistance gene
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
TBE	Tris-Boric acid-EDTA
TE	Tris-HCl+Na_2-EDTA
TEMED	Tetramethylethylenediamine
TM	Tunicamycin
UPR	Unfolded protein response

1. Introduction

Plants are confronted with a plethora of pathogenic viruses, bacteria or fungi. But surprisingly, plants are resistant against the majority of them and disease is the exception. In order to ensure proper development and reproduction, plants have evolved defense strategies to stop pathogenic colonization. In addition, plants form mutualistic associations with microorganisms (Harrison, 2005; Hause and Fester, 2005; Kogel et al., 2006). Both partners benefit from such symbioses as examplified by the improved nutrient state in plant root-arbuscular mycorrhiza interactions. *Piriformospora indica,* a representative fungus in the recently defined order *Sebacinales,* also forms mutualistic symbioses thereby transferring various benefits to its hosts such as abiotic and biotic stress tolerance, improved plant growth, and an increased seed production (Varma et al., 1999; Waller et al., 2005; Schäfer et al., 2007). Plant-*P. indica* interactions have been characterized at a molecular and cell biological level (Weiss et al., 2004; Deshmukh et al., 2006). Studies in barley roots indicated that *P. indica* colonization gradually increased with tissue maturation. Additional genetic studies revealed that barley and *Arabidopsis* root colonization coincided with root cell death (Deshmukh et al., 2006; Jacobs, Zechmann, Kogel, Schäfer, unpublished). However, the mechanisms behind cell death initiation and execution during the mutualistic association and the significance of cell death for root colonization are currently unknown.

1.1. Plant-microbe interactions and plant innate immunity

Based on fossil records, early land plants were found to be colonized by fungal symbionts (Gehrig et al., 1996). The evolution of land plants is hypothesized to be a result of microbial interactions with epiphytic, symbiotic or pathogenic microbes (Chisholm et al., 2006). The outcome of these plant-microbe interactions might be considered as neutral, beneficial (mutualistic) or detrimental (pathogenic). In order to avoid pathogenic infection, plants have evolved two levels of innate immune responses against pathogens. The first level recognizes and responds to molecules common to many classes of microbes so called

microbial-associated molecular patterns (MAMPs), which is termed as MAMP-triggered immunity (MTI). The second level responds to pathogen virulent effectors either directly or through their effects on host targets, which is termed as effector-triggered immunity (ETI). (Chisholm et al., 2006; Jones and Dangl, 2006).

1.1.1. Microbial-associated molecular patterns (MAMPs)-triggered immunity

MAMP-triggered immunity (MTI, syn. basal defense) is very effective in stopping pathogens and is thought to be ancient. MTI is activated by conserved microbe-derived molecules so called MAMPs (microbe-associated molecular patterns), which include multiple cell-surface components of bacteria such as lipopolysaccharides, flagellins, peptidoglycans and cell wall components of higher fungi such as chitin or ergosterol (Jones and Takemoto, 2004; Zipfel and Felix, 2005, Boller and Felix, 2009). MAMPs fulfill an essential function in microbial lifestyle and represent highly conserved structures that are found across a range of microbes, but do not exist in hosts (Nürnberger et al., 2004). During pathogen attacks, these MAMPs are recognized by plant plasma membrane-localized pattern recognition receptors (PRRs). The best characterized MAMP and the respective PRRs of *Arabidopsis* are summarized below (Table 1-1).

Table 1-1. MAMPs and PRRs of *Arabidopsis*.

MAMP	PRR	References
Bacterial flagellin/flg22	FLS2	Felix et al.,1999; Gomez-Gomez and Boller,2000; Chinchilla et al., 2006
Bacterial EF-Tu/elf18	EFR	Kunze et al., 2004; Zipfel et al., 2006; Shiu and Bleecker, 2003
Fungal chitin	CERK1	Miya et al., 2007

The perception of MAMPs by PRRs occurs at the sites of microbial invasion and results in the activation of MTI signaling. Immediately downstream of MAMP recognition, mitogen-activated protein kinase (MAPK) signaling pathways are activated, which, in turn,

initiate defense gene expression via transcription factors (e.g. WRKYs) (Nicaise et al., 2009). MTI in *Arabidopsis* also relies on the accumulation of antimicrobial glucosinolates and camalexin (Bednarek et al., 2009; Clay et al., 2009). In addition, Ca^{2+} contributes to MTI as MAMP-induced Ca^{2+} influx activates MAPK- and Ca^{2+}-dependent protein kinase (CDPK) signaling (Boudsocq et al., 2010). CDPKs also regulate the production of reactive oxygen species (ROS), detectable as the oxidative burst (Boudsocq et al., 2010). This ROS burst is generated by the plasma membrane localized NADPH oxidase RBOHD (Zhang et al., 2007). The resulting MTI responses end in callose deposition, (Boller and Felix, 2009) as well as phytohormone synthesis and signaling (e.g. salicylic acid (SA), ethylene (ET), and jasmonate (JA)). Phytohormones regulate the expression of certain pathogenesis-related (PR) proteins, which contribute to plant innate immunity (Tsuda et al., 2009). MTI responses vary in dependence of the life style of attacking microbes. Microbes can follow a biotrophic, necrotrophic or hemibiotrophis life style. The latter means a lifestyle with an initial biotrophic phase followed by a necrotrophic colonization phase. In a simplified model SA-dependent defense pathway restrict colonization by biotrophic microbes while necrotrophic microbes are stopped by JA/ET-dependent signaling. In general, the two signaling pathways seem to act antagonistically (Glazebrook, 2005).

MAMP-triggered immune signaling also results in the generation of damage-associated molecular patterns (DAMPs), which function as endogenous elicitors. For instance, AtPep1 is a peptide, which was isolated in *Arabidopsis* and exhibited characteristics of an endogenous elicitor of the innate immune responses. Pep1 is recognized by plasma membrane localized receptors PEPR1 and PEPR2 and activate defense related genes such as *PDF1.2* and induce the production of H_2O_2 (Huffaker et al. 2006; Yamaguchi et al., 2010). Other known DAMPs include cell wall fragments such as oligogalacturonides (OGAs) and cutin monomers (Boller and Felix, 2009). DAMP signaling is thought to sustain MTI (Ryan et al., 2007).

1.1.2 Pathogen effectors-triggered immunity (ETI)

In order to successfully colonize host plants, pathogenic and mutualistic microbes need to suppress MTI. Microbes have therefore evolved effectors, which are released in order to

promote pathogen virulence. The mode of action of these effectors is quite diverse, but in general, they interfere with the plant surveillance system or disrupt defense signaling. Bacterial pathogens have developed a type III secretion system (TTSS) to inject effectors proteins into plant cells, and, eventually, suppress MTI (Bent and Mackey, 2007; Jones and Dangl, 2006). The transfer of effectors into plant cells by fungi and oomycetes is less clear. Since pathogens obtained the capacity to suppress host defense, plants have evolved more specialized mechanism to detect microbial effectors or effector activites summarized as effector-triggered immunity (ETI, syn. *R* gene-mediated resistance) (Bent and Mackey, 2007; Chisholm et al., 2006; Dangl and Jones 2006). R protein activates immune responses after direct binding to effectors or indirectly by monitoring effector action. Direct protein interactions were detected between *flax* resistance genes and *flax* rust avirulence genes (Dodds et al., 2006). While there are other cases showing that plant R proteins indirectly recognize pathogen effectors by monitoring the integrity of host cellular targets of these effectors (van der Biezen and Jones, 1998; Jones and Dangl, 2001). This latter principle of effector recognition was defined as “guard hypothesis” (Dangl and Jones, 2006; Bent and Mackey, 2007). Eventually, *R*-gene mediated resistance is regarded to result in a faster and more efficient initiation of defense signaling than MTI. A hallmark of *R*-gene mediated defense is the hypersensitive response (HR), a localized programmed cell death, which occurs at the site of infection and inhibits fungal penetration. During evolution of host-microbe interactions, microbes have found ways to circumvent *R* gene-mediated defense responses by modifying or eliminating effectors/effector actions resulting in successful plant colonization and microbial propagation. In turn, plants continuously adapt their R protein repertoire. Nowadays, the evolutionary processes in immune signaling are summarized in the zigzag model (Fig. 1-1) (Jones and Dangl, 2006).

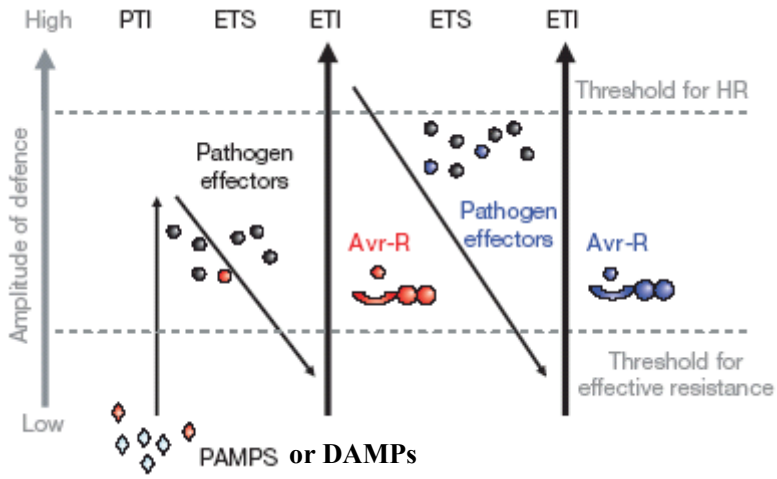


Fig. 1-1: The zigzag model for illustrating the plant innate immune system (Modified from Jones and Dangl, 2006).
In this model, the evolution of disease resistance and susceptibility is divided into 4 phases. In phase 1, plant membrane-localized pattern recognition receptors (PRRs) detect microbial MAMPs and stimulate MAMP-triggered immunity (MTI) in plants. In phase 2, microbes have evolved effectors to promote pathogen virulence and MTI is suppressed, which results in effector-triggered susceptibility (ETS). In phase 3, specific effectors (formerly called Avr proteins) are recognized by host R proteins, resulting in effector-triggered immunity (ETI) and disease resistance. In phase 4, natural selection drives microbes to evolve new effectors that could suppress ETI again. Nevertheless, plants evolve new R protein to counteract effector action and to trigger ETI. In terms of defense intensity, MTI is usually lower than ETI, which is indicated by the amplitude of defense on the y-axis. Therefore, hypersensitive response (HR) is usually not associated with MTI but frequently observed during ETI at the infection site.

1.1.3 Induced resistance

An additional protective strategy of plants to avoid systemic spread of invading microbes is systemically induced resistance and it can be mainly divided into two forms. One is termed as systemic acquired resistance (SAR), which is activated in various plant species by pathogens that cause either HR or disease symptoms (Ryals et al., 1996). This resistance is long-lasting and effective against a broad spectrum of pathogens including viruses, bacteria, fungi and oomycetes. Molecularly, SAR is associated with induction of a large number of pathogenesis-related proteins (PR proteins) in both local and systemic tissues. Salicylic acid (SA) is a crucial component in the SAR signal transduction pathway. Plants, which express the bacterial SA hydroxylase gene *NahG*, cannot longer accumulate SA and are impaired in SAR (Durrant and Dong, 2004; Maleck et al., 2000). The second form of induced resistance is

termed induced systemic resistance (ISR), which is activated by non-pathogenic rhizobacteria. Like SAR, it protects plants against a range of pathogens, but it is independent of SA and *PR* gene induction. The blocking of ISR in *jar1* (*jasmonic acid resistant 1*) and *etr1* (*ethylene resistant 1*) mutants indicated that ISR requires JA/ET signaling components. As a result, defense compounds (e.g. *PDF1.2*) are induced (Van Loon 1997; Pieterse et al., 1998). The protein of Nonexpressor of pathogenesis-related gene 1 (NPR1), also termed as NIM1, is an essential downstream regulator for successful activation of both SAR and ISR. However, NPR1 plays a central role in SAR but a supporting role in ISR. Intriguingly, *NPR1* expression is not increased in plants expressing both SAR and ISR, which indicates that the cellular level of NPR1 is sufficient to activate SAR and ISR (Dong, 2004; Pieterse and Van Loon, 2004).

1.2 Mutualistic symbiotic interactions between plants and microbes

The establishment of plants on land is believed to be supported by the interaction with fungal symbionts (Gehrig et al., 1996). Among all endosymbioses in natural ecosystems, the most widespread endosymbiotic interactions are formed between plants and fungi. Among the best studied symbioses between plant roots and fungi are mycorrhizas, which refers to Greek "mycos" meaning fungus and "rhiza" meaning root. These symbioses occur in several different forms: the first form is called ectomycorrhiza, which is known to be predominant on trees in temperate forests (Parniske 2008). During the interaction with plants, this kind of fungal partner remains outside of plant cells. The second form is called endomycorrhiza, including orchid, ericoid and arbuscular mycorrhiza (AM) (Parniske 2008). Fungi following these symbiotic interaction strategies intracellularly colonize plant cells (Parniske 2008). The symbiosis with AM is formed by 70-90% of land plant species, which is thought to be the most widespread terrestrial symbiosis (Fitter 2005; Smith and Read, 2008). During symbiotic development, tree-shaped subcellular structures, so called arbuscules, are formed within plant cells and they are thought to be the main part for nutrient exchange between the fungus and plant partners. This established symbiotic interaction between plant roots and arbuscular mycorrhizal fungi (AMF) may improve the nutrient state of both partners. The fungi obtain fixed carbon compounds from host plants, while plants benefit from increased nutrient supply

(e.g. phosphorus), or water supply, or enhanced stress tolerance and resistance (Finlay 2008; Solaiman and Saito 1997; Bago et al., 2003). It is estimated that up to 20% of the photosynthesis products of terrestrial plants are consumed by AMF (Bago et al., 2000). Therefore, AM symbiosis is thought to significantly contribute to global phosphate and carbon cycling and to affect productivity in land ecosystems (Fitter, 2005).

The question arises how mycorrhiza fungi can successfully invade plants and how they overcome host innate immunity? Previous studies revealed that AM development is accompanied by an exchange of signaling molecules between both symbionts. On one side, plant hormones so called strigolactones are exudated by plant roots and recognized by AM fungi thereby stimulating fungal metabolism and hyphal branching. On the other side, AM fungi release yet to be isolated signaling molecules, which initiate root symbioses (Akiyama et al., 2005; Gomez-Roldan et al., 2008; Parniske 2008). Furthermore, similar to interactions between plants and parasitic mircrorganisms, AM fungi are confronted with the plant innate immune system. At early interaction stages, molecules secreted by the microbes or derived from physical or chemical cleavage of the plant cell walls can initiate an effective defense response in plants. Similar molecules are released by ectomycorrhizal and AM fungi (e.g. chitin) (Salzer and Boller, 2000). However, previous studies have shown that the defense response in AMF-colonized plants is transient and does not impair initial AMF colonization (Blilou et al., 2000; Garcia-Garrido and Ocampo, 2002; Lambais, 2000). Hence, symbiotic fungi might have developed strategies to achieve compatibility, for example by avoiding to trigger host immunity, by stimulating defense suppressors, by activating counter defense against antimicrobial compounds, by inducing susceptibility factors or by releasing effectors to suppress defense signaling (Lambais, 2000; Salzer et al., 2000; Garcia-Garrido and Ocampo, 2002, Bent and Mackey, 2007). Interestingly, AM fungi cannot only successfully achieve compatibility with plants, but recent studies also demonstrated that the colonization of plant roots with AM fungi resulted in an inhibition of leaf colonization by bacterial pathogens (Liu et al., 2007). It is still an open question whether the increased resistance to pathogens is due to improved plant fitness or due to specific defense responses induced by AM fungi.

1.3 The mutualistic fungus *Piriformospora indica*

1.3.1 *P. indica* - classification and root colonization pattern

The root-colonizing mutualistic fungus *Piriformospora indica* was discovered in association with a spore of the AM fungus *Glomus mosseae* in the rhizospheres of woody shrubs in sandy desert soils of the Thar region of northwest India (Verma et al., 1998). Through ultrastructural analyses of fungal hyphae and sequences analyses of the fungal 18S RNA or nuclear rRNA of the 5´-terminal domain of the ribosomal large subunit (nucLSU), *P. indica* was classified as member of class B of the order *Sebacinales*, which belongs to the class of Agaricomycetes (Basidiomycota) (Verma et al., 1998; Hibbett, 2006). This recently defined order *Sebacinales* within the *Hymenomycetes* encompasses a great multitude of ericoid-, orchid-, jungermannioid mycorrhiza and ectomycorrhizae (Weiss et al., 2004; Setaro et al., 2006). Within the *Sebacinales*, *P. indica* exhibits the closest relationships to *S. vermifera* and multinucleate *Rhizoctonia*, both of which show an obvious host specificity among orchids regarding their beneficial impacts such as supporting seed germination. Previous studies indicated that these fungi could form intracellular hyphal coils (Milligan and Williams 1988; Warcup, 1988) which represents characteristics of orchid mycorrhizas (Peterson and Massicotte, 2004). In addition, members of *Sebacinales* also develop other mycorrhiza-specific colonization types such as hyphal sheaths, Hartig nets, and intracellular coils (Brundrett 2004; Selosse et al., 2007).

Compared to the above mentioned mycorrhizas, *P. indica* follows a divergent colonization type in barley and *Arabidopsis* roots as shown by epifluorescence microscopy (Deshmukh et al., 2006; Jacobs, Zechmann, Kogel, Schäfer, unpublished data). Previous studies revealed that the fungus intercellularly colonizes the barley roots and frequently penetrates and intracellularly colonizes rhizodermal and cortical cells. As colonization proceeds, parts of the root are densely covered with extracellular hyphae and harbor thorough inter- and intracellular networks. Nevertheless, the fungus is never observed to enter vascular tissue and predominantly colonizes the root maturation zone. By contrast, the root elongation and meristmatic zone is rarely colonized. Finally, fungal colonization results in extracellular and

intracellular sporulation, which is indicated by the formation of chlamydospores (Deshmukh et al., 2006).

1.3.2 Beneficial activities of *P. indica* symbioses with host plants

So far, all studied members of the order *Sebacinales*, like *P. indica* and the closely related *Sebacina vermifera* species (*Sebacinales* group B), have been shown to form mutualistic symbioses with a wide spectrum of plants thereby exhibiting an unique biological activity of high ecological and agronomical relevance (Weiss et al., 2004; Waller et al., 2005; Deshmukh et al., 2006). The propagation of *P. indica* and related sebacinoid fungi can be done in axenic cultures (Fig 1-2A, B). *P. indica* has been shown to confer growth promotion to roots and shoots of a broad variety of host plants, including barley and *Arabidopsis* (Varma et al., 1999; Peskan-Berghoefer et al., 2004; Waller et al., 2005). Similar to AM, *P. indica* was recently found to play an important role in improving host plant nutrition. A phosphate transporter of *P. indica* (*PiPT*) is actively involved in phosphate transportation to host plant. Moreover, the growth promoting effects of *P. indica* were shown to depend on this phosphate transport (Yadav et al., 2010). Further studies showed that *P. indica* was also able to enhance tolerance against abiotic stresses in several plants (Sahay and Varma, 1999; Waller et al., 2005). In addition, recent studies showed that *P. indica* is able to mediate resistance against the necrotrophic root pathogens *Fusarium culmorum* and *Cochliobolus sativus* (Waller et al., 2005; Deshmukh and Kogel, 2007) and induce systemic resistance in leaves of barley and *Arabidopsis* against the powdery mildew fungi *Blumeria graminis f.sp. hordei* and *Golovinomyces orontii*, respectively (Waller et al., 2005; Stein et al., 2008). This leaf resistance is thought to base on induced systemic resistance (ISR) (Stein et al., 2008).

Interestingly, the α-proteobacterium *Rhizobium radiobacter* was found to be associated with *P. indica*. Subsequent studies indicated that *R. radiobacter* is capable of conferring growth promotion and systemic resistance against powdery mildew fungi *Blumeria graminis f.sp. hordei* on barley plants, which are similar to those conferred by *P. indica* (Sharma et al., 2008). Since all attempts failed to generate bacteria-free *P. indica*, the contribution of both microbes in symbiotic interactions remains to be answered.

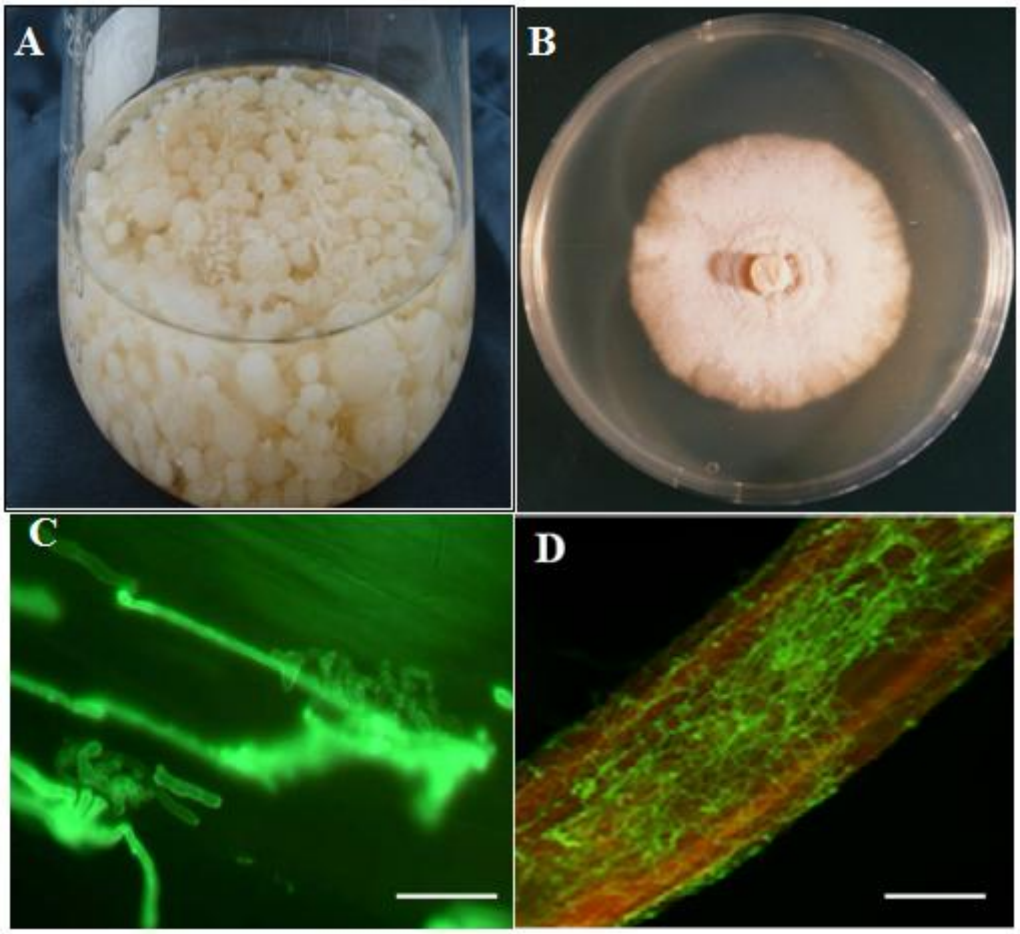


Figure 1-2. *Piriformospora indica*.
(A and B) Cultivation of *P. indica* in axenic culture. (C) *Arabidopsis* root cells colonized by *P. indica* at 7 dai. The fungus was stained with chitin-specific wheat germ agglutinin-Alexa Fluor 488 (WGA-AF 488) and coiled-like hyphae reminiscent of pelotons of orchid mycorrhizas were observed by epifluorescence microscopy. Bar = 20 μm (Schäfer and Kogel, 2009). (D) Barley root colonized by *P. indica.* Fungal hyphae were stained with WGA-AF 488 and plant cell walls were stained by congo red. The root was observed by epifluorescence microscopy.

1.3.3 Cellular colonization strategy of *P. indica*

In the interaction of *Piriformospora indica* with roots from barley and *Arabidopsis,* similar growth promotion and defensive activity have been found as have been orginally reported for

plant-AMF interactions. Recent research demonstrated that after a transient and weak induction at early interaction stages, defense genes were general suppressed (Jacobs, Zechmann, Kogel, Schäfer, unpublished data). Furthermore, at early colonization stages of *Arabidopsis* roots, *P. indica* suppressed MTI to achieve root compatibility (Jacobs, Zechmann, Kogel, Schäfer, unpublished data). Additionally, *P. indica*-colonized barley was shown to exhibit enhanced antioxidative capacities provided by the ascorbate-glutathione cycle, which might enhance ROS (reactive oxygen species) detoxification (Waller et al., 2005). ROS accumulation contributes to plant innate immunity and is considered to coincide with the hypersensitive response (HR) (Apel and Hirt, 2004, Bent and Mackey, 2007).

A main qualitative difference between *P. indica* and other mycorrhizas is that *P. indica* requires cell death for root colonization (Desmukh et al., 2006). Barley plants overexpressing the negative cell death regulator *BAX Inhibitor-1* (*HvBI-1*) displayed a dramatically reduced susceptibility compared to wild-type roots as determined by quantitative PCR (Deshmukh et al., 2006). Accordingly, it was indicated that from 5 dai onwards *HvBI-1* transcript accumulation was significantly suppressed in barley roots in response to *P. indica* colonization. This genetic and molecular analyses in combination with the cytological studies unambiguously document that cell death emergence is associated with and is apparently required for *P. indica* proliferation. Recent transmission electron microscopical studies of the *Arabidopsis* root-*P. indica* interaction indicated that the fungus penetrated and colonized living cells. This biotrophic colonization phase is followed by a cell death-associated colonization phase (Jacobs, Zechmann, Kogel, Schäfer, unpublished data). Hence, the mutualistic *Arabidopsis*-*P. inidca* interaction provided a model system to dissect the mechanisms of cell death-associated colonization and to elucidate its significance for the reported beneficial effects.

1.4 Endoplasmic reticulum quality control (ERQC) and unfolded protein response (UPR)

1.4.1 Protein folding and quality control in ER

Protein folding is a crucial process for protein function in all organisms. During evolution, cells have gained sophisticated mechanisms to ensure the proper folding of proteins and the disposal of irreversibly misfolded proteins (Kaufman 2002). The endoplasmic reticulum (ER) is known to be the site of synthesis, folding and modification of secreted and cell surface proteins as well as resident proteins of the secretory pathway. It is also a convergent point in the processing of glycoproteins destined for secretion. ER quality control (ER-QC) is defined as a surveillance mechanism that permits only correctly folded proteins to exit the ER and to reach their functional sites, e.g. vacuoles, plasma membrane and extracellular matrix (apoplast). By contrast, misfolded proteins are either retaining in the ER lumen for further folding attempts or are directly targeted for ER-associated degradation (ERAD) (Malhotra and Kaufman, 2007). When secretory proteins pass the ER, they attempt to obtain their proper three dimensional structures, which is a prerequisite for their functionality. Protein folding processes are controlled by at least three ER quality control systems, which include the SDF2-ERdj3b-BIP complex, the calreticulin/calnexin cycle, and the protein disulfide isomerase (PDI) system (Anelli and Sitia 2008). After co-translational translocation into the ER, nascent proteins bind to the SDF2-ERdj3b-BIP complex and get *N*-glycosylated through the catalytic oligosaccharide transferase complex (OST). Thereafter, glycoproteins are continuously processed by ER-resident lectin-like chaperones, calreticulins (CRTs) and calnexins (CNXs). After passing this CNX/CRT cycle, natively folded proteins leave the ER and enter the Golgi compartment. In turn, as an essential function of protein folding quality control, the non-native proteins are tagged and re-associated to the CNX/CRT cycle by UDP-glucose:glycoprotein glucosyltransferase (UGGT), which functions to facilitate their ER retention. For certain glycoproteins, intramolecular disulfide isomerization is required for correct folding, which is executed by protein disulfide isomerases (PDIs). ER working load and, thus, ER-QC activities are thought to vary depending on the developmental stage, the type of tissue, or the occurrence of external stresses. ER-QC during early stages of the secretory pathway is summarized below (Fig. 1-3).

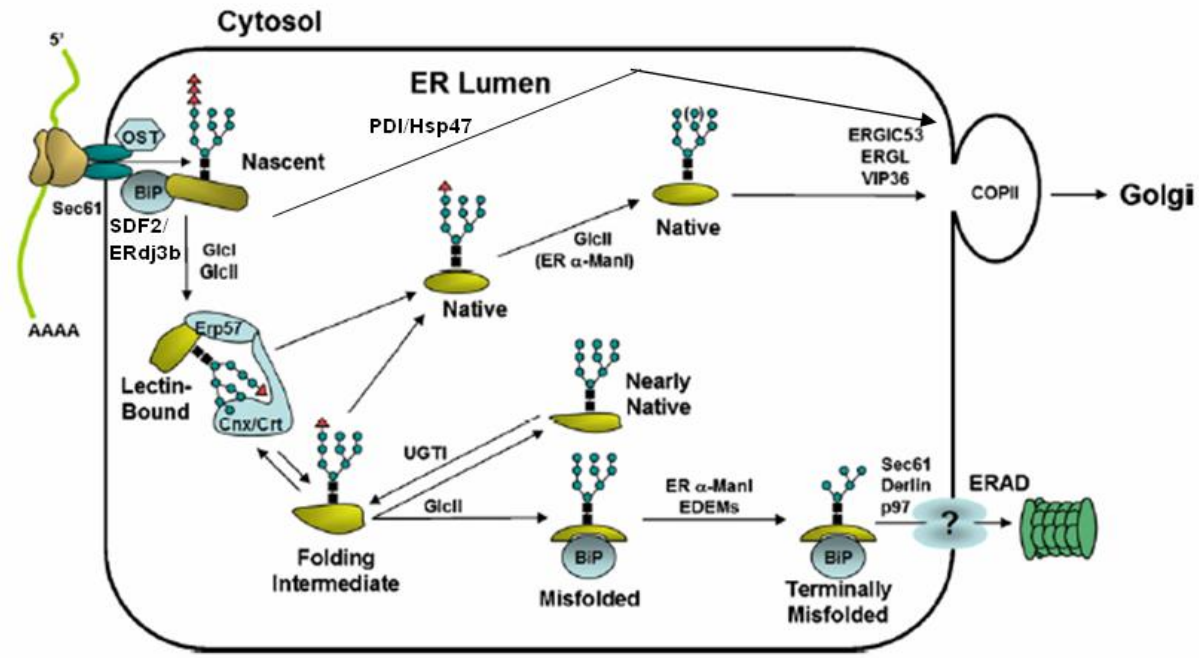


Figure 1-3. Protein quality control in the early secretory pathway. (Modified from Malhotra and Kaufman, 2007)

The scheme illustrates chaperones and folding assistants of the BiP-containing complex, the calnexin (CNX)/calreticulin (CRT) cycle, and the PDI-containing complex. The nascent polypeptides enter ER lumen through the translocon complex Sec61 and are modified by the oligosaccharyltransferase (OST), and then they are further modified with the help of the chaperone complex BiP2/SDF2/ERdj3b or PDI. The modifications of glycoproteins mediated by glucosidase I and II (Glc I and II) are recognized ER lectins CNX and CRT, which initiate protein folding. Release from CNX/CRT that is followed by Glc II cleavage of innermost glucose residue prevents further interaction with CNX and CRT. Here, natively folded polypeptides transit the ER to the Golgi compartment with the possible assistance of mannose-binding lectins such as ERGIC-53, VIPL, ERGL. The non-native polypeptides are tagged for reassociation with CNX/CRT by the UDP-glucose:glycoprotein glucosyltransferase (UGT1) to facilitate their ER retention and to prevent anterograde transport. Misfolded polypeptides are targeted for ER-associated degradation (ERAD) by retrotranslocation, probably mediated by ER degradation-enhancing mannosidase-like protein (EDEM) and Derlins, into the cytosol and delivery to the 26S proteosome. In this figure, triangles represent glucose residues, squares represent N-acetylglucosamine residues, and circles represent mannose residues.

1.4.2 Function of ER-QC in plant innate immunity and plant development

After biotic stress sensing, one common reaction of eukaryotic cells is the activation of defined defense signaling pathways resulting in the pronounced transcription of genes encoding antimicrobial proteins such as pathogenesis-related (PR) proteins. A considerable

number of these glycoproteins have an apoplastic destination and traverse the ER and Golgi apparatus prior to their vesicle-mediated transport and release at the plasma membrane by exocytosis (Jelitto-van Dooren et al. 1999, Lipka et al. 2007). In plants, systemic acquired resistance (SAR) is thought to be established by NPR1-mediated expression of *PR* genes. Wang et al (2005) performed gene expression profiling in *Arabidopsis* and, intriguingly, they found that NPR1 not only controls the expression of *PR* genes, but also the protein secretory machinery including those genes encoding central ER-QC components such as BiP2, DAD1 and Sec61 translocon complex. Their studies further indicated that a coordinated up-regulation of the protein secretory machinery is required for apoplastic transport of PR proteins.

Moreover, recent studies also revealed the significance of ER-QC components for MAMP-triggered immunity (MTI) in that the disturbance of ER-localized processing of the MAMP receptor EFR1 results in impaired MTI responses and enhanced disease susceptibility against bacterial and fungal pathogens (Nekrasov et al. 2009, Saijo et al., 2009). Furthermore, proper function of ER-QC was also shown to be required for plant development as UDP-glucose:glycoprotein glucosyltransferase (UGGT) mediates processing of the brassinosteroid receptor BRI1. The brassinosteroid belongs to a unique class of plant polyhydroxylsteroids that are crucial for plant growth (Clouse and Sasse, 1998; Jin et al. 2007).

1.4.3 Unfolded protein response (UPR)

Unproper function of the ER processing machinery results in the accumulation of unfolded proteins, which initiate activation of an adaptive signaling cascade known as unfolded protein response (UPR) (Malhotra and Kaufman 2007). Perception of ER stress and subsequent activation of UPR signaling pathways are required to maintain ER integrity. The UPR is defined by the induction of ER chaperones, by an increase in ER-associated degradation (ERAD), and by the attenuated translation of secreted proteins (Malhotra and Kaufman 2007). The molecular mechanisms underlying ER-QC and UPR signaling have been described mainly for yeast and mammals. Yeast inositol-requiring enzyme-1 (IRE1) was the first discovered ER stress sensor protein (Cox and Walter, 1996; Mori et al., 2000). IRE1 is an

ER-resident transmembrane receptor protein kinase / ribonuclease. It is activated via oligomerization and autophosphorylation processes elicited in response to ER stress. Thereafter, two more ER stress sensors, activating transcription factor 6 (ATF6) and interferon-induced double-stranded RNA-activated protein kinase-related protein (PERK), were found to activate UPR in mammals (Schröder and Kaufman, 2005). Mammalian ATF6 is a transmembrane protein at the ER membrane which senses ER stress by its C-terminal ER luminal domain. ER stress initiates the translocation of its N-terminal bZIP domain to Golgi bodies where it is cleaved by serine protease site-1 protease (S1P) and metalloprotease site-2 protease (S2P) (Ye et al., 2000). This process is termed regulated intramembrane proteolysis (RIP). The cleaved N-terminal cytosolic bZIP domain is released and translocated to the nucleus in order to induce expression of UPR genes (Yoshida et al., 2000). Mammalian PERK is also an ER-localized transmembrane sensor which senses ER stress through its luminal domain. PERK1 mediates phosphorylation of translation initiation factor-2α (eIF2α) thereby attenuating translation (Harding et al., 2000).

In contrast, prolonged ER stress or malfunctional UPR results in proapoptotic signaling and programmed cell death (PCD), which is mediated by the same set of ER stress sensors that are activating UPR (Schröder 2006, Szegezdi et al. 2006). In mammals, caspase cascades build a backbone of proapoptotic signaling (Szegezdi et al. 2006). The following scheme (Fig. 1-4) exhibits a summary of UPR and ER stress-induced proapoptotic signaling events in mammals.

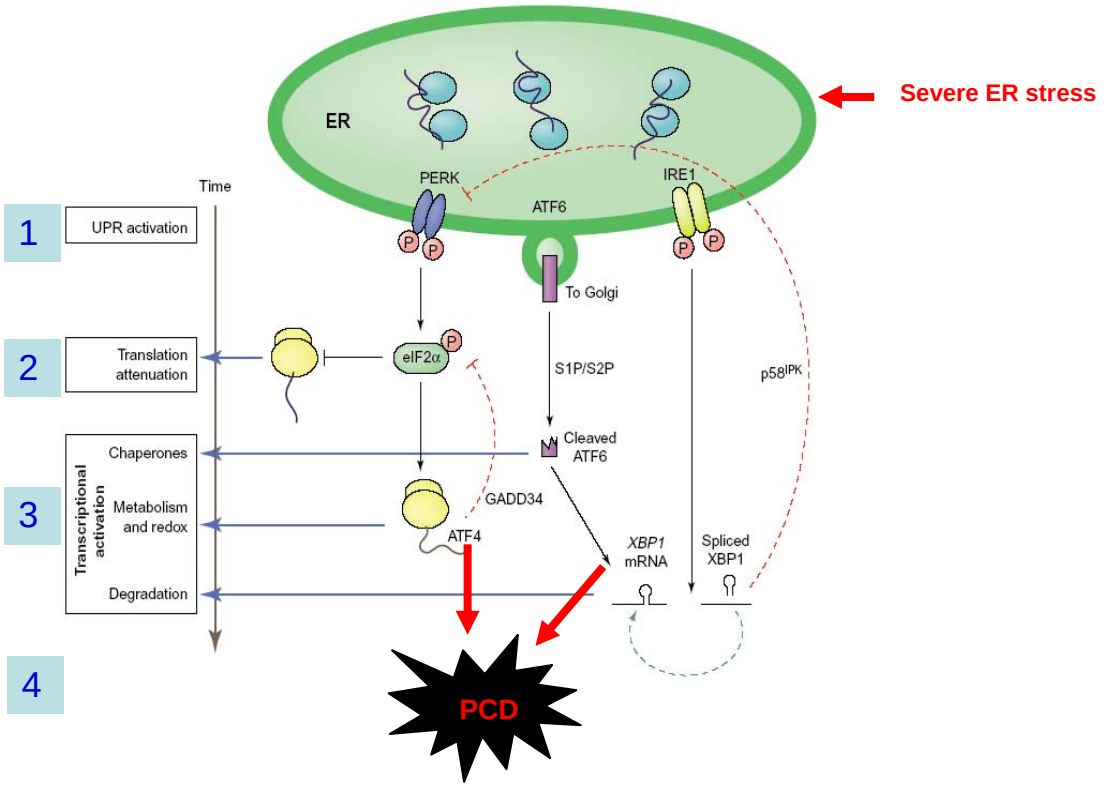


Figure 1-4. Mammalian UPR mediated by different ER stress sensors resident in ER. (Modified from Rutkowski and Kaufman, 2004)
This scheme indicates a temporal dimension of signaling events initiated by ER stress sensing molecules PERK, ATF6 and IRE1. UPR is firstly activated after stress perception by sensors. Thereafter translation is attenuated followed by the transcriptional activation of chaperones, ER metabolism and redox systems as well as components of the ER-associated degradation machinery. ER stress signaling is an interactive response as examplified by the IRE1 pathway whose execution requires the increased production of *XBP1* mRNA by ATF6. The broken arrows represent feedback loops, both positively (green) and negatively (red) act on the UPR. Finally, with the occurrence of severe ER stress, apoptotic signaling is induced and further leads to PCD. Notably, the function of PERK, ATF6 and IRE1 are probably not as mutually exclusive as described in this picture. The activation of some genes might require the action of more than one sensing pathway.

1.5 Programmed cell death

The controlled death of cells is an essential part of growth and development in many eukaryotic organisms, including animals and plants. In addition to its role in generative and vegetative reproduction, cell death also maintains a crucial role in the response to biotic and abiotic stresses. The initiation and execution of the cell death are strictly genetically determined and are therefore defined as programmed cell death (PCD). In eukaryotes, PCD

takes a major role in removing excessive, damaged or infected cells, which might have disadvantageous effects on tissue integrity (Kuriyama and Fukuda, 2002; Hoeberichts and Woltering, 2003; Hückelhoven, 2004).

1.5.1 ER stress-induced apoptosis in animals

The molecular and biochemical processes underlying PCD are far better understood in animals than in plants and one of the best characterized mammalian cell death types is apoptosis. Its specific biochemical and morphological features include activation of caspases cascade, condensation of the nucleus and cytoplasm, fragmentation of genomic DNA into large (50 to 300 kb) and subsequently small (200 bp) nucleosomal fragments (DNA laddering), and fragmentation of the cell into membrane-confined vesicles (apoptotic bodies) (Nooden, 2004; Hoeberichts and Woltering, 2003). Among them, the central component of the apoptotic machinery is the caspases cascade. In animals, caspases activation is triggered by either extrinsic or intrinsic signaling pathways. Specific “death receptors” can, upon activation, directly recruit caspase-activating multimeric protein complexes through what is called extrinsic pathway. By contrast, a diverse range of cellular stresses such as cytotoxic drugs and DNA damage can trigger caspase activation via the intrinsic pathway, which is mediated by cytochrome c release from the mitochondria. Alternatively, ER stress can directly induce caspase activity. Once activated, caspases may process and activate downstream caspases that cleave numerous cellular proteins, eventually leading to cell death (Hengartner, 2000; Hoeberichts and Woltering, 2003; Shiozaki and Shi, 2004).

In mammals, intensive studies have focused on the principles of ER stress-induced proapoptotic signaling, because ER stress-induced apoptosis is implicated in the patho-physiology of several neuro-degenerative and cardiovascular diseases. Szegezdi and colleagues (2006) described that continuous or severe ER stress will activate pro-apoptotic processes. UPR-mediated signals are thought to trigger apoptosis through three distinct phases, which are described as initiation phase, commitment phase and execution phase. PERK, ATF6, and IRE1 are not only mediating UPR but ER stress-induced cell death signaling is also depending on these receptors. In the initiation phase, PERK, ATF6, and IRE1 are fundamental

for the initiation of apoptotic signaling under severe ER stress. In this situation, PERK activates the bZIP transcription factor ATF4, which further induces the transcription factor C/EBP homologous protein CHOP, a well known apoptotic cell death elicitor (Ma et al., 2002; Ron and Habener 1992). The IRE1 and recruited tumor necrosis factor receptor-associated factor 2 (TRAF2) initiate cell death that might depend on the activation of apoptosis signal-regulating kinase 1 (ASK1)/c-Jun amino terminal kinase (JNK) pathway (Urano et al., 2000; Nishitoh et al., 2002). The ATF6 translocates to Golgi apparatus and is activated after cleavage by site-1 and site-2 proteases. Active ATF6 then moves to nucleus and induces genes containing an ER stress response element (ESRE) in their promoter (Schröder and Kaufman, 2005). The transcription factors CHOP and X-box binding protein 1 (XBP1) are identified to be targeted by ATF6. However, these pro-apoptotic signals triggered by PERK, ATF6, and IRE1 do not directly lead to cell death, but initiate the activation of downstream molecules such as CHOP and JNK to accelerate the occurrence of cell death in the subsequent commitment phase. Central to this proapoptotic state is the activation of proteins of the BCL2 family. Among this family, Bcl-2 is an antiapoptotic protein, which is down-regulated by CHOP. By contrast, other members of the BCL2 protein family such as Bax (Bcl2-associated X protein) and Bak (Bcl-2 homologous antagonist/killer) function as regulators of apoptotic cell death (Schröder, 2008). BAX and BIM (BCL2-interacting mediator of cell death) are key components in the execution of apoptosis by enhancing Ca^{2+} release from ER and mitochondria. In addition, BAX and BIM also mediate cytochrome c release from mitochondria thereby activating the apoptosome. Besides, recent studies also showed that reactive oxygen species (ROS) can directly activate ASK1 by disrupting an ASK1 thioredoxin (TDX) through oxidation of TDX, and thereby lead to activation of JNK and cell death (Tobiume et al., 2002). It indicated a significant contribution of oxidative stress to ER stress-induced apoptosis (Malhotra and Kaufman 2007). In the final execution phase, the concerted activation of transcription factors, kinase pathways, and regulation of BCL2 family proteins may lead to the activation of caspase cascades and further result in the ordered and sequential occurrence of cell death (Szegezdi et al. 2006). The complicated process of ER stress-induced apoptosis is summarized in Fig. 1-5 (Malhotra and Kaufman, 2007).

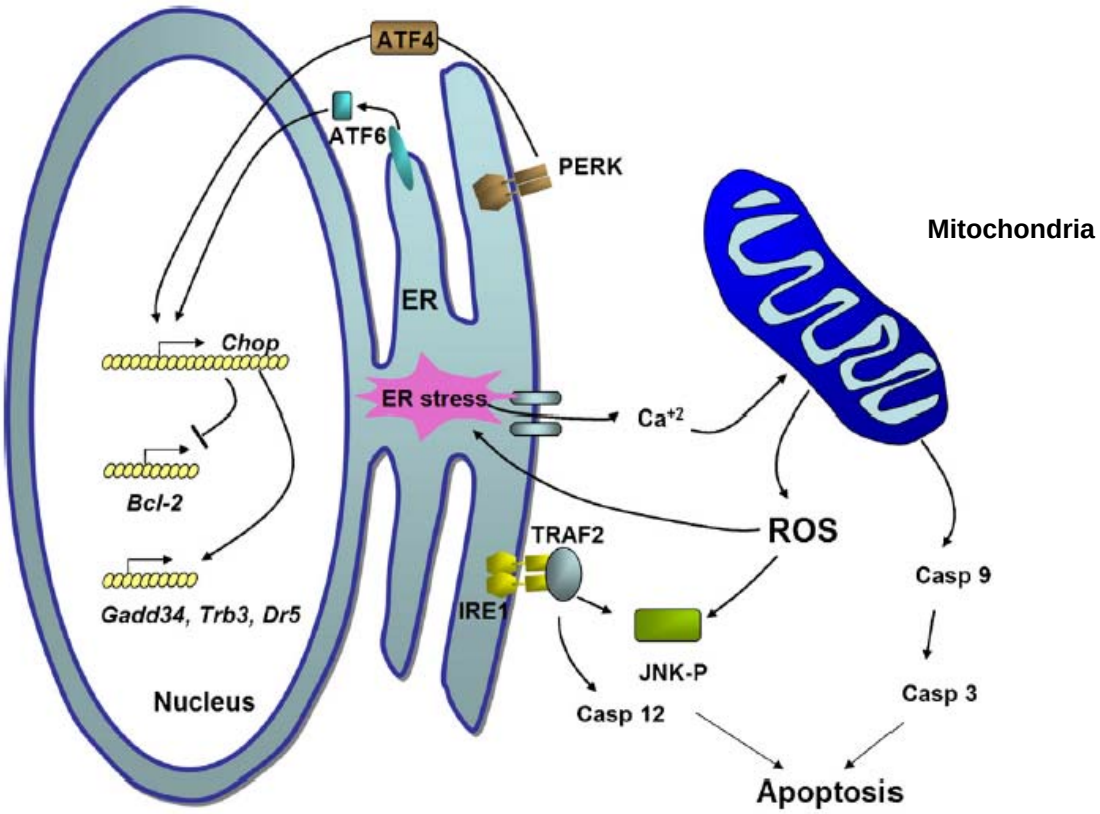


Figure 1-5. Pathways of ER stress-induced mammalian apoptosis. (Modified from Malhotra and Kaufman, 2007)

In this figure, the various ER stress-induced cell death pathways are summarized. The first signaling pathway is the elicitation of JNK phosphorylation and activation by IRE1 and recruited TRAF2. The caspase 12, which is an ER-associated effector in the caspase cascade further activates procaspase 9 to cleave procaspase 3, the main executioner of cell death. A second signaling pathway is mediated by ER stress-triggered transcriptional induction of proapoptotic genes such as *CHOP* through the ER stress sensors PERK, ATF6 and possibly IRE1. The third signally pathway depends on mitochondrial ROS production resulting from ER stress-induced Ca^{2+} release and depolarization of the inner mitochondrial membrane. Consequently, the caspase cascade is triggered to execute apoptosis. Therefore, it is concluded that unresolved ER stress activates cell death via multiple pathways.

1.5.2 ER stress signaling pathways in plants

Since the ER is the side of the production of the majority of nutritional plant proteins such as seed storage proteins of crop plants and therapeutic products as antibodies and vaccines, protein quality control and UPR of plants have recently attracted considerable attention (Urade, 2009). In plants, IRE1-like proteins, *Arabidopsis* IRE1s (AtIRE1-1 and AtIRE1-2) (Koizumi et al., 2001; Noh et al., 2002) and rice IRE1 (OsIRE1) (Okushima et al., 2002) were identified based on the similarity to those in yeast and mammals. However, plant IREs were

not proven to be associated with ER stress signaling. In addition, bZIP transcription factors bZIP28 and bZIP60 were isolated and shown to be ER stress responsive and even act as ER stress sensors or transducers (Liu et al., 2007; Iwata et al., 2005). Both proteins have functions in UPR similar to those reported for mammalian ATF6. Under non-stressed situations, bZIP28 and bZIP60 are ER membrane resident proteins. The accumulation of misfolded proteins results in the release of the N-terminal cytoplasmic domains of bZIP28 and bZIP60 and subsequent translocation into the nucleus. Interestingly, these transcription factors induce UPR genes containing P-UPRE and ERSE regulatory elements in their promoters. Transcription of bZIP28 and bZIP60 is activated by infection with heat stress and pathogens. Therefore, it is speculated that ER stress, heat stress and pathogens infection activate transcription of the same set of UPR genes (Urade, 2009). It still needs to be confirmed whether plant UPR pathways are interacting with other transcription factors in a complex network during various biological processes.

1.5.3 ER stress-induced cell death in plants

In plants, recent work suggested that programmed cell death (PCD) triggered by biotic or abiotic stresses such as pathogen attack, heat shock, ozone exposure, phytotoxin or oxidative stress-inducing agents also exhibits various morphological and biochemical traits of apoptosis, such as nuclear condensation, aggregation of chromatin at the nuclear margins, cell shrinkage, blebbing of the plasma membrane ,and DNA laddering, ROS accumulation, release of cytochrome c from mitochondria, and activation of hydrolytic enzymes such as serine and cysteine proteases and DNase (Lam, 2004; Greenberg and Yao, 2004; Lam, 2008). Despite of the observations of these classic morphological and biochemical features for animal apoptosis in plant PCD systems, sequenced plant genomes revealed the absence of plant homologues to several key regulators in animal PCD, including canonical caspases and Bcl-2 related proteins. Nevertheless, the transient activation of caspase-like protease (CLP) in plant PCD induced by biotic or abiotic stress (Lam and del Pozo, 2000; Woltering, 2004; Bonneau et al., 2008) and the functional Bax-inhibitor-1 protein (BI-1) in plants (Kawai et al., 1999) indicated that plant PCD might be controlled via a distinct set of regulators that performs similar functions to

animal PCD regulators (Watanabe and Lam, 2009).

In plants, two groups of proteases have been proposed as candidates to regulate caspase-like activities: the vacuole processing enzymes (VPEs) and the metacaspases (MCs) (Woltering et al., 2002). VPEs are cysteine protease, which exhibit caspase activities as reported for mammlian PCD. In contrast to the cytosolic localization of mammalian caspases, VPEs mainly localize in vacuoles. *Arabidopsis* maintains four *VPE* genes (*αVPE*, *βVPE*, *γVPE*, *δVPE*), which are classified by their homology and expression patterns (Hatsugai et al., 2006). Recent studies gave direct evidence that VPEs have caspase-like activity in *Nicotiana tabacum* and *Arabidopsis*. Furthermore, VPEs are found to mediate virus-induced hypersensitive cell death and are essential for mycotoxin-induced cell death (Hatsugai et al., 2004; Rojo et al., 2004; Kuroyanagi et al., 2005). In those studies, an ultrastructural analysis and a viability assay with protoplasts showed that disintegration of vacuolar membranes occurred in virus-infected leaves before the cells were dead (Hatsugai et al., 2004). The disintegration of vacuolar membranes continued, resulting in complete vacuolar collapse in association with plasmolysis and formation of cytoplasmic aggregations within the cells. By contrast, VPE-deficient plants prevented the vacuolar collapse followed by cell death after virus infection (Hatsugai et al., 2004). This observation suggests that VPE functions as a key molecule in cell death triggered by vacuolar collapse. So far, VPE is thought to be the first identified vacuolar component that regulates cell death and this vacuolar system-mediated cell death strategy in plants is not seen in animals (Hatsugai et al., 2006).

Several metacaspases of plants and fungi contain caspase domains (Uren et al., 2000). However, plant metacaspases are unable to cleave caspase-specific substrates. Instead, those metacaspases of *Arabidopsis* that have been analyzed were found to cleave arginine/lysine-specific substrates (Vercammen et al., 2004; Watanabe and Lam, 2005; He et al., 2007). In addition, their enzymatic activities are not inhibited by caspase inhibitors (He et al., 2007). Although metacaspases do not have caspase-like activities, several reports indicate that metacaspases play a role in plant PCD. For examples, the down-regulation of a type II metacaspase suppresses PCD in suspensor cells of an embryogenic culture of *Picea abies* (Suarez et al., 2004). The knock out (KO) lines of metacaspase type II exhibited a reduced cell death phenotype after challenge with the plant pathogen *Botrytis cinerea* (Van Baarlen et

al., 2007). The *metacaspase-8* KO lines were reduced in UVC or H_2O_2-triggered cell death and exhibited an increased tolerance to the PCD-inducing herbicide methyl viologen (He et al., 2007; Chen and Dickman, 2004). Taken together, certain metacaspases are obviously involved in the regulation/execution of PCD. It has to be analyzed whether all metacaspase have pro cell death activities and which of the different PCD pathways in plants depend on metacaspases (Bonneau et al., 2008).

Compared to ER stress-induced apoptosis in mammals, the molecular basis of ER stress-induced PCD in plants is less well understood. However, several indications implicate a conservation of ER stress signaling between plants and mammals. Bax inhibitor-1 (BI-1) is an evolutionally conserved protein that predominantly localizes to the ER membrane and acts as a broad spectrum cell death suppressor in mammals, fungi and plants (Watanabe and Lam, 2004; Hückelhoven 2004). Overexpression of BI-1 proteins from a variety of origins was shown to suppress Bax-induced and abiotic stress-induced cell death in numerous of eukaryotes (Watanabe and Lam, 2008b). In *Arabidopsis*, BI-1 was demonstrated to involve ER stress response and its related cell death pathway (Watanabe and Lam, 2008a). The data indicated that ER stress-induced PCD can be manipulated by the disruption of *AtBI1* or overexpressing AtBI1 proteins, which results in accelerated or attenuated PCD. Therefore, AtBI1 is thought to serve as a rheostat that functions to gauge the threshold of misfolded proteins in the ER for PCD activation (Watanabe and Lam, 2008b). Watanabe and Lam (2008b) summarized their data and indicated that AtBI1 is a critical survival factor for suppression of ER stress-induced PCD, thereby allowing the UPR sufficient time to recover cell homeostasis. However, the fundamental question regarding how ER stress-induced PCD pathway is regulated in plant cells still remains to be resolved (Urade, 2007).

1.6 Objective

Previous studies have demonstrated that the root endophytic fungus *Piriformospora indica* requires cell death for colonization during the mutualistic symbiosis with barley and *Arabidopsis* (Deshmukh et al., 2006; Jacobs, Zechmann, Kogel, Schäfer, unpublished). Since the genetic, molecular and biochemical mechanisms of *P. indica*-mediated cell death are still

not known, it is the aim of my work to elucidate the nature of the cell death associated with the colonization of *Arabidopsis* roots by *P. indica*. More specifically, I was interested to determine those processes particpating in the initiation, regulation and execution of colonization-associated cell death. These analyses would help to understand those mechanisms involved in the establishment of plant root-*P. indica* symbioses. Moreover, the studies will help to elucidate the impact of the different stages root colonization on beneficial effects mediated by the fungus. For my studies I followed cytological, genetic, molecular, and biochemical approaches to investigate cell death-associated colonization.

In the first cytological approach, I analyzed the root cell ultrastructural alterations associated with *P. indica* colonization with the assistance of transmission electron microscopy (TEM).and confocal laser scanning microscopy (CLSM). Based on these results I performed genetic and molecular studies to evaluate the fungal colonization in *Arabidopsis* mutants, which are in absence of genes encoding crucial ER-QC components and VPEs by using quantitative real time-PCR (qRT-PCR). Furthermore, I applied qRT-PCR to investigate the expression of UPR marker genes during the fungal colonization in mock- or TM-treated plant roots. By this, I aimed to check how UPR signaling is affected by *P. indica* at transcriptional level. Finally I carried out biochemical studies to dissect the biochemical mechanisms of root-*P.indica* interaction. Through immunoblot analyses, I checked the accumulation of ER-resident proteins in *P. indica* colonized roots. It further indicated how UPR signaling is affected by *P. indica* at translational level. Moreover, pharmaceutical analyses using specific caspase-1 and VPE substrates were performed to investigate the enzyme activities in *P. indica*-colonized roots. Subsequently, a fluorescein diacetate (FDA)-based cell death assay was performed to relate the enzyme activities and colonization studies to the occurrence of cell death.

2. Materials and Methods

2.1 Plant, fungal material and plant inoculation

2.1.1 *Arabidopsis Thaliana*

Arabidopsis (*Arabidopsis thaliana* L.) plants (cultivar Columbia-0) were obtained from the Nottingham Arabidopsis Stock Center. *Arabidopsis* seeds were sterilized with 3% sodium hypochlorite for 10 minutes and rinsed 3 to 4 times with autoclaved water before drying. The sterilized seeds were put on ½ MS medium or ATS medium in squared petri-dishes, incubated at 4°C for 48 hours prior to their transfer to a phyto-chamber for 3 weeks under the following: 8 h light (fluorescent cool white, Toshiba FL40SSW/37, 180 µmol m-2 s-1 photon flux density) / 16 h night, 22°C / 18°C, and 60% relative humidity. In order to guarantee that all plant roots grew on the surface of the medium, petri-dishes with sterilized seeds were vertically arranged. For propagation, *Arabidopsis* seeds were put on top of a 1:3 (v / v) sand : soil mixture (nutrient concentrations of the soil: N:P:K = 150:150:250 mg/L), and were incubated at 4°C for 48 hours before transfer to the greenhouse and their growth under long day conditions.

½MSmedium	**½ MS± medium**
4-5% gelrite	4-5% gelrite
½ concentration of MS salts	½concentration of MS salts
	1% sucrose

ATS medium

Stock solution	Vol. of stock to add for 1L	Micronutrients
1 M KNO_3	5 ml	70 mM H_3BO_3
1 M KPO_4	2,5 ml	14 mM $MnCl_2$
1 M $MgSO_4$	2 ml	0,5 mM $CuSO_4$
1 M $Ca(NO_3)_2$	2 ml	1 mM $ZnSO_4$

Micronutritients	1 ml	0,2 mM Na_2MoO_4 10 mM NaCl 0,01 mM $CoCl_2$

Table 2-1. Lists of applied *Arabidopsis* lines

Lines	Description	Source	Literature
Columbia (Col-0)	Wild type	NASC N1092	
αvpe	*alpha vacuolar processing enzyme*. alpha VACUOLAR PROCESSING ENZYME (VPE) belongs to a novel group of cysteine proteinases (VPEs) that is expressed in vegetative organs and is upregulated in association with various types of cell death and under stressed conditions.	I. Hara-Nishimura, Dep. of cell biology, National Institute for Basic Biology, Okazaki, Japan	Kuroyanagi et al., 2005
βvpe	*beta vacuolar processing enzyme*. Lacks a vacuolar processing enzyme that is expressed specifically in seeds and is essential for the proper processing of storage proteins.	I. Hara-Nishimura, Dep. of cell biology, National Institute for Basic Biology, Okazaki, Japan	Kuroyanagi et al., 2005
γvpe	*gamma vacuolar processing enzyme*. Lacks a vacuolar processing enzyme that is expressed in vegetative organs and is upregulated in association with various types of cell death and under stressed conditions.	I. Hara-Nishimura, Dep. of cell biology, National Institute for Basic Biology, Okazaki, Japan	Kuroyanagi et al., 2005
δvpe	*delta vacuolar processing enzyme*. Lacks a vacuolar processing enzyme with caspase-1-like activity that is specifically expressed in inner integument of developing seeds.	I. Hara-Nishimura, Dep. of cell biology, National Institute for Basic Biology, Okazaki, Japan	Kuroyanagi et al., 2005
vpe-KO	*vacuolar processing enzyme null*. Mutant lacks all four VPEs.	I. Hara-Nishimura, Dep. of cell biology, National Institute for Basic Biology, Okazaki, Japan	Kuroyanagi et al., 2005

Columbia (Col-6)	Wild type for *βvpe*	I. Hara-Nishimura, Dep. of cell biology, National Institute for Basic Biology, Okazaki, Japan	Kuroyanagi et al., 2005
bip2	It encodes Luminal binding protein 2 (BiP2) and functions as ER-resident chaperone.	X. Dong, Dep. of Biology, Duke University, Durham, NC, USA	Wang et al., 2005
dad1	*Defender against apoptotic death1*. It encodes ER-resident co-chaperone DAD1, which is a subunit of the oligosaccharyl transferase (OST) complex..	X. Dong, Dep. of Biology, Duke University, Durham, NC, USA	Wang et al., 2005
sec61α	It encodes a subunit of the SEC61 translocon complex, which provides a channel for proteins to cross the ER membrane.	X. Dong, Dep. of Biology, Duke University, Durham, NC, USA	Wang et al., 2005
bzip28	It encodes membrane-associated basic domain/leucine zipper (bZIP) factor, which is a candidate for ER stress sensors/transducers in plants.	S. Howell, Plant Sciences Institute, Iowa State University, USA	Liu et al., 2007
bip3	It encodes luminal binding protein, which functions as ER-resident chaperone.	C. Zipfel, The Sainsbury laboratory, Norwich Research Park, UK	Li et al., 2009

2.1.2 *Piriformospora indica*

In the work, the isolate of *P. indica* DSM11827 (German collection of microorganisms and cell cultures in Braunschweig, Germany) was applied. *P. indica* was isolate at the Indian Thar desert in 1997 (Verma et al., 1998). The chlamydospores of the applied *P. indica* isolate were stored in glycerin at -80°C, which can be used to prepare master plates. The fungi are subsequently propagated from master plates for experiments. The cultivation of *P.indica* was in modified *Aspergillus* minimalmedium (*complex medium*, modified after Pham et al., 2004) at room temperature for 6 to 8 weeks.

CM medium (Modified *Aspergillus* minimalmedium)

All components (1 liter)	20 x Salt solution	Microelements
20 x Salt solution	120 g $NaNO_3$	6 g $MnCl_2$ x $4H_2O$
20 g Glucose	10,4 g KCl	1,5 g H_3BO_3
2 g Peptone	10,4 g $MgSO_4$ x $7H_2O$	2,65 g ZnSO4 x 7H2O
1 g Yeast extract	30,4 g KH_2PO_4	750 mg KI
1 g Casamino-acid	Add 1 liter $H2O_{dest}$	2,4 mg Na_2MO_4 x $2H_2O$
1 ml Microelements		130 mg $CuSO_4$ x $5H_2O$
950 ml dest. Water		Add 1 liter $H2O_{dest}$
Optional: 15 g Agar-Agar		

2.1.3 Inoculation of plant roots with *Piriformospora indica*

P. indica growing on CM medium plates for 3-4 weeks was ready for preparation of spore suspensions. To collect spores from CM agar plates, sterilized water containing 0,05% Tween-20 was added. Through gently scratching the surface of plates with a spatula, the spores were released and the suspension solution was filtered through miracloth (Calbiochem, Bad Soden, Germany) in order to remove mycelium. After that, spores were collected by centrifuging suspension solution at 3500 rpm for 7 minutes. Then, spores were washed at least 3 times with sterilized Tween-H_2O. By using a hemacytometer in combination with a microscope, spore densities were determined. The spore concentration was adjusted to 500,000 spores / ml with sterilized Tween-H_2O. For inoculation, 1 ml spore suspension was pipetted on top of plant roots in one squared petri-dish.

2.2 Cyto-histochemical techniques

2.2.1 Fixation and staining of *P. indica* in root tissue with WGA Alexa Fluor® 488 (*Wheat Germ Agglutinin*)

The root material was fixed in a solution containing chloroform (20% v:v), ethanol (80% v:v), and trichloroacetic acid 0,15% w:v) for at least 24 h. Before staining, fixed roots were washed 3 x 5 minutes with stilled water. Roots were boiled in 10% KOH for 30 seconds, and then washed 3 x 5 minutes with 1 x PBS buffer (pH 7,4). Subsequently, root segments were incubated in 1 x PBS (pH 7,4) containing 10 µg/ml WGA Alexa Fluor® 488 (Molecular Probes, Karlsruhe, Germany) and 0,02% Silwet L-77. During the incubation, the roots were stained by vacuum infiltration three times for 1 minute at 25 mm Hg and kept in staining solution for 10 minutes. After washing with 1 x PBS buffer(pH 7,4), roots were put on glass slides and WGA-AF 488 was detected with epifluorescence microscopy (Axioplan 2, Zeiss, Oberkochen, Germany) at excitation of 470/20 nm and detection of 505-530 nm.

Staining solution	**1 x PBS buffer (pH 7,4)**
5 ml 1 x PBS buffer (pH 7,4)	0,2 g KCl
50 µg WGA Alexa Fluor® 488	0,2 g KH_2PO_4
0,05% Silwet L-77	1,15 g Na_2HPO_4
	Add 1 liter $H2O_{dest}$

2.2.2 Confocal layer scanning micropcopy

The live root material was stained with WGA-AF 488 as described in chapter 2.2.1. Confocal fluorescence images were recorded on a multichannel TCS SP2 microscope (Leica, Bensheim, Germany). WGA-AF 488 and GFP were excited with a 488-nm laser line and detected at 505–540 nm.

2.2.3 Sample preparation for transmmision electron microscope

Sample preparation for ultra-structural studies was performed according to Zechmann et al. (2007). Small pieces of roots (~2 cm) were cut and fixed in 2,5% glutardialdehyde in 0.06 M sodium phosphate buffer at pH 7.2 (Sørensen, 1909) for 90 min at room temperature (RT). Subsequently, samples were rinsed in 0.06 M sodium phosphate buffer at pH 7.2 (4 times 15

min each) and post-fixed in 0.1 M sodium phosphate buffer (pH 7.2) containing 1% osmium tetroxide for 90 min at RT. Samples were then dehydrated for 20 min each step in increasing concentrations of acetone (50%, 70%, 90%, 100%). Pure acetone was exchanged by propylene oxide and specimen were gradually infiltrated with increasing concentrations (30%, 50%, 70%, 100%) of Agar 100 epoxy resin (Agar Scientific Ltd., Stansted, England) for a minimum of 3 h per step. Samples were finally embedded in fresh resin agar at RT and polymerized at 60°C for 48 h. Ultrathin sections (80 nm) were cut with a Reichert Ultracut S ultramicrotome and post stained for five minutes with lead citrate and for 15 minutes with 1% uranyl acetate at RT before they were observed with a Philips CM10 TEM.

0,06 M sodium phosphate buffer (pH 7,2)

2 ml 0.2 M NaH_2PO_4

9 ml 0.2 M Na_2HPO_4

Add MiliQ H_2O to 35 ml

2,5% glutardialdehyde in 0.06 M sodium phosphate buffer (pH 7,2)

3,5 ml glutardialdehyde

31,5 ml 0.06 M sodium phosphate buffer (pH 7.2)

1% osmium tetroxide in 0,1 M sodium phosphate buffer (pH 7,2)

Add 5 ml 4% osmium tetroxide to 0,1 M sodium phosphate buffer (pH 7,2).

Agar 100 epoxy resin (prepare freshly for each step)

Agar 100 epoxy resin	20 ml	10 ml	5 ml	2,5 ml
DDSA (Hardener)	16 ml	8 ml	4 ml	2 ml
MNA (Hardener)	8 ml	4 ml	2 ml	1 ml
BDMA /(Accelerator)	1,3 ml	0,625 ml	0,312 ml	0,156 ml

All the components are vortexed in a closed plastic cup and heated up in microwave for 5 to 10 seconds until 60°C. Thereafter, the mixture is shaked thoroughly for a few seconds before adding BDMA (Accelerator) and propylenoxide. Subsequently, the plastic cup is closed and mixed carefully by hand. Because of an over pressure, the components should be thoroughly mixed after releasing the pressure.

2.3 Molecular biological standard methods

2.3.1 DNA-Extraction

Genomic plant DNA was extracted with the CTAB method. In addition, for screening the *Arabidopsis* mutants, a quick-dirty plant genomic DNA isolation method was applied.

- DNA extraction using CTAB method

Plant material was grinded to fine powder with liquid nitrogen of which 100 mg were mixed with 900 µl warm CTAB extraction buffer (65°C) by gentle inversion. This mixture was incubated for 60-90 minutes at 65°C with continuous gentle rocking. After that, 450 µl of chloroform / isoamylalcohol (24:1) was added to the cooled-down samples and mixed gently on a shaker for 5-10 minutes. Then the samples were spinned in a table-top centrifuge (Eppendorf, Hamburg, Germany) for 15 minutes with a speed of 10,000 rpm at room temperature. The supernatant was collected and 30 µg of RNase A was added. After incubation for 30 minutes at room temperature, 600 µl of isopropanol was added (20 minutes, RT) in order to precipitate DNA. The DNA pellet was obtained by centrifugation (13.000 rpm, 30 minutes, RT), and washed with WASH 1 solution (76% ethanol, 0,2 M sodiumacetate) as well as WASH 2 solution (76% ethanol, 10 mM ammoniumacetate) for 20 minutes. Finally, the dry DNA pellet was dissolved in 20-50 µl TE-buffer and the concentration was measured using a NanoDrop ND-1000 (peqLab Biotechnology GmbH, Erlangen, Germany).

CTAB Extraction buffer

100 mM Tris (pH 7,5)

700 mM NaCl

50 mM EDTA (pH 8,0)

1% $CTAB_2$

140 mM β-mercaptoethanol

TE-buffer

5 mM Tris-HCl (pH 8,0)

1 mM EDTA

Note: The buffer was freshly prepared and warmed to 65°C before adding the CTAB and β-mercaptoethanol.

- Quick-dirty plant genomic DNA isolation method

A small leaf from six-weeks-old *Arabidopsis* was crushed with a small pestle in liquid nitrogen and 500 µl of DNA extraction buffer was added. After vigorously vortex, the samples were incubated at room temperature for 10 minutes before adding 500 µl of chloroform. The samples were centrifuged at 13,000 rpm for 10 minutes and supernatants were removed to a new tube and precipitated by 500 µl isopropanol (2 minutes, RT). The DNA pellet was obtained by centrifugation (13,000 rpm, 10 min, RT) and washed with 70% ethanol. Subsequently, the dry pellet was dissolved in 20-50 µl TE-buffer and can be directly used for PCR.

DNA extraction buffer

200 mM Tris (pH 7,5)

250 mM NaCl

25 mM EDTA

0,5% SDS

H_2O_{dest}

Before RNA extraction, *Arabidopsis* roots were grinded to fine powder with liquid nitrogen and stored at -80°C until use. The extraction was performed as described by Logemann et al. (1987) or by using TRIzol (Invitrogen, Karlsruhe, Germany). DNase-I treatment was done (Fermentas, Germany) in order to remove DNA-contamination from extracted RNA.

- RNA-Extraction method (Logemann et al., 1987)

In the descibed protocol, 200 mg of grinded powder was added to 1 ml of RNA extraction buffer. After vigorously vortex, 200 µl of chloroform was added and shaked for 10 minutes at room temperature before centrifugation (13,500 rpm, 15 min, 4°C). The supernatant was transferred to a new tube and 850 µl chloroform was added. Then the samples were vortexed and again centrifuged (13,500 rpm, 15 min, 4°C). Subsequently, the supernatant was collected and precipitated by isopropanol (overnight, -20°C). The RNA pellet was obtained through centrifugation (13,500 rpm, 30 min, 4°C) and washed with 70% ethanol (2 times for 10 min). The dry RNA pellet was dissolved in 23-53 µl of H_2O_{DEPC}. The dissolved RNA was centrifuged for 10 minutes, and 20-50 µl of RNA was transfered to new tubes. Finally, the RNA concentration was determined by NanoDrop ND-1000 (peqLab Biotechnology GmbH, Erlangen, Germany).

RNA Extraction buffer

38 ml phenol

11,82 g guanidin thiocyanat

7,6 g ammonium thiocyanat

3,34 ml sodiumacetate (3 M)

5 ml glycerin

Add 100 ml H_2O_{DEPC}

H_2O_{DEPC}

$H2O_{dest}$+0.1% (v : v) DEPC

(Diethylpyrocarbonate)

- RNA extraction with TRIzol reagent

For the extraction of RNA with TRIzol, 50-100 mg of grinded material was well mixed in 1 ml TRIzol reagent and incubated for 5 minutes at RT in order to completely dissociate nucleoprotein complexes. Then 200 µl of chloroform was added. After vigorously shaking by hands for 15 seconds and incubation at RT for 2 to 3 minutes, the samples were followed by centrifugation (14,000 rpm, 20 min, 4°C). The RNA-containing aqueous supernatant together with 500 µl of isopropanol was incubated for 30 min at RT followed by centrifugation (14,000 rpm, 30 min, 4°C) in order to get pellet. Subsequently, the RNA pellet was washed with 75% ethanol and the dry pellet was dissolved in 30 µl of RNase-free water for 5 min at 65°C.

- **DNase-I digestion of RNA**

In order to remove DNA contamination in RNA, DNase-I digestion was performed in a 10 µl-system containing 1 µl 10 X DNase-I buffer (Fermentas, Germany), 1 µl DNase-I

(Fermentas, Germany), 0,25 µl RNase inhibitor (Fermentas, Germany) and 500 ng to 2 µg RNA. The mixture was incubated at 37°C for 30 minutes; then the reaction was inactivated by adding 2 µl EDTA and incubated at 70°C for 10 minutes. Finally, the concentration of cleaned RNA was measured by NanoDrop ND-1000 (peqLab Biotechnology GmbH, Erlangen, Germany).

2.3.2 Standard Polymerase Chain Reaction (PCR)

The regular standard PCR was performed in a 25 µl volume. The reaction mixture contained 1,25 U DCS-Pol DNA-polymerase (DNA Cloning Service, Hamburg, Germany), 2,5 µl 10 X Reaction buffer BD, 2,5 mM $MgCl_2$, 200 µM dNTPs (Amersham Pharmacia Biotech, Freiburg, Germany), 10 pmol relative oligonucleotides together with 100 pg to 100 ng DNA template The standard PCR amplification was proceeded in $T_{Professional}$ or $T_{Personal}$ cyclers (Biometra, Göttingen, Germany). The PCR scheme is described below. If necessary, the PCR products were mixed with 10 X DNA-loading buffer and then separated by gel electrophoresis. According to the size of PCR products, the concentration of TBE-gels was ranged from 1%-2% containing 0,75 v % ethidiumbromid (stock solution: 10 mg/ml). Subsequently, the visualisation of PCR products on the gel was performed 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).

PCR Scheme

	5 min	94℃	Denaturation
26 - 45	30 sec	94℃	Denaturation
cycles	30 sec	52 - 60℃	Annealing
	30 - 120 sec	72℃	Elongation
	7 – 10 min	72℃	Final elongation
	For ever	4℃	Reaction termination

10 X TBE

900 mM Tris
900 mM Boric acid
25 mM EDTA
Add 1L $H_2O_{dest,}$ pH 8,0

10 X DNA-Loading buffer

0.25% (w/v) Bromphenol blue
40% Sucrose

2.3.3 Revers transcriptional polymerase chain reaction (RT-PCR)

Different from standard PCR, the template of RT-PCR is RNA. The RT-PCR was performed with either one-step method that directly started from RNA or two-step method which cDNA was firstly produced from RNA, and then PCR was preceded using cDNA as template.

2.3.3.1 One-step RT-PCR

For one-step RT-PCR, QIAGEN OneStep RT-PCR kit was applied. According to the protocol, a master mix was prepared with 10 µl 5 X QIAGEN OneStep RT-PCR buffer, 400 µM of each dNTP, 0,6 µM specific oligonucleotides, 2 µl QIAGEN OneStep RT-PCR enzyme mix and the template RNA in the range of 1 pg to 2 µg per reaction was applied. Subsequently, the total 50 µl of mix was preceded in $T_{Professional}$ or $T_{Personal}$ cyclers (Biometra, Göttingen, Germany) and the running programs were described below. Finally, the amplified products could be detected by gelelectrophoresis as described in chapter 2.3.3.

One-step RT-PCR Scheme

	Reverse transcription:	30 min	50°C
	Initial PCR activation step:	15 min	95°C
	3-step cycling		
25-45 cycles	Denaturation:	0,5-1 min	94°C
	Annealing:	0,5-1 min	50-68°C
	Extension:	1 min	72°C
	Final extension:	10 min	72°C

2.3.3.2 cDNA synthesis

The transcription of total RNA to cDNA was performed with qScript^{TM} cDNA Synthesis kit (Quanta Biosciences, Gaithersburg, USA). 4 µl 5 X qScript reaction mix, 1 µl qScript reverse transcriptase and 500 ng of DNase-I treated RNA was mixed and incubated. When the reaction was completed, 80 µl $H2O_{DEPC}$ was added. For PCR-based analyses, 2 µl cDNA were applied.

Reverse transcription scheme

1 cycle: 22°C 5 min

1 cycle: 42°C 30 min

1 cycle: 85°C 5 min

2.3.4 Quantitative Real-Time PCR (qRT-PCR)

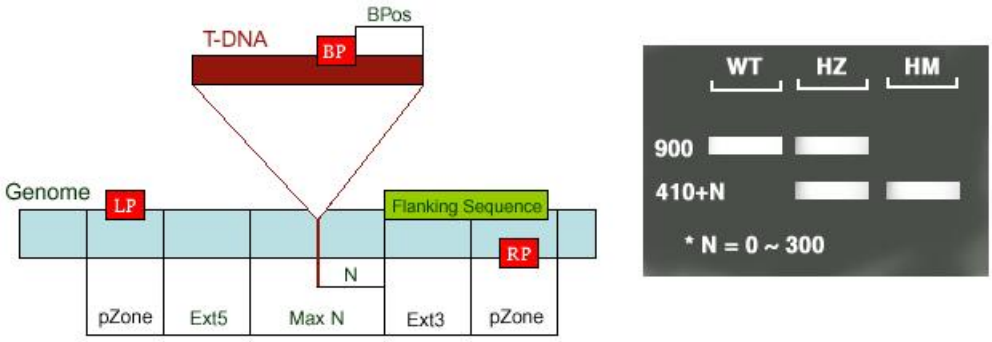
The quantitative real-time PCR was applied either to quantify the relative amount of *P. indica* genomic DNA and plant genomic DNA in colonised *Arabidopsis* roots or to analyse the relative expression level of candidate transcripts in cDNA samples. In each reaction (15 µl), 40 ng of genomic DNA or 10 ng of cDNA was used as template and added to 7.5 µl SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) and 350 nM relative oligonecleotides. In order to minimize the operating errors, each sample was triplicately pipetted. The amplification was performed in cyclers of Stratagene (Stratagene Mx3000P QPCR

SystemMx3000P; Stratagene Research, La Jolla, CA, USA) or of Applied Biosystems (Applied Biosystems 7500 Fast Real Time PCR, Applied Biosystems Inc., CA, USA). The running program started from the first step of denaturation (95°C, 7 min), and then continued with 40 amplification cycles with the annealing temperature of specific oligonuleotides (95°C 30 s, AT 30 s, 72°C 30 s). Finally, the SYBR fluorescence was measured for each cycle and then the PCR dissociation curve was recorded in the range of 65-95°C by 0,5°C steps. The cycle threshhold (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).

2.4 Forward genetic screening of cell death mutants and ER Stress mutants

2.4.1 Screening out homozygous lines of *Arabidopsis* mutants

The screened *Arabidopsis* mutants are listed in Table. 2-1. If the mutant stock seeds were heterozygous, a PCR-based screening method was applied to select out homozygous plants for further experiments. Ten seeds from the stock were separately grown in a sand-soil mixture (1:3). Small leaves from six-weeks-old plants were separately harvested and genomic DNA was extracted with a quick and dirty protocol (see 2.3.1). The T-DNA left border primer (LBb1,3), the left and right genomic primers (LP and RP) of T-DNA inserted mutants were designed by the T-DNA primer design software of the SALK Institute.



MaxN - Maximum difference of the actual insertion site and the sequence

Figure 2-1. SALK T-DNA primer design protocol. T-DNA fragment was inserted into genomic DNA. For SALK lines, by using primers of LB, genomic LP and RP, the wildtype (WT), heterozygous (HZ), and homozygous (HM) lines will be distinguished. WT lines, with no insertion, should get a product of 900-1100 bps (from LP to RP). HM lines, with insertions

in both chromosomes, will get a product of 410+N bps (300+N bases: from RP to insertion site, plus 110 bases: from LBb1.3 to left border of the vector). HZ lines, with insertion in one of the pair chromosomes, will get both products. LP, RP-left, right genomic primers; BP-T-DNA border primer LB; N-Difference of the actual insertion site and the flanking sequence position; MaxN-Maximum difference of the actual insertion site and the sequence; pZone-Regions used to pick up primers; Ext5, Ext3-Regions between the MaxN to pZone, reserved not for picking up primers. (http://signal.salk.edu/tdnaprimers.2.html)

Two pairs of PCR were set up: LBb1.3+RP (PCR1) and LP+RP (PCR2). In PCR1, a band at the size of 410+N bps in HM or HZ lines was expected. In PCR2; a product of 900-1100 bps was expected in WT or HZ lines. The PCR programme was according to 2.3.4. The sequence of LB1.3 and applied T-DNA line-specific LP and RP primers are listed in Table 2. The HM lines that were confirmed by PCR1 and PCR2 were continuously grown until the seeds were harvested for further experiments.

Table 2-2. Primers used for genotyping of T-DNA insertion lines

Mutants, AGI	Primers	AT(°C)	Sequences
αvpe	HtRV	55	5′-CGAAGCTTATGCCAGAAATGGACAA-3′
	LB3-1	55	5′-TAGCATCTGAATTTCATAACCAATCTCG-3′
βvpe	scrF	55	5′-TTCTCGCTGCCATGGCTAAG-3′
	CAPS	55	5′-CACCATCTCTTACACGATAGAT-3′
γvpe	Forward	57.3	5′-AGTGGGAAGGTTGTGGATAG-3′
	Reverse	52.0	5′-CTTTTCCCAAAAATGAACAAG-3′
δvpe	kForward	55	5′-TCATGCAAGGTGCTTATGTGTGA-3′
	kReverse	55	5′-CGTCCGTACCGTACAGTTGGTA-3′
	P06RB	55	5′-TTCCCTTAATTCTCCGCTCATGATC-3′
bip2, AT5G42020	LP	59.76	5′-CGACAGGTGCGACTAAAAATC-3′
	RP	60.02	5′-AGGTCACATTTGAAGTGGACG-3′
dad1,	LP	60.31	5′-AATGGCAGTTTTTCATCCCTC-3′

AT1G32210	RP	59.78	5′-TTTGTTCTCGTGCACGTAATG-3′
sec61α, AT2G34250	LP	59.96	5′-TCTTTGAAATTTGGAACGGTG-3′
	RP	59.85	5′-AGGAACAACAAATGGTGATGC-3′
	LBb1.3	52.4	5′-ATTTTGCCGATTTCGGAAC-3′

2.4.2 Production of *γvpe dad1* double mutant

In order to produce the *γvpe dad1* double mutant, both *γvpe* and *dad1* mutants were grown in a soil-sand mixture (3:1) in the greenhouse for at least 6 weeks. Prior to performing crossings, maturing flowers must be present in the bolting *Arabidopsis* plants. After crossings, homozygous *γvpe dad1* lines and the relative wild-type lines were screened out at the T2 generation.

2.4.2.1 Mutant crossings

During the crossing, *γvpe* was chosen to be the recipient line. An inflorescence from *γvpe* mutant was selected and all flowers that were either too young or already showing white petals were removed. 3-10 flowers were left to proceed with emasculation. With the help of a very fine forcep (INOX1), sepals, petals, and anthers were carefully removed without touching the stigma or style. Subsequently, the mature flowers from *dad1* mutants were used to rub or dab the stigma of emasculated *γvpe* plant. In order to avoid pollen contamination, the forcep was always cleaned with 70% ethanol after touching flowers of the *γvpe* and *dad1* mutants. Once crosses were made, the meristem and all smaller buds were pinched out to prevent further development. Finally, crossed inflorescences were labelled. After 2-3 days, elongations indicated successful crosses. Before harvesting crossed T1 seeds, ovaries were kept developing until they started yellowing.

2.4.2.2 Screening out homozygous and wild-type lines of *γvpe dad1* mutant

For identification of *γvpe dad1* mutants, a PCR-based screening method was applied. After crossing, the *γvpe dad1* T1 seeds were obtained from successful crosses. Around 10 T1 seeds were further grown on a soil-sand mixture (3:1) for at least 6 weeks. Thereafter, small leaf pieces were separately harvested from each line and DNA was extracted with a quick and dirty protocol (see 2.3.1). Using the programme of PCR1 and PCR2 together with genotyping primers of *γvpe* and *dad1* mutants described in 2.4.1, the positive *γvpe dad1* T1 lines were screened out. Subsequently, positive lines were grown for 4 weeks and seeds were harvested. Because of segregation of T1 seeds in T2 generation (WT : HZ : HZ : HM = 9:3:3:1), 40 seeds of a positive T1 line were further grown. By using the PCR-based screening method, 3 *γvpe dad1* T2 HM lines were successfully screened out. The seeds from *γvpe dad1* T2 HM and WT lines were used for further experiments.

2.4.3 Quantification of fungal colonization by qPCR

For each analysis, mutants as well as their parent lines were prepared at the same time. The seeds were sterilized, put on ½ MS or ATS medium in squared petri-dishes (see 2.1.1). Around 1 cm of medium in squared petri-dishes was cut before putting the seeds on the plates. Three-weeks-old plants were inoculated, 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 days after inoculation (dai), roots were harvested (3-4 plates / time points) and DNA was extracted by using the described CTAB method (see 2.3.1). Subsequently, the colonization level of plant roots by *P. indica* was analysed by qPCR (see 2.3.5). For each sample, 40 ng of genomic DNA served as template. At*UBQ5*-specific primers were used to amplify plant genomic DNA, while the of Pi*ITS* (Intragenic transcribed spacer)-specific primers were used to amplify *P. indica* DNA. The cycle threshold (Ct) values of At*UBQ5* and Pi*ITS* were extracted and the colonization levels of mutants and wildtype were calculated as described above. The sequences of At*UBQ5*-specific primers were 5′-CCAAGCCGAAGAAGATCAAG-3′ and 5'-ACTCCTTCCTCAAACGCTGA-3’. The sequences of Pi*ITS*-specific primers were 5′-CAACACATGTGCACGTCGAT-3′ and 5′-CCAATGTGCATTCAGAACGA-3′.

2.5 Gene expression analyses of *Arabidopsis* roots in the colonization of *P. indica*

In order to investigate how and to which extent *P. indica* interferes with the ER stress response / UPR (unfolded protein response), the kinetic analyses of UPR marker genes were performed in both tumicamycin-treated plants and tunicamycin-treated plants, which were inoculated with *P. indica* at 3 days prior to tunicamycin treatment. Gene expression patterns were compared in the two kinetic studies.

Seeds of *Arabidopsis* ecotype Columbia-0 were grown on ½ MS medium for 3 weeks. Non-colonized and *P. indica*-colonized roots were treated with tunicamycin (5 µg / ml) or DMSO (mock). For fungal treatment, roots were inoculated with *P. indica* chlamydospores (500,000 spores ml^{-1}) three days prior to tunicamycin and mock treatment. Roots were harvested as follows:

Table 2-3. Treatment and harvest of roots

	Treatment 1	**Treatment 2**	**Harvest time points**
$Kinetic_{TM}$ (I, II, III)	Tunicamycin (5 µg / ml)		0, ½, 1, 2, 3 days after treatment 1
$Kinetic_{Mock\ of\ TM}$ (I, II, III)	Tween H_2O (with DMSO)		0, ½, 1, 2, 3 days after treatment 1
$Kinetic_{P.\ indica+TM}$ (I, II, III)	*P. indica* (500,000 spores/ml)	Tunicamycin (5 µg / ml) (3 days after Treatment 1)	0, 3 days after treatment 1 and ½, 1, 2, 3 days after treatment 2
$Kinetic_{Mock\ of\ P.\ indica+TM}$ (I, II, III)	*P. indica* (500,000 spores/ml)	Tween H_2O (with DMSO) (3 days after Treatment 1)	0, 3 days after treatment 1 and ½, 1, 2, 3 days after treatment 2

Root material was harvested at the indicated time points. Firstly, one plant root from each plate was selected to be fixed and stained with WGA-AF 488 and forwarded to microscopical inspection of root colonization. *P. indica* colonization was detected by epifluorescence microscopy (see 2.2.1). Secondly, the other root material was grinded to fine powder with liquid nitrogen. The RNA was extracted with TRIzol reagent (Invitrogen, Karsruhe, country) and, subsequently, cDNA was synthesized as described (see 2.3.4.2). The relative expression levels of candidate transcripts were quantified by qRT-PCR using 10 ng cDNA template, 10 µl of SYBR Green and 350 nM specific oligonucleotides (see 2.3.5). The sequences of applied

primers are listed below.

Table 2-4. Sequences of applied qPCR primers

AGI code	Primer	Description	Sequences	Tm (°C)	Product size (bp)
	AtUBQ5	Ubiquitin	Fwd 5′-CCAAGCCGAAGAAGATCAAG-3′	60	139
			Rev 5'-ACTCCTTCCTCAAACGCTGA-3’		
AT1G09080	AtBIP3	ERSE-like, XBP1-BS-like	Fwd 5′-GGAGAAGCTTGCGAAGAAGA-3′	54	156
			Rev 5′-ATAACCGGGTCACAAACCAA-3′		
At5g07340	AtCNX2	Calnexin2	Fwd 5′-AGACTTTGAGCCTCCGTTGA-3′	58	249
			Rev 5′-TCTTCCTCGTCATCCCAATC-3′		
AT2G47470	AtPDI	ERSE-like, XBP1-BS-like	Fwd 5′-ATACGAAGCGAGGAGACGAA-3′	56	152
			Rev 5′-GCAGCCTTCTCGTACTCAGG-3′		
At1g77510	AtSPDI	ERSE-like	Fwd 5′-GCCACTAAGGCGATGATGTT-3′	56	129
			Rev 5′-GCTCTCTGCATCACCAACAA-3′		
AT3G10800	AtbZIP28	Membrane-associated basic domain/leucine zipper factor 28	Fwd 5′-GCCAGTGATCCTCTCTTTGC-3′	58	187
			Rev 5′-CAGAAGACAGTGCACCAGGA-3′		
AT2G40950	AtbZIP17	Membrane-associated basic domain/leucine zipper factor 17	Fwd 5′-ACAGGAGATCGGGAGAGGAT-3′	58	135
			Rev 5′-GCTCCTCGACGTAATGCTTC-3′		
AT3G56660	AtbZIP49	Membrane-associated basic domain/leucine zipper factor 49	Fwd 5′-CAAAACCACAGTCCCGAGTT-3′	56	105
			Rev 5′-TGGTCATGAGATGAGGGACA-3′		
AT1G42990	AtbZIP60	Membrane-associated basic domain/leucine zipper factor 60	Fwd 5′-GGCTGATAAAGTTCTAACCGT-3′	56	188
			Rev 5′-CCGCATCTCTATTTCTTACTCTC-3′		
AT5G50460	AtSEC61	Membrane-associated translocon complex	Fwd 5′-TTCACGAAAGTTGCAGTTCG-3′	55	105
			Rev 5′-ACCGACGATGATGTTGTTGA-3′		
AT5G47120	AtBI-1	*Arabidopsis* Bax inhibitor-1	Fwd 5′-GCAGCAGCAATGTTAGCAAG-3′	57	177
			Rew 5′-CACCACCATGTATCCCACAA-3′		

2.6 Investigation of MAMP-triggered immunity (MTI) in *Arabidopsis* mutants

In order to investigate whether the applied mutants still have regular MAMP-triggered defense responses, series of experiments were performed.

2.6.1 Analyses of flagellin-induced seedling growth inhibition

The seeds of *bip2*, *dad1*, and *sec61α* mutants in addition to seeds of wild-type Col-0 were sterilized and grown on ½ MS medium for 2 weeks. Subsequently, seedlings were transferred to 4-well multidishes containing liquid full MS medium containing 1% sucrose (10 seedlings per line, 1 seedling per well). For flagellin treatment, 1 µM flg22 was added to the medium. For mock treatment, mock solution was added to the medium. At 10 days after treatment (dat), fresh weight of both flg22- and mock-treated seedlings were measured.

Dissolving solution

0,1% BSA / 0,1 M NaCl

1,5 g BSA + 8,766 g NaCl

Add dH_2O to 100 ml.

1 µM flg22-solution	**Mock-solution**
1,5 µl flg22-stock was dissolved in 1 ml dissolving solution and added to 14 ml liquid full MS (+ 1% sucrose).	1 ml dissolving solution was added to 14 ml liquid full MS (+1% sucrose).

Note: Both of the flg22-solution and mock-solution are sterilized with filter membrane.

2.6.2 Analyses of flagellin- or chitin- induced oxidative burst

bip2, *dad1*, and *sec61α* mutants in addition to wild-type Col-0 were grown on a sand : soil-mixture (V/V=1:3) for around six weeks at short day conditions. Leaves of 6-weeks-old plants were cut to obtain 25 mm^2 leaf discs. Leaf discs were incubated in glass tubes

containing water overnight. Water was removed and 195 µl luminol solution together with 5 µl phosphate buffer was added. Samples were mixed and either 0.1 µM flg22 or 1 µM *N*-acetylchitooctaose added. Oxidative burst was measured using Berthold Lumat LB 9501 (Berthold, Bad Widbach) for 40 minutes.

1 mM luminol stock (5-Amino-2,3-Dihydro-1,4-Phthalazinedione)

Dissolve 1,77 mg luninol in 1 ml 10 mM NaOH (with sonication for 10-30 sec).

Add 9 ml H_2O_{dest}.

Horseradish peroxidase stock

1 mg/ml stock solution (2U / ml)

Luminol assay solution

2 ml Lumino stock

100 µl Horseradish peroxidase stock

Add $H2O_{dest}$ to 100 ml

Wrap the tube with aluminium foil and keep solution in dark.

Phosphate buffer

200 mM Na_2HPO_4 81%, 7,12g / 200 ml

200 mM NaH_2PO_4 19%, 5,52g / 200 ml

Adjust solution to pH 8.0

2.7 Investigation of ER stress response in *Arabidopsis* plant roots colonized by *P. indica*

2.7.1 Tunicamycin assay on mock-treated and *P.indica*-colonized *Arabidopsis* Columbia-0 plant roots

Col-0 seeds were sterilized and grown on squarred petri dishes with full MS gelrite containing 1% sucrose for 10 days. Thereafter, plants were either inoculated with *P. indica* or mock-treated. At 3 dai, seedlings were transferred to liquid MS solution containing 1% sucrose. 25 ng/ml tunicamycin or DMSO was added to these plants. Plant fresh weights were

measured at 7 dat.

2.7.2 Investigation of BIP protein accumulation in *Arabidopsis* roots in response to tunicamycin treatment and in dependence of *P. indica* colonization

2.7.2.1 Plant preparation and protein extraction

Arabidopsis Col-0 seeds were sterilized and grown on ½ MS medium under short day conditions. Roots of three-weeks-old plants were performed the following treatments. Firstly, the roots were inoculated with *P. indica* or mock-treated and harvested at 0, 3 and 7 days after treatment; secondly, the roots were treated with 5 µg/ml tunicamycin solution and harvested at 1 dat; thirdly, the roots were inoculated with *P. indica* at 3 days prior to tunicamycin or DMSO (mock) treatment, and root samples were harvested at 2 days after treatment.

All root samples harvested at indicated time points were grinded to fine powder using liquid nitrogen. For each sample, around 100 mg root material was added to 400 µl extraction buffer. After centrifugation at 14,000 rpm and at 4°C for 20 minutes, the supernatant was collected containing the protein extract. Protein concentrations were measured with the Bradford assay. Therefore, a standard curve with bovine serum albumin (BSA) was prepared. Protein absorbance was measured at 595 nm using a spectrometer (Biorad, Munich, Germany) in order to estimate proteins concentrations. Subsequently, 20 µg of each protein sample was obtained using 5 X sample reducing buffer (Pierce, Rockford, USA) and MiliQ H_2O. Samples were denatured at 95°C for 5 minutes. Samples were stored at -20°C until use.

Protein extraction buffer

250 mM sucrose

50 mM HEPES-KOH pH 7.5

5% glycerol

1 mM Na_2MoO_4

25 mM NaF

10 mM EDTA

2 mM DTT

0,5% Triton X-100

Protease inhibitor cocktail tablet (Roche, Mannheim, Germany) was freshly added and can be stored at -20°C for at least 12 weeks.

2.7.2.2 SDS-PAGE and western blotting analysis of BIP protein

The acrylamide gels (1 mm, 10%) including stacking gel and separation gel were prepared as described below. PAGE system was assembled and filled with 1 X SDS running buffer. The denatured samples were loaded on the gel and run at 100 V for ~1,5 h until bromophenol blue front reached the end of the gel. Thereafter, the apparatus was dissembled and gels were transferred to 1 X TOWBIN buffer.

For western blot analysis, Roti-PVDF membrane with a pore size of 0.45 µm (Karl Roth, Karlsruhe, Germany) was cut and activated by methanol. Subsequently, the western sandwich was assembled as described below and put in cold 1 X TOWBIN buffer.

Cathode

Black grid of clamp
Sponge
2 Watman paper
Gel (facing the cathode)
PVDF membrane
2 Watman paper
Sponge
Transparent/red grid of clamp

Anode

After blotting (80 V for 2h or 25 V overnight, 4°C), proteins are transferred to PVDF membrane. Thereafter, the membrane was blocked with 1 X TBS-Tween 20 / 5% milk powder buffer (2h at RT or overnight at 4°C) before further incubation with anti-BIP antibody (Santa Cruz, Heidelberg, Germany; Dilution factor = 1:1000) and alkaline phosphatase-conjugated anti-rabbit antibody (Sigma-Aldrich, Munich, Germany; Dilution factor = 1:5000). After washing of incubated membranes with 1 X TBS-Tween 20 (4 times, 5 minutes each), the membrane was further washed in alkaline phosphatase (AP) buffer for 10 minutes. Finally, by

incubating the membrane with Immunstar AP substrate (Biorad, Munich, Germany) for 5 minutes, signals were detected using X-ray films.

Polyacrylamide gel (for 2 mini-gels, 10%)

Seperationgel:	Stacking gel:
2,5 ml lower gel 4 X buffer	2,5 ml upper gel 4 X buffer
4,9 ml $H2O_{DEPC}$	6,6 ml $H2O_{DEPC}$
2,5 ml acrylamide solution 40%	0,8 ml acrylamide solution 40%
50 µl 10% ammonium persulfate	100 µl 10% ammonium persulfate
5 µl TEMED	10 µl TEMED

Note: Ammonium persulfate is always freshly prepared.

10x SDS running buffer (pH 8,3)	**1 X TOWBIN buffer**
250 mM Tris	25 mM Tris
1,92 M Glycine	192 mM Glycine
1% SDS	20% Methanol
Add H_2O_{dest} to 1 Liter	Add H_2O_{dest} to 1 Liter

20xTBS-T (Tris Buffered Saline Tween)	**Alkaline PhosphataseBuffer (AP buffer)**
3 M NaCl	100 mM TRIS pH = 9.5
200 mM Tris pH=8	100 mM NaCl
1% Tween-20	50 mM $MgCl_2$

2.8 Investigation of caspase-dependent vacuolar cell death in *Arabidopsis* plant roots colonized by *P.indica*

2.8.1 Caspase 1 and vacuolar processing enzyme activity assays

In this assay, *bip2*, *dad1*, *γvpe*, *vpe-KO*, *γvpe dad1* mutants together with wild-type Col-0 plants were grown on ½ MS medium for three weeks. Subsequently, three-weeks-old plant roots were inoculated with *P. indica* or mock-treated, and roots were harvested at 7 dai. A buffer containing 100 mM sodium acetate (pH 5,5), 100 mM NaCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride was applied and root material homogenized. Root extracts

were collected after centrifugation at 5500 rpm for 5 minutes. In order to measure caspase 1 and VPE activity, 1 mM fluorogenic caspase 1 substrate (Ac-YVAD-MCA) and VPE substrate (Ac-ESEN-MCA) (Peptide Institute, Osaka, Japan) were added to the root extracts. After incubation for 15 minutes and 30 minutes, fluorescence intensities were measured at 465 nm after excitation at 360 nm using a fluorescence microplate reader (TECAN infinite® 200, Männedorf, Switzerland).

2.8.2 Cell death assay

In this assay, *bip2*, *dad1*, *γvpe*, *vpe-KO*, *γvpedad1* mutants together with wild-type Col-0 plants were grown on ½ MS medium for two weeks. Subsequently, two-weeks-old plant roots were either treated with tunicamycin solution (5 µg / ml) or inoculated with *P. indica.* At 3 days after tunicamycin treatment and 7 days after *P. indica* inoculation, root samples were segmented to 1.5 cm pieces (8 segments / line) and transferred to liquid ½ MS medium containing 2 µl of fluorescein diacetate (FDA) stock solution. After 10 minutes incubation, FDA-stained root segments were washed 5 times and the fluorescence intensities were measured at 535 nm after excitation at 485 nm using a fluorescence microplate reader (TECAN infinite® 200, Männedorf, Switzerland).

Fluorescein diacetate stock solution

Dissolve 5 mg fluorescein diacetate in 1 ml aceton.

3. Results

3.1 *P. indica* impairs the integrity of the endoplasmic reticulum

3.1.1 Ultrastructural studies on *P. indica*-colonized *Arabidopsis* root cells by transmission electron microscopy

In order to observe and monitor subcellular changes associated with *P. indica* colonization of *Arabidopsis* root cells, transmission electron microscopy (TEM) was performed. These studies revealed an initial biotrophic colonization up to 3 days after inoculation (dai), which was followed by a cell death-associated colonization (> 3 dai) of *Arabidopsis* roots by *P. indica*. Cell death-associated colonization was characterized by the lysis of cytoplasm, and then followed by swelling of the endoplasmic reticulum as well as tonoplast rupture, which is an indication of vacuolar collapses. Nevertheless, plastids and mitochondria remained ultrastructurally unaltered (Fig. 3-1A, B).

3.1.2 Confocal laser-scanning microscopy studies on *P. indica*-colonized *Arabidopsis* roots

The TEM studies indicated ER disintegration by *P. indica*. In order to substantiate the indications of impaired ER integrity, colonization of *Arabidopsis* line GFP-tmKKXX was analyzed by confocal laser-scanning microscopy. The KKXX is a C-terminal signal of tomato *Cf-9* disease resistance gene, which encodes a type I membrane protein carrying a cytosolic dilysine motif. Previous studies on mammals and plants demonstrated that this motif promotes the retrieval of type I membrane proteins from the Golgi apparatus to the ER. In the above line, green fluorescent protein was fused to the transmembrane domain of Cf-9 and expressed in *Arabidopsis* (Benghezal et al. 2000). In this study, *P. indica*-colonized GFP-tmKKXX roots were stained with WGA-AF488 and the fungal intracellular hyphae were visible. The confocal fluorescence images indicated ER structure collapses in colonized root

cells. This collapse was restricted to *P. indica*-colonized root cells as ER remained intact in adjacent non-colonized root cells (Fig. 3-1C).

Taken together, the cytological studies indicated that the integrity of the ER was impaired by *P. indica*.

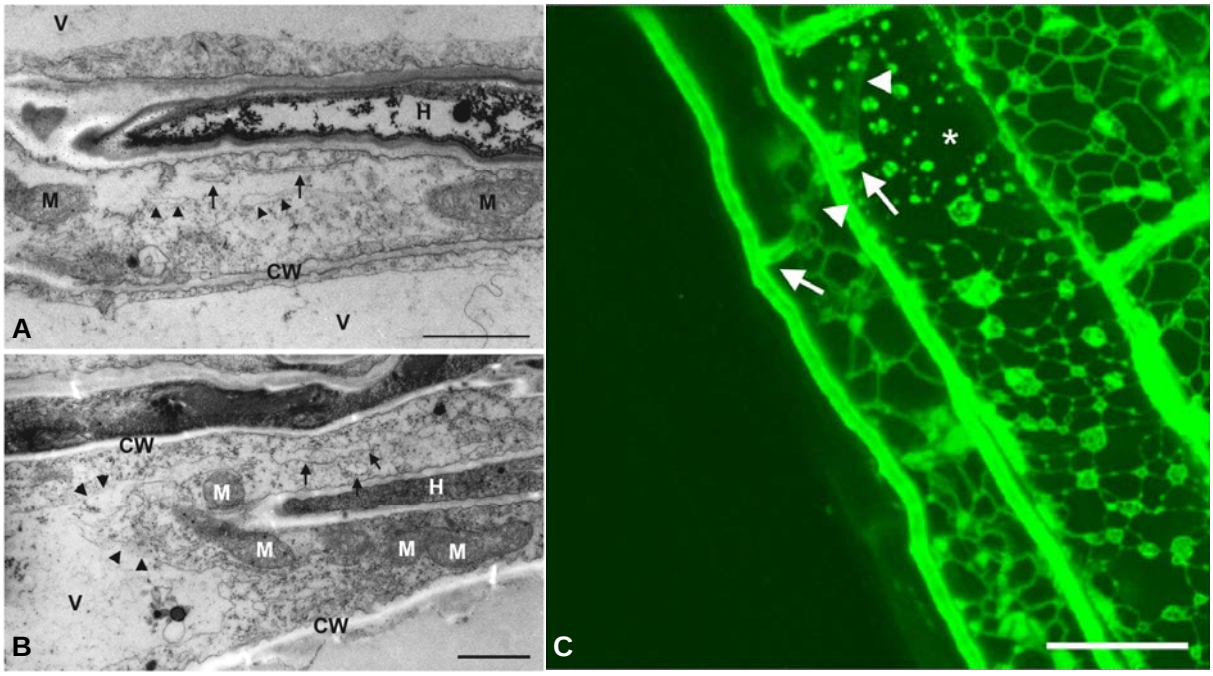


Figure 3-1. ER disintegration in *Arabidopsis* root cells during cell death-associated colonization by *P. indica*.

A, B: Transmission electron micrographs (TEM) show intracellular fungal hyphae. A: Cell death-associated colonization at which the ER is partially swollen (arrowheads). Intact ER is also visible (arrows). Tonoplast of non-colonized neighbouring cells is intact. B: At later cell death stages, the ER disintegrates (arrows) and vacuolar collapse (arrowheads) is visible. H, hyphae; CW, cell wall; M, mitochondria; V, vacuole. Bars = 2 µm. C: Confocal microscopy of *P. indica*-colonized GFP-tmKKXX (ER marker) expressing plant roots. The fungus penetrated (arrows) two cells and intracellular hyphae are visible (arrowheads). The ER of the lower cell is still intact, while ER disintegration is associated with colonization of the upper cell. Note the ER of surrounding, non-colonized cells is intact. *P. indica* was stained with WGA-AF488. Intracellular hyphae are faintly stained due to limited dye diffusion. WGA-AF488 and GFP were excited with a 488 nm laser line and detected at 505-540 nm using a TCS SP2 CLSM (Leica). Bar = 20 µm.

3.2 Impaired ER function improves mutualistic root colonization

3.2.1 Quantification of *P. indica* colonization on roots by qRT-PCR

As the cytological analyses demonstrated colonization-associated ER impairment, the question arose to what extend the disturbed ER function would affect fungal colonization success. Therefore, *Arabidopsis* mutants lacking central components of the ER processing machinery were selected. These mutants, which were comprimised in the ER-resident chaperone BIP2 (luminal binding protein 2, *bip2*), the DAD1 (defender against apoptotic death 1, *dad1*, a subunit of the oligosaccharide transferase complex [OST]), or in a component of the SEC61 translocon complex (SEC61α, *sec61α*), were analyzed for *P. indica* colonization. To this end, *bip2*, *dad1*, *sec61α*, and their parent line Col-0 were checked for altered colonization at 3 and 7 dai with *P. indica*. The relative amount of fungal DNA in plant roots was determined by qRT-PCR. All mutants exhibited enhanced fungal colonization rates at 7 dai compared with Col-0 (Fig. 3-2).

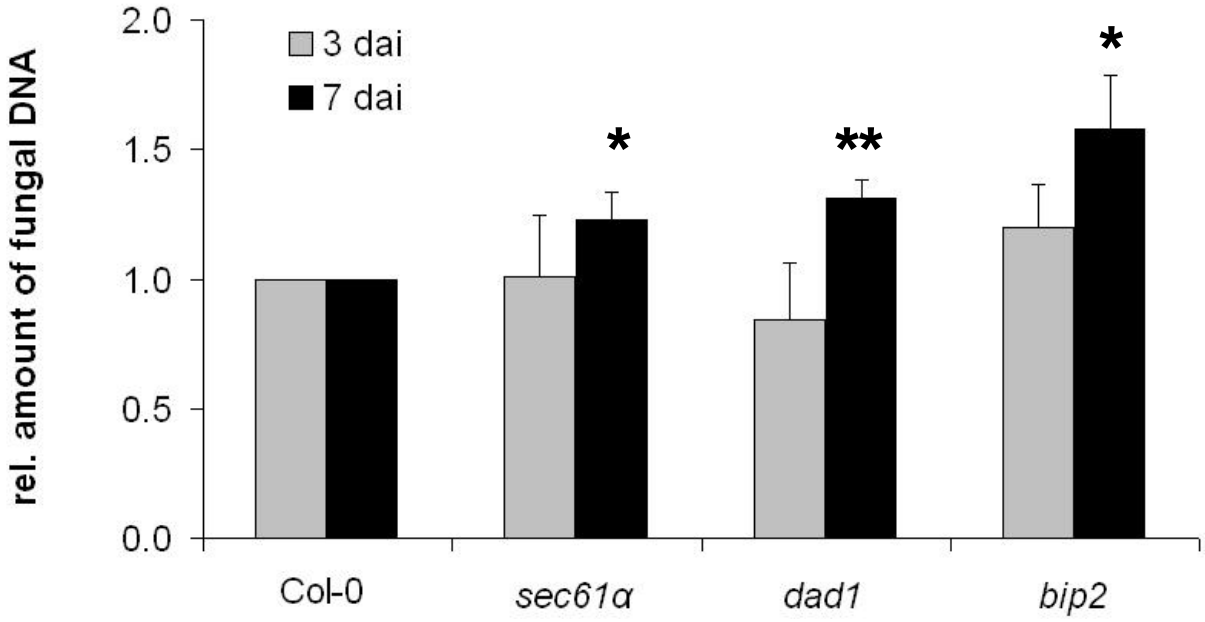


Figure 3-2. Increased *P. indica* colonization of mutants *sec61α*, *dad1,* and *bip2* at the cell death-associated colonization stage (7 dai).
Three-week-old *Arabidopsis* Col-0 plants as well as *sec61α*, *dad1,* and *bip2* mutants were inoculated with *P. indica*. Root samples were harvested at 3 and 7 dai. The fungal biomass were determined by qRT-PCR at biotrophic (3 dai) and cell death-associated colonization stages (7 dai) using *AtUBI5* and *PiITS*-specific primers. The root fungal colonization levels in all mutants were normalized to Col-0 colonization. Results shown are means of three

independent experiments. For each experiment, around 200 plants were analyzed per line at each time point. Asterisks indicate significance at $P < 0.05$ (*), 0.01 (**) analyzed by Student's *t*-test.

3.2.2 Analyses of MAMP-triggered immune responses on investigated *Arabidopsis* mutants

Altered colonization might be explained by malfunctional immunity in these ER processing mutants. Therefore, it was necessary to elucidate their immune responsiveness to microbe-associated molecular patterns (MAMPs). A series of experiments were performed. In a first assay, two-week-old plant roots from *sec61α*, *bip2*, *dad1* mutants and their parent line Col-0 were treated with flg22 or respective mock solution. Subsequently, flg22-induced seedling growth inhibition (SGI) was analyzed in all plants. Plant biomass of Col-0 and all mutants was significantly reduced by flg22 treatment, as opposed to flg22-insensitive *fls2c* mutant (Fig. 3-3). This indicated sensitivity of mutant seedlings to the MAMP flg22.

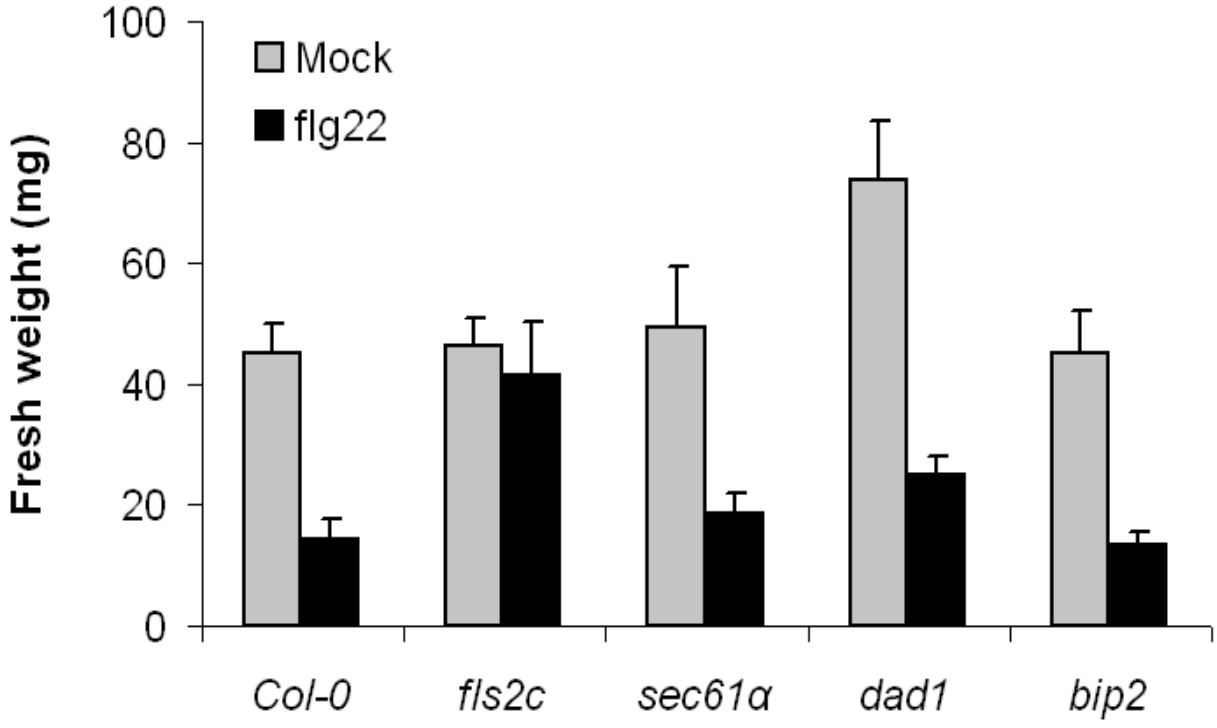
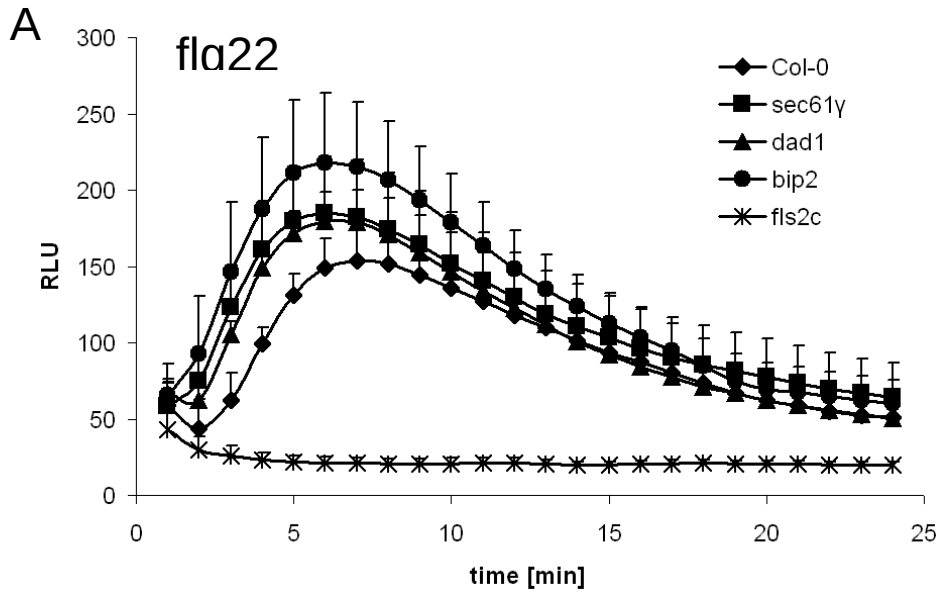


Figure 3-3. flg22-triggered growth inhibition in seedlings of *sec61α*, *dad1* and *bip2* mutants.
Roots of two-week-old WT plants (Col-0) together with *sec61α*, *dad1*, *bip2*, and *fls2c* (flg22 insensitive) mutants were challenged with 10 µM flg22. All mutants displayed WT-like growth inhibition. *fls2c* mutants served as flg22-insensitive control. Plant fresh weights were determined 10 days after flg22 treatment. Data represents mean values of three independent biological experiments.

Secondly, the occurrence of flg22-, or chitin-induced oxidative burst in mutants and wildtype Col-0 roots were analysed. Transient root oxidative bursts upon treatment with flg22 or chitin were observed in *bip2*, *dad1*, *sec61α* mutants and Col-0 but not in flg22- and chitin-insensitive mutants *fls2c* and *cerk1-2*, respectively (Fig. 3-4). Taken together, the data indicated the responsiveness of the mutants to chitin and flg22-triggered immunity.



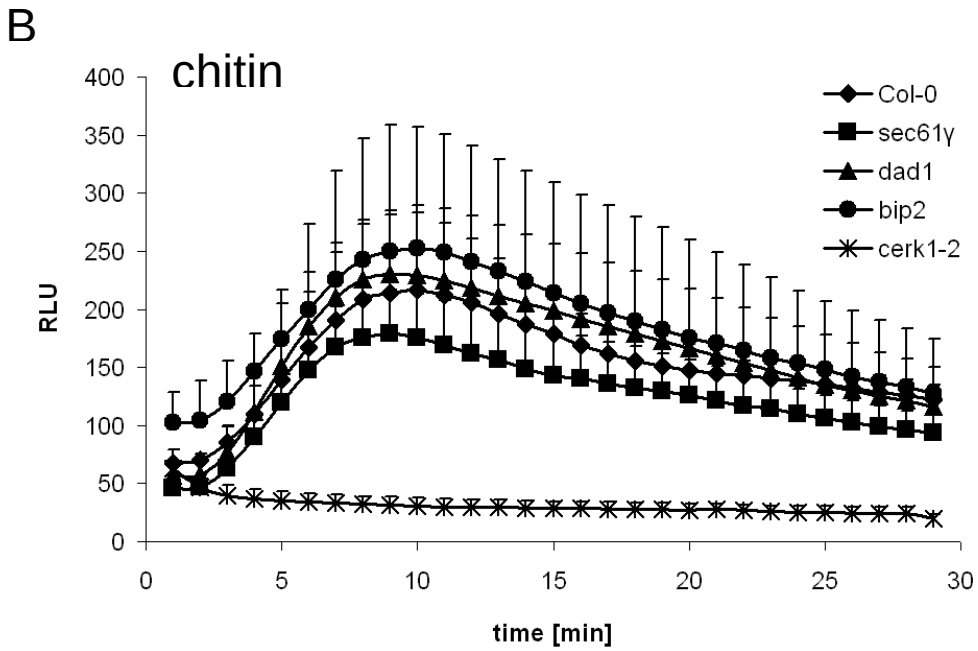


Figure 3-4. flg22- or chitin-induced ROS production in *sec61α*, *dad1,* and *bip2* mutants. Roots of two-week-old WT plants together with *sec61α*, *dad1*, *bip2*, *fls2c* (flg22 insensitive), and *cerk1-2* (chitin insensitive) mutants were challenged with 0.1 µM flg22 (A) or 1 µM *N*-acetylchitooctaose (chitin) (B). *fls2c* and *cerk1-2* mutants served as flg22- or chitin-insensitive control, respectively. Oxidative bursts were measured in 10 mg root segments (1 cm each segment) by a luminol-based assay directly after application of respective MAMPs. Values are given as relative light units (RLU) over time. Data displayed are means with standard errors of four independent measurements per treatment of one biological experiment. Experiments were repeated thrice with similar results.

3.3 *P. indica*-colonized plants are hypersensitive to ER stress

Since fungal colonization analyses indicated improved colonization of mutants lacking crucial components of the ER processing machinery, the question arose whether *P. indica* colonization affects ER stress tolerance of plants. A pharmaceutical approach with the application of ER stress inducer tunicamycin was performed. Tunicamycin specifically blocks dolichol synthesis and thereby inhibits protein *N*-glycosylation. In this assay, both of *P. indica*-colonized (3 dai, biotrophic stage) and mock-treated (3 dat) Col-0 plants were treated with tunicamycin (TM, 25 ng/ml) or DMSO (control). Plant fresh weights were determined at 7 days after treatment. In non-colonized plants, TM treatment resulted in a ~ 20% reduction of fresh weight compared to DMSO-treated plants. While in *P. indica*-colonized plants, TM

reduced the plant biomass significantly by ~ 60% (Fig. 3-5). This indicated that *P. indica*-colonized plants were hypersensitive to ER stress.

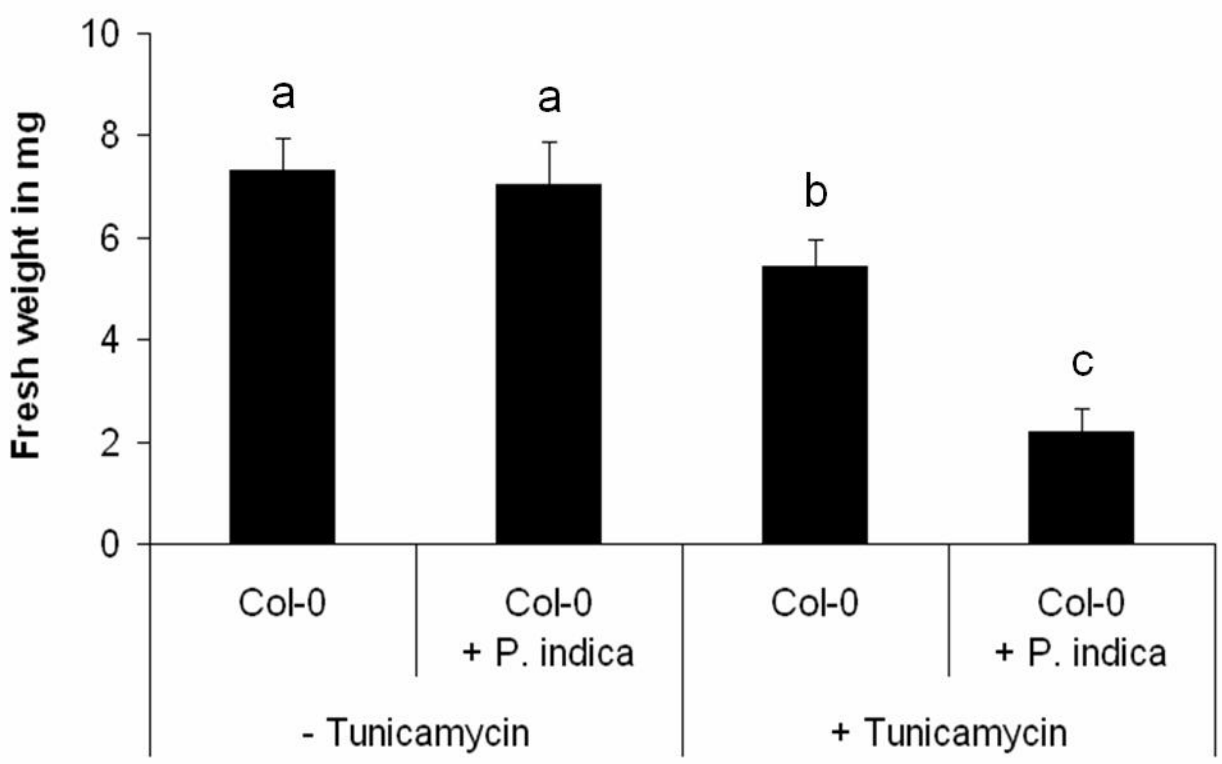


Figure 3-5. *P. indica*-colonized plants are hypersensitive to ER stress.
Arabidopsis Col-0 plants were grown on MS gelrite plates containing 1% sucrose for 10 days. Thereafter, plants were inoculated with *P. indica* or mock-treated. Three days later, seedlings were transferred to liquid MS solutions (+ 1% sucrose) containing 25 ng/ml tunicamycin or DMSO (mock). Fresh weights of seedlings were determined at 7 days after treatments. The experiment was repeated three times with similar results. For each experiment, 10 plants were analyzed per treatment. Letters indicate significance analyzed by Student's *t*-test.

3.4 The unfolded protein response is suppressed by *P. indica*

The cytological and pharmaceutical data indicated the occurrence of ER stress in plant roots during *P. indica* colonization; it prompted us to investigate whether root colonization resulted in an activation of ER stress signaling. In order to elucidate this question, the unfolded protein response (UPR) in *P. indica*-colonized roots was analyzed at transcriptional and translational levels.

3.4.1 Gene expression analyses of ER stress sensors and UPR markers in *P. indica*-colonized *Arabidopsis* roots

Arabidopsis Col-0 plant roots were inoculated with *P. indica* or treated with mock solution. *P. indica*-colonized and mock-treated roots were harvested at 1, 3, and 7 dai in order to monitor gene expression levels of putative ER stress sensors (*bZIP17*, *bZIP28*, *bZIP60*) and markers of the unfolded protein response (UPR) (*sPDI*, *BIP3*, *CNX2*) by qRT-PCR. Interestingly, none of the tested genes was induced during fungal colonization (Fig. 3-6). Moreover, expression levels of *bZIP28*, *BIP3*, and *CNX2* were even suppressed in colonized roots at various time points (Fig. 3-6).

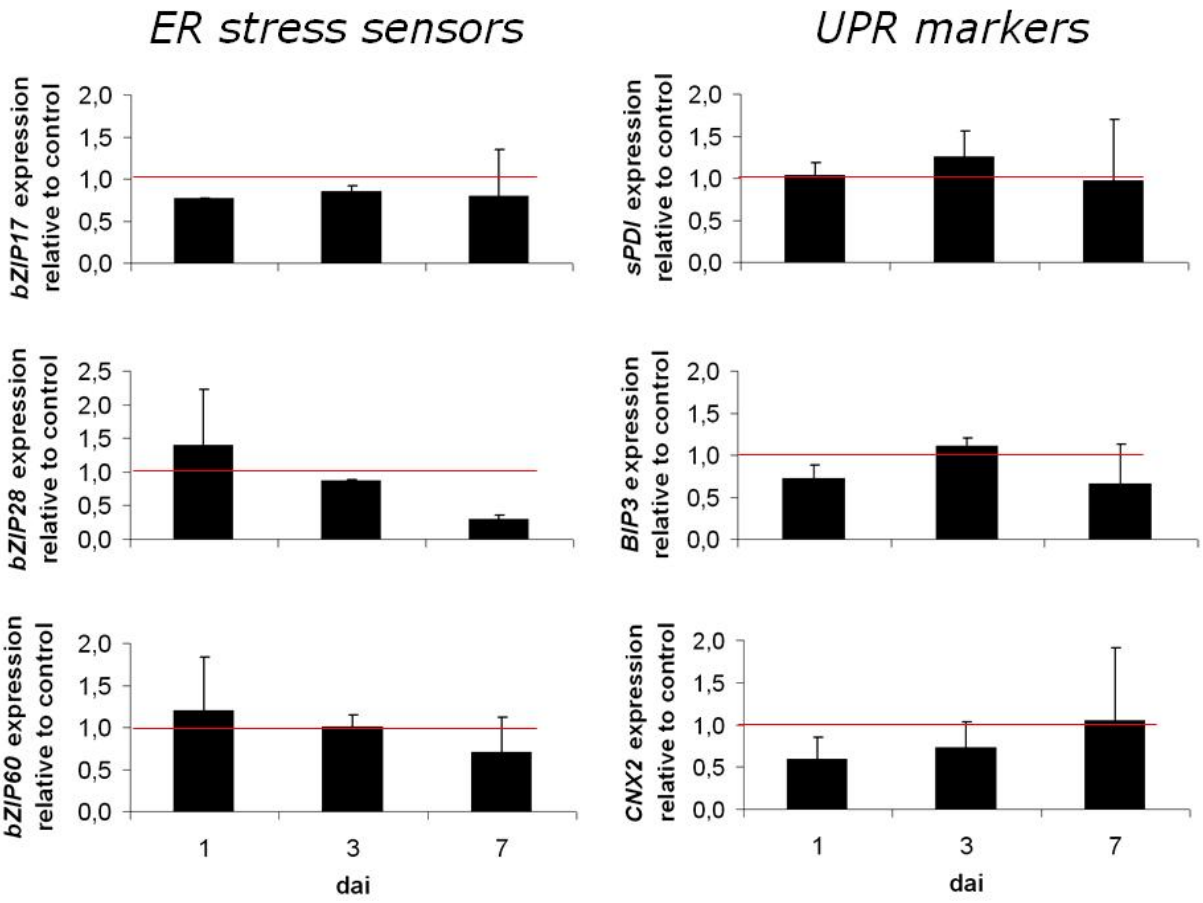


Figure 3-6. ER stress signaling is not induced during root colonization by *P. indica*. Expression of ER stress sensors (*bZIP17*, *bZIP28*, *bZIP60*) and markers for the unfolded protein response (*sPDI*, *BIP3*, *CNX2*) was measured by qRT-PCR. For the analyses, three-week-old *Arabidopsis* WT plants were inoculated with *P. indica* or mock-treated. Root samples were harvested at 1, 3 and 7 dai. The obtained Ct thresholds of the candidate genes

were related to the Ct thresholds of the housekeeping gene *AtUBI5* using the $^{\Delta\Delta}$Ct method. Data shown represent fold changes of candidate genes and display the ratio of candidate expression in colonized roots relative to mock-treated roots. The values are means with standard error and base on three independent biological experiments.

Subsequently, in order to elucidate if the selected ER stress markers were induced by ER stress in roots and if *P. indica* might suppress ER stress signaling, another experiment was set up, in which both non-colonized and *P. indica*-colonized roots (3 dai, biotrophic stage) were treated with tunicamycin (TM, 5 µg/ml) or DMSO (control). The root samples were harvested at 1 and 3 days after TM treatment and the expression levels of ER stress sensors (*bZIP17*, *bZIP28*, *bZIP60*) and UPR markers (*sPDI*, *BIP3*, *CNX2*, *BI-1, SEC61γ*) were analyzed by qRT-PCR. The analysis indicated that all tested genes (except *bZIP28*) were induced by TM treatment (Fig. 3-7). By contrast, all genes (except *bZIP28*) exhibited a reduced induction in *P. indica*-colonized roots upon TM treatment (Fig. 3-7).

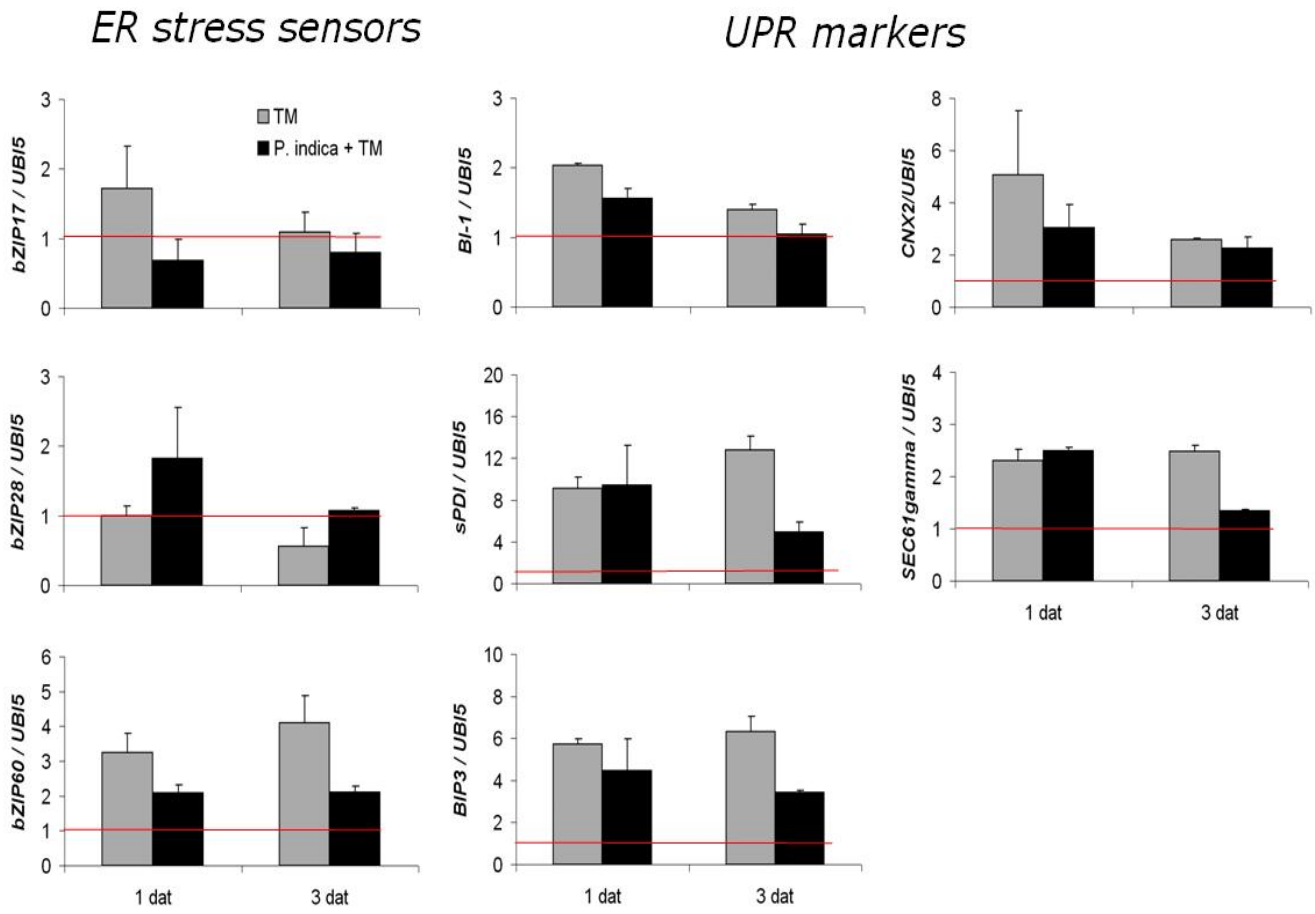


Figure 3-7. Tunicamycin-induced ER stress is suppressed by *P. indica*.
Three-week-old *Arabidopsis* WT plants were inoculated with *P. indica* (*Pi*) or mock-treated. Three days later, inoculated and mock-treated plants were treated with tunicamycin (TM, 5 µg/ml) or DMSO (control). Root samples from different treatments (*Pi* + TM, *Pi* + control; mock + TM, mock + control) were harvested at 1 and 3 dat. Data represent the Ct thresholds of the indicated candidate genes relative to the Ct thresholds of the housekeeping gene *AtUBI5* using the $^{\Delta\Delta}$Ct method. $^{\Delta\Delta}$Ct values obtained from *Pi* + TM samples were divided by $^{\Delta\Delta}$Ct values of *Pi* + control to obtain the displayed fold changes. Similarly, $^{\Delta\Delta}$Ct values of samples mock + TM were divided by $^{\Delta\Delta}$Ct values of mock + control. The values are means with standard error and base on three independent biological experiments.

3.4.2 Accumulation of UPR marker BIP in *P. indica*-colonized *Arabidopsis* roots

In complementary analyses, we investigated if impaired ER stress signaling was also detectable at the protein level. The regulatory protein, luminal binding protein (BIP), is one of ER-localized chaperones. It functions in cotranslational folding and modification of secreted proteins (Koizumi, 1996). The expression of BIP is induced in reponse to tunicamycin or heat-shock stress (Koizumi, et al., 1999). In the presented study, BIP accumulation was investigated in *Arabidopsis* roots during *P. indica* colonization. First, I tested the accumulation of the luminal binding protein (BIP) in dependence of *P. indica* colonization as described above for transcriptional analyses (Fig. 3-8A). *P. indica*-colonized and non-colonized roots were harvested at 0, 3, and 7 dai and forwarded to immunoblot analyses using the *Arabidopsis* polyclonal anti-BIP antibody. In analogy to our gene expression analyses by qRT-PCR, BIP protein levels were reduced in colonized roots compared to non-colonized roots at 3 and 7 dai (Fig. 3-8A). In addition, BIP accumulation after TM treatment in dependence of *P. indica* colonization was monitored. For this, *P. indica*-colonized (3 dai) and non-colonized roots were treated with TM or DMSO (control) and roots were harvested two days after treatment. The immunoblot analyses with anti-BIP antibody revealed that BIP accumulation was clearly increased by TM treatment in non-colonized roots, which indicated translational induction of UPR (Fig. 3-8B). By contrast, BIP accumulation was obviously suppressed by *P. indica* in TM-treated roots.

Taken together, the analyses indicated that *P. indica* is affecting ER stress signaling at transcriptional and translational levels.

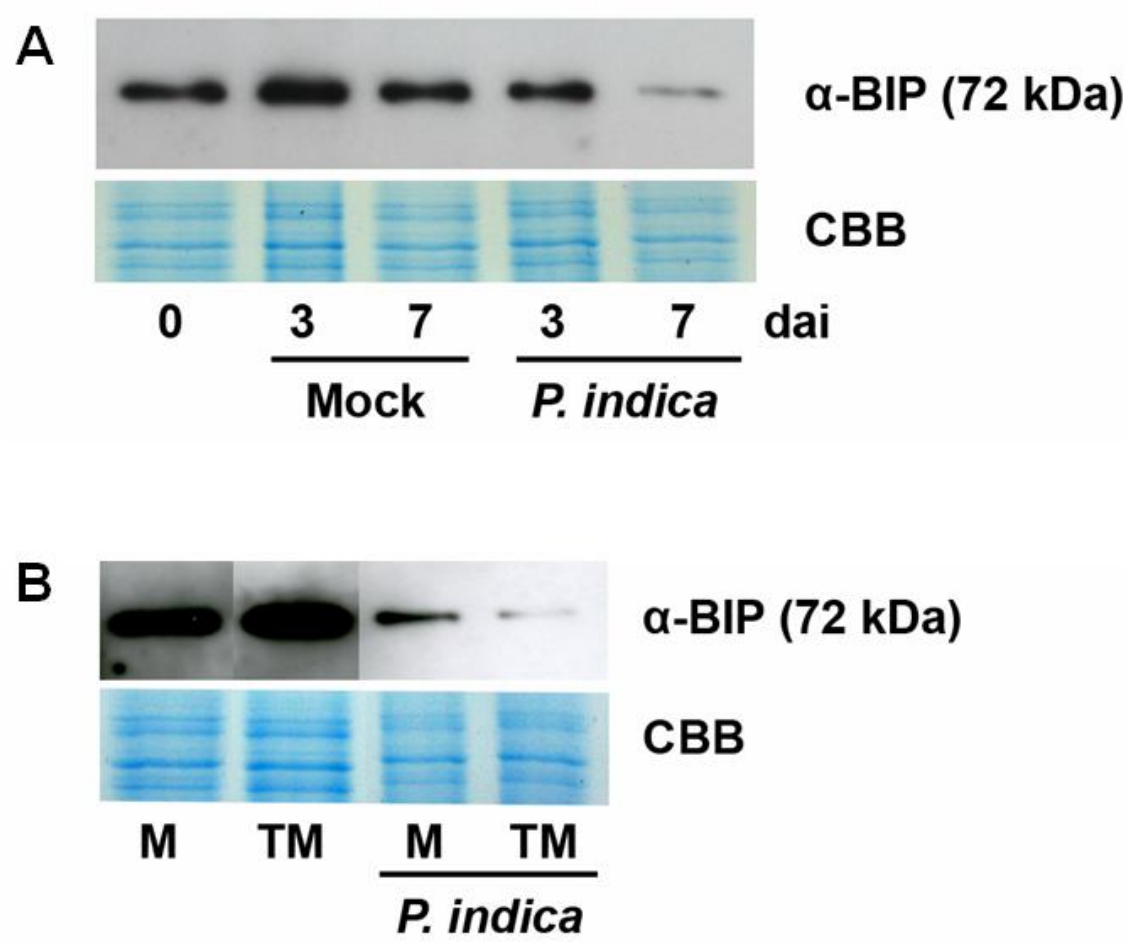


Figure 3-8. **ER-localized BIP protein accumulation is impaired by *P. indica*.**
A: BIP protein accumulation during *P. indica* colonization. For the analyses, *Arabidopsis* WT roots were inoculated with *P. indica* or mock-treated and harvested at 0, 3 and 7 dai for protein extraction. B: BIP protein accumulation indicative of ER stress in dependence of TM treatment and *P. indica* inoculation. Samples were run on the same blot but the lanes were rearranged for presentation. For the analyses, *Arabidopsis* WT roots were inoculated with *P. indica* or mock-treated. At 3 dai (biotrophic stage), roots were treated with TM (5 µg /ml) or DMSO (control) and harvested 2 dat. For all experiments, 20 µg total protein per sample was separated by 10% SDS-PAGE. Immunoblot analyses were performed with *Arabidopsis* anti-BIP antibodies. The staining with coomassie Brilliant Blue indicates equal loading of all samples.

3.5 Vacuole-mediated cell death is downstream of ER stress induction and affects mutualistic root colonization

The previous TEM studies indicated the co-occurrence of ER swelling and vacuolar collapse in colonized cells at later interaction stages (Fig. 3-1B). Therefore, I followed the question

whether vacuole collapse is essential for cell death-associated root colonization. Vacuolar processing enzymes (VPEs) are summarized in a small gene family consisting of four members (αVPE, βVPE, γVPE, δVPE). VPEs possess proteolytic activities and cleave caspase-1-specific substrates (Hatsugai et al., 2006). In mammalian system, programmed cell death depends on caspase activities (Cohen, 1997). In plants, it was reported that VPEs mediate vacuolar collapse and are important for the execution of virus-induced cell death (hypersensitive response) (Hatsugai et al. 2004). In a first experiment, fungal colonization of all *vpe* mutants was determined at 3 and 7 dai by qRT-PCR.

3.5.1 Deficiencies in vacuolar processing enzymes alter *P. indica* colonization of roots

In this assay, we quantified root colonization of *αvpe*, *βvpe*, *γvpe*, *δvpe*, and the null mutant *vpe-KO* at 3 and 7 dai by qRT-PCR. Compared to wild-type plants, altered colonization patterns were observed in *vpe* mutants. In *αvpe* and *γvpe* mutants, the relative amount of fungus was efficiently reduced at 7 dai. In addition, *γvpe* mutant showed an increased fungal colonizaion at 3 dai. *βvpe* and *δvpe* mutants showed an (non-significant) enhancement in fungal colonization at 3 and 7 dai (Fig. 3-9). Notably, the relative fungal colonization in *vpe-KO* mutant revealed a higher colonization level at earlier stages (3 dai) similar to that in *γvpe*, but a lower colonization level at later stages (7 dai) (Fig. 3-9).

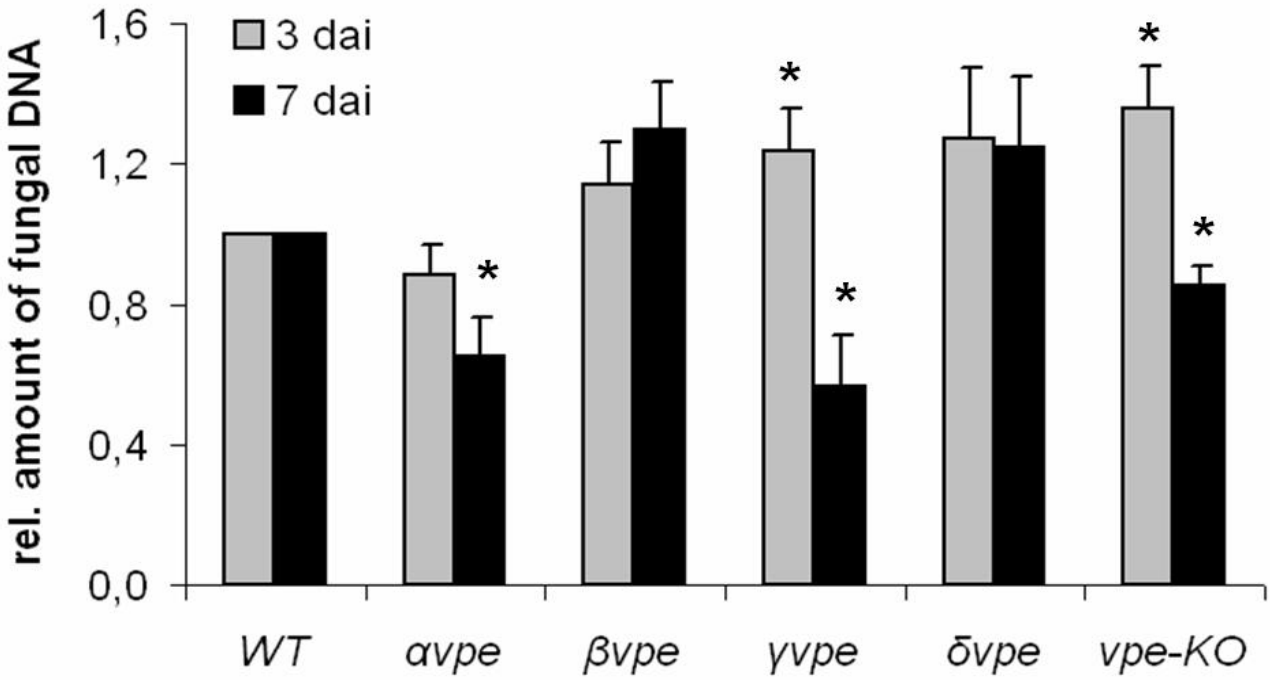


Figure 3-9. ***P. indica*** **colonization of** ***Arabidopsis*** **roots is dependent on vacuolar processing enzymes (VPEs).**
For the analysis, three-week-old *Arabidopsis* WT, *αvpe*, *βvpe*, *γvpe*, *δvpe* and *vpe* null mutants (*vpe-KO*) were inoculated with *P. indica*. Root samples were harvested at 3 and 7 dai. The fungal biomass was determined at both biotrophic (3 dai) and cell death-associated colonization stages (7 dai) by qRT-PCR using *AtUBI5* and *PiITS*-specific primers. The fungal colonization levels of all mutants were normalized to WT colonization. Results shown are means of three independent experiments. For each experiment, around 200 plants were analyzed per line at each time point. Asterisks indicate significance at $P < 0.05$ (*) analyzed by Student's *t*-test.

3.5.2 Analyses of *P. indica* colonization on *γvpedad1* roots

Since severe ER stress results in cell death in mammalian organisms, we were interested if VPE-mediated cell death might be downstream of ER stress induction. In order to elucidate this question, a *γvpe dad1* double mutant was generated. In a first experiment, *P. indica* colonization of *γvpedad1* roots was determined by qRT-PCR.

3.5.2.1 Production of double mutant *γvpedad1* by PCR-based screening analyses

Based on the colonization phenotype of *γvpe* mutant, it was chosen as the recipient line and crossed with *dad1* mutant. Seeds of *γvpe dad1* F1 generation were obtained. Considering the possibility of false crossing, F1 seeds (~10 seeds) were separately grown on sand: soil mixture (1:3) to confirm successful crossing in a PCR-based approach. Therefore, six-week-old plant leaves were separately harvested and genomic DNA of each plant was extracted. Two PCR reactions were performed, which identified *γvpe* and *dad1* mutant-specific T-DNA insertions in the selected lines. The positive lines #2 and #12, which contained *γvpe* and *dad1* T-DNA insertions, were used to receive homozygous F2 seeds (Fig. 3-10).

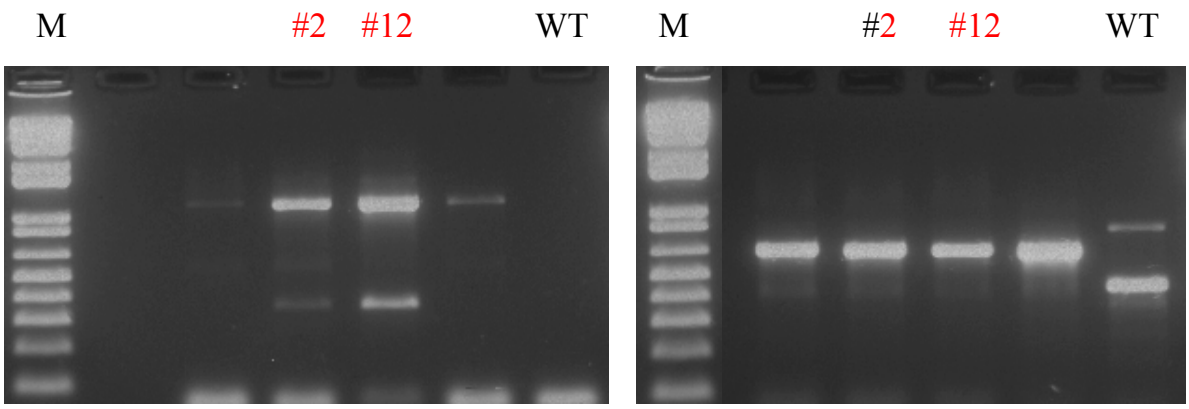
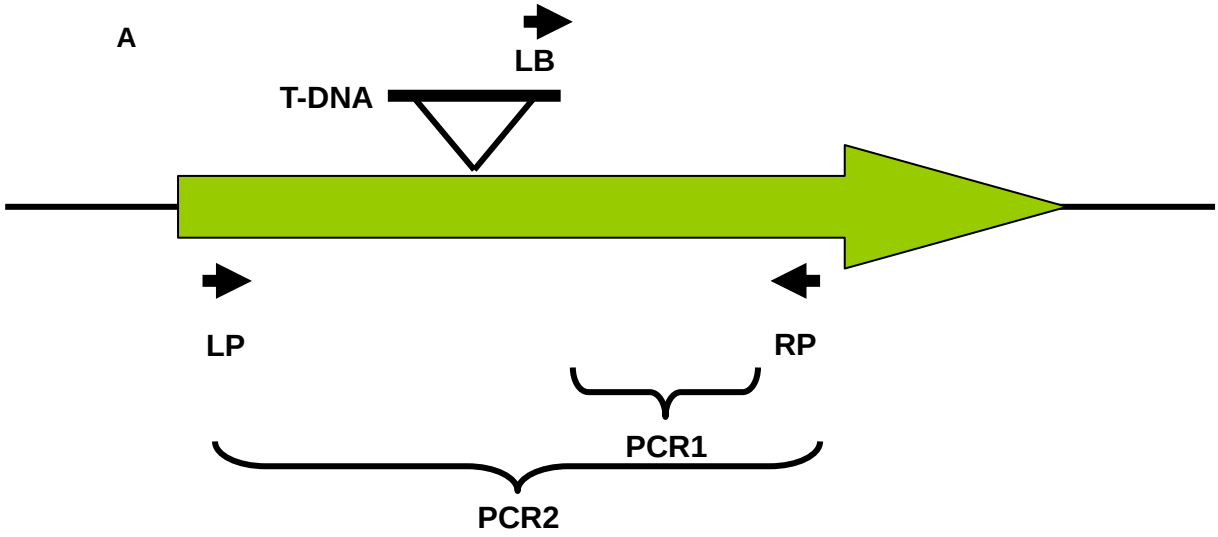


Figure 3-10. PCR-based screening of double mutant *γvpe dad1* F1 seeds.
The F1 seeds were separately grown on sand : soil mixture (1:3) for six weeks. The genomic DNA of each line was extracted and used as PCR template. In PCR1: the *γvpe* mutagenized fragment (Mut. γvpe) was detected by LBb1.3 and RP*γvpe* primers. In PCR1: the *dad1* mutagenized fragment (Mut. dad1) was detected by LBb1.3 and RP*dad1* primers. In both PCR reactions, respective PCR products were obtained for lines #2 and #12. *Arabidopsis* Col-0 plant DNA served as control.

Segregation in the F2 generation is expected as follows: WT:HZ:HZ:HM = 9:3:3:1. F1 *γvpe dad1* lines were confirmed by PCR, the F2 seeds from F1 line #2 were subsequently harvested for further screening of homozygous *γvpe dad1* lines. A 3-Primer-PCR-based screening as described in chapter 2.4.1 was applied. For detection of the *dad1*-specific T-DNA insertion, a PCR product should be amplified in homozygous lines using LB- and RP*dad1*-specific primers. By contrast, amplification should fail using LP- and RP-specific primers. Similarly, a PCR product should be amplified in homozygous *γvpe* T-DNA insertion lines using LB- and RP*γvpe*-specific primers. In homozygous wild-type lines, a PCR product should be amplified using LP- and RP-specific primers. From 20 F2 lines, homozygous *γvpedad1* mutant (#3) and wild-type plant (#17) were identified and forwarded to further experiments (Fig. 3-11).



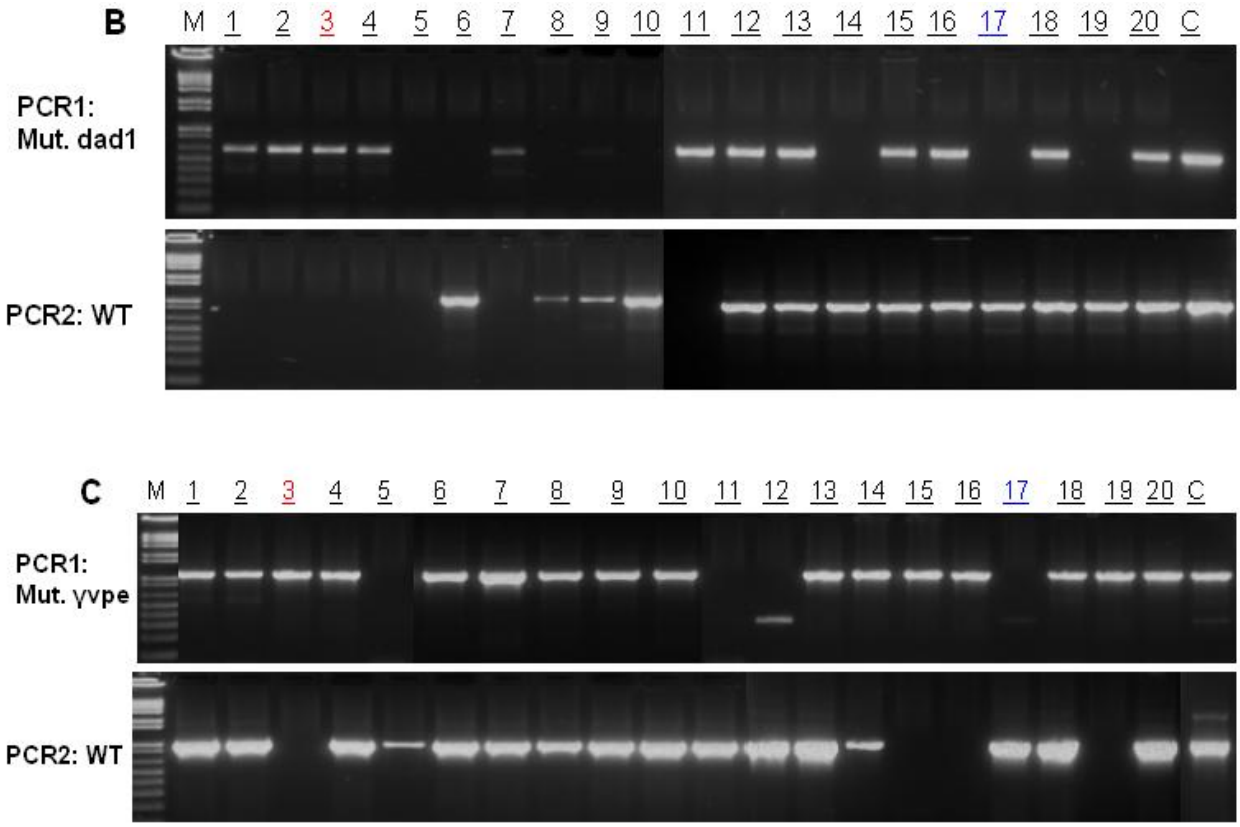


Figure 3-11. Identification of homozygous *γvpedad1* lines by 3-Primer-PCR method.
(A) Schematic representation of a T-DNA insertion. The position of the T-DNA insertion is shown as a black box above the gene that is symbolized by the green arrow. The primers LB and RP are used to detect the presence of the insertion in the progeny of the original mutant (PCR1). The primers LP and RP are used to detect the presence of the wildtype version of the gene (PCR2). (B) PCR analysis for *dad1*-specific T-DNA insertion in 20 F2 plants. Presence of a PCR product in PCR1 but not in PCR2 indicates that the corresponding plant is a homozygous mutant (plants 1, 2, 3, 4, 7, 11). Amplification of a fragment in PCR2, but not in PCR1 implies that the corresponding line is homozygous wildtype (plant 6, 8, 10, 14, 17, 19). The amplification of products in both PCR1 and PCR2 suggests the plant is heterozygous. The DNA of *dad1* mutant and Col-0 are used as respective control in PCR1 and PCR2. (C) PCR analysis for *γvpe*-specific T-DNA insertion in 20 F2 plants. Presence of a PCR product in PCR1 but not in PCR2 indicates that the corresponding plant is a homozygous mutant (plants 3, 15, 19). Amplification of a fragment in PCR2, but not in PCR1 implies that the corresponding line is homozygous wildtype (plant 5, 11, 12, 17). The amplification of products in both PCR1 and PCR2 suggests the plant is heterozygous for the insertion. The DNA of *γvpe* mutant and Col-0 are used as respective control in PCR1 and PCR2.

3.5.2.2 Quantification of *P. indica* colonization on *γvpe dad1* roots

Homozygous *γvpe dad1* (3# line) and wildtype plants (17# line) were inoculated with *P. indica*. Root colonization was determined by qRT-PCR at 3 and 7 dai. Compared with wild-type plants, *γvpe dad1* displayed reduced colonization at 7 dai (Fig. 3-12) which was comparable to the colonization of *γvpe* at this timepoint (Fig. 3-12). These data implicated that *P. indica* might force ER stress-induced cell death during root colonization that might be executed by VPEs.

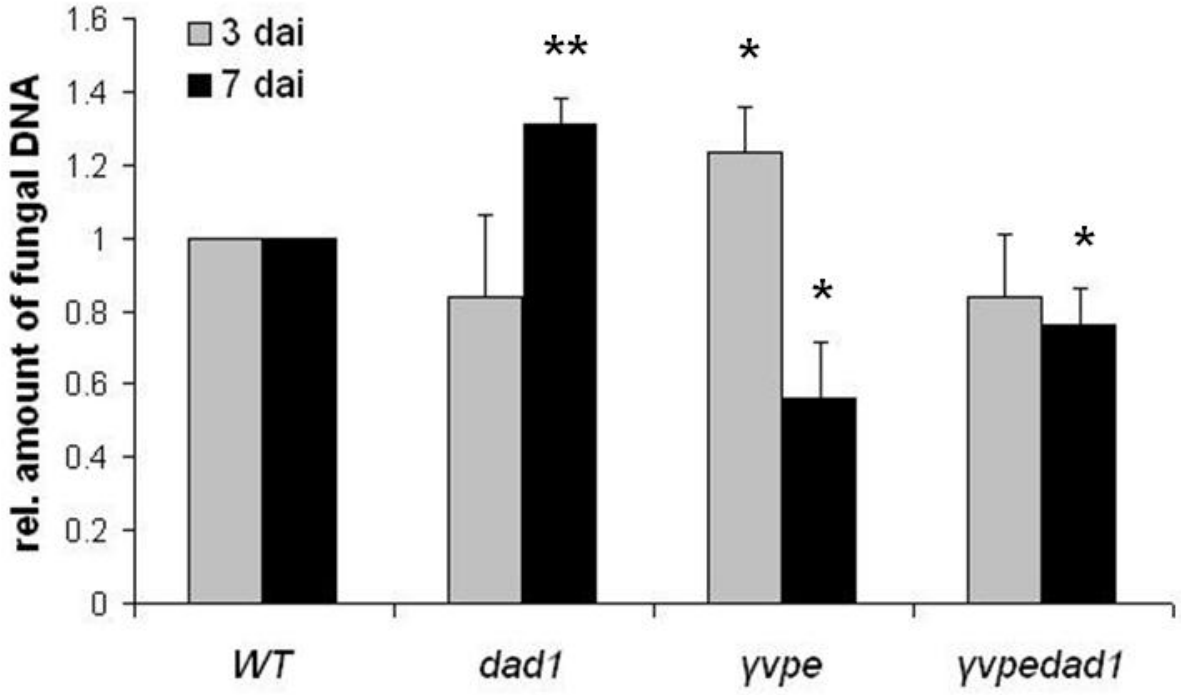


Figure 3-12. *P. indica* colonization of single mutant *γvpe* and *dad1* compared to colonization of the double mutant *γvpe dad1*.
For the analysis, three-week-old *Arabidopsis* WT, *dad1*, *γvpe* and *γvpedad1* mutants were inoculated with *P. indica*. Root samples were harvested at 3 and 7 dai. The fungal biomass were determined by qPCR at both biotrophic (3 dai) and cell death-associated colonization stages (7 dai) using *AtUBI5* and *PiITS*-specific primers. Root colonization levels in all mutants were normalized to WT colonization. Results shown are means of three independent experiments. For each experiment, around 200 plants were analyzed per line at each time point. Asterisks indicate significance at $P < 0.05$ (*) analyzed by Student's *t*-test.

3.6 VPE and caspase-1 activities are enhanced in *P. indica*-colonized roots

VPEs were shown to have protease activities, which are detectable as VPE and caspase-1 activity. These activities were shown to be important for plant cell death execution (Hatsugai et al. 2004, Kuroyanagi et al. 2005). To confirm the significance of vacuolar-mediated cell death for root colonization, VPE- and caspase-1 activities were measured in wild-type, *γvpe*, *vpe-KO*, *dad1*, and *γvpedad1* roots in dependence of *P. indica* colonization. An assay for VPE and caspase activities was set up to measure VPE- and caspase-1 activities in root extracts from *P. indica*-colonized and non-colonized roots during cell death-associated colonization (7 dai). In this assay, either 1 mM of VPE-specific substrate Ac-ESEN-MCA or caspase-1-specific substrate Ac-YVAD-MCA was applied to root extracts. Respective protease activities were determined spectrometrically by measuring VPE- and caspase-1-specific cleavage of the fluorophore 4-metyl-coumaryl-7-amide (MCA). The results indicated that both VPE and caspase-1 activities were increased in *P. indica*-colonized compared to non-colonized roots (Fig. 3-13A). As expected, enzyme activities were weakly detectable in *vpe-KO* (Fig. 3-13A). Similarly, enzyme activities were hardly detectable in *γvpe* roots. Interestingly, although non-colonized *dad1* roots displayed VPE and caspase-1 activities as observed in Col-0 roots, both enzyme activities were strongly enhanced in colonized *dad1* roots and even higher when compared to respective wild-type roots (Fig. 3-13A). In order to confirm whether reduced colonization of *γvpe dad1* might be associated with altered enzyme activities, VPE- and caspase-1 activities were measured in *γvpe dad1* roots in dependence of *P. indica* colonization. Enzyme activities were hardly detectable in colonized or non-colonized *γvpe dad1* roots and resembled enzyme activities detected in *γvpe* (Fig. 3-13B). In summary, the results of the enzyme activity assay correlated with the colonization data (Fig. 3-2, 3-9, 3-12). It indicated that VPE- and caspase-1 activities might be essential for cell death execution and, thus, colonization success.

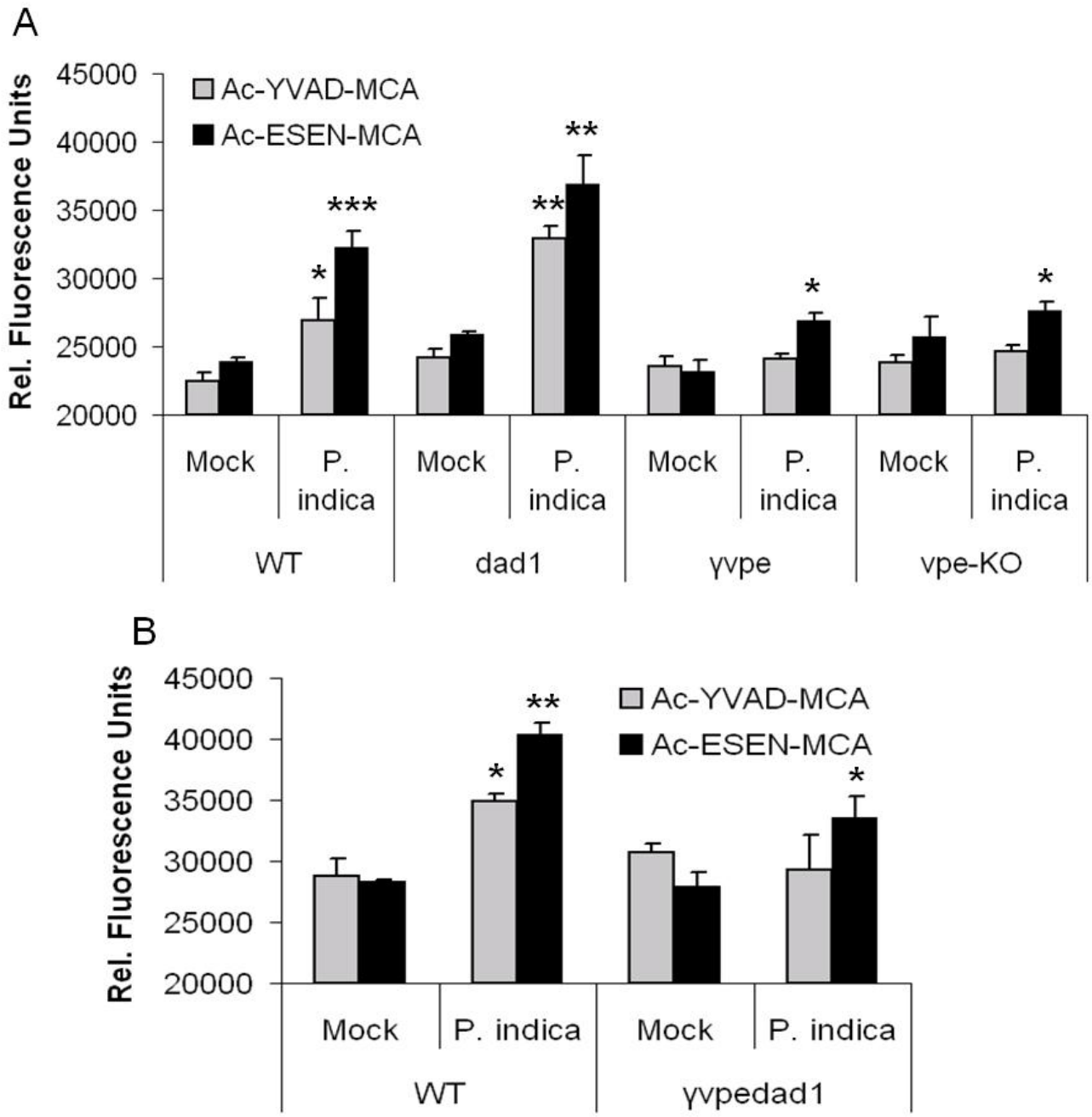


Figure 3-13. VPE and caspase-1 activities are enhanced during root colonization by *P. indica*.

A,B: VPE and caspase-1 activities during cell death-associated colonization of roots by *P. indica*. Three-week-old *Arabidopsis* WT and mutant plants were inoculated with *P. indica* or mock-treated. Root samples were harvested at 7 dai (cell death-associated colonization stage). For the assay, 100 nM VPE substrate of Ac-ESEN-MCA or caspase-1 substrate Ac-YVAD-MCA were added to root extracts to determine VPE and caspase-1 activities, respectively. Fluorescent intensities were spectrometrically detected at 465 nm after excitation at 360 nm. The values are given as relative fluorescence units (RFU). Data displayed are means with standard errors of four independent measurements per treatment of one biological experiment. Experiments were repeated thrice with similar results. Asterisks indicate significance between *P. indica*-colonized and non-colonized roots at $P < 0.05$ (*), 0.01 (**), 0.001 (***) analyzed by Student's *t*-test.

3.7 ER dysfunction enhances *P. indica*- and TM-induced cell death in a VPE-dependent way

In order to relate the enzyme activities and colonization studies to the occurrence of cell death, a fluorescein diacetate (FDA)-based cell death assay was performed. Therefore, root segments were stained with FDA. In viable cells, esterases will cleave off the fluorescein and the degree of cleavage as indication of cell viability can be spectrometrically quantified. In a first assay, *bip2*, *dad1*, *vpe-KO, γvpe, γvpe dad1* mutants as well as respective wild-type plants were treated with tunicamycin (TM) and root segments were stained with FDA at 3 days after treatment (dat). The results showed that *bip2* and *dad1* mutants had ~ 20% less fluorescence intensity than wild-type segments, which indicated a reduced number of living cells (Fig. 3-14A). By contrast, *vpe-KO* and *γvpe* mutants showed an increase of fluorescence intensity (> 30%) compared to wild-type root segments, which was an indication for an elevated number of living cells. Similarly, root segments of *γvpe dad1* also exhibited enhanced fluorescence intensities (Fig. 3-14A). In a second assay, we determined FDA cleavage in the same mutants during cell death-associated colonization by *P. indica* (7 dai). Similar to the results obtained in the TM assay, *bip2* and *dad1* exhibited reduced fluorescence intensities, while fluorescence intensities were enhanced in *vpe-KO, γvpe*, and *γvpe dad1* (Fig. 3-14B). These analyses confirmed enhanced ER stress- and *P. indica*-induced cell death in plants that are impaired in ER function. In addition, ER stress- and *P. indica*-induced cell death is dependent on γVPE function.

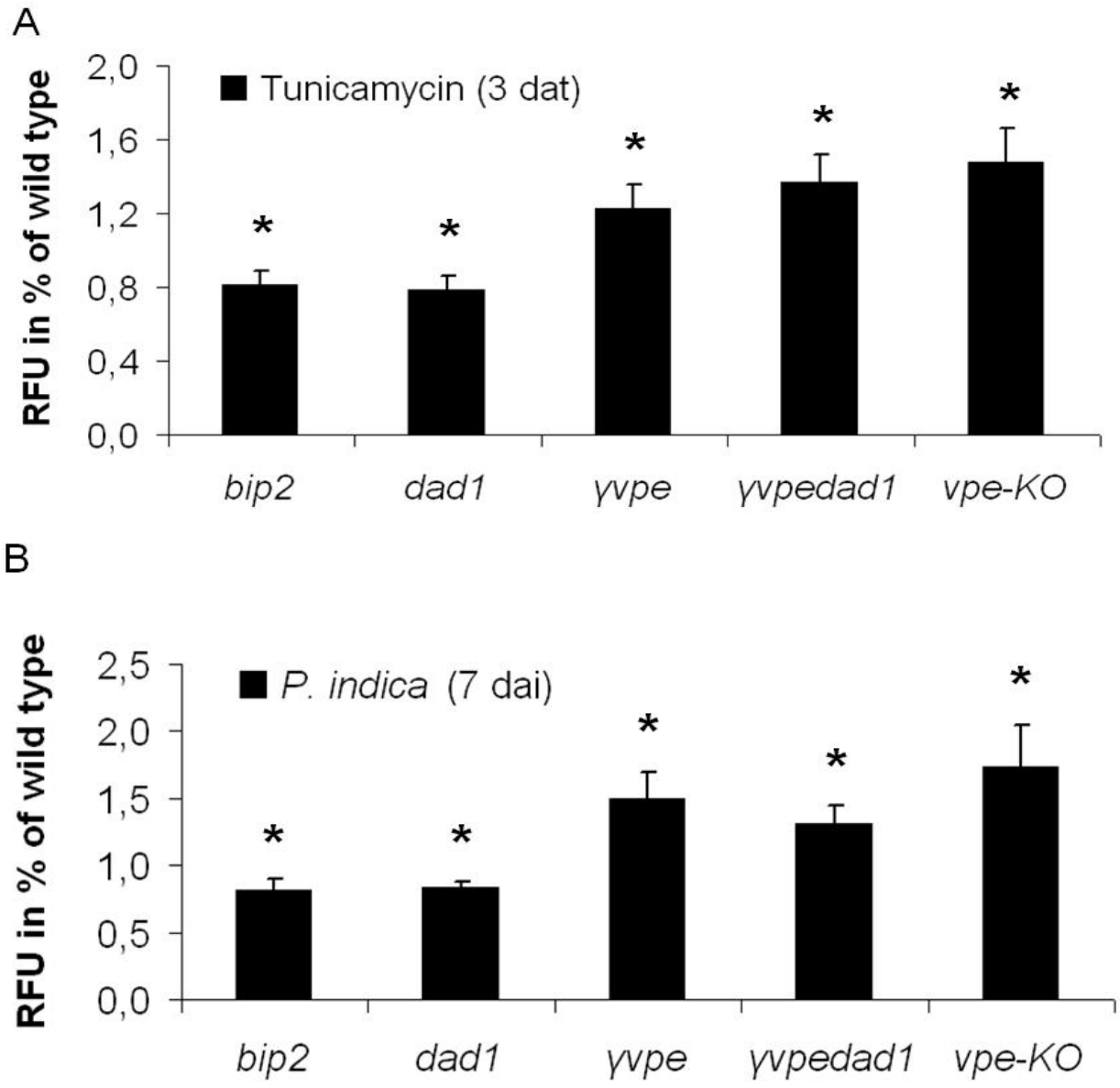


Figure 3-14. Tunicamycin- and *P. indica*-induced root cell death depends on γVPEs and is enhanced in mutants that are impaired in ER function.

A,B,: Fluorescein diacetate (FDA)-based assay indicative of cell death in *dad1*, *bip2*, *γvpe*, *vpe-KO* and *γvpe dad1* roots compared to WT (set to one) after TM treatment (A) or *P. indica* inoculation (B). Two-week-old *Arabidopsis* WT plants, *bip2*, *dad1*, *γvpe*, *vpe-KO*, and *γvpe dad1* mutants were treated with TM (5 µg/ml) or mock-treated (A). Alternatively, WT and mutant plants were inoculated with *P. indica* or mock-treated (B). Roots were harvested at 3 dat (A) or at the cell death-associated colonization stage (7 dai) (B). For either treatment, root segments were cut in 2 cm pieces and stained with fluorescein diacetate (FDA). After staining, roots were washed and fluorescence intensities were spectrometrically determined at 535 nm after excitation at 485 nm. The values are given as fluorescence units (RFU) relative to WT roots. Data displayed are means with standard errors of eight independent measurements per treatment of one biological experiment. Experiments were repeated thrice with similar results. Asterisks indicate significance at $P < 0.05$ (*) analyzed by Student's *t*-test.

4. Discussion

Unlike mammalians, programmed cell death (PCD) pathways in plants are less understood. Studies during the past decades indicate that plant PCD has a pivotal association with plant development (e.g. senescence, xylogenesis) and defense responses (hypersensitive response). The hypersensitive response (HR) is among the best characterized cell death responses in plants. It is commonly controlled by direct or indirect interactions of pathogen avirulence gene products (effectors) with their cognate R gene products. Although the subcellular changes associated with HR have been determined, the molecular process involved in the initiation and execution of HR are fairly unknown (Heath 2000; Mur et al., 2008). In plants, another process so called autophagy is involved in various cellular remodelling processes during plant development and cell differentiation (Bassham et al., 2006). Interestingly, autophagy might be important for the restriction of HR to infected cells thereby inhibiting its uncontrolled spreading (Liu et al., 2005).

Interestingly, root colonization by the mutualistic fungal endophyte *P. indica* is different from the biotrophic life style of mycorrhizal fungi, not at least due to the cell death dependence of this association. First evidence on cell death dependence of root colonization was provided in the barley-*P. indica* system (Deshmukh et al., 2006). Such an activation of cell death pathways was not reported in other mutualistic plant-fungus symbioses. In the present work, cytological, genetic, molecular, and biochemical strategies were applied to analyze the interaction between the endophyte *P. indica* and *Arabidopsis* roots. My aim was to draw a picture of molecular and cellular processes involved in cell death-associated colonization of *Arabidopsis* roots.

4.1 The mutualistic symbiosis of *Arabidopsis* root with *P. indica*

Similar to the symbiotic interaction between barley roots and *P. indica*, a mutualistic symbiosis is also formed between *Arabidopsis* roots and *P. indica*. Here, the fungus colonizes host plant roots, proliferates by inter- and intracellular growth and produces chlamydospores

inside root cells as well as on the root surface (Jacobs, Zechmann, Kogel, Schäfer unpublished). Recent phylogenetic studies have indicated that *P. indica* is a member of the order *Sebacinales*, which exclusively houses mycorrhizal fungi (Weiss et al., 2004). All members of this order that have been examined so far, produce comparable biological activities regarding biomass increase and systemic resistance to biotrophic powdery mildew fungi (Varma et al., 1999; Deshmukh et al., 2006, Stein et al., 2008). Thus, *P. indica* is regarded as a representative of the order of *Sebacinales*, which harbour microbes with prospective agronomical impact (Deshmukh et al., 2006, Weiss et al., 2004).

Former studies in our group demonstrated that *Arabidopsis* root colonization by *P. indica* is characterized by an initial biotrophic lifestyle. These cell biological studies further revealed that biotrophic colonization is transient and followed by a cell death-associated colonization phase (Jacobs, Zechmann, Kogel, Schäfer, unpublished). Moreover, the former analyses detected a broad-spectrum suppression of root innate immunity by *P. indica*. Defense suppression was found to be crucial for root colonization and might be the reason of its wide host range (Jacobs, Zechmann, Kogel, Schäfer, unpublished). In the present study, colonization-associated cell death was analyzed and was demonstrated to be initiated by an uncoupled ER stress response, which meets in a vacuolar-mediated, caspase 1-dependent cell death.

4.2 ER stress occurrence in *Arabidopsis* roots during *P. indica* colonization

The previous transmission electron- (TEM) and confocal laser-scanning microscopy (CLSM)-based studies demonstrated that colonization-associated cell death is characterized by ER swelling (Fig. 3-1A, 1B and 1C), lyses of the cytoplasm (Fig. 3-1B), and vacuolar collapse (Fig. 3-1B). These studies implicated that ER stress occurred during colonization. This was substantiated by the pharmacological analyses with tunicamycin (TM). *P. indica*-colonized plants were hypersensitive to TM-induced ER stress (Fig. 3-5). As mentioned, adaptation and recovery from ER stress is mediated by the unfolded protein response (UPR) (Schröder and Kaufman, 2005). However, cells will die if the mechanisms for stress adaptation are insufficient to relieve the ER stress (Boyce and Yuan, 2006). The

application of TM, which is a known ER stress inducer as it inhibits N-linked glycosylation of proteins, was demonstrated to induce strong PCD phenotypes such as nuclear condensation, DNA laddering and H_2O_2 production in *Arabidopsis* roots (Watanabe and Lam, 2008a). In mammals, apoptosis is known to be activated in response to severe or constant ER stress (Malhotra and Kaufmann, 2007). Therefore, we speculated that *P. indica*-induced ER stress might result in cell death mediated by vacuolar collapse. It prompted me to further dissect to which extent the ER stress response was affected by the fungus and whether this ER stress was the initiator for cell death execution.

4.3 The suppression of ER stress response signaling by *P. indica*

ER stress is generally elicited during plant development or by abiotic and biotic stress (eg. salt stress or pathogenesis). ER stress is the result of an enhanced working load of the ER as stress adaptation is associated with an elevated secretion of proteins. This enhanced working load ends in the accumulation of misfolded proteins, which is sensed through different sensor proteins and results in the activation of the ER stress response or so called unfolded protein response (UPR). UPR encompasses translational attenuation, transcriptional induction of ER chaperones (e.g. BiPs), and elevated degradation of misfolded proteins by the proteasome. By these responses, eukaryotic cells aim to relieve ER stress (Malhotra and Kaufman 2007, Vitale and Boston 2008).

In plants, the UPR was initially described in the *floury*-2 endosperm mutant of maize (*Zea mays*), which produces an aberrant 24-kD α-zein storage protein with a defective signal peptide processing site (Boston et al., 1991; Fontes et al., 1991). The defective storage protein accumulates as an ER membrane-resident protein and provokes an ER stress response with dramatically increased levels of BiP and other ER-resident chaperones (Coleman et al., 1995; Gillikin et al., 1997). Thereafter, studies demonstrated that in response to ER stress plant cells aim to create a more optimal protein-folding environment by the induction of BiP and Ca^{2+}-dependent ER folding proteins (Jelitto-Van Dooren et al., 1999; Martinez and Chrispeels, 2003; Noh et al., 2003; Iwata and Koizumi, 2005a; Kamauchi et al., 2005). Compared with the knowledge on signal transduction mechanisms that trigger UPR in yeast

and mammalian cells, the mechanisms of UPR in plants are much less well understood. The chemical tunicamycin (TM) is commonly used to induce ER stress in yeast and animals. Several studies indicated that TM is able to induce ER stress in plants (Koizumi et al., 1999; Iwata and Koizumi, 2005b; Urade, 2007). Interestingly, homologues of ER stress sensors of animals have also been identified in *Arabidopsis*, which included several genes encoding bZIP transcription factors similar to ATF6 in mammalian cells (Iwata and Koizumi, 2005b; Liu et al., 2007), two IRE1-related genes (Koizumi et al., 2001; Noh et al., 2002), and a number of genes related to PERK. However, the involvement of IRE1 homologues in ER stress responses has not been demonstrated.

Iwata and Koizumi (2005a) described a membrane-associated bZIP transcription factor in *Arabidopsis*, bZIP60, which is upregulated in response to ER stress. *Arabidopsis bZIP60* was shown to be transcriptionally activated by TM. The bZIP60 protein is composed of 295 amino acids and contains a bZIP domain and an adjacent putative trans-membrane domain. They further performed a transient expression assay which demonstrated that expression of *bZIP60ΔC*, a truncated version of *bZIP60* lacking the trans-membrane domain, activated *BiP* and *calnexin* (*CNX*). The analyses suggested that bZIP60 functions in a way analogous to ATF6 during the ER stress response. It was also proposed that bZIP60 is an ER stress sensor/transducer similar to ATF6 (Iwata et al., 2008).

Another bZIP transcription factor, bZIP28, has been reported to be activated by TM and involved in the ER stress response (Liu et al., 2007; Tajima et al., 2008). bZIP28 activated expression of *BiP* genes in response to ER stress. Consistenly, the T-DNA insertion mutant *bZIP28* exhibited reduced induction of all *BiP* genes. Liu and colleagues (2007) described that bZIP28 is an N-glycosylated protein including an N-terminal bZIP domain, a putative transmembrane domain (TMD), and a C-terminal domain. They further showed that *Arabidopsis* bZIP28 tagged with Myc or GFP at its N-terminus resides in the ER membrane under unstressed conditions, but is cleaved in response to TM-induced ER stress. According to the size of the processed AtbZIP N-terminal fragment, it is speculated that similar to ATF6, AtbZIP28 might be cleaved by the plant S1P/S2P-dependent regulated intra-membrane proteolysis (RIP) system under ER stress conditions. Further, both tunicamycin (TM) and dithiothreitol (DTT) treatments obviously increased GFP fluorescence in the nucleus, which

suggested that AtbZIP28 N-terminal fragment is translocated into the nucleus (Liu et al., 2007). Time-lapse imaging experiments further indicated that the N-terminal fragment of AtbZIP28 translocated from the ER into the nucleus in response to ER stress (Tajima et al., 2008). A model for the ER stress-induced activation of bZIP60 and bZIP28 in plants is summarized below (Fig. 4-1). In addition to bZIP28 and bZIP60, bZIP17, which was found to mediate salt stress signaling in a way similar to ER stress signaling, might also function as ER stress sensors (Iwata et al., 2008, Liu et al., 2008).

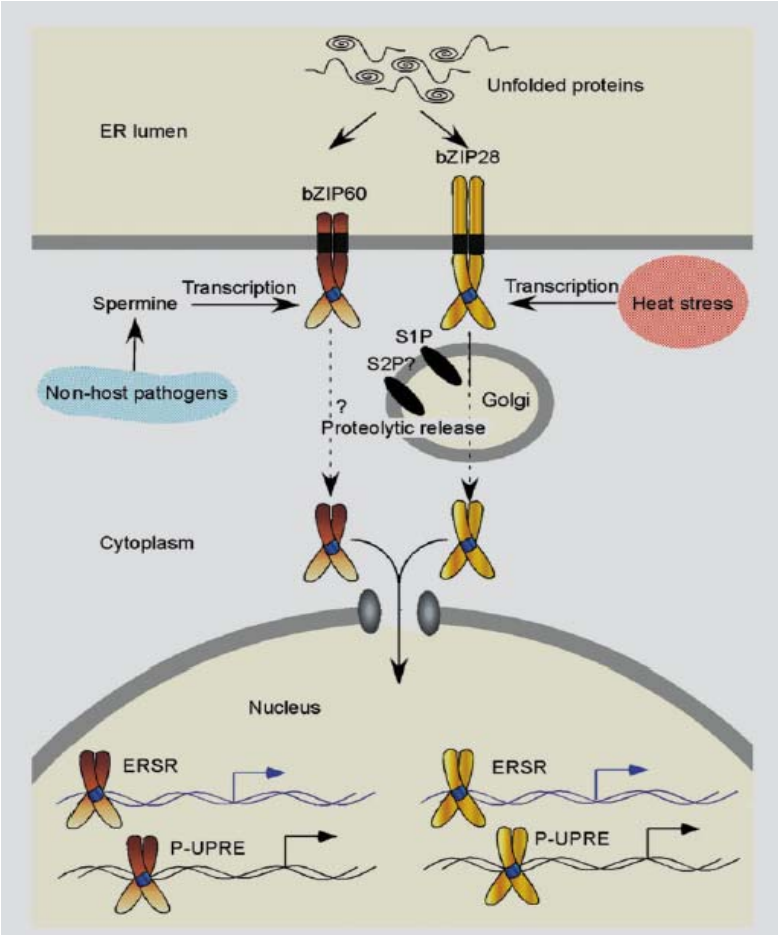


Figure 4-1. Model for the ER stress response mediated by bZIP transcription factors bZIP60 and bZIP28 in plants. (Modified from Urade, 2009)
This model describes that both bZIP60 and bZIP28 proteins localize to the ER membrane. Abiotic or biotic stress ends in the accumulation of unfolded proteins in ER, which activate the processing of ER stress sensors bZIP60 and bZIP28. The N-terminal cytoplasmic domain of bZIP28 protein is proposed to be released from the ER membrane by a S1P/S2P-dependent RIP-like mechanism. In turn, processing of bZIP60 protein was not shown to be dependent on S1P cleavage. Eventually, the cleaved N-terminal cytoplasmic domains of bZIP60 and bZIP28 are translocated to the nucleus and induce transcription of UPR genes.

As indicated earlier, microarray analyses with *Arabidopsis* plants exposed to TM-induced ER stress revealed the induction of a set of genes including those involved in protein folding (e.g. *BIPs*, *CNXs*, *PDIs*), protein degradation (e.g. *HRD1*, *DER1*), and translocation (e.g. *SEC61γ*) (Kamauchi et al. 2005; Iwata et al. 2008). Therefore, these genes can be considered as ER stress marker genes and were applied in my studies. Although my cytological and pharmaceutical studies indicated ER stress associated with *P. indica* colonization, TM-induced ER stress markers were not induced but rather suppressed by *P. indica* (Fig. 3-7). In accordance with this, BiP protein levels were also suppressed during *P. indica* colonization and after TM treatment of *P. indica*-colonized roots (Fig. 3-8). In addition, none of the bZIP transcription factors (bZIP17, bZIP28, bZIP60) were induced and *bZIP28* was even suppressed during *P. indica* colonization. Consistently, UPR genes (e.g. *BIP3*) were not found to be induced by *P. indica* (Fig. 3-6). The application of TM to *P. indica*-colonized roots (3 dai) confirmed the suppression of ER stress signaling (UPR signaling) by *P. indica* (Fig. 3-7). Surprisingly, although expression of *bZIP28* was non-responsive to TM and suppressed during *P. indica* colonization, TM application to *P. indica*-colonized roots caused its induction (Fig. 3-6, 3-7). Therefore, one might speculate that the combined stresses induced by *P. indica* and TM might activate stress pathways, which may recruit bZIP28. These data clearly indicated that the signaling involved in ER stress response is suppressed by *P. indica* at the transcriptional and translational level.

If the UPR fails to restore the protein-folding defect, apoptosis is activated in response to ER stress. The ER might function as a site where apoptotic signals are generated and intergrated to elicit the death response (Malhotra and Kaufman, 2007). The molecular basis of ER stress-induced PCD in plants is less well understood than that in mammals. However, several indications implicate a conservation of ER stress signaling between plants and mammals. So far, the mammalian BCL-2 related proteins, which were identified to control apoptosis are divided into three subfamilies, including pro-survival type Bcl-2 subfamily (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1), pro-apoptotic type Bax subfamily (Bax, Bak and Bok) and BH3 subfamily (Bad, Bid, Bik, Blk and BimL). The Bcl-2 subfamily proteins inhibit apoptosis, whereas Bax subfamily proteins activate apoptosis. BH3 subfamily proteins share a short motif with Bcl-2 family proteins and regulate their activity antagonistically through their

direct interaction (Watanabe and Lam, 2009). However, plant genome sequences do not reveal homologues to several key cell death regulators as described above. It is known that BAX INHIBITOR-1 (BI-1) is a negative cell death regulator in mammals as it antagonizes BAX-induced cell death (Xu and Reed 1998). Although BAX homologues were not found in plants, barley BI-1 and other plant BI-1 proteins suppress BAX-induced cell death *in planta* (Eichmann et al. 2006; Babaeizad et al., 2008; Imani et al., 2006; Hoefle et al., 2009). Moreover, the expression of *Arabidopsis BI-1* (*AtBI-1*) was induced in response to ER stress, while *Arabidopsis* plants overexpressing *BI-1* exhibited enhanced TM tolerance, which indicated its antiapoptotic function in ER-PCD (Kamauchi et al., 2005; Watanabe and Lam, 2008). Therefore, it is speculated that the level of BI-1 might determine plant survival during ER stress in *Arabidopsis* plants. AtBI-1 might not control the UPR directly; in turn, it may act as a cell death suppressor that functions in parallel to the UPR when the ER stress is activated (Watanabe and Lam, 2009). A recent mammalian BI-1 study revealed functional interactions of BI-1 with Bcl-2 and Bax proteins which indicated that BI-1 and Bcl-2 control apoptosis downstream of or parallel to Bax action through regulating Ca^{2+} balance in the ER (Xu et al., 2008). Therefore, Bax might manipulate ER homeostasis through facilitating Ca^{2+} release from the ER. Because there are no obvious homologues to Bcl-2 subfamily proteins in plants, plant BI-1 might play an important role in regulating Ca^{2+} homeostasis during ER stress (Watanabe and Lam, 2009). Interestingly, *P. indica* was shown to suppress *BI-1* transcription during barley root colonization and *BI-1* overexpression resulted in reduced *P. indica* colonization (Deshmukh et al. 2006). Consistently, in the present work, I observed a slight suppression of *AtBI-1* by *P. indica* after TM treatment (Fig. 3-7).

4.4 *P. indica* colonization-associated cell death is executed by a vacuolar-mediated caspase 1-dependent plant cell death

4.4.1 Caspase-dependent mammalian apoptosis

As mentioned above, both the death-receptor- and mitochondrial-mediated mammalian

apoptotic pathways are ultimately executed by the activation of specific subsets of caspases and Bcl-2 family members (Szegezdi et al., 2006), which finally aim to eliminate cells that may be unnecessary or harmful for the organisms. Caspases are known as cysteine proteases which exist within cells as inactive zymogens and are subsequently cleaved to form active enzymes during the induction of apoptosis. Although the caspases associated with ER stress-induced apoptosis have not yet been conclusively established, processing of caspase 12, 2, 3, 4, 7, and 9 have been observed in different ER stress studies (Cheung et al., 2006; Dahmer, 2005; Di Sano et al., 2006; Hitomi et al., 2004). Caspase 12 is associated with the ER membrane and activated by ER stress (Tan et al., 2006). In addition, proapoptotic BCL2 family members Bax and Bak colocalize to the ER membrane and function to activate apoptosis via caspase 12 (Cheung et al., 2006; Dahmer, 2005; Di et al., 2006; Hitomi et al., 2004). Caspase 12 activates caspase 9, which in turn activates caspase 3 leading to cell death (Morishima et al., 2002). *Caspase 12*$^{-/-}$ mice are partially resistant to ER stress-induced apoptosis but sensitive to other death stimuli, suggesting that caspase 12 is a regulator specific to ER stress-induced apoptosis (Nakagawa et al., 2000).

4.4.2 Caspase-mediated cell death pathways in plants

The extent of conservation of plant and mammalian programmed cell death pathways is still not clear. However, extensive studies have shown that PCD in plants and animals share components with caspase-like activities (Cohen, 1997; Lam and del Pozo, 2000; Woltering et al., 2002). These findings indicated the central role of caspases in certain pathways. In addition, it showed that plant PCD partly relies on proteins which exhibit functional homology to PCD signaling components in mammals.

Caspase-like activities have been detected in plants by synthetic substrates and therefore base on consensus cleavage site of mammalian caspases (Table 4-1). Among the detected caspase-like activities in plants YVADase (VPE) and DEVDase activities were best studied and were found to be associated with various PCD pathways (Bonneau et al., 2008).

Table 4-1. Caspase-like activities detected in plants. (Bonneau et al., 2008)

Activity	Species and tissue	Reference
YVADase (VPE)	Tobacco leaf tissue	del Pozo and Lam, 1998
	Barley embryonic suspension cells	Korthout *et al*., 2000
	Tobacco BY2 cells	Mlejnek and Procházka, 2002
	White spruce, germination of seeds	He and Kermode, 2003
	Pisum sativum seedlings	Belenghi *et al*., 2004
	Arabidopsis thaliana seedlings	Danon *et al*., 2004
	Arabidopsis thaliana, fumonisin B-induced, leaf lesion	Kuroyanagi *et al*., 2005
DEVDase	Barley embryonic suspension cells	Korthout *et al*., 2000
	Tobacco (BY2) suspension cells	Tian *et al*., 2000
	Tobacco BY2 cells	Mlejnek and Procházka, 2002
	White spruce, germination of seeds	He and Kermode, 2003
	Pisum sativum seedlings	Belenghi *et al*., 2004
	Norway spruce, embryogenic cell line	Bozhkov *et al*., 2004; Suarez *et al*., 2004
	Avena sativa leaves	Coffeen and Wolpert, 2004
	Arabidopsis thaliana seedlings	Danon *et al*., 2004
	Papaver pollen	Thomas and Franklin-Tong, 2004
IETDase (saspase)	*Avena sativa* leaves	Coffeen and Wolpert, 2004
	Arabidopsis thaliana seedlings	VI Rotari and P Gallois, unpublished data
	SI in *Papaver* pollen	Bosch and Franklin-Tong, 2007
LEHDase	Leaf of *Nicotiana benthamiana*	Kim *et al*., 2003
LEVDase	SI in *Papaver* pollen	Bosch and Franklin-Tong, 2007
TATDase	Tobacco Xanthi, leaves	Chichkova *et al*., 2004
VEIDase	SI in *Papaver* pollen	Bosch and Franklin-Tong, 2007
	Barley seeds	Boren *et al*., 2006
	Norway spruce, embryogenic cell line	Bozhkov *et al*., 2004
	Arabidopsis thaliana seedlings	VI Rotari and P Gallois, unpublished data
VKMDase (saspase)	*Avena sativa* leaves	Coffeen and Wolpert, 2004

In order to prove the requirement of caspase-like activities for PCD execution, caspase inhibitors have been applied in several studies. The baculovirus protein p35, a pan caspase inhibitor, has been demonstrated to effectively block cell death in several systems such as the hypersensitive response (HR) in tobacco plants infected by *Pseudomonas syringae* pv. *phaseolicola* or tobacco mosaic virus (TMV) (del Pozo and Lam, 2003) and UV-induced PCD in *Arabidposis* (Danon et al., 2004). Bonneau et al (2008) summarized inhibitors that efficiently suppress various types of plant cell death (Table 4-2). Taken together, caspase-like activities are thought to be involved in most plant PCD responses. It further indicates that caspase-like activities might represent a central mechanism of plant cell death.

Table 4-2. Application and function of caspase inhibitors in different plant PCD systems.

(Bonneau et al., 2008)

Inhibitor	Inhibition	Experimental system (reference)
BocD-fmk	Yes	*Nicotiana tabacum* cv. Xanthi cell suspension induction using xylanase or staurosporine (Elbaz *et al*., 2002)
Ac-DEVD-CHO	Yes	Cell death in tobacco caused by *Pseudomonas syringae* pv. *phaseolicola* (del Pozo and Lam, 1998)
		Menadione-induced PCD in tobacco protoplasts (Sun *et al*., 1999)
		Induction of PCD in tomato suspension cells using camptothecin, stausporine, and fumonisin-B1 (De Jong *et al*., 2000)
		UV-C-induced PCD in *Arabidopsis thaliana* protoplasts (Danon *et al*., 2004)
		Pollen incompatibility in *Papaver* (Thomas and Franklin-Tong, 2004)
		Elimination of weaker shoots in *Pisum sativum* seedlings (Belenghi *et al*., 2004)
		Ce^{4+}-induced apoptosis of cultured *Taxus cuspidata* cells (Ge *et al*., 2005)
		Heat-shock-induced cell death. Tobacco BY2 (Vacca *et al*., 2006)
		Fusaric-acid-induced PCD on *Crocus sativus* root tips (Samadi and Shahsavan Behboodi, 2006)
		HR induced by *P. syringae* pv. *tabaci* (Krzymowska *et al*., 2007)
	No	HR induced using tobacco mosaic virus (Hatsugai *et al*., 2004)
		HR induced by *P. syringae* pv. *maculicola* (Krzymowska *et al*., 2007)
		N gene-mediated HR in tobacco (Chichkova *et al*., 2004) biotin-DEVD-CHO
Ac-DEVD-cmk	Yes	Mega-gametophyte cells of white spruce seeds (He and Kermode, 2003)
z-DEVD-fmk	Yes	Isopentenyladenosine-induced PCD in tobacco BY2 cells (Mlejnek and Procházka, 2002)
z-LEHD-fmk	No	Embryogenic cell line of Norway spruce (Bozhkov *et al*., 2004)
Biotin-TATD-CHO	Yes	*N* gene-mediated HR in tobacco (Chichkova *et al*., 2004)
z -VAD-fmk	Yes	*Nicotiana tabacum* cv. Xanthi cell suspension induction using xylanase or staurosporine (Elbaz *et al*., 2002)
		Embryogenic cell line of Norway spruce (Bozhkov *et al*., 2004)
		Isopentenyladenosine-induced PCD in tobacco BY2 cells (Mlejnek and Procházka, 2002)
z–VEID-fmk	Yes	Embryogenic cell line of Norway spruce (Bozhkov *et al*., 2004)
Ac-YVAD-CHO	Yes	UV-C-induced PCD in *Arabidopsis thaliana* protoplasts (Danon *et al*., 2004)
		HR induced using tobacco mosaic virus (Hatsugai *et al*., 2004)
		Fusaric acid-induced PCD on *Crocus sativus* root tips (Samadi and Shahsavan Behboodi, 2006)
		Tissue remodelling in lace plant (Gunawardena, 2007)
	No	Xylem formation in *Zinnia* system (Fukuda, 1997)
		Pollen incompatibility in *Papaver* (Thomas and Franklin-Tong, 2004)
		HR induced by *P. syringae* pv. *maculicola* (Krzymowska *et al*., 2007)
Ac-YVAD-cmk	Yes	Cell death in tobacco caused by *Pseudomonas syringae* pv. *phaseolicola* (del Pozo and Lam, 1998)
		Induction of PCD in tomato suspension cells using camptothecin, stausporine, and fumonisin-B1 (De Jong *et al*., 2000)
		NO-induced PCD in *Arabidopsis* suspension cultures (Clarke *et al*., 2000)
		HR induced by *P. syringae* pv. *tabaci* (Krzymowska *et al*., 2007)
z-YVAD-fmk	Yes	Isopentenyladenosine-induced PCD in tobacco BY2 cells (Mlejnek and Procházka, 2002)
Biotin-YVAD-fmk	Yes	Fumonisin B1-induced lesion formation (Kuroyanagi *et al*., 2005)

4.4.3 The proteases vacuolar processing enzymes and their function in plant cell death

Plant vacuolar processing enzymes (VPEs) were initially characterized to cleave the protease substrates ESEN and AAN. VPEs were found to be involved in various protein maturation processes of seeds or leaves (Yamada et al., 2005). Virus-induced silencing of *VPE* genes which correspond to the most abundant *VPEs* in *Nicotiana benthamiana* (*NbVPE-1a* and *NbVPE-1b*) demonstrated that VPEs possessed YVADase/casapase-1 activity (Hatsugai et al., 2004).

In tobacco, infection by tobacco mosaic virus (TMV) is stopped by HR in cultivars that carry the *N* resistance gene (Holmes, 1983). Recent investigations demonstrated that VPE deficient plants exhibited no apparent characteristic of TMV-induced cell death and were markedly more susceptible against TMV (Hatsugai et al., 2004). In addition, PCD induced in plants by

the fungal toxin fumonisin B1 (FB1) (Asai et al., 2000), was found to depend on VPEs. In *Arabidopsis* VPE-null mutants, that lack all four VPEs, FB1-induced cell death was completely abolished (Kuroyanagi et al., 2005). This implicates that both PCD-dependent resistance and susceptibility processes share VPE activities (Hatsugai et al., 2006). In addition, studies using promoter-GUS fusions showed that *αVPE* and *γVPE* were up-regulated in dying cortex cells next to the emerging lateral root (Kinoshita et al., 1999). Moreover, experiments with *Arabidopsis* VPE-null mutant (KO) have shown the requirement of VPE for the developmental cell death in seed integuments (Nakaune et al., 2005). δVPE was found to exhibit caspase 1-like activity using the biotinylated inhibitor biotin-YVAD-fmk (Nakaune et al., 2005) and its expression in *Arabidopsis* plants was restricted to the inner integuments of the seed coat at an early stage of seed development (Nakaune et al., 2005; Hara-Nishimura et al., 2005). Additionally, both *αVPE* and *γVPE* were also up-regulated in senescing tissues (Kinoshita et al., 1999). Notably, γVPE was further indicated to exhibit a caspase 1-like activity and might be recruited in plants to regulate vacuole-mediated cell dismantling during cell death progression in plant-pathogen interaction (Rojo et al., 2004).

Despite the structural and enzymatic similarities between plant VPEs and mammalian caspase 1, caspase 1 is a cytosolic enzyme (Cohen, 1997), while VPE enzymes are supposed to have a vacuolar localization (Kinoshita et al., 1999; Hara-Nishimura et al., 2005). The collapse of the vacuole is considered as a specific and crucial feature of plant PCD (Jones, 2001; van Doorn and Woltering, 2004). VPE-deficient plants did not exhibit vacuolar collapse accompanied with cell death after virus infection (Hatsugai et al., 2004). Thus, it is suggested that VPEs plays a key role in cell death by triggering vacuolar collapse (Hatsugai et al., 2006).

Taken together, all these results suggest that VPEs are involved in various types of cell death associated with plant development, senescence, and plant-microbe interactions.

4.4.4 Vacuolar processing enzymes play an important role in *P. indica* colonization associated cell death

Interestingly, the present studies indicated that not all VPE members support *P. indica*

colonization. In opposite to the reduced colonization of *γvpe*, *αvpe* and *vpe-KO* mutants, *βvpe* and *δvpe* mutants showed a higher (but insignificant) colonization by *P. indica* (Fig. 3-9). Reduced colonization of *αvpe* and *γvpe* mutants at 7 dai (Fig. 3-9) might indicate that αVPE and γVPE function in the same signaling cascade. Notably, the enhanced colonization of *γvpe* at 3 dai (biotrophic stage) might indicate an additional immunity-related function of γVPE. Accordingly, as described before, this γVPE was formerly identified to be participating in vacuolar collapse associated with virus-induced hypersensitive cell death (Hatsugai et al. 2004).

In accordance with the function of caspases as executioner in ER stress-induced PCD in mammals (Szegezdi et al. 2006), my studies indicated that caspase 1 activity, which was found to be mainly associated by γVPE, was enhanced during cell death-associated colonization (7 dai) by *P. indica* (Fig. 3-13) while we did not detect altered caspase 1 activity at the early biotrophic root colonization by *P. indica* (3 dai) (not shown). Consistently, the significance of caspase 1 activity for ER stress-induced PCD during *P. indica* colonization was indicated by a fluorescein diacetate (FDA)-based cell death assays. The analyses displayed a reduction of both colonization-associated and tunicamycin (TM)-induced cell death in *γvpe* as well as *vpe-KO* mutants (Fig. 3-14). Intriguingly, these analyses did not only indicate an essential role of VPE in colonization-associated cell death, but also underlined the significance of ER dysfunction as PCD initiator. I observed that the *dad1* mutant displayed an elevated level of caspase 1 activity compared to roots of wild-type plants during cell death-associated colonization (7 dai) (Fig. 3-13) and an increased cell death occurrence after both TM treatment and *P. indica* colonization (Fig. 3-14). Importantly, the extent of TM-induced and colonization-associated cell death phenotypes was highly similar in *γvpe dad1* double mutant and *γvpe* mutant (Fig. 3-14). Hence my studies revealed that the execution of ER stress-induced PCD and colonization-associated PCD was crucially dependent on γVPE-/caspase 1 activity. Consistently, *γvpe dad1* and *γvpe* showed a similar degree of *P. indica* colonization (Fig. 3-9 and 3-12). It further implies that VPE-mediated vacuolar collapse is downstream of ER stress-induced cell death.

4.5 *P. indica* induced ER stress impairs MAMP-triggered immunity

4.5.1 The role of the ER in transport and secretion in plant-microbe interactions

In plant-microbe interactions, once a pathogen is recognized by plants, an arsenal of defense responses is activated to inhibit microbial enzymes, to strengthen the cell wall, or to kill the pathogen. Several studies demonstrated that plant basal defense infrequently recruits the hypersensitive cell death reaction. In general, cell wall-associated plant defenses are activated to stop pathogens (Hückelhoven, 2007a). It was further shown that fungal attacks also influenced the endomembrane systems. Golgi-derived vesicles were required for secretion of defense-related components into the apoplast (Collins et al., 2003; Wang et al., 2005, Lipka et al., 2005). Consistently, ER and Golgi bodies accumulated at penetration sites of oomycete pathogens such as non-pathogenic *Phytophthora sojae* and *Hyaloperonospora parasitica* isolates Cala2 (avirulent) and Noks1 (virulent) in *Arabidopsis* (Takemoto et al., 2003). In plant leaves, focal secretion processes and formation of locally confined cell wall appositions (CWAs) efficiently control colonization by non-adapted biotrophic pathogens such as barley powdery mildew fungus *B. graminis*. These immune responses base on vesicle-mediated transport processes of compounds partially generated in the ER (Lipka et al., 2005; Hückelhoven, 2007b). Our previous cytological studies of root colonization by *P. indica* indicated a very infrequent appearance of CWAs during the biotrophic phase (Jacobs, Zechmann, Kogel, Schäfer, unpublished). Interestingly, in the present study, we recorded a disturbance of ER-localized proteins at biotrophic-dependent colonization stages (3 dai) (Fig. 3-8). At this biotrophic stage, UPR marker genes were not induced in *P. indica*-colonized roots (Fig. 3-6, 3-7). These data might indicate an impaired generation of MTI components (e.g. antimicrobial proteins, PRRs) by *P. indica*.

4.5.2 The role of receptor proteins quality control in the ER for plant innate immunity

Innate immunity in eukaryotes bases on the prompt detection of non-self structures (MAMPs)

derived from potential microbial invaders. Plants detect potential threats derived from surrounding microbes through two classes of immune receptors, which are the pattern recognition receptors (PRRs) that can directly recognize their cognate MAMPs and the disease resistance (R) proteins that can recognize the activities and structures of pathogen-derived effectors (Chisholm et al., 2006; Jones and Dangl, 2006; Boller and Felix, 2009). The recognition of MAMPs by PRRs was evidenced to maintain a critical function in host defense activity because an increase of pathogen invasion and propagation in *Arabidopsis* plants was observed in the absence of single PRRs (Zipfel et al., 2004; Zipfel et al., 2006).

FLS2 and EFR are two well-known PRRs recognizing the bacterial flagellin and bacterial elongation factor TU (EF-TU) respectively (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). Both of them are plasma membrane-localized glycoproteins that need to transit the secretory pathways for maturation and to reach their final destination at the plasma membrane. Maturation in the ER and Golgi is required for proper receptor function. These processes are regulated by the ER quality control (ERQC) system including the SDF2-ERdj3b-BIP complex, the calreticulin/calnexin cycle, and the protein disulfide isomerase (PDI) system (Reddy et al., 1996; Anelli et al., 2007).

Several studies revealed that specific components of the ER machinery are required to mediate processing of the pattern recognition receptor (PRR) EFR1, which activates innate immunity after recognition of the bacterial MAMP elongation factor TU (Nekrasov et al. 2009; Saijo et al. 2009; Zipfel et al. 2006). Nekrasov and co-workers (2009) demonstrated that the ER protein complex, which comprises stromal-derived factor-2 (SDF2), ERdj3b and BiP, was required for the proper biogenesis of EFR1. Furthermore, they revealed an unexpected differential requirement for ERQC and glycosylation components for the two closely related receptors EFR and FLS2. They identified in *elfin* (elf18-insensitive) population alleles of *STT3A* (*Staurosporin and temperature sensitive -3A*), coding for a component of the ER oligosaccharyltransferase (OST) complex involved in N-glycosylation of nascent proteins (Koiwa et al., 2003). The characterisation of *stt3a* mutants revealed that STT3a-mediated N-glycosylation is indispensable for EFR, but dispensible for FLS2. Although both EFR and FLS2 carry numerous putative glycosylation sites, they may have different glycosylation

states (e.g. position, glycan structures) that could impact their interaction with and dependence on the ER-QC machinery. Similarly, the studies from Saijo and co-workers (2009) revealed a key function of the CRT/UGGT cycle and STT3A-dependent N-glycosylation for two fundamental types of plant immunity - MTI and SA-induced immunity. Recent studies in our lab demonstrated that *P. indica* impairs early root immune signaling. Roots in which MTI was activated were more resistant against *P. indica* (Jacobs, Zechmann, Kogel, Schäfer, unpublished). These analyses revealed the presence of a functional MAMP-triggered immunity (MTI) in plant roots and *P. indica* must suppress root MTI in order to achieve root compatibility.

4.5.3 The ER is crucially required for the secretion of immunity-related compounds

The ERQC components do not only play a critical role in plant MAMP-triggered immunity, but also in the ER-associated secretion of antimicrobial proteins after activation of systemic acquired resistance (SAR) (Wang et al., 2005). The accumulation of the endogenous signaling molecule salicylic acid (SA) is known to be required for the induction of SAR as it activates gene expression via the central regulator protein NPR1 (Non-expressor of pathogenesis-related genes 1, also known as NIM1) (Dong, 2004). NPR1 activation results in the induction of *PR* genes, which encode small, secreted or vacuole-targeted proteins with antimicrobial activities (Van Loon and Pieterse, 1999). The ER participates in the processing and translocation of PR proteins to vacuoles or to the apoplast. In microarray analyses, Wang et al (2005) identified additional NPR1 regulated genes. Intriguingly, among the induced genes, one group consisted of defense genes, such as several *PR* genes, while a second main group was found to include members involved in the protein secretory pathway. Among the latter were genes that are encoding ER-localized proteins, for example components of the Sec61 translocon complex, luminal binding protein (BiP) and defender against apoptotic death 1 (DAD1). All these components are known to be crucially required for the ERQC system. Moreover, through electrophoretic mobility shift assays (EMSA), they found that the corresponding ER chaperone genes share a novel promoter *cis* element *TL1*, which is targeted by a nucleus-translocated transcription factor after salicylic acid treatment. Therefore,

considering the situation of a massive upload of PR proteins in vacuoles and the apoplast during SAR, the basal activity of the protein secretory pathway might not be sufficient to accommodate the exploding increase of newly synthesized PR proteins. It was speculated that a coordinated up-regulation of the ERQC-mediated protein secretory machinery is required to guarantee correct folding, modification and transport of antimicrobial proteins.

4.6 Conclusion

Based on the presented data, the question arises whether impaired ER integrity during *P. indica* colonization only aims to induce PCD in order to improve root colonization? The previous studies revealed the presence of a functional MAMP-triggered immunity (MTI) in plant roots (Millet et al. 2010) and *P. indica* achieves root compatibility by suppressing the root MTI (Jacobs, Zechmann, Kogel, Schäfer, unpublished). In present studies, the disturbance of ER-localized proteins (Fig. 3-8) along with a lack of induction of UPR marker genes were observed in *P. indica*-colonized roots (Fig. 3-6, 3-7) at biotrophic colonization stages (3 dai). All these results indicate that *P. indica*-induced ER dysfunction might also impair root MAMP-triggered immunity. By impairing root defense, the fungus might further enhance root compatibility at early biotrophic colonization. I propose that the disturbance of ER integrity by *P. indica* might follow two pathways to ultimately enhance root compatibility: (1) reduction of processing and secretion of MTI components (eg. PR proteins, PRRs) and (2) induction of ER stress-induced caspase 1-dependent PCD. Additionally, in plant PCD-associated cell-autonomous immunity, vacuoles accumulate a variety of antimicrobial proteins; and through the fusion of a large central vacuole with the plasma membrane, these proteins can be released to intercellular space where they attack bacterial pathogens (Hatsugai et al., 2009). Furthermore, this membrane fusion is triggered by the infection of avirulent bacterial strain *Pseudomonas syringae* pv. *tomato* DC3000 having the *Avr* gene *avrRpm1* (*Pst* DC3000/ *avrRpm1*) (Mackey et al., 2002) and suppressed by the infection of virulent bacterial strain *Pst* DC3000 that does not have *avrRpm1* in a plant caspase 3-like enzyme-dependent manner. This indicates a novel plant defense strategy against bacterial pathogens (Hatsugai et

al., 2009). Therefore, it is tempting to speculate that impaired ER function by *P. indica* also affects vacuole load with antimicrobial proteins. This might explain that VPE-mediated vacuolar collapse was not stopping but even supporting fungal growth at early colonization stages (3 dai) (Fig. 3-9). According to the data presented in this work, I propose the following scenario (Fig. 4-2) to explain *P. indica* colonization-associated ER dysfunction and root cell death processes.

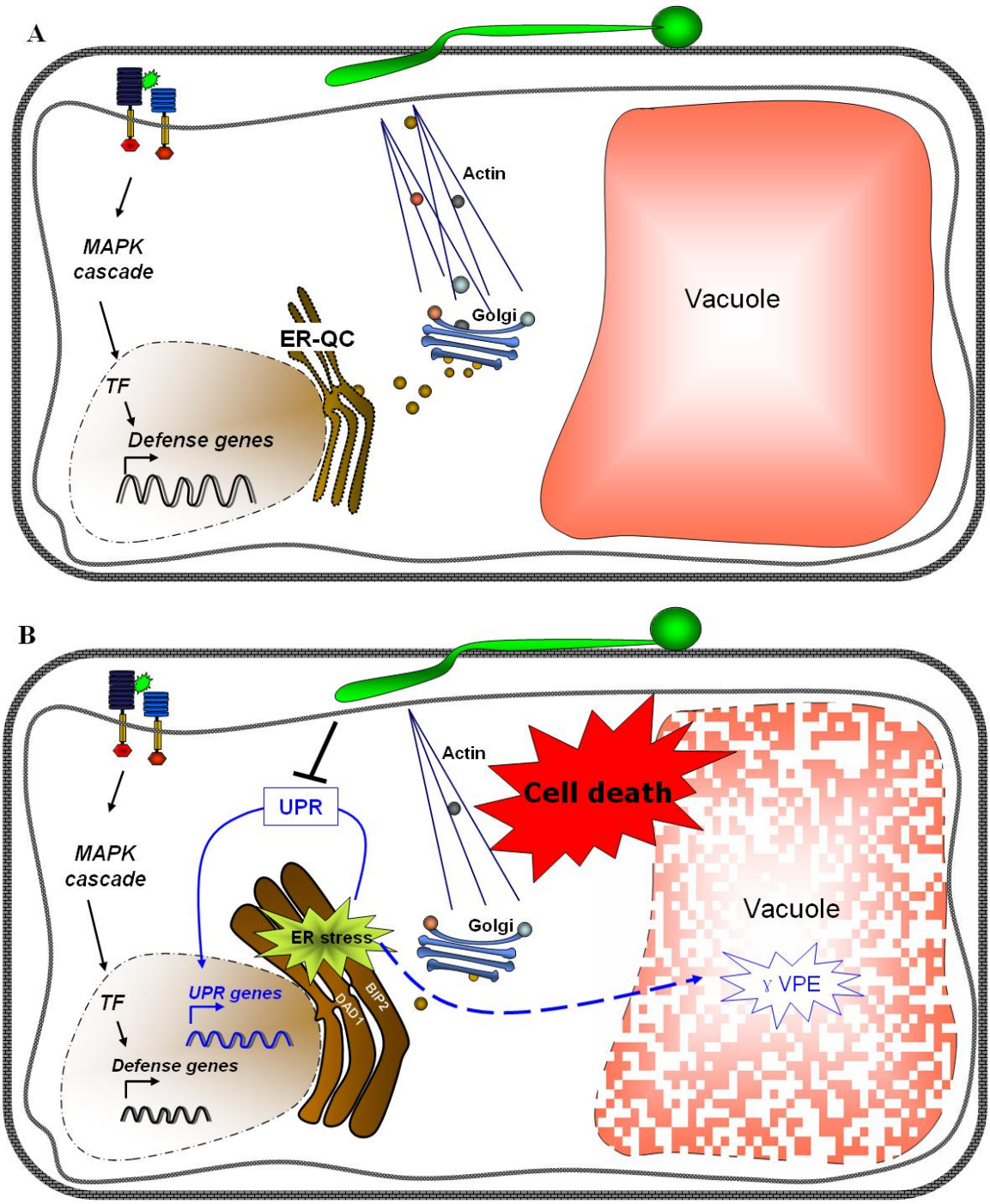


Figure 4-2. Scenario of *P. indica* colonization-associated root cell death processes.
(A) Generally, root MAMP-triggered immunity is activated after pathogen recognition and followed by the MAPK signaling cascade activation. Through the regulation of transcription factors (TF) (e.g. WRKYs), defense genes (e.g. PR genes, PRRs) are induced. The defense-related proteins (PR proteins, PRRs proteins) are synthezied and processed within the ER by ER-QC components (e.g. BIP2, DAD1) and then sequestered in vesicles that transport to the cis-Golgi reticulum and subsequently pass through the Golgi to the *trans*-Golgi network (TGN). Here, the secreted proteins are transported to the plasma membrane in an actin-dependent manner. (B) During *P. indica* colonization, ER function is disturbed by the fungus. This is proposed to result in a reduced ability of the ER to process and secret MTI components (e.g. PR proteins, PRR proteins), which would impair the function of root MTI. In addition, ER stress signaling (UPR) is suppressed by *P. indica*. Therefore, ER stress-induced plant PCD is initiated, and through yet to be defined pro-apoptotic signaling pathways, plant cell death is executed by a γVPE/caspase 1-mediated vacuolar collapse.

5. Summary / Zusammenfassung

5.1 Summary

The basidiomycete *Piriformospora indica* colonizes roots of a multitude of plants thereby transferring several beneficial effects such as growth promotion as well as enhanced local and systemic resistance against pathogens (Varma et al., 1999; Waller et al., 2005; Deshmukh and Kogel, 2007; Stein et al., 2008). The wide host range of *P. indica* implicates that the fungus has developed effective strategies to overcome plant innate immunity. Recent cytological studies on *P. indica*-colonized *Arabidopsis* roots revealed that the fungus has developed an initial biotrophic colonization strategy followed by a cell death-associated colonization pattern (Jacobs, Zechman, Kogel, Schäfer, unpublished). It was the aim of my project to analyze the genetic, molecular, and biochemical mechanisms of *P. indica*-mediated cell death during root colonization. Cell biological analyses uncovered an impaired ER integrity as well as vacuolar collapse at later cell-death-associated fungal colonization stages. Subsequent pharmacological analyses confirmed ER stress induction by *P. indica*, but molecular and biochemical analyses suggested suppression of ER stress signaling, known as unfolded protein response, by the fungus. Based on biochemical and genetic experiments, I provide evidence that colonization-associated cell death is initiated by an uncoupled ER stress signaling. My data raises the possibility that the emerging ER stress activates a cell death programme, whose execution is dependent on the caspase-1 activity mediated by vacuole-localized vacuolar processing enzymes (VPEs). I propose a model in which *P. indica* is impairing ER integrity to induce VPE/caspase 1-dependent cell death. In addition, the fungus might interfere with ER functionality to disturb root MAMP-triggered immunity. The combined inhibitory activity might ultimately enhance the fungus´ ability to colonize roots.

5.2 Zusammenfassung

Der Basidiomyzet *Piriformospora indica* besiedelt Wurzeln einer Vielzahl von Pflanzen, die dadurch ein verbessertes Wachstum sowie erhöhte lokale und systemische Resistenz gegenüber Pathogenen zeigen (Varma et al., 1999; Waller et al., 2005; Deshmukh and Kogel, 2007; Stein et al., 2008). Das breite Wirtsspektrum von *P. indica* weist auf die Entwicklung effektiver Strategien zur Überwindung der Pflanzenabwehr hin. Kürzlich durchgeführte zytologische Studien von durch *P. indica* besiedelter Arabidopsiswurzeln zeigten eine zweiphasige Besiedlungsstrategie, welche durch eine initiale biotrophe gefolgt von einer Zelltod-assoziierten Phase gekennzeichnet war (Jacobs, Zechman, Kogel, Schäfer, unpublished). Auf diesen Ergebnissen aufbauend war das Ziel meiner Arbeit, die genetischen, molekularen und biochemischen Mechanismen des Besiedlung-assoziierten Zelltods zu untersuchen. Zellbiologische Studien zeigten zunächst eine beeinträchtigte ER Integrität als auch einen vakuolären Kollaps während der Zelltod-assoziierten Wurzelbesiedlung. Nachfolgende pharmokologische Analysen bestätigten eine ER Stressinduktion durch *P. indica,* während molekulare und biochemische Untersuchung eine gleichzeitige, Pilz-vermittelte Suppression in der ER Stress-assoziierten Signalgebung nachwiesen. Basierend auf biochemischen und genetischen Experimenten konnte ich schließlich zeigen, dass Besiedlung-assozierter Zelltod durch eine unangepasste ER Stresssignalgebung initiert wird. Die Exekution des daraufhin ausgelösten Zelltodprogramms ist von Caspase 1 Aktivität abhängig, welche durch vakuolär lokalisierte *vacuolar processing enzymes* (VPEs) vermittelt wird. In dem von mir aufgestellten Modell beeinträchtigt *P. indica* die ER Integrität, um VPE/Caspase 1-abhängigen Zelltaod zu induzieren. Daneben könnte eine Störung der Funktionalität des ERs durch den Pilz eine Beeinträchtigung der Wurzelabwehr zur Folge haben. Diese kombinierte inhibitorische Aktivität führt vermutlich zu einer erhöhten Wurzelkompatibilität.

6. References

Anelli, T., Ceppi, S., Bergamelli, L., Cortini, M., Masciarelli, S., Valetti, C., Sitia, R. (2007) Sequential steps and checkpoints in the early exocytic compartment during secretory IgM biogenesis. *EMBO J.* 26:4177–4188.

Anelli, T., and Sitia, R. (2008) Protein quality control in the early secretory pathway. *EMBO J* 27:315-27.

Asai, T., Stone, JM., Heard, JE., Kovtun, Y., Yorgey, P., Sheen, J., Ausubel, FM. (2000) Fumonisin B1-induced cell death in arabidopsis protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. *Plant Cell.* 12:1823-1836.

Apel, K. and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol* 55:373–399.

Akiyama, K., Matsuzaki, K. and Hayashi, H. (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435:824–827.

Babaeizad, V., Imani, J., Kogel, K.-H., Eichmann, R., Hückelhoven, R. (2008) Over-expression of the cell death regulator BAX inhibitor-1 in barley confers reduced or enhanced susceptibility to distinct fungal pathogens. *Theor. Appl. Genet.* 118:455-463.

Bago, B., Pfeffer, PE., Shachar-Hill, Y. (2000) Carbon metabolism and transport in arbuscular mycorrhizas. *Plant Physiol.* 124:949-958.

Bago, B., Pfeffer, PE., Abubaker, J., Jun, J., Allen, JW., Brouillette, J., Douds, DD., Lammers, PJ., Shachar-Hill, Y. (2003) Carbon export from arbuscular mycorrhizal roots involves the translocation of carbohydrate as well as lipid. *Plant Physiol.* 131:1496–1507.

Bassham, DC., Laporte, M., Marty, F., Moriyasu, Y., Ohsumi, Y., Olsen, LJ., Yoshimoto, K. (2006) Autophagy in development and stress responses of plants. *Autophagy* . 2:2-11.

Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doubsky, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina, A., Schulze-Lefert, P. (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 323:101-106

Benghezal, M., Wasteneys, G.O., and Jones, D.A. (2000) The C-terminal dilysine motif confers endoplasmic reticulum localization to type I membrane proteins in plants. *Plant Cell* 12:1179–1201.

Bent, AF. and Mackey, D. (2007) Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annu Rev Phytopathol.* 45:399-436.

Blilou, I., Bueno, P., Ocampo, JA., García-Garrido, JM. (2000). Induction of catalase and ascorbate peroxidase activities in tobaccco roots inoculated with the arbuscular mycorrhizal fungus Glomus mosseae. *Mycological Research* 104:722–725.

Boller, T. and Felix, G. (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu Rev Plant Biol.* 60:379-406.

Bonneau, L., Ge, Y., Drury, GE., Gallois, P. (2008) What happened to plant caspases? *J Exp Bot.* 59:491-499.

Boston, R.S., Fontes, E.B., Shank, B.B., and Wrobel, R.L. (1991) Increased expression of the maize immunoglobulin binding protein homolog b-70 in three zein regulatory mutants. *Plant Cell* 3: 497–505.

Boudsocq, M., Willmann, MR., McCormack, M., Lee, H., Shan, L., He, P., Bush, J., Cheng, SH., Sheen, J. (2010) Differential innate immune signalling via Ca(2+) sensor protein kinases. *Nature* 464:418-22

Boyce, M. and Yuan, J. (2006) Cellular response to endoplasmic reticulum stress: a matter of life or death. *Cell Death Differ.* 13:363-373.

Brundrett, M. (2004) Diversity and classification of mycorrhizal associations. *Bot. Rev.* 79: 473–495.

Chae, H.J., Kim, H.R., Xu, C., Bailly-Maitre, B., Krajewska, M., Krajewski, S., Banares, S., Cui, J., Digicaylioglu, M., Ke, N., Kitada, S., Monosov, E., Thomas, M., Kress, C.L., Babendure, J.R., Tsien, R.Y., Lipton, S.A., and Reed, J.C. (2004) BI-1 regulates an apoptosis pathway linked to endoplasmic reticulum stress. *Mol Cell* 15:355-66.

Chen, S., Dickman, MB. (2004) Bcl-2 family members localize to tobacco chloroplasts and inhibit programmed cell death induced by chloroplast-targeted herbicides. *J Exp Bot.* 55:2617–2623.

Cheung, HH., Lynn Kelly, N., Liston, P., Korneluk, RG. (2006) Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. *Exp Cell Res* 312:2347–2357.

Chisholm, S. T., Coaker, G., Day, B. and Staskawicz, B. J. (2006) Host–microbe interactions: shaping the evolution of the plant immune response. *Cell* 124:803–814.

Clay, NK., Adio, AM., Denoux, C., Jander, G., Ausubel, FM. (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science* 323:95-101

Clouse, SD. and Sasse, JM. (1998) BRASSINOSTEROIDS: Essential Regulators of Plant Growth and Development. *Annu Rev Plant Physiol Plant Mol Biol* 49:427–451.

Cohen, P. (1997) The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Cell Biol.* 7:353-361.

Coleman, C.E., Lopes, M.A., Gillikin, J.W., Boston, R.S., and Larkins, B.A. (1995) A defective signal peptide in the maize high-lysine mutant floury 2. *Proc. Natl. Acad. Sci.* USA 92:6828–6831.

Collins, NC., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, JL., Hückelhoven, R., Stein, M., Freialdenhoven, A., Somerville, SC., Schulze-Lefert, P. (2003)

SNAREprotein-mediated disease resistance at the plant cell wall. *Nature* 425:973–977.

Cox, JS. and Walter, P. (1996) A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell* 87:391–404.

Dahmer, MK. (2005) Caspases-2, -3, and -7 are involved in thapsigargin induced apoptosis of SH-SY5Y neuroblastoma cells. *J Neurosci Res* 80:576–583.

Dangl, J. L. and Jones, J. D. G (2001) Plant pathogens and integrated defence responses to infection. *Nature* 411: 826–833.

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

Danon, A., Rotari, V.I., Gordon, A., Mailhac, N., Gallois, P. (2004) Ultraviolet-C overexposure induces programmed cell death in Arabidopsis, which is mediated by caspase-like activities and which can be suppressed by caspase inhibitors, p35 and Defender against Apoptotic Death. *J. Biol. Chem*. 279:779-787.

Di Sano, F., Ferraro, E., Tufi, R., Achsel, T., Piacentini, M., Cecconi, F. (2006) Endoplasmic reticulum stress induces apoptosis by an apoptosome-dependent but caspase 12-independent mechanism. *J Biol Chem* 281:2693–2700.

Deshmukh, S., Hueckelhoven, R., Schäfer, P., Imani, J., Sharma, M., Weiss, M., Waller, F., and Kogel, K.H. (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proc Natl Acad Sci USA* 103:18450–18457.

Deshmukh, S. and Kogel, K.H. (2007) *Piriformospora indica* protects barley from root rot disease caused by Fusarium. *J. Plant Dis. Prot*. 114:263–268.

del Pozo, O., Lam, E. (1998) Caspases and programmed cell death in the hypersensitive response of plants to pathogens. *Curr Biol* 8:1129–1132.

del Pozo, O., Lam, E. (2003) Expression of the baculovirus p35 protein in tobacco affects cell death progression and compromises N gene-mediated disease resistance response to tobacco mosaic virus. *Molecular Plant–Microbe Interactions* 16:485–494.

Dodds, PN., Lawrence, GJ., Catanzariti, AM., The, T., Wang, CI., Ayliffe, MA., Kobe, B., Ellis, JG. (2006) Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proc Natl Acad Sci* U S A. 103:8888-8893.

Dong, X. (2004) NPR1, all things considered. *Curr Opin Plant Biol.* 7:547-552.

Durrant, W.E. and Dong, X. (2004) Systemic acquired resistance. *Annu. Rev. Phytopathol.* **42:**185–209.

Eichmann, R., Dechert, C., Kogel, K.H., and Hückelhoven, R. (2006) Transient over-expression of barley BAX Inhibitor-1 weakens oxidative defence and MLA12-mediated resistance to *Blumeria graminis* f.sp. *hordei*. *Mol Plant Pathol* 7:543-552.

Finlay, R. D. (2008) Ecological aspects of mycorrhizal symbiosis: with special emphasis on the functional diversity of interactions involving the extraradical mycelium. *J. Exp. Bot.* 59:1115–1126.

Fitter, A.H. (2005) Darkness visible: reflections on underground ecology. *J. Ecol.* 93:231–243.

Fontes, E.B., Shank, B.B., Wrobel, R.L., Moose, S.P., OBrian, G.R., Wurtzel, E.T. and Boston, R.S. (1991) Characterization of an immunoglobulin binding protein homolog in the maize floury-2 endosperm mutant. *Plant Cell* 3:483–496.

García-Garrido, JM., Ocampo, JA. (2002) Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. *J Exp Bot.* 53:1377-1386.

Gehrig, H., Schussler, A., and Kluge, M. (1996) Geosiphon pyriforme, a fungus forming endocytobiosis with Nostoc (cyanobacteria), is an ancestral member of the Glomales: evidence by SSU rRNA analysis. *J. Mol. Evol* 43:71–81.

Gillikin, J.W., Zhang, F., Coleman, C.E., Bass, H.W., Larkins, B.A., and Boston, R.S. (1997) A defective signal peptide tethers the floury-2 zein to the endoplasmic reticulum membrane. *Plant Physiol.* 114:345–352.

Glazebrook, J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43:205-227.

Gomez-Gomez, L., Felix, G., and Boller, T. (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J* 18:227-284.

Gomez-Roldan , V., Fermas, S., Brewer, PB., Puech-Pagès, V., Dun, EA., Pillot, JP., Letisse, F., Matusova, R., Danoun, S., Portais, JC., Bouwmeester, H., Bécard, G., Beveridge, CA., Rameau, C., Rochange, SF. (2008) Strigolactone inhibition of shoot branching. *Nature* 455:189-194.

Greenberg, J. T. and Yao, N. (2004) The role and regulation of programmed cell death in plant–pathogen interactions. *Cell. Microbiol* 6:201–211.

Hara-Nishimura, I., Hatsugai, N., Kuroyanagi, M., Nakaune, S., Nishimura, M. (2005) Vacuolar processing enzyme: An executor of plant cell death. *Curr Opin Plant Biol* 8:404–408.

Harding, HP., Zhang, Y., Bertolotti, A., Zeng, H., Ron, D. (2000) Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell* 5:897–904.

Harrison, MJ. (2005) Signaling in the arbuscular mycorrhizal symbiosis. *Annu. Rev. Microbiol* 59:19–42.

Hatsugai, N., Kuroyanagi, M., Yamada, K., Meshi, T., Tsuda, S., Kondo, M., Nishimura, M., Hara-Nishimura, I. (2004) A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. *Science* 305:855-858.

Hatsugai, N., Kuroyanagi, M., Nishimura, M., Hara-Nishimura, I. (2006) A cellular suicide strategy of plants: vacuole-mediated cell death. *Apoptosis* 11:905–911.

Hatsugai, N., Iwasaki, S., Tamura, K., Kondo, M., Fuji, K., Ogasawara, K., Nishimura, M., Hara-Nishimura, I. (2009) A novel membrane fusion-mediated plant immunity against bacterial pathogens.*Genes Dev.* 23:2496-2506.

Hause, B. and Fester, T. (2005) Molecular and cell biology of arbuscular mycorrhizal symbiosis. *Planta* 221:184–196.

He, R., Drury, GE., Rotari, VI., Gordon, A., Willer, M., Tabasum, F., Woltering, EJ., Gallois, P. (2007) Metacaspase-8 modulates programmed cell death induced by UV and H2O2 in Arabidopsis. *Journal of Biological Chemistry* 283:774–783.

Heath, MC. (2000) Hypersensitive response-related death. *Plant Mol Biol* 44:321-334.

Hengartner, Michael O. (2000) The biochemistry of apoptosis. *Nature* 407:770-776.

Hibbett, DS. (2006) A phylogenetic overview of the Agaricomycotina. *Mycologia.* 98:917-925.

Hitomi, J., Katayama, T., Eguchi, Y., Kudo, T., Taniguchi, M., Koyama, Y., Manabe, T., Yamagishi, S., Bando, Y., Imaizumi, K., Tsujimoto, Y., Tohyama, M. (2004) Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. *J Cell Biol* 165:347–356.

Hoeberichts, FA and Woltering, EJ (2003) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. *Bioessays* 25:47–57.

Hoefle, C., Loehrer, M., Schaffrath, U., Frank, M., Schultheiss, H., Hückelhoven, R. (2009) Transgenic suppression of cell death limits penetration success of the soybean rust fungus Phakopsora pachyrhizi into epidermal cells of barley. *Phytopathology* 99:220-226.

Holmes, KC. (1983) Flexibility in tobacco mosaic virus. *Ciba Found Symp*. 93:116-38.

Holt, BF. 3rd, Belkhadir, Y., Dangl, JL. (2005) Antagonistic Control of Disease Resistance Protein Stability in the Plant Immune System. *Science* 309:929-932.

Huffaker, A., Pearce, G., Ryan, CA. (2006) An endogenous peptide signal in Arabidopsis activates components of the innate immune response. *Proc. Natl. Acad. Sci.* USA 103:10098–10103

Hückelhoven, R. (2004) BAX Inhibitor-1, an ancient cell death suppressor in animals and plants with prokaryotic relatives. *Apoptosis* 9:299–307.

Hückelhoven, R (2007a) Cell wall-associated mechanisms of disease resistance and susceptibility. *Annu Rev Phytopathol* 45:101-127.

Hückelhoven, R (2007b) Transport and secretion in plant-microbe interactions. *Curr Opin Plant Biol.* 10:573-579.

Ihara-Ohori, Y., Nagano, M., Muto, S., Uchimiya, H., Kawai-Yamada, M. (2006) Cell death suppressor, Arabidopsis BI-1, is associated with calmodulin-binding and ion homeostasis. *Plant Physiol*. 143:650-660.

Imani, J., Baltruschat, H., Stein, E., Gengxiang, J., Vogelsberg, J., Kogel, K., Hückelhoven, R. (2006) Expression of barley Bax inhibitor-1 in carrots confers resistance to Botrytis cinerea. *Mol. Plant Pathol.* 7:279-284.

Iwata, Y. and Koizumi, N. (2005a) Unfolded protein response followed by induction of cell death in cultured tobacco cells treated with tunicamycin. *Planta* 220:804–807.

Iwata, Y. and Koizumi, N. (2005b) An Arabidopsis transcription factor, AtbZIP60, regulates the endoplasmic reticulum stress response in a manner unique to plants. *Proc. Natl. Acad. Sci.*

USA 102:5280-5285.

Iwata, Y., Fedoroff, N.V., and Koizumi, N. (2008) *Arabidopsis* bZIP60 is a proteolysis-activated transcription factor involved in the endoplasmic reticulum stress response. *Plant Cell* 20:3107-3121.

Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Likpa, V., Kogel, K.H., and Schäfer, P. Broad suppression of innate immunity is required for colonization of *Arabidopsis* roots by the beneficial fungus *Piriformospora indica*. Unpublished.

Jelitto-Van Dooren, E.P., Vidal, S., and Denecke, J. (1999) Anticipating endoplasmic reticulum stress. A novel early response before pathogenesis-related gene induction. *Plant Cell* 11:1935-1944.

Jin, H., Yan, Z., Nam, K.H., and Li, J. (2007) Allele-specific suppression of a defective brassinosteroid receptor reveals a physiological role of UGGT in ER quality control. *Mol Cell* 26:821-830.

Jones, AM. (2001) Programmed cell death in development and defence. *Plant Physiology* 125:94–97.

Jones, D.A. and Takemoto, D. (2004) Plant innate immunity—direct and indirect recognition of general and specific pathogen-associated molecules. *Curr. Opin. Immunol* 16:48–62

Kamauchi, S., Nakatani, H., Nakano, C., and Urade, R. (2005) Gene expression in response to endoplasmic reticulum stress in *Arabidopsis thaliana*. *FEBS J* 272:3461-3476.

Kaufman, RJ. (2002) Orchestrating the unfolded protein response in health and disease. *J Clin Invest* 110:1389–1398.

Kawai-Yamada, M., Pan, L., Reed, J.C., Uchimiya, H. (1999) Evolutionally conserved plant homologue of the Bax inhibitor-1 (BI-1) gene capable of suppressing Bax-induced cell death in yeast. *FEBS Lett.* 464:143-147.

Kawai-Yamada, M., Saito, Y, Jin, L., Ogawa, T., Kim, K.M., Yu, L.H., Tone, Y, Hirata, A, Umeda, M., Uchimiya, H. (2005) A Novel Arabidopsis Gene Causes Bax-like Lethality in Saccharomyces cerevisiae. *J. Biol. Chem.* 280:39468–39473.

Kinoshita, T., Yamada, K., Hiraiwa, N., Kondo, M., Nishimura, M., Hara-Nishimura, I. (1999) Vacuolar processing enzyme is upregulated in the lytic vacuoles of vegetative tissues

during senescence and under various stress conditions. *The Plant J* 19:43–53.

Kogel, KH., Franken, P. and Hueckelhoven, R. (2006) Endophyte or parasite – what decides? *Curr Opin Plant Biol* 9:358–363.

Koizumi, N. (1996) Isolation and responses to stress of a gene that encodes a luminal binding protein in Arabidopsis thaliana. *Plant Cell Physiol.* 37:862-865.

Koizumi, N., Ujino, T., Sano, H., Chrispeels, MJ. (1999) Overexpression of a gene that encodes the first enzyme in the biosynthesis of asparagine-linked glycans makes plants resistant to tunicamycin and obviates the tunicamycin-induced unfolded protein response. *Plant Physiol*. 121:353-361.

Koizumi, N., Martinez, I. M., Kimata, Y., Kohno, K., Sano, H., and Chrispeels, M. J. (2001) Molecular characterization of two Arabidopsis Ire1 homologs, endoplasmic reticulum-located transmembrane protein kinases. *Plant Physiol*. 127:949–962.

Kuriyama, H. and Fukuda, H. (2002) Developmental programmed cell death in plants.*Curr.Opin.Plant Biol* 5:568-573.

Kuroyanagi, M., Yamada, K., Hatsugai, N., Kondo, M., Nishimura, M., and Hara-Nishimura, I. (2005) Vacuolar processing enzyme is essential for mycotoxin-induced cell death in *Arabidopsis thaliana*. *J Biol Chem* 280:32914-32920.

Lam, E. and del Pozo, O. (2000) Caspase-like protease involvement in the control of plant cell death. *Plant Mol. Biol* 44:417–428.

Lam, E. (2004) Controlled cell death, plant survival and development. *Nature Rev. Mol. Cell Biol*. 5:305-315.

Lam, E. (2008) Programmed cell death in plants: Orchestrating an intrinsic suicide program within walls. *Crit. Rev. Plant Sci*. 27:413-423.

Lambais, MR. (2000) Regulation of plant defence-related genes in arbuscular mycorrhizae. *The American Phytopathological Society*, 45–59.

Lipka, V., Dittgen, J., Bednarek, P., Bhat, R., Wiermer, M., Stein, M., Landtag, J., Brandt, W., Rosahl, S., Scheel, D., Llorente, F., Molina, A., Parker, J., Somerville, S., Schulze-Lefert, P. (2005) Pre- and postinvasion defenses both contribute to nonhost

resistance in *Arabidopsis*. *Science* 310:1180-1183.

Lipka, V., Kwon, C., and Panstruga, R. (2007) SNARE-ware: the role of SNARE-domain proteins in plant biology. *Annu Rev Cell Dev Biol* 23:147-174.

Liu, Y., Schiff, M., Czymmek, K., Tallóczy, Z., Levine, B., Dinesh-Kumar, SP. (2005) Autophagy regulates programmed cell death during the plant innate immune response. *Cell.* 121:567-577.

Liu, J., Maldonado-Mendoza, I., Lopez-Meyer, M., Cheung, F., Town, CD., Harrison, MJ. (2007) Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. *Plant J.* 50:529–544.

Liu, J. X., Srivastava, R., Che, P., and Howell, S. H. (2007) An endoplasmic reticulum stress response in Arabidopsis is mediated by proteolytic processing and nuclear relocation of a membrane-associated transcription factor, bZIP28. *Plant Cell* 19:4111–4119.

Liu, J.X., Srivastava, R., and Howell, S.H. (2008) Stress-induced expression of an activated form of AtbZIP17 provides protection from salt stress in Arabidopsis. *Plant Cell Environ* 31:1735-1743.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*, 4:402-408.

Ma, Y., Brewer, JW., Diehl, JA., Hendershot, LM. (2002) Two distinct stress signalling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J Mol Biol* 318:1351–1365.

Mackey, D., Holt, BF III, Wiig, A., Dangl, JL. (2002) RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. *Cell* 108:743–754.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L. and Dietrich, R.A. (2000) The transcriptome of Arabidopsis thaliana during systemic acquired resistance. *Nat. Genet.* **26**:403–409.

Malhotra, J.D. and Kaufman, R.J. (2007) The endoplasmic reticulum and the unfolded protein response. *Semin Cell Dev Biol* 18:716-731.

Martinez, I.M. and Chrispeels, M.J. (2003) Genomic analysis of the unfolded protein response in Arabidopsis shows its connection to important cellular processes. *Plant Cell* 15:561–576.

Millet, YA., Danna, CH., Clay, NK., Songnuan, W., Simon, MD., Werck-Reichhart, D., Ausubel, FM. (2010) Innate immune responses activated in Arabidopsis roots by microbe-associated molecular patterns. *Plant Cell* 22:973-990.

Milligan, MJ. and Williams PG. (1998) The mycorrhizal relationship of multinucleate *Rhizoctonias* from nonorchids with *Microtis* (Orchidaceae). *New Phytol* 108:205-209.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., Shibuya, N. (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proc Natl Acad Sci U S A* 104:19613-19618

Mori, K., Ogawa, N., Kawahara, T., Yanagi, H., Yura, T. (2000) mRNA splicing mediated C-terminal replacement of transcription factor Hac1p is required for efficient activation of the unfolded protein response. *Proc Natl Acad Sci* USA 97:4660–4665.

Morishima, N., Nakanishi, K., Takenouchi, H., Shibata, T., Yasuhiko, Y. (2002) An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem* 277:34287–34294.

Mur, LA., Kenton, P., Lloyd, AJ., Ougham, H., Prats, E. (2008) The hypersensitive response; the centenary is upon us but how much do we know? *J Exp Bot* 59:501-520

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, BA., Yuan, J. (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 403:98–103.

Nakaune, S., Yamada, K., Kondo, M., Kato, T., Tabata, S., Nishimura, M., Hara-Nishimura, I. (2005) A vacuolar processing enzyme, deltaVPE, is involved in seed coat formation at the early stage of seed development. *The Plant Cell* 17:876–887.

Nekrasov, V., Li, J., Batoux, M., Roux, M., Chu, Z.H., Lacombe, S., Rougon, A., Bittel, P.,

Kiss-Papp, M., Chinchilla, D., van Esse, H.P., Jorda, L., Schwessinger, B., Nicaise, V., Thomma, B.P., Molina, A., Jones, J.D., and Zipfel, C. (2009) Control of the pattern-recognition receptor EFR by an ER protein complex in plant immunity. *EMBO J* 28:3428-38.

Nicaise, V., Roux, M., Zipfel, C. (2009) Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiol.* 150:1638-1647.

Nishitoh, H., Matsuzawa, A., Tobiume, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., Ichijo, H. (2002) ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* 16:1345–1355.

Nooden, LD. (2004) Plant cell death processes. *Elsevier, Inc. Press*.

Noh, S. J., Kwon, C. S., Chung, W. I. (2002) Characterization of two homologs of Ire1p, a kinase/endoribonuclease in yeast, inArabidopsis thaliana. *Biochim. Biophys. Acta.* 1575:130–134.

Noh, S.J., Kwon, C.S., Oh, D.H., Moon, J.S., and Chung, W.I. (2003) Expression of an evolutionarily distinct novel BiP gene during the unfolded protein response in Arabidopsis thaliana. *Gene* 311:81–91.

Okushima, Y., Koizumi, N., Yamaguchi, Y., Kimata, Y., Kohno, K., Sano, H. (2002) Isolation and characterization of a putative transducer of endoplasmic reticulum stress in Oryza sativa. Plant Cell Physiol. 43:532–539.

Parniske, M. (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nat. Rev. Microbiol.* 6:763–775.

Peskan-Berghoefer, T., Shahollaria, B., Giong, PH., Hehl, S., Markerta, C., Blanke, V., Kost, G., Varma, A. and Oelmüller, R. (2004) Association of *Piriformospora indica* with Arabidopsis thaliana roots represents a novel system to study beneficial plant–microbe interactions and involves early plant protein modifications in the endoplasmatic reticulum and at the plasma membrane. *Physiol Plant* 122:465–477.

Peterson, RL. and Massicotte, HB. (2004) Exploring structural definition of mycorrhizas,

with emphasis on nutrient-exchange interfaces. *Can J Bot* 82:1074-1088.

Pham, GH., Kumari, R., Singh, A., Malla, R., Prasad, R., Sachdev, M., Kaldorf, M., Buscot, F., Ölmüller, R., Hampp, R., Saxena, AK., Rexer, KH., Kost, G., Varma, A. (2004) Axenic culture of symbiotic fungus *Piriformospora indica.* In: Varma A, Abbot L, Werner D and Hampp R (eds). *Plant Surface Microbiology*. Springer-Verlag. Berlin, Heidelberg. pp 593-612.

Pieterse, C.M.J., van Wees, S.C.M., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. and van Loon, L.C. (1998) A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell* 10:1571–1580.

Pieterse, C.M.J. and van Loon, L.C. (2004) NPR1: the spider in the web of induced resistance signaling pathways. *Curr. Opin. Plant Biol.* 7:456–464.

Rasheva, V.I. and Domingos, P.M. (2009) Cellular responses to endoplasmic reticulum stress and apoptosis. *Apoptosis* 14:996-1007.

Reddy, P., Sparvoli, A., Fagioli, C., Fassina, G., Sitia, R. (1996) Formation of reversible disulfide bonds with the protein matrix of the endoplasmic reticulum correlates with the retention of unassembled Ig light chains. *EMBO J* 15:2077–2085.

Rojo, E., Martin, R., Carter, C., Zouhar, J., Pan, S., Plotnikova, J., Jin, H., Paneque, M., Sanchez-Serrano, JJ., Baker, B. (2004) VPEgamma exhibits a caspase-like activity that contributes to defense against pathogens. *Curr Biol* 14:1897–1906.

Ron, D. and Habener, JF. (1992) CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev* 6:439–453.

Rutkowski, DT. and Kaufman, RJ. (2004) A trip to the ER: coping with stress. *Trends Cell Biol*. 14:20-28.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.-Y., and Hunt, M.D. (1996) Systemic acquired resistance. *Plant Cell* 8:1809–1819.

Ryan, D., Kendall, M., Robards, K. (2007) Bioactivity of oats as it relates to cardiovascular disease. *Nutr Res Rev.* 20:147-162.

Sahay, N.S. and Varma, A. (1999) Piriformospora indica: a new biological hardening tool for micropropagated plants. *FEMS Microbiol Lett* 181: 297–302.

Saijo, Y., Tintor, N., Lu, X., Rauf, P., Pajerowska-Mukhtar, K., Häweker, H., Dong, X., Robatzek, S., Schulze-Lefert, P. (2009) Receptor quality control in the endoplasmic reticulum for plant innate immunity. *EMBO J* 28:3439-3449.

Smith, S. E. and Read, D. J. (2008) Mycorrhizal Symbiosis (Academic, London)

Salzer, P., Bonanomi, A., Beyer, K., Vögeli, R., Aeschbacher, R.A., Lange, J., Wiemken, A., Kim, D., Cook, D.R., Boller, T. (2000) Differential expression of eight chitinase genes in Medicago truncatula roots during mycorrhiza formation, nodulation and pathogen-infection. *Mol. Plant-Microbe Interact.* 13:763-777.

Schäfer, P. and Kogel, K.H. (2009) The sebacinoid fungus *Piriformospora indica*: an orchid mycorrhiza which may increase host plant reproduction and fitness. In: *The Mycota,* Vol. 5, Plant Relationships (Deising, H.B., Esser, K. eds), Springer-Verlag, Heidelberg. pp. 99–112.

Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nature Prot* 3:1101-1108.

Schröder, M. and Kaufman, R.J. (2005) ER stress and the unfolded protein response. *Mutat. Res.* 569:29-63.

Schröder, M. (2006) The unfolded protein response. *Mol Biotechnol* 34:279-90.

Schröder M. (2008) Endoplasmic reticulum stress responses. *Cell Mol Life Sci.* 65:862-894.

Scorrano, L., Oakes, SA., Opferman, JT., Cheng, EH., Sorcinelli, MD., Pozzan, T. (2003) BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science* 300:135–139.

Selosse, MA., Setaro, S., Glatard, F., Richard, F., Urcelay, C.,Weiss, M. (2007) *Sebacinales* are common mycorrhizal associates of Ericaceae. *New Phytol* 174:864-878.

Setaro, S., Weiss, M., Oberwinkler, F., Kottke, I. (2006) Sebacinales form ectendomycorrhizas with Cavendishia nobilis, a member of the Andean clade of Ericaceae, in the mountain rain forest of southern Ecuador. *New Phytologist* 169:355–365.

Sharma, M., Schmid, M., Rothballer, M., Hause, G., Zuccaro, A., Imani, J., Kampfer, P., Domann, E., Schäfer, P., Hartmann, A. and Kogel, K.H. (2008) Detection and identification of bacteria intimately associated with fungi of the order Sebacinales. *Cell. Microbiol.* 11:2235–2246.

Shiozaki, EN. and Shi, Y. (2004) Caspases, IAPs and Smac/DIABLO: mechanisms from structural biology. *TRENDS in Biochem. Sci*. 39:486-494.

Solaiman, M. D. Z. and Saito, M. (1997) Use of sugars by intraradical hyphae of arbuscular mycorrhizal fungi revealed by radiorespirometry. *New Phytol.* 136:533–538.

Stein, E., Molitor, A., Kogel, K.H. and Waller, F. (2008) Systemic resistance in Arabidopsis conferred by the mycorrhizal fungus Piriformospora indica requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant Cell Physiol*. 11:1747–1751.

Suarez, MF., Filonova, LH., Smertenko, A., Savenkov, EI., Clapham, DH., von Arnold, S., Zhivotovsky, B., Bozhkov, PV. (2004) Metacaspase-dependent programmed cell death is essential for plant embryogenesis. *Curr Biol* 14:339–340.

Szegezdi, E., Logue, S.E., Gorman, A.M., and Samali, A. (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 7:880-885.

Tajima, H., Iwata, Y., Iwano, M., Takayama, S., and Koizumi, N. (2008) Identification of an Arabidopsis transmembrane bZIP transcription factor involved in the endoplasmic reticulum stress response. *Biochem. Biophys. Res. Commun.* 374:242–247.

Takemoto, D., Jones, DA., Hardham, AR. (2003) GFP-tagging of cell components reveals the dynamics of subcellular re-organization in response to infection of Arabidopsis by oomycete pathogens. *Plant J.* 33:775–792.

Tan, Y., Dourdin, N., Wu, C., De, VT., Elce, JS., Greer, PA. (2006) Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. *J Biol Chem* 281:16016–16024.

Tobiume, K., Saitoh, M., Ichijo, H. (2002) Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. *J Cell Physiol* 191:95–104.

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J., Katagiri, F. (2009) Network properties of robust immunity in plants. *PLoS Genet* 5:e1000772.

Urade, R. (2007) Cellular response to unfolded proteins in the endoplasmic reticulum of plants. *FEBS. J*. 274:1152–1171.

Urade R. (2009) The endoplasmic reticulum stress signaling pathways in plants. *Biofactors*. 35:326-331.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, HP., Ron, D. (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287:664–666.

Uren, AG., O'Rourke, K., Aravind, L., Pisabarro, MT., Seshagiri, S., Koonin, EV., Dixit, VM. (2000) Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol Cell* 6:961–967.

Van Baarlen, P., Woltering, EJ., Staats, M., Van Kan, JAL. (2007) Histochemical and genetic analysis of host and non-host interactions of Arabidopsis with three Botrytis species: an important role for cell death control. *Molecular Plant Pathology* 8:41–54.

van der Biezen, E. A. and Jones, J. D. G. (1998) Plant disease resistance proteins and the gene-for-gene concept. *Trends Biochem. Sci*. 23:454–456.

van Doorn, WG., Woltering, EJ. (2004) Senescence and programmed cell death: substance or semantics? *J Exp Bot.* 55:2147–2153.

van Loon, L.C. (1977) Induction by chloroethylphosphonic acid of viral-like lesions, associated proteins, and systemic resistance in tobacco. *Virology* 80:417–420.

Pieterse, CM. and van Loon, LC. (1999) Salicylic acid-independent plant defence pathways. *Trends Plant Sci.* 4:52-58.

Varma, A., Verma, S., Sudha, Sahay, N., Butehorn, B., Franken, P. (1999) *Piriformospora indica*, a cultivable plant-growthpromoting root endophyte. *Appl Environ Microbiol* 65:2741–2744.

Vercammen, D., van de Cotte, B., De Jaeger, G., Eeckhout, D., Casteels, P., Vandepoele, K., Vandenberghe, I., Van Beeumen, J., Inze, D., Van Breusegem, F. (2004) Type II metacaspases Atmc4 and Atmc9 of *Arabidopsis thaliana* cleave substrates after arginine and lysine. *J Biol Chem* 279:45329–45336.

Verma, S., Varma, A., Rexer, K.H., Hassel, A., Kost, G., Sarabhoy, A., Bisen, P., Butehorn, B., Franken, P. (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia* 90:896–903.

Vitale, A., and Boston, R.S. (2008) Endoplasmic reticulum quality control and the unfolded protein response: insights from plants. *Traffic* 9:1581-1588.

Waller, F., Achatz, B., Baltruschat, H. Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., Franken, P., Kogel, KH. (2005) The endophytic fungus Piriformospora indica reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc Natl Acad Sci* USA 102:13386–13391.

Wang, D., Weaver, N.D., Kesarwani, M. and Dong, X. (2005) Induction of protein secretory pathway is required for systemic acquired resistance. *Science* 308:1036-1040.

Warcup, J.H. (1988) Mycorrhizal associations of isolates of *Sebacina vermifera*. *New Phytol.* 110:227– 231.

Watanabe, N. and Lam, E. (2004) Recent advance in the study of caspase-like proteases and Bax inhibitor-1 in plants: their possible roles as regulator of programmed cell death. *Mol Plant Pathol* 5:65–70.

Watanabe, N. and Lam, E. (2008a) BAX inhibitor-1 modulates endoplasmic reticulum stress-mediated programmed cell death in *Arabidopsis*. *J Biol Chem* 283:3200-3210.

Watanabe, N. and Lam, E. (2008b) Arabidopsis Bax inhibitor-1: A rheostat for ER stress-induced programmed cell death. *Plant Signal Behav*. 3:564-566.

Watanabe, N. and Lam, E. (2009) Bax inhibitor-1, a conserved cell death suppressor, is a key molecular switch downstream from a variety of biotic and abiotic stress signals in plants. *Int J Mol Sci* 10:3149-3167.

Weiss, M., Selosse, M-A., Rexer, K-H., Urban, A., Oberwinkler, F. (2004) Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. *Mycol Res* 108:1003–1010.

Woltering, EJ., van der Bent, A., Hoeberichts, FA. (2002) Do plant caspases exist? *Plant Physiol* 130:1764–1769.

Woltering, E.J. (2004) Death proteases come alive. *Trends Plant Sci*. 9:469-472.

Xu, Q., and Reed, J.C. (1998) Bax inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast. *Mol Cell* 1:337-346.

Xu, C., Xu, W., Palmer, A.E., Reed, J.C. (2008) BI-1 regulates endoplasmic reticulum Ca2+ homeostasis downstream of Bcl-2 family proteins. *J. Biol. Chem.* 283:11477-11484.

Yamada, K., Shimada, T., Nishimura, M., Hara-Nishimura, I. (2005) A VPE family supporting various vacuolar functions in plants. *Physiologia Plantarum* 123:369–375.

Yamaguchi, Y., Pearce, G., Ryan, CA. (2006) The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in Arabidopsis, is functional in transgenic tobacco cells. *Proc. Natl. Acad. Sci.* USA 103:10104–10109.

Ye, J., Rawson, RB., Komuro, R., Chen, X., Dave, UP., Prywes, R., Brown, MS., Goldstein, JL. (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol Cell* 6:1355–1364.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., Mori, K. (2000) ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Mol Cell Biol* 20:6755–6767.

Zechmann, B., Müller, M., and Zelling, G. (2007) Membrane associated qualitative differences in cell ultrastructure of chemically and high pressure cryofixed plant cells. *J Struct Biol* 158:370-377.

Zhang, J., Shao, F., Li, Y., Cui, H., Chen, L., Li, H., Zou, Y., Long, C., Lan, L., Chai, J., Chen, S., Tang, X., Zhou, JM. (2007) A Pseudomonas syringae effector inactivates MAPKs tosuppress PAMP-induced immunity in plants. *Cell Host Microbe* 1:175-185

Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, RT., Remotti, H. (1998) CHOP is implicated in programmed cell death in response to impaired function of the

endoplasmic reticulum. *Genes Dev* 12:982–995.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, EJ., Jones, JD., Felix, G., Boller, T. (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 15:764-767.

Zipfel, C. and Felix, G. (2005) Plants and animals: a different taste for microbes? *Curr. Opin. Plant Biol*. 8:353–360.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., and Felix, G. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell 125:749-760.

Zong, WX., Li, C., Hatzivassiliou, G., Lindsten, T., Yu, QC., Yuan, J., Thompson, CB. (2003) Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. *J Cell Biol* 162:59–69.

7. Erklärung

Ich erkläre: Ich habe die vorgelegte Dissertation selbständing und ohne unerlaubete fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „ Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eigehalten.

Gießen, Nov 2010

Xiaoyu Qiang

8. Acknowledgements

Here I would like to thank all dear persons; it is obvious that without all of you, the realization of this work would have been probably more difficult.

I take my first gratitude to Prof. Dr. Karl-Heinz Kogel for providing me the working and studying opportunity in his institute; for his outstanding supervision and support during the past three years.

I would like to show my gratitude to Prof. Dr. Volker Wissemann for the acceptance as my second referee; for his suggestions during the thesis preparation.

I owe my deepest gratitude to Dr. Patrick Schäfer for his excellent and professional supervision. Without his keen support, his creative ideas, his immeasurable help and endless discussion, I would not have produced these kinds of results. Without his contributions to the careful reviewing of this manuscript, I would not have got this thesis in a limited time.

I would like to thank Dr. Bernd Zechmann for his professional support in the electron microscopy analyses.

It is a pleasure to thank Christina Neumann, Rebekka Fensch, Dagmar Biedenkopf and Elke Stein for providing me the excellent technical supports.

I sincerely thank to all my dear colleagues in IPAZ for their kind help and support during my stay here. It is my pleasure to work in such a friendly atmosphere.

I owe many chocklates to all my friends, especially Dilin, Puyan, Liang, Bertha and Natalia, for their friendship, trust and always in-time help.

Finally, I would like to express my gratitude to my families, especially my parents, for their unconditional support, encouragement and effort to ensure that I have an excellent education from the beginning till now.

I owe many thanks to my husband Xiang for his endless love, encouragement, understanding and patience.

Thank you for everything!

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

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