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Molecular mechanisms of the colonization of plant roots by the mutualistic fungus *Piriformospora indica* and other mycorrhizal fungi

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1 Preface

As opposed to the floral diversity in natural biotopes, agricultural ecosystems are characterized by the accumulation of genetically homogenous crop plants on a comparably large area. Such conditions favour the selection and propagation of microbes that are enabled to colonize these crops. Insufficient pathogen and pest management strategies and the unavailability of resistant crops, respectively, can result in disease outbreaks that can significantly reduce crop yield and yield quality. Root diseases represent devastating stresses in crop production, whose control is often limited due to the reduced availability of resistant germplasms as well as of chemical protectants and application strategies. The observed disease symptoms are often caused by local colonization-associated tissue disintegration and by systemic effects on green parts due to malnutrition as a result of root dysfunction and blockage of the vascular system. In contrast to pathogenic interactions, root colonization by mutualistic microbes adds multiple beneficial effects to plants: local and systemic protection against pathogens, increased plant biomass, and enhanced seed production (Verma et al., 1998; Varma et al., 1999; Peskan-Berghöfer et al.; 2004, Waller et al., 2005; Liu et al., 2007a). However, the determinants of successful root colonization by mutualistic microbes are only partly understood (Parniske et al., 2008). Additional complexity is added as microbial interactions and composition at the rhizosphere might significantly affect mutualistic symbioses - even more as it is currently hypothesized that plants determine the microbial composition at their rhizosphere (Bressan et al., 2009; Bednarek et al., 2010). A first step towards a successful application of mutualistic microbes in the field is to unravel the molecular concept of such interactions. Such studies need to follow specific aspects such as the identification of plant and microbial factors that affect mutualistic symbioses. Equally crucial are the questions: Do mutualists recruit the same mechanisms and follow the same strategies as pathogens to colonize roots? How do mutualists overcome plant defense and are they harmed by root defense? Do breeding efforts against leaf and root pathogens affect mutualistic interactions in roots and what might be the consequence of such side effects for agricultural production? Can we stabilize mutualistic root colonization under a broad environmental variability in order to sustain crop production?

2 Plant-microbe interactions

A plethora of microbes is found at the phylloshere and at the rhizosphere of plants. Among these microbes, a considerable number has evolved abilities to invade plants in order to utilize plant-derived nutrients for reproduction. These interactions range from pathogenic to mutualistic. In dependence on their life style, pathogens are categorized as biotrophs, necrotrophs or hemibiotrophs. These categories reflect the varying infection strategies as biotrophic pathogens colonize living host cells, while necrotrophs make use of toxins to kill host tissue prior to colonization. Hemibiotrophic pathogens follow a combined strategy consisting of an initial biotrophic and a subsequent necrotrophic colonization phase. Mutualistic interactions are considered to be biotrophic. Hemi-/Biotrophism is thought to represent a higher degree of adaptation as colonized host cells have to be maintained in a viable mode. Such microbes have developed specific feeding structures (e.g. haustoria of powdery mildew fungi, arbuscules of arbuscular mycorrhizal fungi) to absorb plant nutrients. This provokes an exposure to the plant's surveillance system and respective microbes are thought to dependent on an effective arsenal of defense-suppressing means to enhance their virulence (Bent and Mackey, 2007; Boller and Felix, 2009). In addition, biotrophs have to manipulate host metabolism for adequate nutritient delivery and for preprocessing nutrients in order to allow their uptake by microbes (Vögele et al., 2001). In all cases, successful plant colonization is a status in which a highly adapted microbe must have overcome the innate immune system of its host before. This adaptation is an evolution-driven specialization process of microbes and commonly determines host ranges and infectable plant tissues (e.g. roots, leaves) (Bent and Mackey, 2007; Jones and Dangl, 2006). Biotrophism is generally regarded as displaying a higher degree of specialization, which might explain the rather narrow host ranges among such microbes compared to necrotrophic microbes. Notably, irrespective of the underlying life style, plant colonization is the exception as plants possess a highly effective multilayered defense system, designated as innate immunity (Jones and Dangl, 2006; Boller and Felix, 2009). In addition to pathogens, mutualists are confronted with innate immune responses and should need to overcome it in order to establish compatible interactions (Garcia-Garrido and Ocampo, 2002).

2.1 Mutualistic plant-microbe symbioses

Plant-microbe interactions are categorized by their impact on the interacting partners. In contrast to the devastating effects of pathogens, plants gain benefits in mutualistic symbioses with endophytes, such as abiotic stress tolerance or biotic stress resistance in roots and shoots. Mutualistic symbioses are further described by the bidirectional nutritient flow between the interacting partners, which is unidirectional in pathogenic interactions. Per definition, mycorrhizal symbioses are characterized by the exchange of nutrients between the interacting partners. Based on structural characteristics mycorrhizal symbioses are categorized as arbuscular mycorrhizas (AM), ectomycorrhizas, ectendomycorrhizas, ericoid mycorrhizas, arbutoid mycorrhizas, monotropoid mycorrhizas, and orchid mycorrhizas (Peterson and Massicotte, 2004). Based on fossil records it is hypothesized that the settlement on land by plants was achieved in symbiosis with arbuscular mycorrhizal fungi (AMF) (Remy et al., 1994). Mycorrhization enhances the absorbance area of plant roots, thereby improving the acquisition of nutrients, mostly phosphate, from the soil via the plant partner. In turn, mycorrhizas are C-heterotroph and therefore are depedent on carbohydrates delivered by the host plant. Environmental factors determine mutualistic root interactions as, for example, mycorrhization gradually decreases with the plant accessibility of phosphorus in the soil. This indicates the regulatory capacity of the plant on mycorrhization. Root colonization by mutualistic symbionts needs to be seen as a strong dependence on the successful modification of several complex cellular and molecular events inside the host. Root cell penetration and colonization is preceded by fungal recognition in which the receptor SYMRK (Endre et al., 2002; Stracke et al., 2002) is essentially involved. Subsequently, nuclear Ca^{2+} spiking is observed for which the proteins CASTOR, POLLUX (Imaizumi-Anraku et al., 2005) as well as the nucleoporins NUP85 and 133 (Kanamori et al., 2006, Saito et al., 2007) are required. A Ca^{2+}-CALMODULIN-DEPENDENT PROTEIN KINASE (CCaMK) is essential for AM development (Levy et al., 2002; Mitra et al., 2002) and might translate the Ca^{2+} spiking into cell responses by its interaction with CYCLOPS, which represents a protein of unknown function (Yano et al., 2008). Intriguingly, these proteins are also essentially required for root nodulation by rhizobia (Parniske et al., 2008). After fungal recognition, intracellular accomodation is initiated by the formation of a prepenetration apparatus (PPA) in host cells. The PPA is build by rearranging the cytoskeleton and the endoplasmic reticulum (ER) to form a tunnel, which is used like an infection thread to intracellularly colonize host cells (Genre

et al., 2005; 2008). These studies indicate the complexity of processes participating in the establsihment of host-mutualists interactions.

2.2 The fungal order *Sebacinales*

The fungal order Sebacinales was recently defined by phylogenetic studies using the nuclear DNA sequence of the large ribosomal subunit. This order takes a central position within the Hymenomycetidae of the fungal phylum Basidiomycota. The order Sebacinales exclusively harbours an extraordinary diversity of beneficial fungi, which includes ectomycorrhizas, orchid mycorrhizas, ericoid mycorrhizas, cavendishioid mycorrhizas and jungermannioid mycorrhizas in liverworts (McKendrick et al., 2002; Selosse et al., 2002; 2007; Kottke et al., 2003; Urban et al., 2003; Weiß et al., 2004; Setaro et al., 2006). Based on this mycorrhizal diversity, the Sebacinales are thought to significantly support terrestrial ecosystems (Weiß et al. 2004).

The phylogenetic studies divided Sebacinales into two subgroups of which subgroup A consists of ectomycorrhizas and orchid mycorrhizas. These mycorrhizas colonize roots of achlorophyllous or rather heterotrophic orchids by forming hyphal sheaths and intracellular hyphae (Weiß et al. 2004). A similar colonization pattern was found in ectendomycorrhizal sebacinoids that were isolated from Ericaceae (Selosse et al. 2007). Subgroup B is more heterogenic as it habours *Sebacina vermifera* isolates from autotrophic orchids, ericoid mycorrhizas associated with *Gaultheria shallon*, cavendishioid mycorrhizas, and liverwort-associated jungermannioid mycorrhizas (Weiß et al. 2004; Selosse et al. 2007). The various *S. vermifera* isolates are particularly interesting. Warcup (1988) isolated several orchid symbionts of this *S. vermifera* complex. In contrast to sebacinoid mycobionts of group A, these fungi can be axenically cultivated. *S. vermifera* isolates were hypothesized to represent a conglomerate of species rather than one diverse species (Warcup 1988; Weiß et al. 2004) and all members of subgroup B might belong to the *S. vermifera* complex (Weiß et al. 2004).

Although saturated sampling has not been performed, Sebacinales might have a global distribution (Verma et al. 1998; Weiß et al. 2004; Setaro et al. 2006; Selosse et al. 2007) as members were isolated in Australia, Europe and North America (Weiß et al. 2004; Selosse et al. 2007). It is currently unknown whether all members can be regarded as beneficial organisms. Mutualistic activities were found to be mediated by those members that were analyzed, including *S. vermifera* isolates and *Piriformsopora indica*. Colonization of monocotyledonous and dicotyledonous plants by these isolates

resulted in growth promotion and/or enhanced disease resistance (Barazani et al., 2005; 2007; Waller et al. 2005).

2.3 *Piriformospora indica*

The fungus *Piriformospora indica* is embedded within *Sebacinales* group B with the closest relationship to *S. vermifera* isolates and multinucleate *Rhizoctonia* (Weiß et al. 2004). Multinucleate *Rhizoctonia* was originally characterized by its morphological traits. Based on recent phylogenetic studies, this fungus was identified as a member of the *Sebacinales* and it is not related to the pathogenic *Rhizoctonia solani* spp. and binucleate *Rhizoctonia* spp., which belong to the *Ceratobasidiales* (Ogoshi 1987; Weiß et al. 2004; Gonzalez et al. 2006). *P. indica* was isolated in the rhizosphere of two shrubs of the Indian Thar desert, northwest Rajasthan (Verma et al. 1998). However, a physical interaction of *P. indica* with these plants was not reported. The natural host of *P. indica* as well as multinucleate *Rhizoctonia* has not been determined (Williams 1985), while *S. vermifera* isolates were sampled from diverse orchid plants (Warcup 1988; Weiß et al. 2004). *P. indica* occasionally produced intracellular coils in maize roots (Varma et al. 1999), reminiscent of hyphal pelotons, which represent intracellular infection structures of orchid mycorrhizas. This observation was supported by Blechert et al. (1999) as colonisation of protocorms and roots of autotrophic *Dactylorhiza* spp. (Orchidaceae) by *P. indica* was associated with the formation of hyphal coils (pelotons). In orchid mycorrhizas, these pelotons are surrounded by perifungal membranes and interfacial matrices separating them from the host cytoplasm. These complexes at the plant-fungus interfaces function specifically in nutrient exchange (Peterson and Massicotte 2004). It is unknown whether intracellular hyphae of *P. indica* possess perifungal membranes and interfacial matrices enabling these organs to exchange nutrients, as reported for orchid mycorrhizas. However, recent studies monitored an improved phosphate supply to maize shoots by *P. indica* (Yadav et al., 2010) as reported for arbuscular mycorrhizal symbioses (Parniske et al., 2008). In addition, knock-down of a phosphate transporter of *P. indica* resulted in reduced phosphate transfer to the maize shoot and correlated with the inability of the fungus to promote plant growth (Yadav et al., 2010).

The beneficial effects conveyed by *P. indica* to host plants have been extensively studied in barley (Waller et al. 2005), in wheat (Serfling et al., 2007), in *Arabidopsis thaliana* (Shahollari et al. 2005; Sherameti et al. 2005), and other plants (Varma et al.,

1999). In all cases, growth promotion was observed in colonized plants. *P. indica*-mediated growth promotion might not entirely rely on phosphate transfer. A cell wall extract (CWE) of *P. indica* induced, although to a lesser extent, growth promotion in *Arabidopsis*. This CWE was observed to increase cytosolic Ca^{2+} currents and to activate MITOGEN-ACTIVATED PROTEIN KINASE 6 (MPK6), which was shown to be required for CWE-induced growth promotion (Vadassery et al., 2009). In addition, ethylene and cytokinine signaling were shown to contribute to growth promotion (Vadassery et al., 2008; Camehl et al., 2010). In barley, wheat, and *Arabidopsis*, root colonization resulted in systemic resistance in leaves against adapted powdery mildew fungi (Waller et al., 2005; Serfling et al., 2007; Stein et al., 2008). For *Arabidopsis*, this phenomenon was proven to depend on induced systemic resistance (ISR) (Stein et al., 2008). In addition, *P. indica*-colonized plants acquired improved disease resistance towards the necrotrophic root pathogens *Fusarium culmorum* (Waller et al. 2005), *F. graminearum* (Deshmukh and Kogel 2007) as well as stem base-colonizing pathogen *Pseudocercosporella herpotrichoides* (teleomorph: *Tapesia yallundae*) (Serfling et al., 2007).

P. indica possesses an extraordinary broad host range among mono- and dicotyledonous plants (Verma et al., 1998; Blechert et al., 1999; Varma et al., 1999; Waller et al., 2005). Nonhost plants have not been detected, yet. Plant factors as well as fungal factors participating in root colonization are almost unknown. *Arabidopsis* plants lacking PYK10 are more susceptible to *P. indica*. PYK10 encodes a β-glucosidase/myrosinase and is a homolog of PENETRATION 2 (PEN2), which is involved in glucosinolate-associated defense in *Arabidopsis* leaves against *Golovinomyces orontii* (Lipka et al., 2005; Sherameti et al., 2008). Further, ethylene signaling was shown to restrict *P. indica* colonization (Camehl et al., 2010). Although these studies implicated that *P. indica* colonization is controlled by plant innate immunity, it remains elusive, which components and signaling pathways of the immune system are activated by the fungus and which of them control the mutualistic interaction. Since *P. indica* can be genetically transformed (Zuccaro et al., 2009; Yadav et al., 2010), future studies will reveal, which fungal factors participate in the manipulation of host plants.

3 Plant innate immunity

In the past decades, substantial knowledge has been gained on the molecular mechanisms that initiate, regulate, and execute plant innate immunity. Plant innate immunity is based on a two-branched receptor-dependent system. The recognition of microbes is achieved by the specific detection of conserved microbial structures, such as fungal chitin, as well as bacterial molecules such as flagellin, elongation factor TU, peptidoglycans, or lipopolysaccharides, by plasma membrane-localized pattern-recognition receptors (PRRs) (Gomez-Gomez et al. 1999; Kunze et al., 2004; Zeidler et al., 2004; Zipfel et al., 2006; Gust et al., 2007; Miya et al., 2007; Wan et al., 2008; Petutschnig et al., 2010). These components are termed microbe-associated molecular patterns (MAMPs, syn. elicitors). Therefore, the first branch of plant innate immunity is called MAMP-triggered immunity (MTI, syn. basal defense) (Jones and Dangl, 2006). It was the evolution-driven capacity of plant pathogens to develop molecules designated effectors (syn. avirulence and virulence proteins) that interfere with MTI and thus guarantee successful reproduction on plants. Effector recognition is mediated by RESISTANCE (R) proteins, which results in the activation of immune responses termed effector-triggered immunity (ETI, syn. R protein-mediated resistance) (Jones and Dangl, 2006). Several studies indicated an arms race between plants and microbes. As microbes evolve new effectors to overcome ETI, plants develop adequate R proteins to re-establish immunity. However, the inability of microbes to colonize the majority of plants is mediated by nonhost resistance (NHR) (Heath, 2000). NHR is genetically determined and its high effectiveness and sustainability is based on the fast and concerted activation of multilayered plant defense responses (Lipka et al., 2005). NHR is currently believed to be activated by processes determined by MTI and possibly ETI (Jones and Dangl, 2006; Lipka et al., 2008).

3.1 MAMP-triggered immunity

MAMP-triggered immunity (MTI) is a complex defense response (Boller and Felix, 2009) that significantly contributes to resistance against pathogens (Zipfel et al., 2004; 2006). In most cases, MTI is already sufficient to stop plant colonization by microbes. MAMP recognition is a highly specific process. The currently known PRRs have been mostly identified in *Arabidopsis*. Immune responses activated after recognition of chitin by CERK1, flagellin (or its active epitope flg22) by FLS2, and elongation factor TU (or its active epitope elf18) by EFR are among the best studied. Interestingly, microarray-

based studies indicated a high similarity in innate immune responses irrespective of the applied MAMP (Wan et al., 2008). Common to MTI signaling is the immediate phosphorylation and thus activation of MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascades after MAMP recognition (Desikan et al., 2001; Asai et al., 2002). The various MAPK pathways activate different transcription factors such as those of the WRKY family (e.g. WRKY22, WRKY29, WRKY33, WRKY53) (Colcombet and Hirt, 2008). WRKYs are known regulators of defense gene expression; for instance WRKY33 has been demonstrated to induce PAD3 transcription, which encodes a protein involved in the synthesis of the phytoalexin camalexin (Qiu et al., 2008). MAMP recognition immediately induces ion influxes (e.g. Ca^{2+}). Ca^{2+} has been shown to activate CALCIUM-DEPENDENT PROTEIN KINASES (CDPKs), which parallels MAPK phosphorylation (Boudsocq et al., 2010). As recently demonstrated, CDPKs activate a set of transcription factors in a MAPK-dependent, -independent or synergistic manner (Boudsocq et al., 2010). CDPKs further participate in the activation of plasma membrane-bound NADPH oxidases. Among them, RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) is responsible for the rapid production of reactive oxygen species, known as oxidative burst (Zhang et al., 2007). The ROS burst is discussed to have direct antimicrobial activity and to participate in the defense-associated hypersenstive response, which represent a single cell death to stop pathogen invasion (Apel and Hirt, 2004). Furthermore, OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1) is thought to integrate ROS signaling by phosphorylating the MAPKs MPK3 and MPK6 (Rentel et al., 2004). In addition to camalexin, indole glucosinolates (IGS) represent another group of antimicrobial metabolites that are produced after MAMP recognition and significantly contribute to halt microbial invasions (Bednarek et al., 2009, Clay et al., 2009). IGS synthesis is mediated by the transcription factor MYB DOMAIN PROTEIN 51 (MYB51) (Gigolashvili et al., 2007). Microbial attack also results in the generation of endogenous elicitors defined as damage-associated molecular patterns (DAMPs) such as fragments of the plant cell wall or oligogalacturonides (Galletti et al., 2008). Recently, *Arabidopsis* WALL-ASSOCIATED KINASE 1 (WAK1) was identified as oligogalacturonide receptor that, upon stimulation, triggers defense responses against fungal and bacterial pathogens (Brutus et al., 2010). Interestingly, MTI includes the induction of *PRECURSOR OF PEPTIDE 1* (*PROPEP1*) and its homologs (*PROPEP2-7*) in *Arabidopsis*, which encode PEP1-PEP7. The plasma membrane-localized receptors PEPR1 and PEPR2 recognize PEP1-PEP6 and PEP1-PEP2, respectively, thereby

activating immune pathways similar to flg22 (Huffaker et al. 2006; Yamaguchi et al., 2010). Moreover, pretreatment of leaves with PEP1-PEP7 elevates resistance against bacterial invasion, which is dependent on functional PEPR1 and PEPR2 (Yamaguchi et al., 2010). Nowadays, DAMP signaling is thought to sustain MTI (Ryan et al., 2007). In addition, phytohormones such as ethylene (ET), salicylic acid (SA), and jasmonic acid (JA) significantly contribute to and sustain innate immunity (Tsuda et al., 2009). SA synthesis is induced downstream of MAPK activation (Tsuda et al., 2008) as well as after Ca^{2+} influx via the CALMODULIN-BINDING PROTEIN 60g (CBP60g) (Wang et al., 2009). SA is well known to regulate defense genes (Wiermer et al., 2005; Tsuda et al., 2008). Although a SA receptor has not been identified, several signaling components have been isolated. ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) are early mediators of SA-related defense signaling (Wiermer et al., 2005). Downstream of both proteins lies NONEXPRESSOR OF PR1 (NPR1), which takes a central role in SA-related gene regulation (Dong, 2004). Under non-stress conditions, NPR1 is found as oligomer in the cytosol. In response to SA, NPR1 monomerizes and interacts with the nuclear bZIP transcription factor TGA1, thereby enhancing its DNA-binding activity and the regulation of *PR* genes. The NPR1-TGA1 interaction is driven by the cellular redox state. and nitric oxide is essentially involved (Mou et al., 2003; Tada et al., 2008, Lindermayr et al., 2010). JA is another hormone that is synthesized in response to microbial attack. JA synthesis starts from α-linolenic acid, which is processed in successive enzymatic reactions to JA (Browse, 2009). JASMONATE RESISTANT 1 (JAR1) is encoding an amino acid transferase that converts JA to its defense activating derivative JA-isoleucine (JA-Ile) (Staswick and Tiryaki, 2004). JA signaling is initiated by binding of JA-Ile to the F-BOX protein CORONATINE INSENSITIVE 1 (COI1). COI1 binding to JASMONATE ZIM-DOMAIN (JAZ) proteins, which functions as repressors of JA signaling, results in their ubiquitination and subsequent degradation via the 26S proteasome (Chini et al., 2007; Thines et al., 2007). For instance, JAZ3 directly binds to JASMONATE INSENSITIVE 1 (JIN1), which encodes the transcription factor MYC2, and JAZ3 degradation induces *JIN1*-mediated JA responses (Chini et al., 2007). The *Arabidopsis* genome encodes 12 JAZ proteins, whose targeted degradation is thought to give specificity to JA responses (Browse 2009). In terms of plant immunity, JA- and ethylene (ET)-related defense signaling are interwoven (Galzebrook 2005, Pieterse et al., 2009). In *Arabidopsis*, ethylene perception is mediated by five ER membrane-bound receptors, of which

ETHYLENE TRIPLE RESPONSE 1 (ETR1) is taking a dominant role. In the absence of ET, these receptors activate the Raf-like kinase CONSITUTIVE TRIPLE RESPONSE 1 (CTR1). thereby negatively regulating the ET response pathway (Kieber *et al*., 1993). The receptors are inactivated after ET binding, resulting in the deactivation of CTR1, while signaling of downstream effectors like ETHYLENE INSENSITIVE 2 (EIN2) is initiated (Guo and Ecker, 2004; Wang *et al*., 2002). In turn, EIN2 activation interferes with the degradation of the positive ET regulators, transcription factors ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-like 1 (EIL1), by the F-BOX proteins EIN3-BINDING F-BOX 1 and 2 (EBF1, EBF2) (Guo and Ecker, 2003). The transcription factors ETHYLENE RESONSE FACTOR 1 (ERF1) and OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59 (ORA59) have been found to integrate ET- and JA-related plant immune signaling (Lorenzo et al., 2003; Pré et al., 2008). Interestingly, plants impaired in ethylene signaling display an impaired flg22 response in *Arabidopsis* (Mersmann et al., 2010). It was demonstrated that EIN3 directly regulates *FLS2* in a positive feedback loop, which is required for proper flg22-triggered immunity (Boutrot et al., 2010).

JA/ET defense is generally regarded to effectively stop necrotrophic pathogens, while SA defense restricts plant invasions by biotrophic microbes. Several studies indicated the antagonism between both hormone pathways in that SA signaling suppresses JA/ET responses and vice versa (Glazebrook 2005). Microbes were even found to hijack this antagonism to enhance plant susceptibility. For instance, plant pathogenic bacteria *Pseudomonas syringae* produce the JA mimicking molecule coronatine (Bender et al., 1991) to suppress the otherwise antimicrobial activity of SA signaling (Feys et al., 1994; Kloek et al., 2001). In plants, transcription factors WRKY70, TGA2, 5, and 6 as well as GLUTAREDOXIN 480 (GRX480) have been identified as positive regulators of SA signaling that suppress the JA pathways (Ndamukong et al., 2007; Li et al., 2004; Zander et al., 2010). In turn, JIN1 is required to suppress SA signaling (Laurie-Berry et al., 2006). Gibberellic acid (GA) represents a hormone that modulates JA-SA crosstalk. GA signaling is blocked by DELLA proteins. Upon perception of GA, the GA receptor GA INSENSITIVE DWARF1 (GID1) binds to DELLA proteins in order to mediate their ubiquitination and proteasome-mediated degradation (Sun, 2008). Interestingly, flg22 was found to stabilize DELLA protein REPRESSOR OF GA (RGA) in a FLS2-dependent manner even in the presence of GA (Navarro et al., 2008). Bacteria stabilized DELLA proteins to enhance plant susceptibility. DELLA proteins negatively

regulate SA defense. By contrast, plants lacking DELLA proteins show enhanced resistant against *P. syringae*, which is associated with a pronounced SA defense. Consistently, GA insensitive plants are more susceptible (Navarro et al., 2008). In the same studies, GA signaling was shown to have opposite effects on defense signaling against the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola*. Plants lacking DELLA proteins were more susceptible and GA insensitive plants were more resistant against both pathogens. It was found that JA signaling was strongly impaired in the *della* mutants, while genetically determined GA insensitivity resulted in enhanced JA signaling (Navarro et al., 2008). In addition to GA, abscisic acid (ABA) modulates JA-SA crosstalk as it supports JA but inhibits SA signaling (Asselbergh et al., 2008). ABA deficient tomato plants are more resistant against *B. cinerea* (Audenaert et al., 2002, Asselbergh et al., 2007). Interestingly, the resistance is associated with enhanced SA defense indicating ABA suppression of SA signaling (Audenaert et al., 2002). In addition, ABA suppresses basal and JA/ET-related defense, while ABA-deficiency leads to enhanced resistance against *Fusarium oxysporum* in *Arabidopsis* (Anderson *et al.*, 2004). However, ABA does not have a unidirectional impact on plant-pathogen interactions. In dependence of the interaction stage, ABA has opposite effects on *Arabidopsis* leaf colonization by *P. syringae* pv. *tomato* (*Pst*). Stomata closure is arranged after bacterial recognition, which is mediated by ABA and represents an effective barrier to stop *Pst* entry into the intercellular space. To counteract this immune response, *Pst* was shown to release coronatine to suppress ABA signaling resulting in stomata reopening (Melotto et al., 2006). By contrast, ABA mediates susceptibility at later interaction stages. *Pst* actively elevates ABA and JA in leaves by secreting effectors into host cells, thereby abrogating callose deposition and MTI (de Torres-Zabala *et al*., 2007). In addition, ABA mediates a reduction of lignin- and SA synthesis as well as the suppression of the phenylpropanoid pathway and various defense-related genes (Ward *et al*., 1989; Mohr and Cahill, 2007). In sum, these studies draw a network of phytohormones in balancing and substantiating MTI.

3.2 Effector-triggered immunity

Effector-triggered immunity (ETI) is described as a second branch of innate immune signaling. On the molecular base, ETI is highly similar to MTI although the immune signaling and responses are stronger and faster (Navarro et al., 2004; Jones and Dangl, 2006). Effectors are released by microbes in order to manipulate host targets and thus

to establish compatibility and to enhance their virulence in susceptible hosts. This process is termed effector-triggered susceptibility (Jones and Dangl, 2006). Effectors might have apoplastic or cytoplasmic host targets (Kamoun, 2006). ETI is activated by direct binding of microbial effectors to NB-LRR type receptors or by recognition of effector-mediated alterations on host targets (syn. microbe-induced modified self pattern) by the same type of receptors (Jones and Dangl, 2006). These receptors contain a nucleotide binding (NB) and leucin-rich repeat (LRR) domain and are encoded *R* genes. In contrast to PRRs, which detect conserved molecules that are found in a plethora of microbes, R proteins detect race-specific effectors and thus mediate race-specific resistance (Jones and Dangl, 2006). Direct detection of effectors follows the principles of the gene-for-gene model. The recognition of modified self patterns due to effector activity is described by the guard model. In this model, the effector target, termed guardee, is guarded by the R protein, which detects target modifications. By this means, it is hypothesized that one R protein might guard multiple guardees (Dangl and Jones, 2001). Importantly, manipulation of the host target is thought to improve pathogen fitness and colonization success. There is now mounting evidence that this might not always hold true. In some cases, manipulation of the effector target does not enhance pathogen fitness. It is proposed that plants have evolved decoys to trap effectors in order to prevent effector action on the operative (true) host target. In the proposed decoy model, these decoys are guarded by R proteins in order to monitor effector action and to induce ETI (van der Hoorn and Kamoun, 2008). Surveillance of modifications of guardees or decoys offers an essential advantage as it induces ETI in response to effector action. The pathogen might avoid ETI by discarding the respective effector or adjust its activity (Dangl and Jones, 2006). In sum, recognition of microbe-induced modified self patterns might be a more effective strategy of plants compared to direct effector binding in terms of surveillance and protection.

3.3 The endoplasmic reticulum in plant innate immunity

Biotic stress responses of plants include an increased synthesis and release of proteins participating in immune signaling or with a direct antimicrobial activity. A considerable number of these proteins enter the secretory pathway to reach their final destination. The ER is the first organelle of the secretory pathway and is essential for proper processing and folding of glycoproteins or secreted proteins. After cotranslational translocation via the SEC61 translocon complex, most of the native proteins are *N*-

glycosylated by the OLIGOSACCHARYLTRANSFERASE complex (OST). In plants OST consists of DAD2, OST1, OST2 (DAD1), OST3, OST4, STTA3a, STTA3b, SWP1, WBP1 (Kelleher and Gilmore, 2006). At this stage, native proteins are attached to LUMINAL BINDING PROTEINS (BIPs) to guarantee their retention in the ER for further processing. After deglucosylation of the two outermost glucose units of the *N*-linked oligosaccharide by GLUCOSIDASE I and II, native proteins enter the CALNEXIN/CALRETICULIN (CNX/CRT) cycle for proper protein folding. In addition, free thiol groups are converted into intramolecular disulfide bonds by PROTEIN DISULFIDE ISOMERASE (PDIs). The process of CNX/CRT-mediated folding is supervised by UDP-GLUCOSE:GLYCOPROTEIN GLYCOSYLTRANSFERASE (UGGT). Incorrectly folded proteins are reglucosylated to reenter the CNX/CRT cycle. Properly folded proteins enter the Golgi apparatus for further processing. Misfolded proteins are determined for ER-associated degradation (ERAD), which is initiated by ER DEGRADATION-ENHANCING α-MANNOSIDASE-LIKE LECTINS (EDEMs). EDEMs recognize α-1,6-linked mannose of oligosaccharides and prevent protein aggregation. All processes associated with ER-localized protein folding and assembly, are summarized as ER-Quality Control (ER-QC).

A considerable number of proteins involved in immune signaling (e.g. PRRs) or with a direct antimicrobial activity (e.g. several pathogenesis-related (PR) proteins) are passing the secretory pathway. Malfunction of components of the secretory pathway were found to impair plant innate immunity. For instance, disturbance of ER-QC affected immune signaling as STT3a, CRT3 (and partially CRT1, CRT2), UGGT, GLUCOSIDASE IIα and IIβ as well as STROMAL-DERIVED FACTOR 2 (SDF2) and ERdJ3B, which build a complex with BIPs, were essentially required for processing of the PRR ELONGATION FACTOR-TU RECEPTOR (EFR) (Li et al., 2009; Lu et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009). Interestingly, this ER-QC impairment did not influence the PRRs CERK1 or FLS2 function. In addition, SA application, which results in the fast induction of defense genes, up-regulated components of the ER-QC (Wang et al., 2005). This induction was mediated or facilitated by NPR1. Promoter studies revealed a significant enrichment of the *cis* element *TL1* in promoters of the up-regulated ER-QC genes. Promoter mutagenesis proved the relevance of the *TL1*-element for gene induction. However, the corresponding transcription factor remains elusive (Wang et al., 2005). Consistently, SA-dependent defense against the bacterial

pathogen *P. syringae* pv *maculicola* (ES4326) was dependent on UGGT and STT3a (Saijo et al., 2009).

ER working load and, thus, ER-QC activities vary depending on the developmental stage or the occurrence of external stresses. In case ER-QC does not meet the demand of protein processing, ER stress occurs triggered by the enrichment of misfolded proteins. ER stress is perceived by ER-localized transmembrane receptors, which activate the unfolded protein response (UPR). UPR is regarded as an adaptive process of cells to reestablish ER-QC and to relieve ER stress (Malhotra and Kaufman 2007). In mammals, the UPR comprises the induction of ER chaperones (e.g. BIPs), elevated ER-associated degradation (ERAD), and attenuated translation of secreted proteins (Malhotra and Kaufman 2006). A comparable UPR was also observed in plants (Kamauchi et al., 2005; Iwata et al., 2008). However, compared to mammalian systems, processes associated with UPR signaling are less well understood. In plants, bZIP28 and bZIP60 were shown to be important for UPR regulation and, in addition to bZIP17, might function as ER stress sensors (Iwata et al., 2008; Liu et al., 2008). This is an astonishing analogy to mammals, in which, in addition to PERK1 and IRE, the bZIP transcription factors XBP1, ATF4 and ATF6 take a central role (Malhotra and Kaufman, 2006). The same sensors also initiated programmed cell death (PCD) under conditions of severe or prolonged stress (Szegedi et al., 2006). In contrast, the role of plant bZIPs in PCD regulation is unknown, although severe ER stress ends in an genetically determined cell death, in which the negative cell death regulator BAX INHIBITOR-1 (BI-1) is essentially involved (Malerba et al., 2004; Watanabe and Lam, 2008). BI-1 is thought to control Ca^{2+} transfer from the ER into the cytosol (Ihara-Ohori et al., 2007), a characteristic response of ER stress-induced PCD in mammals (Rasheva and Domingos, 2009). Recently, a link of ER-induced PCD and innate immunity was given as an ER-localized Ca^{2+}-ATPase was shown to negatively regulate PCD as part of MTI, ETI, and nonhost resistance against viral and bacterial pathogens (Zhu et al., 2010).

3.4 Root innate immunity

The knowledge and proposed models of innate immunity almost exclusively describe the situations in leaves. It is currently almost unknown how root innate immunity is organized and whether it features common or distinct characteristics of leaf defense. This lack of information bears potential drawbacks as crop plants benefit from interactions with mutualistic root colonization. Therefore, improving leaf or root

resistance of crop plants against antagonists might have detrimental effects in crop production. Furthermore, studies of the barley and rice root colonization by *Magnaporthe grisea* revealed the ability of a leaf pathogen to enter aboveground tissue via the root (Sesma and Osbourn, 2004). Notably, in dependence of the attacked tissue, *M. grisea* displayed different colonization strategies. For this tissue-adaptive invasion, *M. grisea* obviously required a different set of host proteins (Dufresne and Osbourn, 2001).

Recent studies indicated a MAMP-induced surveillance system in *Arabidopsis* roots comparable to leaf MTI (Millet et al., 2010). It was demonstrated that root immunity was activated in response to the MAMPs flg22, elf18, and chitin similar to the situation in leaves. In addition, phytohormones have been reported to affect root colonization by pathogens. *Arabidopsis* root colonization by the oomycete *Phytophthora parasitica* was associated with the transient activation of SA- and JA-related defenses, while ET defense was constitutively induced. All hormones contributed to plant defense as respective mutants impaired in hormone synthesis or signaling were more susceptible to the oomycete (Attard et al., 2010).

In mycorrhizal symbioses, early colonization of pea and rice roots was accompanied by a transient increase in SA synthesis. An involvement of SA-related defense in restricting mutualistic colonization was indicated in rice roots. Here, root treatment with SA resulted in reduced root colonization (Blilou et al., 2000). Accordingly, incompatibility of a pea mutant to arbuscular mycorrhizal (AM) colonization was associated with prolonged SA synthesis (Blilou et al., 1999), while tobacco NahG plants that were SA deficient displayed an increase in AM invasion and arbuscule formation (Herrera Medina et al., 2003). It is proposed that AM fungi suppress initial SA-related defense during early colonization stages (Gutjahr and Paszkowski, 2009). Comparable to the situation in leaves, JA synthesis and signaling might be recruited by mycorrhizal fungi to counteract SA-induced activities. JA levels were increased in roots of *Medicago truncatula* and barley during mycorrhizal colonization (Hause et al., 2002; Isayenkov et al., 2005; Stumpe et al., 2005). Moreover, JA deficiency in *M. truncatula* mutants resulted in reduced AM colonization (Isayenkov et al., 2005).

All the above mentioned studies indicated MTI in roots during mutualistic or pathogenic interactions and the observed responses resemble MTI in leaves. Interestingly, R protein-mediated resistance (ETI) has not been observed in roots interacting with oomycetes or bacteria. So far, root ETI was just reported for the rice-*M. grisea* interaction (Sesma and Osbourn, 2004). Future studies will clarify whether root ETI

against pathogens is the exception or whether our current knowledge reflects a deficit in scientific investigations. Notably, ETI is commonly observed in roots of various plants colonized by parasitic root-knot and cyst nematodes (Fuller et al., 2008). Mi-1-mediated resistance against the root-knot nematode *Meloidogyne javanica* is among the best studied examples for ETI in roots, which is associated with HR. Similar to foliar R proteins, Mi-1 resistance against *M. javanica* is dependent on the chaperone *HSP90* as plants silenced in this protein exhibited a compromised Mi-1 immunity (Bhattarai et al., 2007). In sum, roots obviously possess the mechanistic, genetic and biochemical repertoire to perform ETI. It remains to be answered whether ETI is also a more common response to stop root pathogens.

4 Specific comments on this work

The articles 5.1-5.7 describe the colonization strategy of *P. indica*. In molecular studies *P. indica* was found to exert a broad-spectrum suppression of root innate immunity and to differentially regulate genes participating in the synthesis and signaling of various phytohormones (5.1, 5.3, 5.4). The phytohormones gibberellic acid (5.1, 5.3) and ethylene (5.2) essentially contributed to plant root colonization. Especially GA might counteract salicylic acid-related defense, which was found to effectively restrict colonization of *Arabidopsis* roots by *P. indica* (5.4). Successful root colonization was dependent on the suppression of root innate immunity and this ability might explain the extraordinary broad host range of *P. indica* (5.4). Cell biological studies indicated that *P. indica* colonized living cells of *Arabidopsis* roots (5.4). By contrast, at later interaction stages, successful root colonization was dependent on root cell death as observed in *Arabidopsis* and barley (5.5-5.7). The cell death programme was found to be initiated by an uncoupled ER stress signaling response that was induced by *P. indica*. This ER stress resulted in the execution of a vacuole-mediated capase-dependent cell death (5.7).

The fungus *Piriformospora indica* transfers several benefits to colonized plants including systemic resistance in leaves against the fungus *Blumeria graminis* f.sp. *hordei* as well as salt tolerance. The articles 5.8-5.11 describe research on the benefits mediated by *P. indica* and closely related *Sebacina* sp. in barley, *P. indica*-mediated salt tolerance was associated with reduced lipid peroxidation, metabolic heat production, and fatty acid desaturation in addition to an increase in the antioxidative capacities in colonized barley (5.8). Array-based studies identified genes that were systemically induced in barley leaves as a result of root colonization by *P. indica*. HSP70 might be regarded as marker for root colonization by *Sebacina* sp. as it was additionally induced in leaves in response to root colonization by three *Sebacina vermifera* isolates, which are closely related to *P. indica* (5.9). *P. indica* and other sebacinoid fungi showed species-specific and endosymbiotic associations with bacteria. These bacteria mediated, although to weaker extent, beneficial effects to co-cultured plants as reported for their fungal hosts. The beneficial effects observed in plants colonized by sebacinoid fungi might therefore partially rely on the activities of the endosymbiotic bacteria (5.10, 5.11). The state-of-the-art in the field of *P. indica* and sebacinoid fungi-related research is comprehensively discussed in 5.11. Special emphasis is given to the close relationship of *P. indica* to orchid mycorrhizas (5.11).

In order to functionally analyze plant proteins involved in symbiotic and pathogenic root-microbe interactions, a fast and robust system for generation of stable transformation of barley roots was developed (5.12). This system allows examining proteins within 6-8 weeks. A prerequisite for the release and cultivation of genetically modified crop plants is the elucidation of transgenic effects on the plant metabolome and transcriptome. In field-based studies, barley plants were released that express a fungal chitinase or a chimeric bacterial β-glucanase. These studies indicated that the targeted introduction of a transgene has little if any effects on the plant metabolome and transcriptome and were almost undetectable in comparison to the transfer of traits by classical breeding strategies (5.13).

5 List of articles presented in this work

5.1 **Schäfer, P.**, Pfiffi, S., Voll, L.M., Zajic, D., Chandler, P.M., Waller, F., Scholz, U., Pons-Kühnemann, J., Sonnewald, S., Sonnewald, U., Kogel, K.H. (2009) Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*. *The Plant Journal* 59: 461-474.

5.2 Khatabi, B., Molitor, A., Lindermayr, C., Pfiffi, S., Durner, J., von Wettstein, D., Kogel, K.H., **Schäfer, P.** Ethylene supports colonization of plant roots by the beneficial fungus *Piriformospora indica*. Submitted to *Journal of Experimental Botany* (MS ID: JEXBOT/2010/055780).

5.3 **Schäfer, P.**, Pfiffi, S., Voll, L.M., Zajic, D., Chandler, P.M., Waller, F., Scholz, U., Pons-Kühnemann, J., Sonnewald, S., Sonnewald, U., Kogel, K.H. (2009) Phytohormones in plant root-*Piriformospora indica* mutualism. *Plant Signaling & Behavior* 4: 669-671.

5.4 Jacobs, S., Zechmann, B., Molitor, A., Trujillo, M., Petutschnig, E., Lipka, V., Kogel, K.H., **Schäfer, P.** Broad spectrum suppression of innate immunity is required for colonization of Arabidopsis roots by the beneficial fungus *Piriformospora indica*. Submitted to *Plant Physiology* (MS ID: PLANTPHYSIOL/2010/167395).

5.5 **Schäfer, P.**, Khatabi, B., Kogel, K.H. (2007) Root cell death and systemic effects of *Piriformospora indica*: a study on mutualism. *FEMS Microbiology Letters* 275: 1-7.

5.6 Deshmukh, S.D., Hückelhoven, R., **Schäfer, P.**, Imani, J., Sharma, M., Weiß, M., Waller, F., Kogel, K.H. (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *Proceeding of the National Academy of Sciences of the United States of America* 103: 18450-18457.

5.7 Qiang, X., Zechmann, B,. Kogel, K.H., and **Schäfer, P.** Endoplasmic reticulum stress induction by *Piriformospora indica* initiates a caspase-dependent cell death to achieve root compatibility. Submitted to *Cell Host and Microbe* (Under review at the Editorial Board).

5.8 Baltruschat, H., Fodor, J., Harrach, B.D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K.H., **Schäfer, P.**, Schwarczinger, I., Zuccaro, A.,

Skoczowski, A. (2008). Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytologist* 180: 501-510.

5.9 Waller, F., Mukherjee, K., Achatz, B., Deshmukh, S., Sharma, M., **Schäfer, P.**, Kogel, K.H. (2008) Local and systemic modulation of plant responses by *Piriformospora indica* and related *Sebacinales* species. *Journal of Plant Physiology* 165: 60-70.

5.10 Sharma, M., Schmid, M., Rothballer, M., Hause, G., Zuccaro, A., Imani, J., Kämpfer, P., **Schäfer, P.**, Hartmann, A., Kogel, K.H. (2008) Detection and identification of mycorrhiza helper bacteria intimately associated with representatives of the order *Sebacinales*. *Cellular Microbiology* 10: 2235-2246.

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

5.12 Imani, J., Li, L., **Schäfer, P.***, Kogel, K.H.* STARTS – a stable root transformation system for the functional study of proteins in biotic stress in the monocot model barley. *Shared senior authorship. (Intended to be submitted by the end of November 2010).

5.13 Kogel, K.H., Voll, L.M., **Schäfer, P.**, Jansen, C., Wu, Y., Langen, G., Imani, J., Hofmann, J., Schmiedl, A., Sonnewald, S., von Wettstein, D., Cook, R.J., Sonnewald, U. (2010) Transcriptome and metabolome profiling of field-grown transgenic barley lack induced differences but show cultivar-specific variations. *Proceeding of the National Academy of Sciences of the United States of America* 107: 6198-6203.

5.1 **Schäfer, P.**, Pfiffi, S., Voll, L.M., Zajic, D., Chandler, P.M., Waller, F., Scholz, U., Pons-Kühnemann, J., Sonnewald, S., Sonnewald, U., Kogel, K.H. (2009) Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*. *The Plant Journal* 59: 461-474.

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Manipulation of plant innate immunity and gibberellin as factor of compatibility in the mutualistic association of barley roots with *Piriformospora indica*

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SUMMARY

Fungi of the order *Sebacinales* (Basidiomycota) are involved in a wide spectrum of mutualistic symbioses with various plants, thereby exhibiting unique potential for biocontrol strategies. *Piriformospora indica*, a model organism of this fungal order, is able to increase the biomass and grain yield of crop plants, and induces local and systemic resistance to fungal diseases and tolerance to abiotic stress. To elucidate the molecular basis for root colonization, we characterized the interaction of *P. indica* with barley roots by combining global gene expression profiling, metabolic profiling, and genetic studies. At the metabolic level, we show that fungal colonization reduces the availability of free sugars and amino acids to the root tip. At the transcriptional level, consecutive interaction stages covering pre-penetration-associated events and progressing through to root colonization showed differential regulation of signal perception and transduction components, secondary metabolism, and genes associated with membrane transport. Moreover, we observed stage-specific up-regulation of genes involved in phytohormone metabolism, mainly encompassing gibberellin, auxin and abscisic acid, but salicylic acid-associated gene expression was suppressed. The changes in hormone homoeostasis were accompanied with a general suppression of the plant innate immune system. Further genetic studies showed reduced fungal colonization in mutants that are impaired in gibberellin synthesis as well as perception, and implicate gibberellin as a modulator of the root's basal defence. Our data further reveal the complexity of compatibility mechanisms in host–microbe interactions, and identify gibberellin signaling as potential target for successful fungi.

Keywords: compatibility, gibberellin, plant defence, plant hormone, symbiosis, mutualism.

INTRODUCTION

Despite the intensive measures taken to protect crops from diseases and pests, recent evaluations have shown that continuously increasing total crop production is accompanied by increased yield losses due to biotic and abiotic stresses (Oerke and Dehne, 2004; Lobell and Field, 2007). One solution to this problem is to improve crop production strategies to make them more reliable for the producer and safer for consumers and the environment (Cook, 2006). A key to this is to increase knowledge of the intricate and dynamic communications between crop plants and their interacting parasitic or beneficial microbial partners (Khush, 2005). By elucidating 'compatibility mechanisms', i.e. mechanisms of either disease or beneficial symbiosis development and the molecular networks supporting microbial virulence, key processes can be identified and exploited to develop more sustainable measures based on either novel chemicals or genetically improved crop plants.

In nature, plants are generally colonized by a range of fungal microbes that may have detrimental, neutral or

beneficial effects on their hosts. For a unifying and balanced view on compatibility mechanisms, it is essential to study the parasitic lifestyles of biotrophs and hemi-biotrophs as well as those of mutualistic fungi (Kogel *et al.*, 2006; Kogel, 2008; and references therein). Compatibility in host–microbe systems depends on biochemical interplay between molecules of the interacting partners, resulting in host recognition, host invasion, microbial nutrition, host colonization and microbial reproduction (Vögele and Mendgen, 2003; Hückelhoven, 2005, 2007; O'Connell and Panstruga, 2006; Robert-Seilaniantz *et al.*, 2007; Speth *et al.*, 2007). More specifically, conserved microbe-associated molecular patterns (MAMPs) and microbe-induced molecular patterns (MIMPs), lead to recognition of invaders by the plant (Jones and Dangl, 2006; Bent and Mackey, 2007). MAMP/MIMP recognition is achieved by plasma membrane-localized pattern recognition receptors (PRRs) initiating MAMP-triggered immunity (MTI). Successful *in planta* development of biotrophic and hemi-biotrophic pathogens and most probably microbial symbionts is entirely dependent on the release of effector molecules that specifically interfere with MTI and result in the phenomenon called effector-triggered susceptibility (Jones and Dangl, 2006). This distinct early phase of plant defence suppression is followed by a second phase of effector-mediated metabolic reprogramming of the host tissue that eventually results in successful microbial establishment (Cui *et al.*, 2005; Göhre and Robatzek, 2008).

The root-colonizing basidiomycete *Piriformospora indica* is the archetype of the recently established mycorrhizal order *Sebacinales* (Weiss *et al.*, 2004). Hallmarks of the mutualistic symbioses formed by these fungi with a broad range of mono- and dicotyledonous plants are growth promotion, yield increases, enhanced resistance to root and leaf pathogens, and abiotic stress tolerance (Waller *et al.*, 2005; Deshmukh and Kogel, 2007; Shahollari *et al.*, 2007; Stein *et al.*, 2008). The colonization patterns of the various root regions show some qualitative differences that distinguish *P. indica* from obligate biotrophic arbuscular mycorrhizal fungi. The highest fungal biomass was found in the differentiation and root hair zones, and the meristematic zone was less extensively colonized (Deshmukh *et al.*, 2006). In contrast, arbuscular mycorrhizal fungi are known to preferentially colonize younger root parts, as physiological activity of host cells is a prerequisite for efficient nutrient exchange between the symbiotic partners (Karandashov and Bucher, 2005). Indeed, one of the main qualitative differences between arbuscular mycorrhizal fungi (Glomeromycota) and *P. indica* mycorrhiza (Basidiomycota) is the dependence on cell death for root colonization at late interaction stages (>5 days after inoculation). However, this cell death-associated colonization does not lead to root necrotization as seen for hemi-biotrophic or necrotrophic fungi. Therefore, the term necrotrophy is misleading, and 'cell death-dependent colonization' is a more precise description of this interaction phase (Schäfer and Kogel, 2009). The dependence on host cell death was also shown in barley plants constitutively over-expressing the negative cell death regulator *BAX INHIBITOR-1*. As a result of the genetically increased cell viability, fungal root colonization was significantly reduced in these transgenic plants (Deshmukh *et al.*, 2006). However, recent transmission electron microscopic analyses have revealed an initial biotrophic phase preceding the cell death-dependent colonization stage (P. Schäfer and B. Zechmann, unpublished results).

In the present study, we have assessed the response of barley roots to *P. indica* colonization by transcriptional and metabolic profiling. The most significant changes were observed in genes associated with signal perception and transduction, secondary metabolism, plant defence and hormone metabolism. These studies revealed complex interplay of *P. indica* with its host, during which gibberellin may be recruited to manipulate plant defence and to initiate the mutualistic symbiosis.

RESULTS

The transcriptome reflects a biphasic colonization of barley roots by *Piriformospora indica*

In an initial microscopic study, extracellular colonization of roots was seen within 1–2 days after inoculation (dai) with *P. indica* chlamydospores, during which fungal hyphae frequently fused in order to form an initial extracellular network. By 3 dai, intercellular hyphae were visible and single rhizodermal cells were penetrated without the formation of specific penetration organs. By 7 dai, large areas of the root surface were covered with *P. indica* mycelium, and inter- and intra-cellular hyphae were abundant in the rhizodermis and cortex. Fungal sporulation was most frequently initiated at approximately 14 dai (Figure 1). Root colonization was generally not accompanied by the emergence of structural and biochemical defence barriers, and did not coincide with tissue necrotization even at later cell death-associated interaction stages.

The time points 1, 3 and 7 dai were chosen for further analyses, as distinct interaction stages were covered: extracellular fungal development (1 dai), penetration-associated and early colonization events (3 dai), and progressive root cell colonization (7 dai). For transcriptome profiling, a custom-made 44K Agilent microarray was designed, consisting of approximately 40 000 probe sets (see Experimental procedures). Of these, 392 (1 dai), 459 (3 dai) and 509 (7 dai), respectively, were differentially regulated *P. indica*-colonized roots compared to mock-treated roots (Figure 2), while 1107 genes were differentially regulated at one of the three time points at least [Table S1, complete data accessible at the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (Edgar *et al.*, 2002), accession GSE13756]. However, fewer than

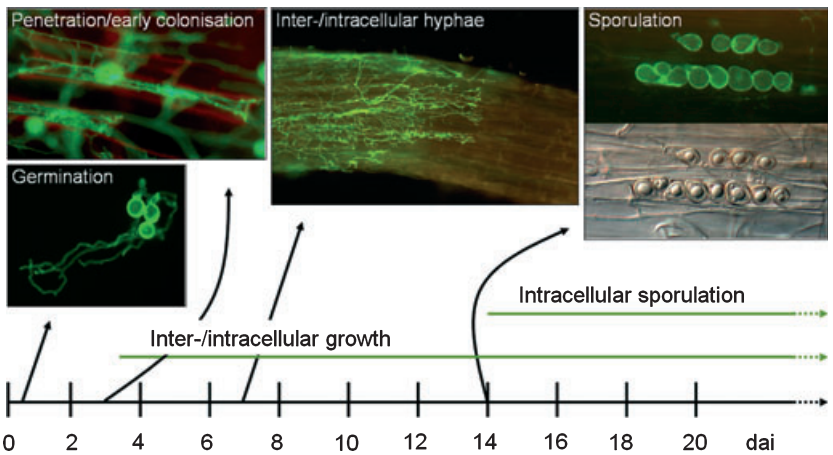


Figure 1. Schematic overview of barley root colonization by *Piriformospora indica*.
After chlamydospore germination (at approximately 12 h after inoculation), the fungus started to penetrate rhizodermal cells and intercellularly colonize the root cortex (3 dai). Subsequently, the fungus infests the root extra-, inter- and intracellularly. At approximately 7 dai, the fungus builds inter-/intra- and extra-cellular networks. Fungal sporulation is most frequently observed at approximately 14 dai. Fungal structures were stained using WGA-AF 488.

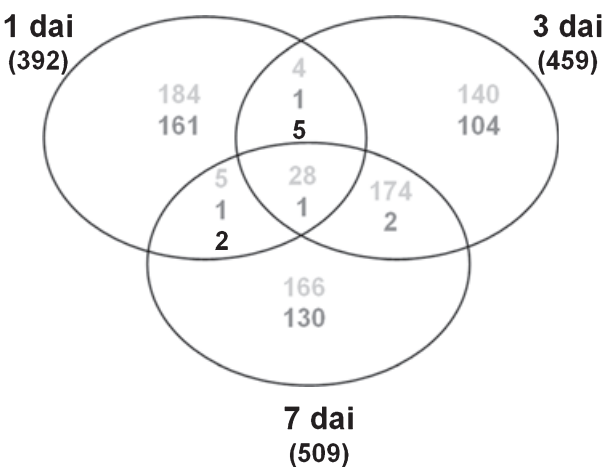


Figure 2. Number of *Piriformospora indica*-responsive barley root genes.
Numbers of differentially regulated genes after root inoculation with fungal chlamydospores at 1, 3 and 7 dai, displayed as a Venn diagram. Light grey, induced genes; grey, suppressed genes; black, genes that are induced at one time point but suppressed at the other given time point.

10% of the identified genes at 1 dai showed altered expression at 3 (9%) and 7 dai (9.5%). In contrast, more genes displayed an overlapping expression pattern between 3 and 7 dai (approximately 40–50%). Interestingly, although approximately 50% of the genes were induced or suppressed at 1 dai, approximately 75% of genes were induced and only 25% suppressed at 3 and 7 dai.

The Agilent array data were verified by quantitative PCR with gene-specific primers for the genes encoding terpene synthase 7, syn-copalyl diphosphate synthase and a putative abscisic acid-induced protein (genes that showed broad variations in induction/suppression levels; Figure S1).

P. indica-colonized roots displayed pronounced alterations in expression of genes involved in stress responses

Annotation of differentially regulated genes resulted in 15 functional groups and two groups comprising either unknown ESTs (380 genes, 34.3%) or genes that could not be assigned to any group (34 genes, 3.1%) (Figure 3, Table 1 and Table S1).

Genes involved in plant defence/stress responses represent the largest group of differentially regulated genes (151 genes, 13.6%). Likewise, many signalling components were affected by *P. indica* (90 genes, 8.1%). Genes participating in secondary metabolism (58, 5.2%), those encoding transporters/channels/pumps (53, 4.8%), and those involved in transcription/protein biosynthesis (48, 4.3%) also showed pronounced transcriptional alteration. In contrast, nutrient storage (13, 1.2%), cellular trafficking/cytoskeleton (20, 1.8%), cell-wall metabolism (21, 1.9%) and DNA metabolism/genome organization (23, 2.1%) were only weakly influenced.

Most functional groups showed a stage-dependent expression profile. Genes associated with transcription/protein biosynthesis and signalling were strongly transcriptionally altered at 1 dai. Components of secondary metabolism and transporters/channels/pumps showed the greatest differences at 3 dai. Finally, transcripts of receptors and proteins involved in plant defence/stress responses exhibited a higher degree of differential regulation at 3 and 7 dai (Table 1).

P. indica interferes with plant defence and affects signal perception and transduction

The diverse set of defence/stress-responsive genes induced or suppressed by *P. indica* encoded 'defence-related' proteins (e.g. R proteins, PR proteins) as well as genes encoding 'stress-responsive' enzymes (e.g. laccases, late-embryogenesis-abundant proteins/dehydrins), indicating that *P. indica* elicited a rather non-specific defence reaction. As approximately 4% of all ESTs on the array were defined as defence/stress-related, their pronounced differential regulation cannot be explained by their exceeding presence on the array. At 1 dai, 11% of differentially regulated genes (25 induced/18 suppressed) were classified in this category,

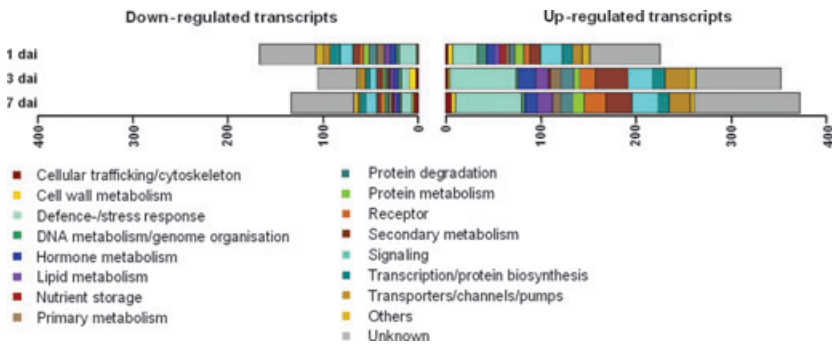


Figure 3. Functional categories of genes in barley roots that were differentially regulated upon *Piriformospora indica* colonization.
Coloured bars illustrate the absolute number of genes that were down- or up-regulated within the various categories at 1, 3 and 7 dai. See inset for colour code.

Table 1 Distribution of differentially regulated genes within each functional category

			1 dai		3 dai		7 dai		1 dai	3 dai	7 dai
	Number of genes	Percentage of genes	Up	Down	Up	Down	Up	Down	Percentage of genes[a]	Percentage of genes[a]	Percentage of genes[a]
Cell wall metabolism	21	1.9	5	1	2	8	4	2	1.5	2.2	1.2
Cellular traffic/cytoskeleton	20	1.8	3	1	3	2	7	5	1.0	1.1	2.4
Defence/stress response	151	13.6	25	18	69	8	69	11	11.0	16.8	15.7
DNA metabolism/genome organization	23	2.1	9	4	3	3	4	2	3.3	1.3	1.2
Hormone metabolism	38	3.4	9	5	18	5	13	3	3.6	5.0	3.1
Lipid metabolism	36	3.3	5	5	13	4	16	3	2.6	3.7	3.7
Nutrient storage	13	1.2	7	1	3	1	0	1	2.0	0.9	0.2
Primary metabolism	33	3.0	4	8	10	2	8	4	3.1	2.6	2.4
Protein degradation	34	3.1	5	8	14	2	13	3	3.3	3.5	3.1
Protein metabolism	33	3.0	9	6	6	2	12	2	3.8	1.7	2.8
Receptor	42	3.8	7	4	17	2	25	4	2.8	4.1	5.7
Secondary metabolism	58	5.2	11	8	34	5	27	4	4.8	8.5	6.1
Signalling	90	8.1	23	13	26	7	28	11	9.2	7.2	7.7
Transcription/protein biosynthesis	48	4.3	11	11	13	5	11	6	5.6	3.9	3.3
Transporter/channels/pumps	53	4.8	10	7	25	6	23	2	4.3	6.8	4.9
Others	34	3.1	8	8	8	2	4	5	4.1	2.2	1.8
Unknown	380	34.3	74	59	89	42	111	66	33.9	28.5	34.8
Total	1107	100	225	167	353	106	375	134	100	100	100
Total percentage	–	–	57.4	42.6	76.9	23.1	73.7	26.3	–	–	

[a]Total % of genes regulated at indicated time points.

while 16.8% (69 induced/eight suppressed) and 15.7% (69 induced/11 suppressed) were identified at 3 and 7 dai, respectively. In total, only five genes encoding two putative PR10s, a putative laccase 18, a putative germin A and a putative syringolide-induced protein were differentially regulated at all time points. Based on their expression pattern, the 151 defence/stress-responsive genes were divided into four regulation clusters (Figure S2a–d). Cluster A consists of genes that were suppressed by *P. indica* (35 genes, 23%). Genes in cluster B showed a transient induction profile at 1 or 3 dai (47 genes, 31%). All genes that showed transient induction at 3 dai but lower up-regulation at 7 dai (12 genes, 8%) were assigned to cluster C. Finally, cluster D encompasses genes that were steadily up-regulated (31 genes) at 3 and 7 dai or exclusively induced at 7 dai (26 genes). Based on their expression pattern, the genes of cluster D might exclusively code for proteins that effectively restrict root colonization, and several germins, which are known to restrict powdery mildew infection of barley leaves (Zimmermann *et al.*, 2006), were represented in this cluster. Alternatively, cluster D might include genes involved in regulation of cell death, which is frequently observed at 7 dai (Deshmukh *et al.*, 2006), or genes that are activated by cell death-derived signals released by dying cells. However, of these 57 genes, 35% showed a fold change induction >4. This is in accordance with the generally moderate induction level of defence/stress-associated genes: approximately 70% of the genes showed a less than fourfold induction level at all time points. Interestingly, the highest induction values were found at the pre-penetration stage (1 dai) (Figure S2).

A high number of differentially abundant transcripts encoded receptors (42 ESTs) and signal transducers (90

ESTs) (encompassing transcription factors, DNA-binding proteins and protein kinases) (Table S1). Again, receptor gene expression overlap was mainly observed between 3 and 7 dai (52–68%), and to a minor extent at 1 dai (10–20%). Similarly, approximately 45% of the genes involved in cell signalling showed congruent expression between 3 and 7 dai.

P. indica-induced changes in auxin, ABA, and brassinosteroid synthesis and signalling

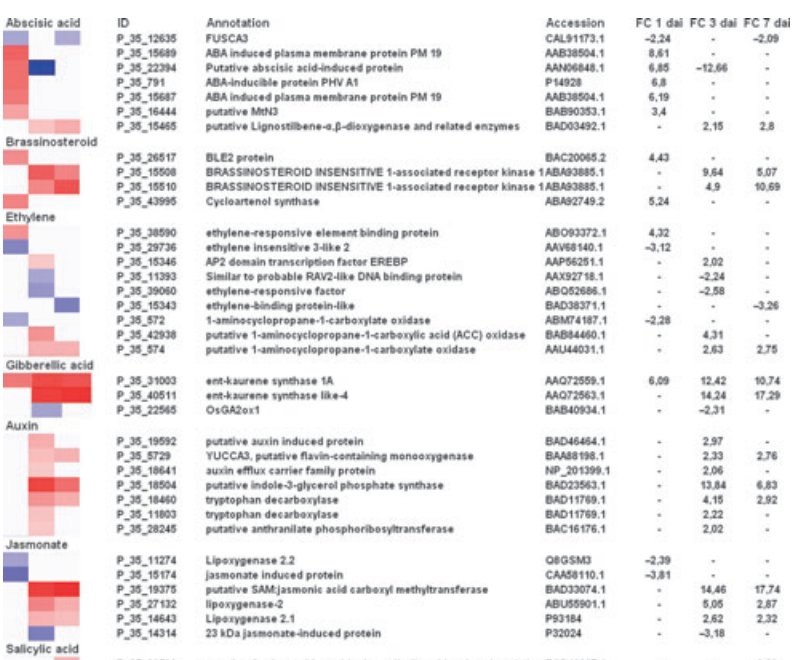
Genes encoding for a tryptophan decarboxylase and a putative indole-3-glycerol phosphate synthase involved in L-tryptophan synthesis, an immediate precursor of IAA (Ljung *et al.*, 2005), were up-regulated at 3 and 7 dai. In addition, a second tryptophan decarboxylase and a putative anthranilate phosphoribosyl transferase that might be involved in tryptophan synthesis show maximum expression at 3 dai (Figure 4). Further, an auxin-induced protein and a flavin-containing mono-oxygenase (YUCCA3) were up-regulated and an auxin-repressed protein was down-regulated at 3 dai and/or 7 dai suggesting that auxin biosynthesis and signalling might be activated during symbiotic colonization.

Changes in *P. indica*-induced hormone balance were also seen for the sesquiterpenoid ABA (Figure 4). The hormone plays a crucial role in abiotic and biotic stress responses (Finkelstein and Rock, 2002), and often shows antagonistic activity for other hormones (Asselbergh *et al.*, 2008). Four ABA-responsive proteins of unknown function were induced at 1 dai, but repressed at later time points. The identification of several genes encoding late embryogenesis abundant (LEA) proteins/dehydrins might also be due to ABA accumulation, as several members of that gene family are ABA-responsive (Hundertmark and Hincha, 2008).

The gene encoding cycloartenol synthase (CS), which that contributes to the synthesis of brassinosteroid (BR) precursors, and *BLE2*, which encodes a BR-responsive nine transmembrane protein, were induced at 1 dai. In addition, two *BAK1* genes encoding brassinosteroid insensitive 1-associated receptor kinases 1 that are involved in BR signalling were induced at 3 and 7 dai.

P. indica modifies the expression of genes involved in oxylipin synthesis

Root colonization by *P. indica* is associated with transcriptional changes in genes associated with lipid metabolism (Table S1). Hydrolysis of phospholipids by lipases leads to the release of unsaturated fatty acids, which can serve as substrates for the synthesis of oxylipins (Feussner and Wasternack, 2002; Meijer and Munnik, 2003; Shah, 2005). The micoarray analysis indicated that genes encoding four oleate Δ12-desaturases that convert oleate to linoleic acid were differentially regulated (three probe sets at 3 and 7 dai, and one transcript at 3 dai) and a gene encoding cytochrome b_5, which is required as an electron donor for desaturation, was similarly induced (see lipid metabolism, Table S1). Central to oxylipin synthesis is the action of lipoxygenases (LOXs) that convert linoleic or α-linolenic acid to the oxylipin precursors (9*S*)- and (13*S*)-hydroperoxide (Feussner and Wasternack, 2002). Two *LOX* genes, *LOX2.1* and *LOX2*, which catalyse the oxidation of linoleic acid to 13-hydroxyoctadecadienoic acid (Peng *et al.*, 1994; Vörös *et al.*, 1998), were induced at 3 and 7 dai (Figure 4). Inter-



ID	Annotation	Accession	FC 1 dai	FC 3 dai	FC 7 dai
P_35_12635	FUSCA3	CAL91173.1	-2,24	-	-2,09
P_35_15689	ABA induced plasma membrane protein PM 19	AAB38504.1	8,61	-	-
P_35_22394	Putative abscisic acid-induced protein	AAN06848.1	6,85	-12,66	-
P_35_791	ABA-inducible protein PHV A1	P14928	6,8	-	-
P_35_15687	ABA induced plasma membrane protein PM 19	AAB38504.1	6,19	-	-
P_35_16444	putative MtN3	BAB90353.1	3,4	-	-
P_35_15465	putative Lignostilbene-α,β-dioxygenase and related enzymes	BAD03492.1	-	2,15	2,8
P_35_26517	BLE2 protein	BAC20065.2	4,43	-	-
P_35_15508	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1	ABA83885.1	-	9,64	5,07
P_35_15510	BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1	ABA83885.1	-	4,9	10,69
P_35_43995	Cycloartenol synthase	ABA92749.2	5,24	-	-
P_35_38590	ethylene-responsive element binding protein	ABO93372.1	4,32	-	-
P_35_29736	ethylene insensitive 3-like 2	AAV68140.1	-3,12	-	-
P_35_15346	AP2 domain transcription factor EREBP	AAP56251.1	-	2,02	-
P_35_11393	Similar to probable RAV2-like DNA binding protein	AAX92718.1	-	-2,24	-
P_35_39060	ethylene-responsive factor	ABQ52686.1	-	-2,58	-
P_35_15343	ethylene-binding protein-like	BAD38371.1	-	-	-3,26
P_35_572	1-aminocyclopropane-1-carboxylate oxidase	ABM74187.1	-2,28	-	-
P_35_42938	putative 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase	BAB84460.1	-	4,31	-
P_35_574	putative 1-aminocyclopropane-1-carboxylate oxidase	AAU44031.1	-	2,63	2,75
P_35_31003	ent-kaurene synthase 1A	AAQ72559.1	6,09	12,42	10,74
P_35_40511	ent-kaurene synthase like-4	AAQ72563.1	-	14,24	17,29
P_35_22565	OsGA2ox1	BAB40934.1	-	-2,31	-
P_35_19592	putative auxin induced protein	BAD46464.1	-	2,97	-
P_35_5729	YUCCA3, putative flavin-containing monooxygenase	BAA88198.1	-	2,33	2,76
P_35_18641	auxin efflux carrier family protein	NP_201399.1	-	2,06	-
P_35_18504	putative indole-3-glycerol phosphate synthase	BAD23563.1	-	13,84	6,83
P_35_18460	tryptophan decarboxylase	BAD11769.1	-	4,15	2,92
P_35_11803	tryptophan decarboxylase	BAD11769.1	-	2,22	-
P_35_28245	putative anthranilate phosphoribosyltransferase	BAC16176.1	-	2,02	-
P_35_11274	Lipoxygenase 2.2	Q8GSM3	-2,39	-	-
P_35_15174	jasmonate induced protein	CAA58110.1	-3,81	-	-
P_35_19375	putative SAM:jasmonic acid carboxyl methyltransferase	BAD33074.1	-	14,46	17,74
P_35_27132	lipoxygenase-2	ABU55901.1	-	5,05	2,87
P_35_14643	Lipoxygenase 2.1	P93184	-	2,62	2,32
P_35_14314	23 kDa jasmonate-induced protein	P32024	-	-3,18	-
P_35_30509	putative S-adenosyl-L-methionine:salicylic acid carboxyl methyltra	BAD12867.1	-	-	2,69

Figure 4. Changes of transcripts involved in hormone signalling during *Piriformospora indica* colonization.
Genes involved in hormone metabolism and responses that are differentially regulated at one time point at least are shown. Colours represent fold changes of each gene, which are either up-regulated (red) or down-regulated (blue) compared to mock-inoculated roots. Fold changes (FC) were calculated by dividing antilog signal intensities obtained from arrays hybridized with cDNA of mock- and *P. indica*-ztreated roots.

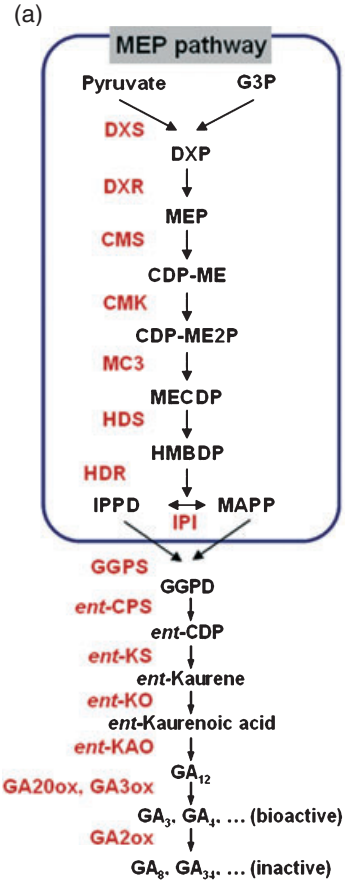
estingly, oxylipin synthesis is apparently suppressed at the pre-penetration phase (1 dai), as *LOX2.2* and a gene encoding a jasmonate-induced protein (involved in the downstream JA response) were found to be suppressed at 1 dai. Suppression of *JIP23* encoding 23 kDa jasmonate-induced protein at 3 dai might suggest synthesis of oxylipins other than jasmonate.

Alterations in the methylerythritol phosphate (MEP) pathway and synthesis of secondary metabolites

Terpenoids derive from the C_5 precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are synthesized either via mevalonate or the MEP pathway. Almost all genes of the MEP pathway are induced by *P. indica* at late stages (Figure 5). Genes encoding two putative geranylgeranyl diphosphate synthases, which produce geranylgeranyl diphosphate (GGDP) from IPP and DMAPP, were induced at 3 and 7 dai. GGDP, in turn, is the precursor for mono-, di- and sesquiterpenes, the family to which anti-microbial phytoalexins, carotenoids or phytohormones such as gibberellins (GA) or ABA belong. Downstream of GGDP, the most strongly induced gene was one encoding a terpene synthase (44-fold at 3 dai; see also Figure S1). In parallel, a gene encoding a putative syn-copalyl diphosphate synthase (syn-CDS) mediating the cyclization of GGDP and a 10-deacetylbaccatin III-10-*O*-acetyl transferase-like gene, whose homologue is associated with taxol synthesis in *Taxus* x *media* (Guo *et al.*, 2007), were induced at all time points. A high number of cytochrome P450 mono-oxygenases of unknown function were also induced by *P. indica*. Various members of this enzyme family are involved in the production of both diterpene phytoalexins (Okada *et al.*, 2007) and GA (Yamaguchi, 2008) in rice.

As carotenoids represent one major product class of the MEP pathway, we examined whether transcriptional induction of this pathway and of geranylgeranyl diphosphate synthase (GGPS) would result in elevated carotenoid production in *P. indica*-colonized roots. We found that barley roots contained minute amounts of carotenoids. Of the 10 detected carotenoids, six low-abundance carotenoid species could be reliably quantified. Violaxanthin and neoxanthin were the most abundant of these, and are also precursors in ABA synthesis (Finkelstein and Rock, 2002). The violaxanthin content was slightly lower in inoculated compared to non-inoculated roots at 1 dai, but not at later stages (Figure 6i). The amount of total neoxanthin (*cis*- and *trans*-neoxanthin, Figure 6j), the major carotenoid in barley roots, was reduced at 1 dai but elevated at 7 dai, indicating increased productivity of the MEP pathway in proximal root segments.

(a)



(b)

Gene	1 dai	3 dai	7 dai
DXS1		11,3	14,0
DXS2		2,80	3,30
DXR		4,6	4,8
CMK1		2,5	2,4
CMK2		2,3	2,3
HDS1		5,4	4,50
HDS2		4,0	5,4
HDR		3,1	3,10
GGPS		2,5	3,20
ent-KS1a	6,1	12,4	10,7
ent-KS-like 4		14,2	17,3
GA2ox1		−2,3	

Figure 5. *Piriformospora indica* induces the methylerythritol phosphate (MEP) pathway and gibberellic acid (GA) synthetic genes in barley.
(a) Scheme of the MEP pathway and GA biosynthesis. Products/substrates: glyceraldehyde-3-phosphate (G3P); 1-deoxy-D-xylulose 5-phosphate (DXP); 2-C-methyl-D-erythritol 4-phosphate (MEP); 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-ME); 2-phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-ME2P); 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MECDP); 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (HMBDP); isopentenyl diphosphate (IPP); dimethylallyl diphosphate (DMAPP); geranylgeranyl diphosphate (GGDP); copalyl diphosphate (CDP); gibberellin A12 (GA_{12}); gibberellin A3 (GA_3); gibberellin A4 (GA_4); gibberellin A8 (GA_8); gibberellin A34 (GA_{34}). Enzymes: DXP synthase (DXS); DXP reductoisomerase (DXR); CDP-ME synthase (CMS); CDP-ME kinase (CMK); MECDP synthase (MCS); HMBDP synthase (HDS); HMBDP reductase (HDR); IPP isomerase (IPI); GGDP synthase (GGPS); *ent*-CDP synthase (*ent*-CPS); *ent*-kaurene synthase (*ent*-KS); *ent*-kaurene oxidase (*ent*-KO); *ent*-kaurenoic acid oxidase (*ent*-KAO); gibberellin 3 oxidase (GA3ox); gibberellin 20 oxidase (GA20ox); gibberellin 2 oxidase (GA2ox).
(b) Genes involved in the MEP pathway and GA biosynthesis that are differentially regulated in *P. indica*-colonized barley roots at 1, 3 and 7 dai. Numbers indicate fold induction of respective genes in *P. indica*-colonized versus mock-treated roots.

Barley mutants impaired in gibberellin synthesis or perception are less extensively colonized by *P. indica*

As GA is produced from GGDP, we searched for genes involved in GA biosynthesis downstream of GGDP, and found two differentially expressed genes encoding putative *ent*-kaurene synthases at 3 and 7 dai (Figure 5). Accordingly, a *GA2ox* gene mediating inactivation of active GA (Yamaguchi, 2008) was down-regulated in response to *P. indica* at 3 dai. These results suggest that GA biosynthesis is raised by *P. indica*.

In order to determine the impact of GA synthesis and signalling on *P. indica* colonization, we analysed barley GA mutants. M117 has a low endogenous GA content, probably caused by a block at either geranylgeranyl diphosphate synthase or *syn*-copalyl diphosphate synthase, as it fails to accumulate *ent*-kaurene in the presence of tetcyclacis (which prevents further oxidation to kaurenoic acid, J.R. Lenton and P.M.C., unpublished results; Chandler and Robertson, 1999). M121 is GA-insensitive due to a mutation in the gene encoding the GA receptor GID1 (Chandler *et al.*, 2008). Significantly, both mutants showed reduced colonization by *P. indica* (Figure 7a), which was cytologically detectable as a reduced amount of fungal hyphae at 7 dai and reduced intracellular sporulation at 21 dai. However, structural defence responses (e.g. cell-wall fortifications) were not detected in either mutant. As GA has been shown to affect the balance between other phytohormones (Navarro *et al.*, 2008), we monitored defence responses known to be associated with SA, JA and ethylene during *P. indica* colonization. Alterations in barley root GA homoeostasis were associated with elevated expression of *PR10* and the SA-responsive gene *PR1B* at 3 and 7 dai in both mutants (Figure 7b). Similarly, the defence-associated gene *PR5* was induced by the fungus at 3 dai. Ethylene-responsive *RAF1* expression was not affected (data not shown).

The availability of C and N assimilates is decreased in barley roots colonized with *P. indica*

In contrast to defence/stress-associated genes, phytohormone signalling and secondary metabolism, genes involved in primary metabolism showed only minor differences in transcript levels at all time points (Table 1). Nevertheless, we expected a shift in assimilate availability in response to *P. indica* colonization due to additional sink activity by the fungus. Therefore, we assessed the amounts of free sugars and amino acids. In general, differences in hexose, sucrose and amino acid contents followed a developmental pattern in the proximal parts of harvested roots (Figure 6a,b,d). Hexose and amino acid contents decreased sharply with increasing root age, and starch contents exhibited a slight decrease at 7 dai, but sucrose contents increased strongly from 1 to 3 dai. Phloem transport of amino acids such as glutamine and asparagine decreased by 90% from 1 to 3 dai

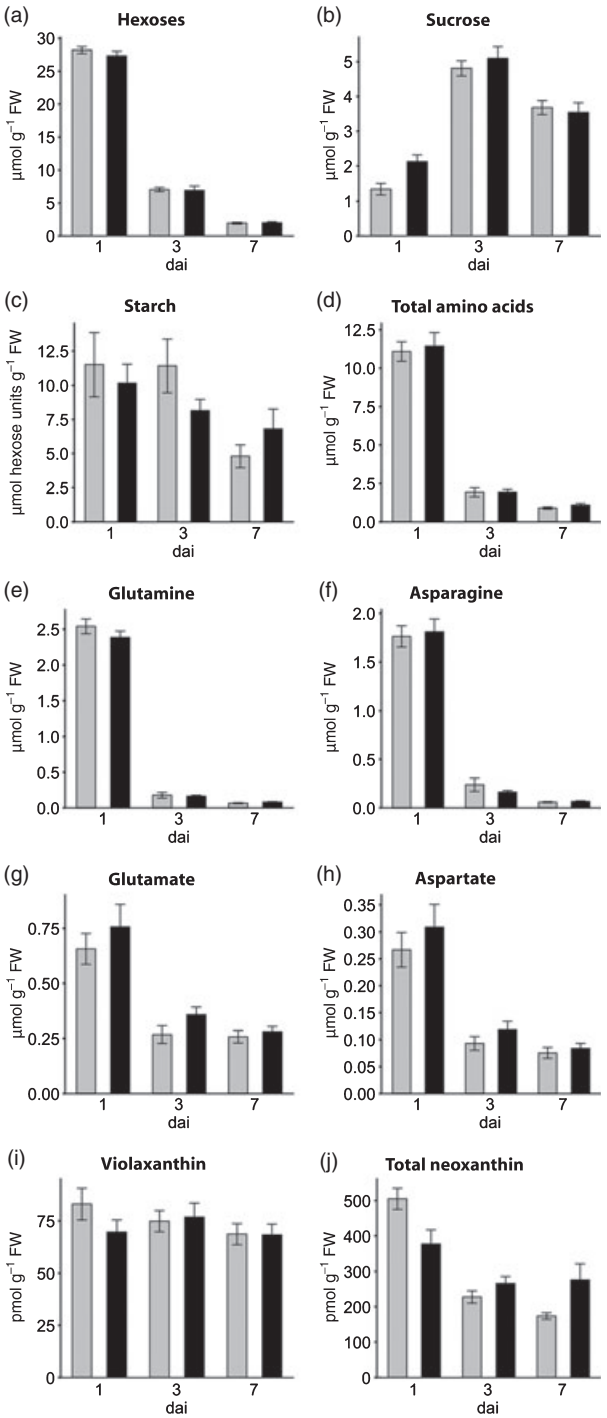


Figure 6. Carbohydrate, amino acid and carotenoid contents in barley roots colonized by *Piriformospora indica*.
For analysis, the whole root (1 dai) or proximal 3 cm of the roots (3 and 7 dai) from *P. indica*-inoculated and control plants were analysed. Contents of hexoses (a), sucrose (b) and starch (c), total amino acid content (d), and contents of glutamine (e), asparagine (f), glutamate (g), aspartate (h), violaxanthine (i) and neoxanthine (j) in the proximal 3 cm of control roots (grey) and *P. indica*-colonized roots (black) at 1, 3 and 7 dai are shown. Data are the means of 12 independent samples from three independent experiments; error bars represent the standard error.

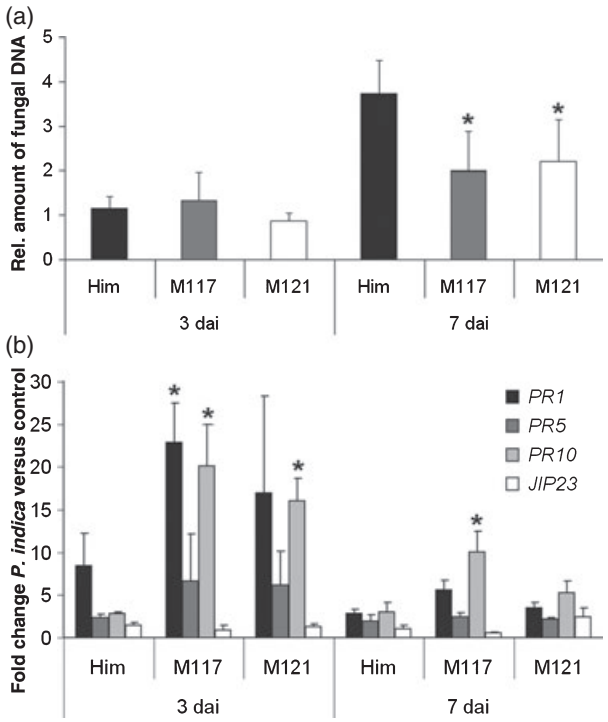


Figure 7. Amount of fungal colonization of GA mutants and alterations in expression of defence genes in roots.
(a)Relative amount of fungal DNA in GA mutants at 3 and 7 dai as determined by quantitative PCR.
(b) Relative expression of *PR1B*, *PR5*, *PR10* and *JIP23* in roots of M117 and M121 in response to *Piriformospora indica* at 3 and 7 dai. The data are based on three independent biological experiments. The barley (cv. Himalaya) mutant lines used were impaired in GA synthesis (M117) or defective in the GA receptor GID1 (M121). Data were analyzed by analysis of variance (ANOVA) using a block design. Asterisks indicate significance at $P < 0.05$.

(Figure 6e,f), as did that of many minor amino acids (data not shown). In contrast, transport of another group of amino acids (e.g. glutamate and aspartate) did not decrease as strongly (Figure 6g,h).

As the results of these experiments suggested a developmental decline in sugar and amino acid contents with increasing degree of differentiation of the harvested root portion, we determined the sugar and amino acid contents in distal root regions encompassing the calyptra and the meristematic (0–0.5 cm), elongation (0.5–1 cm) and differentiation zones (1–1.5 and 1.5–3 cm). *Piriformospora indica* colonization led to a decrease of hexose content in all sampled segments (Figure 8a), but no changes were observed for sucrose (Figure 8c). The sucrose/hexose ratio (Figure 8e) exhibited a stronger decrease in *P. indica*-colonized roots compared to mock-treated roots when moving from proximal root segments towards the root tip, indicating that the supply of sugars to the sink tissue at the root tip is diminished in the presence of *P. indica*. The starch content along the root axis followed a similar pattern to the hexose content (Figure 8g).

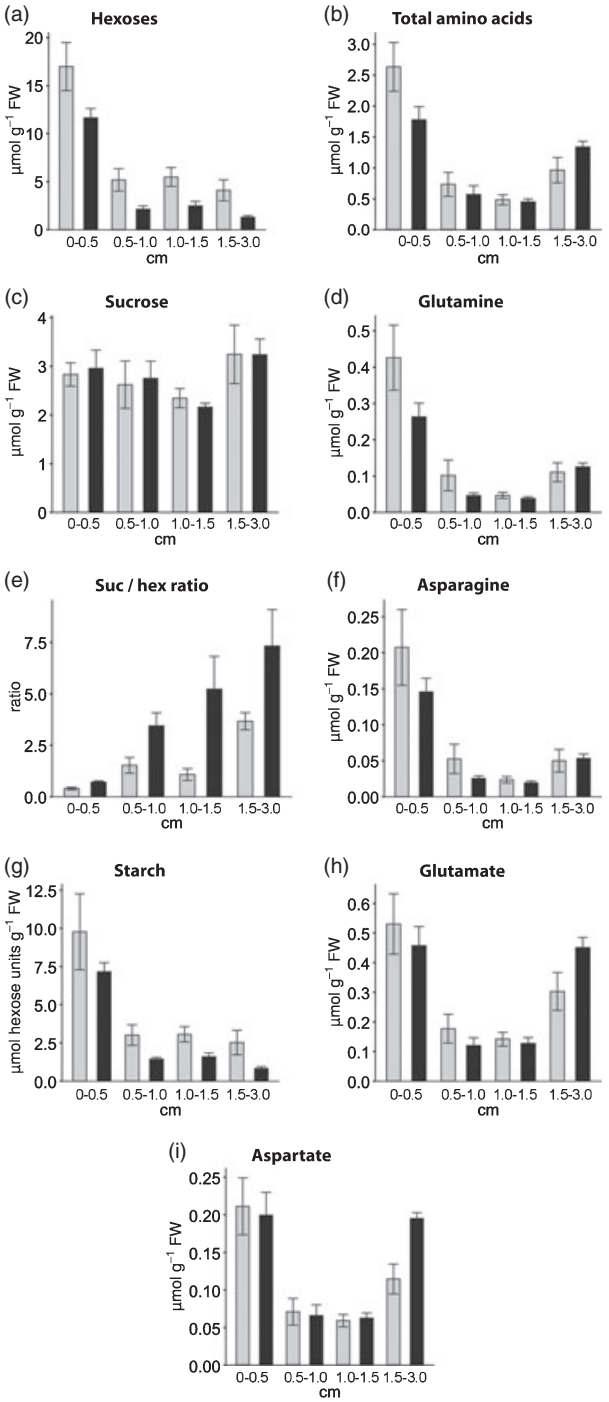


Figure 8. Carbohydrate and amino acid contents in segments of barley roots colonized by *Piriformospora indica.*
For analyses, roots were divided into four segments (0–0.5 cm, calyptra and meristematic zone; 0.5–1 cm, elongation zone; 1.0–1.5 and 1.5–3 cm, differentiation zones), and were harvested from *P. indica*-colonized plants and control plants at 3 dai. The contents of hexoses (a) and sucrose (c), the sucrose/hexose ratio (e) and the starch content (g), as well as the total amino acid content (b) and contents of glutamine (d), asparagine (f), glutamate (h) and aspartate (i) in control roots (grey) and *P. indica*-colonized roots (black) are shown. The results shown are those of one representative experiment out of two. Data are the means of four independent samples, and error bars represent the standard error.

Similar to hexose and starch, *P. indica*-colonized roots contained fewer total free amino acids in the root tip and elongation zone (Figure 8b), due to a decrease in glutamine (Figure 8d), asparagine (Figure 8f) and glutamate (Figure 8h). In contrast, the higher total amino acid contents in the differentiation zone upon colonization with *P. indica* resulted from increased amounts of glutamate and aspartate (Figure 8h,i).

DISCUSSION

Regulation of defence/stress-related genes

Although stress-related genes represent the largest group among *P. indica*-induced genes in barley roots (151 genes), induction levels are generally moderate (Figure 3, Figure S2 and Table 1). In addition, only 31 genes of cluster D were constitutively induced, while the remaining genes were suppressed or displayed a transient induction (Figure S2). In general, the defence/stress-responsive genes affected by *P. indica* are associated with abiotic as well as biotic stress. This gene spectrum is rather broad and reminiscent of MTI responses that are non-specific and moderate in terms of the activation level of responses (Zipfel *et al.*, 2004; Jones and Dangl, 2006; Wan *et al.*, 2008). Over time, the gene spectrum alters most significantly between 1 dai and the later time points, while approximately 50% of the differentially regulated genes overlap between 3 and 7 dai. These expression profiles most probably reflect extracellular fungal development at 1 dai compared to inter-/intra-cellular colonization at the later time points. The high induction levels of defence/stress-responsive genes at 1 dai might indicate recognition of the fungus by the plant innate immune system. It is reasonable to speculate that fungal MAMPs (e.g. chitin) lead to defence activation. In turn, reduced induction or even suppression of respective genes at 3 and 7 dai might indicate active manipulation of the plant surveillance system and respective defence signalling cascades by the fungus. The differences recorded between 3 and 7 dai corroborate cytological studies that indicated an initial biotrophic phase followed by a cell death-dependent phase (P. Schäfer and B. Zechmann, unpublished results). Using transmission electron microscopy, *P. indica* was shown to colonize living Arabidopsis root cells by invaginating the plant plasma membrane. As the interaction proceeded, colonized cells died. However, adjacent non-colonized root cells were not affected or impaired in viability, suggesting that the fungus does not release cytotoxic molecules in order to kill cells ahead of penetration. As later colonization stages were not accompanied by tissue necrotization, it is more appropriate to use the term 'cell death-dependent', instead of the 'necrotrophic colonization phase' (Schäfer and Kogel, 2009). In conclusion, those genes induced or suppressed at 7 dai but not at 3 dai might participate in cell-death regulation or their expression might be modified by signals originating from dying cells. Liu *et al.* (2005) showed that cell death-derived signals might be translocated in neighbouring cells and exhibit pro-apoptotic activity. These authors found that a malfunctional autophagy pathway did not restrict tobacco mosaic virus-induced hypersensitive response cell death to the initial infection site and resulted in a spreading cell death phenotype. In our study, among the genes with highest transcript abundance at 3 and 7 dai are several members of the germin multi-gene family, some of which are developmentally regulated in the roots and leaves of seedlings under non-stress conditions and are thought to function in cell-wall metabolism (Zimmermann *et al.*, 2006). In barley leaves, *GerA*, *Ger4c* and *Ger4d* are strongly induced after powdery mildew attack, and contribute to fungal growth arrest (Zimmermann *et al.*, 2006). Hence, germin induction in roots may restrict *P. indica* invasion.

It is appealing to speculate that genes categorized as 'defence/stress-responsive' support the plant in balancing the mutualistic colonisation by *P. indica*. For instance, two LysM receptor-like kinases were found to be induced. The extracellular lysin motifs of plant LysM receptor-like kinases signify such proteins as receptors (Zhang *et al.*, 2007). Recently, *CERK1* was identified, which encodes a LysM receptor-like kinase that participates in chitin recognition and MTI (Miya *et al.*, 2007; Wan *et al.*, 2008). In contrast, NFR1 and NFR5 from *Lotus japonicus* were identified as crucial components for rhizobial nodulation by binding to Nod factors released by N_2-fixing bacteria (Limpens *et al.*, 2003; Radutoiu *et al.*, 2007). It remains to be investigated whether either or both LysM receptor-like kinases identified in our study support or restrict establishment of the sebacinoid symbiosis.

In addition, an Arabidopsis homologue of the two *BAK1* genes identified in our study was previously shown to be involved in basal defence triggered by flagellin (Chinchilla *et al.*, 2007). Hence, *BAK1* induction might be involved in MTI responses triggered by *P. indica* rather than in brassinolide signalling.

Reduced GA synthesis represses root compatibility

Our microarray analyses revealed comprehensive induction of the MEP pathway, which delivers precursors for GA synthesis (Figure 5). This is in line with the induction of two putative *ent*-kaurene synthases that may be involved in GA synthesis, and suppression of *GA2ox1*, the product of which inactivates GA (Figure 5). Subsequent genetic studies revealed reduced colonization by *P. indica* of two mutants, M117 and M121, that are impaired in GA synthesis or perception (Figure 7a). These phenotypes might be partially explained by an altered defence response, as the mutants showed elevated expression of *PR1*, *PR5* and *PR10* (Figure 7b). Recently, Navarro *et al.* (2008) demonstrated that Arabidopsis mutants blocked in GA signalling show enhanced resistance against necrotrophic *Alternaria*

brassicicola. In contrast, quadruple DELLA mutants that show constitutive GA signalling exhibited increased susceptibility (Navarro *et al.*, 2008). Interestingly, the JA/ethylene-responsive gene *PDF1.2* showed delayed expression in *DELLA* in response to *A. brassicicola* or methyl jasmonate treatment (Navarro *et al.*, 2008). These results suggest a direct connection between GA signalling and SA/JA responses. In analogy altered GA homoeostasis might explain induction of *PR1B* in the barley–*P. indica* interaction. However, *JIP23* expression was affected only marginally (Figure 7b). Further biochemical and genetic studies are required to elucidate at which level (synthesis, perception or signal transduction) *P. indica* affects GA chomoeostasis in barley roots, and to what extent altered compatibility in GA mutants is a consequence of a modified defence response (e.g. suppression of SA-triggered responses).

Impact of *P. indica* on salicylic acid, jasmonate and ethylene signalling

The phytohormones salicylic acid, jasmonate and ethylene are components of the plant innate immune system, and have a considerable impact on pathogenic as well as mutualistic interaction partners (Glazebrook, 2005; Loake and Grant, 2007). Interestingly, genes encoding enzymes of the phenylpropanoid pathway, which is involved in the synthesis of SA, phytoalexin and lignin precursors, are weakly or transiently induced at 3 dai or down-regulated by *P. indica* (Table S1), which is consistent with the results of cytological studies, which rarely showed cell-wall lignification during *P. indica* colonization (Schäfer and Kogel, 2009; P.S., unpublished results).

Root colonization by *P. indica* is also accompanied by altered expression patterns of genes that are known to participate in oxylipin metabolism. Lipoxygenases (LOXs) catalyse the dioxygenation of linoleic or α-linolenic acid to (9*S*)- and (13*S*)-hydroperoxides, which are precursors of various oxylipins (Feussner and Wasternack, 2002). As oxylipins can act in plant defence as bioactive messengers (Blee, 2002; Feussner and Wasternack, 2002), as anti-microbial compounds (Weber *et al.*, 1999), or as cell death-promoting agents (Rusterucci *et al.*, 1999; Vollenweider *et al.*, 2000), *P. indica*-responsive LOXs might affect root colonization. The results for JA synthesis/signalling are contradictory, as a putative *S*-adenosyl-L-methionine: jasmonic acid carboxyl methyltransferase mediating methyl jasmonate synthesis was induced at 3 and 7 dai, but JA marker genes (e.g. *JIP23*) were suppressed at 3 dai. As the enzymatic activity of the putative *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase has not yet been demonstrated, synthesis of certain oxylipins other than JA or methyl jasmonate might be induced by *P. indica*.

The microarray data paint a similar picture regarding ethylene synthesis and signalling at later interaction stages. Two ESTs encoding 1-aminocyclopropane-1-carboxylic acid oxidase (ACC oxidase), which is involved in ethylene synthesis, were induced by *P. indica* at 3 and/or 7 dai. In contrast, genes encoding two transcription factors (ethylene-responsive factor, RAV2-like DNA binding protein) and an ethylene binding protein-like gene were down-regulated at 3 or 7 dai. This contradiction in ethylene synthesis and signalling might indicate induction of other yet to be identified ethylene signalling components. Alternatively, the ACC oxidases might be post-transcriptionally or post-translationally inactivated, thereby preventing ethylene synthesis.

ABA and auxin may act as negative regulators of root innate immunity

In addition to initiation after *de novo* ABA synthesis, ABA signalling is facilitated by interaction of phospatidic acid with the repressor ABI1 (Zhang *et al.*, 2004), and active ABA can be rapidly recruited from the glucose-conjugated ABA pool (Lee *et al.*, 2006). ABA regulates expression of members of the dehydrins/LEA protein family, various members of which were strongly induced at 1 dai. Dehydrins/LEAs function as chaperone-like proteins and maintain cellular functions under stress conditions (Hundertmark and Hincha, 2008). In Arabidopsis, ABA mediates susceptibility against *Pseudomonas syringae* pv. *tomato*. Type 3 effectors released by *P. syringae* pv. *tomato* cause elevation of ABA and JA in leaves, thereby abolishing callose deposition and MTI (De Torres-Zabala *et al.*, 2007). Previous studies have shown ABA-mediated reduction of lignin and SA synthesis, and suppression of the phenylpropanoid pathway and various defence-related genes (Ward *et al.*, 1989; Mohr and Cahill, 2007). ABA has also been shown to suppress basal and JA/ET-related defences, while ABA deficiency led to enhanced resistance against *Fusarium oxysporum* in Arabidopsis (Anderson *et al.*, 2004). Our data are consistent with the hypothesis that ABA signalling might be used by *P. indica* to overcome initial host defence and to prepare for cell penetration and host colonization.

In addition, genes participating in auxin signalling and synthesis were induced by *P. indica*. As auxin mediates lateral root initiation and formation (Ljung *et al.*, 2005), and *P. indica* enhances lateral root formation and primary root emergence (S. Jacobs and A. Molitor, unpublished results), the induction of auxin biosynthetic genes at 3 and 7 dai might support plant growth. Genetic studies in Arabidopsis have further demonstrated reduced bacterial growth in plant mutants that are repressed in auxin signalling (Navarro *et al.*, 2006). Interestingly, *P. indica* was also reported to produce auxin (Sirrenberg *et al.*, 2007). Taken together, these results suggest that *P. indica* might increase auxin signalling in order to (i) change the root morphology, thereby improving root accessibility, and/or (ii) impair plant defence.

P. indica influences primary metabolite distribution in barley roots

When we assessed the major carbohydrate and amino acid contents at various time points after root colonization by *P. indica*, most changes could be attributed to metabolite gradients along the root axis rather than fungal colonization. There were no consistent effects on the transcriptional regulation of primary metabolism, which contradicts our initial assumption that the presence of the fungus would affect metabolite redistribution. We observed high hexose and amino acid contents at 1 dai (Figure 6), confirming that the meristematic zone in the root tip represents a strong sink tissue (Herbers and Sonnewald, 1998). The high starch content in the most distal root segment is due to the presence of amyloplasts in the calyptra. Because our data suggest that most differences between *P. indica*-colonized and control roots might be obscured by metabolite gradients along the root axis, we assessed metabolite contents in various root segments at 3 dai. We found a decrease in hexose, glutamine and asparagine contents from tip to base (Figure 8), supporting the view that the hexose and amino acid contents depend on root differentiation. The tips of colonized roots showed a decrease in hexose, glutamine and asparagine contents, suggesting that sink strength is decreased by *P. indica*, and the sucrose/hexose ratio, an indicator of lower sink strength, was higher in colonized segments. There are two possible explanations for this. First, the availability of assimilates transported via the phloem might be lower at the root tip due to competition with *P. indica*, which predominantly resides in the differentiation zone (Deshmukh *et al.*, 2006). Uptake of hexoses and amino acids from the root has been demonstrated for arbuscular mycorrhizal fungi (Pfeffer *et al.*, 1999; Govindarajulu *et al.*, 2005), and it is known that host cells and symbiont can compete for carbon when the supply from the phloem is limiting (Son and Smith, 1988). Second, cell death (Deshmukh *et al.*, 2006) and auxin synthesis/signalling correlated with root colonization, which might explain the initiation of lateral roots in response to the fungus. Therefore, the supply to the primary root tip could be lower due to increased distribution to competing lateral root primordia.

EXPERIMENTAL PROCEDURES

Plant and fungal material

For all experiments, barley seeds (*Hordeum vulgare* L. cv. Golden Promise, cv. Himalaya and GA mutants M117, M121) (Chandler and Robertson, 1999; Chandler *et al.*, 2008) were surface-sterilized, pre-germinated, inoculated with chlamydospores or mock-treated as described previously (Deshmukh *et al.*, 2006). For the transcriptome and metabolome experiments, seedlings were grown in a 2:1 mixture of Seramis expanded clay (Mars, http://www.seramis.de/) and Oil Dri (Damolin, http://www.damolin.dk/) under 16 h light (60 mmol m^{-2} sec^{-1} photon flux density) at 22/18°C (day/night) and 60% relative humidity. Three independent biological experiments were carried out. Barley roots were harvested at 1, 3 and 7 dai by carefully removing the seedlings from the substrate. Because of the higher colonization of older root parts, the upper 3 cm of the root (next to the kernel base) were collected at 3 and 7 dai, and aliquots were quick-frozen in liquid nitrogen. At 1 dai, the whole root was harvested. For each sample, 96 plants were harvested and divided into four subsets, which were used for metabolome analyses. For the transcriptome analyses, the roots of the four subsets were pooled and used for RNA isolation. Aliquots of homogenized frozen root material were used to quantify fungal biomass in inoculated roots by quantitative PCR, and for metabolite analyses, RNA or DNA isolation (see below). For the GA mutant analyses and metabolome studies of apical root segments, inoculated plants were grown on modified plant nutrient medium (0.5 mM KNO_3, 2 mM $MgSO_4$, 0.2 mM $Ca(NO_3)_2$, 0.43 mM NaCl, 0.14 mM K_2HPO_4, 2 ml/l Fe-EDTA [20 mM $FeSO_4$, 20 mM Na_2EDTA]) under the same growth chamber conditions as described above. GA mutant roots were harvested 3 and 7 dai. For determination of metabolites in apical root segments, roots of *P. indica*-inoculated and mock-treated cv. Golden Promise were removed from 1l glass jars at 3 dai and dissected into four segments (the apical first 0.5, 0.5–1, 1–1.5 and 1.5–3 cm from the tip). Pooled material from the individual segments was separately shock-frozen in liquid nitrogen and analysed for metabolite content (see below).

For all experiments, roots were cytologically analysed after tissue fixation in trichloroacetic acid solution and staining with WGA-AF488 (Molecular Probes, http://www.invitrogen.com/) for fungal colonization and the absence of fungal contaminants in mock-treated roots by epifluorescence microscopy as described previously (Deshmukh *et al.*, 2006).

Quantification of fungal colonization by quantitative PCR

Genomic DNA of wild-type and GA mutant plants was extracted from approximately 100 mg root material using a plant DNeasy kit (Qiagen, http://www1.qiagen.com/) according to the manufacturer's instructions. Aliquots (10 ng) of total DNA were used as the template for quantitative PCR analyses. Amplifications were performed in 20 μl SYBR Green JumpStart *Taq* ReadyMix (Sigma-Aldrich, http://www.sigmaaldrich.com/) with 350 nM oligonucleotides, using an Mx3000P thermal cycler with a standard amplification protocol (Stratagene, http://www.stratagene.com/). The $2^{-\Delta C_t}$ method (Livak and Schmittgen, 2001) was used to determine the degree of root colonization. Cycle threshold (C_t) values were obtained by subtracting the raw C_t values for the *P. indica Tef* gene (Bütehorn *et al.*, 2000) from the raw C_t values for plant-specific ubiquitin (see Table S2 for the specific oligonucleotide primers used). Data were analysed using the 'lm' statistical procedure (linear model) in R using a block design. The marginal means were compared for all variables (*a*, *b*, *c*).

Design of barley oligonucleotide arrays

The 444 652 barley EST sequences publically available at http://www.harvest-web.org in June 2007 were assembled into 28 001 consensus sequences, leaving 22 937 singletons. The assembly is available on the HarvEST website (assembly 35, http://www.harvest-web.org). For calculation of a 44K 60-mer oligonucleotide array using the Agilent eArray algorithm (http://earray.chem.agilent.com/earray), 13 265 singletons with a significant hit (E-value $<10^{-10}$) in Arabidopsis or rice and the 28 001 consensus sequences were used, together with 2600 replicate probes and internal controls. The oligonucleotide sequences spotted on the 44K array are available at http://www.biologie.uni-erlangen.de/bc/xkriptom.html. The latest annotation can be obtained at http://www.harvest-web.org.

Transcriptome analysis

For transcriptome studies, RNA was extracted from homogenized root material using TRIzol (Invitrogen, http://www.invitrogen.com/) as described by the manufacturer. Aliquots (1 μg) of total RNA were used for cDNA synthesis with a qScript cDNA synthesis kit (Quanta Biosciences, http://www.quantabio.com/). RNA quality was analysed using an Aligent 2100 bioanalyser (Agilent, http://www.agilent.com/). Probe synthesis and labelling were performed according to Agilent's protocol for One-Color Microarray-Based Gene Expression Analysis (version 5.0.1). Labeled probes were hybridized to custom-designed Agilent barley 44K microarrays, and raw data were generated using an Agilent microarray scanner and feature extraction software.

For confirmation of array data, total RNA isolation and cDNA synthesis were performed as described above using the same root material as for the array experiments. Aliquots of 10–20 ng cDNA were used as the template for quantitative PCR using primers specific for individual genes (Table S2), and constitutively expressed ubiquitin served as the internal standard.

Data analysis was performed using Bioconductor/R (http://www.bioconductor.org/). The Limma package (Smyth, 2004) of Bioconductor was used for expression analysis of differentially regulated genes. Therefore, data were read by read.maimage, filtered by flags, and normalized using quantile normalization of background-corrected $\log_2$-converted intensities (normalizeQuantiles). Using lmFit (Linear Model for Series of Arrays), a linear model was fitted to the $\log_2$ expression data for each probe, and contrasts.fit (Compute Contrasts from Linear Model Fit) was used to obtain coefficients and standard errors for contrasts of the coefficients of the original model. An empirical Bayes method, ebayes (Empirical Bayes Statistics for Differential Expression), was used to calculate the moderated *t* statistics. A table of the top-ranked genes from the linear model fit was extracted using topTable (Table of Top Genes from Linear Model Fit). Genes with a *P* value ≤0.05 that were at least twofold regulated at one time point were filtered and displayed by heatmap. The data discussed here have been deposited in NCBI's Gene Expression Omnibus database (Edgar *et al.*, 2002), and are accessible through GEO Series accession number GSE13756 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13756).

Gene expression analyses

For elucidation of candidate gene expression in GA mutants, RNA was extracted, reverse-transcribed to cDNA, and used to analyse the expression patterns of defence-related genes (Table S2) using standard quantitative PCR protocols. Data were analysed using the statistical procedure 'lm' (linear model) as described above.

Determination of carbohydrates and free amino acids

Frozen samples were extracted, and glucose, fructose and sucrose were quantified using a coupled optical test at 340 nm as described by Stitt *et al.* (1989) in a total assay volume of 200 μl using a microtiter plate reader (BioTek, http://www.biotek.com/).

Amino acids were derivatized using the fluorophore 6-aminoquinolyl-*N*-hydroxysuccimidyl carbamate (AccQ Tag, http://www.waters.com/) and subsequently resolved by HPLC analysis using a reverse-phase column (Luna C18; particle size 5 μm, length 250 mm, internal diameter 4.6 mm; Phenomex, http://www.phenomenex.com) as described by van Wandelen and Cohen (1997) with modifications as described by Abbasi *et al.* (2007).

Quantitation of carotenoids

Frozen root tissue (50 mg) was ground to a fine powder and extracted with 400 μl methanol by homogenization. During the following steps, samples were shielded from light and kept on ice. After incubation for 5 min at 4°C, 400 μl 50 mM Tris/HCl pH 8.0/1 M NaCl were added, and the mixture was incubated for 5 min before addition of 800 μl chloroform for extraction of the carotenoids from the methanol phase. Samples were inverted for 5 min, incubated for 10 min, and then centrifuged at 13 000 rpm for 5 min to achieve phase separation. Extraction with chloroform was repeated, and the lower phases were pooled and vacuum dried in a speed-vac.

For reverse-phase chromatography, extracts were dissolved in 200 μl eluent B (methanol:acetonitrile:isopropanol:water, 73:20:5:2), and 20 μl aliquots were resolved on a Dionex Acclaim PA C16 column (internal diameter 4.6 mm, length 150 mm, particle size 5 μm) using a Dionex Ultimate 3000 HPLC system (http://www.dionex.com) connected to an ICS 2600 photodiode array detector (Hamamatsu, http://sales.hamamatsu.com) with a gradient as described by Fraser *et al.* (2000). Pigments were detected and quantified based on their absorption at 450 nm, and identified according to their specific 3D spectra between 300 and 700 nm.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Verification of microarray data by quantitative PCR-based analysis of expression patterns of selected candidates.

Figure S2. Regulation of genes associated with defence/stress upon *P. indica* colonization.

Table S1. Transcript profiles of genes differentially regulated in barley roots by *P. indica* at 1, 3 and 7 dai.

Table S2. Sequences of primer pairs.

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5.2 Khatabi, B., Molitor, A., Lindermayr, C., Pfiffi, S., Durner, J., von Wettstein, D., Kogel, K.H., **Schäfer, P.** Ethylene supports colonization of plant roots by the beneficial fungus *Piriformospora indica*. Submitted to *Journal of Experimental Botany* (MS ID: JEXBOT/2010/055780).

Ethylene Supports Colonization of Plant Roots by the Beneficial Fungus *Piriformospora indica*

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ABSTRACT

The mutualistic basidiomycete *Piriformospora indica* colonizes roots of mono- and dicotyledonous plants, thereby transferring various benefits to its hosts. Given its capability for colonizing an extraordinarily broad range of hosts, it must be anticipated that the fungus evolved efficient strategies for overcoming plant immunity and establishing a suitable environment for nutrient acquisition and reproduction. Global gene expression studies with barley identified various ethylene synthesis and signaling components that were differentially regulated in *P. indica*-colonized roots. Based on these findings we initiated work to elucidate the effects of ethylene in early steps of the symbiotic association. The data presented here propose an increase in ethylene synthesis in barley and *Arabidopsis* roots during *P. indica* colonization. Our data indicate the ineffectiveness of ethylene-related defense in stopping *P. indica* colonization. Accordingly, *Arabidopsis* plants exhibiting constitutive ethylene signaling, -synthesis or ethylene-related defense showed enhanced susceptibility against *P. indica*. Based on our data ethylene might contribute to the extraordinary ability of *P. indica* to colonize a multiplicity of hosts.

Key words

Compatibility, ethylene, mutualism, oxidative burst, root symbioses, root innate immunity,

INTRODUCTION

Ethylene plays a prominent role in senescence, plant development, and plant immunity (Bleeker and Kende, 2000; Broekaert *et al*., 2006). In *Arabidopsis thaliana*, ethylene is perceived by ER membrane-bound receptors (e.g. ETR1). In the absence of ethylene, the receptors activate a Raf-like kinase (CTR1), which negatively regulates the downstream ethylene response pathway (Kieber *et al*., 1993). Binding of ethylene inactivates the receptors, resulting in the deactivation of CTR1, which allows downstream effectors like EIN2 to function as positive regulators of ethylene signaling (Guo and Ecker 2004; Wang *et al*., 2002) by activating transcription factors EIN3 and EIN3-like 1 (EIL1) (Kendrick and Chang 2008). Constitutive ethylene signaling is observed in *ctr1* (Kieber *et al.*, 1993) and in *ethylene overproducer 1* (*eto1*) mutants. ETO1 negatively regulates ethylene synthesis by inactivating and/or degrading 1-aminocyclopropane-1-carboxylic acid synthase 5 (ACS5) and probably other ACS isoforms such as ACS4, ACS8, and ACS9 (Chae *et al.,* 2003; Chae and Kieber 2005; Wang *et al*., 2004).

Plant immunity is activated after perception of conserved microbial molecules, so called microbe-associated molecular patterns (MAMPs), by specific pattern recognition receptors (PRRs) (Boller and Felix, 2009). The recognition of bacterial flagellin by the PRR FLS2 results in the activation of an array of immune responses summarized as MAMP-triggered immunity and includes the rapid production of ethylene and reactive oxygen species (ROS) (Felix *et al*., 1999). Both, ethylene and ROS have immune signaling properties and contribute to the restriction of pathogen invasions (Tsuda *et al*., 2009; Mersmann *et al.,* 2010). In the ethylene pathway,

EIN3 and EIL1 drive the expression of primary ethylene transcriptional activators, e.g. *ERF1*. ERF1 regulates ethylene responsive-genes including defense-associated genes such as *PR3* and *PDF1.2* (Adie *et al*., 2007). Recently, ethylene signaling was shown to be essential for flagellin-triggered ROS production as EIN3 (and probably EIL1) directly mediates the regulation of *FLS2* (Boutrot *et al*., 2010; Mersmann *et al*., 2010).

Piriformospora indica is a root-colonizing basidiomycete that colonizes mono- and dicotyledonous plants, including barley (*Hordeum vulgare*) and *Arabidopsis*, in which the fungus elicits benefits such as yield increase and adaptation to abiotic and biotic stress (Schäfer and Kogel 2009; Varma *et al*., 1999; Verma *et al*., 1998; Waller *et al*., 2005). Cytological and genetic studies have shown that the colonization of roots is dependent on cell death. This cell death-dependent colonization is preceded by an initial biotrophic growth phase which extends to 3 days after inoculation (Deshmukh *et al*., 2006; Kogel, Schäfer, unpublished data). The fungus exhibits an immune suppressing activity, which is essential for root colonization, and may partially explain its remarkably broad host range (Schäfer *et al*., 2009). Microarray analysis of barley roots colonized by *P. indica* detected the differential expression of genes participating in ethylene synthesis and signaling (Schäfer *et al*., 2009). In the present study, we analyzed the effect of ethylene on the colonization of *Arabidopsis* and barley roots by *P. indica*. We demonstrate that *P. indica* affects ACC synthesis and that ethylene signaling is not detrimental to fungal growth. We discuss the possibility that ethylene is a positive modulator of the mutualistic plant root-*P. indica* symbiosis.

MATERIALS AND METHODS

Plant material and fungal inoculation.

Seeds of *Arabidopsis thaliana* ecotype Col-0 and mutants *eto1-1* (N3072), *ein2-1* (N8844), *ctr1-1* (N8057), *etr1-3* (N3070), *35S*::*ERF1* (N6142) and *ACS*::*GUS* reporter plants (N31379, N31380, N31381, N31382, N31383, N31385, N31386, N31387) were obtained from the European Arabidopsis Stock Centre (NASC). All *Arabidopsis* plants and the respective parents were grown on ½ Murashige and Skoog medium on squared petri dishes, which were vertically positioned. Plants were grown at 22/18°C day/night cycle under short-day conditions (10 hours light) at 60% rel. humidity in a growth chamber. Three-weeks-old plants were inoculated with *P. indica* (500,000 spores ml^{-1}) and harvested at indicated timepoints. For barley, all experiments were conducted with cultivar Golden Promise. Plants were inoculated as described previously (Deshmukh *et al*., 2006) and transferred to 1.5 l glas jars containing $PNM_{(1/10)}$ (Schäfer *et al*., 2009). Barley root treatment and harvest were performed as described below.

Cyto-histological observations and GUS.

For cytological examinations, the fungus was stained with wheat germ agglutinin-Alexa Fluor 488 (WGA-AF 488) as previously described (Deshmukh *et al*., 2006). *Arabidopsis ACS*::*GUS* plants were harvested at indicated timepoints. GUS staining was performed as described previously (Sundaresan *et al*., 1995). Briefly, roots were stained with staining solution (50 mM phosphate buffer, pH 7.0, 0.5 mM potassium ferricyanide, 0.2% Triton X-100, 0.5% DMSO, 20% methanol, 2 mM EDTA, 1 mM

X-Gluc) and incubated overnight at 37°C. The staining reaction was stopped by incubation in 70% ethanol. The roots were microscopically analyzed using an Axioplan 2 microscope (Carl Zeiss, Jena, Germany). WGA-AF 488 was detected at 470/20 nm (excitation) and 505-530 nm (emission).

Application of ACC and 1-methylcyclopropene (MCP).

Two-days-old barley seedlings (cv. Golden Promise) were inoculated with *P. indica* and transferred to $PNM_{(1/10)}$ (Schäfer *et al*., 2009) supplemented with 100 µM ACC (Sigma-Aldrich, Munich, Germany). ACC was dissolved in water and filter-sterilized prior to its addition to autoclaved plant growth media. For MCP (Rohm and Haas Company, Philadelphia, USA) treatment, inoculated plants were transferred to glass jars (volume 1.5 l) in which a vial containing 16 mg MCP (0.14% active ingredient) dissolved in 200 µl water and placed into a jar. The final concentration of 1-MCP in the gas phase of the jar was expected to be about 500 ppt (Tamaoki *et al.*, 2003). Roots were harvested at 3 and 7 dai, frozen in liquid nitrogen and subjected to DNA isolation (see below).

Determination of ACC content.

Two-day-old barley plants (cv. Golden Promise) were inoculated with *P. indica* or mock treated and transferred to jars containing $PNM_{(1/10)}$ (Schäfer *et al*., 2009). Roots were harvested at 0, 1, 3, and 7 dai. At 3 and 7 dai, the upper two centimeters (basal part) were separately harvested form the lower apical part. Plant material was ground in liquid nitrogen and extracted according to Langebartels et al. (1991). Free

ACC and total ACC released by acid hydrolysis (2 N HCl for 3 h at 120°C) were determined according to Lizada and Yang (1979) as described by Langebartels et al. (1991). The amount of conjugated ACC was calculated by subtracting the amount of ACC from total ACC.

Quantitation of *P. indica* colonization by quantitative real time PCR.

Genomic DNA of wild type and *Arabidopsis* mutant roots as well as ACC-/MCP- and mock-treated barley roots was extracted from ~100 mg root material using Plant DNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Ten ng of total DNA served as template for qRT-PCR analyses. Amplifications were performed in 20 µl SYBR green JumpStart *Taq* ReadyMix (Sigma-Aldrich, Munich, Germany) with 350 nM oligonucleotides, using an Mx3000P thermal cycler with a standard amplification protocol (Stratagene, La Jolla, USA). To determine the degree of root colonization, cycle threshold (Ct) values were obtained by subtracting the raw Ct values of the *P. indica Tef* gene (*PiTef*) from the raw Ct values of *Arabidopsis* using the *PiTef*-specific primers 5'-TCGTCGCTGTCAACAAGATG-3' and 5'-ACCGTCTTGGGGTTGTATCC-3' (Bütehorn *et al.*, 2000), *AtUBQ5*-specific primers 5'-CCAAGCCGAAGAAGATCAAG-3' and 5'-ACTCCTTCCTCAAACGCTGA-3', or *HvUBQ60*-specific primers 5'-ACCCTCGCCGACTACAACAT-3' and 5'-CAGTAGTGGCGGTCGAAGTG-3'.

Chitin-induced root oxidative burst.

Three-days-old barley seedlings were either treated with *P. indica*, *Rhizoctonia solani* AG8, or mock treatment. For determination of oxidative burst, at 3 dai roots were cut in 1 cm long pieces (10 mg per assay) and floated in water over night. The roots were transferred to tubes with 20 µM luminol (Sigma-Aldrich, Munich, Germany) and 1,5 µg horseradish peroxidase (Roche Diagnostics, Mannheim, Germany). 1 mg ml^{-1} chitin (Sigma-Aldrich, Munich, Germany) was used as elicitor for a luminol-based assay as described by Gomez-Gomez et al., (1999). Luminescence measurements were performed for 30 min in a Berthold Lumat LB 9501 (Berthold, Bad Wildbach, Gemany).

Statistical analysis.

All experiments were conducted at least in triplicate and standard errors were calculated for all mean values. Levels of significance were calculated using a Student's *t*-test.

RESULTS

Impaired ethylene signaling reduces colonization of barley roots by *P. indica*

In global transcriptome analyses, we identified the differential regulation of components with a putative function in ethylene synthesis and signaling (Schäfer *et al*., 2009). Among the ethylene synthesis genes were three *1-aminocyclopropane-1-carboxylic acid (ACC) oxidases* (Tab. 1). The six signaling genes encoded putative transcription factors: *ethylene-responsive element binding protein, ethylene insensitive 3-like 2, AP2 domain transcription factor EREBP*, a putative *RAV2-like DNA binding protein, ethylene-responsive factor,* and *ethylene-binding protein-like* (Tab. 1). Interestingly, while ethylene synthesis genes were mostly induced, signaling components were generally suppressed during *P. indica* colonization (Tab. 1).

The data raised the possibility that ethylene modulates *P. indica*‘s ability to colonize plant roots. Because barley mutants with compromised ethylene biosynthesis and signaling were not available, we conducted pharmacological experiments in order to determine the significance of ethylene for a successful symbiosis. To this end, two-days-old barley seedlings were transferred to agar containing 100 µM of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), or transferred to a jar containing a vial with 1 mM of the ethylene antagonist 1-methylcyclopropene (MCP), which blocks ethylene signaling by interacting with ethylene receptors (Sisler and Serek, 2003). Seedlings were inoculated with *P. indica* (500.000 spores ml^{-1}) and fungal colonization was determined at 3 and 7 days after inoculation (dai) by quantitative real time PCR (qRT-PCR). We found a tendency for improved root

colonization after ACC treatment while, in contrast, treatment with the MCP resulted in an approximately 50% reduction in the amount of fungal DNA at 7 dai (Students *t*-test, $P < 0.05$) (Fig. 1). Neither of the compounds had adverse effects on morphology or growth of *P. indica in vitro* (not shown). These data raised the possibility that the colonization of barley roots by *P. indica* is supported by ethylene signaling during the cell death-associated growth phase.

The ACC level is increased in colonized barley roots

While blockage of ethylene signaling reduced fungal colonization, higher doses of ACC, the immediate precursor of ethylene, had no effect on the interaction. One explanation could be that the ACC levels were per se enhanced in roots during colonization. If ethylene signaling was indeed saturated, treatment with ACC would not further impact ethylene synthesis or fungal root colonization. To test this hypothesis, we determined ACC contents in *P. indica*-colonized roots. Since a fraction of the ACC is usually malonylated, and therefore not available for ethylene synthesis, we measured free and malonylated ACC at 1, 3, and 7 dai. Because previous studies indicated that *P. indica* colonizes preferentially the maturation zone of roots (Deshmukh *et al*., 2006), the upper two centimeter of the root (basal part = maturation zone) were analyzed separately from the remaining apical root tissue (apical part). In all samples, the amount of malonylated ACC was higher than free ACC (Fig. 2A, B), and there were no significant changes in malonylated ACC upon fungal colonization (Fig. 2B). By contrast, we found slightly higher amounts of free

ACC in all colonized roots (Fig. 2A), with a significant increase in the apical root part during the cell-death associated growth phase (7 dai).

MAMP-triggered root oxidative burst is suppressed by *P. indica*

Global gene expression analysis has previously demonstrated that *P. indica* does not trigger strong defence responses in barley roots during early colonization stages (Schäfer *et al.*, 2009). Ethylene signaling is essentially required for MAMP-triggered oxidative burst, one of the earliest innate immune responses (Boutrot *et al.*, 2010; Mersmann *et al.*, 2010). The finding that *P. indica* increases the ACC levels in root tissue induced us to reassess the fungus' ability to suppress chitin-induced oxidative burst. To this end, we determined chitin-induced root oxidative burst in non-colonized and *P. indica*-colonized roots at 3 days after inoculation. In non-colonized roots, a strong accumulation of H_2O_2 was measured after chitin treatment (Fig. 3) as was reported earlier for leaves. By contrast, chitin-induced root oxidative burst was almost completely abolished in *P. indica*-colonized roots. This finding corroborates the earlier results showing that *P. indica* exhibits a strong capability in suppression plant defence responses (Schäfer *et al.*, 2009). To exclude the possibility that the suppressing activity is a general attribute of root colonizing fungi, we also tested the ROS-suppressing acitivty of *Rhizoctiona solani*. This fungus is a pathogenic root colonizing basidiomycete that also displays a broad host range. We found that *R. solani* could not suppress the chitin-induced ROS accumulation suggesting that ROS-suppressing activity is associated with the symbiotic potential

of *P. indica*. Moreover, this data suggest that the ACC inducing acitivity of the fungus is not part of a general MAMP-trigered immune response. (Fig. 3).

Colonization-associated regulation of ACC synthases in *Arabidopsis* roots

ACC quantification in barley roots did not allow for any cellular resolution of ACC production as ACC reporter lines are not available for this cereal. Because we wanted to study colonization-associated ACC production in more detail, we switched to the *Arabidopsis-P. indica* system. *Arabidopsis* root colonization by *P. indica* is highly similar as indicated by cell biological analyses (Kogel, Schäfer, unpublished data). We used *Arabidopsis*-reporter plants for ACC synthesis that express β-glucuronidase (GUS) fusions with promoters of genes encoding *1-aminocyclopropane-1-carboxylic acid synthases* (*ACS*). ACS are the rate limiting enzymes in ethylene synthesis (Tsuchisaka and Theologis 2004a). In *Arabidopsis*, nine *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS*) genes (*ACS1*, *ACS2*, *ACS4*, *ACS5*, *ACS6*, *ACS7*, *ACS8*, *ACS9*, and *ACS11*) have been identified (Tsuchisaka and Theologis 2004a). Using the respective reporter lines allows for monitoring the spatio-temporal expression of the respective *ACS* genes upon *P. indica* colonization. To this end, *Arabidopsis* (reporter) plants were analyzed by fluorescence and bright field microscopy at 7 dai upon double-staining for GUS activity and for fungal hyphae with WGA-AF 488. Based on the AREX database (Brady *et al*., 2007; Cartwright *et al.*, 2009), all the nine *ACS* genes are expressed in the meristmatic, elongation, and maturation zone, but differ in their expression level as well as site (Tab. 2). Among all tested lines, only *ACS1*::*GUS* and *ACS8*::*GUS*

lines showed a response to *P. indica* (Fig. 4, 5). We observed *ACS1* induction at primordia and the base of lateral roots after colonization by *P. indica* (Fig. 4). Most obviously, the root tip region of *P. indica*-colonized *ACS1*::*GUS* and *ACS8*::*GUS* plants showed a strong GUS activation at 7 dai (Fig. 5); however, staining pattern slightly differed among both lines. GUS activity in *ACS1*::*GUS* was detected in the elongation zone, while GUS accumulated in ACS8::*GUS* also in the meristem (Fig. 5). Fungal mycelium was not detected in the elongation and meristmatic zone (data not shown). GUS accumulation pattern did not differ in any line in non-colonized compared to *P. indica*-colonized roots at 3 dai. The regulation of ACC synthases *ACS1* and *ACS8* at 7 dai conincided with the enhanced ACC contents determined in barley root tips at 7 dai (Fig. 2).

Ethylene signaling enhances colonization of *Arabidopsis* roots by *P.* indica at the cell death-associated interaction stage

In order to confirm in *Arabidopsis* that ethylene affects *P. indica* colonization, fungal growth was analyzed in the *Arabidopsis* mutants *ethylene insensitive2-1* (*ein2-1*), *ethylene triple response1-3* (*etr1-3*), and *constitutive triple response1-1* (*ctr1-1*), all of which are impaired in ethylene signaling. In addition, the ethylene synthesis mutant *ethylene overproducer 1-1 (eto1-1*) was tested. qRT-PCR-based quantification of the amount of fungal DNA at 3 (biotrophic colonization) and 14 dai (cell death-associated colonization) showed higher colonization of mutants that displayed constitutive ethylene signaling (*ctr1-1*) or enhanced ethylene synthesis (*eto1-1*) during cell death-associated colonization. Colonization of *etr1-3* was not

altered as compared to the wild-type, while *ein2-1* displayed a reduced colonization at 3 dai (Fig. 6). Subsequently, we analyzed the colonization of *35S*::*ERF1* plants in order to monitor the influence of ethylene-mediated gene transcription on fungal development. ERF1 is a transcription factor that is central to ethylene-associated defense signaling in *Arabidopsis* (Berrocal-Lobo *et al*., 2002). Like *ctr1-1* and *eto1-1*, plants overexpressing *ERF1* were significantly more colonized by *P. indica* at 14 dai (Fig. 6).

DISCUSSION

In plant-microbe interactions, ethylene like jasmonate and salicylic acid effectively sustains MAMP-triggered immune responses against pathogens (Broekaert *et al.*, 2006; van Loon *et al*., 2006; Tsuda *et al*., 2009). In a model proposed by Boutrot *et al.* (2010), flagellin recognition by FLS2 results in MAP kinase (MAPK) 3 and 6 activation that, in turn, phosphorylates and thereby stabilizes ACS2, ACS6, and EIN3 (Liu and Zhang, 2004; Yoo *et al*., 2008). Consequently, rapid ethylene production takes place after MAMP recognition and, due to the regulation of *FLS2* by EIN3, a steady-state level of FLS2 at the plasma membrane is achieved (Boutrot *et al*., 2010; Mersmann *et al*., 2010). By contrast, impaired ethylene perception and signaling disturbs FLS2 regulation, thereby impairing MAPK3/6 activation and ROS production. This activity might also explain the impact of ethylene on mutualistic symbioses, since it inhibits plant root mycorrhization and rhizobial nodulation of legumes (Penmetsa and Cook, 1997; Guinel and Geil 2002; Penmetsa *et al*., 2008; Riedel *et al*., 2008). In addition, ethylene mediates resistance against necrotrophic pathogens through the activity of the transcription factor ERF1 (Berrocal-Lobo *et al*., 2002; Broekaert *et al*., 2006).

The spatio-temporal events of root colonization in barley and *Arabidopsis* by *P. indica* are very similar (Deshmukh *et al*., 2006; Schäfer *et al*., 2009; Kogel, Schäfer, unpublished data). Our analyses revealed the significance of ethylene for *P. indica* colonization although we observed species-specific effects of ethylene signaling on plant root colonization (Fig. 1, 6). First, blockage of ethylene signaling by MCP in barley roots resulted in reduced root colonization. Similarly, colonization of

Arabidopsis signaling mutant *ein2-1* was compromised. Second, *ctr1-1*, *eto1-1* and *35S*::*ERF1* plants that display constitutive ethylene signaling, synthesis or defense, respectively, were significantly better colonized by *P. indica*. The stunted root morphology of *eto1-1* and *ctr1-1* might contribute but cannot entirely account for improved colonization, as we also observed increased colonization in *35S*::*ERF1* plants, which possess an unaltered root phenotype.

In parts, our data are inconsistent with a recent report in which *ein2-1* and 35S::*ERF1* seedlings did not display altered colonization by *P. indica* at 12 dai (Camehl *et al*., 2010). We established a robust and sensitive screening system in which plants were grown on medium in squared petri dishes to which a defined amount of spores (500.000 spores ml^{-1}) was directly applied to roots thereby avoiding detachment of seedlings (see Material and Methods). In addition, fungal colonization was determined in a direct approach by quantifying fungal DNA in relation to plant DNA via qRT-PCR. Hence, the differences might be explained by the employed screening systems and quantification methods.

Ethylene synthesis and signaling is a stimuli-dependent process as examplified by the spatio-temporal expression of ACC synthases (ACS) in aerial plant (Tsuchisaka and Theologis, 2004a) and in root tissues (Tab. 2). Plant root colonization by *P. indica* revealed a spatially distinct ACC synthesis pattern. In *Arabidopsis* and barley, we observed increased ACC synthesis in apical root tissue during cell death-associated colonization (Fig. 2, 5). In *Arabidopsis*, *P. indica* root colonization was associated with increased ACC synthesis, which was presumably mediated by ACS1 and ACS8 as indicated by their induction in the elongation zone or whole root tip

region, respectively (Fig. 5). In addition, *ACS1* regulation was slightly enhanced at the base of lateral roots and root primordia colonized by *P. indica* (Fig. 4). *ACS1* induction has not been reported in roots (Tsuchisaka and Theologis, 2004a) but is in accordance with the AREX database prediction (Tab. 2). The analyses revealed systemic regulation of both genes as the fungus was not detected at these root sites (data not shown). The absence of *P. indica* at the root tip implicates an immune-related function of ACS1 and ACS8. Based on leaf expression data in Genevestigator (Zimmermann *et al*., 2004), *ACS1* and *ACS8* are generally not responsive to biotic stress. ACS1 is nonfunctional as homodimer, but can regain activity by heterodimerizing with immune-responsive ACS2 or ACS6 (Tsuchisaka and Theologis 2004b; Liu and Zhang, 2004). However, we did not detect *ACS2* and *ACS6* induction at root tips during *P. indica* colonization (data not shown). The pronounced GUS accumulation in *P. indica*-colonized *ACS8*::*GUS* roots (Fig. 5) is reminiscent of the previously reported *ACS8* induction in *Arabidopsis* roots after auxin treatment (Tsuchisaka and Theologis 2004a). Notably, auxin stimulates the activities of several ACS (Ruzicka *et al*., 2007; Stepanova *et al*., 2007) and antagonizes SA-mediated defense (Pieterse *et al*., 2009). In turn, SA defense was found to restrict *P. indica* colonization, while jasmonic acid signaling, in analogy to ethylene signaling supported root colonization (Kogel, Schäfer, unpublished data). It is tempting to speculate that auxin might be synthesized by the plant (Schäfer *et al*., 2009), and/or by the fungus as was recently hypothesized (Sirrenberg *et al*., 2007), thereby regulating *ACS8* expression and impairing SA-related immune processes. An antagonistic activity of ethylene against SA-related defense has been

demonstrated in 35S::*ERF1* seedlings (Berrocal-Lobo and Molina, 2004), which displayed enhanced susceptibility against *P. indica* (Fig. 6).

Taken together, we demonstrated that ethylene supports root colonization by *P. indica*. The enhanced ACC contents in *Arabidopsis* and barley might not be associated with MAMP-triggered immunity as indicated by the suppression of chitin-induced root oxidative burst by *P. indica*. Furthermore, improved colonization of *35S*::*ERF1* indicates the ineffectiveness of ethylene-triggered defense against *P. indica*. These findings are in accordance with recent studies in which mutants impaired in jasmonic acid synthesis and signaling displayed reduced *P. indica* colonization (Kogel, Schäfer, unpublished data). As ethylene supports colonization in both barley and *Arabidopsis*, the phytohormone might be recruited by *P. indica* to establish root compatibility. We further speculate that ethylene contributes to *P. indica*'s extraordinary ability to colonize a multiplicity of hosts.

ACKNOWLEDGEMENTS

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TABLES

Table 1 List of barley genes differentially regulated by *P. indica* and involved in ethylene synthesis or signaling.

		Fold change (dai)				
Gene	**Acc. number**	**1**	**3**	**7**	**Process**	**Published**
1-aminocyclopropane-1-carboxylate oxidase	ABM74187.1	-2,3	-	-	synthesis	Schäfer et al. 2009
putative 1-aminocyclopropane-1-carboxylic acid oxidase	BAB84460.1	-	4,3	-	synthesis	Schäfer et al. 2009
putative 1-aminocyclopropane-1-carboxylate oxidase	AAU44031.1	-	2,6	2,8	synthesis	Schäfer et al. 2009
AP2 domain transcription factor EREBP	AAP56251.1	-	2,0	-	signaling	Schäfer et al. 2009
ethylene-binding protein-like	BAD38371.1	-	-	-3,3	signaling	Schäfer et al. 2009
ethylene insensitive 3-like 2	AAV68140.1	-3,1	-	-	signaling	Schäfer et al. 2009
ethylene-responsive element binding protein	ABO93372.1	4,3	-	-	signaling	Schäfer et al. 2009
ethylene-responsive factor	ABQ52686.1	-	-2,6	-	signaling	Schäfer et al. 2009
Similar to probable RAV2-like DNA binding protein	AAX92718.1	-	-2,2	-	signaling	Schäfer et al. 2009

Table 2 Regulation of *ACC synthase* genes in *Arabidopsis* roots according to the AREX database.

	ACS1	ACS2	ACS4	ACS5	ACS6	ACS7	ACS8	ACS9	ACS11
	AT3G61510	AT1G01480	AT2G22810	AT5G65800	AT4G11280	AT4G26200	AT4G37770	AT3G49700	AT4G08040
Lateral Root Primordia		+	+		++	+	++		+
Phloem CCs		+	+		++		+		
Meta/Proto Phloem		+	+	+	++		++	+	+
Meta/Proto Xylem		+	+		++		++		
Phloem Pole Pericycle		++	+		++	+	++		+
Xylem Pole Pericycle		+	+		++		++		+
Procambium	++		++	++			+	++	+
Endodermis	+	++	+	+	++	+	++	+	+
Cortex		+	+		++	+	+	+	+
Non-Hair	+	+	++	+	++	+	+	+	+
Hair		+	++	+	++	+	+	+	+
Quiescent Center		++	+		+		+		
Lateral Root Cap	+	+	+	+	+	+	+	+	+
Columella		+	+		++		+		
Meristmatic Zone	+	+	+	+	+	+	+	+	+
Elongation Zone	+	+	+	+	++	+	+	+	+
Maturation Zone	+	+	++	+	++	++a	+b	+	+c

+ Min. Exp. (≥ 0.5)
++ Max. Exp. (≥ 2)
a predominant expression in maturation zone 9-11
c predominant expression in maturation zone 12
b predominant expression in maturation zone 11

FIGURE LEGENDS

Fig. 1. Colonization of barley cultivar Golden Promise by *P. indica* in response to ACC and MCP.

Two-day-old seedlings were inoculated with *P. indica* and subsequently treated with 100 µM 1-aminocyclopropane-1-carboxylic acid (ACC) or 500 ppt 1-methylcyclopropene (MCP) as described in Materials and Methods. *P. indica* colonization was significantly inhibited by MCP at 7 dai. The values are normalized to colonization in mock-treated roots (set to one). The data base on three independent biological experiments. Student's *t*-test indicated a significant difference in *P. indica*-colonization of MCP-treated roots ($p < 0.05$).

Fig. 2. ACC contents in barley roots in dependence of *P. indica* colonization.

Free (A) and malonylated (B) 1-aminocyclopropane-1-carboxylic acid (ACC) contents were determined in *P. indica* and mock-treated roots at 1, 3, and 7 days after treatments. At 1 dai, the complete roots were harvested and forwarded to ACC measurements. At 3 and 7 dai, the upper two centimeters (basal part) and the remaining part of the roots (apical part) were separately analyzed. Absolute values are given in nM • g FW^{-1} for mock-treated and *P. indica*-colonized roots. (A) Free ACC levels were steadily but insignificantly increased in *P. indica*-colonized apical and basal tissue at all time points. Free ACC content was significantly enhanced at 7 dai in the apical zone as indicated by Students *t*-test (** $p < 0.01$). (B) Malonylated ACC was not significantly altered during *P. indica* colonization at any timepoint or

in any tissue. Data show the mean content of four biological experiments (with at least two technical repetitions per experiment) and bars indicate standard errors.

Fig. 3. Suppression of chitin-induced oxidative burst by *P. indica*.

Chitin was applied to barley root segment of seedlings harvested at 3 days after *P. indica*- or *Rhizoctonia solani* inoculation or mock-treatment, respectively. Values are given as relative light units (RLU) over time as means with standard errors of two biological experiments with three independent measurements per treatment and experiment.

Fig. 4. GUS accumulation in roots of *ACS1*::*GUS* reporter plants colonized by *P. indica*.

Arabidopsis line *ACS1*::*GUS* was harvested at 7 dai and, after GUS and WGA-AF 488 staining, cytologically analyzed. (A, B) *ACS1*::*GUS* colonization at the base of lateral roots or primordia was associated with enhanced GUS accumulation. *P. indica* was visualized by staining with WGA-AF 488. (C) In mock-treated *ACS1*::*GUS*, GUS staining was weakly detectable e.g. at the lateral root base. Bars = 60 µm.

Fig. 5. GUS accumulation in roots of *ACS1*::*GUS* and *ACS8*::*GUS* reporter plants colonized by *P. indica*.

Arabidopsis line *ACS1*::*GUS* and *ACS8*::*GUS* were harvested at 7 dai and, after GUS and WGA-AF 488 staining, cytologically analyzed. GUS staining was more

pronounced in root tip regions of colonized roots compared to mock-treated roots (upper images). At 7 dai, *P. indica*-colonized roots of both lines showed a significant increase of dark blue tips and a significant reduction in pale blue tips compared to mock-treated roots. GUS staining did not colocalize with colonization sites of *P. indica* or extracellular fungal growth. The data base on at least two biological experiments. Asterisks indicate significant differences between control and *P. indica*-colonized roots according to Students *t*-test (* $p < 0.05$, ** $p < 0.005$).

Fig. 6. Colonization of ethylene synthesis and signaling mutants by *P. indica*.

Three-weeks-old plants were inoculated with *P. indica* and fungal biomass in *ein2-1, etr1-3, eto1-1, ctr1-1,* and *35S*::*ERF1* was determined by qRT-PCR at 3 and 14 dai. The values were related to Col-0 (set to one). While *ein2-1* showed reduced colonization at 3 dai, significantly more fungal biomass was detected in *ctr1-3, eto1-1* and 35S::*ERF1* plants at 14 dai. Colonization of *etr1-3* was unaltered over all timepoints. The data base on at least three independent experiments. Students *t*-test indicated significant difference in *P. indica*-colonization (* $p < 0.05$, ** $p < 0.005$).

Figure 1

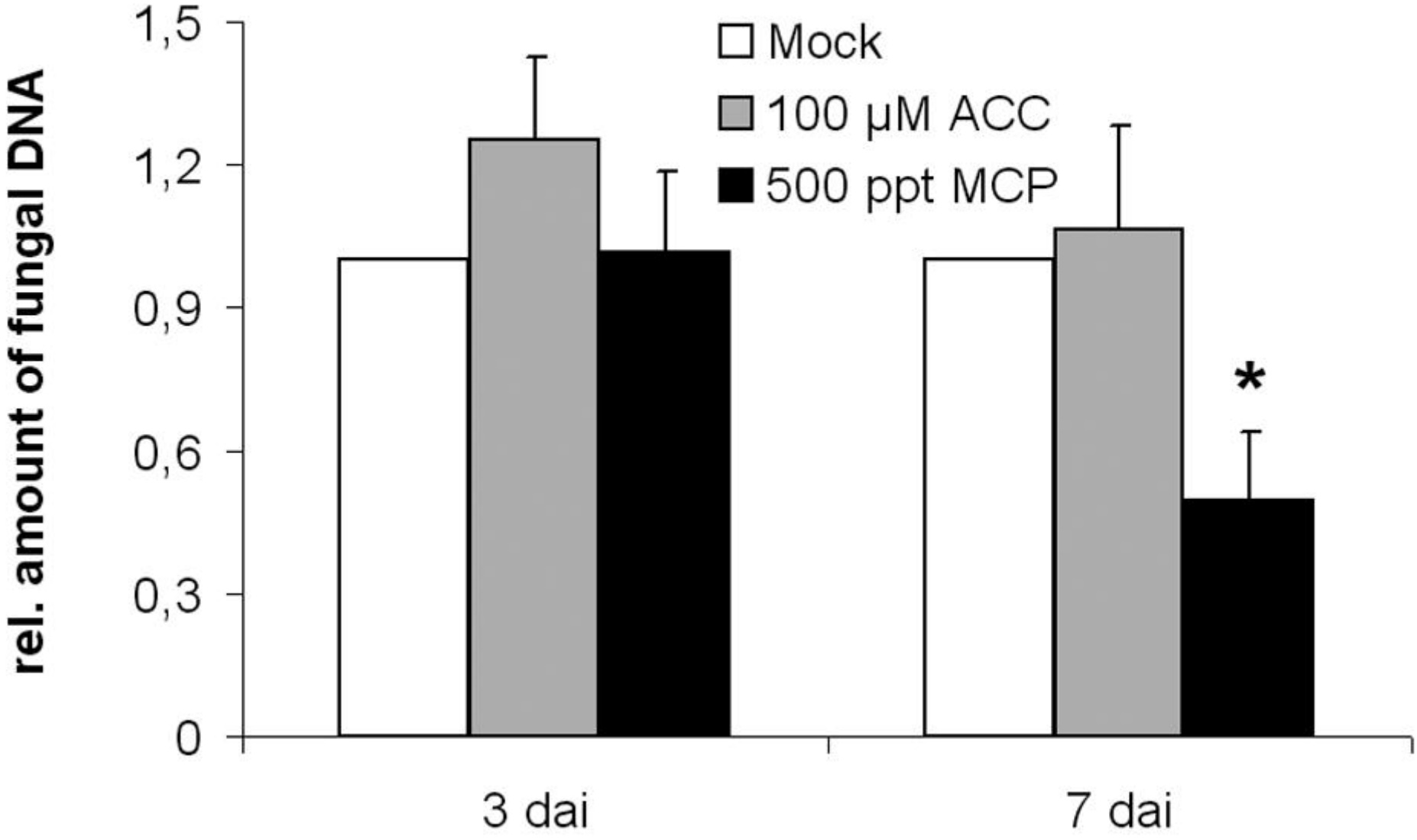

rel. amount of fungal DNA
1,5
1,2
0,9
0,6
0,3
0
Mock
100 µM ACC
500 ppt MCP
*
3 dai
7 dai

Figure 2

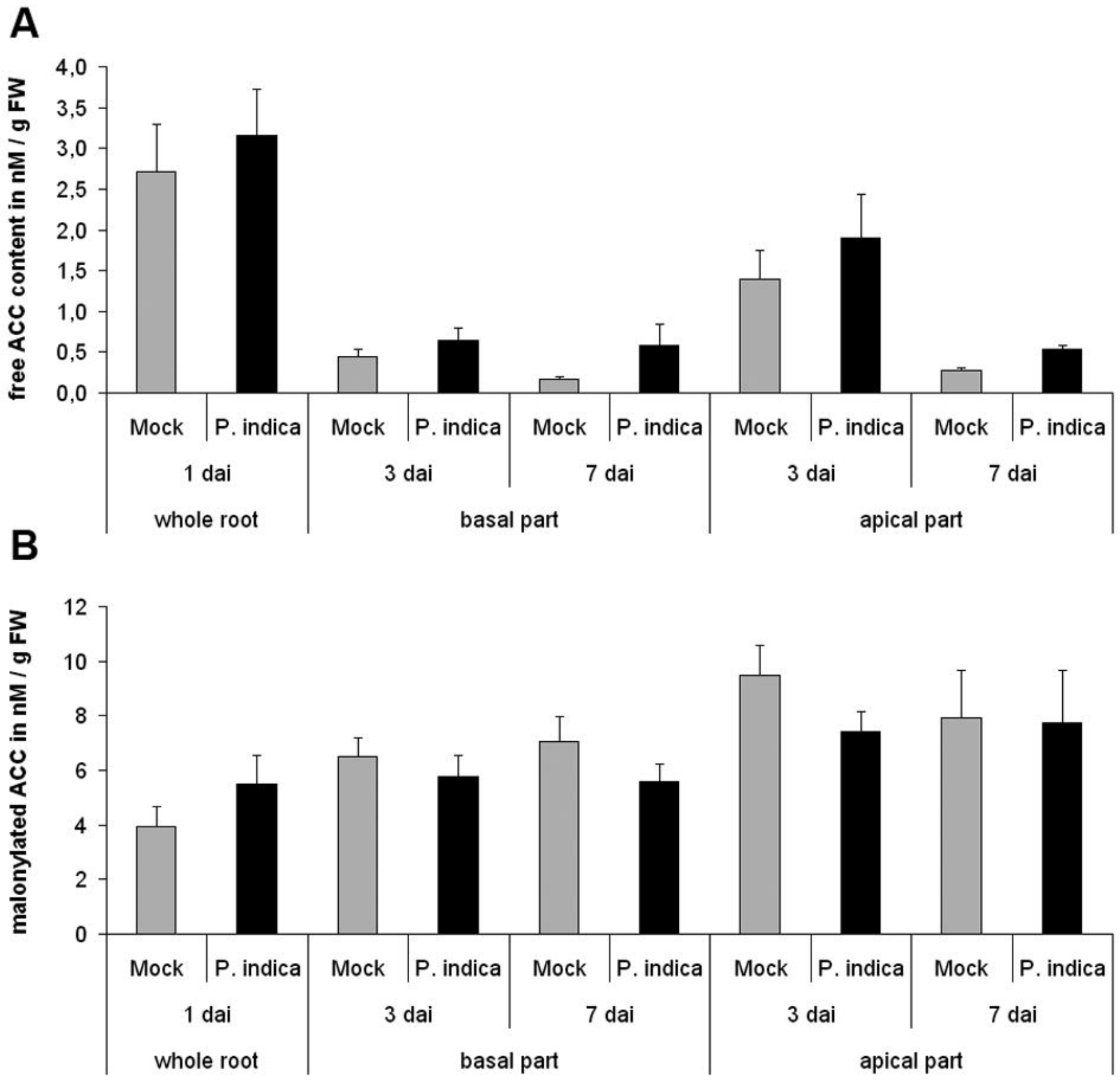

A
free ACC content in nM / g FW
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Mock
P. indica
1 dai
whole root
3 dai
7 dai
basal part
apical part
B
malonylated ACC in nM / g FW
12
10
8
6
4
2
0

Figure 3

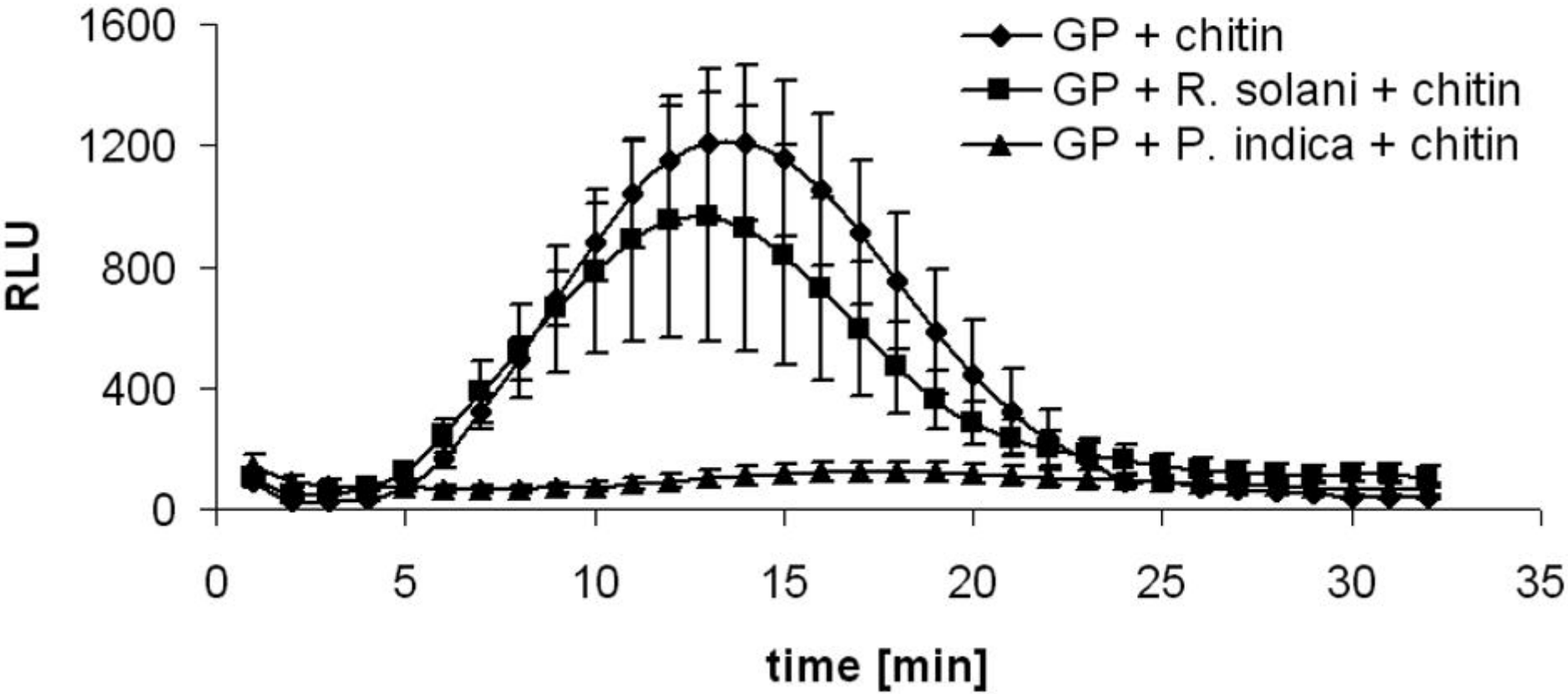

1600
1200
800
400
0
RLU
0
5
10
15
20
25
30
35
time [min]
GP + chitin
GP + R. solani + chitin
GP + P. indica + chitin

Figure 4

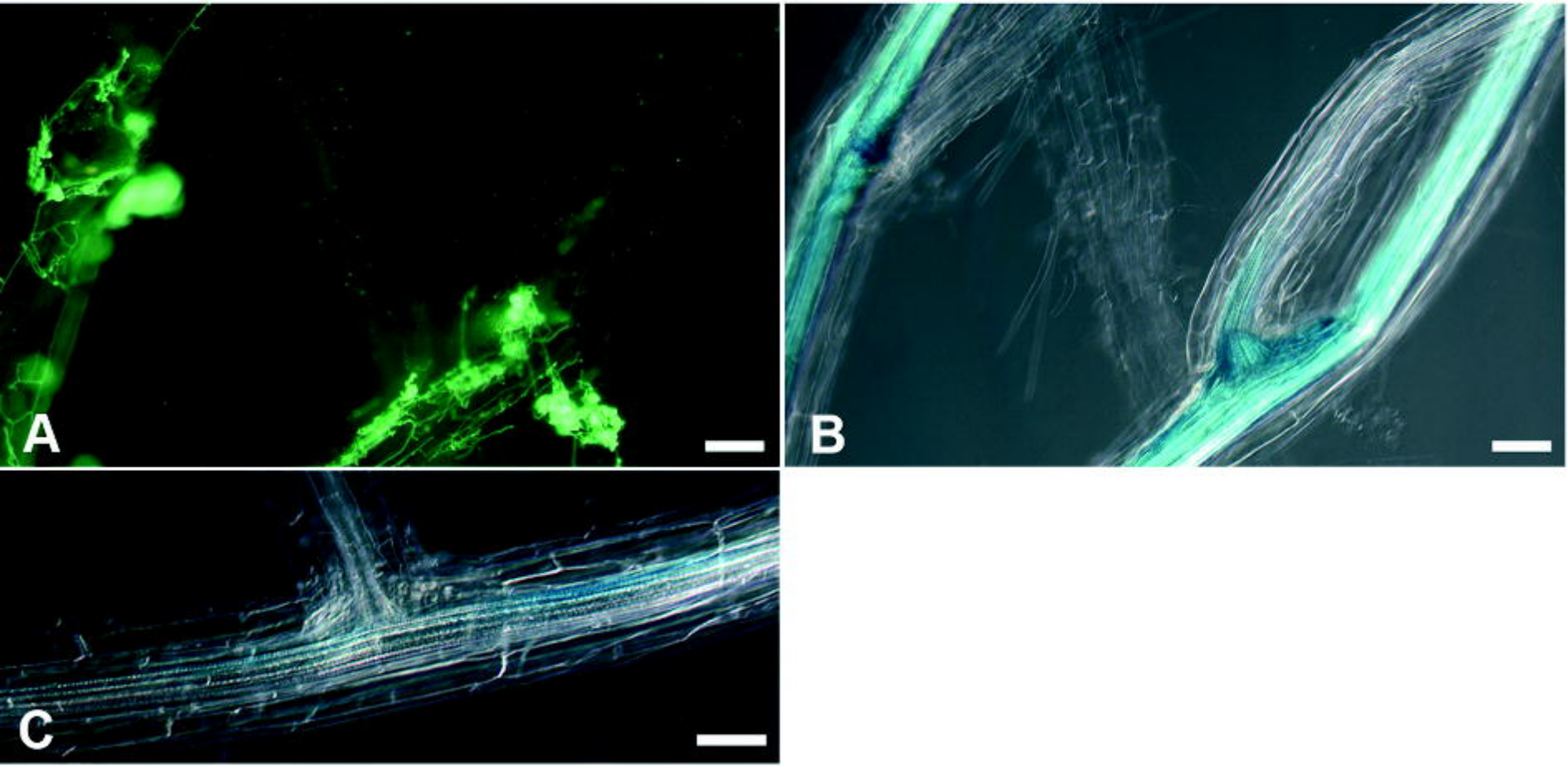
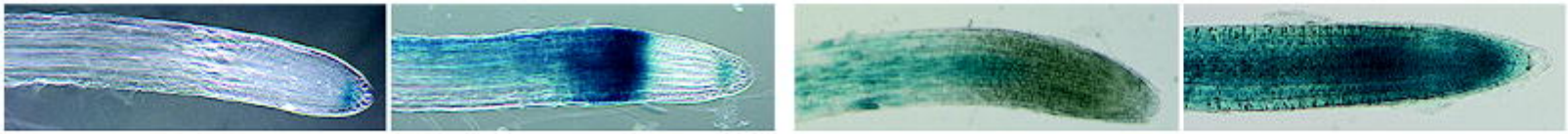


Figure 5



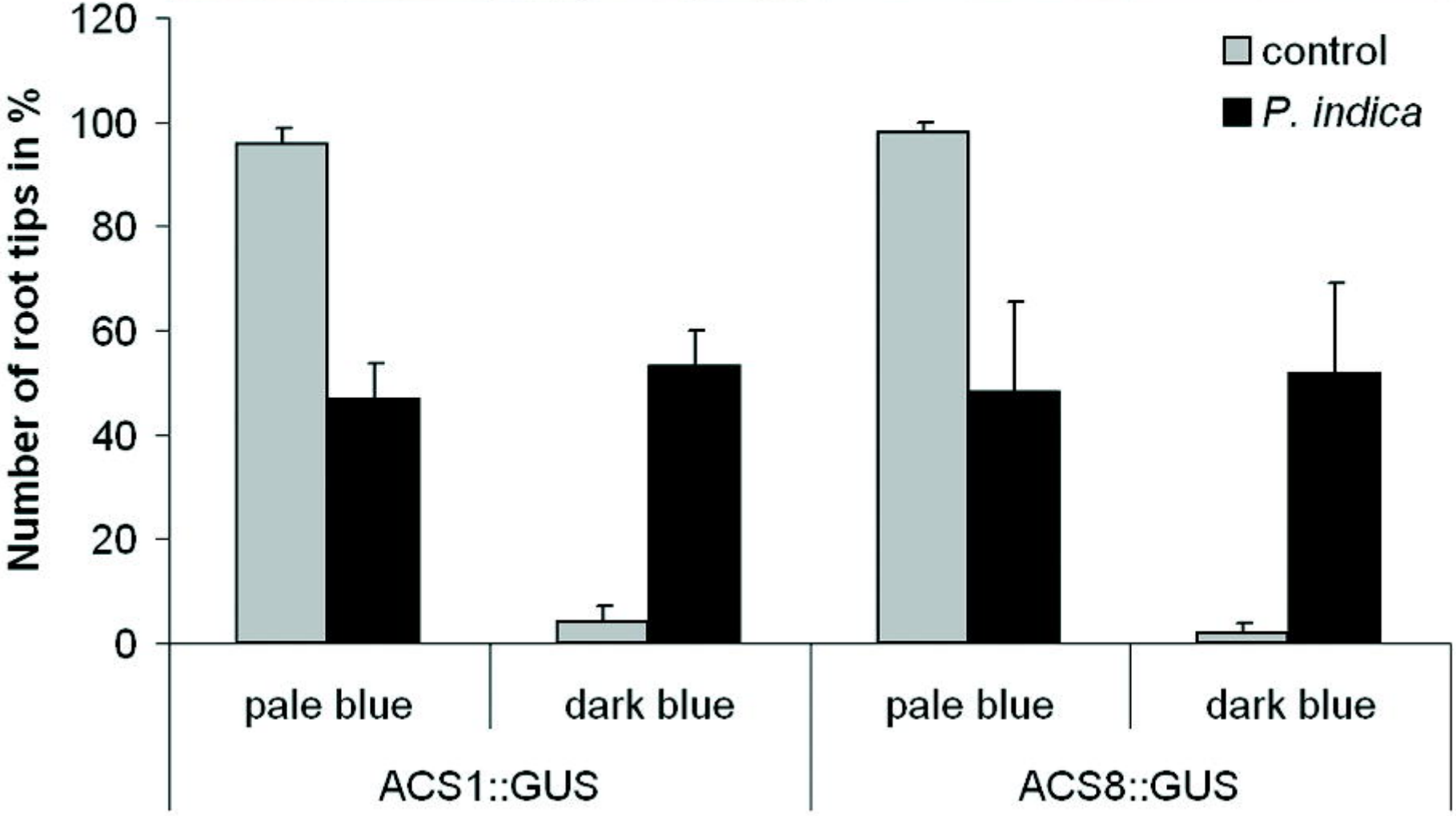
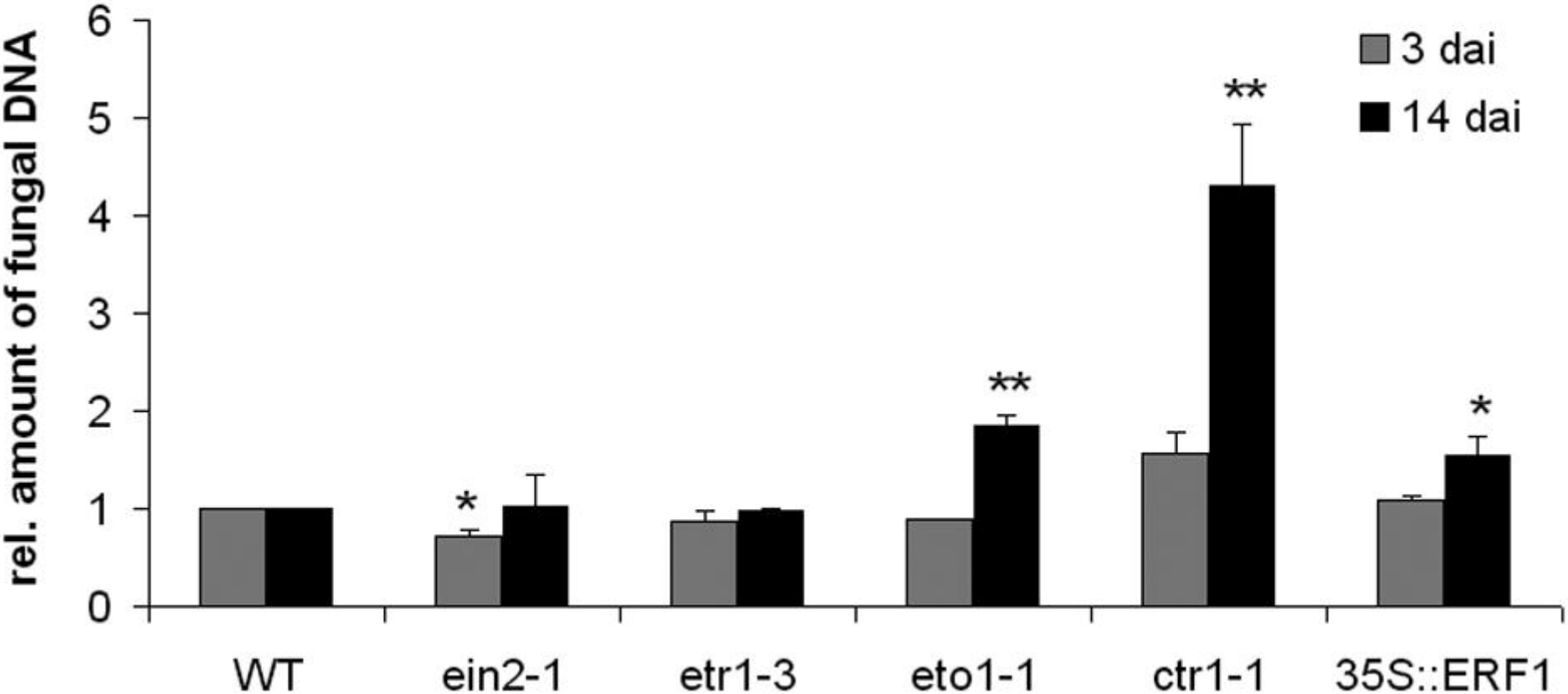

Number of root tips in %
120
100
80
60
40
20
0
control
P. indica
pale blue
dark blue
pale blue
dark blue
ACS1::GUS
ACS8::GUS

Figure 6


rel. amount of fungal DNA
6
5
4
3
2
1
0
3 dai
14 dai
*
**
**
*
WT
ein2-1
etr1-3
eto1-1
ctr1-1
35S::ERF1

5.3 **Schäfer, P.**, Pfiffi, S., Voll, L.M., Zajic, D., Chandler, P.M., Waller, F., Scholz, U., Pons-Kühnemann, J., Sonnewald, S., Sonnewald, U., Kogel, K.H. (2009) Phytohormones in plant root-*Piriformospora indica* mutualism. *Plant Signaling & Behavior* 4: 669-671.

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Article Addendum

Phytohormones in plant root-*Piriformospora indica* mutualism

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Key words: compatibility, plant defense, gibberellic acid, symbiosis, plant hormones

Piriformospora indica **is a mutualistic root-colonising basidiomycete that tranfers various benefits to colonized host plants including growth promotion, yield increases as well as abiotic and biotic stress tolerance. The fungus is characterized by a broad host spectrum encompassing various monocots and dicots.[1,2] Our recent microarray-based studies indicate a general plant defense suppression by *P. indica* and significant changes in the GA biosynthesis pathway.[3] Furthermore, barley plants impaired in GA synthesis and perception showed a significant reduction in mutualistic colonization, which was associated with an elevated expression of defense-related genes. Here, we discuss the importance of plant hormones for compatibility in plant root-*P. indica* associations. Our data might provide a first explanation for the colonization success of the fungus in a wide range of higher plants.**

Introduction

As sessile organisms plants have to cope with their environment and have developed efficient strategies to face harmful abiotic or biotic challenges. The underlying molecular network is extremely complex due to the multitude of perception and signaling systems combined with a multilateral crosstalk. Phytohormones are embedded in these signaling events and are well known integrators of stress responses as observed by their challenge-responsive synthesis and signaling.[4-8] Salicylic acid (SA), jasmonate (JA) and ethylene are the best characterized phytohormones in terms of averting invasions by plant pathogens. In a simplified model, SA is seen as a resistance mediator against biotrophic organisms, while JA and ethylene are involved in effective defense responses against necrotrophic pathogens.[9,10] Other studies could also decipher the significance of abscisic acid (ABA) and gibberellins (GAs) for the outcome of plant-microbe interactions by modulating defense responses.[4,11-14] Plant hormones are known to have antagonistic acitivity in plant developmental processes and this antagonism is also observed in plant defense responses (e.g., SA-JA-, GA-ABA antagonism).[5,8,15,16] In contrast, other hromones (e.g., JA and ethylene) act mostly synergistically in defense processes.[17,18] Hence, it is not further astonishing that microbes have evolved sophisticated strategies to efficiently establish compatible interactions by synthesising and mimicking phytohormones, or directly manipulating hormone signaling.[4,5,19,20]

The mutualistic root colonising fungus *Piriformospora indica* has been characterized as a exceptionally efficient organism as indicated by its ability to colonize a broad variety of monocot and dicot plant species.[1,2] Interestingly, a nonhost has not been identified. Related to our recent studies, we discuss GA and other hormones as significant components for the colonization success of this mutualist.[3]

Hormone Synthesis and Signaling during Mutualistic Root Colonization

The molecular and biochemical events activated in plants in response to *P. indica* colonisation are mostly unknown. First cytological studies draw a more complex picture on these mutualistic interactions as was initially believed. In Arabidopsis, the fungus was shown to colonize root cells by an initial biotrophic phase followed by a later cell death-dependent colonization phase (Schäfer P and Zechmann, unpublished data).[1,21] At the biotrophic phase the plasma membranes of colonized cells is invaginated and the cell is alive. A similar infection strategy is expected for barley roots. Hence, the fungus is not simply colonising dead root cells or killing cells prior to or during penetration. Provided that the fungus is certainly recognized by plasma membrane localized pattern recognition receptors, which perceive microbe-derived molecules (e.g., chitin), the fungus should activate innate immune signaling. Interestingly, our microarray studies revealed defense

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suppression by *P. indica* during barley root colonization. As this corrumption obviously supports fungal establishment, it remains to be determined by which means the fungus suppresses plant defense.

In recent studies, ABA and GA were shown to affect Arabidopsis colonization by fungal and bacterial pathogens. ABA was shown to suppress basal defense in Arabidopsis thereby facilitating leaf colonization by *Pseudomonas syringae* pv. *tomato*.[13] In addition, Arabidopsis mutants blocked in GA signaling showed enhanced susceptibility against *Pseudomonas syringae* DC3000 and enhanced resistance against two necrotrophic pathogens (*Alternaria brassicicola*, *Botrytis cinerea*).[4] In contrast, quadruple DELLA mutants with a constitutive GA signaling phenotype were more resistant against the bacterium but highly susceptible against both necrotrophs. Interestingly, the quadruple mutant displayed enhanced levels of free SA after *P. syringae* DC3000 attack, which was also reflected by elevated *PR1* and *PR2* transcripts, while the JA/ethylene-responsive *PDF1.2* exhibited a delayed expression.[4] This indicates a direct connection of GA signaling with SA and JA responses.

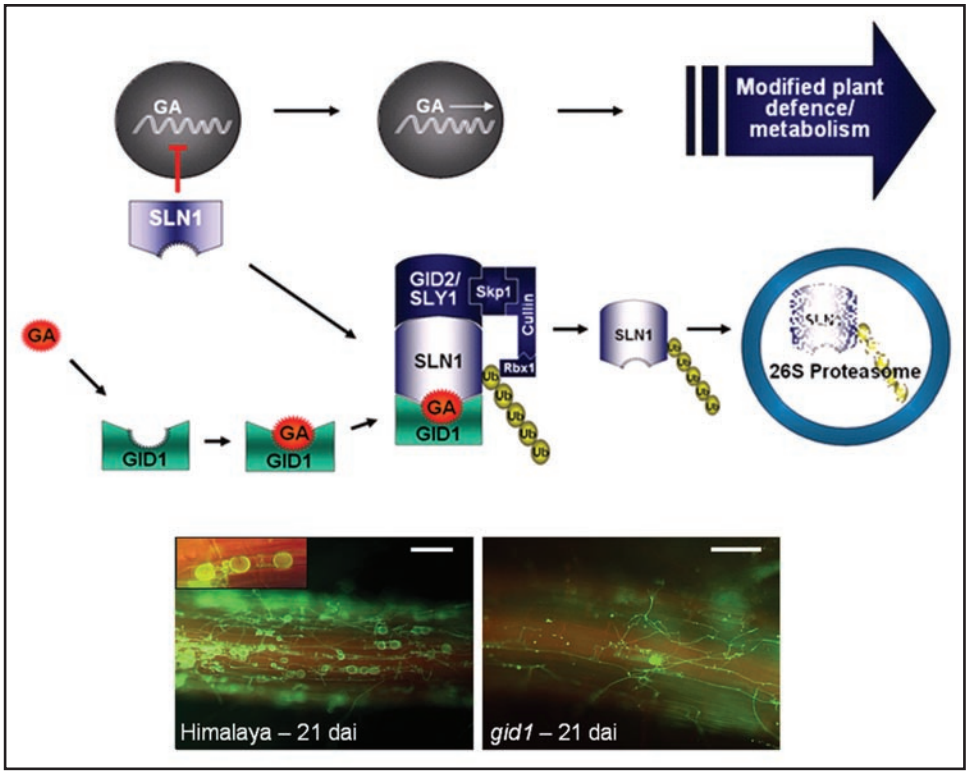

Figure 1. Impaired GA perception reduces barley root colonisation by *P. indica*. GA-responsive gene transcription is inhibited by SLENDER1 (SLN1) in the absence of GA (upper). After GA synthesis, GA gets attached to the GA receptor GID1. Thereafter, SLN1 binds to GID, which results in SLN1 ubiquitination and its proteasomal degradation. By removing SLN1, GA-responsive transcription is initiated that is thought to modify plant defense signaling and metabolism (upper). In the barley mutant *gid1*, GA-responsive transcritption is inhibited and *P. indica* root colonisation is markedly reduced at 21 dai (lower right) compared to parent line Himalaya (lower left). Intracellular sporulation was almost absent in *gid1* (inset, lower right). The fungus was stained with wheat germ agglutinin-Alexa Fluor 488 (WGA-AF 488) and visualized by fluorescence microscopy (Bars = 20 µm).

Barley root colonization was also accompanied by changes in plant hormone metabolism. During extracellular fungal development (1 day after inoculation, dai) the expression of ABA-responsive genes was induced. This, however, changed at penetration/early colonization (3 dai) and progressed colonization stages (7 dai). At these time points GA synthesis was observed to be obviously elevated as indicated by the induction of almost all genes of the non-mevalonate pathway and two putative kaurene synthases. In contrast, SA and JA-related defense genes only showed a weak and transient induction pattern or were even suppressed.[3] It is tempting to speculate that ABA might be recruited by *P. indica* to suppress defense at pre-penetration stages while GA is taking over this job at subsequent interaction stages (3, 7 dai). However, our studies showed the GA-dependence of barley root colonisation by *P. indica* as a GA synthesis mutant and the GA receptor mutant *gid1* were significantly less colonized by the mutualist. Moreover, *PR* gene expression was significantly elevated by *P. indica* in both mutants compared to wild type roots at 3 dai.[3] Subsequent, cytological studies showed a substantial reduction extracellular fungal growth and of extra- und intracellular sporulation in *gid1* compared to wild type Himalaya (Fig. 1). In *gid1*, fungal colonization might be stopped at the penetration stage. The resulting restriction in nutrient acquisition might explain its impaired extracelullar development and sporulation.

In summary, plant hormone signaling is obviously recruited by *P. indica* in order to manipulate plant defense and most probably plant metabolism. Plant hormones might further be a key to explain the broad host spectrum of *P. indica*. Current studies are directed to decipher the phytohormonal state and signaling during plant colonization by *P. indica*.

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Title

Broad Spectrum Suppression of Innate Immunity Is Required for Colonization of *Arabidopsis thaliana* Roots by the Fungus *Piriformospora indica*

Authors

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Abstract

Piriformospora indica is a root colonizing basidiomycete that confers a wide range of beneficial traits to its host. This mutualistic fungus was discovered in the Indian Thar desert. Despite or maybe due to its natural habitat, the fungus evolved an extraordinary capacity in the colonization of diverse plants. Its broad host spectrum encompasses gymnosperms, mono- as well as dicotyledonous angiosperms, which implies an efficient interference with the host immunity. Moreover, our studies showed a biotrophic colonization of *Arabidopsis thaliana* roots by *P. indica*, a colonization strategy that has not yet been reported for fungi in roots of Arabidopsis. This biotrophic stage is transient and followed by a cell death-associated colonization phase. Although our molecular and genetic data clearly indicate that *P. indica* is confronted with a functional root innate immune system, defense responses are only marginally induced during colonization. Our studies exclude an evasion of defense activation by the fungus. Instead, *P. indica* exhibits an efficient and broad-spectrum suppression of root immunity triggered by fungal or bacterial microbe-associated molecular patterns (MAMPs). This extraordinary efficiency in the suppression of root immunity is mandatory for mutualistic colonization and could explain *P. indica*'s capability to infect a so far unlimited range of plant hosts. Our studies clearly indicate that mutualists like pathogens are confronted with an effective innate immune system and that colonization success essentially depends on the evolution of immune-suppressive strategies.

Introduction

The success of microbes to invade plants reflects their ability to manipulate immune responses and reprogram host metabolism (O'Connell et al., 2006).These activities are antagonized in plants by a two layered immune system. The first induced defense instance relies on the recognition of conserved microbial structures, so called microbe-associated molecular patterns (MAMPs), and is defined as MAMP-triggered immunity (MTI). Microbes subverting MTI are confronted with the second, more costly but highly efficient effector-triggered immunity (ETI). ETI is activated after recognition of virulence factors, called effectors, or the products of their activity, by intracellular receptors (Jones and Dangl, 2006).

Bacterial flagellin, elongation factor-TU (EF-TU) or fungal chitin are well-defined MAMPs that are recognized by the pattern recognition receptors (PRRs) FLS2, EFR, or CERK1, respectively, leading to the activation of MTI (Gomez-Gomez et al., 1999; Kunze et al., 2004; Zipfel et al., 2004; Miya et al., 2007). MAMP perception leads to the activation of mitogen-activated protein kinase (MAPK) pathways and subsequent defense gene expression regulated by transcription factors (e.g. WRKYs). MTI also relies on the accumulation of antimicrobial glucosinolates and camalexin (Bednarek et al., 2009; Clay et al., 2009). 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) that is thought to be generated by plasma membrane localized NADPH oxidase RBOHD (Zhang et al., 2007). MTI is further substantiated by the action of phytohormones like salicylic acid (SA), ethylene (ET), and jasmonate (JA) (Tsuda et al., 2009).

MTI warrants a basic protection as it determines the nonhost status of plants towards most pathogens (He et al., 2006) and restricts disease progression in compatible interactions (Zipfel et al., 2004). Models of innate immunity are based almost exclusively on analyses of interactions with foliar pathogens, and it is currently unknow to what extent MTI is tissue-/organ-specific or nonspecific. Compared to other plant organs, roots are confronted with the highest diversity of microbes. Plant health and development are intimately associated with proper nutrient and water acquisition by roots. In crop production, root diseases are of global concern due to a lack of resistant germplasms and their limited accessibility for chemical protection. Not until recently the presence of a sensitive MAMP-triggered immune system was

described in *Arabidopsis thaliana* roots (Millet et al., 2010). However, organization and protective properties of root innate immunity are almost unknown. Furthermore, we do not know if root surveillance systems discriminate between mutualists and pathogens or if colonization success rather reflects the efficiencies of microbial strategies to deactivate innate immunity.

The root-colonizing basidiomycete *Piriformospora indica* represents a useful model to study principles of root-symbiont interactions in terms of mutualism and root colonization. As mutualist, it confers beneficial traits such as increased yield, abiotic stress tolerance, and disease resistance to plants (Varma et al., 1999; Waller et al., 2005). Similar to mycorrhizal interactions, fungal phosphate supply to roots is imperative for growth promotion (Yadav et al., 2010). *P. indica* is an efficient root colonizer as indicated by its exceptionally broad host range, and a nonhost plant has not yet been discovered. This suggests an enormous capacity to evade or suppress plant innate immune responses.

In this work we aimed at elucidating the colonization strategy of *P. indica* and at identifying crucial components of root MTI by taking advantage of its ability to colonize Arabidopsis. By employing transmission electron- and epifluorescence microscopy along with reporter and mutant plants, we describe for the first time a biotrophic colonization of Arabidopsis roots by a fungus. This interaction stage is followed by a cell death-associated colonization phase that needs to be distinguished from necrotrophy. Our genetic and molecular analyses demonstrate the efficiency of the root innate immune system to halt microbial colonization and indicate the significance of root MTI suppression rather than evasion by *P. indica* as prerequisite for successful colonization. Furthermore, our data reveal similarities between leaf and root immunity and that mutualistic like pathogenic colonization success is intimately dependent on efficient immune suppression strategies.

Results

The biphasic lifestyle of *P. indica*: biotrophy followed by cell death. Microscopic studies revealed the following spatio-temporal chronology of colonization of Arabidopsis roots by *P. indica* (Fig.1). Root colonization started after spore germination with inter- and intracellular penetration of rhizodermal and cortical tissue at 2 days after inoculation (dai). At 3 dai, intracellular colonization of rhizodermal-, cortical-, and root hair cells was prominent (Fig. 1A, B). Fungal hyphae consequently branched (Fig. 1A) and occasionally formed whorls (Fig. 1B) as reported for orchid mycorrhiza (Peterson and Massicotte, 2004; Schäfer and Kogel, 2009). Finally, external and intracellular sporulation started in the maturation zones (MZ) I and II at 7 and 14 dai, respectively (Fig. 1C). Growth of *P. indica* was restricted to rhizodermal and cortical cells, and colonization increased with tissue age and reached its highest level in MZ II. In contrast, meristematic and elongation zones remained free of hyphae except for occasional infection of an epidermal cell.

Transmission electron microscopy revealed that the intracellular colonization is initiated by plasma membrane invagination (Fig. 2A). The well preserved ultrastructure of the cytosol, plasma membranes, and all organelles at early colonization stages (3 dai) demonstrated that colonized cells were alive (Fig. 2A, B). Biotrophic colonization by *P. indica* was substantiated with Arabidopsis line GFP-Chi, in which ER and ER bodies are tagged with the green fluorescent protein (Flückiger et al., 2003). The integrity of ER and nucleus were confirmed in living root cells in MZ II by confocal microscopy (3 dai) (Fig. 3A-C, Video S1A, B). Evidence for cell integrity was also provided by failed staining of intracellular hyphae (Fig. 3B) with chitin-specific dyes WGA-AF488 or -633 in contrast to extracellular hyphae (Fig. 3A, C) and intracellular hyphae in dead cells lacking an intact plasmalemma (Fig. S2). Cell death as indicated by the absence of ER and nucleus (Fig. S2) was frequently observed in MZ II of GFP-Chi plants at later interaction stages (>3 dai). Adjacent non-colonized rhizodermal cells displayed intact nuclei and ER (Fig. S2). The subcellular sequence of cell death started with the disintegration of cytoplasm and the endomembrane system (Fig. S3A). The plasma membrane, while still present, showed inversions (Fig. S3B) reminiscent of membrane blebbing (*in sensu* Mittler et al., 1997). Adjacent non-colonized cells were intact as demonstrated by well preserved cytoplasm (Fig. S3C). We did not find tissue necrosis or browning in colonized root areas (not shown) and cell death was not accompanied by whole cell autofluorescence.

***P. indica* suppresses a conserved set of tissue-nonspecific MTI responses.** In leaves, invasion by biotrophic fungi is controlled by locally confined cell wall appositions (CWAs) and single cell hypersensitive response (HR) (Lipka et al., 2005; Hückelhoven, 2007). In clear contrast, we rarely detected CWAs or HR-like responses during biotrophic root colonization of *P. indica* (Fig. S4). This observation prompted us to test whether *P. indica* evades or suppresses basal immune responses. In leaves, the active epitopes of bacterial flagellin (flg22), of elongation factor-TU (elf18), or the octamer of fungal chitin (*N*-acetylchitooctaose, Glc8) trigger the oxidative burst and induce defence gene transcription (Gomez-Gomez et al., 1999; Kunze et al., 2004; Zipfel et al., 2004; Miya et al., 2007). In addition, plantlets treated with flg22 or elf18 exhibit growth inhibition. Accordingly, a series of experiments were conducted, in which *P. indica*-colonized roots were treated 3 dai (biotrophic stage) with flg22. *P. indica* abolished the flg22-mediated growth inhibition as indicated by unimpaired seedling root length and fresh weight as compared to non-colonized controls (Fig. 4A). As reported for leaves, we detected a transient oxidative burst in non-colonized roots upon treatment with flg22. By contrast, *P. indica*-colonized roots were almost non-responsive to flg22 (Fig. 4B). To test the range of the suppression we also tested the sensitivity of colonized roots to Glc8 and elf18. *P. indica* was also able to abolish Glc8-induced oxidative burst (Fig. 4C) as well as the elf18-induced seedling growth inhibition and oxidative burst (Fig. S5A, B). Next, we tested whether suppression of MAMP-triggered responses was also evident on the basis of defense marker gene expression. To this end, at 3 dai with *P. indica*, roots were treated with flg22, harvested at 2, 24, and 72 hours after treatment (hat), and analyzed for transcriptional activation of marker genes by quantitative real time PCR (qRT-PCR). Consistent with earlier results, marker genes for MTI (*WRKY22*, *WRKY29*, *WRKY33*, *WRKY53*) (Colcombet and Hirt, 2008), oxidative stress (*OXI1*, *RBOHD*, *RBOHF*), (Rentel et al., 2004; Torres et al., 2005) salicylic acid (SA) (*CBP60g*, *SID2*) (Wang et al., 2009), and jasmonic acid (JA) (*PDF1.2*), were induced either at 2, or at 2 and 24 hat in non-colonized roots (Fig. 4D, S6). In addition, MYB51, which participates in the biosynthesis of antimicrobial indole glucosinolates (Clay et al., 2009), was induced. In marked contrast, the transcriptional induction of all tested genes by flg22 was suppressed by *P. indica* colonization. Interestingly, although *PDF1.2* was upregulated

in colonized roots at all time points, the induction level was clearly reduced in *P. indica*-colonized roots treated with flg22.

MAMPs of mutualistic microbes can activate a highly effective root MTI. Our results revealed that *P. indica* efficiently suppresses MAMP-triggered responses. We subsequently wanted to elucidate the significance of root MTI in the mutualistic symbiosis. Two-weeks-old seedlings were flg22- or mock-treated at 1 day prior to inoculation. Fungal growth was quantified at biotrophic (3 dai) and at cell death-associated colonization stages (7 dai). flg22 treatment led to reduced colonization at both time points (Fig. 5A). To underline the role of MTI in *P. indica* colonization, we employed two mutants with altered MTI responses. The plant U-box type E3 ubiquitin ligase (PUB) triple mutant *pub22/23/24* does not exhibit a constitutive defense response but MTI is hyperactivated after MAMP application (Trujillo et al., 2008). Colonization of *pub22/23/24* was significantly reduced in comparison to Col-8 (Fig. 5B). By contrast, the *cerk1-2* (*chitin elicitor receptor kinase 1-2*) mutant, which is impaired in perception of chitin and a yet unidentified danger signal associated with bacterial attack (Gimenez-Ibanez et al., 2009) allowed higher colonization (Fig. 5B). The reduced colonization of *pub22/23/24* could be caused by reduced suppression of MTI by *P. indica*. Therefore, we determined the oxidative burst in the *pub22/23/24* mutant. In contrast to the parent line Col-8, the flg22-triggered oxidative burst increased in colonized *pub22/23/24* roots (Fig. 5C, S7). We also analyzed the expression of the same set of stress genes in *pub22/23/24* and Col-8 roots at 1, 3, and 7 days after *P. indica*- or mock-inoculation. As expected, MTI markers were not induced in non-colonized roots of *pub22/23/24*. While *WRKY29*, *WRKY33*, *CBP60g* and *MYB51* were moderately upregulated at 7 dai in Col-8, all tested genes displayed a stronger induction in *pub22/23/24* at 1 or at 1 and 7 dai (Fig. 5D, S8). Interestingly, in Col-8, opposite to the increased expression of the SA marker *CBP60g*, induction of JA marker *PDF1.2* gradually decreased. Similarly, *CBP60g* and *SID2* induction in *pub22/23/24* was associated with the suppression of *PDF1.2*.

Hormones balance root colonization by *P. indica*. The induction of SA-, JA-, and glucosinolate marker genes by *P. indica* in Col-8, prompted us to test their significance for symbiosis. Fungal biomass was quantified in SA signaling mutants *npr1-1* (*nonexpressor of PR1*), *eds1* (*enhanced disease susceptibility 1*), in SA

synthesis mutant *sid2-2* (*salicylic acid-inducible defective 2-2*), as well as in JA signaling mutants *jin1-1* (*jasmonate insensitive 1-1*), *coi1-16* (*coronatine insensitive 1-16*), and in JA synthesis mutant *jar1-1* (*jasmonate resistant 1-1*). Except for *npr1-1*, all SA mutants showed higher colonization at 3 and 7 dai (Fig. S9). By contrast, root colonization was reduced in *jar1-1* and *jin1-1* at 7 dai (Fig. S9). *coi1-16* colonization was comparable to wild type. *coi1-16* bears a second mutation in *PEN2* (Westphal et al., 2008), which might compensate the expected colonization decrease in *coi1-16*. Consistently, *pen2-1* displayed a higher colonization at 7 dai (Fig. S9). Finally, we examined *pad3* (*phytoalexin deficient3*), which shows a marked reduction in the synthesis of antimicrobial camalexin (Glazebrook and Ausubel, 1994). *pad3* colonization was similar to that of wild type.

Discussion

Based on the presented data, we propose a root colonization model consisting of four consecutive colonization stages (Fig. 6): (i) Extracellular colonization of the root surface by *P. indica* (~1 dai). (ii) Biotrophic colonization phase (< 3dai), at which hyphae colonize living rhizodermal and cortical cells (Fig. 2, 3, Video S1A, B). After penetration, hyphae grow into cells but remain extracytosolic by invaginating the plasma membrane. (iii) Cell death-associated colonization phase (>3 dai) and (iv) fungal reproduction by extracellular (~7 dai) and intracellular sporulation (~14 dai). Biotrophy is underlined by the lack of ultrastructural changes (e.g. lysis of the cytosol or ruptured tonoplast), which start to occur at the cell death-associated colonization stage (Fig. S2, S3). The biotrophic cell colonization demonstrates that fungal development is neither dependent on dead cells nor is cell death a prerequisite for penetration. Importantly, biotrophic colonization did not coincide with any defense responses. Several indications contradict an immunity-related role of cell death associated with colonization: (i) the absence of whole cell autofluorescence or browning caused by the accumulation of phenolic and antimicrobial compounds as reported for HR cells (Heath, 2000) and as rarely observed in young root tissue (Fig. S4G, H), (ii) the absence of HR hallmarks such as mitochondria swelling, elevated vesicle formation, vacuolization of the cytoplasm, and protoplast shrinkage (Heath, 2000; Mur et al., 2008), (iii) the non-deleterious impact of cell death on *P. indica* colonization as reported for barley (Deshmukh et al., 2006) and indicated by transcellular fungal growth (Fig. S3). Together, these findings implicate a profound adaptation of *P. indica* to Arabidopsis roots.

The intracellular colonization of living cells provokes an extended exposure of fungal structures to plasma membrane-localized pattern recognition receptors (PRRs) and the subsequent activation of immune responses. The ubiquitous expression of PRRs such as FLS2 throughout different tissues (Robatzek et al., 2006), indicates that signaling processes are conserved in leaves and roots. First evidences for this were shown by Millet and colleagues (Millet et al., 2010), and more characteristics of root MTI are presented here (Fig. 4, S6). The activation of MAMP-triggered responses in wild type roots by flg22 (Fig. 4) or in *pub22/23/24* roots by *P. indica* (Fig. 5) implicates a conserved immune system in leaves and roots. *P. indica* effectively counteracts immune signaling as seen by the total abolishment of MAMP-triggered seedling growth inhibition (Fig. 4A, S5A) as well as by the suppression of the MAMP-induced

oxidative burst (Fig. 4B, C, S5B) and induction of gene expression (Fig. 4D, S6). The suppression of *WRKY22*, *WRKY29*, *WRKY33*, and *WRKY53* transcription in *P. indica*-colonized roots by flg22 implies that the fungus impairs a broad set of MAPK-mediated signaling pathways and thus, the backbone of MTI (Colcombet and Hirt, 2008). MTI suppression by *P. indica* requires further temporal and spatial elucidation – temporal because *P. indica* suppresses both early (oxidative burst) and later immune responses (gene induction), and spatial because these responses are activated at distinct subcellular locations. More significantly, *P. indica* nullifies immune responses usually triggered by diverse bacterial (flg22, elf18) and fungal MAMPs (chitin) (Fig. 4, S5). It was demonstrated that these MAMPs activate a similar set of MAPKs and induce an overlapping gene set (Wan et al., 2008). Therefore, *P. indica* must target a component or process, which is upstream of the immune signaling cascade(s) and is recruited by different perception systems. A straightforward explanation would be the deactivation of a major hub that integrates multiple PRR signals. Alternatively though, *P. indica* might secrete a plethora of effectors, which are functional in distinct and evolutionary distantly related hosts, to silence multiple key components required by different MAMP-triggered responses. The reduced colonization and enhanced MTI activation in *pub22/23/24* excludes an impairment of PRR function by *P. indica*. The ubiquitin ligases PUB22, PUB23, and PUB24 negatively regulate leaf MTI. These U-Box proteins are thought to control PRR-derived signaling upstream of the MAPK cascade. By targeting the activity of the ligases or immediate downstream signaling components, *P. indica* would be able to impair defense signaling. Irrespective of the existence of a major or several immune signaling integrator/s, these targets should be conserved in *P. indica* hosts. Supporting this view, we observed suppression of defense gene expression and chitin-induced oxidative burst by *P. indica* in barley roots (Schäfer et al., 2009, unpublished data).

Few studies have described the existence of root MTI (Attard et al., 2010; Millet et al., 2010). However, the present study demonstrates its involvement in halting microbial root colonization as evidenced by the reduced colonization of flg22 pre-treated roots and *pub22/23/24* by *P. indica* as well as the enhanced colonization of *cerk1-2* (Fig. 5A, B). The reduced colonization of flg22-treated WT plants and failed inhibition of MTI in *pub22/23/24* suggest that *P. indica* follows a strategy of suppression rather than evasion of MTI. It further indicates that the perception of MAMPs released by the mutualistic symbiont triggers immunity similar to pathogen MAMPs. *pub22/23/24*

is an excellent tool to detect such responses as it does not show elevated MTI under normal growth conditions (Trujillo et al., 2008). Reduced *pub22/23/24* colonization correlated with an elevated oxidative burst, induction of MAPK-activated genes (*WRKY22*, *WRKY29*), and also genes participating in ROS metabolism (*RBOHD*, *OXII*). This might reflect elevated signaling activities of existing receptors or an elevated *de novo* synthesis of receptors as a consequence of *P. indica* colonization. Alternatively, it is tempting to speculate, that the PUB triplet is targeted by effector proteins, which mediate their stabilization in a similar manner as *P. infestans* AVR3a stabilizes the U-Box protein CMPG1, thereby suppressing host cell death during biotrophic colonization (Bos et al., 2010).

In leaves, SA, JA, camalexin, and glucosinolates substantiate MTI (Bednarek et al., 2009; Tsuda et al., 2009). This might also hold true for root MTI as suggested by the improved colonization of *eds1*, *sid2-2*, and *pen2*, respectively, by *P. indica* (Fig. S9). In turn, camalexin and JA, which stop necrotrophic pathogens (Thomma et al., 2009), are not effective root MTI components against *P. indica* as indicated by unaltered *pad3* and reduced colonization of *jar1-1* and *jin1-1*. In disagreement with our former results (Stein et al., 2008), *npr1-1* did not exhibit an altered colonization in five independent experiments. As our former system was prone to root injuries during soil detachment from Arabidopsis roots prior to *P. indica* inoculation, we introduced an agar-based screening system, thereby eliminating root injuries and enhancing screening sensitivity. Hence, SA-mediated defense against the fungus was NPR1-independent (Fig. S9) as has been reported for *dnd1*, *cpr1*, *cpr5*, *cpr6*, and *cpr30*, which exert a constitutive SA defense (Clarke et al., 2000; Genger et al., 2008; Gou et al., 2009). Given that *PDF1.2* expression decreases stepwise (JA marker), and that simultaneously *CBP60g* (SA marker) is upregulated in Col-8 (Fig. 5D), the fungus might recruit JA signaling to counterbalance SA defense. JA-SA antagonism is known from studies in *coi1* and *jin1*, where both mutants displayed an increased SA signaling after bacterial challenge (Kloek et al., 2001; Laurie-Berry et al., 2006). Hence, reduced colonization of *jar1-1* and *jin1-1* might reflect increased SA defense in these mutants triggered by *P. indica*. In accordance, *P. indica* strongly induced SA markers *CBP60g* and *SID2* in *pub22/23/24* roots, while *PDF1.2* expression was suppressed (Fig. 5D, S8).

In conclusion, based on our analyses, we propose a model (Fig. 6) in which root MAMP-triggered immunity efficiently restrict penetration and root colonization of the

mutualist *P. indica*. This immune barrier can be overcome by *P. indica* by the manipulation of MAMP-triggered responses. The gene expression data (Fig. 5D) and mutant analysis (Fig. S9) suggest that SA and glucosinolates represent MTI components that efficiently control root colonization. The elimination of root-based immunity is not only pivotal for the colonization success but might also explain *P. indica*'s broad host spectrum. Furthermore, the *pub22/23/24* mutant studies implicate that the mutualist *P. indica* releases MAMPs thereby activating immune responses as reported for pathogens. In future, it will be interesting to elucidate to which extent the root surveillance system discriminates between pathogens and mutualists. Based on our studies it is apparent that roots possess a perception system and immune repertoire similar to leaves.

Materials and Methods

Plant material and inoculation. Seeds of *Arabidopsis thaliana* ecotype Col-0, Col-8, Col-3gl1, mutants *jin1-1* (N517005), *jar1-1* (N8072), *pad3* (N3805), *npr1-1* (N3726), and A5 (N84735) were obtained from the Nottingham Arabidopsis Stock Center. *coi1-16* seeds were kindly provided by B. Hause, Halle, *eds1* by J. Parker, Cologne, GFP-Chi seeds by G. P. di Sansebastiano, Lecce, and *sld2-2* seeds by F. M. Ausubel, Boston. *cerk1-2*, *pen2-1*, and *pub22/23/24* were published earlier (Lipka et al., 2005; Trujillo et al., 2008; Gimenez-Ibanez et al., 2009). All plants were grown on ½ MS medium without sucrose at 22/18°C day/night cycle (8 hours light) at 60% rel. humidity. For fungal quantification and gene expression analysis, roots of three-weeks-old plants were inoculated with *P. indica* (500,000 spores ml^{-1}) and harvested at the indicated time points.

Quantitation of fungal colonization by qRT-PCR. Genomic DNA was extracted from roots with the Plant DNeasy Kit (Qiagen). Ten ng DNA served as template for real-time quantitative PCR analyses by using 20 µl SYBR green JumpStart *Taq* ReadyMix (Sigma-Aldrich) with 350 nM oligonucleotides and a 7500 FAST thermal cycler with a standard protocol (Applied Biosystems). Fungal colonization was determined by the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008) by subtracting the raw Ct values of *P. indica ITS* from those of *AtUBQ5* (for primer sequences see Table S1). Data were analyzed by Student's *t*-test.

Cytological analyses. Root samples were either fixed or directly stained with chitin-specific WGA-AF488 and/or -633 (Molecular Probes) as described (Deshmukh et al., 2006). Images were taken using an Axioplan 2 microscope (Zeiss). WGA-AF488 and GFP emission was detected at 505-530 nm (excitation: 470/20 nm). Cell autofluorescence emission was detected at 546/12 nm (excitation: 590 nm). Confocal images were recorded on a TCS SP2 microscope (Leica). WGA-AF488 and GFP were excited with a 488 nm laser line and detected at 505-540 nm, WGA-AF633 was excited with a 546 nm laser line and detected at 600-660 nm. For ultrastructural studies, roots were embedded as described (Zechmann et al., 2007) and ultrathin sections (80 nm) were investigated after post-staining with uranyl acetate and lead citrate with a Philips CM10 TEM.

Gene expression analysis by qRT-PCR. For gene expression analysis, plants were inoculated with *P. indica* or mock-treated. Three days later, plants were transferred to liquid MS medium containing 0.1 µM flg22 or mock solution. Roots were harvested at 2, 24, and 72 hat. RNA was extracted using TRIzol (Invitrogen) and aliquots were used for cDNA synthesis with a qScript cDNA synthesis kit (Quanta Biosciences). Ten ng cDNA were used as template for qRT-PCR as described above for fungal quantification. The $2^{-\Delta Ct}$ method was used to determine differential gene expression (for primer sequences see Table S1).

MAMP-induced root oxidative burst and growth retardation. Two-weeks-old plant roots were either treated with 1 µM flg22, 1 µM *N*-acetylchitooctaose, or 1 µM elf18, respectively, at 3 dai with *P. indica* or mock treatment. For determination of the oxidative burst, roots were cut in 1 cm long pieces (10 mg per assay) and subjected to a luminol-based assay as described (Gomez-Gomez et al., 1999). For the growth retardation assay, plants were treated with 10 µM flg22 or 1 µM elf18 at 3 dai with *P. indica* or mock treatment. Plant fresh weight was determined ten days after treatment. flg22 and elf18 peptide sequence was used as described (Gomez-Gomez et al., 1999; Kunze et al., 2004).

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Figure legends

Figure 1. Colonization of an *Arabidopsis* root by *P. indica* in the meristematic, elongation and maturation zones (MZ). Colonization is restricted to rhizodermal, root hair, and cortical cells while the root endodermis (brown cells) and root vasculature is not colonized. The letters and arrows in the middle panel points to interactions sites displayed in images A-C. A: Epifluorescence image of epidermal root cells that are penetrated (arrows) without specialized penetration organs. Intracellular hyphae (arrowheads) show a branched morphology.
B: Epifluorescence image of intracellular hyphae that are characterized by a distinct globular structure (arrowheads). Penetration sites are indicated by arrows. C: Bright field image of intracellular sporulation that starts around 14 dai in the MZ II. Fungal hyphae were stained with WGA-AF488. Bars = 20 µm.

Figure 2. Early biotrophic stages of the *Arabidopsis-P. indica* interaction. Transmission electron micrographs show intact root cells of *Arabidopsis* ecotype Col-0 colonized with fungal hyphae (H). A, B: Root cells with dense cytosol, intact plastids (P), mitochondria (M), vacuoles (V), ER (arrowheads in A), dictyosome (arrow in image B), and cell walls (CW). A: Image shows parts of a root cell with the plasma membrane closely surrounding the hyphae (arrows). Bars = 0.2 µm (A), 1 µm (B).

Figure 3. Biotrophic colonization by *P. indica* in MZ II at 3 dai. A-C: Confocal microscopy of living cortical cell (CC) from colonized *Arabidopsis* line GFP-Chi with GFP-tagged ER. ER bodies (+), and nucleus (*). Extracellular (arrows) but not intracellular hyphae (arrowheads) are stained with WGA-AF488 in A and with WGA-AF633 in C. Bars = 15µm.

Figure 4. *P. indica* suppresses MAMP-triggered growth retardation, oxidative burst, and gene transcription during biotrophic colonization (3dai). For all analyses, MAMPs were applied to two-weeks-old plants at 3 days after *P. indica* inoculation. A: Suppression of flg22-induced growth retardation by *P. indica*. B, C: Suppression of flg22- and *N*-acetylchitooctaose (Glc8)-induced root oxidative burst by *P. indica*. Values are given as relative light units (RLU) over time as means with standard errors

of four independent measurements per treatment of one experiment. Experiments were repeated thrice with similar results. Asterisks indicate significance at $P < 0.001$ (***) analyzed by Student's *t*-test. D: Suppression of flg22-induced gene transcription determined by RT-qPCR. Three days after *P. indica* inoculation or mock-treatment, roots were treated with flg22 or mock and harvested 2, 24 and 72 h after treatments. Induced transcript suppression was observed for markers of MTI *WRKY29*, of salicylic acid (*CBP60g*) and of jasmonic acid (*PDF1.2*) as well as glucosinolate (*MYB51*). The values represent means with standard error and are based on three independent biological experiments.

Figure 5. MAMP-triggered immunity restricts colonization of *Arabidopsis* roots by *P. indica*. Three-weeks-old plants were inoculated with *P. indica* and fungal biomass was determined during biotrophic (3dai) and cell death-associated colonization stages (7dai) by RT-qPCR. A: Col-0 roots were treated with 10 µM flg22 or mock-treated 1 day prior to *P. indica* inoculation. flg22 pre-treatment led to a reduced colonization at 3 and 7 dai. [Standard errors are from two independent experiments.] B: Reduced colonization of MAMP-hyperresponsive triple mutant *pub22/23/24* and enhanced root colonization of chitin-insensitive mutant *cerk1-2*. [Three independent experiments with 200 plants per mutant, wild type and time point; significance at $P < 0.05$ (*)] C: flg22-induced root oxidative burst in the *pub22/23/24* mutant is not suppressed by *P. indica*. Values are given as relative light units (RLU) over time. [Standard errors are from four independent measurements per treatment in one experiment]. The experiment was repeated thrice with similar results. D: Failed suppression of flg22-induced defense by *P. indica* in mutant *pub22/23/24*. Three-weeks-old Col-8 wild type or *pub22/23/24* mutant were inoculated with *P. indica* and analyzed at 1, 3 or 7 dai with RT-qPCR for transcription of *WRKY29* (MTI marker), *CBP60g* (SA marker), *PDF1.2* (JA marker), and *MYB51* (marker for antimicrobial glucosinolates). Expression values were calculated by the $^{\Delta\Delta}$Ct method by relating Ct thresholds of candidates to those of the housekeeping gene *ubiquitin 5*. The values are given as fold changes (FC) and represent means with standard error and are based on two independent biological experiments.

Figure 6. Model of the spatio-temporal colonization pattern of *Arabidopsis* roots. Root colonization by *P. indica* can be divided into four stages. After germination of

the spores and extracellular growth, hyphae penetrate epidermal or cortical cells and establish an early biotrophic colonization phase. Biotrophic stages can be preceded by intercellular colonization. The early and late biotrophic stages are characterized by complete intactness of the cell organelles (e.g. nucleus, blue) and plasma membrane invagination (dark grey lines inside cells). Biotrophically colonized cells die (light grey filling of cells) during subsequent cell death-associated colonization. Host cell death is indicated by organelle disruption, while the plasma membrane (dark grey lines inside the cell) still surrounds intracellular hyphae. Intracellular sporulation takes place in epidermal and cortical cells at about 14 dai. Endodermis cells (brown color) are not colonized. CC, central cylinder; E, endodermis; C, cortex; R, rhizodermis.

MTI is restricting root colonization by *P. indica* from early through late interaction stages. The fungus achieves biotrophic root colonization by the suppression of early MTI. Salicylic acid (SA) -mediated defense and antimicrobial indole glucosinolates (IGS) participate in MTI and are not suppressed by the fungus. As indicated by mutant colonization (Fig. S9) and gene expression profiles (Fig. 3D), SA and IGS might take a dominant role at later colonization stages at which *P. indica* might recruit JA signaling and other yet to be defined pathways to counteract SA-supported MTI.

Video S1A Fluorescent channel detection of the biotrophic colonization of a cortical cell by *P. indica*.

Video S1B Transmission channel detection of the biotrophic colonization of a cortical cell by *P. indica*.

Figure S2 Cell death-associated colonization by *P. indica* in the maturation zone II.

Figure S3 Cell death-associated colonization of Arabidopsis roots by *P. indica*.

Figure S4 Defense responses during early stages of the Arabidopsis-*P. indica* interaction.

Figure S5 Suppression of elf18-triggered responses by *P. indica*.

Figure S6 Suppression of flg22-induced gene expression by *P. indica* in Arabidopsis roots.

Figure S7 flg22-induced oxidative burst in *pub22/23/24* is not suppressed by *P. indica*.

Figure S8 Enhanced defense gene induction in *pub22/23/24* roots by *P. indica*.

Figure S9 SA and glucosinolate defense restricts colonization of Arabidopsis roots by *P. indica*.

Table S1 Primers used for qRT-PCR

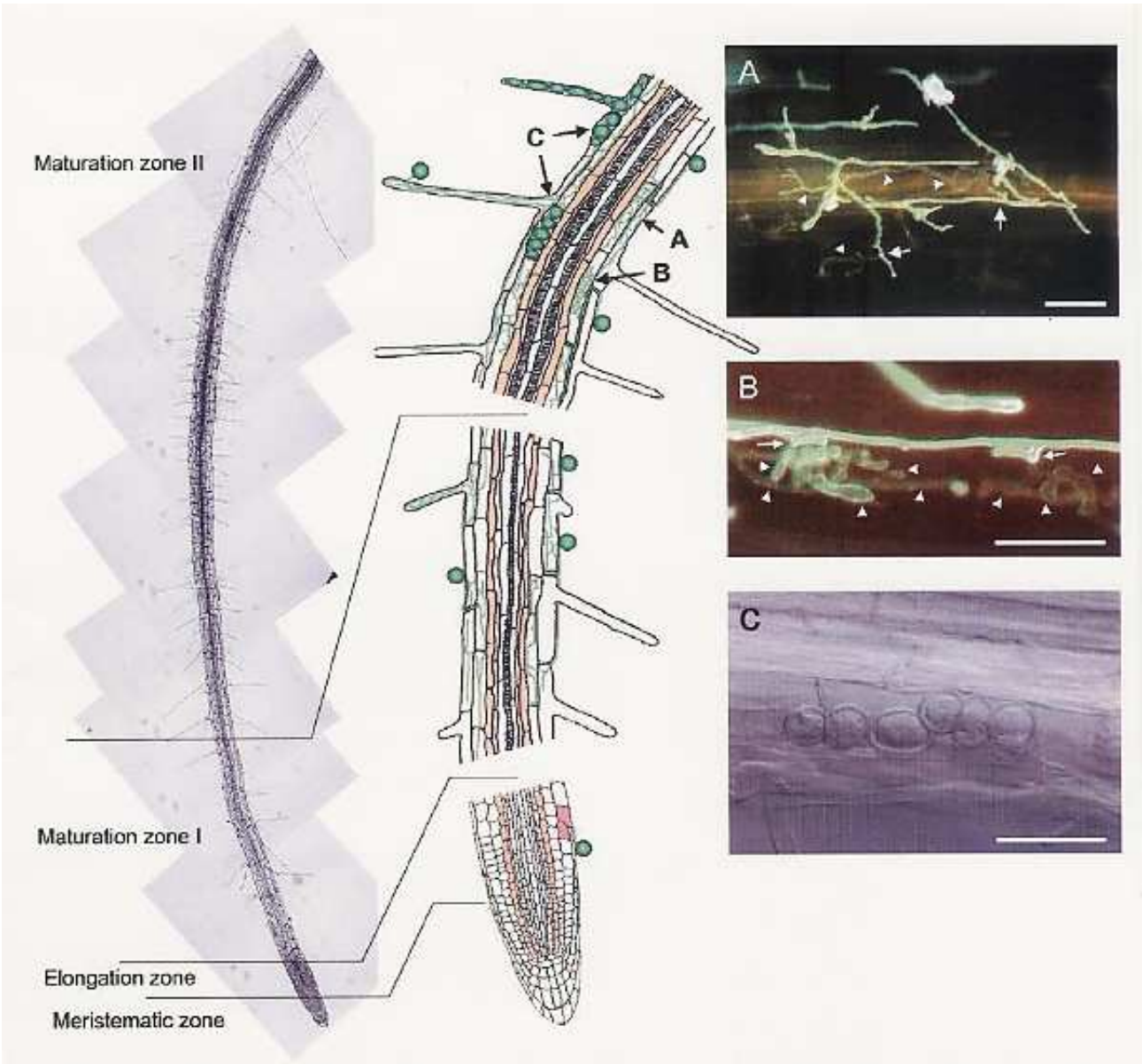

Figure 1. Colonization of an Arabidopsis root by P. indica in the meristematic, elongation and maturation zones (MZ). Colonization is restricted to rhizodermal, root hair, and cortical cells while the root endodermis (brown cells) and root vasculature is not colonized. The letters and arrows in the middle panel points to interactions sites displayed in images A-C. A: Epifluorescence image of epidermal root cells that are penetrated (arrows) without specialized penetration organs. Intracellular hyphae (arrowheads) show a branched morphology.
B: Epifluorescence image of intracellular hyphae that are characterized by a distinct globular structure (arrowheads). Penetration sites are indicated by arrows. C: Bright field image of intracellular sporulation that starts around 14 dai in the MZ II. Fungal hyphae were stained with WGA-AF488. Bars = 20 µm.

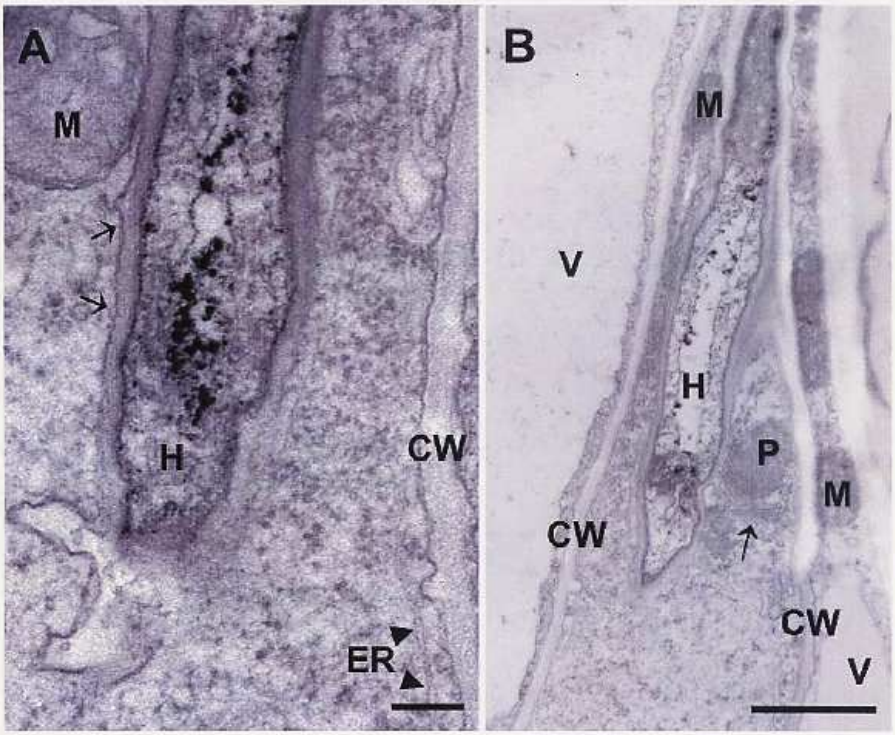

Figure 2. Early biotrophic stages of the Arabidopsis-P. indica interaction. Transmission electron micrographs show intact root cells of Arabidopsis ecotype Col-0 colonized with fungal hyphae (H). A, B: Root cells with dense cytosol, intact plastids (P), mitochondria (M), vacuoles (V), ER (arrowheads in A), dictyosome (arrow in image B), and cell walls (CW). A: Image shows parts of a root cell with the plasma membrane closely surrounding the hyphae (arrows). Bars = 0.2 µm (A), 1 µm (B).

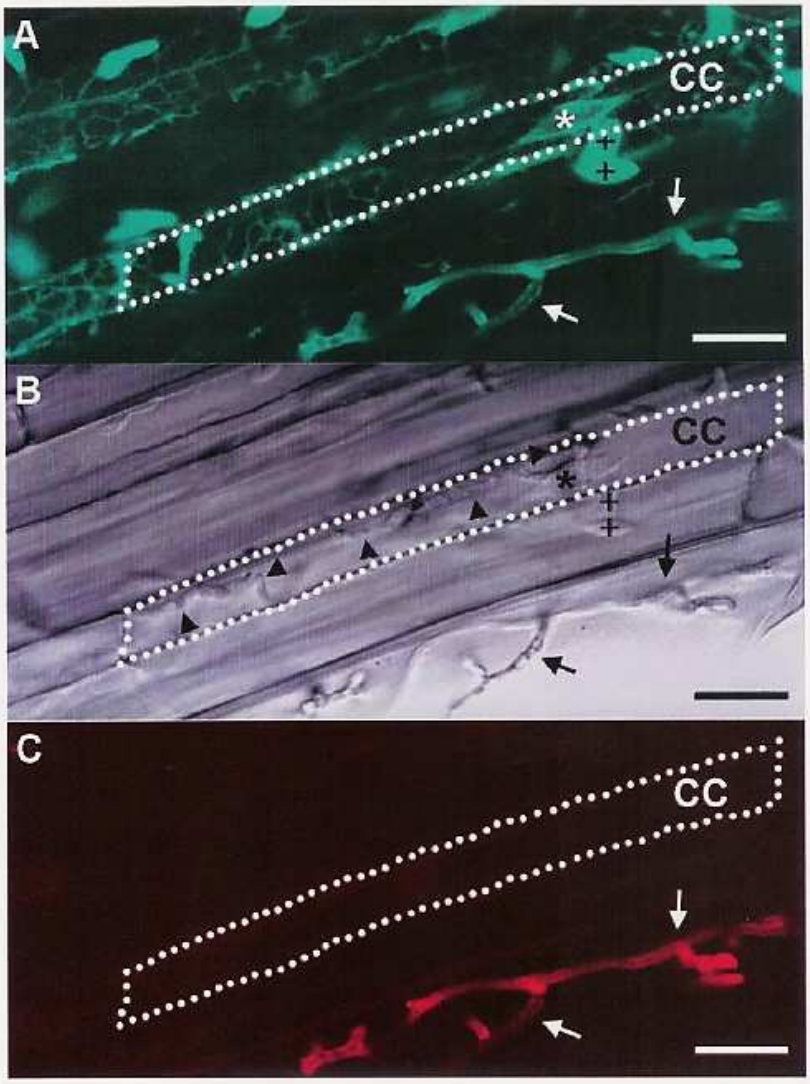

Figure 3. Biotrophic colonization by P. indica in MZ II at 3 dai. A-C: Confocal microscopy of living cortical cell (CC) from colonized Arabidopsis line GFP-Chi with GFP-tagged ER, ER bodies (+), and nucleus (*). Extracellular (arrows) but not intracellular hyphae (arrowheads) are stained with WGA-AF488 in A and with WGA-AF633 in C. Bars = 15µm.

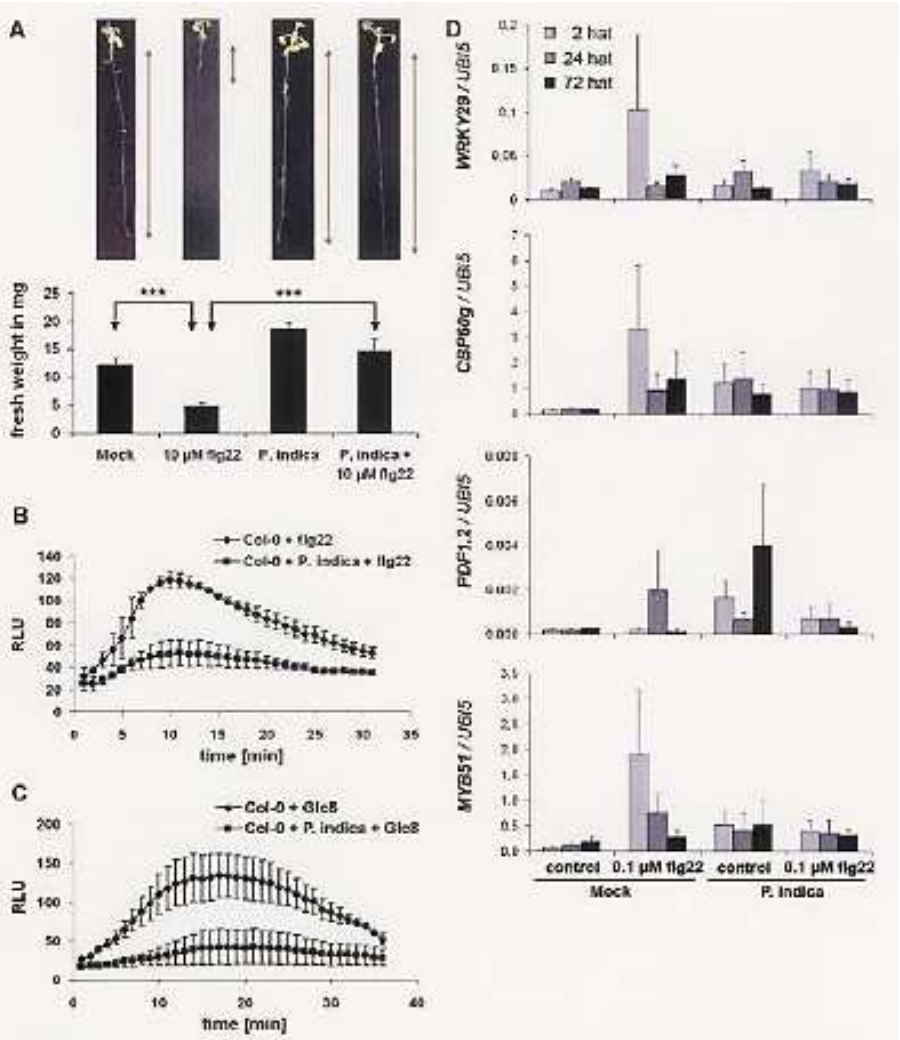

Figure 4. P. indica suppresses MAMP-triggered growth retardation, oxidative burst, and gene transcription during biotrophic colonization (3dai). For all analyses, MAMPs were applied to two-weeks-old plants at 3 days after P. indica inoculation. A: Suppression of flg22-induced growth retardation by P. indica. B, C: Suppresssion of flg22- and N-acetylchitooctaose (Glc8)-induced root oxidative burst by P. indica. Values are given as relative light units (RLU) over time as means with standard errors of four independent measurements per treatment of one experiment. Experiments were repeated thrice with similar results. Asterisks indicate significance at $P < 0.001$ (***) analyzed by Student's t-test. D: Suppression of flg22-induced gene transcription determined by qRT-PCR. Three days after P. indica inoculation or mock-treatment, roots were treated with flg22 or mock and harvested 2, 24 and 72 h after treatments. Induced transcript suppression was observed for markers of MTI WRKY29, of salicylic acid (CBP60g) and of jasmonic acid (PDF1,2) as well as glucosinolate (MYB51). The values represent means with standard error and are based on three independent biological experiments.

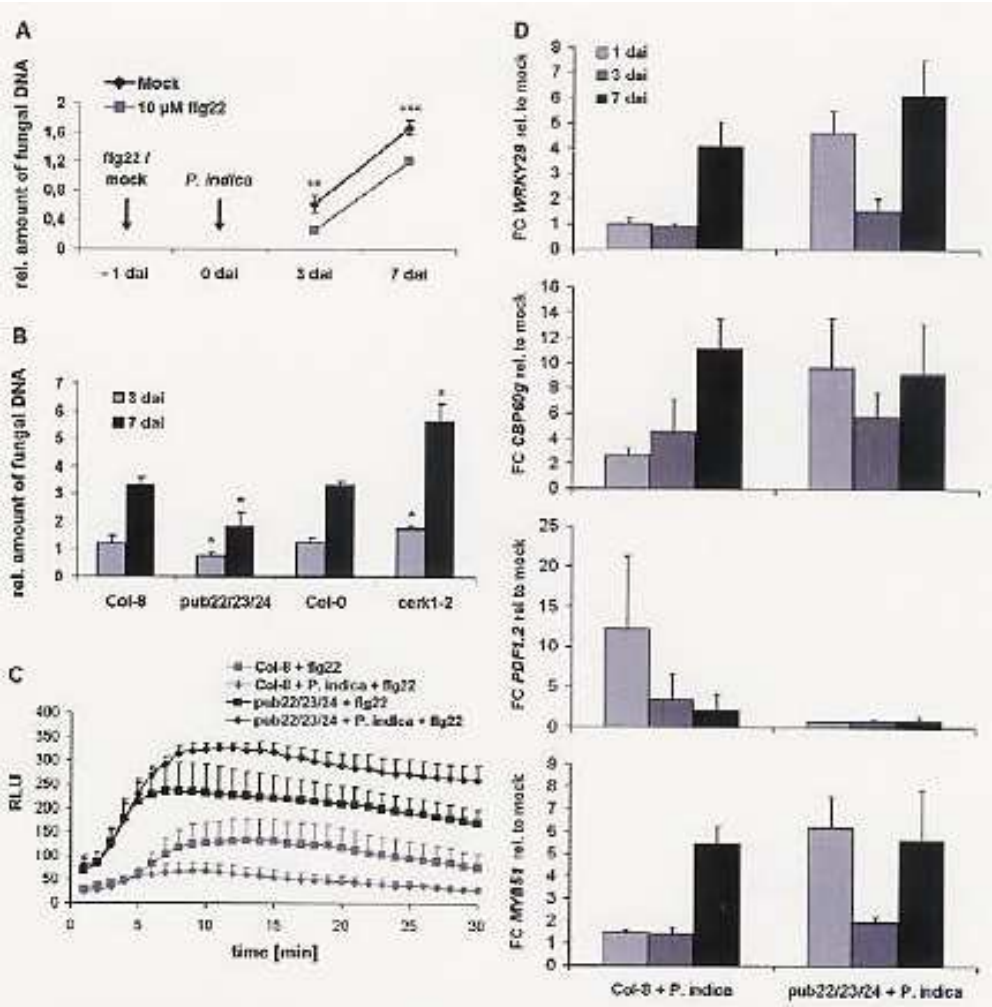

Figure 5. MAMP-triggered immunity restricts colonization of Arabidopsis roots by P. indica. Three-weeks-old plants were inoculated with P. indica and fungal biomass was determined during biotrophic (3dai) and cell death-associated colonization stages (7dai) by qRT-PCR. A: Col-0 roots were treated with 10 µM flg22 or mock-treated 1 day prior to P. indica inoculation. flg22 pre-treatment led to a reduced colonization at 3 and 7 dai. [Standard errors are from two independent experiments.] B: Reduced colonization of MAMP-hyperresponsive triple mutant pub22/23/24 and enhanced root colonization of chitin-insensitive mutant cerk1-2. [Three independent experiments with 200 plants per mutant, wild type and time point; significance at $P < 0.05$ (*)] C: flg22-induced root oxidative burst in the pub22/23/24 mutant is not suppressed by P. indica. Values are given as relative light units (RLU) over time. [Standard errors are from four independent measurements per treatment in one experiment]. The experiment was repeated thrice with similar results. D: Failed suppression of flg22-induced defense by P. indica in mutant pub22/23/24. Three-weeks-old Col-8 wild type or pub22/23/24 mutant were inoculated with P. indica and analyzed at 1, 3 or 7 dai with qRT-PCR for transcription of WRKY29 (MTI marker), CBP60g (SA marker), PDF1.2 (JA marker), and MYB51 (marker for antimicrobial glucosinolates). Expression values were calculated by the ΔΔCt method by relating Ct thresholds of candidates to those of the housekeeping gene ubiquitin 5. The values are given as fold changes (FC) and represent means with standard error and are based on two independent biological experiments.

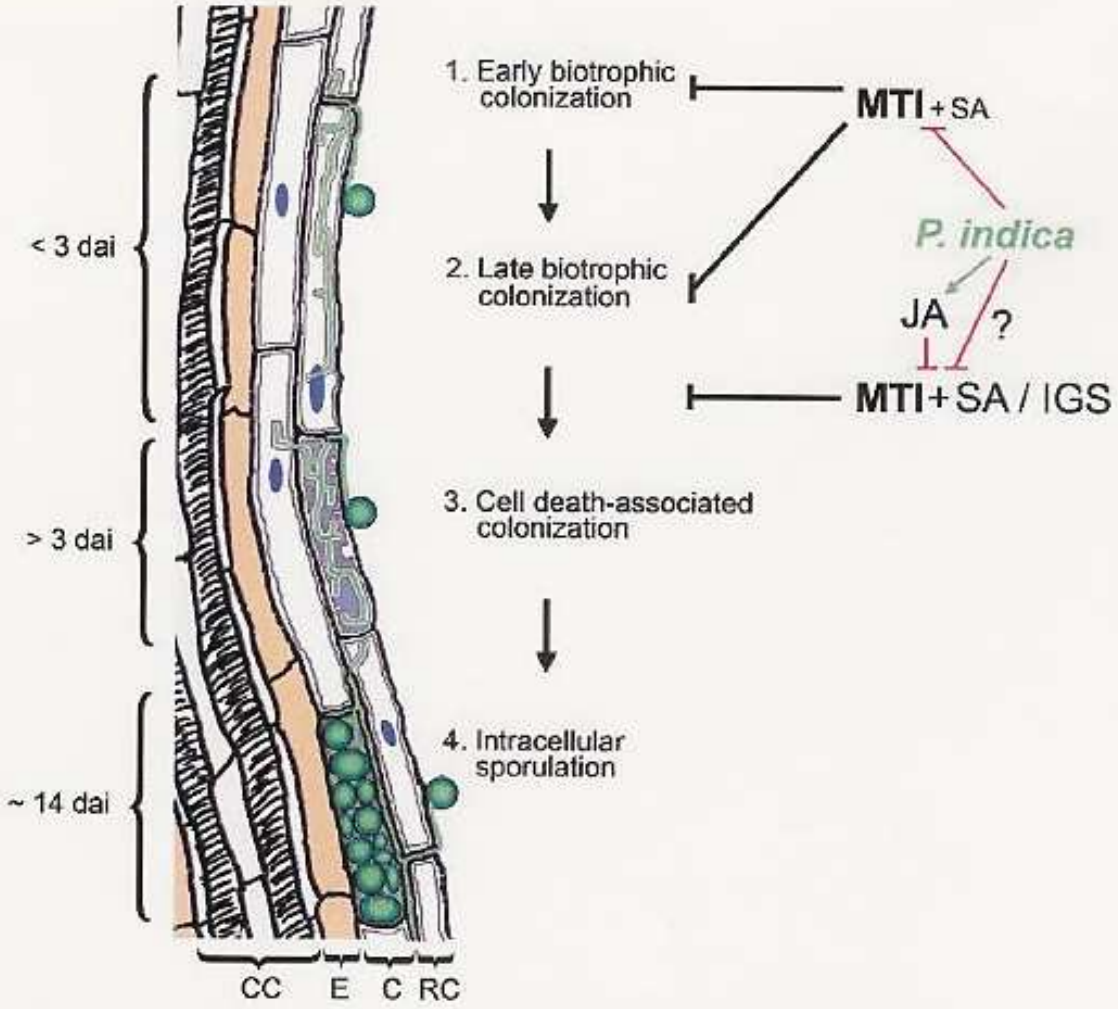

Figure 6. Model of the spatio-temporal colonization pattern of Arabidopsis roots. Root colonization by P. indica can be divided into four stages. After germination of the spores and extracellular growth, hyphae penetrate epidermal or cortical cells and establish an early biotrophic colonization phase. Biotrophic stages can be preceded by intercellular colonization. The early and late biotrophic stages are characterized by complete intactness of the cell organelles (e.g. nucleus, blue) and plasma membrane invagination (dark grey lines inside cells). Biotrophically colonized cells die (light grey filling of cells) during subsequent cell death-associated colonization. Host cell death is indicated by organelle disruption, while the plasma membrane (dark grey lines inside the cell) still surrounds intracellular hyphae. Intracellular sporulation takes place in epidermal and cortical cells at about 14 dai. Endodermis cells (brown color) are not colonized. CC, central cylinder; E, endodermis; C, cortex; R, rhizodermis.
MTI is restricting root colonization by P. indica from early through late interaction stages. The fungus achieves biotrophic root colonization by the suppression of early MTI. Salicylic acid (SA) -mediated defense and antimicrobial indole glucosinolates (IGS) particiapte in MTI and are not suppressed by the fungus. As indicated by mutant colonization (Fig. S9) and gene expression profiles (Fig. 5D), SA and IGS might take a dominant role at later colonization stages at which P. indica might recruit JA signaling and other yet to be defined pathways to counteract SA-supported MTI.

Supporting Information

Supplemental Figure Legends

Video S1A Fluorescent channel detection of the biotrophic colonization of a cortical cell by *P. indica*. The movie consists of 29 z-stacks taken with a multichannel TCS SP2 confocal laser-scanning microscope (Leica). Displayed is the interaction site described in Fig. 3. See description in Fig. 3 for identification of fungal and plant structures. The movie displays a colonization site in maturation zone II of Arabidopsis line GFP-Chi in which the ER, ER bodies, and nucleus are GFP-labeled. Optical sections were taken after excitation with laser line 488 and 633 nm, which were merged for the movie.

Video S1B Transmission channel detection of the biotrophic colonization of a cortical cell by *P. indica*. The movie consists of 29 z-stacks taken with a multichannel TCS SP2 confocal microscope (Leica). Displayed is the interaction site described in Fig. 3. See description in Fig. 3 for identification of fungal and plant structures. The movie displays a colonization site in maturation zone II of Arabidopsis line GFP-Chi. Optical sections were taken by transmission channel.

Figure S2 Cell death-associated colonization by *P. indica* in the maturation zone II. A-C: Confocal microscopy of colonized dead rhizodermal cell (RC) lacking ER and nucleus. P. indica was double stained with WGA-AF488 (A) and WGA-AF633 (C). Arrows indicate fungal penetration sites of rhizodermal cell (RC). RC is dead as indicated by the absence of ER and nucleus. Neighboring rhizodermal cells are alive as ER bodies (cross in A, B) and ER are visible (top cell) as well as the nucleus (asterisk, out of focus) in a lower cell. B: Transmission channel image of A. Labels are as described in A. C: Same as A. Bars = 15 µm.

Figure S3 Cell death-associated colonization of Arabidopsis roots by *P. indica*. Transmission electron micrographs show root cells with disintegrated cytosol (*) and fungal hyphae (H). A: The early stage of cell death-associated colonization (CAD) is characterized by intact mitochondria (M), the presence of lipid bodies (LB), and multivesicular bodies (MVB). The cytosol (*) is slightly dissolved. B: Late stage of CAD. Image shows two root cells. The cell in the left part is completely pervaded by the hypha, whereas the cell in the right part of the image is partly colonized. Penetration of the latter takes place directly through the cell walls

as plasmodesmata cannot be seen at the penetration site (arrowhead). The plasma membrane is visible and appears intact throughout the colonized cells (arrows) although the cytosol is disintegrated in some areas of the cell (*). Mitochondria and plastids (P) are still intact. Inset shows a close up of a plasma membrane protrusion close to the penetration site reminsicent of membrane blebbing. C: Four root cells separated by cell walls (CW). The middle cell is colonized with hyphae and contains a partly dissolved cytosol (*), whereas the two cells below and one upper cell, that are not colonized, show a dense cytosol (CY), intact plastids and tonoplasts (V). Bars = 1 µm (A, B, C), 0.5 µm (inset in B).

Figure S4 Defense responses during early stages of the Arabidopsis-*P. indica* interaction. *P. indica* induced very infrequently structural and biochemical defense responses in Arabidopsis roots. A: Epifluorescence image displays the rare observation of cell wall appositions (arrowheads) associated with fungal penetration attempts of a rhizodermal cell. B: Bright field image of A showing cell wall appositions (CWAs, arrowheads). C, D: Epifluorescence and bright field image of fungal penetration attempts associated with CWAs (black arrowhead). Focal accumulation of peroxisomes was visualized using Arabidopsis line A5 in which peroxisomal tetrafunctional protein is GFP-tagged [Cutler SR, Ehrhardt DW, Griffitts JS, Somerville CR (2000) Random GFP::cDNA fusions enable visulaization of subcellular structures in cells of Arabidopsis at a high frequency. *Proc Natl Acad Sci USA* 97:3718-3723]. White arrowheads indicate penetration attempts and focal peroxisome accumulation in another focal plane. E, F: Epifluorescence and bright field image of fungal penetration sites in the absence of CWAs (arrowheads) indicate the reduced focal accumulation of peroxisomes in comparison to C. Note the lower number of peroxisomes in the absence of CWAs in E compared to C. Arrows in D and F show extracellular hyphae. G, H: Epifluorescence image of WGA-AF488 stained intracelluar hyphae in elongating cells (G, arrowheads) showing strong autofluorescence (H, arrowheads) indicative of a HR-like defense response at 7 dai. G: Fungal intracellular colonization is restricted to single epidermal cells. Arrowheads indicate intracellular hyphae at another focal plane that were confronted with an identical response. Bars = 20 µm.

Figure S5 Suppression of elf18-triggered responses by *P. indica*. *P. indica* suppresses elf18-triggered responses such as seedling growth retardation and oxidative burst. A: Roots of two-weeks-old Col-0 plants were inoculated by *P. indica* or mock-treated and subsequently challenged with 1 µM elf18 or with a control treatment at 3 dai. Plant fresh weight was

determined 10 days after treatment. Data represent mean values of three biological experiments. B: Roots of two-weeks-old Col-0 plants were either inoculated with *P. indica* or mock-treated and challenged with 0.1 µM elf18. Oxidative bursts was measured in 10 mg root segments (1 cm each segment) by a luminol-based assay directly after application of elf18. 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. Asterisks indicate significance at $P < 0.001$ (***) analyzed by Student's *t*-test.

Figure S6 Suppression of flg22-induced gene expression by *P. indica* in Arabidopsis roots. *P. indica* suppresses flg22-triggered gene transcription as evidenced by qRT-PCR analysis. Roots of two-weeks-old plants were inoculated with *P. indica*. Three days after inoculation roots were either mock-treated or treated with flg22 and harvested at 2, 24, or 72 hat. Data displays the Ct thresholds of the indicated gene candiates relative to the Ct thresholds of the housekeeping gene *ubiquitin 5* using the $^{\Delta\Delta}$Ct method. The values are means with standard error and are based on three independent biological experiments.

Figure S7 flg22-induced oxidative burst in *pub22/23/24* is not suppressed by *P. indica*.
Roots of two-weeks-old Col-8 or *pub22/23/24* plants were either inoculated with *P. indica* or mock-treated. The oxidative burst was measured in root segments by a luminol-based assay directly after application of 0.1 µM flg22. A: Sum of the relative light units (RLUs) obtained in Fig. 4C. B: Peak RLUs recorded in Fig. 4C. Data displayed are means with standard errors of four independent measurements per treatment of one biological experiment. The experiment was repeated thrice with similar results.

Figure S8 Enhanced defense gene induction in *pub22/23/24* roots by *P. indica*. For qRT-PCR analysis of the indicated genes, three-weeks-old Col-8 or *pub22/23/24* plants were mock-treated or inoculated with *P. indica*. Roots were harvested at 1, 3, or 7 hat. The obtained Ct thresholds of the candiates were related to the Ct thresholds of the housekeeping gene *ubiquitin 5* using the $^{\Delta\Delta}$Ct method. Displayed are the fold changes of the genes relative to mock-treated roots. The values are means with standard error and are based on two independent biological experiments.

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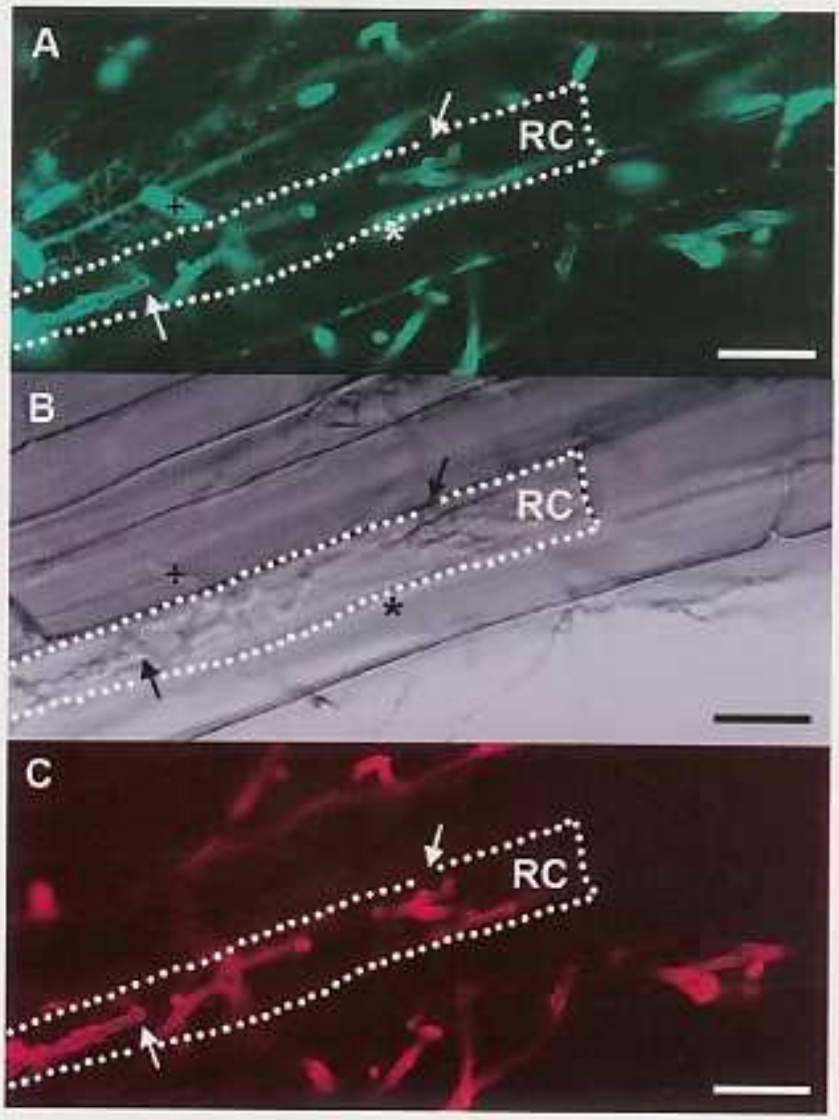

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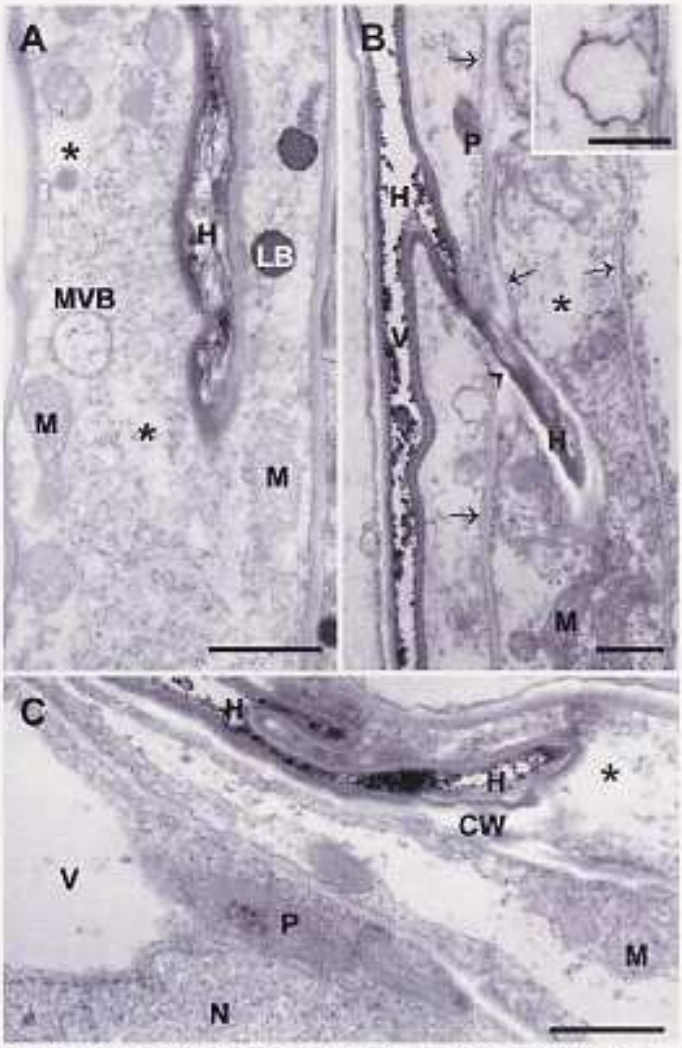

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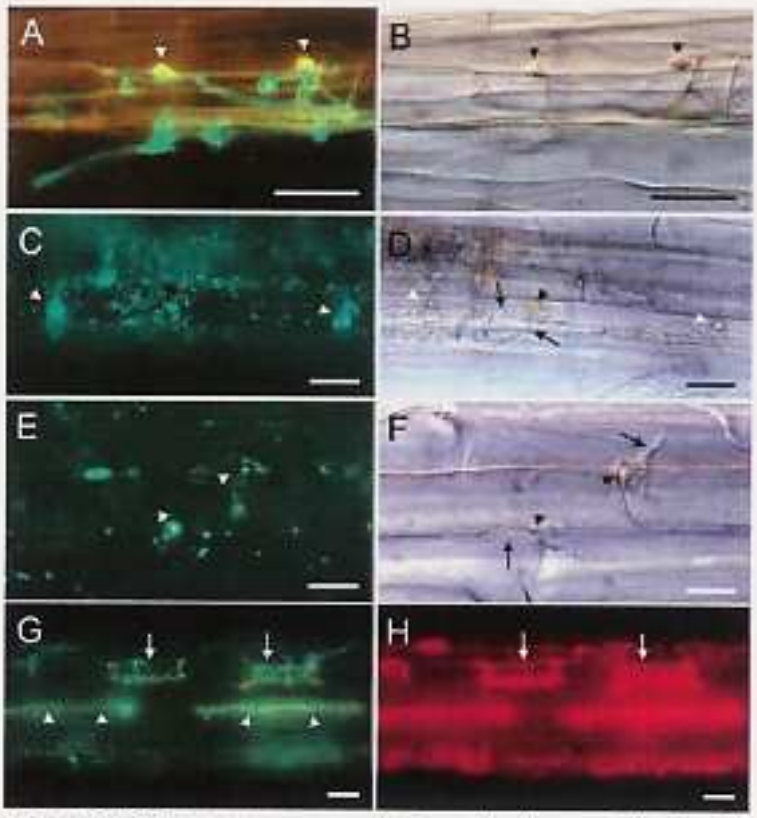

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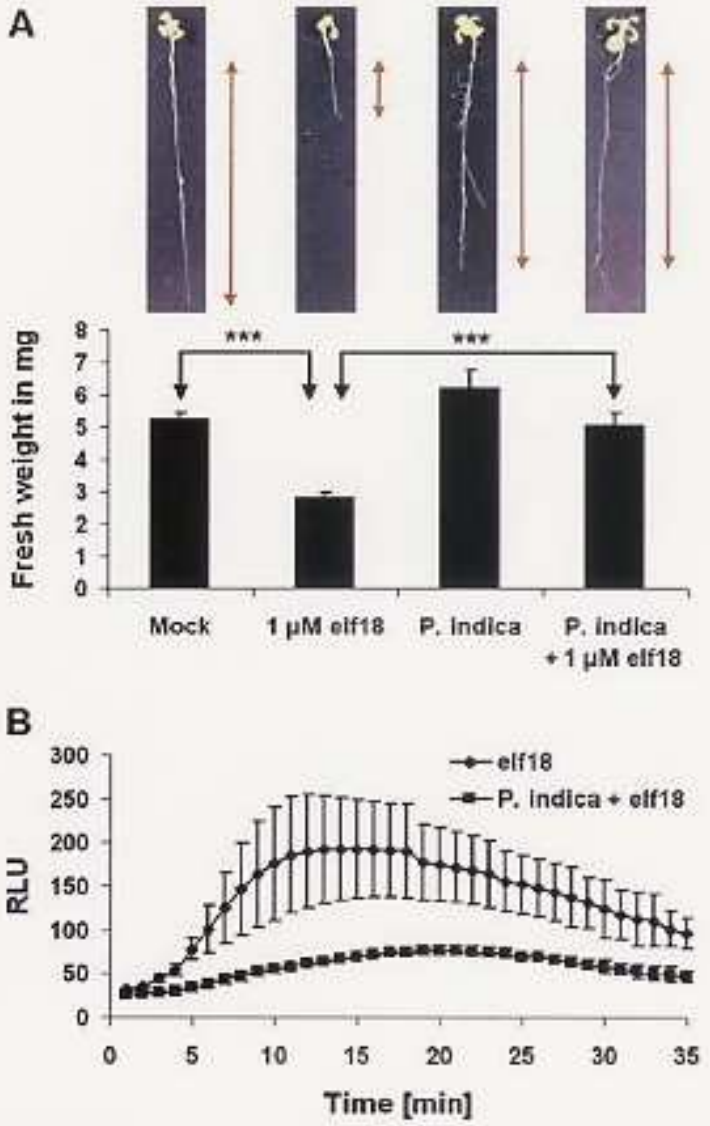

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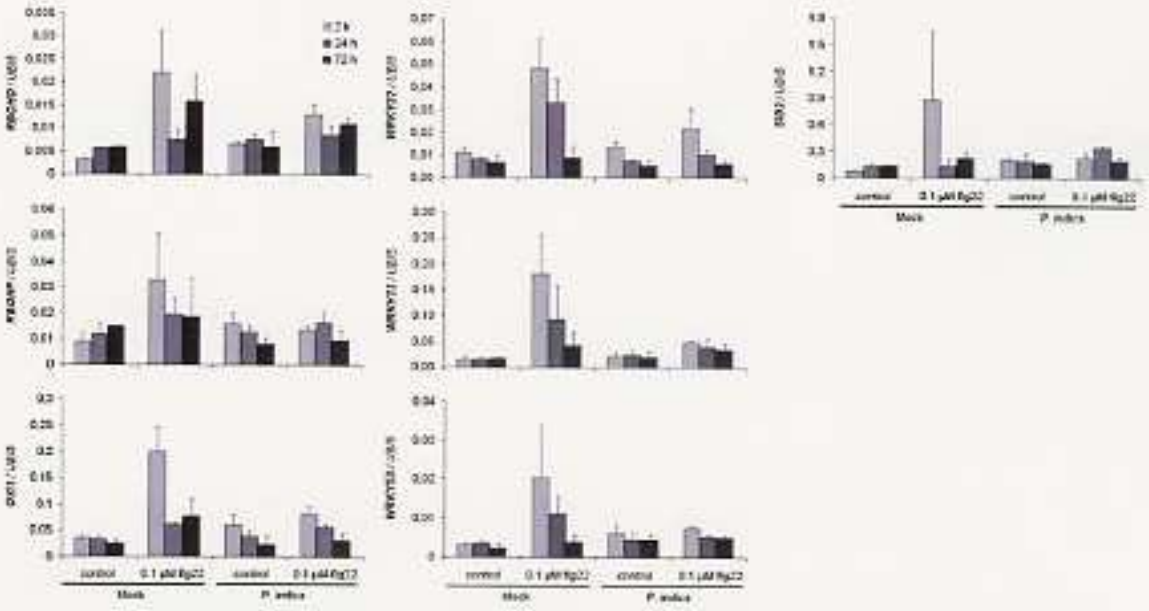

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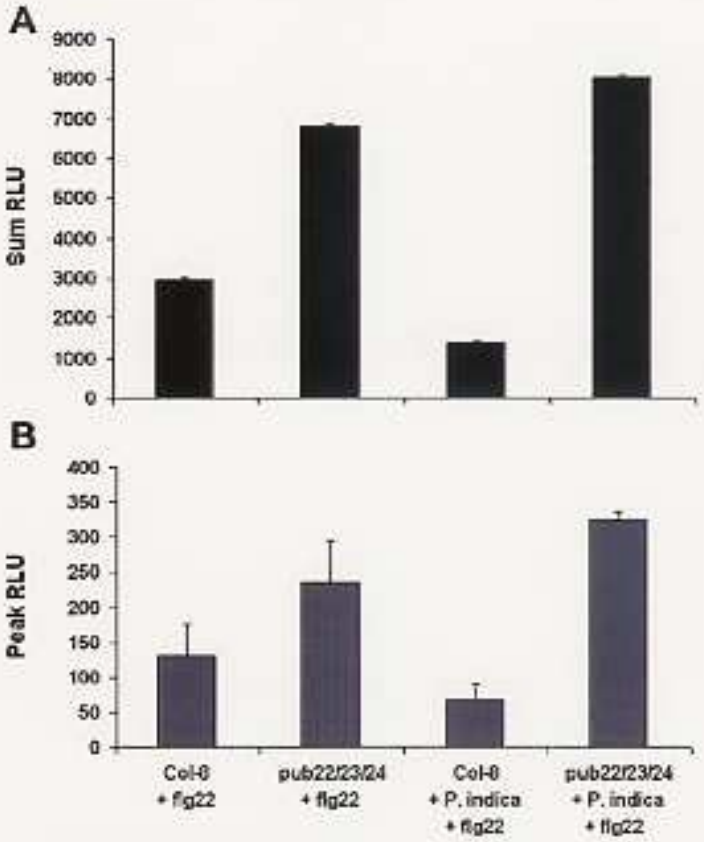

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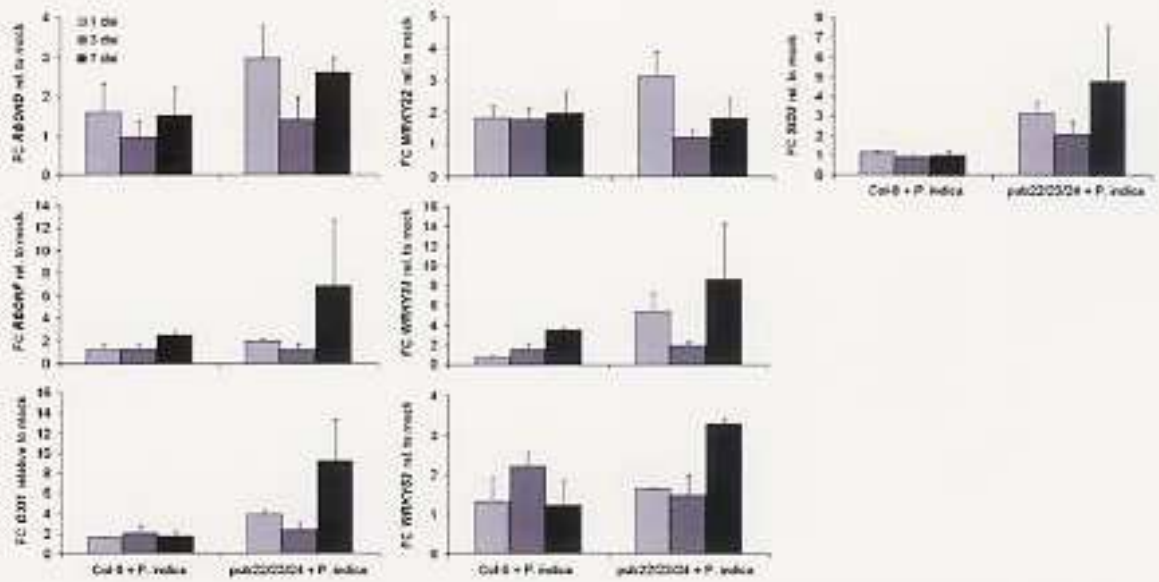

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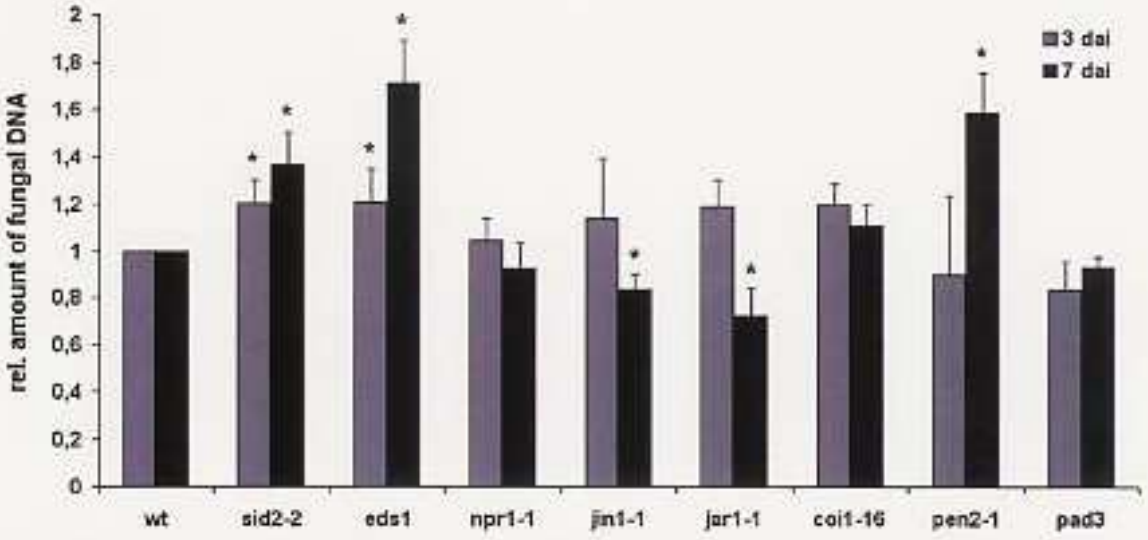

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Supplemental Tables

Table S1 Primers used for qRT-PCR

Gene	AGI	Forward	Reverse
RBOHD	AT5G47910	CCTCAACAACACCACCTCCT	GTAAGAGGCCGTTGGAATCA
RBOHF	AT1G64060	AGCAGAACGAGCATCACCTT	GGATTCGATCTCGGATTTCA
OXI1	AT3G25250	TCATCTACATTGGCCGTGTC	CGTCGCTCCATACAACATCT
WRKY22	AT4G01250	ATCTCCGACGACCACTATTG	TCATCGCTAACCACCGTATC
WRKY29	AT4G23550	TCCGGTACGTTTTCACCTTC	AGAGACCGAGCTTGTGAGGA
WRKY33	AT2G38470	CAAAGGAAAGGAGAGGATGG	GTAGACTGAGGTTTAGGATGG
WRKY53	AT4G23810	GCAACGAAACAAGTCCAGAG	GTCTTTACCATCATCAAGCCC
CBP60g	AT5G26920	AAGAAGAATTGTCCGAGAGGAG	GGCGAGTTTATGAAGCACAG
SID2	AT1G74710	TCCGTGACCTTGATCCTTTC	ACAGCGATCTTGCCATTAGG
PDF1.2	AT5G44420	AACCTTGAAGGAGCCAAACA	CACACGATTTAGCACCAAAGA
MYB51	AT1G18570	ACCAACCTCGAATCTTCTCTG	TTTCAACACAAGACTCCTCCA
UBI5	AT3G62250	CCAAGCCGAAGAAGATCAAG	ATGACTCGCCATGAAAGTCC
ITS	-	CAACACATGTGCACGTCGAT	CCAATGTGCATTCAGAACGA

5.5 **Schäfer, P.**, Khatabi, B., Kogel, K.H. (2007) Root cell death and systemic effects of *Piriformospora indica*: a study on mutualism. *FEMS Microbiology Letters* 275: 1-7.

MINIREVIEW

Root cell death and systemic effects of *Piriformospora indica*: a study on mutualism

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mutualism; programmed cell death; *Sebacinales*; arbuscular mycorrhizal fungi; growth promotion; resistance induction.

Abstract

The root systems of most terrestrial plants are confronted with a huge variety of invasive microorganisms that either can cause detrimental effects or in case of mutualistic symbiosis provide benefits for the host. In either case, establishment of the parasitic or mutualistic interaction is the result of a highly sophisticated cross-talk between the partners. Despite the ecological importance of mutualistic symbioses, the molecular events accompanied by this phenomenon are far from being understood. *Piriformospora indica* represents a recently discovered fungus that transfers considerable beneficial impact to its host plants. In this review, the current knowledge on this novel symbiosis is summarized by focusing on its biological effects in hosts and the role of programmed cell death in the establishment of the mutualistic interaction.

Introduction

Plants are exposed to various abiotic and biotic stresses and therefore evolved defensive strategies to ensure reproduction. In contrast to hostile confrontations with pathogenic organisms plants commit mutualistic associations with equally invasive microorganisms, resulting in bilateral benefits for the interacting partners (Harrison, 2005; Hause & Fester, 2005; Kogel *et al.*, 2006). The processes involved in the establishment of mutualistic symbioses, which may comprise microorganism recognition events in addition to sophisticated counter defense strategies and host cell reprogramming conducted by the mutualist, are only partially understood (Gianinazzi-Pearson, 1996; Garcia-Garrido & Ocampo, 2002; Parniske, 2004; Harrison, 2005).

The root-colonizing fungal mutualist *Piriformospora indica* was discovered in association with woody shrubs in the Indian Thar desert in 1997. Since then, the fungus has been shown to confer growth promotion to a broad spectrum of host plants, including *Arabidopsis* (Varma *et al.*, 1999; Peškan-Berghöfer *et al.*, 2004; Waller *et al.*, 2005). The commonly observed enhanced stress tolerance in colonized plants (Sahay & Varma, 1999) is mirrored in barley by an enhanced salt tolerance (Waller *et al.*, 2005). In addition, the fungus is able to induce resistance systemically in leaves of barley and *Arabidopsis* against the powdery mildew fungi *Blumeria graminis* f.sp. *hordei* and *Golovinomyces orontii*, respectively (Waller *et al.*, 2005; E. Stein, A. Molitor, K.-H. Kogel & F. Waller, unpublished data). The interaction of *Arabidopsis* and *P. indica* together with the phenomenon of systemically induced resistance provides a significant combination to unravel processes involved in the establishment of a mutualistic association and beneficial consequences thereof. Furthermore, this association may be supportive for the investigation of the genetic basis of programmed cell death in roots as a consequence of microorganism invasion. It is intriguing that *P. indica* is able to colonize large areas of the root without provoking tissue necrotization although root colonization and sporulation occur together and strongly depend on root cell death (Deshmukh *et al.*, 2006). We discuss here the current

knowledge of molecular and biochemical mechanisms supporting root colonization and systemic effects mediated by the endophyte along with its status as a member within the order *Sebacinales*.

Biological activities of *P. indica*

Phylogenetic analyses revealed that *P. indica* belongs to the newly formed order *Sebacinales* within the *Hymenomycetes* in which it shows a close relationship with the majority of *Sebacina vermifera* isolates. The order *Sebacinales* houses a great variety of ericoid, orchid, jungermannioid and ectomycorrhizae, which can be regarded as distributed worldwide although their global occurrence is far from being thoroughly analyzed (Weiß *et al.*, 2004; Setaro *et al.*, 2006). Thus, it is not further surprising that the beneficial effects (growth promotion and induced resistance) conferred by *P. indica* on barley have also been observed with all *Sebacina* sp. investigated so far (Deshmukh *et al.*, 2006). Hence, *P. indica* might be regarded as a representative member of a huge group of microorganisms with a considerable biological activity and a significant agronomic potential.

Like arbuscular mycorrhizal fungi (AMF), *P. indica* is able to transfer growth-promoting activity to its host plants but it possesses a broader host range among mono- and dicotyledonous plants (Verma *et al.*, 1998; Varma *et al.*, 1999; Pham *et al.*, 2004; Barazani *et al.*, 2005; Sherameti *et al.*, 2005; Deshmukh *et al.*, 2006). In spring barley, the enhanced plant biomass is accompanied by grain yield increases of up to 11% (Waller *et al.*, 2005). Apart from these effects on vegetative and generative plant development, *P. indica* mediates stress tolerance to infested plants. Pham *et al.* (2004) reported stimulatory effects on adventitious root formation in ornamental cuttings while enhanced salt tolerance has been observed in barley (Waller *et al.*, 2005). It still has to be elucidated to what extent the host physiology is redirected by the endophyte to cause all the effects mentioned. However, plant growth promotion might be partially explained by an elevated nitrate assimilation and/or starch degradation because a nitrate reductase and glucan-water dikinase have been shown to be stimulated by the fungus in *Arabidopsis* and tobacco roots (Sherameti *et al.*, 2005). In contrast, improved phosphate supply as a fundamental trait of AMF-based symbioses might not be involved. Firstly, a conserved phosphate transporter of potato that is activated by distinct AMF is not induced by *P. indica* (Karandashov *et al.*, 2004) and, secondly, higher transcript abundance of a specific phosphate transporter induced by AMF in barley roots could not be detected after *P. indica* colonization (S. Deshmukh & F. Waller, unpublished data). Recent findings point towards an involvement of auxin in *P. indica*-triggered growth promotion. Oelmüller and colleagues isolated two *P. indica*-responsive *Arabidopsis* proteins showing similarity to a myrosinase-binding and a myrosinase-associated protein, respectively. Both proteins might influence glucosinolate turnover and, thus, raise the amount of indole-3-acetic acid (IAA) in plants (Peškan-Berghöfer *et al.*, 2004; Grubb & Abel, 2006). Moreover, *P. indica*-infested *Arabidopsis* was found to exhibit stunted but highly branched roots on Murashige–Skoog medium containing 2% sucrose, a phenotype that could be mimicked by the application of IAA. Interestingly, these effects were also observed on roots that are growing close to the fungus but are not thought to be in physical contact with it. The detection of sufficient quantities of IAA in culture filtrate of axenically grown *P. indica* and the observation that the culture filtrate caused the described root phenotype implicate a production of auxin by the fungus (Sirrenberg *et al.*, 2007). In addition, the fungus may induce auxin production in the plant.

Pathogen resistance

The general resistance transmitted by *P. indica* against abiotic stress could also be confirmed for biotic strains. In barley, *P. indica* was shown to protect plants from deleterious effects caused by fungal root pathogens, e.g. *Fusarium* spp., *Cochliobolus sativus* and *Rhizoctonia solani* (Waller *et al.*, 2005; S. Deshmukh & K.-H. Kogel, unpublished data).

An additional beneficial effect associated with the mutualistic interaction is a systemic protection against leaf pathogens. In general, systemic resistance represents a defense strategy of plants to restrict microbial invasions to initial infection sites and, hence, to protect yet uninfected plant organs. In barley, *P. indica* triggers respective plant defense responses, which eventually lead to a systemic protection against the leaf pathogen *B. graminis* f.sp. *hordei* (Waller *et al.*, 2005). However, the phenomenon of systemically induced resistance as a consequence of *P. indica* infestation is not restricted to barley. Recently, identical responses could be observed in 'mutualized' *Arabidopsis* toward the leaf pathogen *Golovinomyces orontii*. Using *Arabidopsis* mutants compromised in known defense pathways showed the requirement of an intact *Npr1* gene for *P. indica*-mediated systemic resistance. Significantly, a compromised nuclear localization as caused by the *npr1-3* mutation was not sufficient to abolish the resistance response, suggesting that the jasmonate pathway is also required. In conclusion, the studies of *Arabidopsis* signal transduction mutants support the hypothesis that increased foliar resistance against *G. orontii* conferred by *P. indica* should be regarded as ISR (induced systemic resistance) (E. Stein, A. Molitor, K.-H. Kogel & F. Waller, unpublished data).

Root colonization by *P. indica*

Detailed cyto-histochemical studies have been undertaken to understand the infestation process of *P. indica* in barley roots (Deshmukh *et al.*, 2006). The symbiosis is entirely

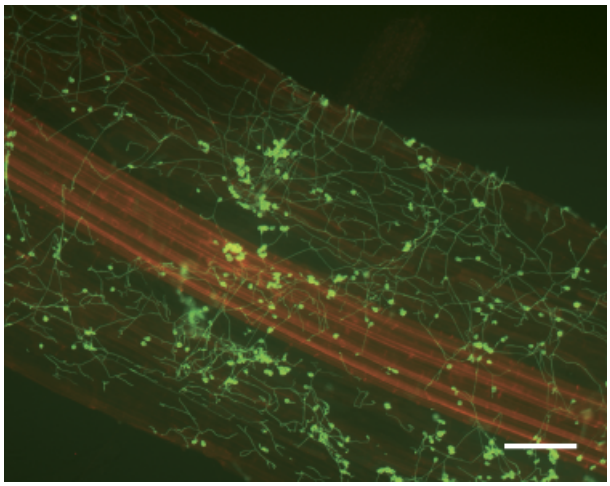

Fig. 1. Barley root 36 h after inoculation (hai) with *Piriformospora indica*. The fungus was stained with Wheat Germ Agglutinin-Alexa Fluor 488 and the root cell walls were stained with congo red. Root colonization starts with the extracellular establishment of the fungus. Globular fungal structures represent chlamydospores that are used for root inoculation (scale bar = 30 μm).

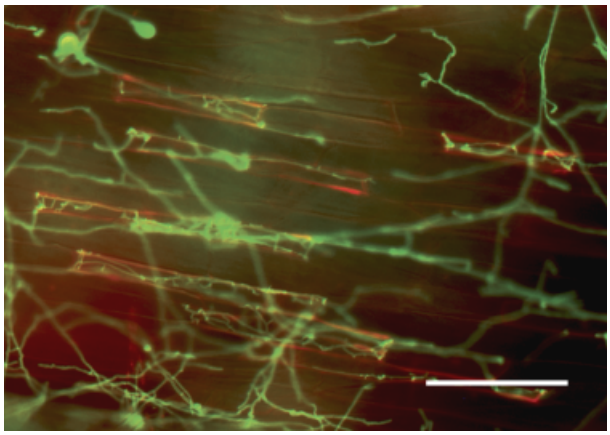

Fig. 2. Barley root 60 hai with *Piriformospora indica*. The fungus was stained with Wheat Germ Agglutinin-Alexa Fluor 488 and the root cell walls were stained with congo red. Single cells are infested by the fungus. These cells are entirely packed with intracellular hyphae before the colonization of neighboring cells. Eventually, the fungus will build a net of inter- and intracellular hyphae (scale bar = 30 μm).

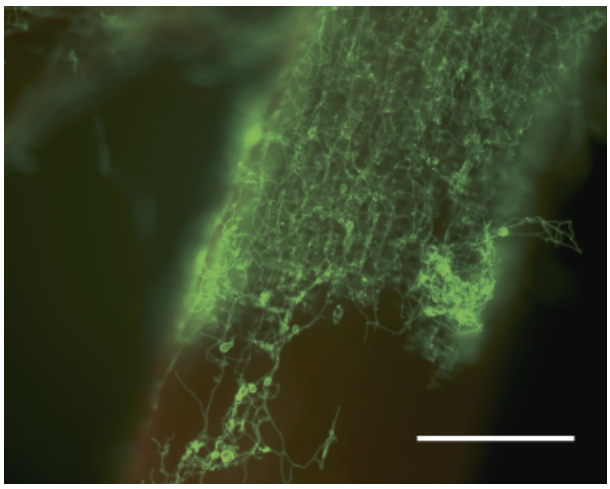

Fig. 3. Barley root colonization 8 dai with *Piriformospora indica*. The fungus was stained with Wheat Germ Agglutinin-Alexa Fluor 488 and the root cell walls were stained with congo red. The fungus has developed a dense network of extra-, inter- and intracellular hyphae (scale bar = 200 μm).

different from plant–AMF associations. It is characterized by extensive cellular and extracellular fungal growth in epidermal and cortical tissue, although fungal mycelium never reaches the stelar tissue. Plant colonization starts with the germination of chlamydospores on the root surface and subsequently, fungal mycelia derived from different spores frequently fuse as observed during cytological studies (Fig. 1). At 24–36 h, the fungus starts to invade host tissue by penetrating epidermal cells via the anticlinal cell walls or by colonizing the intercellular space between cells (Fig. 2). Fungal intracellular colonization patterns greatly depend on tissue maturation. While the infestation of the younger differentiation zone initiates from single cells that are completely packed with fungal hyphae before the penetration of adjacent cells, cells of the root hair zone are quickly traversed by single hyphae, which apparently use plasmodesmata for cell-to-cell movement (Deshmukh *et al.*, 2006). Besides, root hairs are frequently invaded by the endophyte. If at all, the physiologically highly active root tip is solely intercellularly infested. PCR-based quantification of *P. indica* revealed that root colonization gradually increases with tissue maturation. Concomitantly, the fungus preferentially sporulates in cortical, epidermal and root hair cells of older root tissue. *Piriformospora indica* infestation progresses rapidly (Fig. 3) with sporulation starting around 7 days after germination (Deshmukh *et al.*, 2006).

Molecular study of the *Arabidopsis–P. indica* interaction

The mutualistic *Arabidopsis–P. indica* association is an intriguing new model system for the elucidation of the molecular mechanisms responsible for host recognition, root colonization and subsequent beneficial activities accompanied by microbial plant symbiosis. The symbiosis leads to morphological, physiological and molecular changes in host plants (Peškan-Berghöfer *et al.*, 2004).

The introduction of proteomic approaches has led to the identification of several *P. indica* responsive *Arabidopsis* proteins. Among them, a MATH [meprin and tumor necrosis factor receptor-associated factor (TRAF) homology] domain containing protein (Oelmüller *et al.*, 2005), a leucine-rich repeat protein and a leucine-rich repeat

containing receptor kinase (Shahollari *et al.*, 2005, 2007) are the most thoroughly described ones. These proteins show a transient expression pattern during early interaction stages and are localized at the plasma membrane and endoplasmatic reticulum, respectively, of *Arabidopsis* root cells. Hence, the proteins are thought to be involved in signal perception or transduction and to be required for the establishment of a compatible association (Shahollari *et al.*, 2005, 2007). In mammals, MATH-domain containing proteins are known to exert proteolytic activity and to be essential for signal transduction, suggesting an analogous function in recognition processes preceding *P. indica* colonization (Oelmüller *et al.*, 2005). Other proteins comprise a glycosidase II α subunit and a DnaJ-chaperone domain containing protein, which might be involved in cell wall synthesis and plant growth via a role in protein processing (Peškan-Berghöfer *et al.*, 2004). The function of several identified *P. indica*-stimulated proteins obviously depends on their posttranslational modifications, e.g. the MATH-domain containing protein, a glycosyl hydrolase and a glutathione *S*-transferase (Peškan-Berghöfer *et al.*, 2004). Interestingly, the structural alteration of the MATH-domain containing protein was not detected in *P. indica*-insensitive *Arabidopsis* mutants that do not exihibit a growth promotion phenotype (Oelmüller *et al.*, 2005). One of these mutant plants was shown to carry a deletion in a leucine-rich repeat protein (Shahollari *et al.*, 2007).

Plant programmed cell death and fungal lifestyle

The gradual increase of fungal biomass with tissue maturation was found to reflect the occurrence of host cell death. The coincidence of host cell death with fungal proliferation demonstrates a new type of mutualistic interaction attributed to *P. indica* (Deshmukh *et al.*, 2006). In general, programmed cell death (PCD) is considered as being an active process of plants to face physiological constraints provoked by internal or external stimuli (Hückelhoven, 2004; Lam, 2004). For instance, PCD is a metabolically regulated mechanism (e.g. nutrient recycling) important for plant development or in case of the hypersensitive response (HR) has a protective function in local and systemic tissue, characterized by defense gene expression to avert pathogen ingress (Heath, 2000; Hoeberichts & Woltering, 2003). Interestingly, the kind of cell death occurring during *P. indica* infestation in barley may not be regarded as a microorganism-antagonizing plant response as defense marker genes (e.g. PR1b, PR5 and 1,3-β-glucanase) are weakly and transiently up-regulated solely at early interaction stages (Deshmukh & Kogel, in press) as opposed to their pronounced induction during pathogen-induced HR (Heath, 2000). In addition, accumulation of reactive oxygen species (ROS), e.g. hydrogen peroxide (H_2O_2), as well as whole-cell autofluorescence mediated by phenolic compounds as hallmarks of HR (Lamb & Dixon, 1997; Heath, 2000; Apel & Hirt, 2004) have never been detected in colonized roots. In contrast, ROS accumulation has been observed in AMF-colonized *Medicago truncatula*, *Nicotiana tabacum* and *Zea mays* (Salzer *et al.*, 1999; Fester & Hause, 2005) as well as in nodulated legume root cells (Matamoros *et al.*, 2003), and is thought to reflect a plant repsonse to control symbiotic infestation and to restrict cellular plant–symbiont associations (Fester & Hause, 2005; Puppo *et al*, 2005). Hence, the observation that *P. indica* suppresses the accumulation of H_2O_2 in colonized tissue may be crucial to achieve and maintain compatibility (P. Schäfer, unpublished data). Together, the endophyte might recruit host pathways used by developmentally regulated suicide program (e.g. senescence, xylogenesis) in order to guarantee its reproduction. However, this strategy faces certain limitations controlled by host roots, resulting in the sharp restriction of the fungus to the root cortex (Deshmukh *et al.*, 2006). To what extent the induction of the antioxidative ascorbate glutathione cycle is involved in host cell death regulation and the pattern of infestation is a focus of the present research (see below). However, the observed coincidence of tissue colonization and root cell death is regarded as being actively regulated by the fungus rather than being a fungal sensing of dead root tissue before infestation. A first evidence has been given by introducing the DNA-binding fluorescent dye 4′,6-diamidin-2′-phenylindoldihydrochlorid (DAPI). Intriguingly, heavily infested root regions consist of DAPI-negative/nucleus-free host cells as proof for cell death while adjacent noninfested cell clusters contain DAPI-positive nuclei. Secondly, cellular occupation was observed to coincide with the absence of the host cytoskeleton as compared with noncolonized adjacent cells. Final evidence comes from the regulation and expression of antiapoptotic BAX INHIBITOR-1 (BI-1). The expression of *BI-1* in *P. indica*-infested barley is repressed from 5 days after inoculation (dai) onwards, which coincides with cellular mycelial proliferation and in contrast does not match with earlier root penetration events. Constitutive overexpression of *HvBI-1* in barley leads to a significant reduction in fungal biomass at 20 dai compared with wild-type plants. These findings are in accordance with the significant role of BI-1 in plant defense and cell survival. In barley, overexpression of *HvBI-1* caused enhanced susceptibility to the biotrophic leaf pathogen *B. graminis* f.sp. *hordei* (Hückelhoven *et al.*, 2003; Hückelhoven, 2004). Enhanced susceptibility correlated with a decrease in local H_2O_2 accumulation at the penetration site and reduced HR (Eichmann *et al.*, 2006; V. Babaeizad *et al.*, unpublished data). Consistently, *HvBI-1* overexpression elevates resistance in carrot against *Botrytis cinerea* (Imani *et al.*, 2006), a fungal pathogen that exerts a

necrotrophic life style and thus relies on host cell death for successful host colonization.

Host antioxidative activities and defense responses under *P. indica* infestation

The mutualistic symbiosis of barley and *P. indica* is accompanied by an increased antioxidative status conferred by the ascorbate–glutathione cycle. *Piriformospora indica*-infested barley showed an increased accumulation of ascorbate in roots while the glutathione contents were only significantly elevated in leaves in comparison with noninfested plants (Waller *et al.*, 2005). Colonized plants display an enhanced salt and drought stress tolerance, which is in accordance with the findings of Eltayeb *et al.* (2007). The latter authors demonstrate that the transgenic enhancement of reduced ascorbate in tobacco increases tolerance to ozone, salt and drought. In addition, the tobacco plants were significantly reduced in H_2O_2 (Eltayeb *et al.*, 2007) as were *P. indica*-infested barley roots (P. Schäfer, unpublished data).

Although the ascorbate–glutathione cycle functions as a scavenging system facing ROS accumulation and, hence, to prevent oxidative damage in cells, ascorbate and glutathione are not regarded as simple cell protecting guards but thought to act as key signaling compounds by linking (oxidative) stress perception with cellular responses (Ball *et al.*, 2004; Foyer & Noctor, 2005). Ascorbate is known to be an important cofactor of enzymes (e.g. ascorbate peroxidase, 2-oxoglutarate-dependent oxygenase, ACC oxidase) involved in ROS scavenging or in the biosynthesis of cell wall compounds, defense-related secondary metabolites (e.g. phenylpropanoids, alkaloids) and hormones (e.g. systemins, ethylene, abscic acid) (Wolucka *et al.*, 2005). In contrast, ascorbate deficiency was shown to result in elevated abscisic acid and salicylate glycoside contents in *Arabidopsis* mutants (*vtc1*, *vtc2*) as well as in increased defense gene expression accompanied by an enhanced resistance against *Pseudomonas* ssp. and *Peronospora parasitica* (Pastori *et al.*, 2003; Barth *et al.*, 2004; Pavet *et al.*, 2005). It is tempting to speculate that ascorbate supports root colonization by *P. indica* due to its antioxidative activity or via ethylene synthesis, which is known to be involved in root cell death regulation associated with aerenchyma formation or xylogenesis (Kuriyama & Fukuda, 2000; Jones, 2001). However, the reported unchanged status of PR gene expression in *P. indica* infested roots (Deshmukh & Kogel, in press) implies that the defensive potential of ascorbate might not be triggered by the mutualist.

The glutathione metabolism is thought to affect antioxidative and stress-related gene expression (Ball *et al.*, 2004). Glutathione is required for fundamental physiological processes such as plant growth, flowering, the termination of a plant's life cycle as well as cell-death intrinsic developmental events like senescence and tracheary element differentiation (Ogawa, 2005). The impact of glutathione on plant defense responses has been shown in the *Arabidopsis* lesion mimick mutant lsd1 in which the regulation of *PR-1* expression and the restriction of the runaway cell death phenotype are dependent on total glutathione contents (Senda & Ogawa, 2004). Its diverse action on these processes is thought to be mediated via the glutathionylation of proteins and phytohormones, thereby altering and/or activating proteins as well as creating signaling metabolites (Ogawa, 2005). Alternatively, glutathione might directly or indirectly reduce disulfide groups to convert protein properties as described for the monomerization and concomitant activation of the defense regulator NPR1, whose monomeric state allows its translocation to nuclei as a prerequisite for defense gene induction (Mou *et al.*, 2003). However, defense gene expression appears to be unaffected in leaves of *P. indica* infested barley (Waller *et al.*, 2005) despite the accumulation of glutathione.

Conclusion and perspectives

Piriformospora indica has substantial relevance as a mutualistic symbiont regarding two aspects: first, its significance as a beneficial representative within the new order *Sebacinales* that exhibits a considerable potential regarding agronomic application. This is particularly manifested by its influence on vegetative and generative reproduction, its impact on biotic and abiotic stress tolerance as well as on its compatibility with a broad spectrum of mono- and dicotyledonous plants. Second, its association with *Arabidopsis* makes it a unique model system to investigate the basic molecular and genetic processes that are defining this mutualistic symbiosis. The system offers vast opportunities compared with other mutualistic systems due to the wealth of genetic and molecular tools available for *Arabidopsis*. In addition, *P. indica* can easily be reproduced in axenic culture, so that the fungus can easily be used for genetic dissections and manipulation. Its broad host range enables comparative studies on molecular mechanisms required for root colonization and systemically induced resistance in mono- and dicotyledonous plants.

A crucial step in deciphering the symbiotic interaction surely lies in understanding the underlying mechanisms related to the invasive character of the fungus. Right now, the system appears to be paradoxical with respect of the detrimental effects of *P. indica* on infested root tissue although the root does appear to be the initiation site of all beneficial effects. Just one of several questions left to be answered is how the fungus can redirect root cells to accept its invasive and PCD-dependent growth without provoking the plant defense machinery.

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The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley

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Fungi of the recently defined order Sebacinales (Basidiomycota) are involved in a wide spectrum of mutualistic symbioses (including mycorrhizae) with various plants, thereby exhibiting a unique potential for biocontrol strategies. The axenically cultivable root endophyte *Piriformospora indica* is a model organism of this fungal order. It is able to increase biomass and grain yield of crop plants. In barley, the endophyte induces local and systemic resistance to fungal diseases and to abiotic stress. To elucidate the lifestyle of *P. indica*, we analyzed its symbiotic interaction and endophytic development in barley roots. We found that fungal colonization increases with root tissue maturation. The root tip meristem showed no colonization, and the elongation zone showed mainly intercellular colonization. In contrast, the differentiation zone was heavily infested by inter- and intracellular hyphae and intracellular chlamydospores. The majority of hyphae were present in dead rhizodermal and cortical cells that became completely filled with chlamydospores. In some cases, hyphae penetrated cells and built a meshwork around plasmolyzed protoplasts, suggesting that the fungus either actively kills cells or senses cells undergoing endogenous programmed cell death. Seven days after inoculation, expression of barley *BAX inhibitor-1* (*HvBI-1*), a gene capable of inhibiting plant cell death, was attenuated. Consistently, fungal proliferation was strongly inhibited in transgenic barley overexpressing GFP-tagged HvBI-1, which shows that *P. indica* requires host cell death for proliferation in differentiated barley roots. We suggest that the endophyte interferes with the host cell death program to form a mutualistic interaction with plants.

biodiversity | mycorrhiza | rhizosphere | Sebacinales | systemic resistance

Most plants studied in natural ecosystems are infested by fungi that cause no disease symptoms. These endophytic fungi are distinguished from pathogens that lead to disease and reduce the fitness of their host plants (1). In many cases, endophytes form mutualistic interactions with their host, the relationship therefore being beneficial for both partners. Mutualism frequently leads to enhanced growth of the host. The beneficial effects for the plant can be a result of an improved nutrient supply by the endophyte as known for arbuscular mycorrhizal symbiosis, the most intensely studied mutualistic plant–fungus interaction (2). In addition to providing mineral nutrients, endophytes also can improve plant resistance to pathogens as demonstrated for arbuscular mycorrhiza fungi (AMF) in roots (3) and for a highly diverse spectrum of ascomycete endophytes in leaves (4, 5).

Mutualism requires a sophisticated balance between the defense responses of the plant and the nutrient demand of the endophyte. Hence, a mutualistic interaction does not imply absence of plant defense. Defense-related gene expression has been well studied during host colonization by obligate biotrophic AMF. Induction of defense genes was most prominent at early time points during penetration (6) but could also be detected during arbuscule development (7). On the other hand, there is clear evidence for impeded defense reactions during the establishment of mycorrhization. It is therefore a rather fine-tuned balance that keeps a mutualistic interaction in a steady state without disadvantages for both partners (8).

In the present work we aimed at studying fungal development and host reactions in the mutualistic symbiosis of the fungal root endophyte *Piriformospora indica* and barley (9, 10). The basidiomycete is a model organism for species of the recently described order Sebacinales, fungi that are involved in a uniquely wide spectrum of mutualistic symbioses (mycorrhizae) with plants (11). The axenically cultivable *P. indica* increases biomass and grain yield of crop plants. In barley, the endophyte induces root resistance against *Fusarium culmorum*, one of the fungal species causing head blight, and systemic resistance to barley powdery mildew *Blumeria graminis* f.sp. *hordei* via an unknown mechanism probably independent of salicylate or jasmonate accumulation. Moreover, *P. indica* protects barley from abiotic stress, such as high salt concentrations (10).

P. indica was originally discovered in the Indian Thar desert in northwest Rajasthan. *In vitro* experiments have shown a broad host spectrum of the fungus (12), including members of the Brassicaceae, like *Arabidopsis*, which are not colonized by AMF. As in barley, *P. indica* enhances seed yield, reduces the time for seed ripening, and increases tolerance to abiotic stress in *Arabidopsis* (13). How the fungus penetrates plant roots, how roots are eventually colonized, or whether the mutualistic fungus has a facultative biotrophic or a necrotrophic lifestyle are issues that have not yet been studied. In *Arabidopsis*, mycelium covers the surface of the roots. Hyphae penetrate root hairs and rhizodermis cells and eventually form chlamydospores in these cells (13). Our previous observations in barley revealed that the fungus, in contrast to obligate biotrophic AMF, colonizes dead root cells, suggesting a previously uncharacterized type of mutualism. Here

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Abbreviations: AMF, arbuscular mycorrhiza fungi; Ct, cycle threshold; dai, days after inoculation; nucLSU, nuclear gene coding for the large ribosomal subunit; PCD, programmed cell death; WGA-AF 488, wheat germ agglutinin-Alexa Fluor 488; WGA-TMR, wheat germ agglutinin-tetramethylrhodamine.

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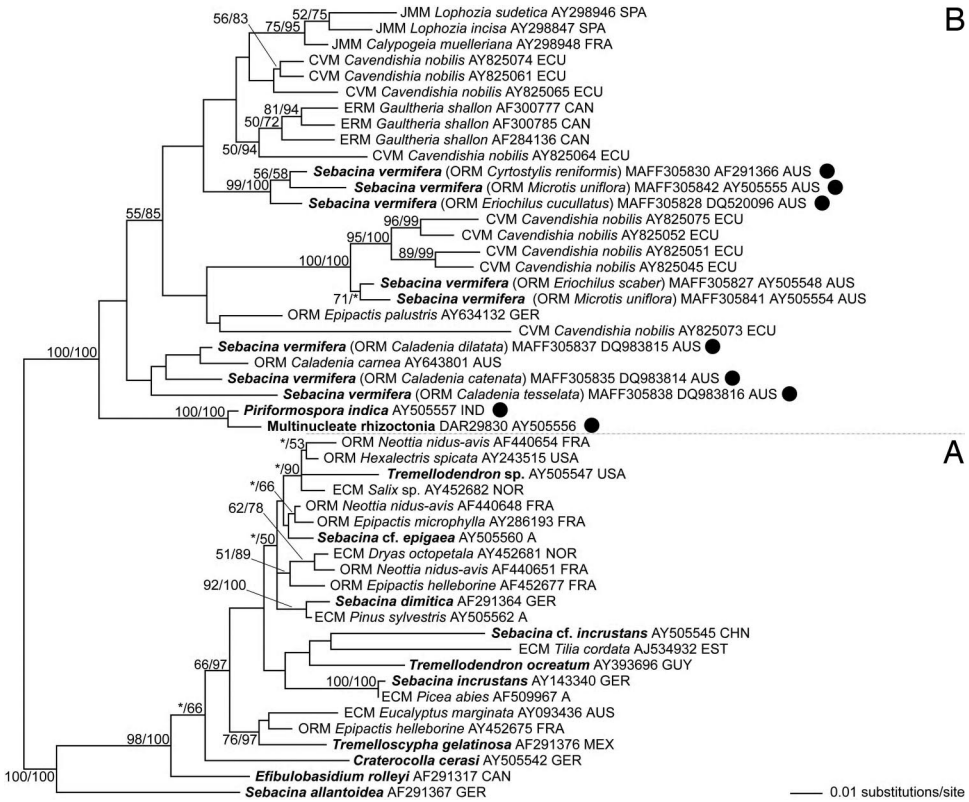

Fig. 1. Phylogenetic placement of the strains tested in this study within the Sebacinales, estimated by maximum likelihood from an alignment of nuclear rDNA coding for the 5′ terminal domain of the ribosomal large subunit. Branch support is given by nonparametric maximum likelihood bootstrap (first numbers) and by posterior probabilities estimated by Bayesian Markov chain Monte Carlo (second numbers). Support values of <50% are omitted or indicated by an asterisk. The tree was rooted according to the results of ref. 11, and subgroups discussed in ref. 11 are denoted with ''A'' and ''B.'' Sequences of the strains used in this study are indicated by black circles. Sequences from morphologically determined specimens or cultures are printed in bold. Sebacinalean sequences obtained from mycorrhizal plant roots are assigned to mycorrhizal types by the following acronyms: CVM, cavendishioid mycorrhiza (14); ECM, ectomycorrhiza; ERM, ericoid mycorrhiza; JMM, jungermannioid mycorrhiza; and ORM, orchid mycorrhiza. Proveniences are given as follows: A, Austria; AUS, Australia; CAN, Canada; CHN, People's Republic of China; ECU, Ecuador; EST, Estonia; FRA, France; GER, Germany; GUY, Guyana; IND, India; MEX, Mexico; NOR, Norway; and SPA, Spain.

we provide cytological and molecular evidence that *P. indica* proliferates in dead host cells and that colonization gradually increases with tissue maturation. The expression level of the cell death regulator *BAX inhibitor-1* (*HvBI-1*) appears critical for *P. indica* development in barley, suggesting that the recently discovered endophyte interferes with the host cell death machinery.

Results

***P. indica* Belongs to the Recently Defined Order Sebacinales.** Based on the nuclear genes coding for the large ribosomal subunit (nucLSU), available strains of the *Sebacina vermifera* species complex (Sebacinales group B) are closely related to *P. indica* (Fig. 1). We addressed the question whether strains of the *S. vermifera* complex exhibit comparable biological activities as *P. indica*. To this end, barley seedlings were inoculated with *P. indica* or different isolates of *S. vermifera* and shoot length and biomass were determined (Table 1). Despite obvious variation, we found consistent biological activities in the same order of magnitude as with *P. indica*. To determine the potential for systemic induction of resistance, barley third leaves from endophyte-colonized and noncolonized, 21-day-old plants were inoculated with the conidia of *B. graminis* f.sp. *hordei*, and powdery mildew pustules were counted after 7 days. We found consistent resistance-inducing activity of all strains of the *S. vermifera* complex, although there was considerable variation of the fungal activity of the different isolates (Table 1). These data support the view that the order Sebacinales is a source of endophytes with a feasible agronomical impact.

Endophytic Development in Barley Roots. To track endophytic development in barley, root penetration and colonization were analyzed by fluorescence microscopy. In general, we observed a gradual increase of fungal colonization and proliferation associated with root maturation (Fig. 2*a*). Colonization initiates from chlamydospores, which, upon germination, finally form a hyphal network on and inside the root. Hyphae enter the subepidermal layer through intercellular spaces where they branch and continue to grow (Fig. 2 *b–e*). In young differentiated root tissue, the fungus then often colonizes and completely fills up single cells (Fig. 2 *f* and *g*) before adjacent cells are colonized, whereas an unrestricted net-like intra- and intercellular colonization pattern is observed in mature parts. Intracellulary growing hyphae show necks at sites where the fungus traverses a cell wall (Fig. 2*h*). Occasionally, subepidermal hyphae penetrate the space between the cell wall and plasma membrane of rhizodermal or cortical cells. After branching, these hyphae enwrapped protoplasts, which showed cytoplasmic shrinkage (Fig. 3*a*). At later colonization stages, fungal hyphae excessively occupied rhizodermal and cortical cells. In some cases, transverse cell walls of adjoining cortical cells were absent, with the protoplasts covered by a dense meshwork of fungal hyphae. Eventually arrays of single spores developed from intracellular hyphal tips (Fig. 3 *b* and *c*). The fungus also penetrated basal parts of root hair cells, in which branching hyphae form large numbers of chlamydospores starting from the base of the root hair until a stack of spores fills the root hair (data not shown). In addition to this intracellular spore

Table 1. Effect of different Sebacinales species on barley biomass and systemic resistance to powdery mildew

Species/isolate	Increase in shoot length, %	Increase in shoot fresh weight, %	Reduction in leaf infection by *B. graminis*, %
P. indica	13.66**	26.45**	70.85**
S. v./MAFF305830	23.25**	48.24**	79.45**
S. v./MAFF305842	16.87**	15.48*	56.36*
Multinucleate *Rhizoctonia*/DAR29830	7.56**	10.76*	56.27*
S. v./MAFF305828	14.97**	28.72**	10.89
S. v./MAFF305837	16.34**	32.01**	58.19**
S. v./MAFF305835	7.80*	9.82	50.74*
S. v./MAFF305838	7.72**	6.41	44.89*

Species/isolates are shown with their culture collection numbers. Isolates of *Sebacina vermifera* (*S. v.*) were obtained from the National Institute of Agrobiological Sciences (Tsukuba, Japan); the isolate DAR29830 was kindly provided by Karl-Heinz Rexer (University of Marburg, Marburg, Germany). Values are means of three independent experiments, each consisting of 60 endophyte-inoculated and mock-inoculated plants, respectively. Powdery mildew infection was calculated from the number of fungal colonies developing on third leaf segments 7 dai with *B. graminis* f.sp. *hordei,* race A6 (15). Asterisks denote statistically significant differences between the respective values of endophyte-colonized and noncolonized plants (*, $P < 0.05$, Student's *t* test; **, $P < 0.01$, Student's *t* test).

formation, chlamydospores also were generated in the mycelial mats at the root surface.

***P. indica* Proliferates in Dead Cells.** We addressed the question of whether cortical and rhizodermal cells heavily occupied by fungal hyphae and chlamydospores were alive. In a cell viability assay with the fluorescent marker fluorescein diacetate, colonized cells did not show enhanced green fluorescence, suggesting that they were dead. In addition, these cells did not show any visible cytoplasmic streaming. Staining of colonized root hairs with an Alexa Fluor-488-labeled anti-actin antibody failed to show any host cytoskeleton, whereas noncolonized root hairs showed intact actin filaments (data not shown). To confirm that fungal colonization associates with dead cells, we double-stained root segments with DAPI for intact plant nuclei and wheat germ agglutinin-Alexa Fluor 488 (WGA-AF 488) for fungal chitin. We found a close spatial association of strong fungal colonization (Fig. 3 *d* and *f*) and DAPI-negative cells (Fig. 3 *e* and *g*), further suggesting that massive development of *P. indica* takes place in dead host cells.

Microscopic analyses demonstrated a fungal colonization pattern that strongly associated with the developmental stage of the host tissue (Fig. 2*a*). To substantiate this finding, we determined the amount of *P. indica* in different root zones by quantitative PCR using *P. indica* genomic DNA as a template for the quantification of the *P. indica* translation elongation factor gene *Tef* relative to the plant ubiquitin gene. Ten days after inoculation, the roots were cut into 0.5-cm-long apical segments of the root tip with the root cap and a basipetal segment including the differentiation zone. Consistent with the cytological data, we found a 5-fold higher relative amount of *P. indica* in the differentiation zone as compared with the apical root segment (2.53 ± 0.23 compared with 0.52 ± 0.12).

Analysis of fungal growth in the apical elongation zone revealed fungal development in intercellular spaces and formation of subepidermal intercellular hyphal mats. In contrast to its development in the differentiation zone, neither host cell wall degradation nor heavy fungal sporulation could be observed in this tissue, supporting the notion that there is a correlation between root tissue and fungal development. Juvenile tissue, which is considered to display less developmental cell death, is thus less occupied by *P. indica*. To support this observation, we tested for genomic DNA fragmentation by probing gel blots of high-molecular-weight DNA isolated from different root segments with radioactively labeled DNA probes. Genomic DNA fragmentation results from programmed cell death (PCD). As expected, the proportion of low-molecular-weight DNA fragments resulting from DNA fragmentation was lower in root tips than in mature parts of the root. *P. indica* did not change the amount of DNA fragmentation in root tips, whereas a small increase of 5–9% low-molecular-weight DNA was detected in the mature zone 10 days after inoculation with *P. indica*. To visualize DNA fragmentation in the root tissue, we used *in situ* DNA nick-end labeling and observed DNA fragmentation in nuclei of protoplasts enwrapped by *P. indica* (Fig. 3*h*). However, this was a rare event perhaps indicating a transient status before nuclei completely dissolved in invaded cells. Taken together, these results indicate that invasive growth of *P. indica* mainly occupies dead and dying cells in barley roots. Consistently, the fungus infested only dead cells of the root cap at the root tip zone, whereas the central meristematic tissue was always free of fungal hyphae (Fig. 3*i*). In adjacent cortical tissue, the fungus was present in the intercellular spaces of cells differentiating into cortical and epidermal tissue apparently without affecting differentiation. Accordingly, lateral root development from cambial cells that differentiate in root tip meristems was not compromised in roots infested by *P. indica*.

We measured the ratio of fungus to plant DNA (fungus/plant DNA ratio, FPDR) over time to check whether *P. indica* overgrows barley roots at late interaction stages. We observed an early moderate increase of the FPDR (1.8-fold) followed by a decrease and a final steady state (data not shown). This pattern reflects the symbiotic interaction in which the fungus develops moderately, subsequently induces plant growth (reflected in a decrease of FPDR), and finally reaches a steady-state level of fungal structures in the plant root. This growth pattern indicates a final balance of root growth and fungal proliferation.

Balancing of Host Cell Death and Impact of the Cell Death Regulator BAX Inhibitor-1. Because the cytological analysis of root colonization suggested that *P. indica* proliferates in dead host cells, we addressed the question of whether root invasion by *P. indica* interferes with the host's cell death machinery. Therefore, we kinetically analyzed expression of barley *HvBI-1*. BI-1 is one of the few conserved cell death suppressor proteins that apparently controls PCD in all eukaryotes and is considered a regulator of endoplasmic reticulum-linked Ca^{2+} signaling. In plants, *BI-1* is often activated in response to biotic or abiotic stresses (15–17).

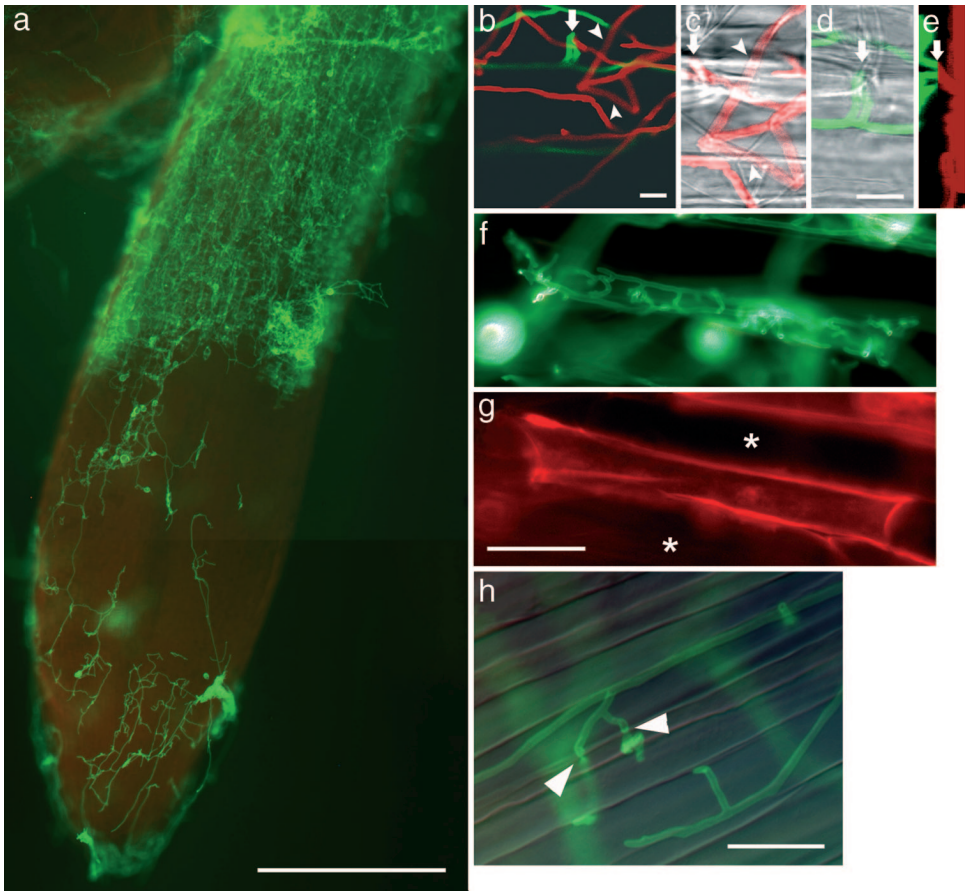

Fig. 2. Infestation pattern of *P. indica* in barley roots. (*a*) By 8 dai, hyphae excessively occupy rhizodermal and cortical cells of the differentiation zone. The elongation zone is less colonized, with occasional intercellular subepidermal hyphal structures. The root cap is heavily infested with hyphae. (*b–e*) After penetration (arrows) fungal hyphae colonize the subepidermal layer. (*b*) To better visualize the position of hyphae in the *z* axis, a confocal laser scanning image consisting of 30 frames of adjacent focal planes (*z* axis) was displayed as a maximum projection with the fluorescent signal of the wheat germ agglutinin-stained fungal hyphae displayed in red for the upper (abaxial) 15 frames and in green for the lower (adaxial, subepidermal) 15 frames. (*c* and *d*) For visualization of plant cell walls, two close-up bright-field images of two different focal planes are superimposed with the respective frames of the fluorescence images. Intercellular hyphae start branching and proliferate within the subepidermal space. (*c*) Subepidermal hyphae crossing cell walls (arrowheads) without exhibiting morphological changes (e.g., neck formation, as in *h*) revealing their periclinal localization. (*d*) The upper focal plane is characterized by hyphae penetrating the anticlinal space of adjacent rhizodermis cells. (*e*) Projection of the fluorescent signals of *c* and *d* in the *y* axis (vertical) and *z* axis (horizontal). Absence of fluorescent signals between adaxial (green) and abaxial hyphae (red) indicates a layer of rhizodermal cells free from hyphae. The penetration site is indicated by an arrow. (*f*) Colonization of a single cell within young differentiated tissue. After penetration, the cell is completely filled with intracellular hyphae before the colonization of adjacent cells. (*g*) The cell wall of the colonized cell is strongly stained with Congo red because of better dye accessibility compared with noncolonized neighbor cells (asterisks). Penetrated cells did not show autofluorescence. (*h*) Intracellular mycelium in mature root tissue. Overlay of bright-field image and fluorescence image. Intracellular hyphae form necks (arrowheads) at sites of cell wall crossing. Fungal structures are visualized by WGA-AF 488. [Scale bars: *a*, 300 μm; *b* and *d*, 10 μm (*c* and *d* are of the same scale); *f–h*, 30 μm.]

Quantitative PCR analysis of *HvBI-1* expression showed it slowly increasing during root development throughout the course of the experiment (Fig. 4*a*). In contrast, when roots were colonized by *P. indica*, *HvBI-1* expression was significantly reduced as compared with noncolonized roots from 7 days after inoculation (dai) onwards (Fig. 4*a*). These data support the idea that *P. indica* interacts with the host cell death machinery for successful development but does not cause plant stress.

To gain evidence for a role of host PCD and requirement of *HvBI-1* down-regulation for fungal success, we overexpressed a functional GFP–HvBI-1 fusion protein in barley under control of the constitutive cauliflower mosaic virus 35S promoter and analyzed fungal development. GFP–HvBI-1 expression was confirmed by PCR and by observation of the fluorescence of GFP–HvBI-1 at the nuclear envelope and in the endoplasmic reticulum in all transgenic plants used for further analysis (Fig. 5, which is published as supporting information on the PNAS web site). Root development in all independent GFP–HvBI-1 barley lines tested was macroscopically indistinguishable from wild type. We microscopically observed development of *P. indica* in GFP–HvBI-1 barley. Fungal epiphytic growth and sporulation were not strongly affected by GFP–HvBI-1. In contrast, invasive inter- and intracellular fungal growth was significantly reduced in GFP–HvBI-1 roots at 20 dai. To quantify the impact of GFP–HvBI-1 on fungal proliferation, the amount of *P. indica* was measured by quantitative PCR. At 20 dai, the relative amount of *P. indica* DNA in transgenic plants was only 20–50%, compared with wild-type plants depending on the transgenic line tested (Fig. 4*b*).

Discussion

P. indica and barley form a mutualistic symbiosis in which the endophyte colonizes the plant root, proliferates by inter- and intracellular growth and produces chlamydospores in dead root tissue. After establishment of the symbiosis the fungus confers improved growth, disease resistance and abiotic stress tolerance

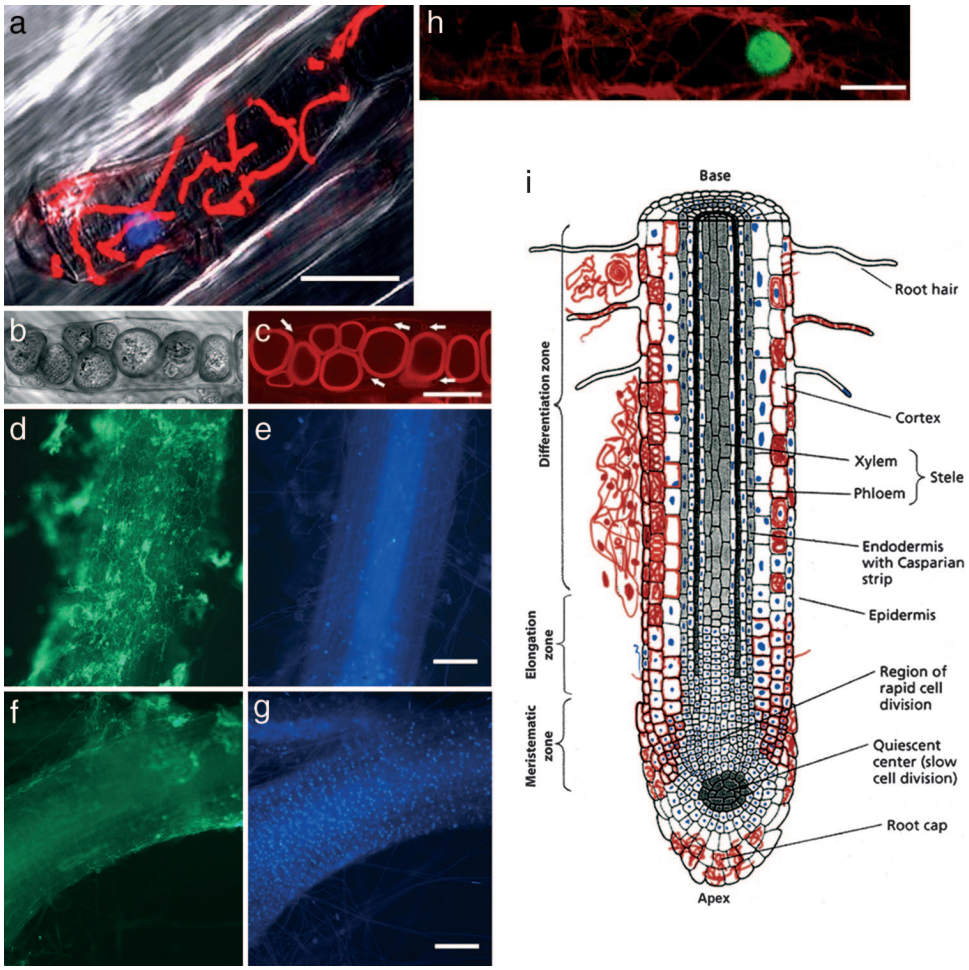

Fig. 3. Association of fungal structures with living and dead cells of the host tissue. (*a*) Fungal hyphae swathe a plant protoplast, which undergoes cytoplasmic shrinkage. Hyphae and nucleus stained with WGA-TMR and DAPI, respectively, are superimposed with the bright-field image. (*b*) Bright-field interference contrast image of chlamydospores in a root cortex cell. (*c*) Fluorescence image of the same cell stained with fuchsin-lactic acid. Arrows indicate hyphae on which the chlamydospores are formed. (*d–g*) Root colonization spatially associated with the absence of intact plant nuclei. Root segments (60 hours after inoculation) double-stained for intact plant nuclei (DAPI; *e* and *g*) and fungal hyphae (WGA-AF 488; *d* and *f*). (*d* and *e*) A root segment heavily colonized by fungal hyphae (*d*) contains only a few DAPI-stained nuclei (*e*). (*f* and *g*) A root segment with minor fungal colonization (*f*) contains a high number of DAPI-stained nuclei (*g*). (*h*) Hyphae swathing a cortical cell protoplast with a TUNEL-positive (green) nucleus. (*i*) Schematic drawing of a *P. indica*-infested root showing the different tissues and the associated colonization pattern, with hyphae depicted in red and DAPI-positive plant nuclei depicted in blue. (Scale bars: *a*, 30 μm; *c*, 10 μm; *d–g*, 300 μm; and *h*, 20 μm.) [Modified from ref. 37 (Copyright 1998, Sinauer, Sunderland, MA).]

to the host plant. Based on the nucLSU sequences our data show that strains of the *S. vermifera* species complex (Sebacinales group B) are closely related to *P. indica* (Fig. 1). These strains yield comparable biological activities in terms of biomass increase and systemic resistance to the biotrophic powdery mildew fungus (Table 1). Hence, the order Sebacinales, of which *P. indica* is considered a model organism, is a source of endophytes with a prospective agronomical impact.

To gain a better understanding of the cellular events leading to the establishment of the mutualistic symbiosis, we microscopically analyzed the interaction of the fungus with the root during the first days of development. After germination of chlamydospores, fungal hyphae grow closely aligned to the topography of rhizodermal cells before penetration of the root at the anticlinal interface of adjacent rhizodermal cell walls (Fig. 2 *b–e*). At such sites, hyphal branching initiates the formation of subepidermal intercellular networks. Intercellular growth is followed by the penetration of rhizodermal cells, which preferentially occurs in differentiated tissue. In young differentiated tissue, single penetrated cells are completely filled with fungal hyphae (Fig. 2 *f* and *g*). Such cells may provide resources for further invasive fungal growth. Mature root tissue is occupied by a network of intracellular hyphae, whose cell to cell "movement" is indicated by hyphal constrictions ("necks"; see Fig. 2*h*). In either case, fungal colonization proceeds by intra- and intercellular infestation of surrounding tissue and gradually increases with tissue maturation. Further proliferation of fungal hyphae finally leads to the development of extra- and intraradical "mats" of hyphae. At this stage, we visualized a clear spatial association of dead root tissue with strong mycelial growth. Dead tissue is characterized by the absence of intact plant nuclei, which were detectable in adjacent, less infected tissue (Fig. 3 *d–g*). This close association of host cell death with massive fungal growth suggests that the fungus contributes to host cell death. Although *P. indica* can induce cell death in poplar under specific conditions on artificial medium (18), fungal culture filtrate did not show any phytotoxic activity on barley (data not shown). At particular interaction sites, we obtained cytological evidence that the fungus can attack and enwrap living (DAPI-positive) protoplasts (Fig. 3*a*). Because *P. indica* can grow between and penetrate into living cells, we suggest that close association of the fungus with living tissue contributes to host reprogramming and, finally, cell death. The

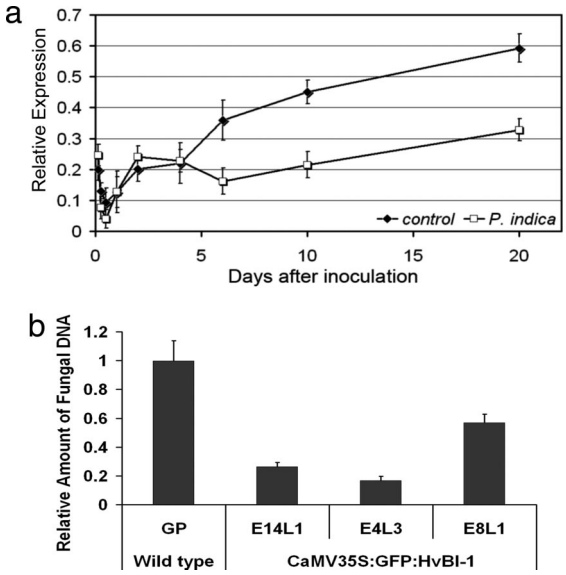

Fig. 4. Influence of HvBI-1 on the development of *P. indica* in barley roots. (*a*) Quantitative PCR analysis of *HvBI-1* expression. As compared with noncolonized roots, expression of the gene is significantly lower from 7 dai onward up to 20 dai. Error bars represent standard deviations. (*b*) The relative amount of *P. indica* DNA in transgenic GFP-HvBI-1 roots was determined at 20 dai. Error bars represent standard deviations. GP represents wild-type plants (Golden Promise). E14L1, E4L3, and E8L1 represent independent transgenic GFP-HvBI-1 GP lines, with five plants tested per line. All three lines were significantly different from the wild type ($P < 0.005$, Student's *t* test). Similar results were obtained in three experiments with plants of an independent transgenic GFP-HvBI-1 line.

inter- and intracellular growth pattern indicated that the fungus is also able to digest plant cell walls and we observed the elimination of transverse cell walls of adjoining cortex cells colonized by the fungus and/or filled with spores (data not shown). In summary, these observations indicate the fungus' capacity to attack and enter host tissue and to proliferate and sporulate in dead cells, most notably in the root differentiation zone (Figs. 2*a* and 3*i*). To obtain molecular evidence for a requirement of host cell death regulation, we analyzed the role of the cell death regulator HvBI-1. Although levels of *HvBI-1* mRNA slowly increased during barley root development, *P. indica*-colonized roots showed a significant reduction of *HvBI-1* mRNA levels compared with noncolonized roots from 7 dai onward (Fig. 4*a*). These data suggest a lowered threshold for PCD in endophyte-colonized roots and support the idea that *P. indica* influences intrinsic plant PCD. In barley leaves, *HvBI-1* is strongly expressed in incompatible interactions with the obligate biotrophic leaf pathogen *B. graminis* (15) and may have a role in restricting resistance-associated hypersensitive cell death reactions. The fact that *P. indica* attenuates expression of *HvBI-1* therefore indicates that PCD observed in the interaction with *P. indica* is different from hypersensitive cell death in pathogen defense. It remains to be shown what kind of PCD might be controlled by *P. indica*.

Previous work showed that HvBI-1 has a central role in the outcome of host–pathogen interactions (16, 19, 20). To functionally confirm the role of host PCD and a requirement of *HvBI-1* down-regulation for fungal proliferation, we constitutively overexpressed a GFP–HvBI-1 fusion protein in barley. All transgenic lines showed enhanced resistance to cell death induced by transient expression of mouse BAX in epidermal leaf cells (R. Eichmann, unpublished results). Comparison of the transgenic plants with the respective wild type showed a significant reduction of invasive growth of *P. indica* in GFP–HvBI-1 barley at 20 dai, when fungal proliferation is in a steady state. In contrast, transient overexpression of *HvBI-1* in barley leaf epidermis supported early biotrophic invasion of *B. graminis* into resistant barley (15, 21). Additionally, all GFP–HvBI-1 lines that restricted proliferation of *P. indica* showed enhanced susceptibility to a virulent isolate of *B. graminis*. This effect relied on a lower ability of the plant to stop the fungus by hypersensitive cell death, which is involved in basal barley disease resistance (V. Babaeizad, R. Eichmann, and J.I., unpublished results). Hence, the expression level of HvBI-1 might inhibit or support fungal proliferation depending on the microbial lifestyle. Quantification of *P. indica* confirmed that fungal growth was significantly restricted in GFP–HvBI-1 barley (Fig. 4*b*). Taken together, we provide genetic evidence that *P. indica* requires host cell death for successful proliferation. We suggest that the mutualistic symbiosis between *P. indica* and barley involves a sophisticated regulation of the plant's cell death machinery. The close spatial association of root cell death with massive infestation by *P. indica* might reflect the fungus' success to manipulate host cell PCD. Thereby *P. indica* might take advantage of naturally occurring root cell death in mature parts of the root. However, the main part of the root further develops and is not necrotized when colonized by the fungus.

Conclusion and Perspectives

The mutualistic symbiosis of crop plants and Sebacinales has a great potential for sustainable agriculture. In contrast to AMF, *P. indica* and other members from the same order mediate resistance to root pathogens and systemic resistance to biotrophic leaf pathogens. From an agronomical point of view, it is most promising that *P. indica* can enhance crop yield in cereals (10). Exploitation of endophytic fungi like *P. indica* may, however, not only complement crop production strategies, which presently rely on a high input of fungicides, but additionally may be an eminent source of molecular traits affecting both disease resistance and grain yield in cereals. For future utilization, it is important to gain additional information on effective application strategies (e.g., spore formulation), growth conditions, and the influence of environmental factors. The prospected huge biodiversity in the Sebacinales (11) and the physiological variation between the Sebacinales strains yield the perspective that for a given crop plant an optimal sebacinalean mutualist might become available. This latter notion is supported by the results of our molecular phylogenetic analysis (Fig. 1), which shows that the type of the interaction between Sebacinales and their plant hosts is probably influenced to a greater extent by the plant than by the fungus. Strains of the *S. vermifera* species complex that interacted with barley similar to *P. indica* were originally isolated from Australian orchids (11). In orchid mycorrhizae, however, the fungus invades vital cortical root cells of the host to form intracellular hyphal coils. The strains tested in the present study also are closely related to members of the Sebacinales that form cavendishioid mycorrhizas (14) with certain hemiepiphytic ericads (Fig. 1). In this mycorrhizal association, the fungal partner also predominantly invades vital cortical cells. It is evident that the mutualistic symbiosis between plants and fungi of the Sebacinales is a treasure chest to discover mechanisms to protect plants from biotic and abiotic stresses. Although evidence has been provided that the plant's antioxidant system plays a pivotal role in the *P. indica*-mediated stress tolerance (10), the precise mechanism and underlying signaling pathways remain to be elucidated. In this respect, *P. indica* has another important advantage: In contrast to AMF, *P. indica* colonizes *Arabidopsis*, and our recent results provide evidence that the fungus induces systemic resistance in this model plant similar to the resistance provided to the powdery mildew fungus in barley (our unpublished data). The power of the *Arabidopsis* signal transduction mutants available and reverse genetics will soon accelerate

disclosure of the molecular basis of the symbiosis and its beneficial effects on the host. Despite this perspective, differences in signaling pathways relevant for agronomically important traits exist between *Arabidopsis* and cereals, justifying strong emphasis on future cereal research.

Materials and Methods

Plant and Fungal Material and Plant Inoculation. Barley (*Hordeum vulgare* L.) cultivar Golden Promise was obtained from Jörn Pons-Kühnemann (University of Giessen, Giessen, Germany). *P. indica* isolate *WP2* was propagated as described (10). *S. vermifera* isolates (culture collection numbers; see Table 1) were propagated in MYP medium (aqueous solution of 7 g/liter malt extract, 1 g/liter peptone and 0.5 g/liter yeast extract).

For inoculation, barley kernels were sterilized with 6% sodium hypochloride, rinsed in water, and germinated for 2 days. Subsequently, seedling roots were immersed in an aqueous solution of 0.05% Tween-20 containing 5×10^5 ml^{-1} *P. indica* chlamydospores or homogenized mycelial solution (1 g/ml) of *S. vermifera*, respectively. Inoculated seedlings were grown in a 2:1 mixture of expanded clay (Seramis; Masterfoods, Verden, Germany) and Oil Dri (Damolin, Mettmann, Germany) (10).

Molecular Phylogenetic Analysis. We used nuclear DNA sequences coding for the 5′ terminal domain of the ribosomal large subunit to estimate the phylogenetic position of the Sebacinales strains used in the present study. An alignment covering a representative sampling of nucLSU sequences available for this fungal group was constructed with MAFFT 5.850 (22). The alignment was analyzed by using heuristic maximum likelihood as implemented in PHYML 2.4.4 (23), with a general time-reversible model of nucleotide substitution and additionally assuming a percentage of invariant sites and Γ-distributed substitution rates at the remaining sites (GTR+I+G; the Γ distribution approximated with four discrete rate categories), starting from a BIONJ tree (24). All model parameters were estimated by using maximum likelihood. Branch support was inferred from 1,000 replicates of nonparametric maximum-likelihood bootstrapping (25), with model parameters estimated individually for each bootstrap replicate. Additionally we performed a Bayesian Markov chain Monte Carlo analysis with MrBayes 3.1 (26). We ran two independent Markov chain Monte Carlo analyses, each involving four incrementally heated chains over two million generations, using the GTR+I+G model of nucleotide substitution and starting from random trees. Trees were sampled every 100 generations, resulting in an overall sampling of 20,000 trees per run, from which the first 5,000 trees of each run were discarded (burn in). The remaining 15,000 trees sampled in each run were pooled and used to compute a majority rule consensus tree to get estimates for the posterior probabilities. Stationarity of the process was controlled by using the Tracer program (27).

Generation of Transgenic Barley Plants. For constitutive overexpression and for tagging expression, we cloned a cDNA fusion of GFP and *HvBI-1* by digestion of pGY1-CaMV35S::GFP–HvBI-1 (15, 21) into appropriate sites of the binary vector pLH6000 (DNA Cloning Service, Hamburg Germany), which was then introduced into *Agrobacterium tumefaciens* strain AGL1 (28) to transform barley cultivar Golden Promise as described (29, 30). PCR analysis was used to confirm integration of the transfer DNA. The GFP reporter was visualized with either a standard fluorescence microscope or a confocal laser scanning microscope as described below.

Root Fixation, Staining and Microscopy, and DAPI Staining. Root segments were fixed as described in ref. 31, with noted exceptions. Fixed root segments were transferred to an enzyme solution containing 10 mg/ml driselase and chitinase, 16 mg/ml β-D-glucanase (InterSpex Products, San Mateo, CA) and 1 mg/ml BSA (Sigma, St. Louis, MO) dissolved in 25 mM phosphate buffer (PB) (4.0 g of NaCl/0.1 g of KCl/0.7 g of Na_2HPO4 $2H_2O$/0.1 g of KH_2PO4 in 500 ml water, pH 6.8) at room temperature for 15 min. After rinsing in PB, roots were further treated with 0.5% Triton X-100 in PB for 10 min. After additional rinsing in PB, plant nuclei were stained with 1 μg/ml DAPI for 30 min. During incubation, segments were vacuum-infiltrated three times for 1 min at 25 mmHg (1 mmHg = 133 Pa) and then rinsed with PB. Additionally, root material was stained with WGA-AF 488 as described below. All segments were analyzed with an Axioplan 2 microscope (excitation 365 nm and emission 420–540 nm; Zeiss, Jena, Germany).

A TUNEL assay was performed using an *in situ* cell death detection kit (Fluorescein; Roche Applied Science, Penzberg, Germany) according to the instruction manual. Root segments were fixed as described above. In addition, root segments were dehydrated and dewaxed by passage for 15 min through series of increasing concentrations of ethanol in water (from 10% to 100% in 10% increments) and back from 100% to 0% in 10% increments). Subsequently, segments were incubated in 50 μl of TUNEL reaction mixture. Grade 1 DNase I-treated roots were used as positive controls. Solutions were vacuum-infiltrated as described above and incubated for 60 min at 37°C in humidified atmosphere in the dark. Subsequently, segments were washed and transferred to 1× PB (pH 7.4) for destaining. Destained segments were counterstained with wheat germ agglutinin-tetramethylrhodamine (WGA-TMR) as described below. TUNEL-positive nuclei were excited at 488 nm and detected at 505–540 nm. Fluorescein diacetate vitality staining and actin staining of barley root was performed according to refs. 32 and 33, respectively.

Staining of *P. indica* in Root Tissue. Hyphae in root segments were either stained by 0.01% acid fuchsin-lactic acid (10) or with the chitin-specific dyes WGA-AF 488 and WGA-TMR (Molecular Probes, Karlsruhe, Germany). Depending on the studies, root material was either fixed for some experiments, dehydrated as described above, or transferred to trichloroacetic acid fixation solution [0.15% (wt/vol) trichloroacetic acid in 4:1 (vol/vol) ethanol/chloroform]. Subsequently, segments were incubated at room temperature for 10 min in 1× PBS (pH 7.4) containing each respective dye at 10 μg/ml. During incubation, segments were vacuum-infiltrated three times for 1 min at 25 mmHg. After rinsing with 1× PBS (pH 7.4), segments were mounted on glass slides. In cases that Congo red (Merck, Darmstadt, Germany) was used for counterstaining, it was added to WGA-AF 488 staining solution at a final concentration of 10 μg/ml. Confocal fluorescence images were recorded on a multichannel TCS SP2 confocal microscope (Leica, Bensheim, Germany). WGA-AF 488 was excited with a 488-nm laser line and detected at 505–540 nm. WGA-TMR was excited with a 543-nm laser line and detected at 560–630 nm. All segments that were analyzed with an Axioplan 2 microscope were either excited at 470/20 nm and detected at 505–530 nm for WGA-AF 488 or excited at 546/12 nm and detected at 590 nm for Congo red.

Genomic DNA Isolation, Real-Time PCR, and Transcript Analysis. The degree of root colonization was determined by using the $2^{-\Delta Ct}$ method (34). Cycle threshold (Ct) values were generated by subtracting the raw Ct values of the *P. indica* internal transcribed spacer or *Tef* gene (35) from the raw Ct values of plant-specific ubiquitin.

Roots were harvested, frozen, and ground in liquid nitrogen, and genomic DNA was isolated from ≈100 mg of root powder with the Plant DNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For quantitative PCR, 5–10 ng of total DNA was used. Amplifications were performed in 20

μl of SYBR green JumpStart *Taq* ReadyMix (Sigma–Aldrich, Munich, Germany) with 350 nM oligonucleotides, using an Mx3000P thermal cycler (Stratagene, La Jolla, CA). After an initial activation step at 95°C for 7 min, 40 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 82°C for 15 s) were performed, and a single fluorescent reading was obtained after the 82°C step of each cycle. A melting curve was determined at the end of cycling to ensure amplification of only a single PCR product. Ct values were determined with the Mx3000P V2 software supplied with the instrument.

For quantitative two-step RT-PCR, 2 μg of total RNA were reverse-transcribed to first-strand cDNA with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Aliquots of 20 ng of first-strand cDNA were subsequently used as a template for quantitative PCR with gene-specific primers. The plant-specific ubiquitin gene served as a control for constitutive gene expression in roots. Ubiquitin expression was consistent after inoculation with *P. indica* when compared with the amount of 18S ribosomal RNA. Specific PCR conditions were as described above, and comparative expression levels ($2^{-\Delta Ct}$) were calculated according to ref. 36. Expression levels are relative to the level of ubiquitin expression, which was constant in all RNA samples used and was set to 1. Values are the means of four samples of one biological experiment (infected roots) assayed by quantitative PCR in triplicate. The oligonucleotides used were as follows: ubiquitin (accession no. M60175), 5′-CAGTAGTGGCGGTCGAAGTG-3′ and 5′-ACCCTCGCCGACTACAACAT-3′; *P. indica* Tef (accession no. AJ249911) 5′-ACCGTCTTGGGGTTGTATCC-3′ and 5′-TCGTCGCTGTCAACAAGATG-3′; Bax inhibitor-1 (accession no. AJ290421) 5′-GTCCCACCTCAAGCTCGTTT-3′ and 5′-ACCCTGTCACGAGGATGCTT-3′; and *P. indica* ITS (accession no. AF 019636) 5′-CAACACATGTGCACGTCGAT-3′ and 5′-CCAATGTGCATTCAGAACGA-3′

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5.7 Qiang, X., Zechmann, B,. Kogel, K.H., and **Schäfer, P.** Endoplasmic reticulum stress induction by *Piriformospora indica* initiates a caspase-dependent cell death to achieve root compatibility. Submitted to *Cell Host and Microbe* (Under review at the Editorial Board).

Induction of ER Stress by *Piriformospora indica* Initiates a Caspase-Dependent Cell Death to Achieve Root Compatibility in Arabidopsis

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Running Title

Mutualistic cell death in *Arabidopsis* roots

SUMMARY

Colonization of *Arabidopsis* roots by the mutualistic sebacinoid fungus *Piriformospora indica* comprises an initial biotrophic followed by a host cell death-associated interaction phase where fungal reproduction takes place. We aimed to clarify the molecular base of colonization-associated cell death which is unique to sebacinoid symbiosis. Cytological analysis revealed endoplasmic reticulum (ER) swelling and vacuolar collapse indicative of ER stress induction during symbiotic *P. indica* colonization. Consistently, tunicamycin-triggered ER stress was enhanced in colonized roots although canonical markers for ER stress sensing (e.g. *bZIP60*) and the unfolded protein response (UPR) (e.g. *BIP3*) were at the same time suppressed. *Arabidopsis* mutants compromised in caspase 1 activity and cell death-mediating vacuolar processing enzymes (VPEs) showed reduced fungal colonization and concomitantly decreased cell death incidences in colonized roots. We hypothesize that *P. indica* intervenes with ER stress signalling thereby activating a VPE-mediated cell death, which is required for establishing a successful symbiosis with *Arabidopsis.*

Highlights

Piriformspora indica disturbs ER integrity during root colonization
ER stress induction results in the activation of programmed cell death (PCD)
ER stress-induced PCD is dependent on vacuolar processing enzymes (VPEs)
Root colonization by *P. indica* is dependent on VPE-/caspase-mediated PCD

INTRODUCTION

The endoplasmic reticulum (ER) is a convergent point in the processing of glycoproteins destined for secretion. These secretory proteins pass the ER in order to obtain their proper three dimensional structures, which is a prerequisite for their functionality. The ER essentially contributes to the protein composition of the plasma membrane, extracellular matrix (apoplast), and vacuoles. Because of these functions, the ER is intimately involved in plant developmental processes as well as plant innate immune responses. For instance, the proper function of the plasma membrane-localized immune receptor EFR is dependent on proper ER processing (Nekrasov et al., 2009; Saijo et al., 2009). In addition, defective proteins are recognized by the ER machinery and are discarded as demonstrated for the plasma membrane standing brassinosteroid receptor BRI-1 (Jin et al., 2007). Moreover, a considerable number of anitmicrobial proteins have an apoplastic destination and traverse the ER and Golgi apparatus prior to their vesicle-mediated transport and exocytosis at the plasma membrane (Jelitto-van Dooren et al., 1999; Wang et al., 2005; Lipka et al., 2007; Kwon et al., 2008). Accurate protein folding is controlled by at least three systems, which are mutually defined as ER quality control (ER-QC): 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 are *N*-glycosylated by the oligosaccharide transferase complex (OST) and bind to the SDF2-ERdj3b-BIP complex. Thereafter, protein folding is mediated by the composed action of calreticulins (CRTs) and calnexins (CNXs). In certain glycoproteins, intramolecular disulfide isomerization is eventually required for correct folding, which is executed by proteins disulfide isomerases (PDIs). Misfolded proteins are sorted and degraded by cytosolic proteasomes (Anelli and Sitia, 2008; Vitale and Boston, 2008). The ER processing machinery is apparently highly conserved among eukaryotes including plants (Liu and Howell, 2010). ER working load and, thus, ER-

QC activities varies depending on the developmental stage, the type of tissue, or the occurrence of external stresses. In case ER-QC does not meet the demand of protein processing, ER stress is triggered by the enrichment of misfolded proteins. In mammals, ER stress is sensitized by the ER membrane standing receptors inositol requiring protein-1 (IRE1), activating transcription factor-6 (ATF6), and protein kinase RNA (PKR)-like ER kinase (PERK), which activates the unfolded protein response (UPR) (Schröder, 2006). UPR functions as an adaptive process of cells to enhance quality control and to relieve ER stress (Malhotra and Kaufman, 2007). In mammals, the UPR consists of induction of ER chaperones, elevated ER-associated degradation (ERAD), and attenuated translation of secreted proteins (Malhotra and Kaufman, 2007). 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. ER stress-induced apoptosis relies on the activation of a set of cell-death associated cysteine proteases, so called caspases (Szegezdi et al., 2006; Rasheva and Domingos, 2009). In contrast to the knowledge on plants' ER-QC, ER stress sensing/-signaling and ER stress-induced cell death are less well understood albeit molecular studies implicate that ER stress sensing/signaling and UPR is conserved in plants (Kamauchi et al., 2005; Vitale and Boston, 2008). Recent studies identified the transcription factors bZIP28 and bZIP60 as ER membrane-localized ER stress sensors that are involved in the induction of UPR (Liu et al., 2007; Iwata et al., 2008). Both proteins are very similar to mammalian bZIP transcription factor ATF6 (Urade, 2009). Moreover, the ER is known to participate in plant PCD pathways as it modulates drought stress-induced PCD (Duan et al., 2009) or even initiates PCD as a result of ER dysfunction (Malerba et al., 2004; Watanabe and Lam, 2006). While a regulatory role of the ER in plant cell death initiation has been demonstrated (Watanabe and Lam, 2009), the molecular basis of PCD initiation and execution in response to ER stress remained elusive.

In the present study, we investigate the colonization strategy of the mutualistic fungus *Piriformospora indica* in *Arabidopsis* roots. Our recent studies indicated a biphasic colonization strategy (Schäfer, unpublished data): After an initial colonization of living cells (biotrophic phase), a cell-death associated colonization strategy was observed which is consistent with previous reports on interactions of *P. indica* with barley (Deshmukh et al., 2006). In barley, the latter phase involved regulation of ER membrane-localized Bax-Inhibitor 1 (BI-1) raising the possibility that the fungus recruits a host cell death program in order to establish a successful interaction. Based on cytological and molecular studies, we suggest here that *P. indica* successfully colonizes *Arabidopsis* roots by triggering ER stress and simultaneously suppressing the adaptive UPR. Furthermore we hypothesize that the inability of colonized plant cells to relieve ER stress results in the induction of a caspase-dependent vacuolar cell death program. We propose a model in which fungal colonization success is intimately dependent on ER stress-induced cell death.

RESULTS

P. indica Impairs the Integrity of the Endoplasmic Reticulum thereby Improving Mutualistic Root Colonization

We performed transmission electron microscopy, to analyze subcellular changes during cell death-associated colonization of *Arabidopsis* roots by *P. indica*. Colonization-associated cell death was characterized by the lysis of cytoplasm, followed by swelling of the ER, and tonoplast rupture. In contrast, plastids and mitochondria remained ultrastructurally unaltered (Figures 1A and 1B). As these studies indicated ER disintegration by *P. indica*, colonization of *Arabidopsis* line GFP-tmKKXX, in which the ER is GFP-tagged (Benghezal et al., 2000), was analyzed by confocal laser-scanning microscopy. While ER structures in non-colonized cells of this line remained intact, *P. indica*-colonized cells showed ER collapse (Figure 1C).

As our cytological analyses implied an impairment of the ER by the fungus, we examined to what extend the disturbed ER function would affect colonization success. *Arabidopsis* mutants were selected that lack central components of the ER-QC. These mutants, which were deficient in the chaperone BIP2 (binding protein 2, *bip2*), in a subunit of the oligosaccharide transferase DAD1 (defender against apoptotic death 1, *dad1*), or in a component of the SEC61 translocon complex (SEC61α, *sec61α*), were analyzed for fungal colonization. To this end, *bip2*, *dad1*, *sec61α*, and parent line Col-0 were checked for altered colonization at 3 and 7 days after inoculation (dai) with *P. indica* by qRT-PCR. All mutants exhibited enhanced fungal colonization rates at 7 dai as compared to Col-0 (Figure 1D). suggesting that impaired ER-QC supports fungal growth in the roots. As altered colonization might be explained by malfunctional immunity in these ER-QC mutants, we examined their responsiveness to microbe-associated molecular patterns (MAMPs). In the first assay, we analyzed the flg22-induced seedling growth inhibition (SGI) in Col-0 and mutant plants. The plant biomass of Col-0 and all mutants was significantly reduced by flg22 treatment, as opposed to flg22-

insensitive *fls2c* mutant (Figure S1A). Secondly, we analysed the occurrence of flg22-, or chitin-induced oxidative burst in mutant roots. Consistently, we observed transient root oxidative bursts upon treatment with flg22 or chitin in *bip2*, *dad1*, *sec61α* mutants and Col-0 but not in flg22- and chitin-insensitive mutants *fls2c* and *cerk1-2*, respectively (Figure S1B, C), indicating intactness of MAMP-triggered immunity in the mutants.

P. indica-Colonized Plants Are Hypersensitive to ER Stress but Disturbed in the Unfolded Protein Response

Since analysis of fungal growth in roots indicated improved colonization of mutants lacking crucial components of the ER-QC, we investigated whether *P. indica* affects tolerance of the colonized plants to ER stress. We applied ER stress inducer tunicamycin (TM), which specifically blocks UDP-*N*-acetylglucosamine:dolichol phosphate *N*-acetylglucosamine-1-P transferase and thereby inhibits protein *N*-glycosylation in the ER (Pattison and Amtmann, 2009). *P. indica*-colonized (3 dai, biotrophic stage) and mock-treated Col-0 plants were treated with TM or DMSO (control). Plant fresh weights were determined 7 days after treatment. In non-colonized plants, TM treatment resulted in a ~ 20% reduction of fresh weight compared to DMSO-treated plants. Significantly, in *P. indica*-colonized plants, TM reduced the plant biomass by ~ 60% as compared to untreated controls (Figure 2A). This suggests that *P. indica*-colonized plants are hypersensitive to ER stress.

Since our data suggest induction of ER stress by *P. indica*, we next tested whether colonization-associated ER stress might result in both activation of ER stress sensing and subsequent UPR. To this end, we harvested *P. indica*-colonized and non-colonized roots at 1, 3, and 7 dai and monitored gene expression levels of putative ER stress sensors (*bZIP17*, *bZIP28*, *bZIP60*) and markers of the UPR (*sPDI*, *BIP3*, *CNX2*) by qRT-PCR. Unexpectedly, none of the tested genes was induced during colonization (Figure 2B). Instead, *bZIP28*, *BIP3*, and *CNX2* were even suppressed in colonized roots at some of the time points (Figure 2B). 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, we treated non-colonized and *P. indica*-colonized roots (3 dai, biotrophic stage) with tunicamycin 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*) were analyzed by qRT-PCR. All genes (except *bZIP28*) were induced by TM treatment (Figure 2C). By contrast, all candidates (except *bZIP28*) exhibited a reduced induction by TM treatment in *P. indica*-colonized roots (Figure 2C). To further support this finding, we investigated if impaired ER stress signaling was also detectable at the protein level. First, we tested the accumulation of the luminal binding protein (BIP) in dependence of *P. indica* colonization as described above. *P. indica*-colonized and non-colonized roots were harvested at 0, 3, and 7 dai and subjected to immunoblot analyses using a polyclonal anti-BIP antibody. Consistent with our qRT-PCR analyses, BIP protein levels were reduced in colonized roots particularly at 7 dai as compared to non-colonized roots (Figure 2D). In addition, we monitored BIP accumulation after TM treatment in dependence of *P. indica* colonization. For this, *P. indica*-colonized (3 dai) or non-colonized roots were treated with TM or DMSO (control) and roots were harvested two days later. BIP accumulated in response to TM treatment in non-colonized roots (Figure 2E). By contrast, this accumulation was clearly suppressed by *P. indica*. Taken together, the analyses suggest that the fungus is affecting ER stress signaling.

Vacuole-Mediated Cell Death Is Downstream of ER Stress Induction and Affects Mutualistic Root Colonization

Our electron microscopical studies indicated co-occurrence of ER swelling and vacuolar collapse in colonized cells at later interaction stages (Figure 1B). This prompted us to test whether vacuole collapse is essential for root colonization. Vacuolar processing enzymes (VPEs) were shown to mediate vacuolar collapse and execution of virus-induced cell death

(hypersensitive response) in tobacco (Hatsugai et al., 2004). VPEs have a small gene family consisting of four members (αVPE, βVPE, γVPE, δVPE) (Hatsugai et al., 2006). In a first assay, we quantified root colonization of *αvpe, βvpe, γvpe, δvpe,* and the quadruple mutant *vpe-null*, which is deficient in the four VPEs, at 3 and 7 dai by qRT-PCR. We observed significantly reduced colonization of *αvpe*, *γvpe*, and *vpe-null* mutants at 7 dai, while *βvpe* and *δvpe* mutants showed little if any enhancement in colonization (Figure 3), indicating that VPE-mediated activities contribute to successful fungal colonization. Interestingly, *γvpe* and *vpe-null* mutants showed higher fungal colonization at earlier stages (3 dai), consistent with the view that cell death-mediating enzymes are obstructive on the biotrophic phase. Since severe ER stress results in cell death in mammalian organisms, we were also interested to test if VPE-mediated cell death acts downstream of ER stress induction. Therefore, we generated *γvpe dad1* double mutants and quantified *P. indica* colonization by qRT-PCR. *γvpe dad1* displayed reduced colonization at 7 dai, which resembled the colonization phenotype of *γvpe* (Figure 3). These data suggest that ER stress-induced cell death might be executed by VPEs.

VPE- and Caspase 1 Activities Are Enhanced in *P. indica*-Colonized Roots

In addition to VPE activity, VPEs were shown to have caspase 1 activity. These protease activities was important for vacuolar-medicated plant cell death execution (Hatsugai et al., 2004; Kuroyanagi et al., 2005). To confirm the significance of vacuolar-mediated cell death for root colonization, we measured VPE- and caspase 1 activities in wild type, *γvpe*, *vpe-null*, *dad1*, and *γvpe dad1* roots in dependence of *P. indica* colonization. To this end, we set up an assay 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). We applied either 1 mM of VPE-specific substrate Ac-ESEN-MCA or caspase 1-specific substrate Ac-YVAD-MCA to root extracts and spectrometrically measured VPE-mediated cleavage of MCA. Both, VPE and caspase 1 activities were increased in *P. indica* colonized compared to non-colonized

roots (Figure 4A). As expected, enzyme activities were weakly detectable in *vpe-null* (Figure 4A). We could hardly detect caspase 1 activity in *γvpe* roots and VPE activity was strongly reduced as compared to wild type roots suggesting that in roots γVPE might be mainly responsible for both enzyme activities. Interestingly, although non-colonized *dad1* roots displayed basal levels of VPE and caspase 1 activities, enzyme activities were strongly enhanced in colonized *dad1* roots and even higher when compared to respective wild type roots (Figure 4A). In order to confirm whether reduced colonization of the double mutant *γvpe dad1* might be associated with altered enzyme activities, we measured VPE- and caspase 1 activities in dependence of *P. indica* colonization. Both enzyme activities were hardly detectable in colonized or non-colonized *γvpe dad1* roots and resembled enzyme activities detected in *γvpe* (Figure 4B) In summary, the results of the enzyme activity assay are consistent with the colonization phenotypes (Figures 1D and 3) suggesting that VPE- and caspase 1 activities might be essential for cell death execution and successful colonization.

ER Dysfunction Enhances *P. indica*- and TM-Induced Cell Death in a VPE-Dependent Way

In order to relate our enzyme and colonization studies to the occurrence of cell death, we performed a fluorescein diacetate (FDA)-based assay. In viable cells, esterases will cleave off the fluorescein and the degree of cleavage can be quantified spectrometrically. In a first assay, we treated *bip2*, *dad1*, *vpe-null*, *γvpe, γvpe dad1* mutants and respective wild type plants with TM and stained root segments with FDA at 3 days after treatment (dat). *bip2* and *dad1* mutants showed ~ 20% less fluorescence intensity than wild type segments, which indicated a reduced number of living cells (Figure 4C). By contrast, *vpe-null* and *γvpe* mutants showed an increase of fluorescence intensity (> 30%) compared to wild type root segments. Similarly, root segments of *γvpe dad1* double mutant exhibited enhanced fluorescence intensities (Figure 4C). 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-null*, *γvpe*, and *γvpe dad1* (Figure 4D). Together, this data confirm enhanced ER stress- and *P. indica*-induced cell death in mutants impaired in ER integrity. Our data identify γVPE as a key factor in the execution of ER stress-induced cell death triggered by fungal colonization in the mutualistic interaction of *P. indica* and *Arabidopsis*.

DISCUSSION

We formerly showed that plants root colonization by the mutualistic fungus *Piriformospora indica* is based on an initial biotrophic followed by a cell death-associated colonization phase (Deshmukh et al. 2006; Schäfer, unpublished data). In the present study, we propose that the colonization-associated cell death in *Arabidopsis* roots is initiated by an uncoupled ER stress response and the suppression of the UPR, which ends up in a vacuolar-mediated, caspase 1-dependent cell death (Figure 5). ER stress during mutualistic colonization was evident from cytological and pharmacological analyses (Figures 1A-C and 2A). Most probably, fungal suppression of ER stress signaling (Figures 2B-E), known as the unfolded protein response (UPR), was essential for cell death initiation. UPR encompasses translational attenuation, induction of ER chaperones (e.g. BIPs), and elevated degradation of misfolded proteins in the ER by the proteasome. By these means, eukaryotic cells aim to relieve ER stress that occurs under abiotic and biotic stress conditions as well as at certain developmental stages (Malhotra and Kaufman, 2007; Vitale and Boston, 2008). Microarray studies 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*) and protein degradation (e.g. *HRD1*, *DER1*) (Kamauchi et al., 2005; Iwata et al., 2008). We found that *P. indica* suppresses TM-induced ER stress as indicated by the expression levels of randomly selected members of the protein folding machinery (*BIP3*, *sPDI*, *CNX2*) in *P. indica*-colonized (3 dai; biotrophic stage) compared to non-colonized roots. In accordance with this, constitutive and TM-triggered BIP protein levels were suppressed by *P. indica*. bZIP28 and bZIP60 are important for UPR regulation and might function as ER stress sensors (Iwata et al., 2008; Liu et al., 2008). However, none of these transcription factors were induced and the expression of *bZIP28* was even suppressed during *P. indica* colonization (Figure 2B). In accordance with studies on leaves, we found only *bZIP60* (and transiently *bZIP17*) induced by TM (Iwata et al.,

2008; Liu et al., 2007) while expression of *bZIP28* was non-responsive to TM (Iwata et al., 2008). Although *bZIP28* was suppressed by *P. indica*, TM application to *P. indica*-colonized roots caused its induction (Figures 2B and C). An explanation might be that the combined stress induced by *P. indica* and TM activates stress pathways, which recruit bZIP28. For instance, bZIP17 was found to sense ER stress induced by salt stress in a way similar to ER stress signaling. Importantly, bZIP28-induced UPR genes (e.g. *BIP3*) were not induced by *P. indica* (Figure 2C).

Failed ER stress adaptation or severe ER stress have been shown to result in the activation of cell death (Szegezdi et al., 2006). In mammals, the principles of ER stress-induced proapoptotic signaling have been intensively studied (Szegezdi et al., 2006; Rasheva and Domingos, 2008). Here, the same plasma membrane localized ER stress sensors that induce UPR also initiate apoptotic signaling under severe ER stress by activating the bZIP transcription factor ATF4, the c-Jun N-terminal kinase (JNK) pathway, and a caspase cascade. Central to this proapoptotic state is the activation of BAX and BIM, which contribute to the execution of apoptosis by enhancing Ca^{2+} release from ER and mitochondria. In addition, BAX and BIM mediate cytochrome *c* release from mitochondria thereby activating the apoptosome (Szegezdi et al., 2006). By contrast, the molecular basis of ER stress-induced PCD in plants is less clear. Several studies implicate, however, a conservation of ER stress signaling between plants and mammals. 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 have not been found in plants, barley BI-1 and other plant BI-1 proteins suppress BAX-induced cell death *in planta* (Eichmann et al., 2006; Watanabe and Lam, 2009). Although its antiapoptotic mode of action is unknown, BI-1 controls Ca^{2+} release from the ER under stress in plants and mammals (Chae et al., 2004; Watanabe and Lam, 2009). *Arabidopsis* plants overexpressing *BI-1* exhibited enhanced TM tolerance, which indicates its antiapoptotic function in ER-PCD (Watanabe and Lam, 2008). In barley, *P.*

indica suppresses *BI-1* transcription and *BI-1* overexpression resulted in reduced *P. indica* colonization (Deshmukh et al., 2006). Consistently, we observed a slight suppression of *AtBI-1* by *P. indica* after TM application (Figure 2C) but did not observe altered *P. indica* colonization in *atbi-1* roots (data not shown). In accordance with mammlian ER-PCD (Szegezdi et al., 2006), caspase 1 activity was enhanced during cell death-associated colonization (7 dai) which mainly relied on γVPE (Figure 4A). Instead, we did not detect altered caspase 1 activity during the biotrophic phase (at 3 dai, not shown). The significance of caspase 1 activity for *P. indica*-induced ER-PCD execution was further shown by the reduced colonization-associated- and TM-induced cell death in *γvpe* (Figure 4C and D). These analyses also underlined the significance of ER dysfunction as ER-PCD initiator. *dad1* mutants displayed an elevated caspase 1 activity during cell death-associated colonization and an increased cell death after TM treatment. However, execution of ER-PCD was crucially dependent on γVPE-/caspase 1 activity as indicated by the highly similar TM- and colonization-induced cell death phenotypes in *γvpe dad1* double mutants and *γvpe* (Figure 4C and D). Consistently, *γvpe dad1* and *γvpe* showed a similar degree of *P. indica* colonization at 7 dai (Figure 3). However, *γvpe dad1* displayed a colonization phenotype comparable to *dad1* at 3 dai being consistent with the alternative hypothesis that ER stress induction is not upstream of γVPE-mediated PCD. But this hypothesis would contradict the virtually identical caspase 1 and VPE activities in *γvpe* and *γvpe dad1* (Figure 4A and B). Moreover, both mutants consistently show reduced cell death after TM application and during late *P. indica* colonization (7 dai) (Figure 4C and D). Therefore, we speculate that, in the biotrophic root colonization (3 dai), *dad1* might counteract a yet unknown process in *γvpe* that is unlinked to ER-PCD. Our studies further indicated that not all VPE members support *P. indica* colonization. *βvpe* and *δvpe* mutants even showed a higher (but insignificant) colonization by *P. indica*. Future studies will reveal if these enzymes have caspase activities or even have an antiapoptotic function. However, αVPE and γVPE might function in the same signaling

cascade as indicated by the similar colonization at 7 dai. The enhanced colonization of *γvpe* at 3 dai (biotrophic stage) might indicate an additional immunity-related function of γVPE. Accordingly, this enzyme was formerly identified to participate in vacuolar collapse associated with virus-induced hypersensitive response (Hatsugai et al., 2004).

Impairment of ER integrity might not only serve PCD induction and, thus, improvement of root compatibility. The ER takes a crucial role in plant innate immunity by processing antimicrobial proteins (Wang et al., 2005). In leaves, focal secretion processes and formation of locally confined cell wall appositions (CWAs) efficiently control colonization by non-adapted biotrophic pathogens. These immune responses base on vesicle-mediated transport processes of compounds partially generated in the ER (Lipka et al., 2005; Hückelhoven, 2007). In addition, specific components of the ER machinery were identified to mediate processing of the pattern recognition receptor (PRR) EFR (Nekrasov et al., 2009; Saijo et al., 2009), which activates innate immunity after recognition of the bacterial MAMP elongation factor TU (Zipfel et al., 2006). Recently, we found that *P. indica* is suppressing early root immune signaling and that this is essential for root compatibility (Schäfer, unpublished data). In the present study, we recorded a disturbance in ER homeostasis as early as 3 dai (Figures 2C-E). It will be interesting to see in future studies to which extent ER dysfunction in colonized roots impair generation and secretion of MTI components (e.g. antimicrobial proteins, PRRs). Furthermore, it is tempting to speculate that impaired ER function also affects vacuole load with antimicrobial proteins. This would explain that VPE-mediated vacuolar collapse is not stopping fungal growth as reported for bacterial pathogens (Hatsugai et al., 2009) but even supporting root colonization by *P. indica*.

EXPERIMENTAL PROCEDURES

Plant, Fungal Material and Plant Inoculation

bip2, *dad1*, and *sec61α* mutants were provided by X. Dong, *αvpe*, *βvpe*, *γvpe*, *δvpe*, and *vpe-null* were provided by I. Hara-Nishimura, *cerk1-2* mutant was provided by V. Lipka, *fls2c* mutant was provided by C. Zipfel, and GFP-tmKKXX was provided by A. Hardham. *γvpe dad1* mutant was generated within this study by crossing *dad1* and *γvpe*. The isolate of *P. indica* DSM11827 was obtained from German collection of microorganisms and cell cultures in Braunschweig, Germany. For inoculation, all *Arabidopsis* seeds were sterilized in 3% sodium hypochlorite and grown on ½ Murashige and Skoog under 8 h light (180 µmol m^{-2} s^{-1} photon flux density)/16 h night, 22°C/18°C, and 60% relative humidity. If not stated otherwise, 3-week-old plant roots were inoculated with *P. indica* chlamydospores at a concentration of 500,000 spores ml^{-1}.

Quantification of Fungal Colonization by QPCR

Genomic DNA was isolated from 100 mg root material with the Plant DNeasy Kit (Qiagen, Hilden, Germany). In QPCR analysis, 40 ng of genomic DNA served as template. The amplification reaction was performed using 10 µl of SYBR green JumpStart Taq ReadyMix (Sigma-Aldrich, Munich, Germany) and 350 nM of oligonucleotides. The standard quantification program from 7500 fast thermal cycler (Applied Biosystems, Heidelberg, Germany) was applied. To determine the relative amount of *P. indica* in plant roots, the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008) was used. The cycle threshold (Ct) values were obtained by subtracting the raw Ct values of *P. indica ITS* from the raw Ct values of *AtUBI5*. The sequences for *AtUBQ5*-specific primers were 5´-CCAAGCCGAAGAAGATCAAG-3´ and 5'-ACTCCTTCCTCAAACGCTGA-3'. The sequences for *P. indica ITS*-specific primers were 5´-CAACACATGTGCACGTCGAT-3´ and 5´-CCAATGTGCATTCAGAACGA-3´.

Gene Expression Analysis by qRT-PCR

For gene expression studies, three-weeks-old plants were inoculated with *P. indica* or mock-treated and harvested at 1, 3, and 7 dai. For tunicamycin treatment, inoculated or mock-treated plants were treated with tunicamycin (TM) (5 μg ml^{-1}) or DMSO (control) at 3 dai. Roots were harvested at 12, 24, 48 and 72 hours after TM treatment. RNA was extracted from homogenized root material using TRIzol (Invitrogen, Darmstadt, Germany). For cDNA synthesis, 500 ng of RNA was DNase-I digestion and transcribed into cDNA using a qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, USA). For QPCR analysis, 10 ng of cDNA was used as template for determing the amplification of candidate genes. As described above the $2^{-\Delta Ct}$ method was applied to evaluate the level of gene expression. Primers were used as listed.

Table 1. List of primers used in this study.

Gene	AGI	Forward	Reverse
BIP3	AT5G28540	GGAGAAGCTTGCGAAGAAGA	ATAACCGGGTCACAAACCAA
BI-1	AT5G47120	GCAGCAGCAATGTTAGCAAG	CACCACCATGTATCCCACAA
CNX2	AT5G07340	AGACTTTGAGCCTCCGTTGA	TCTTCCTCGTCATCCCAATC
sPDI	AT1G77510	GCCACTAAGGCGATGATGTT	GCTCTCTGCATCACCAACAA
bZIP17	AT2G40950	ACAGGAGATCGGGAGAGGAT	GCTCCTCGACGTAATGCTTC
bZIP28	AT3G10800	GCCAGTGATCCTCTCTTTGC	CAGAAGACAGTGCACCAGGA
bZIP60	AT1G42990	CGGAGGAATTTGGAAGCATA	TGCTGATCCAATTCCACAAA

Growth Retardation Assays

For the growth retardation assay with tunicamycin (TM), *Arabidopsis* seedlings were grown on ½ MS medium containing 1% sucrose for 14 days. Plants were inoculated with *P. indica* or mock-treated and transferred to liquid ½ MS medium containing 1% sucrose and TM (25 ng ml^{-1}) or DMSO (control). Seedling fresh weight was determined 7 days after TM/DMSO treatment. For the growth retardation assay with flg22, plants were grown on ½ MS medium

containing 1% sucrose for 14 days and, thereafter transferred to liquid ½ MS medium containing 1% sucrose and 10 µM flg22. Plant fresh weight was determined ten days after treatment. flg22 peptide sequence was used as described (Gomez-Gomez et al., 1999).

MAMP-Induced Root Oxidative Burst and Growth Retardation

Two-week-old plant roots were either treated with 1 µM flg22 or 1 µM *N*-acetylchitooctaose. For determination of the oxidative burst, roots were cut in 1 cm long pieces (10 mg per assay) and subjected to a luminol-based assay as described (Gomez-Gomez et al., 1999).

Protein Extraction and Immunodetection of BIP

Roots were inoculated with *P. indica* or mock-treated and harvested at 1, 3, and 7 dai. For tunicamycin assays, *P. indica* or mock-treated roots were treated with TM 5 µg/ml or DMSO (control) at 3 dai and harvested at 2 dat. Total protein was extracted with a buffer containing 250 mM sucrose, 50 mM HEPES-KOH, 5% glycerine, 1 mM Na_2MoO_4 x $2H_2O$, 25 mM NaF, and 10 mM EDTA. Subsequently, 20 µg of each protein sample was separated by SDS-PAGE and transferred to Roti®-PVDF membrane (Roth, Germany). The proteins were probed with an anti-rabbit IgG-alkaline phosphate antibody (Sigma-Alderich, Germany) matching the *Arabidopsis* anti-BIP antibody (Santa Cruz Biotechnology). The gels were stained in a solution containing (v:v) 20% Coomassie Brilliant Blue R250 (Roth, Germany) and 20% methanol and were later de-stained with a solution of (v:v:v) 40% methanol / 10% glacial acid / 50% water.

Cell Death Assay

Root segments (1.5 cm) from two-week-old plants were transfered to ½ MS containing fluorescein diacetate (FDA). After 10 minutes incubation, 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).

Caspases and VPE Activity Assays

Three-week-old roots were inoculated with *P. indica* or mock-treated, and the roots were harvested at 7 dai. A buffer containing 100 mM sodium acetate (pH5.5), 100 mM NaCl, 1 mM EDTA and 1mM phenylmethylsulfonyl fluoride was applied to get root extracts. 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, Japan) were added to the root extracts. Fluorescence intensities were measured at 465 nm after excitation at 360 nm using a fluorescence microplate reader (TECAN infinite® 200).

Cytological Analyses

Root samples were either fixed or directly stained with chitin-specific WGA-AF488 (Molecular Probes) as described (Deshmukh et al., 2006). Confocal images were recorded on a TCS SP2 microscope (Leica). WGA-AF488 and GFP were excited with a 488 nm laser line and detected at 505-540 nm. For ultrastructural studies, roots were embedded as described (Zechmann et al., 2007) and ultrathin sections (80 nm) were investigated after post-staining with uranyl aceate and lead citrate with a Philips CM10 TEM.

Statistical Analyses

Results are expressed as means ± SD and represent at least three similar experiments. Differences were analyzed with the Student´s t-test. $p < 0.05$ was considered as significant.

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FIGURE LEGENDS

Figure 1. Impaired ER Integrity in *Arabidopsis* Root Cells during Late Colonization Phases (7 dai) of *P. indica* is Correlating with Improved Colonization

(A and 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). Tonoplasts of non-colonized neighbouring cells are intact (asterisk). 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) transgenic plant roots. The fungus penetrated (arrows) two cells and intracellular hyphae are visible (arrowheads). The ER of the upper colonized cell is still intact, while ER disintegration is associated with colonization of the lower cell (asterisk). 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.

(D) Three-week-old *Arabidopsis* WT plants and *sec61α*, *dad1*, and *bip2* mutants were inoculated with *P. indica*. Root samples were harvested at 3 and 7 dai. The fungal biomass at both biotrophic (3 dai) and cell death-associated colonization stages (7 dai) were determined by qRT-PCR using *AtUBI5* and *PiITS*-specific primers. The root fungal colonization levels in all mutants were normalized with 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 $P < 0.05$ (*), 0.01 (**) analyzed by Student's *t*-test.

Figure 2. *P. indica*-Colonized Plants Are Hypersensitive to ER Stress but Disturbed in the Unfolded Protein Response

(A) *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 solution (+ 1% sucrose) containing 25 ng ml^{-1} tunicamycin (TM) 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 significantly reduced biomass of the sample compared to samples without TM analyzed by Student's *t*-test; $p < 0.05$ (a), $p < 0.001$ (b).

(B) 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.

(C) 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 (5 µg ml^{-1}) 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.

(D) BIP protein accumulation during *P. indica* colonization. For the analyses, *Arabidopsis* WT roots were inoculated with *P. indica* or mock-treated and harvested for protein extraction.

(E) 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 arranged 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^{-1}) or DMSO (control) and harvested 2 dat. For all experiments, 20 µg total protein was separated per sample by SDS-PAGE. Immunoblot analyses were performed with anti-BIP antibodies. The staining with Coomassie Brilliant Blue indicates equal loading of all samples.

Figure 3. **Colonization of *Arabidopsis* Roots by *P. indica* Is Dependent on Vacuolar Processing Enzymes (VPEs).**

For the analysis, three-week-old *Arabidopsis* WT, *αvpe*, *βvpe*, *γvpe*, *δvpe* and *vpe* null mutants were inoculated with *P. indica*. Root samples were harvested at 3 and 7 dai. The fungal biomass at both biotrophic (3 dai) and cell death-associated colonization stages (7 dai) were determined by qRT-PCR using *AtUBI5* and *PiITS*-specific primers. The root fungal colonization levels in all mutants were normalized with 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.

Figure 4. Vacuolar Processing Enzymes Are Required for *P. indica* Colonization Associated and Tunicamycin-Induced Cell Death

(A and 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 the root extracts to determine VPE and caspase 1 activities, respectively. Fluorescence 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 twice with similar results. Letters indicate significance of respective enzyme activities between *P. indica*-colonized and non-colonized roots at $P < 0.05$ (a), 0.01 (b), 0.001 (c) analyzed by Student's *t*-test.

(C and D) Fluorescein diacetate (FDA)-based assay indicative of cell death in *dad1*, *γvpe*, *vpe-null* and *γvpe dad1* roots compared to WT (set to one) after TM treatment (C) or *P. indica* inoculation (D). Two-week-old *Arabidopsis* WT plants, *dad1*, *γvpe*, *vpe-null* and *γvpe dad1* mutants were treated with TM (5 μg ml^{-1}) or mock-treated (C). Alternatively, WT and mutants plants were inoculated with *P. indica* or mock-treated (D). Roots were harvested at 3 dat (C) or at the cell death-associated colonization stage (7 dai) (D). For either treatment, root segments were cut in 2 cm pieces and stained with fluorescein diacetate (FDA). After staining, root segments were washed and the fluorescence intensities were spectrometrically determined at 535 nm after excitation at 485 nm. The values are given as relative 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. Letter (a) indicate significance at $P < 0.05$ analyzed by Student's *t*-test.

Figure 5. Overview of Molecular Events Associated with Cell Death-Dependent Colonization of *Arabidopsis* Roots by *P. indica*.

We formerly demonstrated partial suppression of immune signaling by *P. indica* (indicated by dashed line) downstream of its recognition by the root surveillance system (Schäfer, unpublished). Based on the presented data, *P. indica* is inducing ER stress but is simultaneously suppressing the unfolded protein response (UPR) in colonized cells. This results in ER swelling and the subsequent activation of a γVPE/caspase 1-mediated cell death program, which is preceded by vacuolar collapse. Impaired ER integrity is thought to disturb vesicular protein secretion. TF, transcriptions factors (e.g. WRKYs).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. **MAMP-induced responses in Seedlings of *sec61α, dad1,* and *bip2* Mutants.**

(A) Roots of two-week-old WT plants 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 (n = 20 plants per treatment and experiment). Data represents mean values of three independent biological experiments.

(B and C)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 (B) or 1 µM *N*-acetylchitooctaose (chitin) (C). *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 three times with similar results.

Figure
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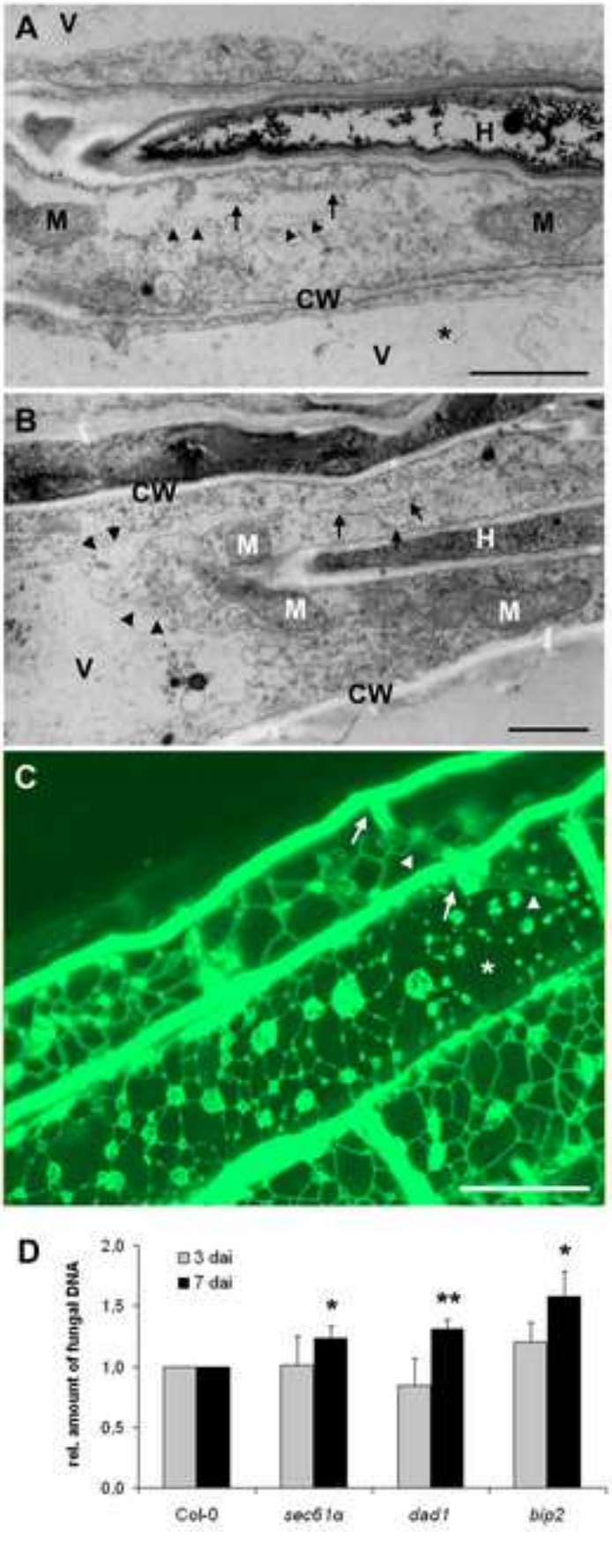
Figure 1



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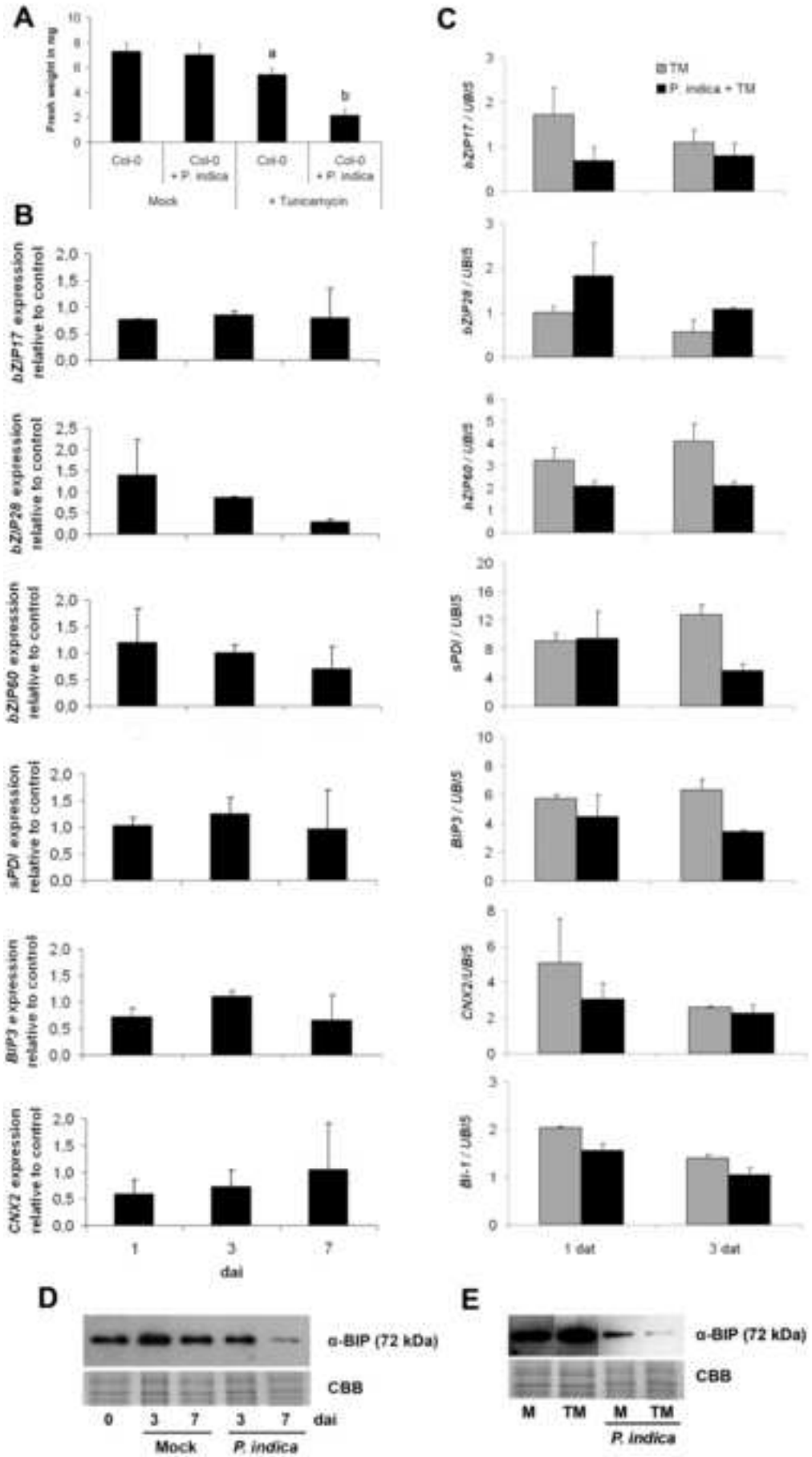

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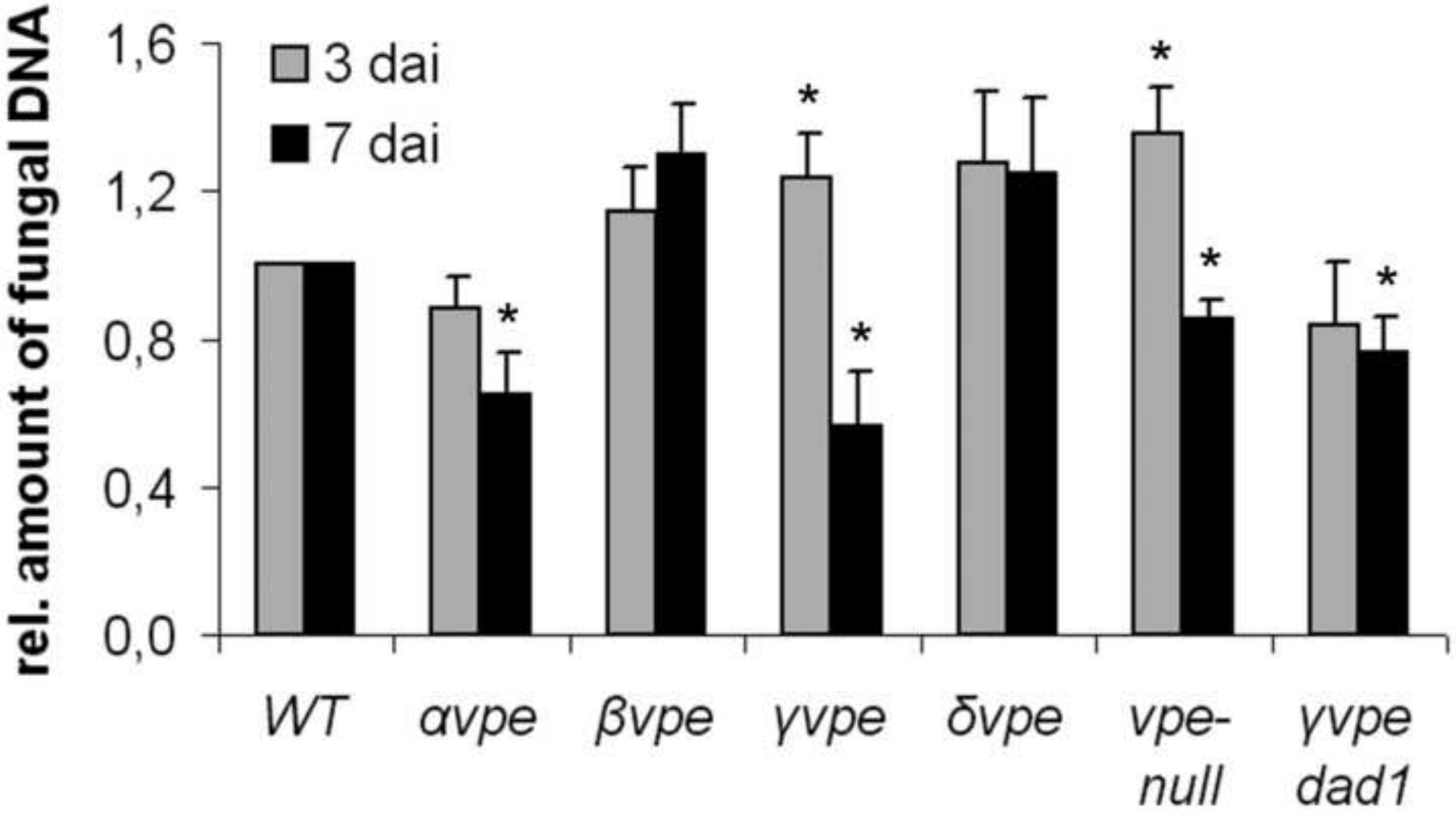

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Figure 4

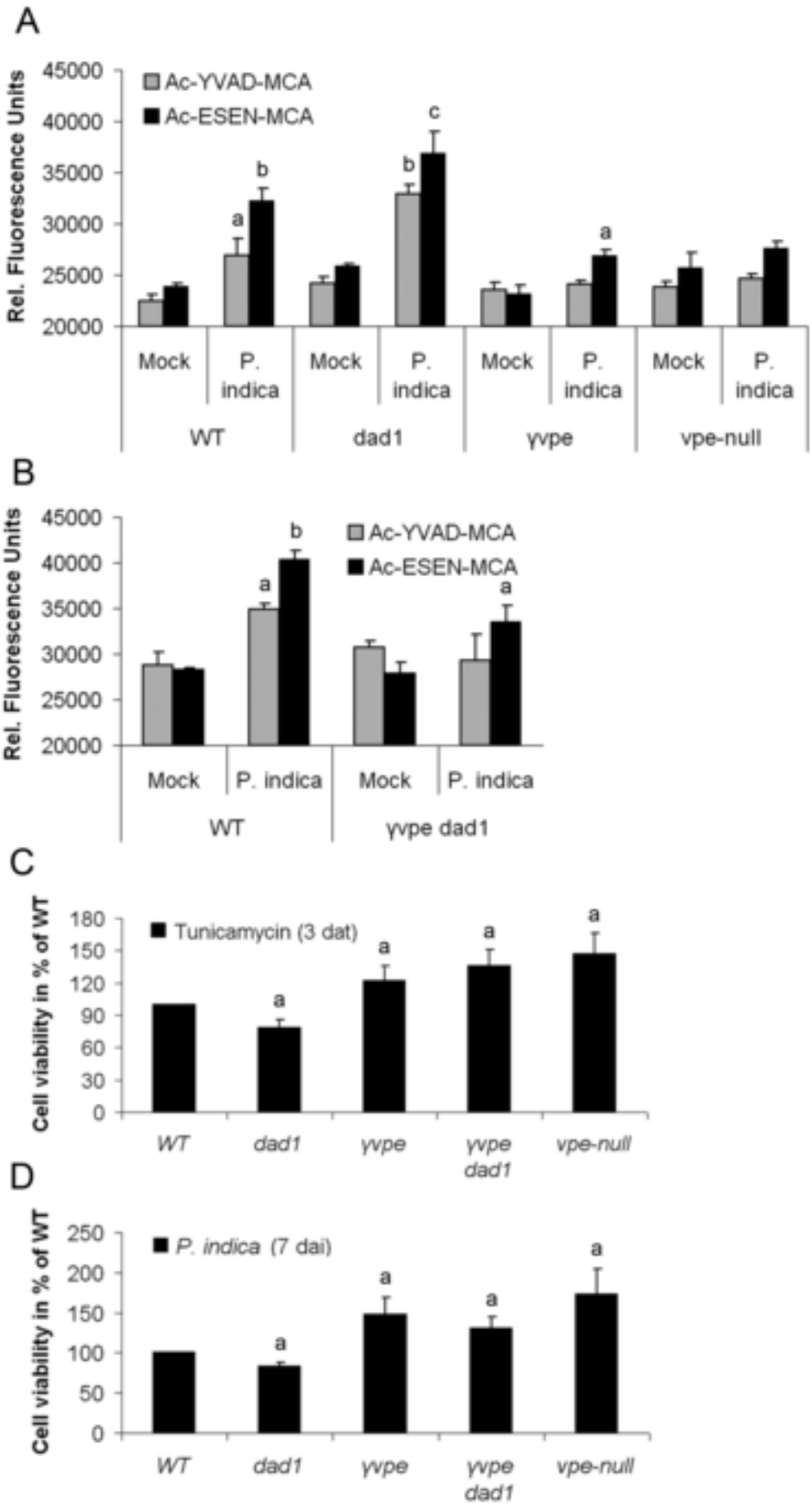

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Figure 5

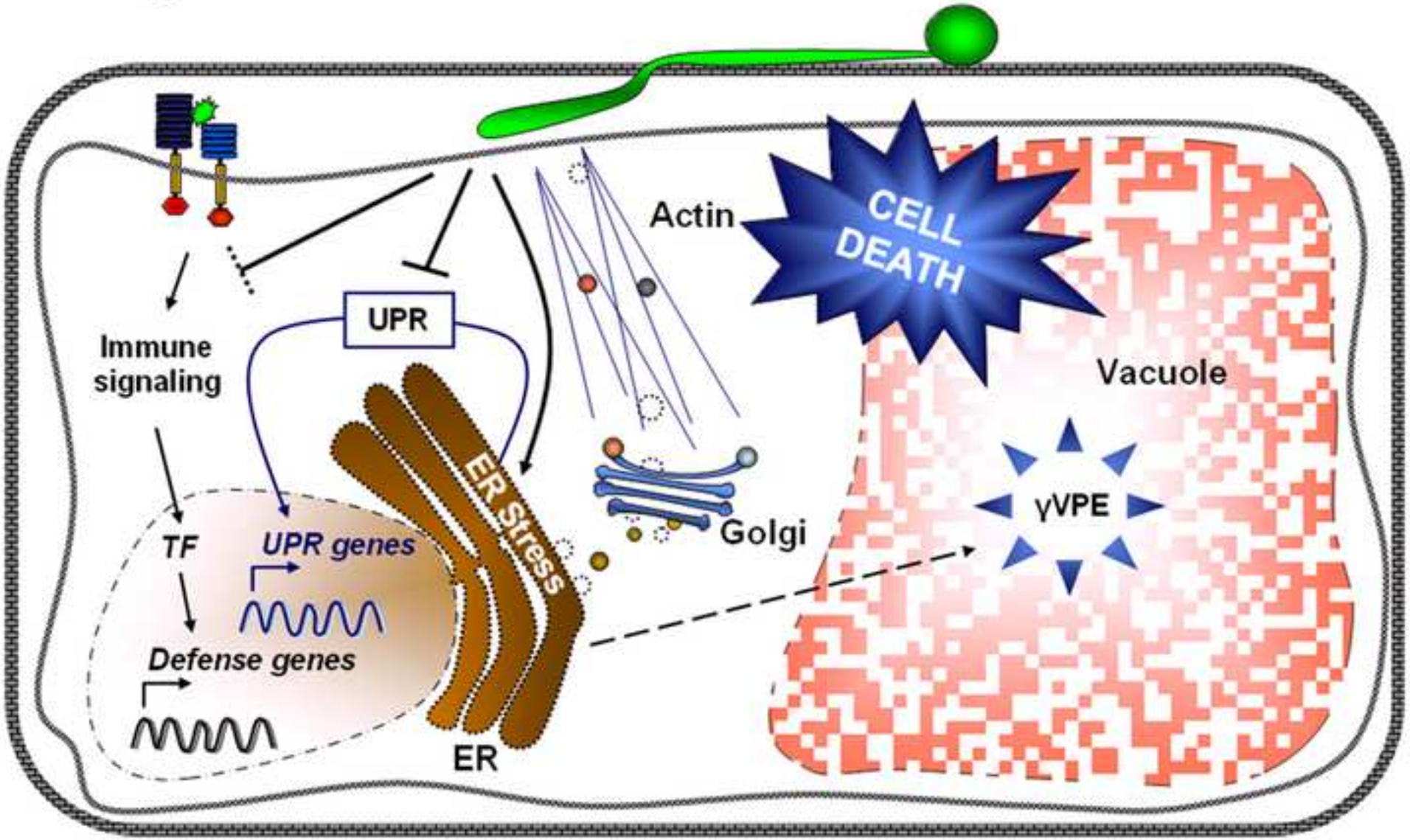

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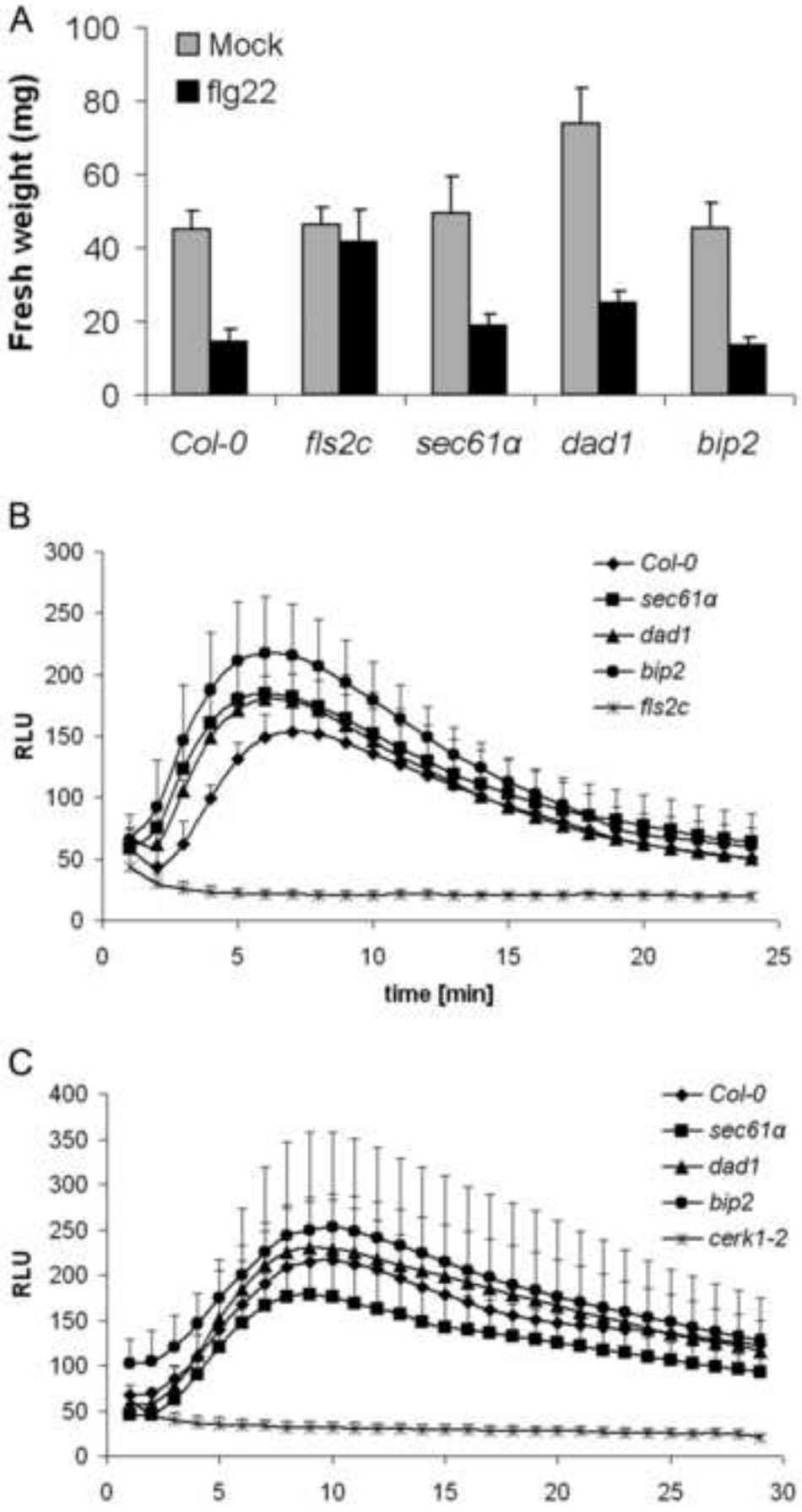
Figure S1



Inventory to Figure S1

Figure S1 is related to Figure 1D as it indicates that the used mutants are not impaired in general responses induced by microbe-associated molecular patterns (MAMPs)such as flg22 and chitin. This implicates that improved colonization of mutants as displayed in Figure 1D might not be related to an impaired plant defense (syn. MAMP-triggered immunity).

5.8 Baltruschat, H., Fodor, J., Harrach, B.D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K.H., **Schäfer, P.**, Schwarczinger, I., Zuccaro, A., Skoczowski, A. (2008). Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytologist* 180: 501-510.

Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants

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Summary

• The root endophytic basidiomycete *Piriformospora indica* has been shown to increase resistance against biotic stress and tolerance to abiotic stress in many plants.
• Biochemical mechanisms underlying *P. indica*-mediated salt tolerance were studied in barley (*Hordeum vulgare*) with special focus on antioxidants. Physiological markers for salt stress, such as metabolic activity, fatty acid composition, lipid peroxidation, ascorbate concentration and activities of catalase, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase enzymes were assessed.
• Root colonization by *P. indica* increased plant growth and attenuated the NaCl-induced lipid peroxidation, metabolic heat efflux and fatty acid desaturation in leaves of the salt-sensitive barley cultivar Ingrid. The endophyte significantly elevated the amount of ascorbic acid and increased the activities of antioxidant enzymes in barley roots under salt stress conditions. Likewise, a sustained up-regulation of the antioxidative system was demonstrated in NaCl-treated roots of the salt-tolerant barley cultivar California Mariout, irrespective of plant colonization by *P. indica*.
• These findings suggest that antioxidants might play a role in both inherited and endophyte-mediated plant tolerance to salinity.

Key words: antioxidant enzymes, ascorbic acid, calorimetry, ethane release, fatty acid unsaturation, *Hordeum vulgare* (barley), *Piriformospora indica*, salt stress.

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Introduction

High salt concentrations in soil and irrigation water are a major threat to agricultural production in arid and semiarid regions. The presence of excess ions in the rhizosphere causes injury to plant roots, followed by their gradual accumulation in the aerial parts with heavy damage to plant metabolism, which leads to stunted growth and reduced yield (Shannon, 1997). Plants have evolved complex mechanisms to counter NaCl toxicity and low water potential in soil caused by salinity as well as drought (reviewed by Munns & Tester, 2008). Furthermore, mutualistic symbiosis with mycorrhizal and endophytic fungi can confer salt tolerance to plants and decrease yield losses in cultivated crops grown in saline soils (Rodriguez

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et al., 2004). Recently, a root-endophytic basidiomycete, *Piriformospora indica*, has been shown to improve plant resistance against root and leaf diseases and alleviate salt stress in barley (Waller *et al.*, 2005).

Piriformospora indica was isolated from the rhizosphere of *Prosopis juliflora* and *Zizyphus nummularia* in the Thar Desert in Rajasthan, India (Verma *et al.*, 1998). This fungus colonizes roots and increases the biomass of both monocot and eudicot plants (Varma *et al.*, 1999). In contrast to arbuscular mycorrhizal fungi, *P. indica* can be easily grown on synthetic media allowing for large-scale propagation and a possible use in plant production.

The aim of this study was to investigate the *P. indica*-mediated protective plant responses to moderate (100 mM NaCl) and high (300 mM NaCl) salt stress in barley. In order to elucidate physiological responses of *P. indica*-colonized barley plants to salinization, we measured important indicators of salt stress, such as metabolic heat production, lipid peroxidation and fatty acid composition; furthermore, we analysed antioxidant activities.

Earlier studies have demonstrated that salt-treated barley shows reduced metabolic activity and respiration rates (Criddle *et al.*, 1989; Jolivet *et al.*, 1990). Thus, calorimetrical determination of heat output can serve as a valuable tool for screening plants for salt tolerance (Criddle *et al.*, 1989; Schabes & Sigstad, 2004).

Lipid peroxidation is associated with cellular membrane damage elicited by salinity stress (Fadzilla *et al.*, 1997). NaCl treatment resulted in higher rates of lipid peroxidation in salt-sensitive plants than in salt-tolerant cultivars (Hernández *et al.*, 1995; Yang *et al.*, 2004). These observations suggest that the rate of lipid peroxidation can also be used to characterize how effectively *P. indica*-treated plants cope with salt stress.

Fatty acid desaturation is associated with salt stress in plants as well (Elkahoui *et al.*, 2004; Liang *et al.*, 2005). Previously, Berberich *et al.* (1998) have found that ω-3 desaturase genes are induced in roots of maize under high salt conditions. In agreement with this result, it has been shown that linolenic acid plays a pivotal role in the tolerance of tobacco plants to salt stress (Im *et al.*, 2002). Therefore, composition of fatty acids was analysed in leaves of uncolonized and *P. indica*-colonized salt-sensitive barley plants under salt stress conditions to characterize fatty acid desaturation.

Drought, salt and temperature extremes all induce the accumulation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals (Apel & Hirt, 2004). Plants are endowed with an array of radical scavengers and antioxidant enzymes that act in concert to alleviate oxidative stress. An imbalance between antioxidant defences and the amount of ROS results in cellular injury (Foyer & Noctor, 2000). An increasing body of evidence suggests that high salinity induces oxidative stress in plants that is at least partly responsible for tissue damage (Hernández *et al.*, 2000; Mittova *et al.*, 2004). Several studies have demonstrated that salinity increases antioxidant activities in salt-tolerant plants above the levels found in salt-sensitive plants (Gossett *et al.*, 1994; Gueta-Dahan *et al.*, 1997; Mittova *et al.*, 2004).

It has been previously shown that *P. indica* also induces antioxidants: the amount of ascorbic acid, the ratio of reduced to oxidized ascorbate and the activity of dehydroascorbate reductase were elevated in barley roots (Waller *et al.*, 2005). We addressed the question of whether antioxidants play a role in *P. indica*-mediated protection of barley against salt stress. Cultivated barley is a relatively salt-tolerant crop but there is a rather high variability among barley cultivars in this trait (Epstein *et al.*, 1980). Two contrasting genotypes, the salt-tolerant cultivar California Mariout and the salt-sensitive cultivar Ingrid, were chosen for this study to define antioxidant responses.

Materials and Methods

Plant inoculation and NaCl treatment

Seeds of salt-sensitive barley (*Hordeum vulgare* L.) cv. Ingrid and salt-tolerant cultivar California Mariout (Epstein *et al.*, 1980) were surface-sterilized for 10 min in 0.25% sodium hypochlorite, rinsed with water and germinated at 22°C on sheets of Whatman No. 1 filter paper in Petri dishes. After 2 d, one part of the germinating seeds was transferred to pots and grown in a 2 : 1 mixture of expanded clay (Seramis, Masterfoods, Verden, Germany) and Oil-Dri (equivalent to Terra Green, Damolin, Mettmann, Germany) in a growth chamber at 22 : 18°C day : night cycle, 60% relative humidity and a photoperiod of 16 h (200 μmol m^{-2} s^{-1} photon flux density), and fertilized weekly with 0.1% Wuxal top N solution (Schering, Düsseldorf, Germany, N : P : K, 12 : 4 : 6). The other part of the seeds was inoculated with *P. indica*: developing roots of 2-d-old germinating seeds were immersed in *P. indica* homogenate before transferring to pots and grown under the same conditions.

Piriformospora indica was propagated in liquid *Aspergillus* minimal medium (Peškan-Berghöfer *et al.*, 2004). Fungal mycelium was prepared for root inoculation as described by Druege *et al.* (2007). Root colonization was determined in 1-wk-old plants by the magnified intersections method (McGonigle *et al.*, 1990) after staining root fragments with 0.01% (w/v) acid fuchsin in lactoglycerol (Kormanik & McGraw, 1982). Fungal structures were visualized in the roots with a Zeiss Axioplan 2 microscope.

Salt-treated sets of uncolonized and *P. indica*-infected plants were exposed to salt from the age of 3 wk, continuously bottom-watered with sterile water containing 100 or 300 mM NaCl. Leaf and root samples were harvested after 1, 2, 3 and 4 wk periods of salt treatment. Control sets of barley plants were irrigated with sterile water.

Isothermal microcalorimetry

Four-centimetre-long apical leaf tips were excised from the youngest fully expanded leaves of 5-wk-old plants. Two leaf cuttings from different barley plants were placed into a sample

ampoule and heat production was recorded by a Thermal Activity Monitor LKB-2277 (Thermometric, Järfälla, Sweden) as described by Fodor *et al.* (2007).

Lipid extraction and separation

Leaf tissue (1.5 g) was ground in 7 ml of methanol–chloroform (2 : 1) with a mortar and pestle at 0–4°C, and vortexed thoroughly. The homogenate was centrifuged at 2000 ***g*** for 20 min at 7°C and the supernatant fluid was transferred to a clean tube. The residual pellet was extracted a second time with 2 ml of the same extraction mixture, vortexed and centrifuged as before. Subsequently, the supernatants were combined. Phase separation and isolation of particular lipid fractions was performed according to Żur *et al.* (2002).

Analysis of fatty acids

Fatty acid composition of phospholipids was analysed by a gas chromatograph (Hewlett Packard 5890 Series II) using capillary column GS-Alumina (30 m length, 0.542 mm in diameter purchased from J&W Scientific, Folsom, CA, USA) as described previously (Żur *et al.*, 2002). The relative amount of particular fatty acids was compared with internal standards (C17:0, Sigma-Aldrich, Munich, Germany). Double bond index was calculated by dividing by 100 the sum of the percentages of the unsaturated fatty acids, each multiplied by the number of its double bonds.

Ethane assay

Lipid peroxidation was monitored by detection of thermally produced ethane. Leaf samples from the youngest fully developed leaves of 5-wk-old plants (*c.* 400 mg) were placed into a 16 ml flask and sealed under nitrogen atmosphere. *In situ* decomposition of ω-3 unsaturated hydroperoxy fatty acids into ethane was accelerated by a brief heat treatment of the samples using a microwave oven according to Degousée *et al.* (1995). Gas chromatographic measurements were carried out as described by Fodor *et al.* (2007). Ethane was quantified by comparison to an authentic standard (Sigma-Aldrich).

Antioxidant assays

Activities of ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), and the concentration of reduced and oxidized forms of ascorbic acid were detected in root extracts spectrophotometrically as described earlier (Harrach *et al.*, 2008).

Monodehydroascorbate reductase (MDHAR) activity was determined in 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM ascorbate, 0.1 mM NADH and 0.2 U ml^{-1} ascorbate oxidase (Hossain *et al.*, 1984). The reaction was started by the addition of ascorbate peroxidase and followed by monitoring the consumption of NADH at 340 nm.

Statistical analysis

At least three independent experiments were carried out in each case. Statistical analysis was performed using Student's *t*-test and MANOVA. Differences were considered to be significant at $P < 0.05$.

Results

Piriformospora indica enhances shoot biomass under salt stress

Hyphal colonization of 1-cm-long root segments was estimated to be 50–60% in Ingrid barley and only the colonized plants were used in each experiment. The rate of colonization was not affected significantly by 3 wk exposure to salt stress (data not shown).

Barley plants irrigated with saline water for 2 wk showed stunted growth and underwent early senescence. The biomass of the youngest developed leaves slightly decreased under saline conditions, while older leaves exhibited chlorosis and subsequent necrosis. Mild salt stress (100 mM NaCl) caused a slight, but not significant, reduction in shoot fresh weight of barley plants. However, high-salt (300 mM NaCl) treatment caused substantial biomass reduction in uncolonized and *P. indica*-colonized cv. Ingrid and cv. California Mariout plants as well (Fig. 1).

Compared with uncolonized plants, shoot fresh weight of *P. indica*-colonized barley cv. Ingrid was enhanced about twofold under both control and saline conditions (Fig. 1). Even after exposure to 300 mM NaCl, *P. indica*-colonized plants produced shoot biomass comparable to uncolonized Ingrid barley grown under nonsaline conditions. Among plants grown in a highly saline environment, shoot fresh weight of salt-tolerant cv. California Mariout was significantly higher compared with the uncolonized cv. Ingrid, but the highest shoot biomass production was detected in *P. indica*-colonized Ingrid plants.

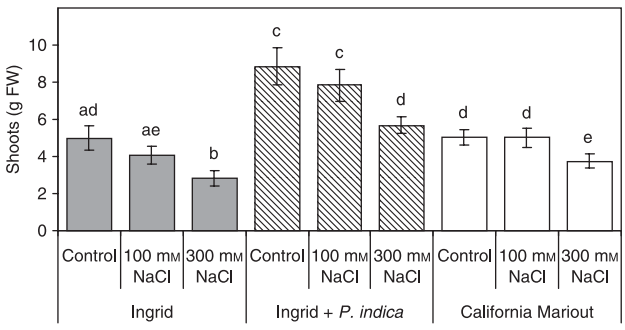

Fig. 1 Shoot fresh weight of 5-wk-old barley (*Hordeum vulgare*) plants, untreated (control) or treated with NaCl from 3 to 5 wk after germination. Ingrid is a salt-sensitive cultivar, California Mariout is a salt-tolerant cultivar, and plants of cv. Ingrid were uncolonized or *Piriformospora indica*-colonized. Letters indicate significant differences among treatments ($P < 0.05$).

Piriformospora indica counteracts the salt-induced decrease in heat efflux

The metabolic heat rates of leaf samples were reduced by *c.* 30% when Ingrid plants were exposed to 300 mM NaCl for 2 wk (Fig. 2a). Infection of roots with *P. indica* did not cause significant changes in heat production of leaves under nonsaline conditions.

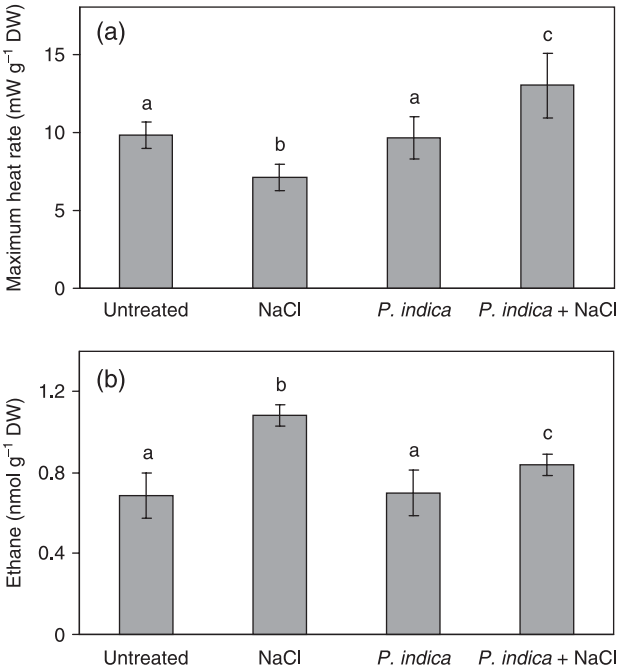

Fig. 2 Effects of salt treatment on metabolic heat efflux detected by isothermal calorimetry (a) and on lipid peroxidation estimated by thermally produced ethane (b) in leaves of 5-wk-old barley (*Hordeum vulgare*) cv. Ingrid plants. Control, untreated 5-wk-old barley; *P. indica*, *Piriformospora indica*-colonized plants; NaCl, plants treated with 300 mM NaCl from 3 to 5 wk after germination; DW, dry weight. Letters indicate significant differences among treatments ($P < 0.05$).

When *P. indica*-colonized Ingrid plants were grown in a high-saline environment, the amount of heat production was significantly ($P < 0.05$) above that observed in uninfected plants.

Changes in fatty acid composition

Fatty acid composition of phospholipid fractions prepared from leaves of salt-sensitive Ingrid barley is listed in Table 1. The major fatty acid species were palmitic (C16:0), palmitooleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids. Analysis of fatty acid composition in barley leaves indicated that the fully saturated C16:0 palmitic acid was the predominant C16 fatty acid, whereas C18 fatty acids mostly consisted of unsaturated species (Table 1). We found a slight salt-induced shift from C16 fatty acids to C18:3 fatty acid upon high-salt treatment. This increase was accompanied by a small but significant rise in the overall proportion of unsaturated fatty acids, in the ratio of C18:3 to C18:2 fatty acids and in the double bond index, which is a more precise indicator of fatty acid desaturation (Table 1).

In leaves of *P. indica*-colonized plants, the proportion of C16:1 fatty acid increased, whereas the molar percentage of C18:1 fatty acid significantly decreased compared with the uninfected plants (Table 1). The proportion of linolenic acid and the derived values for indicators of fatty acid desaturation were slightly elevated upon inoculation with the endophyte. Interestingly, when *P. indica*-inoculated Ingrid plants were subjected to salt, we could not find further changes in the molar percentages of C16 or C18 fatty acids, except for C16:1, which was again down-regulated to the concentration detected in leaves of salt-treated uninfected plants (Table 1).

Piriformospora indica reduces lipid peroxidation in leaves of salt-treated barley

High salinity stress induced the peroxidation of membrane lipids as demonstrated by the emission of thermally produced

Table 1 Fatty acid composition in phospholipids isolated from leaves of barley (*Hordeum vulgare*) cv. Ingrid

Fatty acid	Untreated	NaCl	*Piriformospora indica*	*P. indica* + NaCl
16:0	16.9 ± 0.7	15.4 ± 0.7*	15.8 ± 1.0	16.3 ± 1.1
16:1	2.3 ± 0.2	1.8 ± 0.4	2.8 ± 0.2*	1.9 ± 0.4
18:0	2.4 ± 0.5	2.3 ± 0.3	2.2 ± 0.5	2.3 ± 0.6
18:1	2.7 ± 0.2	2.5 ± 0.2	1.9 ± 0.3*	2.2 ± 0.1*
18:2	25.9 ± 2.2	23.0 ± 1.2	22.6 ± 4.1	23.1 ± 0.9
18:3	49.9 ± 2.5	55.0 ± 1.9*	54.7 ± 5.6	54.1 ± 2.5*
18:3:18:2	1.95 ± 0.25	2.39 ± 0.19*	2.47 ± 0.70	2.32 ± 0.19*
U:S	4.17 ± 0.29	4.65 ± 0.28*	4.54 ± 0.39	4.33 ± 0.46
DBI	2.06 ± 0.04	2.15 ± 0.04*	2.13 ± 0.08	2.12 ± 0.06

Molar percentages of fatty acids ± SD are shown. NaCl, treatment with 300 mM NaCl from 3 to 5 wk after germination; 18:3 : 18:2, ratio of linolenic to linoleic acid; U : S, ratio of unsaturated to saturated fatty acids; DBI, double bond index = Σ(mol % fatty acid × number of double bonds)/100.

*Significant difference between treated and control plants at $P < 0.05$ level.

Table 2 Statistical analysis (MANOVA) for testing the effect of salt concentration, time-point of sampling and root colonization by *Piriformospora indica* on activities of APX, CAT, GR, DHAR and MDHAR antioxidant enzymes in roots of barley (*Hordeum vulgare*) plants

	F					
Factors	APX	CAT	GR	DHAR	MDHAR	df
Salt	**79.15**	**639.74**	**324.52**	**1042.74**	**527.26**	2.69
Time	**22.04**	**58.15**	**54.16**	**93.84**	**12.46**	3.69
P. indica	**144.55**	**335.00**	**30.12**	**279.90**	**124.46**	1.69
Salt × time	**24.95**	**81.22**	**26.78**	**87.67**	**24.78**	6.69
P. indica × salt	**77.40**	**48.39**	**61.41**	**26.41**	**93.78**	2.69
P. indica × time	**38.93**	**174.46**	2.29	**6.80**	**5.09**	3.69

Plants were treated with NaCl between the ages of 3 and 7 wk after germination. The salt factor has three concentrations: 0, 100 and 300 mM NaCl, the time factor has four levels: 1, 2, 3, 4 wk after NaCl treatment; the *P. indica* factor has two levels: uncolonized and *P. indica*-colonized cv. Ingrid; df, degrees of freedom; APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase; DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase. Significant ($P < 0.05$) *F*-values are indicated by bold characters.

ethane derived from the decomposition of the 16-hydroperoxide of linolenic acid. *P. indica* by itself did not affect the emission of ethane from leaves of cv. Ingrid (Fig. 2b). The rate of ethane release from the leaves of salt-treated Ingrid plants increased by 60% compared with the unsalinized control. However, high salt exposure accelerated the rate of lipid peroxidation by only 20% in leaves of *P. indica*-colonized plants (Fig. 2b).

Piriformospora indica further increases antioxidant enzyme activities induced by salt treatment in barley roots

Statistical analysis revealed significant ($P < 0.05$) effects of salt concentration, duration of salt treatment and root colonization by *P. indica* on the activities of antioxidant enzymes (Table 2). Enzyme activities were affected in barley roots by NaCl in the following order (from highest to lowest effect): DHAR, CAT, MDHAR, GR and APX. MDHAR activity was the least affected by the time points. On the other hand, GR activity was the least affected by *P. indica*, which exerted a very high effect on CAT and DHAR activities. Changes in salt concentration significantly affected the time-dependent responses of plants, as evaluated by enzyme activities. Furthermore, root colonization by the endophyte also had significant time-dependent effects on enzyme activities, particularly on CAT and APX, and to a lesser extent on DHAR and MDHAR. Its effect on GR activity was not significant.

In roots of uncolonized Ingrid plants, enzyme activities were markedly increased after salt treatment, peaked at 1 wk after salt exposure and then gradually returned to the corresponding basal levels over the next 3 wk. Only MDHAR activity was found to be enhanced by salt throughout the experiment (Fig. 3). Both the increase and then the decline of enzyme activities were modest when the plants were exposed to 100 mM NaCl compared with the plants subjected to high salt.

In *P. indica*-colonized Ingrid and in California Mariout plants, the salinity-induced changes in enzyme activities were different from those associated with salt stress in uninfected Ingrid barley. First, the time for antioxidant enzymes to reach the peak activities was longer: 3 wk after salt exposure. Second, the ceiling rates of the enzyme activities were significantly higher. Third, a less pronounced decrease was observed in enzyme activities at 4 wk after salt treatment (Fig. 3).

Piriformospora indica enables barley roots to maintain ascorbate in its reduced state under salt stress

Colonization of barley by *P. indica* enhanced both ascorbic acid concentration and the ratio of reduced to oxidized ascorbate about twofold in plant roots after saline exposure (Fig. 4). We could not detect ascorbate in *P. indica* grown axenically in liquid medium.

Strikingly, salt treatment had the opposite effect on ascorbic acid concentrations in uncolonized than in *P. indica*-colonized Ingrid plants (Fig. 4), and therefore salt did not affect significantly the amount of ascorbate (Table 3). However, the *P. indica*-dependent response of ascorbate to salinization was highly significant. The amount of reduced ascorbate strongly declined in uninfected roots after 1 wk of high-salt treatment. By contrast, salinization further increased the ascorbate concentration in the colonized plants at the first time-point of sampling (Fig. 4a). The amount of ascorbate then gradually decreased but still remained above the values recorded for the control plants grown under nonsaline conditions. Furthermore, *P. indica*-colonized plants maintained efficient redox balance of ascorbate even after 3 wk of salt treatment (Fig. 4b). Statistically significant ($P < 0.05$) time-dependent or endophyte-dependent effect of salinization was not observed for the ascorbate : DHA ratio (Table 3). Nevertheless, both *P. indica* and salinity exerted significant effect on ascorbate redox state (Table 3). Remarkably, in uninfected plants, a strong decrease in the ratio of reduced to oxidized form of ascorbate was already detectable 1 wk after salinization: the ascorbate : DHA ratio decreased by *c.* 80% (Fig. 4b).

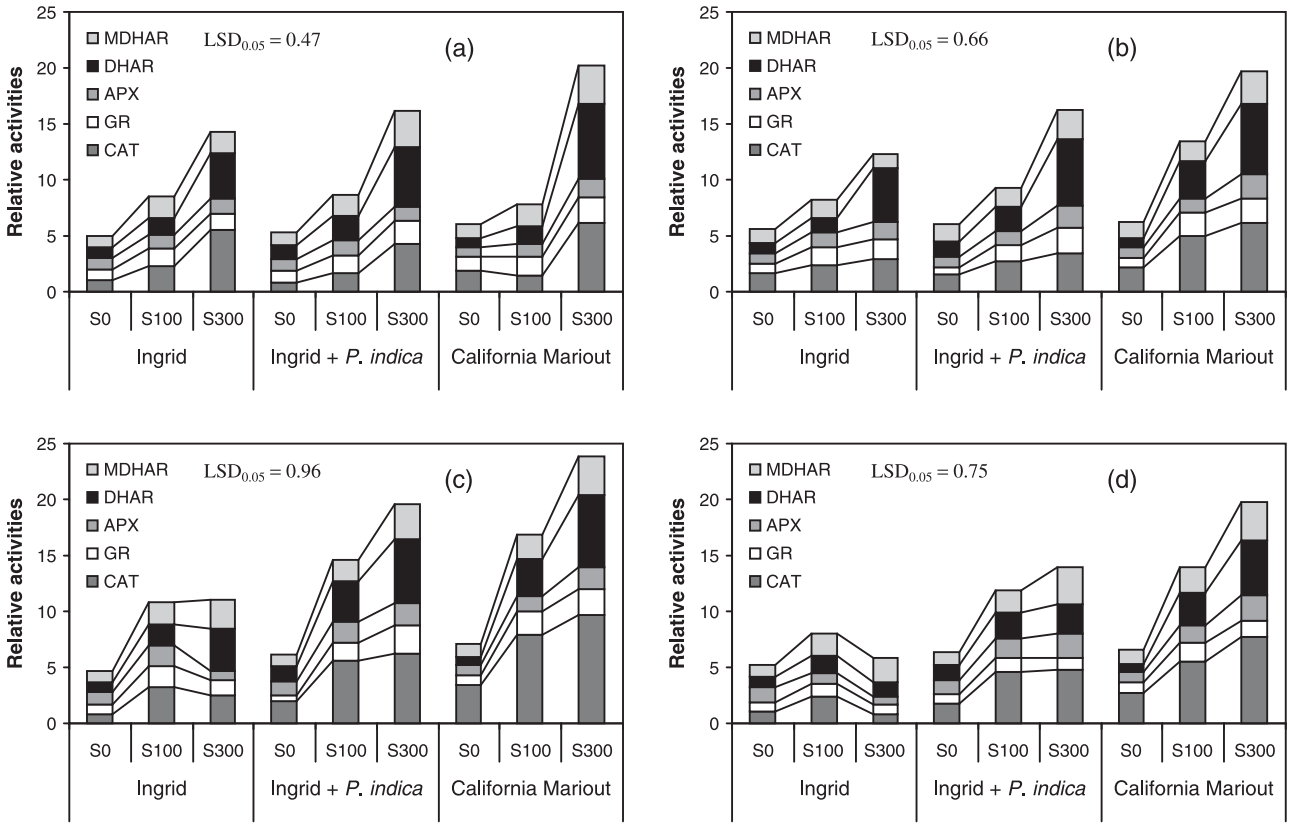

Fig. 3 Relative enzyme activities of catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) in roots of salt-sensitive barley (*Hordeum vulgare*) cv. Ingrid, *Piriformospora indica*-colonized cv. Ingrid and salt-tolerant cv. California Mariout after 1 (a), 2 (b), 3 (c) and 4 wk (d) of salt exposure. Plants were treated with NaCl from 3 to 7 wk after germination. Enzyme activities were normalized to the activities of enzymes measured in roots of unsalinized (S0) Ingrid plants at 1 wk after treatment. Activity level of 1 represents 72.25, 0.34, 1.15, 0.83 and 0.60 mmol g^{-1} FW min^{-1} activities of CAT, GR, APX, DHAR and MDHAR, respectively. S0, S100, S300, treated with 0, 100 and 300 mM NaCl, respectively; $LSD_{0.05}$, least significant difference between means at $P = 0.05$.

Table 3 Statistical analysis (MANOVA) for testing the effect of salt concentration, time-point of sampling and root colonization by *Piriformospora indica* on ascorbic acid content and ratio of reduced ascorbate to oxidized ascorbate in roots of barley (*Hordeum vulgare*) cv. Ingrid

	F		
Factors	ASC	ASC : DHA	df
Salt	0.38	**194.78**	1.77
Time	**30.53**	**26.16**	2.77
P. indica	**454.30**	**208.49**	1.77
Salt × time	**11.88**	0.67	2.77
P. indica × salt	**105.53**	0.03	1.77
P. indica × time	**20.40**	**8.07**	2.77

Barley plants were treated with NaCl from 3 to 6 wk after germination. ASC, ascorbic acid; DHA, dehydroascorbic acid; df, degrees of freedom. The *P. indica* factor has two levels: uncolonized and *P. indica*-colonized cv. Ingrid; the time factor has three levels: 1, 2 and 3 wk after NaCl treatment; the salt factor has two levels: 0 and 300 mM NaCl. Significant ($P < 0.05$) *F*-values are indicated by bold characters.

Discussion

As a result of the symbiosis with *P. indica*, barley tolerates a moderate salt stress (100 mM NaCl) in hydroponic culture (Waller *et al.*, 2005). Here we could show that *P. indica* protects barley even from high salt stress (300 mM NaCl). However, the mechanism of *P. indica*-induced salt tolerance has not yet been investigated.

In order to get a better understanding of the impact of *P. indica* on the establishment of salt tolerance, we assessed biochemical markers for salt stress, such as metabolic activity, fatty acid composition and lipid peroxidation. Previous studies have demonstrated a salt-induced increase in lipid peroxidation (Hernández *et al.*, 1995; Yang *et al.*, 2004) and a marked reduction in metabolic heat production (Criddle *et al.*, 1989) in salt-sensitive plants, while these parameters were unaltered in salt-tolerant cultivars. We provide clear evidence that salt-induced responses indicated by heat emission and ethane production in the *P. indica*-infected salt-sensitive barley cv. Ingrid resemble those found in salinity-tolerant plants. Our calorimetric studies indicated that the rate of metabolic activity

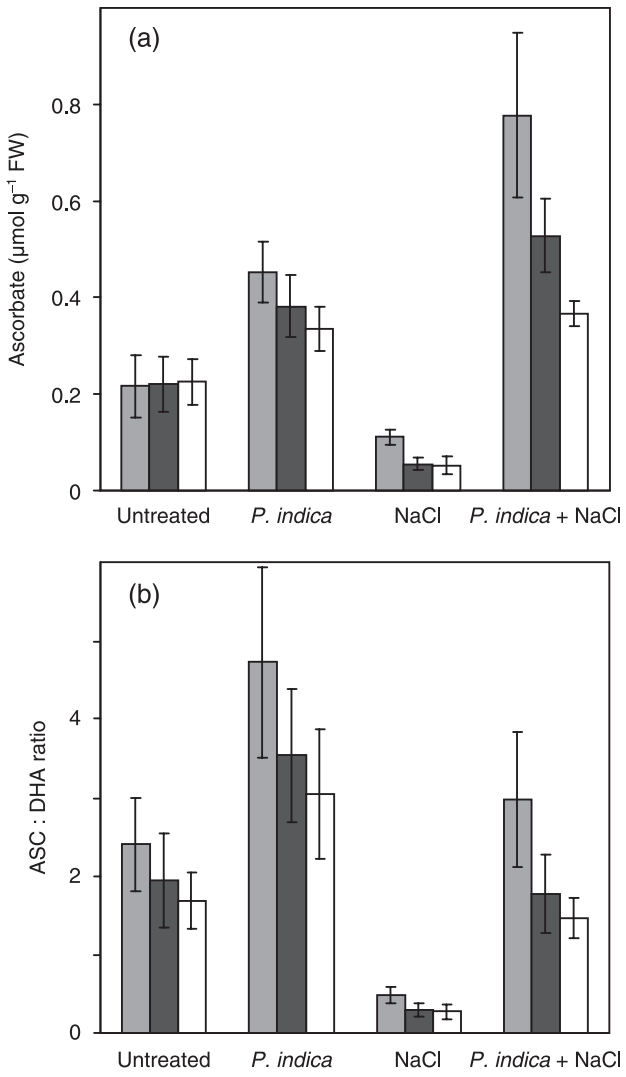

Fig. 4 Amount of reduced ascorbate (a) and ratio of reduced to oxidized ascorbate (b) in roots of salt-sensitive barley (*Hordeum vulgare*) cv. Ingrid plants after 1 (grey bars), 2 (black bars) and 3 (white bars) wk of salt exposure. The plants were untreated or treated with 300 mM NaCl from the age of 3 wk, and uncolonized or *Piriformospora indica*-colonized. ASC, reduced ascorbic acid; DHA, dehydroascorbic acid.

increased in leaves of *P. indica*-infected plants after salt treatment. Therefore, the endophyte seemed to overcompensate the salt-induced inhibition of leaf metabolic activity. Previous results have shown that the extent of natural herbicide resistance of wild oat biotypes is tightly correlated with the rate of heat production upon herbicide exposure, owing to the activation of metabolic pathways required for defence responses (Stokłosa *et al.*, 2006). This suggests that enhanced tolerance to salt stress can be associated with higher metabolic activity in *P. indica*-colonized barley.

Previous studies have shown that exogenously applied unsaturated fatty acids can protect barley during NaCl-induced stress (Zhao & Qin, 2005). Thus, lipid desaturation could be an important component of plant tolerance in response to salt stress. *P. indica* colonization leads to a significant reduction in the proportion of oleic acid in barley leaves, as was previously found in salt-treated barley roots (Zhang *et al.*, 2002; Liang *et al.*, 2005). Similar to salinity, *P. indica* slightly increased the proportion of C18:3 fatty acid in the phospholipid fraction isolated from barley leaves. With one exception (C16:1), *P. indica* induces changes in fatty acid compostion similar to those induced by salinity. Such effects on the fatty acid composition of host plants may display a symbiotic adaptive strategy mediated by the endophyte to cope with salt stress in hostile environments (Rodriguez *et al.*, 2008). We speculate that *P. indica* might induce similar effects on fatty acid composition of the host plants in its original habitat, the arid Thar desert.

Salt-induced lipid peroxidation was significantly attenuated in *P. indica*-treated plants. Cellular membrane damage as a result of salt stress is associated with an accumulation of ROS (Hernández *et al.*, 1995), which can be toxic to living cells causing oxidative damage to DNA, lipids and proteins. On the other hand, ROS can act as signalling molecules for stress responses (Apel & Hirt, 2004). According to a recent report, endophytic fungi characterized by their broad host ranges can confer effective tolerance to ROS under abiotic stress conditions such as salinity (Rodriguez *et al.*, 2008). Interestingly, the clavicipitaceous fungal endophyte, *Epichloë festucae*, which has a restricted host range, can generate superoxide by a NADPH oxidase to establish a mutualistic association with *Lolium perenne* (Tanaka *et al.*, 2006). In *P. indica*-colonized barley roots, we could not detect H_2O_2 accumulation at penetration sites or in the infected cells (data not shown).

Our previous report demonstrated that *P. indica* enhances the ratio of reduced to oxidized ascorbate and induces DHAR activity in colonized barley (Waller *et al.*, 2005). Since ascorbate was not found in *P. indica*, we can assume that the fungus induces the accumulation of ascorbate in plant root cells. Ascorbic acid acts as a primary substrate in the ascorbate-glutathione cycle for detoxification of hydrogen peroxide. In addition, it acts directly to neutralize oxygen free radicals (Foyer & Noctor, 2000). Under the high salt stress condition, *P. indica*-infected Ingrid plants maintained an efficient redox balance of ascorbate and contained higher ascorbate concentration than the unsalinized control, although the concentration of reduced ascorbate decreased over time in roots of salt-treated infected plants. Strikingly, ascorbate content and the ratio of reduced to oxidized ascorbate dramatically decreased in roots of salt-treated uninfected plants soon after 1 wk of salt exposure. These findings are consistent with those presented by Mittova *et al.* (2004), who found that the ratio of ascorbate to DHA decreased in the salt-sensitive *Lycopersicon esculentum* under salt stress, and increased in the salt-tolerant *Lycopersicon pennellii*. Other investigators have shown that ascorbate content decreased in salt-sensitive and salt-tolerant pea cultivars as well, but the

decline was greater in the NaCl-sensitive plants (Hernández *et al.*, 2000). The importance of ascorbate in cellular protection under salt stress has also been demonstrated on an ascorbate-deficient *Arabidopsis* mutant. Impaired in the ascorbate-glutathione-cycle, it accumulated high amounts of ROS and showed increased sensitivity to salt stress (Huang *et al.*, 2005). Consistently, exogenously applied ascorbate increased the resistance to salt stress and attenuated the salt-induced oxidative burst (Shalata & Neumann, 2001).

Alternatively, ascorbate can improve the tolerance of barley to high salinity via processes related to root growth. Ascorbic acid and high ratio of reduced to oxidized ascorbate accelerate root elongation and increase root biomass (Córdoba-Pedregosa *et al.*, 2005).

Earlier studies have suggested that tolerance of plants to salt stress is associated with the induction of antioxidant enzymes (Hernández *et al.*, 2000; Bor *et al.*, 2003, Sekmen *et al.*, 2007). We found that NaCl increased the activities of CAT, APX, DHAR, MDHAR and GR in roots of salt-stressed barley. Although enzyme activities decreased after an initial induction in both salt-sensitive and -tolerant plants, their decline was delayed and less pronounced in *P. indica*-colonized Ingrid barley and in the salt-tolerant cv. California Mariout. Our data highlight the importance of these enzymes in tolerance of barley to salinity. MDHAR activity remained elevated up to 4 wk under high saline conditions in roots of both salt-sensitive and -tolerant barley cultivars. CAT and APX showed a sustained increase in the activities in *P. indica*-infected Ingrid barley after long-term exposure to NaCl. By contrast, their activities decreased in uninfected Ingrid barley after 4 wk of salt exposure. In agreement with these data, overexpression of CAT, APX or DHAR in transgenic plants enhanced tolerance to salt stress (Badawi *et al.*, 2004; Ushimaru *et al.*, 2006; Nagamiya *et al.*, 2007). Surprisingly, *Arabidopsis* double mutant plants deficient in cytosolic and thylakoid APX also show enhanced tolerance to salinity, suggesting that ROS such as H_2O_2 could be responsible for activation of an abiotic stress signal that leads to enhanced stress tolerance (Miller *et al.*, 2007).

The mechanism responsible for *P. indica*-mediated up-regulation of the plant antioxidant system is not known. It has been shown recently that *P. indica* is able to produce auxin when associated with plant roots (Sirrenberg *et al.*, 2007). Exogenous auxin has been found to transiently increase the concentration of ROS and then prevent H_2O_2 release in response to oxidative stress (caused by paraquat) and enhance APX activity, while decreasing CAT activity (Joo *et al.*, 2001; Pasternak *et al.*, 2007). On the other hand, *P. indica* increased the amount of methionine synthase, which plays a crucial role in the biosynthesis of polyamines and ethylene (Peškan-Berghöfer *et al.*, 2004). Transgenic tobacco plants overproducing polyamines also have enhanced tolerance toward salt stress, and salt treatment induces antioxidant enzymes such as APX, superoxide dismutase and glutathione S-transferase more significantly in these transgenic plants than in wild-type controls (Wi *et al.*, 2006). *Sebacina vermifera*, an endophyte closely related to *P. indica*, down-regulates ethylene production in *Nicotiana attenuata* (Barazani *et al.*, 2007). Interestingly, our preliminary results suggest that *P. indica* induces ethylene biosynthesis in barley roots. Ethylene signalling may be required for plant salt tolerance (Cao *et al.*, 2006), and ethylene may induce some antioxidant enzymes when plants are exposed to heat stress (Larkindale & Huang, 2004). However, further experiments are necessary to clarify the function of phytohormones in *P. indica*-induced salt tolerance in barley.

In conclusion, our results demonstrated that a high-saline environment is well tolerated by salt-sensitive barley when previously inoculated with the mutualistic basidiomycete *P. indica*. This endophyte appears to confer tolerance to salt stress, at least partly, through the up-regulation of ascorbate and antioxidant enzymes. Our observations are only correlative but supported by the fact that elevated antioxidant activities are also demonstrated under saline conditions in barley cv. California Mariout, which is genetically tolerant to salt. However, several possible symbiotic mechanisms could account for salt tolerance. For example, root endophytes may act as a biological mediator allowing symbiotic plants to activate stress response systems more rapidly and strongly than non-symbiotic plants (Rodriguez *et al.*, 2004). Since *P. indica* has a broad host range and can easily be propagated in axenic culture on a large scale, we emphasize the high potential of the endophyte in protecting crops against salt stress in arid and semiarid agricultural regions.

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Systemic and local modulation of plant responses by *Piriformospora indica* and related *Sebacinales* species

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Summary

Piriformospora indica is a fungus of the order *Sebacinales* (Basidiomycota) infesting roots of mono- and dicotyledonous plants. Endophytic fungal colonization leads to enhanced plant growth while host cell death is required for proliferation in differentiated root tissue to form a mutualistic interaction. Colonization of barley roots by *P. indica* and related *Sebacina vermifera* strains also leads to systemic resistance against the leaf pathogenic fungus *Blumeria graminis* f.sp. *hordei* due to a yet unknown mechanism of induced resistance. In order to elucidate plant response pathways governed by these root endophytes, we analyzed gene expression in barley plants exhibiting an established symbiosis with *P. indica* 3 weeks after inoculation. *P. indica*-colonized roots showed no induction of defence-related genes, while other genes showed a differential regulation pattern indicating a faster *P. indica*-dependent root development. Gene expression analysis of leaves detected only few systemically induced mRNAs. Among differentially regulated transcripts, we characterized the pathogenesis-related gene *HvPr17b* and the molecular chaperone *HvHsp70* in more detail. HvPr17b shows similarity with TaWCI5, a wheat gene inducible by chemical resistance inducers and salicylate, and was previously proven to exhibit antifungal activity against *B. graminis*. HvHsp70 is the first gene found to systemically indicate root colonization with endophytic fungi of the order *Sebacinales*. Both genes are discussed as markers for endophytic colonization and resulting systemic responses.

Abbreviations: AMF, arbuscular mycorrhizal fungi; cv., cultivar; hai, hours after inoculation; ISR, induced systemic resistance; JA, jasmonic acid; MeJA, methyl jasmonate; SA, salicylic acid, SAR, systemic acquired resistance; WGA, wheat germ agglutinin

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Introduction

As a member of the fungal order Sebacinales (Basidiomycota; Weiß et al., 2004), *Piriformospora indica* infests roots of a broad range of mono- and dicotyledonous plants (Verma et al., 1998; Pham et al., 2004). Endophytic root colonization by this fungus confers enhanced growth to the host plant (Varma et al., 1999; Peškan-Berghöfer et al., 2004) and provides protection against biotic and abiotic stresses. We have shown that *P. indica* reprogrammes barley to salt stress tolerance, resistance to diseases and higher yield (Waller et al., 2005). Disease resistance is provided not only to the roots but also to the shoots. As endophytic growth of *P. indica* is restricted to the root, the fungus is able to provide systemic protection due to a yet unknown mechanism of induced resistance. As *P. indica* can easily be cultured without a host plant (Varma et al., 1999), it is suitable as a model system to study both compatible plant–microbe interactions and to identify potentially new modes of systemic regulation of resistance. Due to its broad host range, resistance induction in crop plants and the availability of related fungi of the order Sebacinales exhibiting a similar activity in host plants, the system also presents a promising potential for applications in agriculture.

To analyse the mechanism of the root endophyte *P. indica* to systemically influence the leaf's defence status, two main approaches have been undertaken: (1) A microscopic study of the interaction of *P. indica* with barley root cells and the requirements for fungal development in the root. (2) An analysis of putative defence signalling pathways by means of candidate marker gene expression studies.

Deshmukh et al. (2006) reported co-localization of *P. indica*-infested barley root areas with dead cortical root cells and a proliferation of the fungus in such cells, being in contrast to the differentiation processes that take place in living cortical root cells of the arbuscular mycorrhiza. A reduced fungal biomass was observed in transgenic barley roots over-expressing the cell death inhibitor Bax-inhibitor 1 (BI-1; Hückelhoven, 2004) providing genetic evidence that *P. indica* requires host cell death for successful colonization. In addition, barley BI-1 transcripts were down-regulated in *P. indica*-infested barley roots 7 days after inoculation (dai), suggesting that the mutualistic symbiosis between *P. indica* and barley involves a tight regulation of the plant cell death machinery. Spatial association of root cell death with massive infestation by *P. indica* might thus reflects successful fungal manipulation of programmed cell death in host root cells. At the same time, cell walls of colonized root tissue did not show elevated autofluorescence using short-wavelength excitation light in an epifluorescence microscope, indicating the absence of a plant defence reaction. As root development in general was not negatively affected, Deshmukh et al. (2006) conclude that the compatible interaction of *P. indica* and barley depends on a sophisticated bi-directional regulation of the plant cell death machinery.

A different approach was to study the expression of genes indicative of signalling pathways known to play important roles in induced resistance. This type of resistance against pathogens is defined as an activation of plant defence prior to contact with a challenging microbe, and includes 'priming', defined as a more rapid expression of defence due to a 'state of alert' of the plant (Sticher et al., 1997; Conrath et al., 2002; Kogel and Langen, 2005). The phenomenon has been studied extensively in salicylic acid (SA)-mediated *systemic acquired resistance* (SAR) in dicotyledonous plants triggered by necrotizing pathogens (Durrant and Dong, 2004), and in *induced systemic resistance* (ISR) triggered by non-pathogenic rhizobacteria (Verhagen et al., 2004). In contrast to SAR, ISR does depend on both NPR1 and the jasmonate (JA)/ethylene pathway, but not on SA (Pieterse et al., 1998). Cereal plants share many components of these resistance pathways, e.g. resistance induction by SA analogues (Kogel et al., 1994; Kogel and Langen, 2005) and functional NPR 1 homologs that have been identified in rice (Chern et al., 2005).

Waller et al. (2005) determined expression levels of SA- and JA-induced genes in leaves of *P. indica*-infested barley plants. Both JA-induced protein-23 (JIP-23) and pathogenesis-related 5 (PR5) mRNAs were not consistently stronger expressed in *P. indica*-infested plants. Therefore, constitutively elevated SA or JA levels are unlikely to be required for the observed systemic resistance. Together with the observation that elements of the antioxidative system were greatly enhanced in leaves of *P. indica*-infested plants, a metabolic modulation of systemic tissues due to a yet unknown signalling pathway was assumed.

To gain more insight into the mechanism of this modulation, we analysed gene expression both in roots and in leaves of *P. indica*-infested and non-infested barley. Gene expression reflected a faster development of roots when infested by *P. indica*, and the absence of an induction of defence-related transcripts. In systemic leaves, a pilot experiment using the Affymetrix Barley 1 gene chip revealed a low number of regulated genes due to *P. indica* root colonization. We detected two genes specifically

induced in systemic tissues by *P. indica*, which can now be used as markers to study and dissect pathways involved in systemic modulation of defence responses in barley.

Materials and methods

Plant and fungal material

Barley (*H. vulgare* cv. Ingrid) was grown in pots with a 2:1 mixture of expanded clay (Seramis, Masterfoods, Verden, Germany) and Oil-Dri (Damolin, Mettmann, Germany) as described in Waller et al. (2005). For root experiments, barley cultivar Maresi was used, which shows no significant differences in phenotype and *P. indica* infestation levels compared with cv. Ingrid. Isolates of *S. vermifera* were obtained from the National Institute of Agrobiological Sciences (Tsukuba, Japan). For inoculation with *P. indica* or *S. vermifera* isolates, 2 g of crushed mycelium was added to 300 g of substrate before sowing. *P. indica* and *S. vermifera* isolates were propagated in liquid *Aspergillus* minimal medium (Peškan-Berghöfer et al., 2004) on a rotary shaker at 18–22 °C. Mycelium from liquid culture was washed with water to remove remaining traces of medium, and crushed using a Waring Blendor (VWR International, Darmstadt, Germany).

Macroarray preparation, hybridization and data analysis

The cDNA macroarray used for root gene expression analysis contained 4608 cDNA fragments, with 1536 cDNA fragments from each of the cDNA libraries GW, GCW, GNW, which were prepared from unchallenged barley roots and *Fusarium culmorum*-challenged roots of a highly susceptible and a less susceptible barley cultivar (Eichmann et al., 2006). Filter hybridization and data acquisition were performed as described in Sreenivasulu et al. (2002) and Eichmann et al. (2006). Genes that were more than 2.5-fold up- or down-regulated 20 days after *P. indica* inoculation compared with non-inoculated controls in at least two of three biological experiments were considered as *P. indica*-regulated. We further enhanced stringency of data analysis by statistics. Normalized signal intensities were transformed to mean differences in signal intensities that were the basis for statistical analysis. We performed a one-sample *t*-test over the three pairs of *P. indica* versus control probes (Zierold et al., 2005). Only genes with *p*-values <0.05 were considered as up- or down-regulated upon *P. indica* inoculation.

Expression analysis using the Affymetrix Barley 1 Gene Chip

In three independent experiments, RNA was extracted from second and third leaves of 3-week-old barley plants either infested or non-infested with *P. indica*. Labelling was performed using the Affymetrix 'One cycle labelling kit' according to the manufacturer's instructions. Hybridization followed the Affymetrix protocol, Barley1 arrays (Close et al., 2004) were processed using the EukGE-WS2 protocol on an Affymetrix GeneChip Fluidics Station 400, and scanned on an Affymetrix GCS3000. After normalization, GeneChip Operating Software (GCOS, Microarray Analysis Suite 5, Affymetrix) was used for comparison expression analysis. As three chips were hybridized each for control plants and *P. indica*-infested plants, nine pair-wise comparisons were performed. For each Contig (representing one cDNA) in each chip-to-chip comparison, the presence of a significant hybridization signal (detection call), a statistical value for detected differences (change call) and the fold-difference between the two calculated hybridization signals (fold change) was obtained. To identify significant changes in gene expression, we considered only those Contigs which (1) were detected as present in at least three of the six chips, (2) showed in at least six out of nine comparisons a change call as 'induced' for induced candidates or as 'repressed' in down-regulated candidates and (3) showed an at least 2-fold change of the calculated means of the three biological repetitions.

RNA isolation and expression analysis by quantitative RT-PCR and gel-blot analysis

Total RNA was isolated from plant material harvested in liquid nitrogen according to Logemann et al. (1987). For quantitative two-step reverse-transcription polymerase chain reaction, 500 ng of total RNA was reverse transcribed to first-strand cDNA using the Superscript™ cDNA Synthesis Kit (Invitrogen GmbH, Karlsruhe, Germany). Aliquots of 10 ng first-strand cDNA were subsequently used as template for qPCR with gene-specific primers. The plant-specific Ubiquitin gene (M60175) served as control for constitutive gene expression. PCR amplifications were performed as described in Deshmukh et al. (2006). Expression levels ($2^{-\Delta Ct}$) were calculated according to Livak and Schmittgen (2001) and are relative to the level of ubiquitin expression. For RNA gel blot experiments, procedures were as described by Eichmann et al. (2006), except that 20 μg of total RNA was used.

Oligonucleotides used: ubiquitin (M60175): 5′-CAGTAGTGGCGGTCGAAGTG-3′, 5′-ACCCTCGCCGACTACAACAT-3′, 18S rRNA: 5′-GCGAGCACCGACCTACTC-3′, 5′-GGACCGGAATCCTATGATGTT-3′; HvPr17b: 5′-CGAGGTTCCTCGACTACTGC-3′, 5′-ATCACATTCAGCCTCCGAAC-3′; HvHsp70: 5′-CCAAGAAGTCGCAGGTTTTC-3′,5′-GGAATGCCAGAAAGGTCAAA-3′ PIP1;3: 5′-CGGCTAGTGGACCAATCAGT-3′, 5′-ATGCACGCTGGATTAAATGG-3′; PIP1;2: 5′-CATCGGGTACAAGCACCAGT-3′, 5′-CCGTACCGCACGTAGTAGGA-3′; drought-induced protein: 5′-TGCYGCTTCTTCACATGGTC-3, 5′-TCTTCCTCAAGTTCGGATGC-3′; horcolin: 5′-GCTCCTAGTGGGAACAAGCA-3′, 5′-TTCTGCCAGCTAACAAAGGTG-3′.

Microscopic procedures

Hyphae in root segments were stained with WGA-AF 488 (Molecular Probes, Karlsruhe, Germany) and analyzed by fluorescence microscopy as described in Deshmukh

et al. (2006). For Figure 1A, Congo red (Sigma Aldrich, Munich, Germany) was included in the staining procedure in order to visualize plant cell walls.

Results

Characteristics of *Sebacinales* interacting with barley roots

Using fluorescence microscopy, the development of *P. indica* stained with fluorescently labelled wheat germ agglutinin (WGA-AF 488) was followed over time. Chlamydospores germinate on the root surface, with the growing hyphae closely attached to rhizodermal cell walls. About 24–36 hours after inoculation (hai), hyphae penetrating intercellular spaces between rhizodermal cells grow in sub-rhizodermal areas before accessing rhizodermal cells and filling these cells with a network of hyphae. Figure 1A shows barley roots 36 h after inoculation stained with WGA-AF 488 (detecting the chitin of fungal structures) and Congo red (to visualize plant cells). Intra- and intercellular hyphae originating from extracellular fungal mycelium and chlamydospores are visible. In developmentally 'older' root areas, a meshwork of intra- and intercellular hyphal growth is observed. In addition, *P. indica*-related fungi belonging to the order *Sebacinales* were shown to promote growth and systemic resistance to barley (Deshmukh et al., 2006). Therefore we analyzed the development of two *Sebacina vermifera* isolates (MAFF 305830, 305828) in roots to assess possible differences in fungal development in the host tissue. In general, development of these isolates with respect to extracellular growth, hyphal structures and infestation patterns was similar to *P. indica*, while some variation was observed for the speed of fungal development. For instance, *S. vermifera* (305828) shows intercellular growth 6 dai, with hyphae growing intercellularly between rhizodermal cells (Figure 1B), while *S. vermifera* (305830) at the same time point is already growing intracellularly in several adjacent rhizodermal cells with a meshwork of hyphae (Figure 1C).

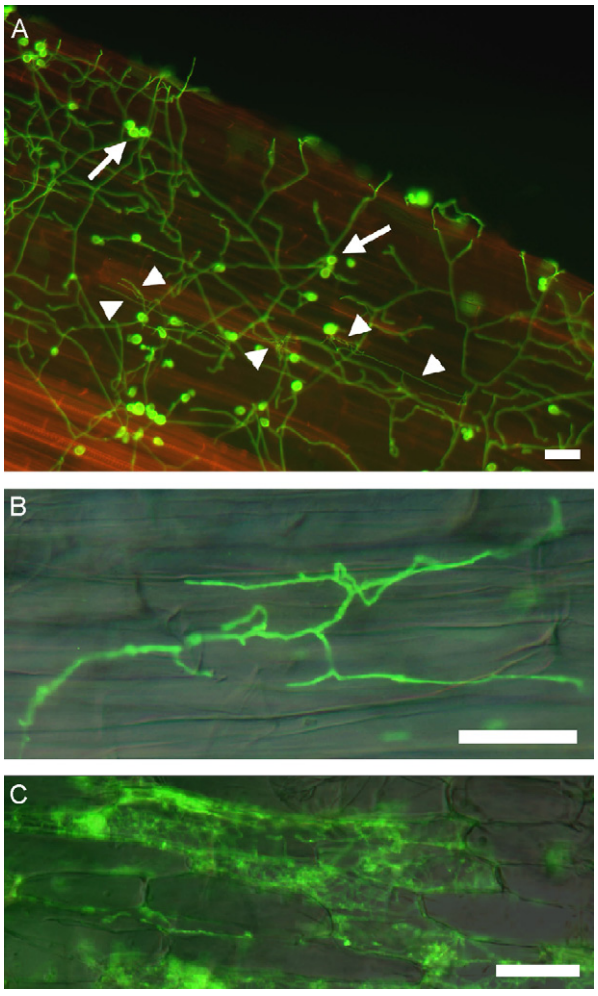

Figure 1. Barley root infestation with Sebacinales. (A) Barley root 36 h after inoculation with *P. indica*. Fungal structures are stained with WGA-AF 488, while plant cell walls are stained with Congo red. This section of an infested barley root is focused on intra- and intercellular hyphae (arrowheads). Extracellular fungal mycelium and chlamydospores (arrows) are in a different focal plane. (B) Barley root 6 days after inoculation with *Sebacina vermifera* (MAFF305828) and (C) 6 days after inoculation with *S. vermifera* (MAFF305830). Fungal structures are stained with WGA-AF 488. Overlays of the bright-field image with the fluorescent image are shown. Scale bars in A–C represent 20 μm.

Gene expression patterns in *P. indica*-infested roots indicate a root-age-dependent regulation of host mRNA levels

Three-week-old barley plants show a steady-state level of *P. indica* root infestation, representing an established symbiosis (Deshmukh et al., 2006). At this stage, barley is displaying an increased systemic resistance against powdery mildew (Waller et al., 2005). To analyze whether *P. indica*-infested roots are showing an enhanced expression of defence-related transcripts, we used a cDNA macroarray containing 4806 cDNAs from cDNA libraries of barley roots, including libraries of roots challenged with the root pathogenic fungus *Fusarium culmorum* (see Materials and methods). Roots from 3-week-old plants inoculated or mock-inoculated with *P. indica* 3 days after seed germination were used as probes. This procedure was performed in three independent experiments. In general, a low number of genes was found to be differentially regulated, with moderate induction levels and a bias towards down-regulated genes

in *P. indica*-infested samples (Figure 2A). Applying stringent selection criteria (see Materials and methods), 18 cDNAs were identified to be repressed and 3 to be induced. Tests by northern blot or quantitative PCR confirmed the expression pattern for 11 of these candidates (Table 1). Sequence homology of candidate cDNAs did not indicate typical defence-related transcripts. Five randomly picked cDNAs of identified candidates were then tested in a northern blot hybridization experiment for expression levels in roots of 10-, 20- and 30-day-old plants infested with *P. indica*. All candidates showed a root-age-dependent regulation of mRNA levels (as exemplified in Figure 2B for an ATP sulphurylase and a LIM domain protein), providing molecular evidence for the observed faster development of *P. indica*-infested roots. Differences in gene expression in 20-day-old roots detected in the cDNA macroarray screen might therefore rather reflect faster root development than a strong constitutive higher or lower level of transcripts induced by *P. indica* (Figure 2B).

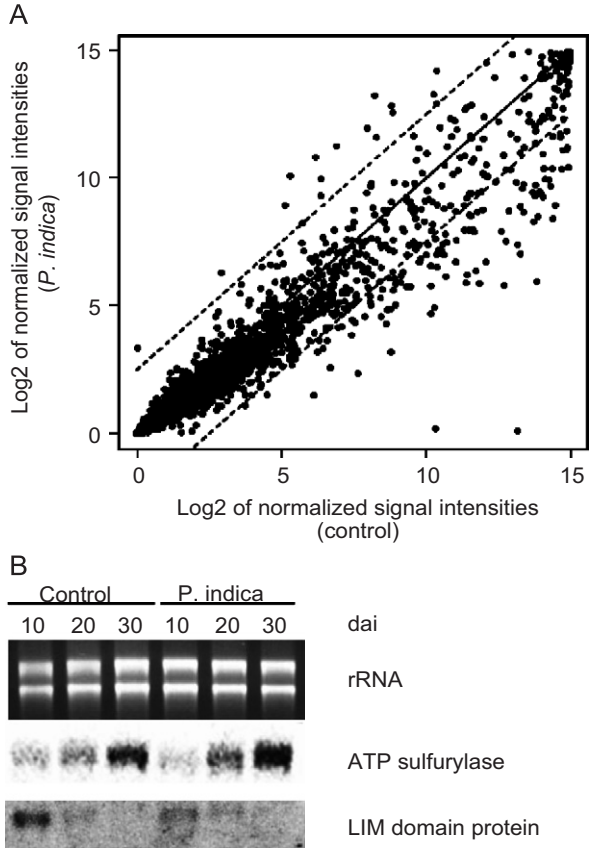

Figure 2. (A) Normalized signal intensities of control and *P. indica*-treated barley root samples hybridized to the cDNA macroarray. Radioactive-labelled cDNAs from one experiment with *P. indica*-inoculated and non-inoculated roots 20 days after inoculation or control treatment were each hybridized to a cDNA macroarray. Log 2 of normalized signal intensities of all 4806 cDNAs (*x*-axis: control, *y*-axis: *P. indica*-inoculated) are plotted. Outer diagonal lines include the area of hybridization signals varying less than 2.5-fold. (B) Northern blot hybridization of two root gene candidates identified in the cDNA macroarray screen for *P. indica*-regulated genes. Samples are from *P. indica*-infested and non-infested barley roots harvested 10, 20 and 30 days after inoculation with *P. indica* 3 days after germination. Upper panel: ethidium-bromide-stained gel image before blotting shows equal loading of RNA samples. Middle and lower panel: gel blots hybridized with radioactive-labelled probes derived from amplified cDNA fragments from clones 3GNW00415 (ATP sulphurylase) and 2GCW001E14 (LIM domain protein), respectively.

Systemic induction of gene expression in leaves of *P. indica*-inoculated plants

To elucidate possible signal transduction pathways responsible for *P. indica*-induced systemic resistance in barley, we analysed gene expression

Table 1. Root gene candidates identified in a cDNA macroarray screen for *P. indica*-regulated genes

cDNA clone on the macroarray	Sequence homology to	Detected regulation, confirmed by RNA gel blot (Blot) or qPCR (qPCR)
3GNW004I5	ATP Sulphurylase	↑(Blot)
3GNW003N8	Peroxidase	↑(Blot)
2GCW001K11	Unknown protein	↓(Blot)
2GCW001E14	LIM domain protein	↓(Blot)
3GNW004P16	TDP glucose dehydratase	↓(Blot)
1GW001G8	Plasma membrane intrinsic protein (PIP) 1;3 (Aquaporin)	↓(Blot)+(qPCR)
1GW003E1	Vacuolar ATPase subunit G	↓(Blot)
1GW001B5	Ascorbate peroxidase	↓(Blot)
1GW00101	PIP1;2 (Aquaporin)	↓(qPCR)
3GNW001I15	Drought-induced protein	↓(qPCR)
2GCW003B1	Horcolin	↓(qPCR)

Genes that were more than 2.5-fold up- or down-regulated 20 days after *P. indica* inoculation compared with mock-inoculated controls in at least two of three biological experiments were considered as *P. indica*-regulated. Sequence homology is given as the closest homolog after performing a BLASTn homology search (Altschul et al., 1997) with the cDNA sequence of the respective clone. All *p*-values for these homologies were lower than 6.5e−47, except 3GNW003N8 (Peroxidase) for which it was 3.1e−05.

using the Affymetrix Barley 1 gene chip (Close et al., 2004). Leaves from 3-week-old barley plants with roots inoculated or mock-inoculated with *P. indica* 3 days after seed germination were used to extract RNA, labelled and taken as probes on the gene chips. Applying stringent selection criteria (see Materials and methods), 27 cDNAs were identified to be systemically induced (19) or repressed (8) in the leaf by *P. indica* root colonization. We tested the expression of seven of the induced candidate genes by quantitative PCR, using RNA from five independent experiments. Each of these experiments consisted of four treatments: *P. indica* non-infested and infested plants, and, in addition, *P. indica*-infested and non-infested plants that were harvested 12 h after inoculation with powdery mildew (*Blumeria graminis* f.sp. *hordei* (*Bgh*)). Two genes showed consistently higher expression levels in *P. indica*-colonized plants: (1) probe set Barley1_49394, corresponding to a *Hordeum vulgare* Hsp70 (heat-shock protein 70) precursor (Chen et al., 1994; Genbank accession L32165) and (2) probe set Barley1_00590, corresponding to *H. vulgare* pathogenesis-related 17b (HvPR17b) (Christensen et al., 2002).

Without *Bgh* challenge, Hsp70 consistently showed a two-fold higher expression in leaves of *P. indica*-infested plants as compared with the non-infested control (Figure 3A). Twelve hours after challenge with *Bgh*, Hsp70 transcript levels were elevated 4.1-fold compared with plants not challenged with *Bgh*. Comparison of Hsp70 levels between *Bgh*-treated *P. indica* non-infested plants and *Bgh*-treated *P. indica*-infested plants showed slightly, but not significantly, elevated levels of Hsp70 transcript in response to *P. indica* infestation.

HvPR17b mRNA levels were slightly, but not significantly, elevated in unchallenged plants infested with *P. indica* (Figure 3B). In plants not infested with *P. indica*, challenge with *Bgh* leads to a 5.5-fold induction of transcript levels 12 hai. At this time point, after *Bgh* challenge, we observed a consistent 1.5-fold higher expression in the *P. indica*-infested plants as compared with non-infested plants.

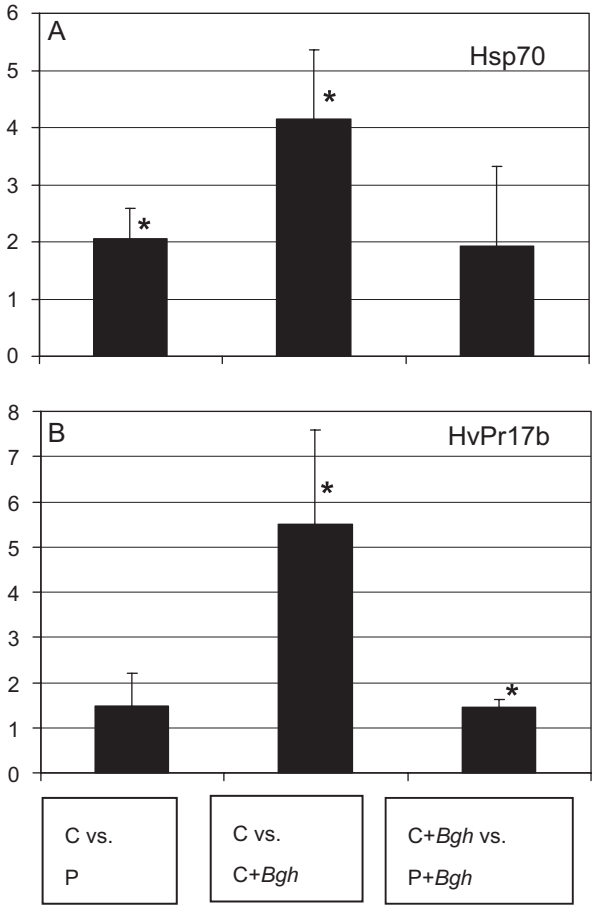

Figure 3. Relative expression of *HvHsp70* and *HvPr17b* in barley leaves. Quantitative real-time PCR experiments from second and third leaves of 3-week-old plants inoculated with *P. indica* 12 h after challenge inoculation with *Blumeria graminis* are shown. Expression levels of the genes were calculated relative to the constitutively present 18S rRNA amplified from the same cDNA. Fold-induction of detected expression levels was calculated for three comparisons: control plants (C) vs. *P. indica*-infested plants (P), control plants vs. *Bgh*-inoculated plants (12 hai) and *Bgh*-inoculated plants vs. *P. indica*-infested *Bgh*-inoculated plants (12 hai). Values shown represent average values from four independent biological experiments, with error bars depicting standard errors and asterisks indicating $p<0.05$ in a one-sample *t*-test. (A) *HvHsp70*. (B) *HvPr17b*.

An Hsp70 precursor mRNA is systemically induced in barley by related fungi of the order *Sebacinales*

Fungi of the order Sebacinales closely related to *P. indica* have also been shown to promote growth and resistance against powdery mildew in barley too (Deshmukh et al., 2006). To analyze whether these fungi induce similar plant responses, we quantified the expression of *HvHsp70* in leaves of barley plants infested with three *S. vermifera* strains (MAFF 305830, 305828 or 305835 (Table 2)). *P. indica*-infested plants showed a 2.3-fold induction of *HvHsp70* transcripts, compared with non-infested controls, whereas induction of the three tested *S. vermifera* strains was 2.7-, 2.6- and 3.8-fold. This systemic induction of *HvHsp70* in leaves of *S. vermifera*-infested barley plants was observed in two independent biological experiments. In

Table 2. Relative expression of HvHsp70 in leaves of barley plants infested with different *Sebacinales* root endophytes

Barley infested with	Fold expression of *HvHsp70* in leaves relative to control plants
Control (non-infested)	1
Piriformospora indica	2.3
Sebacina vermifera MAFF 305830	2.7
Sebacina vermifera MAFF 305828	2.6
Sebacina vermifera MAFF 305835	3.8

Expression of Hsp70 relative to ubiquitin in third leaves of 3-week-old barley plants non-infested (control), or infested with *P. indica*, *Sebacina vermifera* 305830, 305828 or 305835 was assessed by quantitative PCR. Shown are results of the qPCR with expression levels relative to the constitutively expressed barley ubiquitin gene. Fold-induction was calculated relative to expression levels in non-infested plants set to 1. Similar expression and induction levels were obtained in two independent experiments.

parallel, we also quantified expression of the pathogen-induced pathogenesis-related gene 1b (Pr1b) in leaves. Similar to *P. indica*, *S. vermifera*-colonized barley showed low levels of Pr1b expression (data not shown).

Discussion

P. indica root colonization has been shown to severely affect the outcome of an interaction of the biotrophic pathogen *B. graminis* f.sp. *hordei* with barley leaves (Waller et al., 2005). To analyze the mechanism behind this phenomenon, we compared gene expression patterns of *P. indica* infested and non-infested barley plants, both in the root and in the shoot.

Host root interaction – an undercover fungus?

P. indica-infested plants are characterized by enhanced growth, accompanied by elevated salt stress tolerance and biotic stress resistance (Varma et al., 1999; Waller et al., 2005). Deshmukh et al. (2006) showed evidence for a requirement of plant cell death for fungal proliferation, while it is not clear whether *P. indica* actively kills host cells. Interestingly, the endophyte is able to digest plant cell walls and to induce plant cell death under certain axenic conditions related to ammonium supply (Kaldorf et al., 2005). However, fungal culture filtrate is not phytotoxic. Moreover, the fungus grows both inter- and intracellularly within root cortex cells (Figure 1A), without inducing visible cell wall reinforcements typical for a host defence response.

In line with these microscopic observations, gene expression analysis using a cDNA macroarray with 4806 cDNAs revealed that no typical defence-related transcripts were up-regulated in the established *P. indica*–barley symbiosis in the root (Table 1). Several transcripts identified in this screen are regulated depending on root-age, suggesting an effect of fungal colonization on plant development (Figure 2B). We speculate that *P. indica*-mediated differential gene expression is probably the cause or consequence of a faster development of *P. indica*-infested roots. Thus, the pattern might reflect first molecular evidence for the consistent faster growth and development of *P. indica*-infested plant roots (Varma et al., 1999). Faster root development could be also advantageous for the fungus, which relies rather on developmentally older root tissue for its own propagation (Deshmukh et al., 2006).

PR1b, a gene known to be induced 10–100-fold by pathogens, is only slightly (about 3-fold) up-regulated in the roots between 1 and 3 days after *P. indica* inoculation, with even lower expression compared with non-infested control roots 6 dai (Deshmukh and Kogel, 2007). These data suggest that *P. indica* is rather able to suppress expression of PR genes once it is established in the host root. On the other hand, fungal growth is restricted to the root cortex and especially newly formed roots are often free of fungal structures. Hence, it is likely that the plant exerts some control on the degree of endophytic colonization.

Both a limited or absent defence reaction and a fungal proliferation limited to specific tissues are also characteristic for the mutualistic arbuscular mycorrhizal fungi (Hause and Fester, 2005). Liu et al. (2003) identified 67 genes from a set of 2268 cDNAs to be specifically regulated during the interaction of the AM fungus *Glomus versiforme* with *Medicago truncatulata* roots. A set of defence and pathogen-related genes had elevated expression levels in the early phase of interaction and were suppressed in the established interaction (Liu et al., 2003). This corresponds to the absence of *P. indica*-induced defence-related genes in the root identified in this study, which was performed with roots 3 weeks after inoculation. While AMF induced, for example, 224 genes in a whole-genome transcriptome analysis of rice roots (Güimil et al., 2005), the number of *P. indica*-induced genes in barley roots was low. This is not unexpected, as

AMF form nutrition organs in living host cells and induce specific morphological changes in the host root, such as plasma membrane extensions to form an interface for nutrient exchange, which has not been observed for *P. indica*. An important beneficial effect of AMF is an improved nutrient uptake, specifically for phosphate provided to the host (Smith and Read, 1997; Smith et al., 2003), which does not seem to cause the observed beneficial effects of *P. indica* provided to barley (Karandashov et al., 2004; B. Achatz, unpublished results). In summary, root colonization by *P. indica* is characterized by faster root development, colonization of dead root cells and a spatially restricted endophytic growth with only weak elicitation of a defence response.

To identify specific signals of the obvious communication between the endophyte and the root tissue, a gene expression profiling experiment during the early phase (establishment of the fungus) in parallel with a metabolome analysis is currently performed within the DFG research group FOR-666.

Reprogramming and systemic resistance induction

Although interaction with the barley powdery mildew fungus is severely disturbed in *P. indica*-colonized plants, transcripts of *PR5* and *BCI1* known to be induced by elevated SA levels were not increased in leaves of infested barley plants (Waller et al., 2005). Alike, *JIP23*, a marker gene for elevated JA and MeJA levels was not responding to *P. indica*. A pilot experiment utilizing the Barley 1 Gene Chip aimed to identify systemically induced genes. A small number of genes were identified, and induction levels, compared with non-infested control plants, were low. Using specific primers in four independent experiments, consistent systemic expression of two genes could be confirmed. *HvHSP70* is up-regulated in the leaves of *P. indica*-infested plants and is therefore a useful tool to analyze systemic effects of fungal colonization. A second gene, coding for PR17b, is up-regulated in *P. indica*-infested plants only after challenge with powdery mildew fungus, indicating that priming might be a mechanism involved in *P. indica*-mediated plant responses.

An Hsp70 gene as a marker gene for systemic modulation of plant responses

An mRNA coding for a HSP70 was identified as systemically up-regulated due to the presence of *P. indica* in the roots (Figure 3). *S. vermifera* strains that induce systemic resistance in barley (Deshmukh et al., 2006) revealed elevated expression levels of *Hsp70* as well (Table 2). Heat-shock proteins play important roles as 'molecular chaperones' assisting in the correct folding of proteins and are known to play important roles in stress responses. They are induced either by high temperature or by biotic stress (e.g. Gjetting et al., 2004). *Hsp70* homolog Contig590_at shows an elevated expression from 8 h after inoculation of barley leaves both with compatible and incompatible powdery mildew fungus (*Bgh* isolates K1 and 5874) (Caldo et al., 2004). On the other hand, barley spikes inoculated with *Fusarium graminearum* and sampled at 24, 48, 72, 96 and 144 h after treatment did not show a significant up-regulation of *HvHsp70* (Barley Base experiment BB9; Boddu et al., 2006; Shen et al., 2005). Therefore, it is possible that *Hsp70* is regulated locally not in response to necrotrophic but only to biotrophic fungi. Up-regulation of Hsp70 could also be an indirect consequence of metabolic reprogramming, such as the changed antioxidant status (Waller et al., 2005) caused by the root endophyte. The observed powdery mildew-dependent up-regulation may indicate a role in defence, as shown for a HSP90 required for Mla13-mediated race-specific powdery mildew resistance (Hein et al., 2005). However, two *Hsp70* cDNAs different from the *HvHsp70* shown here were expressed stronger in barley single epidermal cells infected by *Bgh* as compared with resistant cells (Gjetting et al., 2007), suggesting a possible role in compatibility.

HvPR17b is a marker gene of *P. indica*-infested barley systemically up-regulated after powdery mildew challenge inoculation

HvPR17b was first identified as an mRNA induced by powdery mildew challenge in barley leaves (Christensen et al., 2002). Its amino acid sequence contains typical hydrophobic signal peptides. Several homologous plant proteins exist, with a highly conserved part of members of this protein family showing similarity to the active site and to the peptide-binding groove of the exopeptidase aminopeptidase N from eukaryotes and the endopeptidase thermolysin from bacteria. Gjetting et al. (2007) report that *HvPR17b* is up-regulated 18 h after *Bgh* inoculation both in cells in which a haustorium initial is formed and in cells producing a papilla stopping fungal penetration. *HvPR17b* is not only induced by *Bgh* but also induced in barley spikes challenged with *F. graminearum* from 48 hai,

with the highest induction 72 hai (Boddu et al., 2006; Barley Base experiment BB9; Shen et al., 2005).

HvPr17b belongs to the same family of PR genes as *WCI5* (wheat chemically induced 5), which is induced by the chemical resistance inducer BTH (benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester), an SA analog in wheat (Görlach et al., 1996). Recently, Pasquer et al. (2005) identified *WCI5* as being induced not only by BTH but also by the fungicides fenpropimorph and azoxystrobin 24 h and 1 week after treatment, indicating that these chemicals exploit resistance inducing side effects. When transiently over-expressed in wheat epidermal cells as a GUS fusion protein, WCI5 reduced the relative penetration efficiency of *B. graminis* f.sp. *tritici* by about 20% (Schweizer et al., 1999). With HvPr17b amino acid identity of 66% to WCI5 and highly conserved protein domains, it is thus possible that HvPR17b is directly involved in *P. indica*-mediated powdery mildew control. The protein might exhibit direct antifungal activity. However, protease activity of HvPR17b could not be detected *in vitro*, leading Christensen et al. (2002) to propose that HvPR17b might influence cell wall metabolism, or is involved in recognition and signalling in the cell wall, e.g. by releasing components of particular pathogens, thereby producing elicitors.

We suggest that enhanced systemic expression of *HvPR17b* after powdery mildew challenge indicates a 'state of alert' in the plant and is therefore a molecular marker for priming by a fungal root endophyte. In addition, *HvPR17b* expression shows that there are conserved elements in resistance induction effective against powdery mildew in cereals, both by *P. indica* infestation and in plants treated with SA.

As *P. indica* obviously is not changing large sets of genes in systemic tissue, it resembles ISR (Pieterse et al., 1998), which in the absence of a challenging pathogen is accompanied with systemic up- or down-regulation of rather low numbers of transcripts in *Arabidopsis* (Cartieaux et al., 2003; Verhagen et al., 2004; Wang et al., 2005). Pathogen resistance induced by AMF occurs in plant roots and has been discussed to be similar to ISR, as the JA pathway is induced in AM roots (Hause et al., 2002) and seems to play a role in protecting systemic tomato roots against *Phytophthora parasitica* (Pozo et al., 2002). A second well-studied mechanism of induced resistance is the SA-mediated SAR, which is thought to be induced by pathogens causing necrotic lesions and/or leading to elevated local SA levels (Durrant and Dong, 2004). However, in barley no typical defence-related transcripts could be identified in roots 20 dai, and systemic up-regulation of genes typically implicated in SA responses was not observed. Therefore, an SAR mechanism seems to be rather unlikely. This notion is strongly supported by experiments exploiting *Arabidopsis* mutants, which demonstrated that *P. indica*-mediated resistance is not affected by a compromised SAR pathway (Stein et al., unpublished). Still, up-regulation of the *WCI5* homolog H*vPR17b* can be accomplished by SA treatment and a convergence of pathways mediated by SA, JA and other compounds upstream of *HvPr17b* is possible. A promoter analysis of systemic *P. indica*-induced genes such as *HvPr17b* will help to clarify signal transduction pathways of systemic resistance induced by the compatible interaction of cereal plants with fungal root endophytes.

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Detection and identification of bacteria intimately associated with fungi of the order *Sebacinales*

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Summary

Because of their beneficial impact on plants, the highly diverse mycorrhizal fungi grouped in the order *Sebacinales* lay claim to high ecological and agricultural significance. Here, we describe for the first time associations of Sebacinoid members with bacteria. Using quantitative PCR, denaturating gradient gel electrophoresis and fluorescence *in situ* hybridization, we detected an intimate association between *Piriformospora indica* and *Rhizobium radiobacter*, an α-Proteobacterium. The stability of the association, vertical transmission of the bacteria during asexual fungal reproduction and fungal plant colonization was monitored using *R. radiobacter*-specific primers. Treatment of mycelium or fungal protoplasts with antibiotics highly efficient against the free bacteria failed to cure the fungus. Barley seedlings dip-inoculated with *R. radiobacter* showed growth promotion and systemic resistance to the powdery mildew fungus *Blumeria graminis* comparable to *P. indica* inoculation. By screening additional isolates of the *Sebacina vermifera* complex, three species-specific associations with bacteria from the genera *Paenibacillus*, *Acinetobacter* and *Rhodococcus* were found. These findings suggest that *Sebacinales* species regularly undergo complex interactions involving host plants and bacteria reminiscent of other ectomycorrhizal and endomycorrhizal associations.

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Introduction

In natural ecosystems, plants experience complex interactions with microorganisms on physical, metabolic and functional levels, and hardly a single plant family has been recognized, which is not living in symbiosis with microorganisms (Smith and Read, 1997; Frey-Klett *et al.*, 2007). Plant symbiotic microbes can grow, propagate and interact not only in form of individual cells but also as multitrophic communities. In many cases this might be the key for the widespread success of host–microbe symbioses because of the immense biochemical and physiological diversity among microbial species and the ability of microbial cells and microbial communities to precisely sense and properly respond to changing environments.

Here we describe symbiosis of barley with species of the fungal order *Sebacinales* with emphasis on fungus-associated bacteria. *Sebacinales*, the most basal Basidiomycota group with known mycorrhizal members, are ubiquitously distributed and are found on all continents in temperate and subtropical climates associated with orchids, liverwort thalli and Ericaceae as ectomycorrhizal and endomycorrhizal fungi (Selosse *et al.*, 2007; Schäfer and Kogel, 2008). Recent studies indicate that they also form a novel type of mutualistic symbiosis with a broad spectrum of monocotyledonous and dicotyledonous plants (Weiss *et al.*, 2004; Matheny *et al.*, 2007), including crop plants such as barley, maize, tomato and – in contrast to arbuscular mycorrhiza fungi – *Brassicaceae* (Varma *et al.*, 1999; Peskan-Berghoefer *et al.*, 2004, Deshmukh *et al.*, 2006). *Sebacinales* are divided into two clades (Selosse *et al.*, 2007). Clade A consists of ectomycorrhizae and ectendomycorrhizae species whereas Clade B includes ericoid along with cultivable orchid root colonizing mycorrhiza species of the complex *Sebacina vermifera* and *Piriformospora indica* (Varma *et al.*, 1998; Weiss *et al.*, 2004). Plants colonized by these

species display improved growth and fitness. Barley plants colonized by *P. indica* show higher grain yield and superior resistance against various root and leaf pathogens (Waller *et al*., 2005; Deshmukh *et al*., 2006). Because of its remarkable beneficial activity the *Sebacinales* have attracted considerable interest as potential biocontrol agents, especially because these fungi are not obligate biotrophs and thus axenically cultivable.

Many recent reports on mycorrhizal interactions suggest that biological activities brought about by the symbiosis ought to be considered under the premise of a more complex tripartite interplay of the host plant with the mycorrhiza fungus and fungus-associated bacteria. These bacteria can associate with fungal spores (Walley and Germida, 1997), hyphae (Nurmiaho-Lassila *et al*., 1997; Sbrana *et al*., 2000), or are present as endobacteria inside fungal cells (Bertaux *et al*., 2005; Lumini *et al*., 2006). In many cases these associations show a certain type of specificity (Artursson *et al*., 2006). In 1994, Garbaye (1994) introduced the term mycorrhization helper bacteria for bacteria associated with mycorrhizal fungi which consistently promote mycorrhizal development. There is a wide variety of beneficial effects mediated by these bacteria to the mycorrhizal partner which include inhibition or promotion of germination and alterations to foraging behaviour, hyphal branching (fungal architecture), growth, survival, reproduction, exudate composition and production of antibacterial metabolites (Rainey *et al*., 1990; Frey-Klett and Garbaye, 2005; Aspray *et al*., 2006; Riedlinger *et al*., 2006; Frey-Klett *et al*., 2007). Although most studies of bacterial interaction have been conducted in ectomycorrhizal systems, the interaction between bacteria and arbuscular mycorrhizal (AM) symbioses has also been shown (Bianciotto *et al*., 1996; Duponnois and Plenchette, 2003). Bacteria have been shown to increase the germination and growth of AM fungi thus helping in the symbiosis (Artursson *et al*., 2006). These beneficial mycorrhizal associates belong to diverse bacterial groups, including Gram-negative Proteobacteria, e.g. *Agrobacterium*, *Azospirillum*, *Azotobacter, Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Pseudomonas, Klebsiella* and *Rhizobium*, Gram-positive Firmicutes like *Bacillus*, *Brevibacillus* and *Paenibacillus*, as well as Gram-positive *Actinobacteria* (*Rhodococcus*, *Streptomyces* and *Arthrobacter*). The contribution of the bacterial partners on plant's physiology and – on another scale – on natural ecosystems is not understood.

We demonstrate here stabile associations of fungal species of the order *Sebacinales* with bacteria known to interact with ectomycorrhiza and endomycorrhiza. We show that *P. indica* is associated with a strain of the species *Rhizobium radiobacter* and that this bacterium exhibits a considerable biological activity to the host plant.

Results

Piriformospora indica *is associated with* R. radiobacter

Under standard culture conditions as used for fungal propagation, *P. indica* develops spherical fungal colonies within 3–4 weeks in the transparent medium. However, crushing the mycelium with a fine blender and subsequent microscopic examination of the supernatant upon bacterial live–dead staining indicated the presence of rod-shaped bacteria of 1–1.5 μm in length. This initial observation suggested that there is a tight association of *P. indica* with bacteria. To check this notion, we traced the presence of specific bacteria in the original *P. indica* isolate *P. indica*-DSM11827 deposited in 1997 immediately after the discovery of the fungus in the Indian Thar desert (Table 1). Using universal primers, almost full-length fragments of the entire bacterial 16S-rRNA gene were amplified from the fungal metagenomic DNA of *P. indica*-DSM11827 as well as in other fungal cultures

Table 1. *Sebacinales* analysed for bacterial presence.

Isolate	Host	Associated bacteria
Piriformospora indica-DSM11827[a]	*Prosopis julifora* and *Zizyphus nummularia*[b]	*Rhizobium radiobacter* (GenBank Accession No. EU669179)
S. vermifera-MAFF305838[c]	*Caladenia tesselata*	*Paenibacillus* sp. (GenBank Accession No. EU669180)
S. vermifera-MAFF305828[c]	*Eriochilus cucullatus*	*Acinetobacter* sp. (GenBank Accession No. EU669181)
S. vermifera-MAFF305835[c]	*Caladenia catenata*	*Rhodococcus* sp. (GenBank Accession No. EU669182)
S. vermifera-MAFF305837[c]	*Caladenia dilatata*	P[d]
S. vermifera-MAFF305830[c]	*Crytostylis reniformis*	P[d]
S. vermifera-MAFF305842[c]	*Microtis uniflora*	P[d]
Multinucleate *Rhizoctonia*-DAR29830[e]		P[d]

a. Type species *Piriformospora indica* was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.
b. Fungus was originally isolated from rhizosphere of these plants.
c. Culture collection numbers: Isolates of *Sebacina vermifera* were obtained from the National Institute of Agrobiological Sciences, Tsukuba, Japan.
d. Present (P) but bacterial identity not yet determined.
e. The isolate DAR29830 was kindly provided by Karl-Heinz Rexer, University of Marburg, Germany.

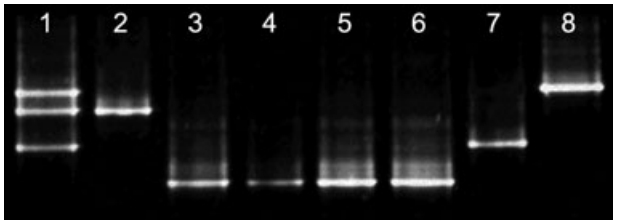

Fig. 1. Detection of *P. indica*-associated bacteria by DGGE. The 16S-rRNA gene fragments of 500 bp were obtained from the metagenome of bacterial communities associated with different isolates of *P. indica*. Lane 1, reference pattern composed with three known bacterial strains (*E. coli*, *H. frisingense* Mb11, *Acinetobacter* sp.); lane 2, *Acinetobacter* sp.; lane 3, *P. indica*-DSM11827; lane 4, *P. indica*-JE1; lane 5, *R. radiobacter* PABac-DSM isolated from *P. indica*-DSM11827; lane 6, *R. radiobacter* PABac-JE isolated from *P. indica*-JE1; lane 7, *E. coli*; lane 8, *Herbaspirillum frisingense Mb11*. Sequence data from the bands in lanes 3 and 4 showed 100% identity with the 16S-rRNA gene sequences of the bacterial isolates PABac-DSM and PABac-JE in lanes 5 and 6.

including *P. indica*-JE1, *P. indica*-HA and *P. indica*-ND (Fig. S1). Fragments showed identical 16S-rRNA gene-coding sequences, designating the bacterium as α-Proteobacterium of the genus *Rhizobium* (GenBank Accession No. EU669179) with the highest similarity to the species *R. radiobacter* (synonym *Agrobacterium radiobacter or Agrobacterium tumefaciens*) (Young *et al.*, 2001). These data suggested that *P. indica* contains a single bacterial strain. To confirm this finding, we employed denaturing gradient gel electrophoresis (DGGE) of the seminested PCR products of the 16S-rRNA-coding gene. DGGE revealed the presence of a single high intensity band in *P. indica*-DSM11827 and *P. indica*-JE1 (Fig. 1). Direct PCR amplification also showed the same pattern of bands (not shown). DNA sequences of the PCR product were identical to a 500 bp region of the 16S-rRNA gene obtained from metagenomic DNA of *P. indica*-DSM11827.

For further characterization we attempted to isolate bacteria from the fungal mycelium of *P. indica*-DSM11827 and *P. indica*-JE1. Growth of isolated bacteria – named PABac-DSM and PABac-JE respectively – was observed 3 days after inoculation of bacteria released from mechanically sheared fungal hyphae in Luria–Bertani (LB) medium supplemented with 0.8% sucrose. Microscopic analysis revealed the presence of a pure culture of rod-shaped bacteria of 1–1.5 μm in length (Fig. S2). By using the primer pair 27f and 1495r to amplify the 16S-rRNA gene, a PCR product of the expected size was obtained (Fig. S1). Cloning, sequencing and comparative 16S-rRNA gene sequence analysis showed that all obtained sequences were identical to each other and were also identical to those isolated from metagenomic DNA of different *P. indica* isolates. After BLAST search and phylogenetic analysis with ARB software (Fig. 2) and EzTaxon server (Chun *et al.*, 2007), the sequence was identified as belonging to an α-Proteobacterium of the genus *Rhizobium* (GenBank Accession No. EU669179) with the highest similarity and 100% homologue to the species *R. radiobacter*-AJ389909. The phenotypic characters (data not shown) were congruent with those reported for *R. radiobacter* (Young *et al.*, 2001 and references therein).

Denaturing gradient gel electrophoresis profiles of a direct and seminested PCR assay using 16S-rRNA gene

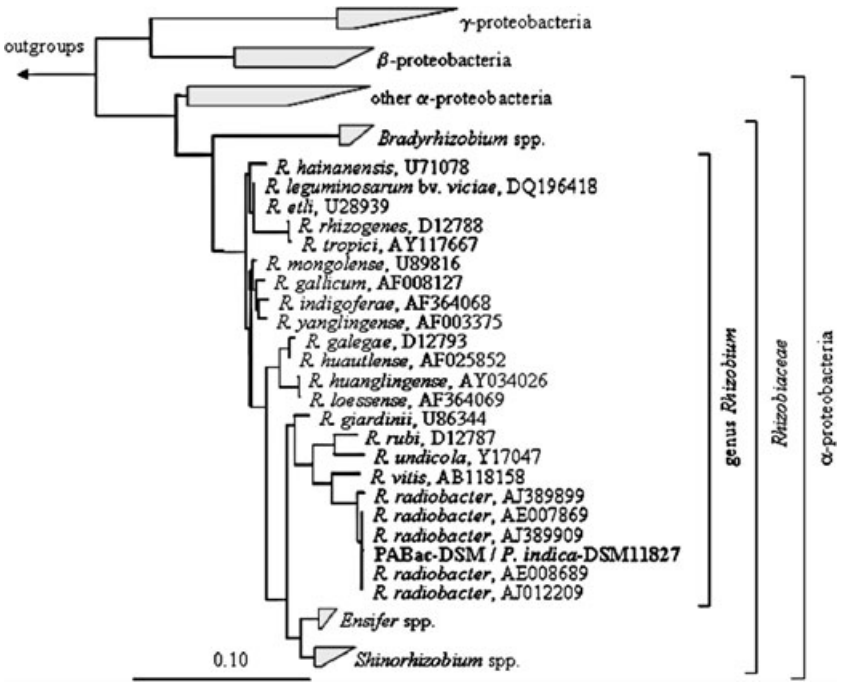

Fig. 2. Phylogenetic classification of *P. indica*-associated bacteria. Phylogenetic tree based on comparative sequence analysis of 16S-rRNA-coding genes of *P. indica*-associated bacteria and representatives of related *Rhizobium radiobacter*/*Agrobacterium tumefaciens* group. The bar indicates 10% sequence divergence.

Table 2. Efficacy of antibiotics against *R. radiobacter*.

Antibiotic	OD_{600} after 48 h
Spectinomycin (300 μg ml^{-1})	0.000
Spectinomycin (200 μg ml^{-1})	0.010
Ciprofloxacin (200 μg ml^{-1})	0.013
Ciprofloxacin (100 μg ml^{-1})	0.017
Cefatoxime (300 μg ml^{-1}) + Ticarcillin (150 μg ml^{-1})	0.019
Oxytertacycline (100 μg ml^{-1})	0.030
Rifampicin (100 μg ml^{-1})	0.032
Cefatoxime (500 μg ml^{-1})	0.050
Oxytertacycline (200 μg ml^{-1})	0.070
Cefatoxime (250 μg ml^{-1})	0.091
Ticarcillin (150 μg ml^{-1})	0.501
Lincomycin (100 μg ml^{-1})	2.160
Carbenicillin (100 μg ml^{-1})	2.243
Gentamicin (100 μg ml^{-1})	2.510
Gentamicin (50 μg ml^{-1})	2.450
Control	2.499

Bacteria (PABac-DSM) were grown in LB medium containing various antibiotics at 25°C. OD600 was measured 48 h after inoculation.

primers corroborated presence of a single high intensity band in PABac-DSM and PABac-JE identical in electrophoretic mobility and proved to have the same sequence as obtained from the metagenomic DNA of *P. indica*-DSM11827 and *P. indica*-JE1 (Fig. 1). The quantification of the ratio of bacterial to fungal DNA in different fungal cultures by real-time quantitative PCR showed an average of 0.035 ng of *R. radiobacter* DNA per 100 ng of *P. indica* DNA.

Treatments for potential curing P. indica *from* R. radiobacter

With the aim of eliminating *R. radiobacter* from *P. indica*-DSM11827, bacteria (*R. radiobacter* PABac-DSM) were first cultivated in LB medium in the presence of various antibiotics while the efficacy of the chemicals was proved by OD_{600} measurement after 48 h (Table 2). Among other antibiotics spectinomycin and ciprofloxacin were found to be highly efficient in completely inhibiting the growth of *R. radiobacter* PABac-DSM *in vitro*. However, after axenic culturing of *P. indica*-DSM11827 for 2 months at 25°C in the presence of ciprofloxacin (200 μg ml^{-1}) and/or spectinomycin (300 μg ml^{-1}) bacteria were neither eliminated from the hyphae nor eliminated from chlamydospores as evidenced by PCR analysis. In analogy to the strategy followed in arbuscular mycorrhiza (Lumini *et al*., 2007), *P. indica* was intended to be cured from bacteria by producing successive vegetative generations starting from single chlamydospores. Five generations of single spores (G1, G2, G3, G4, G5) were grown on agar plates containing 300 μg ml^{-1} spectinomycin and 10 colonies from each generation were tested for bacterial presence by conventional PCR using universal eubacterial primers. However, bacteria were detected in all generations (data not shown). In a complementary approach, young growing hyphae were transferred to freshly prepared plates containing antibiotics every fourth day for five times. In all seven tested *P. indica* colonies, conventional PCR with universal primes as well as real-time quantitative PCR with primers specific for the *Rhizobium/Agrobacterium* intergenic transcribed spacer resulted in PCR products of the appropriate sizes, confirming the presence of *R. radiobacter*. In another attempt, hyphal protoplasts were isolated and subsequently regenerated on plates containing *Aspergillus* minimal medium with 0.3 M sucrose, spectinomycin (300 μg ml^{-1}) and ciprofloxacin (300 μg ml^{-1}) at 30°C and growing mycelium was transferred every second day for eight consecutive days, to fresh medium plates containing antibiotics. All seven independent regenerated *P. indica* colonies gave positive signals after real-time quantitative PCR analysis for the presence of bacterial 16S-23S rRNA intergenic transcribed spacer (ITS) sequences.

Piriformospora indica *is intimately associated with* R. radiobacter

In order to locate bacteria associated with *P. indica*, fungal preparations from axenic cultures of *P. indica*-DSM11827 as well as cells of a pure culture of strain PABac-DSM were stained by fluorescence *in situ* hybridization (FISH). The presence of bacterial rRNA in the fungal mycelium was proven by application of a probe specific for eubacteria (EUB-338-mix) (Table 3). Concomitantly, a colocalized signal was detected by using the Rh-1247 probe, which is specific for rRNA of bacteria belonging to the *Rhizobium* group (Fig. 3A-C). In order to exclude the detection of unspecific hybridizations EUK-516 probes were introduced that are specific for 18S-rRNA of eukaryotes. These analyses further implicated association of bacteria with mycelium and chlamydospores of *P. indica* and further confirmed the low number of associated bacteria as was already indicated by real-time PCR-based quantification.

Rhizobium radiobacter *induces growth promotion and disease resistance in barley*

Most substantial biological activities of *P. indica* and related *S. vermifera* species in various host plants are growth promotion and systemic induced resistance to fungal pathogens (Deshmukh *et al*., 2006). To assess the biological activity of isolated bacteria, roots of 3-day-old barley seedlings were dip-inoculated with PABac-DSM (OD_{600} 1.6). Upon 3 weeks, treated plants showed an increase in shoot fresh weight of 17% over control plants demonstrating the growth promoting activity of the bacterium (Table 4). Moreover, the same plants were more

Table 3. Phylogenetic oligonucleotide probes used for FISH analyses.

Probe[a]	Target[a]	Position[a,b]	Sequence (5′-3′)[a]	FA (%)[a,c]	Specificity[a]
EUK-516	18S-rRNA	502–517	ACCAGACTTGCCCTCC	0–50	*Eukarya*
EUB-338	16S-rRNA	338–355	GCTGCCTCCCGTAGGAGT	0–50	Most bacteria
EUB-338 II	16S-rRNA	338–355	GCAGCCACCCGTAGGTGT	0–50	*Planctomycetales*
EUB-338 III	16S-rRNA	338–355	GCTGCCACCCGTAGGTGT	0–50	*Verrucomicrobiales*
LGC-354-a	16S-rRNA	354–371	TGGAAGATTCCCTACTGC	35	Firmicutes (Gram-positive bacteria with low DNA G + C content)
LGC-354-b	16S-rRNA	354–371	CGGAAGATTCCCTACTGC	35	Firmicutes (Gram-positive bacteria with low DNA G + C content)
LGC-354-c	16S-rRNA	354–371	CCGAAGATTCCCTACTGC	35	Firmicutes (Gram-positive bacteria with low DNA G + C content)
Rh-1247	16S-rRNA	1247–1252	TCGCTGCCCACTGTG	35	*Rhizobium* sp., *Agrobacterium* sp., *Ochrobactrum* sp., some *Azospirilla* sp., few *Sphingomonas* sp.

a. Data taken from probe base (http://www.microbial-ecology.net/probebase) (Loy *et al*., 2003; 2007).
b. Position according to Brosius *et al*. (1981).
c. Formamide in the hybridization buffer.

resistant to the biotrophic fungal leaf pathogen *Blumeria graminis* f.sp. *hordei*. Barley leaves showed a decrease in the frequency of powdery mildew pustules of 64% over control (plants treated only with LB medium; Table 4). To compare the bacteria-mediated activity with that from the fungus, we inoculated roots with *P. indica*. We found an increase in fresh shoot biomass (27%) and a decrease in the number of powdery mildew pustules (54%) as compared with non-colonized plants (Table 4).

In order to further characterize the bacterium, we grew *R. radiobacter* PABac-DSM in mineral salt medium supplemented with 0.5% glucose and 500 μg ml^{-1} of tryptophan. Under this cultivation conditions, the bacterium exhibited a substantial production of indole-3-acetic acid (IAA) up to 40 μg ml^{-1} after 24 h at 25°C in the presence of tryptophan. In the absence of this amino acid, however, there was no production of IAA demonstrating tryptophan-dependent IAA synthesis by *R. radiobacter* PABac-DSM.

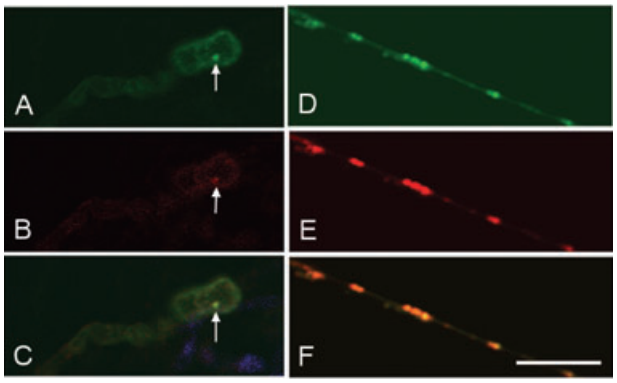

Fig. 3. Detection of fungus-associated bacteria by fluorescence *in situ* hybridization (FISH). Fixed fungal mycelia of *P. indica*-DSM11827 (A–C) and *S. vermifera*-MAFF305838 (D–F) were used for FISH analysis. FISH was performed with EUB-338-mix-FITC (green), specific for the domain *Bacteria* (A and D) and with either Rh-1247-Cy3 probe (red), specific for the *Rhizobium* group (B–C) or LGC-mix-Cy3 (red), specific for the Firmicutes group to which *Paenibacillus* sp. belongs (E–F). While Fig. 3A and D shows the green channel, Fig. 3B and E shows the red channel and Fig. 3C and F shows the superimposed images. In Fig. 3C and F, the composed rgb-images result in a yellow colour for the bacteria, indicating colabelling by EUB-338-mix-FITC and Rh-1247-Cy3 (C) and colabelling by EUB-338-mix-FITC and LGC-354-Cy3 (F). In A– C, arrows indicate the bacterial signals. Scale bar = 10 μm.

Bacterial associations are common in Sebacinales

The hypothesis was followed that other species of the order *Sebacinales* commonly contain endocellular bacteria. Therefore, seven *S. vermifera* isolates were selected, which were originally collected from different autotrophic orchids in Australia (Warcup, 1988). All the strains were previously proven to exhibit beneficial biological activity in barley (Deshmukh *et al*., 2006). After PCR and sequencing analyses using bacterial universal primers we provided evidence that all *S. vermifera* isolates contained bacteria although from different genera (Table 1). We studied the bacterial association of *S. vermifera*-MAFF305838 in more detail. Using FISH and confocal laser scanning microscopy on axenically grown fungal cultures, we detected an intracytoplasmic localization of bacterial cells within hyphae (Fig. 3D–F). Spherical bacteria of 0.5–1 μm size were detected with the probe EUB-338-mix, indicative for almost all bacteria, and colocalized with probe LGC-354-mix,

Table 4. Biological activity conferred by *R. radiobacter* in barley.

Treatment	Increase in shoot weight over control (%)	Decrease in powdery mildew pustules over control (%)
R. radiobacter	17.07**	63.90**
P. indica	27.35**	53.56**

Asterisks denote statistically significant differences between the respective values of endophyte-colonized and non-colonized plants (**Student's *t*-test $P < 0.001$).

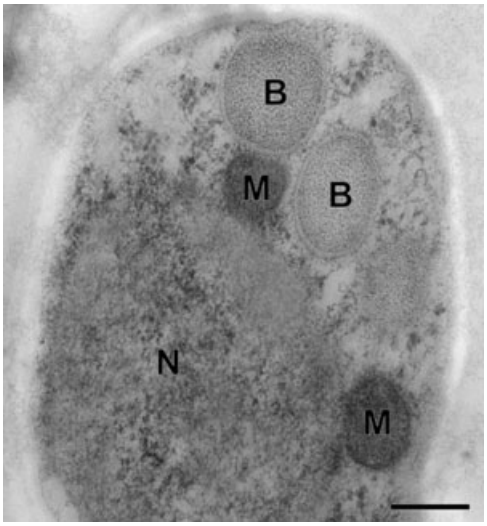

Fig. 4. Transmission electron micrograph of fungal mycelium of *S. vermifera*-MAFF305838 containing endocellular bacteria (B), a nucleus (N) and mitochondria (M) (Scale bar = 200 nm).

specific for the Firmicutes group to which *Paenibacillus* belongs (Table 3). The number of bacteria varied from one to three per hyphal cell. To exclude unspecific probe labelling, the samples were hybridized with EUK-516 probe (specific for 18S-rRNA of eukaryotes). To further substantiate the presence of endobacteria, transmission electron microscopy of *S. vermifera*-MAFF305838 was done. The mycelium from liquid culture was frozen at high pressure and cryosubstituted in glutaraldehyde and uranyl acetate. Examination of the samples confirmed the presence of endobacteria within the cytosol of intact cells of *S. vermifera*-MAFF305838 (Fig. 4).

Discussion

We demonstrate here that species of the *Sebacinales* regularly undergo complex symbioses involving plants and bacteria of different genera. Sebacinoid fungi were formerly shown to possess a broad plant host spectrum (Varma *et al*., 1999; Weiss *et al*., 2004) and, so far investigated, exhibit beneficial biological activities to their hosts (Deshmukh *et al*., 2006). Our finding that the Sebacinoid symbiosis involves bacteria leads to a more complex picture and requires reconsideration of the role played by the fungus in its symbiotic interaction with the plant. However, not only complexity but also intimacy apparently constitutes a crucial trait in bacteria–Sebacinoid associations. First, associations of bacteria with Sebacinoid fungi are rather diverse. We found bacterial species belonging to three different genera, namely *Paenibacillus*, *Acinetobacter* and *Rhodococcus* in three phylogenetically and morphologically distinct members of the *S. vermifera* species complex and *R. radiobacter* closely associated with *P. indica*. Second, isolation and subsequent axenic cultivation of fungus-associated bacteria were successful, so far, only with *R. radiobacter* from *P. indica*. Third, bacteria-free Sebacinoid fungi could not be obtained so far. These tight relational characteristic impedes investigations on the importance of each partner for mediating biological activity and for plant colonization. Intriguingly, these close associations of Sebacinoid fungi with bacteria resemble those previously detected in other mycorrhizal associations (Frey-Klett *et al*., 2007).

Bianciotto *et al*. (2004) recently demonstrated for the AM fungus *Gigaspora margarita* a continuous vertical transmission of its endobacteria from one generation to another guaranteeing the enduring nature of the association. Comparably, *R. radiobacter* was found in developing *P. indica* chlamydospores and in fungal colonies regenerated from single spores or from hyphal tip cells. Moreover, bacteria were still traceable in the fungal cultures after five fungal root passages (data not shown).

Different strategies to obtain bacteria-free fungus failed, which further argues against a rather temporal or loose association. Neither cultivation of hyphae in axenic culture under high antibiotic concentrations nor successive *in vitro* single-spore isolation steps nor the exposure of fungal protoplast to antibiotics in the regenerating medium resulted in bacteria-free *P. indica.* In presence of antibiotics, the fungus grew slowly that may indicate an adverse antibiotic effect on bacteria or on the fungus itself. However, after removing the antibiotics, the amount of bacterium was in range as found in untreated *P. indica*. These findings suggest that either the bacterium is protected inside the fungus or its absence would reduce fungal fitness as reported for *G. margarita* cured from its endocellular bacteria (*Candidatus* Glomeribacter gigasporarum) (Lumini *et al*., 2007). However, *R. radiobacter* could be isolated from fungal mycelium and multiplied in liquid cultures demonstrating that the bacterium is not entirely dependent on the fungus. That the association bases on a critical balance between bacterium and fungus is suggested by an experiment in which *R. radiobacter* was added in abundance to fungal suspension cultures. Here, the bacteria overgrew and entangled the hyphae and the fungus eventually died (data not shown). Interestingly, incubation of crushed hyphae, containing hyphae and bacteria released from mechanical shearing, resulted in clearance of the medium after 2 days. This finding suggests a certain affinity between fungal hyphae and bacteria. Further analysis will show how specific this absorption is.

Using various molecular strategies (16S-rRNA gene sequences analysis, DGGE analysis and real-time quantitative PCR analysis), the intimate association between *P. indica* and *R. radiobacter* was further characterized. We could unequivocally prove the presence of the identical

bacterial strain in the original isolate *P. indica*-DSM11827 deposited in 1997 by A. Varma and in all fungal cultures derived thereof, i.e. *P. indica*-JE1, *P. indica*-HA and *P. indica*-ND. All bacterial sequences from *P. indica* isolates and the bacterial cultures isolated from *P. indica* were identical within the amplified region of the bacterial 16S-rRNA gene. Consistently, DGGE analysis of single-step and seminested PCR assay using 16S-rRNA gene primers revealed a single dominant band for *P. indica*-DSM11827 and *P. indica*-JE1 as well as for PABac-DSM and PABac-JE, further emphasizing that only one distinct bacterial species was associated with *P. indica*.

A cytohistochemical approach using FISH in combination with the *Rhizobium*-specific probe Rhi-1247 showed an association of *R. radiobacter* with spores and hyphae. In general the number of bacteria per hypha was low, which is consistent with the low number of endobacteria (2–20 per cell) present in the ectomycorrhizal fungus *Laccaria bicolor* (Bertaux *et al*., 2003; 2005). Quantitative PCR analysis further supported the microscopic data revealing a ratio of 0.02–0.035 ng of bacterial DNA per 100 ng of *P. indica* DNA. The low number of bacteria within hyphae may explain why we failed to detect *R. radiobacter* by electron microscopy. In contrast, a combined strategy that included electron microscopy and FISH detected the endobacterial nature of *Paenibacillus* sp. in *S. vermifera*-MAFF305838 with roughly 1–3 bacteria per fungal cell. Different to other fungus–endobacterial associations (Partida-Martinez *et al*., 2007), there was no evidence for a focal accumulation of *R. radiobacter* or *Paenibacillus* in fungal hyphae.

It is certainly of interest to clarify the impact of each Sebacinoid symbiosis partner on beneficial effects in colonized plants. A first step has been done by examining the biological potential of *R. radiobacter* (PABac-DSM) in barley. The bacterium qualitatively and quantitatively induced symbiosis phenotypes comparable to those induced by *P. indica*, e.g. growth augmentation and systemic resistance to powdery mildew. Consistently, the potential of specific strains of *R. radiobacter* for improvement of plant performance in integrated production systems has been reported earlier. *R. radiobacter* strain 204 increased barley root and shoot length as well as improved crop yield in barley and wheat leading to its commercial distribution as biofertilizer in Russia (Humphry *et al*., 2007).

Rhizobium radiobacter (syn. *A. tumefaciens*) is well known as a soil-borne bacterium that infects dicotyledonous plants and often causes crown gall disease characterized by the neoplastic growth of infected plant tissue. Tumour induction is dependent on the integration of a bacterial virulence plasmid (Ti) into the plant genome, which contains genes encoding the phytohormones auxin and cytokinin. In our experiments, PABac-DSM did not trigger any harmful effect on barley. The *virD2* gene was detected in PABac-DSM by PCR analyses indicating that the Ti plasmid is present in this strain. However, the *isopentenyltransferase* (*ipt*) gene, which is associated with cytokinin biosynthesis, could not be detected (S. Wagner, unpublished). Hence, the lack of the *ipt* gene may explain the non-pathogenic nature of this bacterial strain. Concordantly, a non-pathogenic strain of *A. radiobacter* containing *virD2* but not *ipt* was previously described by Haas *et al*. (1995). These authors speculated that the strain arose from a pathogenic progenitor through a deletion in the T-DNA. Moreover, coexistence of symbiosis- and pathogenicity-determining genes has been shown to occur in strains of *Rhizobium rhizogenes* enabling this bacterium to induce nodules or tumours in plants (Velazquez *et al*., 2005).

A recent report by Sirrenberg *et al*. (2007) demonstrated production of IAA in liquid culture of *P. indica*, which might induce growth promotion. Because bacteria-free fungus is not available, it remains vague whether the fungus itself, the bacterium or even both partners produced the hormone. We found that *R. radiobacter* (PABac-DSM) produces IAA in the presence of tryptophan. Despite the fact that Salkowski reagent also detects indole pyruvic acid and indole acetamide in addition to IAA (Glickmann and Dessaux, 1995), the method is fairly accurate because IAA is usually known as the main excreted microbial auxin. IAA is best known for its role in plant signal transduction (Quint and Gray, 2006). However, this hormone can act as a signal molecule in bacteria and fungi (Leveau and Preston, 2008) and induce adhesion and filamentation of *Saccharomyces cerevisiae* (Prusty *et al*., 2004). Importantly, IAA has been implicated in plant–microbe compatibility (Robert-Seilaniantz *et al*., 2007). This could be accomplished by suppression of defence reactions otherwise elicited by fungal Microbe-Associated Molecular Patterns (Navarro *et al*., 2006; Wang *et al*., 2007). Notably, in barley and *Arabidopsis*, root colonization by *P. indica* leads to suppression of pathogenesis-related (PR) genes (Deshmukh and Kogel, 2007). Thus, it is tempting to speculate that bacteria-derived auxin contributes to successful root colonization by Sebacinoid fungi. Further research is required to demonstrate that the bacterium is the only source of auxin and that auxin would mimic interaction phenotypes mediated by host plant colonization.

Experimental procedures

Fungal material

Piriformospora indica isolates were obtained from the following sources: *P. indica*-DSM11827 from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany;

P. indica-JE1 from Dr Ralph Ölmüller, Jena, Germany; *P. indica*-HA from Dr Holger Deising, Halle, Germany; *P. indica*-ND from Dr Ajit Varma, New Delhi (Table 1). All isolates stem from one original sample collected in the Thar desert, India in 1997 (Varma *et al*., 1998). They were propagated in liquid *Aspergillus* minimal medium at room temperature (Pham *et al*., 2004). *S. vermifera* strains (Table 1) were propagated in Malt-Yeast-Extract-Pepton medium (aqueous solution of 7 g l^{-1} malt extract, 1 g l^{-1} peptone, 0.5 g l^{-1}, yeast extract).

DNA isolation, PCR and sequencing

High chromosomal weight DNA from 2-week-old *P. indica* cultures was isolated using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) according to the instruction manual. 16S-rRNA gene was amplified by using the bacterial universal primer pair 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495r (5′-CTAC GGCTACCTTGTTACGA-3′). A conventional PCR amplification was performed in a Gene Amp® PCR System 9700 PE Applied Biosystem thermo cycler in a total volume of 25 μl containing 2× PCR Master Mix (Fermantas, Life Sciences, St Leon-Rot, Germany), 75–100 ng of DNA and 1 μM of each primer. After an initial denaturation step at 95°C for 5 min, 34 cycles with denaturation at 95°C for 1 min, primer annealing at 59°C for 1 min, elongation at 72°C for 1.45 min and a final extension at 72°C for 10 min were performed. The obtained PCR products were purified using a Gel Extraction Kit (Promega, Mannheim, Germany) and cloned into the pGEM-T vector (Promega) following the manufacturer's instructions. DNA from 25 plasmids was extracted with Wizard® Plus SV Minipreps (Promega) and submitted for sequencing to AGOWA GmBH, Berlin, Germany. Direct sequencing of PCR products was also performed with the primer pair 27f and 1495r. Sequences were assembled with the Sequencher 3.1.1 software (Gene Codes Corporation) and analysed with the ARB software package (http://www.arb-home.de) (Ludwig *et al*., 2004).

Phylogenetic analysis

The 16S-rRNA-coding gene sequences obtained from the sequenced plasmids and from direct sequencing were added to an existing database of well-aligned small-subunit rRNA gene sequences by using the fast alignment tool implemented in the ARB software package (http://www.arb-home.de) (Ludwig *et al*., 2004). Sequences were proof read according to the chromatograms and wrong positions in the alignments were manually corrected if needed. Phylogenetic analyses were performed by applying maximum likelihood, maximum parsimony and neighbour-joining methods by use of respective tools in the ARB software package.

Isolation of bacteria

Mycelia of 14-day-old *P. indica*-DSM11827 and *P. indica*-JE1 cultures were crushed in Gamborg B5 medium (Duchefa Biochemie, the Netherlands) supplemented with 0.45 M mannitol using a fine blender. Homogenate was filtered through a miracloth (22–25 μm) filter and centrifuged at 100 *g* for 7 min. The supernatant was collected and subsequently centrifuged at 3200 *g* for 10 min. The bacterial cell pellet was resuspended in LB medium containing 0.8% sucrose and inoculated in the same medium at 22°C for 2 days under gentle shaking. The bacterial culture was streaked on LB medium plate and incubated for 2 days at 25°C. Thirty bacterial colonies were randomly picked from the plates and identified by sequencing the 16S-rRNA gene using a universal primer pair 27f and 1495r as described above. Phenotypic characterization and identification at the species level were performed using fatty acid analysis of whole cell extract as described by Kämpfer and Kroppenstedt (1996) and physiological characterization using 90 physiological biochemical tests was performed by the method described by Kämpfer *et al*. (1991).

Denaturing gradient gel electrophoresis

DNA extraction for DGGE analysis was performed using the FastDNA® Spin Kit for soil (MP Biomedicals, LLC., Illkirch, France) according to the manufacturer's protocol. A seminested PCR was performed to amplify a 500 bp region of the bacterial 16S-rRNA-coding gene. First, almost the entire 16S-rRNA gene was amplified using the above mentioned bacterial primer pair. The cycle conditions differ in 25 cycles of amplification. Subsequently, next PCR was performed using 27f (with a 42 bp GC clamp on the 5′ end) and reverse R518 (5′-ATTACCGCGGCTGCTGG-3′) universal primers (Vanhoutte *et al*., 2005). The PCR mix contained (final concentrations) 1× Thermophilic DNA Polymerase Buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 0.025 mM of each dNTP (Fermantas, Life Sciences), 0.25 μM of each primer and 0.05 U μl^{-1} *Taq* DNA polymerase (Promega). One microlitre of first PCR product was used as template in a total volume of 50 μl reaction. Thermal cycling conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 25 amplification cycles with heat denaturation at 94°C for 1 min, primer annealing at 59°C for 45 s and extension at 72°C for 40 s. A final elongation step at 72°C for 10 min completed the reaction. The PCR products were analysed with standard horizontal agarose gel electrophoresis on a 1% agarose gel.

Additionally a single-step PCR amplification of a 500 bp region of the 16S-rRNA was performed using the forward primer 27f or F357 (5′-TACGGGAGGCAGCAG-3′) (Vanhoutte *et al*., 2005), with a 42 bp GC clamp on the 5′ end, in combination with the reverse primer R518. PCR was performed under the above mentioned conditions. The cycle conditions differ only in 32 cycles of amplification.

Denaturing gradient gel electrophoresis analyses were performed using an 8% (wt/vol) acrylamide-bisacrylamide gel (Liqui-Gel™ 37.5:1; MP Biomedicals) with a 35–75% linear urea-formamide (Fluka, Seelze, Germany) denaturing gradient (100% denaturant corresponds to 40% formamide plus 7 M urea). After adding the loading buffer (0.05% bromophenol blue and 0.05% xylene cyanol in 70% glycerol), 20 μl of each sample was loaded on the DGGE gel and submitted to electrophoresis in 1× TAE buffer at 60°C with a constant voltage of 50 V for 20 h using a Bio-Rad DCode™ Universal Mutation Detection System. The gels were stained in the dark for 20 min in ethidium bromide and subsequently washed with 1× TAE buffer. The following bacterial strains were used as reference: *Escherichia coli, Herbaspirillum frisingense* strain Mb11 and *Acinetobacter* sp. The obtained DGGE bands for *P. indica*-DSM11827, *P. indica*-JE1, PABac-DSM and PABac-JE isolate were excised; the DNA fragments were purified using Wizard® Plus SV Minipreps (Promega) and submitted for sequencing to AGOWA GmBH, Berlin, Germany.

Real-time PCR quantification

Genomic DNA of PABac-DSM was used as template to amplify the 16S–23S-rRNA intergenic transcribed spacer region, using primer ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITS_Reub (5′-GCCAAGGCATCCACC-3′) (Cardinale *et al*., 2004). A PCR product of the expected size (1.4 kb) was cloned and sequenced as described earlier. The sequences obtained were used to design the *Rhizobium/Agrobacterium*-specific primer pair ITS_Rhf (5′-TCAGCACATAACCACACCAATCGCG-3′) and ITS_Rhr (5′-TG CTTTGTACGCTCGGTAAGAAGGG-3′). These primers were used in real-time PCR to quantify the amount of bacterium in *P. indica* cultures. Amplifications were performed in 25 μl SYBR® Advantage® qPCR Premix (Clontech Laboratories, CA, USA) according to manufacturer's instructions with 200 nM oligonucleotides, 100–300 ng of fungal genomic DNA, and carried out with a Stratagene-Mx3000P® QPCR SystemMx3000P (Stratagene Research, La Jolla, CA, USA). After an initial activation step at 95°C for 1 min, 45 cycles (95°C for 5 s and 65°C for 25 s) were performed and a single fluorescent reading was obtained at 65°C of each cycle step. A melting curve was determined at the end of cycling to ensure the amplification of a single PCR product. Cycle threshold values were determined with the Mx3000P V2 software supplied with the instrument. A standard curve using different dilutions of bacterial DNA was prepared and was used to calculate the amount of bacterial DNA in fungal samples.

Treatment of P. indica *with antibiotics*

Hyphae were cultured for 5 generations in the presence of either spectinomycin (300 μg ml^{-1}) or ciprofloxacin (200 and 500 μg ml^{-1}) and in combination. Both antibiotics were effective against PABac-DSM *in vitro* (Table 2). Additionally, single-spore culturing was performed in the presence of these antibiotics. Chlamydospores were harvested from 4-week-old plates using a 0.05% Tween-20 solution, and purified three times by centrifugation at 100 g for 7 min. The pellet was resuspended before each centrifugation step in 0.05% Tween-20 containing 300 μg ml^{-1} spectinomycin. Spores were finally treated with spectinomycin (300 μg ml^{-1}) for 3 h and subsequently plated on *Aspergillus* minimal medium plates containing spectinomycin (300 μg ml^{-1}). A single germinating spore was picked using a stereomicroscope (MZ16F, Leica, Germany) and used as inoculum on antibiotic containing agar plates. This was termed generation 1 (G1) of the single-spore culture. Spores were harvested after 3 weeks from these plates and plated to produce further generations of single-spore cultures (G2–G5) in the same way. After every generation, fungal samples were taken and bacterial detection was performed by conventional PCR using universal eubacterial primers and with real-time PCR using specific primers as described above.

Additionally, young growing mycelium from *P. indica* was picked with the help of a stereomicroscope and transferred to new plates containing *Aspergillus* minimal medium with antibiotics (spectinomycin 300 μg ml^{-1} and ciprofloxacin 300 μg ml^{-1}) and incubated at 24°C. Every fourth day the growing mycelium was transferred alternatively to fresh plates or to liquid *Aspergillus* minimal medium containing antibiotics for a total of five times. Finally young mycelium was transferred on *Aspergillus* minimal medium plates and liquid medium without antibiotics, grown for 3 weeks and used for DNA isolation. Seven independent treated colonies were checked for bacterial presence as described above.

Piriformospora indica *protoplast isolation and treatment with antibiotics*

Fresh mycelia from *P. indica*-DSM11827 were crushed and filtered through miracloth. The filtrate was collected by centrifugation and resuspended in liquid *Aspergillus* minimal medium. After 3 days the young mycelium was collected using a miracloth filter, washed twice with 0.9% NaCl and resuspended in SMC buffer (1.33 M sorbitol, 50 mM $CaCl_2$, 20 mM MES buffer pH 5.8) containing 2.5% lysing enzymes from *Trichoderma harzianum* (L1412 Sigma). The suspension was incubated for 1 h at 37°C. The activity of the lysing enzymes was stopped by adding STC buffer (1.33 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris-HCl; pH 7.5). Protoplasts were filtered through miracloth and collected by centrifugation. The pellet was washed three times in STC buffer containing spectinomycin (300 μg ml^{-1}) and ciprofloxacin (300 μg ml^{-1}) and diluted to a final concentration of 1×10^8 cells per ml. The preparation was checked under a phase-contrast microscope to ensure the absence of any mycelial fragments or spores. Liquid *Aspergillus* minimal medium containing 0.3 M sucrose, spectinomycin (300 μg ml^{-1}) and ciprofloxacin (300 μg ml^{-1}) was used for regeneration of protoplasts. Regeneration was observed after 72 h incubation at 30°C. Young growing mycelium from seven colonies were picked with the help of a stereomicroscope and transferred to new plates containing *Aspergillus* minimal medium with antibiotics. Every second day for eight consecutive days the growing mycelium was transferred to fresh *Aspergillus* minimal medium plates containing antibiotics. Finally young mycelium was transferred on medium plates without antibiotics, grown for 3 weeks and used for DNA isolation and checked for bacterial presence as described above.

Fluorescence in situ *hybridization*

Two to four-week-old fungal cultures or overnight grown bacterial cultures were fixed by adding 50% ethanol and incubation at 4°C for 3–4 h. Thereafter, cultures were washed three times in 1× PBS and finally resuspended in a 1:1 mixture of 1× PBS and $Ethanol_{absolute}$. Samples were stored at –20°C. Fixed fungal material was dehydrated in an increasing ethanol series (50%, 80% and 96% ethanol, 3 min each). FISH was performed as described by Manz *et al*. (1996). Hybridization was carried out for 90 min at 46°C, followed by a stringent washing step at 48°C for 10–15 min. All steps of FISH with fungal material were carried out in eppendorf tubes (humid chamber). After the washing step, the material was spread onto glass slides. Two microlitres of the fixed bacterial suspension were immobilized on hydrophobic Teflon-coated slides in 8 mm hybridization wells (Roth GmbH, Karlsruhe) and hybridization was carried out as described above. Before observation, the slides were mounted in AF1 antifading reagent (Citifluor, London, UK).

The fluorescent tagged oligonucleotide probes used in this study were purchased from Thermo Electron Corporation GmbH, Ulm, Germany. These were EUB-338-mix [an equimolar mixture of EUB-338 (Amann *et al.* 1990), EUB-338-II and

EUB-338-III (Daims *et al*., 1999)], LGC-354-mix [an equimolar mixture of LGC-354-a, LGC-354-b and LGC-354-c (Meier *et al*., 1999)], Rhi-1247 (Ludwig *et al*., 1998) and EUK-516 (Amann *et al*., 1990) (Table 3). All of them were labelled either with FITC, Cy3 or Cy5.

Microscopic analysis

Hybridized samples were analysed with a confocal laser scanning microscope (CLSM 510 Axiovert 100 M; Zeiss, Jena, Germany) equipped with an argon laser (laserline 488 nm) and two helium–neon lasers (laserlines 543 and 633 nm), for the excitation of FITC, Cy3 and Cy5 respectively. Plan-Neofluar 100 X/1.3 oil and Apochromat 63 X/1.2 water immersion lenses were used for all analysis and image acquisitions. Monochrome images were taken sequentially at each wavelength to optimize scan conditions and laser settings. Artificial colours were assigned to the fluorescent images resulting from each excitation wavelength: green for 488 nm, red for 543 nm and blue for 633 nm. Superimpositions were processed with the Zeiss software package LSM 510, version 3.5.

Electron microscopy

For ultrastructural studies, cells were fixed and micrographs were taken as described by Straube *et al*. (2006).

Colorimetric assay for indole-3-acetic acid determination

Production of IAA by *R. radiobacter* was determined according to Tsavkelova *et al*. (2007). *H. frisingense* strain Mb11 (aux^+ reference strain) and *Herbaspirillum hiltneri* strain N3 (aux^- reference strain) were included in the analyses as positive and negative control respectively.

Plant materials and growth conditions

Kernels of barley cv. Golden Promise were sterilized with 3% sodium hypochloride for 2 h, rinsed in water and germinated for 3 days. Subsequently, roots of seedlings were inoculated in a homogenized mycelial solution (1 g ml^{-1}) or bacterial suspension in LB medium (OD_{600} 1.6) for 1.5 h. Inoculated seedlings were transferred to pots containing a 2:1 mixture of expanded clay (Seramis®, Masterfoods, Verden, Germany) and Oil-Dri® (Damolin, Mettmann, Germany). Plants were grown in a growth chamber at 22°C/18°C day/night cycle, 60% relative humidity and a photoperiod of 16 h (240 μmol m^{-2} s^{-1} photon flux density). Plants were fertilized once after 2 weeks with 20 ml of a 0.5% Wuxal top N solution (Schering, N/P/K: 12/4/6) per pot containing three plants.

Biological activity of PABac-DSM

Golden Promise plants were harvested 3 weeks after root inoculation with bacteria or *P. indica*, and shoot fresh weight was measured. For the assessment of systemic resistance induction, the youngest leaves were harvested for a detached leaf-segment test with *B. graminis* f.sp. *hordei* (Waller *et al*., 2005).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Detection of bacterial 16S-rRNA gene by agarose gel electrophoresis. Ethidium bromide-stained agarose gel showing PCR products of 16S-rRNA-coding regions amplified with universal bacterial primers 27f and 1495r from *P. indica*'s metagenomic DNA or from bacteria isolated from fungal samples. Lane M, 1 kb plus marker; lane 1, *P. indica*-DSM11827; lane 2, *P. indica*-JE1; lane 3, *P. indica*-HA; lane 4, *P. indica*-ND; lane 5, PABac-DSM isolated *P. indica*-DSM11827; lane 6, PABac-JE isolated from *P. indica*-JE1; lane 7, non-template control.
Fig. S2. Light microscopy of the pure culture of *R. radiobacter* (PABac-DSM). Rod shaped bacteria were observed using epifluorecent microscope (Axioplan 2, Zeiss). (Scale bar = 3 μm).

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

5 The Sebacinoid Fungus *Piriformospora indica:* an Orchid Mycorrhiza Which May Increase Host Plant Reproduction and Fitness

Patrick Schäfer[1], Karl-Heinz Kogel[1]

CONTENTS

I. Introduction

Plants are potential targets (hosts) for a broad spectrum of microbial organisms. The outcome of these associations can be roughly categorised into mutualistic, commensalistic or pathogenic relationships. Interactions with certain mutualistic fungal microbes can benefit plants, resulting for example in an improved plant development even under unfavourable environmental conditions (Chap. 15). Simultaneously, the microbial partners acquire nutrients from the host and can be protected from environmental stress or competitors (Schulz and Boyle 2005). In other cases it is the microbes that primarily profit from the association, with the host fitness being either apparently unaffected (commensalism) or thoroughly impaired (pathogenesis; Redman et al. 2001).

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Prokaryotic or eukaryotic organisms with the capability of colonising plants are generally called endophytes. An endophytic lifestyle was reported among fungi, bacteria, algae, plants and even insects (Schulz and Boyle 2005). This broad defintion of endophytism was later specified to more strongly emphasise infection strategies or the physiological character of interaction types. However, due to the broad spectrum of endophytes and their flexibility (phenotypic plasticity) in host colonisation, along with their ability to adapt to environmental factors and the host's physiological status, a more restrictive general definition does not exist. Focusing on fungal microbes, endophytes were defined as organisms that grow in living plant tissue during their entire life cycle (or a significant part of it) without causing disease symptoms (Petrini 1991; Saikkonen et al. 1998; Brundrett 2004). Schulz and Boyle (2005) broadened this definition by describing endophytes as plant inhabitants that have not yet triggered disease symptoms in plants at the time of detection. This definition excludes the impact of endophytes on host fitness at later interaction stages; depending on their lifestyle in plants or impact on host fitness such fungal endophytes range under this definition from mutualistic to pathogenic microbes (Redman et al. 2001; Schulz and Boyle 2005). In order to simplify this heterogeneity, we follow a rather restricted definition of endophytes encompassing microbes with an asymptomatic lifestyle throughout their interaction with plants. The intention of this definition is to address those fungi whose association and reproduction in plants cause neutral or beneficial rather than detrimental effects in their hosts.

Described in a broad sense, mycorrhizas are highly specialised beneficial associations between plant roots and fungi based on the bilateral exchange of nutrients, defence against pathogens and abiotic stress or an improved water balance. Variations in the benefits for each symbiotic partner gave rise to the terms balanced and exploitive mycorrhizas. Whereas in the former both partners

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benefit equally from each other, the latter type of interaction favours the plant partner. Due to their beneficial potential for plants, mycorrhizal fungi are among the best-characterised fungal symbionts (Chaps. 13, 14). According to the above definition, mycorrhizal fungi would be considered as endophytes displaying mutualistic interactions with plants. However, in order to distinguish mycorrhizas from endophytes, a more precise definition was conceived: Endophytic plant–microbe associations lack a synchronised plant–fungus development, specialised microbial structures serving as localised plant–microbe interfaces and nutrient transfer to the plant (Brundrett 2004). Irrespective of these characteristics and as mentioned above, host plants are well known to benefit from non-mycorrhizal endophytes to their hosts. A common example is the release of toxic or antimicrobial compounds distracting herbivoric and microbial competitors (Schulz et al. 2002; Chap. 15). In other cases plant fitness is enhanced by improved water use efficiency, drought tolerance and enhanced germination rates (Saikkonen et al. 1998; Brundrett 2004). In addition, several endophytes promote plant growth and confer local and systemic induced resistance to plant pathogens (Varma et al. 1999; Schulz and Boyle 2005; Waller et al. 2005).

The fungal basidiomycete *Piriformospora indica* has drawn attention since its discovery in India during the final decade of the past century – not least due to its versatile beneficial effects conferred to a broad variety of host plant species, e.g. barley, maize, parsley, poplar, tobacco and wheat (Sahay and Varma 1999; Varma et al. 1999; Waller et al. 2005; Serfling et al. 2007). This broad host range, combined with its easy handling, makes the fungus a potential agent for protecting plants against abiotic and biotic stresses under greenhouse or field conditions. Hence, *P. indica* could support sustainability in horticulture and agriculture. Because of the reported beneficial effects, it was rather unexpected that colonisation of barley roots was found to be associated with cell death (Deshmukh et al. 2006). In agreement with other endophytic plant–fungus interactions, colonised plants were observed to lack visible disease symptoms (e.g. stunted root and shoot development, or root necrosis). Due to its colonising behaviour, the lack of distinctive colonisation structures and the as yet missing evidence for nutrient transfer to its host plants, *P. indica* was suggested to be a fungal endophyte rather than a representative of the mycorrhizal fungi. In this chapter we discuss current results showing beneficial associations of *P. indica* with plants, especially emphasising its life strategies in host plants. Intriguingly, it has been shown that root colonisation by *P. indica* and its lifestyle in planta may vary depending on environmental factors, the genetic predisposition and the developmental stage of host plants and plant organs, respectively. These findings are discussed in the context of the phylogenetic classification of *P. indica* within the newly defined mycorrhizal order *Sebacinales*.

II. The Mycorrhizal Order *Sebacinales*

Based on morphological and ultrastructural characteristics, members of the order *Sebacinales* were originally classified as wood-decaying basidiomycetes of the order *Auriculariales* (Bandoni 1984; Weiss et al. 2004). However, recent phylogenetic studies using the nuclear DNA sequence of the large ribosomal subunit resulted in the definition of the fungal order *Sebacinales*, occupying a central position within the *Hymenomycetidae*. The order *Sebacinales* exclusively harbours beneficial fungi; however these show an extraordinary diversity, encompassing ectomycorrhizas, orchid mycorrhizas, ericoid mycorrhizas, cavendishioid mycorrhizas and jungermannioid mycorrhizas in liverworts (McKendrick et al. 2002; Selosse et al. 2002, 2007; Kottke et al. 2003; Urban et al. 2003; Weiss et al. 2004; Setaro et al. 2006). Hence, the *Sebacinales* might possess remarkable significance in natural ecosystems (Weiss et al. 2004).

Phylogenetic analysis divided the *Sebacinales* into two subgroups. Subgroup A harbours ectomycorrhizas and orchid mycorrhizas that usually form hyphal sheaths and occasionally intracellular hyphae. Fungi of this group are associated with achlorophyllous or rather heterotrophic orchids (Weiss et al. 2004). Recently, ectendomycorrhizal sebacinoids were isolated from *Ericaceae*. In addition to hyphal sheaths, colonised roots showed intercellular networks as well as intracellular structures (Selosse et al. 2007). Since some members of subgroup A are thought to form tripartite symbioses connecting trees with orchids, it is speculated that most of these fungi are able to form both ecto- and orchid mycorrhizal interactions. Subgroup B

is more heterogenic with respect to the types of mycorrhizal associations. It mainly consists of *Sebacina vermifera* isolates from autotrophic orchids, ericoid mycorrhizas associated with *Gaultheria shallon*, cavendishioid mycorrhizas and liverwort-associated jungermannioid mycorrhizas (Weiss et al. 2004; Selosse et al. 2007). Within this group, isolates of *S. vermifera* represent a particularly interesting complex. These fungi can be axenically cultivated, which distinguishes them from sebacinoid mycobionts of group A. Interestingly, Warcup (1988) isolated several orchid symbionts of the *S. vermifera* complex that were shown to form hyphal coils in orchids. However, only those isolates that were isolated from ectomycorrhizal hosts were able to establish ectomycorrhizal interactions. Furthermore it was confirmed that the symbionts can only colonise a limited number of orchid hosts (Warcup 1988). In conclusion, *S. vermifera* isolates were proposed to represent a conglomerate of species rather than one diverse species (Warcup 1988; Weiss et al. 2004). It is even speculated that all members of subgroup B belong to the *S. vermifera* complex. However, this open question can only be answered when more knowledge on teleomorph stages of jungermannioid and ericoid mycorrhizas becomes available (Weiss et al. 2004).

Although exhaustive fungal sampling has not been performed, *Sebacinales* have been identified worldwide (Verma et al. 1998; Weiss et al. 2004; Setaro et al. 2006; Selosse et al. 2007) with specific branches isolated in Australia, Europe and North America (Weiss et al. 2004; Selosse et al. 2007). To date it is not known whether all *Sebacinales* are beneficial for their hosts. However, those members of the *Sebacinales* (*S. vermifera* isolates, *P. indica*, multinucleate *Rhizoctonia*) that have been examined for their mutualistic activity were able to promote growth and/or enhance disease resistance in monocotyledonous and dicotyledonous plants (Waller et al. 2005; Deshmukh et al. 2006), or support seed germination in orchids (Warcup 1988). These studies revealed that the fungi exhibit broad host specificity, although the majority were isolated from orchids, where they exhibit a rather narrow host range (Warcup 1988).

The recently described fungus *P. indica* was shown to be embedded within this group of mutualistic fungi, with the closest relationship to *S. vermifera* and multinucleate *Rhizoctonia* (Weiss et al. 2004). Although the latter was originally designated as *Rhizoctonia* sp., due to its morphological traits, recent phylogenetic studies clearly identified this fungus as a member of the *Sebacinales*. Hence, this isolate is not closely related with the pathogenic *Rhizoctonia solani* spp. (teleomorphs = *Thanatephorus*) and binucleate *Rhizoctonia* spp. (teleomorphs = *Ceratobasidium*), which are grouped within the *Ceratobasidiales* (Ogoshi 1987; Weiss et al. 2004; Gonzalez et al. 2006). Considering the beneficial effects caused by *P. indica* and the related *Sebacina* spp. or the multinucleate *Rhizoctonia*, *P. indica* might be regarded as a representative member of a huge group of microorganisms with considerable biological activities, significant agronomical potential and high ecological relevance.

III. *Piriformospora indica* – an Orchid Mycorrhizal Fungus?

P. indica was isolated for the first time from an association with a spore of *Glomus mosseae* in the rhizosphere of two shrubs of the Indian Thar desert, northwest Rajasthan (Verma et al. 1998). The fungus shows morphological traits common to members of the *Sebacinales*. In particular it possesses dolipores with imperforated parenthosomes (Verma et al. 1998) and does not have clamp connections. The structure of the basidia is unknown, since teleomorphs have not yet been isolated. However, these ultrastructural characteristics are in accordance with the phylogenetic analyses classifying *P. indica* as a member of the *Sebacinales* (Weiss et al. 2004). Whether *P. indica* coexists with *Glomus* spp. under natural conditions, or if its isolation from *Glomus mosseae* reflects a coincidence of circumstances, has not yet been investigated. It is known that *Sebacinales* often live in association with ascomycetes in their hosts and even colonise the same cells (Selosse et al. 2002; Urban et al. 2003; Setaro et al. 2006); but the reason for this coexistence is not known.

As mentioned above, the order *Sebacinales* harbours almost all mycorrhizal types other than vesicular arbuscular mycorrhizal (AM) fungi, which belong to the phylum *Glomeromycota*. Within the *Sebacinales*, *P. indica* exhibits the closest relationships to *S. vermifera* and multinucleate *Rhizoctonia*. The various *S. vermifera* isolates were sampled from diverse orchid plants and shown to

support orchid seed germination (Warcup 1988; Weiss et al. 2004). The natural mycorrhizal plant partner(s) of multinucleate *Rhizoctonia* has not been definitively determined. Interestingly, the endophyte was isolated from vesicles of *Glomus fasciculatum* in pot cultures of *Trifolium subterraneum* L. (Williams 1985). In analogy to *P. indica*, the interfungal relationship to this AM fungus in nature is unknown. Both *S. vermifera* isolates and multinucleate *Rhizoctonia* exhibit a pronounced host specificity among orchids regarding their beneficial impact, e.g. by supporting seed germination. These fungi were determined to form intracellular hyphal coils (Milligan and Williams 1988; Warcup 1988), which represent characteristic traits of orchid mycorrhizas (Peterson and Massicotte 2004). In contrast to its closest neighbours, *P. indica* was reported to be isolated from the rhizosphere of the shrubs *Zizyphus nummularia* and *Prosopis juliflora* which belong, respectively, to the Rhamnaceae and Fabaceae (Verma et al. 1998).

Specific colonisation types classify each member of the *Sebacinales* to defined mycorrhizal categories. Sebacinoid fungi develop hyphal sheaths, Hartig nets and intracellular coils (ericoid and cavendishioid mycorrhiza, arbutoid ectendomycorrhiza), solely build intracellular coils (orchid mycorrhiza), or even colonise roots intercellularly (ectomycorrhiza ;Brundrett 2004; Peterson and Massicotte 2004; Selosse et al. 2007). Compared to these mycorrhizal types, epifluorescence microscopy revealed a divergent colonisation type for *P. indica* in barley and *Arabidopsis thaliana* roots (Deshmukh et al. 2006; Schäfer, unpublished data). Upon root contact, the fungus starts forming extracellular hyphal mats, which progressively develop. In parallel, it initiates intercellular root colonisation and frequently penetrates rhizodermal and cortical cells. As colonisation proceeds, the root is densely covered with extracellular hyphae and harbours thorough inter- and intracellular networks. However, the fungus never enters vascular tissue. Eventually, fungal colonisation leads to extracellular and intracellular sporulation (formation of chlamydospores; Deshmukh et al. 2006).

Some of these colonisation traits bear similarities to mycorrhizal symbioses. For instance, although the mycelium of *P. indica* is less densely packed and never covers the whole root surface, the extracellular colonisation pattern of *P. indica* is reminiscent of hyphal sheaths (Deshmukh et al. 2006). External hyphal growth was regarded not to be a characteristic of endophytes and rather treated as a mycorrhizal trait (Saikkonen et al. 1998). Some dark septate endophytes (DSE) exhibit an asymptomatic colonisation pattern intringuingly similar to that of *P. indica* (Jumpponen and Trappe 1998). *Phialocephala fortinii*, a representative member of DSE, forms an extensive extracellular hyphal net prior to inter- and intracellular colonisation of rhizodermal, cortical, or root hair cells. Moreover, the fungus often builds intracellular coiled structures in ericaceous plants and even forms Hartig nets or labyrinthine hyphae when associated with ectomycorrhizal hosts. Similarly, *P. indica* was shown to occasionally produce intracellular coils in the monocotyledonous hosts maize and barley (Varma et al. 1999; Deshmukh et al. 2006), reminiscent of hyphal pelotons seen in the cortical cells of orchid mycorrhizas. Similar structures have occasionally been observed in *A. thaliana* (Fig. 5.1). Illustratively, Blechert et al. (1999) ana-

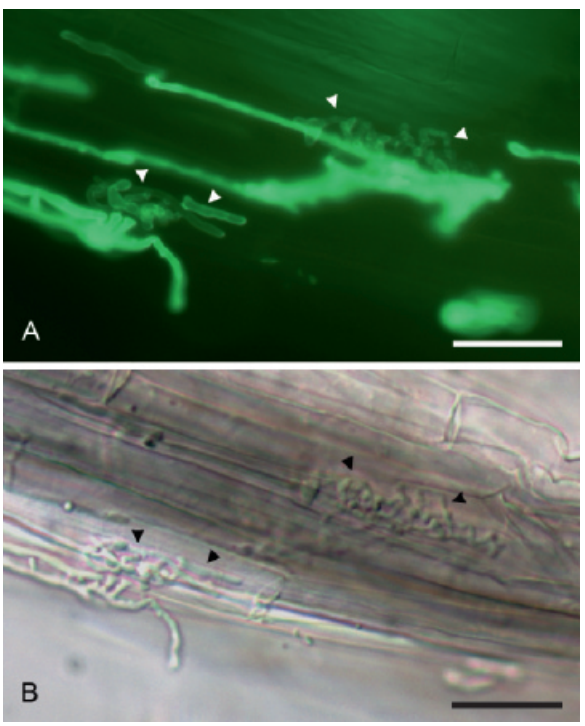

Fig. 5.1. Formation of intracellular coil-like structure of *Piriformospora indica*. Rhizodermal cell of the *Arabidopsis thaliana* root differentiation zone showing intracellular colonisation by *P. indica* at 7 days after inoculation. The fungus has begun to form coil-like hyphal structures (*arrowheads*) that eventually fills the entire plant cell (**A**). *P. indica* was stained with chitin-specific WGA-AF488. **B** Bright-field interference contrast image from the same colonised plant cells. Images were taken using an Axioplan 2 microscope. Root segments were excited at 470/20 nm and detected at 505–530 nm for WGA-AF 488. *Bar* 20 μm

lysed the colonisation of protocorms and roots of autotrophic *Dactylorhiza* spp. (Orchidaceae) by *P. indica* and found hyphal coils (pelotons) to be the typical intracellular structure. In these experiments, *P. indica* was shown to support the development of *D. maculata*. Moreover, comparison of the intracellular pelotons formed in protocorms of two *Dactylorhiza* spp. by *P. indica* were similar in morphology to pelotons formed in naturally grown *D. majalis* by an unknown orchid mycorrhiza. In orchid mycorrhizas, these pelotons are surrounded by perifungal membranes and interfacial matrices separating them from the host cytoplasm. These complexes represent plant–fungus interfaces and function specifically in nutrient exchange (Peterson and Massicotte 2004). In analogy to plant–*P. indica* associations, orchid mycorrhizas do not build the Hartig nets or arbuscules commonly observed, respectively, in ectomycorrhizas or arbuscular mycorrhizas (Peterson and Massicotte 2004). Recapitulating, following the definition of Brundrett (2004) it might be tempting to classify *P. indica* as an orchid mycorrhizal fungus. However, it remains of principal importance to determine whether the coiled or non-coiled intracellular hyphae possess perifungal membranes as well as interfacial matrices enabling these organs to exchange nutrients, as reported for orchid mycorrhizas. Interestingly, most members of the *Sebacinales* exhibit some host flexibility, enabling them to form ectomycorrhizas or orchid mycorrhizas (Warcup 1988; Weiss et al. 2004). It should be emphasised that all of the above-mentioned mycorrhizal traits are variable and depend on environmental factors as well as the colonised host. As a consequence, AM and ectomycorrhizal fungi colonise non-host plants or older root regions of hosts in an endophytic manner, presumably in order to guarantee survival (Brundrett 2004, Johnson et al. 1997). Thus, based on the above presumptions, *P. indica* might be regarded as a mycorrhizal fungus in associations with certain hosts (e.g. orchids), while its endophytic non-mycorrhizal activity might be predominant in alternative hosts such as barley and *A. thaliana*.

IV. Benefits of *P. indica* Symbiosis for Host Plants

The beneficial effects conveyed by *P. indica* and related *Sebacina* spp. to the plant companion have been extensively studied in barley (Waller et al. 2005; Deshmukh et al. 2006). Colonised plant seedlings show up to 30% increase in shoot biomass under greenhouse conditions. Importantly, this positive growth effect is also verifiable under field test conditions: When the spring barley elite cultivar Annabel grown in Mitcherlich test pots is colonised by *P. indica*, both the plant biomass compared to non-colonised plants and the grain yield are increased by about 10%. Unlike arbuscular mycorrhiza, growth promotion governed by *P. indica* has been demonstrated to be unaffected by P or N fertilisation (Achatz and Waller, unpublished data).

P. indica-colonised plants also acquire improved disease resistance towards the necrotrophic root pathogens *Fusarium culmorum* (Waller et al. 2005) and *F. graminearum* (Deshmukh and Kogel 2007). The molecular mechanism of this antifungal activity is not clear, because most of the defence-related *PR* genes in barley roots are only moderately and transiently induced by *P. indica* at early penetration stages (Schäfer et al., unpublished data), and evidence for antimicrobial compounds was not found. Significantly, barley leaves are very efficiently protected from infections by the powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (up to 70% reduction in pustule frequencies), suggesting that a systemic resistance response is elicited by root colonisation. Systemic activation of the plants' defence machinery is corroborated by the detection of *P. indica*-mediated elevation of subcellular plant defence responses, such as cell wall apposition (papillae) and hypersensitive response, in association with attempted infection by *B. graminis*. Likewise, seven *S. vermifera* isolates originating from Australian and European sources confirmed a systemic protection activity in barley seedlings ranging from 10% to 80% reduction of powdery mildew colonies. It remains to be shown to what extent this activity spectrum may reflect a variable constitutive biological potential of single *Sebacina* strains or, alternatively, host cultivar-specific associations and thus varying degrees of specialisation of the mutualistic symbiosis.

Growth promotion as well as enhanced resistance conferred by *P. indica* against pathogens colonising roots (*Fusarium culmorum*), stem bases (*Pseudocercosporella herpotrichoides* (teleomorph: *Tapesia yallundae*) and leaves (*B. graminis* f.sp. *tritici*) were also observed in wheat under greenhouse conditions. Interestingly, the effects were mainly recorded when plants were grown on sand. However, similar effects could not be observed under field conditions, with

the exception of a reduced disease development of *P. herpotrichoides* and a higher straw production in a field with poor soil quality (Serfling et al. 2007). It is noteworthy that the defence potential of *Piriformospora indica* against *Pseudocercosporella herpotrichoides* might rely on systemic effects, since both fungi colonise different plant organs.

Unexpectedly, *A. thaliana* is also among the wide range of host plants of *Piriformospora indica* (Pham et al. 2004). Fungal colonisation influences expression of specific genes in roots of *A. thaliana*, both before and after root contact with the fungal mycelium and promotes plant growth (Shahollari et al. 2005; Sherameti et al. 2005). In addition, colonised plants exhibit better growth performance and are more resistant against *Golovinomyces orontii,* the causal agent of powdery mildew on *A. thaliana* leaves. This systemic character of the induction of disease resistance becomes apparent in the reduced potential of the pathogen to propagate, due to reduced numbers of conidiophores per area unit mycelium and reduced numbers of conidia produced per leaf fresh weight (Stein and Molitor, unpublished data).

V. Cell Death Makes a Difference

Despite extensive colonisation by *P. indica*, barley and *A. thaliana* roots do not display any macroscopic evidence for impairment or even necrotisation (Fig. 5.2). Importantly, the colonisation patterns of the various root regions harbour some quantitative as well as qualitative differences, which additionally distinguish *P. indica* on barley (and *Arabidopsis*) from endomycorrhizal fungi. Fungal root colonisation increases with root maturation and the highest fungal biomass has been found in the differentiation and particularly the root hair zones. Cytological studies revealed the various interaction types of *P. indica* with different barley root regions and showed that the root hair zone (as oldest root zone) was mostly severely colonised by intracellular hyphae. In contrast, cells of the differentiation zone were often filled with fungal hyphae reminiscent of hyphal coils (Deshmukh et al. 2006), while the meristematic zone was barely and solely extracellularly colonised. Root colonisation by *P. indica* differs from that of AM fungi, which are known to preferentially colonise younger root parts, since the physiological activity of host cells is a prerequisite for efficient nutrient exchange between the symbiotic partners.

One of the main qualitative differences between *P. indica* and other mycorrhizas is the requirement of cell death for root colonisation (Deshmukh et al. 2006). Recent transmission electron microscopic studies revealed that cells are not dead at penetration stages, but show ultrastructural changes

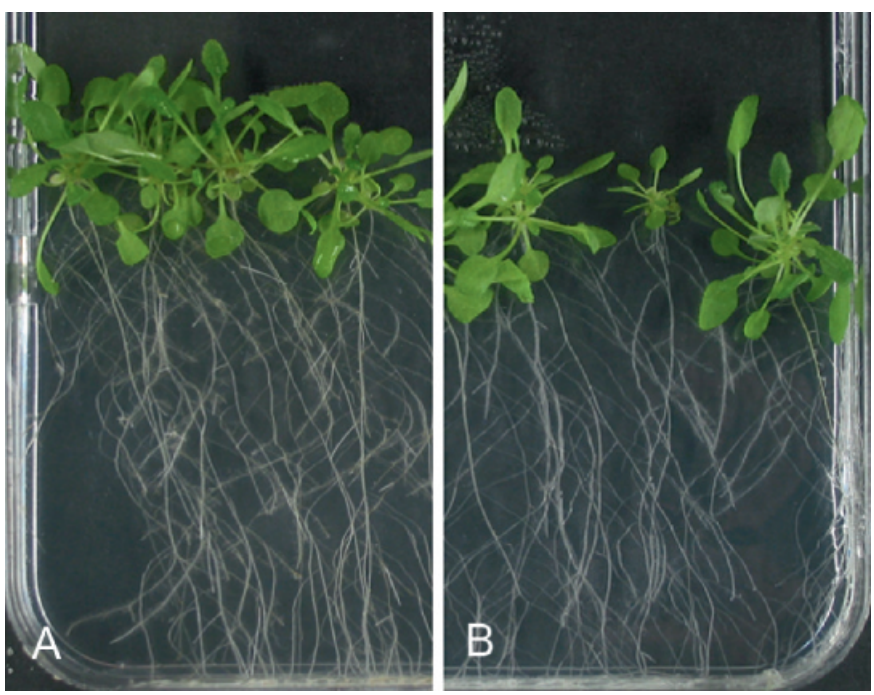

Fig. 5.2. *A. thaliana* root responses towards *P. indica* colonisation. *A. thaliana* plants at 7 days after inoculation with *P. indica* (**A**) or mock-treatment (**B**). Plants were grown for 3 weeks on 0.5 MS medium (mod. 4; Duchefa, The Netherlands) in Petri dishes before inoculation of plants with spore suspension (500 000 spores ml^{-1}) or mock-treatment with 0.02% Tween water. At this stage, roots show intensive inter- and intracellular colonisation without causing visible colonisation symptoms in host roots

as cell colonisation becomes established (Schäfer and Zechmann, unpublished data). These findings suggest that the fungal colonisation strategy is not simply focused on the perception and subsequent colonisation of dead cells. In other words, penetrated host cells obviously die at one defined point of cell colonisation. The fact that this colonisation strategy crucially depends on host cell death at a certain interaction stage was shown in barley plants constitutively overexpressing the negative cell death regulator *Bax Inhibitor-1*. As a result of the genetically increased cell viability, fungal root colonisation was significantly reduced in these transgenic plants (Deshmukh et al. 2006). Conspicuously, in roots of wild-type barley inoculated with *P. indica*, *Bax Inhibitor-1* was found to be suppressed 5 days after inoculation and thereafter. The question arises whether this cell death-associated host response reflects a general colonisation strategy of the endophyte to benefit from plants. Alternatively, it may reflect some kind of imbalanced interaction with unfavourable host plants. As mentioned above, other mycorrhizal fungi are capable of colonising non-host roots and older root regions of host plants in an endophytic manner (Brundrett 2004). Nevertheless, AM fungi are incapable of initiating reproduction in these situations and nutrient supply is apparently not sufficient to guarantee long-term survival. In other words, the AM fungus is changing its life strategy in order to survive hostile conditions (Brundrett 2004). In contrast, *P. indica* is able to sporulate in barley and *A. thaliana* roots and, during the establishment of an initial biotrophic phase, the fungus does not induce apparent molecular and structural defence mechanisms, implying a certain degree of adaptation to these plants (Schäfer and Zechmann, unpublished data).

VI. Parasitic Associations of Plants with *P. indica*

Despite the flexibility of endophytes in colonising plants, the environmental factors, developmental stages and genetic predispositions of the interacting organisms can turn an asymptomatic association into parasitic or incompatible interactions, in which the endophyte either exhibits detrimental growth in plants traceable by disease development (and yield decrease), or has lost the capability to enter the plant tissue. Schulz and Boyle (2005) found that endophytes tended to exhibit a parasitic lifestyle on host plants in laboratory or greenhouse studies, most probably due to unfavourable environmental conditions, whereas symptomless associations were observed in field experiments. This view is supported by investigations on dark septate endophytes in which experimental conditions resulted in a switch to a parasitic lifestyle (Jumpponen and Trappe 1998). Another example is given by *Lophodermium*. This endophyte was shown to asymptomatically colonise young needles of white pines but to switch to a more extensive and parasitic colonisation pattern during needle senescence (Deckert et al. 2001). Experiments with the root endophyte *Epichloë festucae* showed that such switches in endophytic life strategies do not necessarily depend on polygenetic traits. In contrast to the symptomless colonisation of the wild type in the host *Lolium perenne*, endophytic mutants defective in a *NADPH oxidase* (*noxA*) and a proposed regulator (*noxR*) displayed pathogenic colonisation (Takemoto et al. 2006; Tanaka et al. 2006; Chap. 15). Interestingly, the cucurbit pathogen *Colletotrichum magna* was also converted into a fungal mutualist by disruption of a single gene, although the respective gene has not yet been identified (Freeman and Rodriguez 1993; Redman et al. 1999). Hence, endophytism and even parasitism is a matter of harbouring or lacking certain genes or sets of genes. Such rather simplified genetic switches might represent a significant advantage. For example they might support a physiological flexibility under various environmental conditions and, thus, promote fungal reproduction. These genes might represent determinants of the life strategy of these microbes and help them to occupy ecological niches.

Schulz and Boyle (2005) hypothesised a "balanced antagonism" of endophyte–plant interactions, meaning an equilibrium between endophytic virulence factors and host defence responses that enable restricted non-pathogenic tissue colonisation. As soon as external or internal factors are misbalanced the asymptomatic interaction can turn into a parasitic one.

Conditions that are unfavourable for *P. indica*-plant associations, for example an antagonistic genetic background of the host plant or environmental factors, have been reported to impair or even change the outcome of the symbiosis (Kaldorf et al. 2005). However, stunted root development, as recently observed in sterile culture (Sirrenberg et al. 2007), might neither display unfavourable con-

ditions nor be misinterpreted as a parasitic trait of *P. indica*. It rather indicates the auxin-producing capacities of the fungus that are pronounced under certain inoculation conditions. Under natural conditions, similar plant reactions might not be triggered by the fungus. For example, the use of chlamydospores of *P. indica* for plant root inoculation under comparable sterile conditions does not provoke stunted root growth (Fig. 5.2), despite intensive root colonisation. Plant root inhabitation by *P. indica* appears to depend on the developmental stage of the root tissue. In healthy roots, younger root tissue of the meristematic region is barely colonised in *A. thaliana* and barley; and in those rare cases only extracellular colonisation occurs. Deshmukh et al. (2006) comparatively quantified colonisation of root tip regions and the rest of the root and reported on significantly reduced fungal biomass in the former tissue. At the interaction sites of the meristematic zone, where *P. indica* started occupying rhizodermal cells, the plant showed a hypersensitive response-like defence reaction (Fig. 5.3). These defence responses were not detected in equally invaded cells of older root parts (Fig. 5.1; Schäfer, unpublished data). The assumption that mature root zones represent an accumulation of dead or inactive cells, and thus unprotected entry points, can be excluded. By investigating diverse *A. thaliana* plants in which both the structural components of root cells (e.g. actin, tubulin) and the cellular organelles (e.g. nucleus, endoplasmic reticulum, plasma membrane) were tagged with green fluorescing protein, it became obvious that even mature cells were alive at the time of fungal penetration (Schäfer, unpublished data). Taken together, these studies demonstrate a clear preference of the fungus for mature root tissue. Second, the fungus is obviously recognised by its host. The colonisation pattern might be due either to a less active host immunity surveillance system in mature root parts, or to a facilitated access due to the elimination of adverse host activity by the fungus. In contrast, due to the exceptional importance of the meristematic zone for plant survival, this zone might be particularly guarded by the innate immunity system. Obviously the host is capable of restricting fungal colonisation; and this control appears to gradually decrease as root tissue matures.

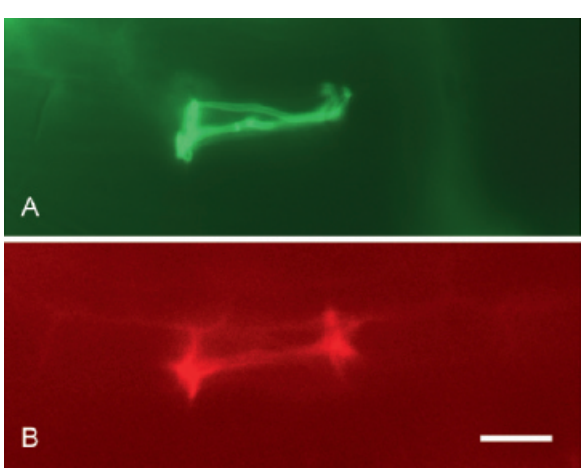

Fig. 5.3. Defence response in a cell colonised by *P. indica*. A rhizodermal cell of the meristematic *A. thaliana* root zone reacts with a hypersensitive-like response after penetration by *P. indica* at 3 days after inoculation. **A** *P. indica* was stained with WGA-AF488. **B** Under UV light the colonised cell shows autofluorescence. Images were taken using an Axioplan 2 microscope. Root segments were either excited at 470/20 nm and detected at 505–530 nm for WGA-AF 488, or excited at 546/12 nm and detected at 590 nm for detection of autofluorescence. *Bar* 20 μm

As discussed above for other plant–endophyte associations, environmental conditions can provoke a pathogenic lifestyle of *P. indica* in host plants, as described by Kaldorf et al. (2005), whose study showed that the beneficial effects of *P. indica* on populus seedlings were redirected into reduced root growth and leaf necrosis when ammonium instead of nitrate was provided as single nitrogen source during plant–fungus co-cultivation. Under these experimental conditions, the fungus exhibited an unrestricted invasion of all plant organs including aerial parts. By adopting the same experimental setup, we reproduced these detrimental effects of *P. indica* in *A. thaliana* and barley (Schäfer and Kogel, unpublished data).

VII. Factors Involved in Plant Colonisation by *P. indica*

As mentioned in the previous sections, the host range of endophytes can be restricted by their genetic predisposition as well as by plant factors. Under natural conditions some endophytes display a certain degree of host specificity, so that not all plant taxa are equally infested. Failed colonisation may be accompanied by the development of disease symptoms (Schulz and Boyle 2005).

So far, *P. indica* has not been shown to possess a distinct host specificity, nor have non-host plants been detected. The fungus colonises monocotyledonous and dicotyledonous plants equally well.

Hosts include orchids (*Dactylorhiza* sp.) and members of the Poaceae (e.g. barley, maize, rice, wheat) and Brassicaceae (e.g. *A. thaliana*; Verma et al. 1998; Blechert et al. 1999: Varma et al. 1999; Waller et al. 2005); and colonisation is asymptomatic, although these plants are both inter- and intracellularly colonised. The question arises to what extent the plant innate immunity is activated by *P. indica*. In barley, defence genes are moderately and transiently induced, as indicated by a marker gene (*PR-1*, *PR-2, PR-5*) expression study (Deshmukh and Kogel 2007; Waller et al. 2008) and microarray-based investigations (Schäfer et al., unpublished data). This is reminiscent of findings reported for plants colonised by AM fungi (Harrison 2005). Some common defence reactions were found in plant–endophyte interactions, e.g. papillae formation, cell wall lignification, H_2O_2 accumulation, enhanced peroxidase activity, or accumulation of phenolic compounds (Schulz and Boyle 2005). Whether these responses significantly contribute to the restriction of endophytic colonisation is unknown. Studies with tobacco and *Nicotiana sylvestris* constitutively expressing different plant chitinases demonstrated that defence-related proteins do not per se exhibit antimicrobial activity against the AM fungus *Glomus mosseae* (Vierheilig et al. 1993, 1995). Certain chitinases are even reported to support mycorrhizal root colonisation by hydrolysing chitin (Salzer et al. 1997), which would otherwise be recognised by the plant innate immunity system and induce pathogen-associated molecular pattern (PAMP)-triggered immunity (Jones and Dangl 2006; Kaku et al. 2006; Miya et al. 2007). Analogously, greenhouse experiments revealed that barley plants constitutively overexpressing an endochitinase of the soilborne fungus *Trichoderma harzianum* were equally well colonised by *G. mosseae* as control plants. Since these plants were shown to synthesise and secrete a highly active recombinant protein, antimicrobial activities of chitinases might not impair the mycorrhizal fungus (Kogel, von Wettstein and Schäfer, unpublished data).

However, recent studies identified some host genes of *A. thaliana* which restrict or support the colonisation of plants by *P. indica*. As reported above, root cell death regulation might be of importance since overexpression of the negative cell death regulator *Bax Inhibitor-1* reduces fungal colonisation in barley and in *A. thaliana* roots (Deshmukh et al. 2006; Schäfer and Kogel, unpublished data). In a genetic screen for host factors regulating fungal colonisation of plant roots, the ethylene-insensitive *A. thaliana* mutant line *etr1-3* was identified (Khatabi and Schäfer, unpublished data). QPCR-based quantification of fungal biomass revealed a lower colonisation of this mutant, which was defective in an ethylene receptor and thus impaired in ethylene-mediated signalling responses (Bleecker et al.1988; Benavente and Alonso 2005). Despite the lower colonisation rate of *etr1-3*, induction of *P. indica*-mediated resistance to powdery mildew was not impaired. Similarly, the *A. thaliana* mutant line *ctr1*, which is defective in a serine/threonine protein kinase and acts as a negative regulator of the ethylene response pathway (Kieber et al. 1993), displayed a constitutive expression of ethylene responsive genes (Zhong and Burns 2003) and showed 3- to 4-fold higher colonisation. The extent to which ethylene production is affected in roots of *A. thaliana* interacting with *P. indica* is currently under investigation.

Recent studies on the *Nicotiana attenuata–S. vermifera* interaction indicated that infested seedlings showed reduced sensitivity to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). After applying ACC to dark-grown seedlings, morphological effects known as the triple response (a shortened and thickened hypocotyl, the inhibition of root elongation growth, a pronounced apical hook) were no longer detected in *S. vermifera*-colonised tobacco plants. Moreover, silencing of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) in *N. attenuata*, which is involved in ethylene synthesis, led to taller plants under non-inoculated conditions. Furthermore, the respective mutants no longer showed growth promoting effects after inoculation with *S. vermifera* (Barazani et al. 2007). Hence, it was postulated that endophyte-triggered growth performance might be the result of impaired ethylene synthesis and/or signalling in colonised plants. Interestingly, Barazani et al. (2005) detected a reduced herbivore resistance in *N. attenuata* leaves after inoculation with *S. vermifera*. After application of an oral secretion of a herbivore to *N. attenuata* leaves, *S. vermifera*-inoculated plants displayed a reduced ethylene burst and suppressed transcript accumulation of ethylene synthesis genes (*NaACS3*, *NaACO1*, *NaACO3*). In these experiments, neither the accumulation of jasmonic acid (JA) and JA-isoleucine nor JA signalling was affected (Barazani et al. 2007). Taken together, the inhibition of ethylene production by *S. vermifera* has positive and negative effects

on the plant. While growth is promoted, herbivore resistance is impaired. The studies of Barazani et al. (2007) and our results with *A. thaliana* may indicate that the beneficial systemic effects, growth promotion of plants and resistance induction against *Golovinomyces orontii*, mediated by sebacinoid mycobionts, are not mediated by the same pathways.

VIII. Impact of Various Plant Mutations on *P. indica*-Induced Resistance

P. indica-induced systemic resistance to the powdery mildew fungus *G. orontii* is largely compromised in *A. thaliana* mutants defective in components of the JA/ethylene (ET) defence pathway. However, this resistance is independent of the salicylate (SA) pathway. *A. thaliana* genotypes showing enhanced resistance to *G. orontii* after *P. indica* colonisation can be clearly distinguished from those with no response to *P. indica*: While induced resistance still occurs in NahG plants not accumulating SA and in the SAR regulatory mutant non-expressor of PR genes1-3 (*npr1-3*), it is abolished in jasmonate response1-1 mutants (*jar1-1*, insensitive to jasmonate; Stein et al., unpublished data). Unlike *npr1-3*, the *npr1-1* null mutant (which exhibits compromised pathways for both salicylate and jasmonate) is also non-responsive to *P. indica* and thus shows a higher susceptibility to *G. orontii*. In contrast to *npr1-1*, the mutant *npr1-3* still supports a cytoplasmic function of NPR1, in spite of the fact that nuclear localisation of this protein is impaired in both mutants. Hence, a compromised defence response in *npr1-1* demonstrates a requirement for the cytoplasmic function of NPR1 for *P. indica*-induced resistance. Since root colonisation with *P. indica* is not compromised in the non-responding mutants, the mutant analyses suggest that JAR1 and NPR1 are genes required for *P. indica*-mediated resistance to powdery mildew. Interestingly, the *jar1-1* mutant is characterised by reduced JA sensitivity, leading to an impaired induced systemic resistance (ISR) reaction and reduced resistance to the opportunistic soil fungus *Pythium irregulare* (Staswick et al. 1992, 1998; Pieterse et al. 1998). JAR1 is able to adenylate JA, an enzymatic step initiating covalent modifications such as coupling to amino acids (Staswick et al. 2002). JA-isoleucine was recently shown to promote the binding of COI1 and JAZ1, crucial elements in JA signalling and possible JA receptor candidates (Chini et al. 2007; Thines et al. 2007). The requirement for JAR1 thus suggests that *P. indica*-mediated resistance requires the formation of JA conjugates. These are active in transmitting several, but not all, JA-mediated responses.

IX. Bacterial Endosymbiotic Associations Within *Sebacinales*

Recent molecular analyses have shown that both *P. indica* and *S. vermifera* are intimately associated with bacteria (Sharma et al. 2008). Based on PCR analyses and sequencing of the 16S ribosomal RNA, an association of *P. indica* with *Rhizobium radiobacter*, a gram-negative α-proteobacterium, was traced back to the original *P. indica* isolate deposited in the culture collection of the German Resource Centre for Biological Material, Braunschweig. This isolate had been deposited immediately after its discovery in the mid-1990s. While bacterial cells are not present in culture filtrates of *P. indica*, they are released after crushing the fungal mycelium, suggesting that *R. radiobacter* is closely associated with the hyphal walls or even lives endosymbiotically. Isolated bacteria show biological activities on barley similar to those mediated by *P. indica*, including systemic resistance induction against powdery mildew and growth promotion. Since *R. radiobacter* has not been successfully eliminated from *P. indica*, it remains an open question to what extent fungus and bacterium contribute to the biological effects on their host plants.

A PCR-based screen of various *Sebacina vermifera* cultures for the presence of bacteria clearly revealed fungal isolates from various original sources to be stably associated with single bacterial species. For instance, *Sebacina vermifera* strain MAFF305838 lives associated with *Paenibacillus* spp. Using fluorescence in situ hybridisation (FISH) with eubacterial fluorescent primers, bacterial cells were localised inside fungal hyphae and chlamydospores (Fig. 5.4). In contrast to *R. radiobacter*, *Paenibacillus* could not be cultivated in axenic cultures. Thus the biological activity of this bacterium and its contribution to a more complex tripartite symbiosis has not been resolved. In essence, the above findings show that the *Sebacinales* undergo complex symbioses involving host plants and bacteria.

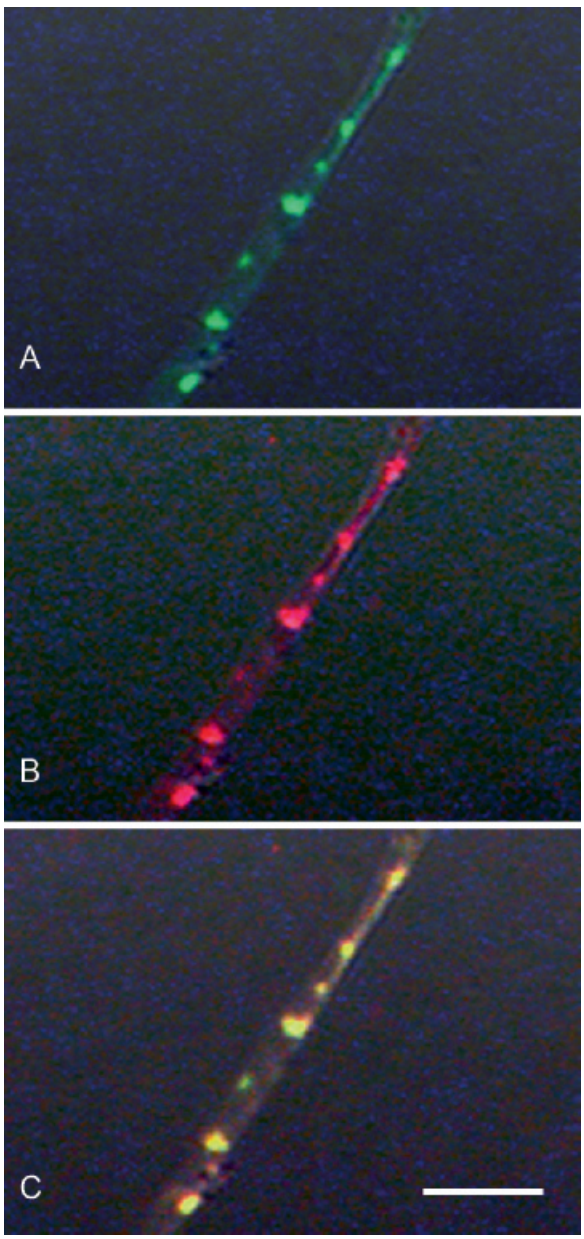

Fig. 5.4. *Sebacina vermifera* strain MAFF305838 harbours endosymbiotic bacteria. Bacteria (*Paenibacillus* sp.) associated with *S. vermifera* strain MAFF305838 were localised within hyphal cells by using fluorescent in situ hybridisation (FISH) and confocal laser-scanning microscopy. Bacteria in association with the fungus were labeled with: (**A**) a EUB-338 FITC-labeled probe mix (excitation/emission: 488 nm/517 nm) for detection of eubacteria and (**B**) a LGC-354 Cy3-labeled probe mix (543 nm/562 nm) for specific detection of Firmicutes, to which *Paenibacillus* sp. belongs. Merged images (**C**) show the congruence of both labels, indicating the endosymbiotic localisation of the bacteria. A EUK-516 Cy5-labeled probe for detecting eucaryotes (633 nm/664 nm) did not bind to endosymbiotic bacteria (data not shown). *Bar* 10 μm

There are several reports showing a mutualistic association of mycorrhizal fungi with bacteria in which, for instance, bacteria improve spore germination and the formation of mycorrhizal interactions. In addition, plant growth-promoting bacteria (PGPR) have been shown to interact physically with fungal hyphae. *Rhizobium* and *Pseudomonas* species attach to germinated AM fungal spores and hyphae (Bianciotto et al. 1996a), but no specificity for either fungal or inorganic surfaces could be detected among the bacteria tested.

True endosymbiotic bacteria have been reported in only a few fungi, including members of the *Glomeromycota* (e.g. *Gigaspora* sp., *Geosiphon pyriforme*) and the ectomycorrhizal basidiomycete *Laccaria bicolor*. For example, endobacteria have been detected in five species of *Gigasporaceae* and various fungal cells, including spores, germtubes and extra- and intraradical hyphae, but not in arbuscules (Bianciotto et al. 1996b). Endosymbiotic bacteria were first identified in the AM fungus *Glomus margarita*, and this association is the best studied interaction of AM fungi and endobacteria (Bianciotto et al. 1996b). Recent studies estimated an average of about 20 000 bacteria per *G. margarita* spore (Bianciotto et al. 2004; Jargeat et al. 2004). Although initially assigned to the genus *Burkholderia*, recent phylogenetic analyses based on 16S ribosomal RNA gene sequences proposed the introduction of a new taxon termed *Candidatus Glomeribacter gigasporarum* (Bianciotto et al. 2003). The small bacterial genomes (about 1.4 Mb) consist of a single chromosome and a single plasmid (Jargeat et al. 2004). Recently, Lumini et al. (2007) published a procedure for dilution of the bacteria by using successive in vitro single-spore inocula. The absence of bacteria severely affected presymbiotic fungal growth with deficiencies in spore shape and hyphal elongation, delays in growth onset of germinating mycelium and in branching after root exudate treatment. These results suggest that endobacteria contribute to regular development of its fungal host.

In the plant pathogen *Rhizopus microsporus* endosymbiotic bacteria play a crucial role in fungal infection strategies. Until recently, *R. microsporus* was thought to produce a toxin that kills plant root cells. However, Partida-Martinez and Hartweck (2005) demonstrated that the toxin was not produced by the fungus but by endogenous bacteria. On the basis of the 16S ribosomal RNA gene sequence, they found that the bacteria belong

to the genus *Burkholderia*, a member of the beta subdivision of proteobacteria. The bacteria and bacteria-free fungus were each isolated in pure culture. There was a strong correlation between the presence of bacteria and the toxin-producing capability of *Rhizopus*. In the absence of endobacteria, *Rhizopus macrosporus* was not capable of vegetative reproduction (Partida-Martinez et al. 2007). Formation of sporangia and spores was restored only upon reintroduction of endobacteria. The motile rod-shaped bacteria appeared to be prone to chemotaxis, since they migrated toward the tips of the hyphae, the region best supplied with nutrients and where sporangia were formed.

X. Conclusions

Present knowledge characterises *P. indica* as a potential orchid mycorrhiza fungus that can be clearly distinguished from ectomycorrhizas or arbuscular mycorrhizas. However, its endophytic life style might be predominant in associations with certain plants. Even during these types of evolutionarily inappropriate interactions, the fungus is able to confer beneficial effects to its hosts; this phenomenon distinguishes *P. indica* from ectomycorrhizal and AM fungi.

P. indica is a model organism of the newly defined order *Sebacinales* within the phylum Basidiomycota, comprising a group of mycorrhizal fungi that form mutualistic symbioses with an as yet widely unrevealed function in natural ecosystems as well as cropping systems. In contrast to AM fungi, *P. indica* and related species of the *S. vermifera* complex confer systemic resistance against root and leaf pathogens to a wide range of monocotyledonous and dicotyledonous plants. Moreover, these fungi bear a significant agronomical potential, since they increase grain yield. Their application in horticulture or agriculture is economically and practically feasible through the facilitated propragation of fungal inoculum using liquid or axenic cultures. The huge prospective biodiversity in the *Sebacinales* provides the perspective that appropriate sebacinalean mutualists might be discovered for many crop plants. Research on *Sebacinales*, however, may not only enable new crop production strategies but additionally may eminently expand our basic knowledge on host–microbe interactions. Recent discovery of fungus-associated endobacteria demonstrated that *Sebacinales* can participate in a more complex symbiosis. Although the exact contributions of the partners are not fully elucidated, it is clear that the bacteria perform activities that were formerly ascribed to the fungal partner. In addition, it is obvious that *P. indica* shows properties that clearly contrast with those ascribed to AM fungi:

1. In comparison to known endophytic strategies, *P. indica* requires host cell death for successful plant colonisation, implying that fungal effector molecules interfere with the host cell death machinery.
2. *P. indica* conveys systemic disease resistance to fungal leaf pathogens, which has rarely been observed in monocotyledonous plants.
3. *P. indica* is the sole fungal mutualist identified to date that colonises *A. thaliana* and mediates a type of systemic resistance to powdery mildew which depends on jasmonate signal pathways.

The power of available *A. thaliana* signal transduction mutants and reverse genetics will further accelerate disclosure of the molecular basis of the symbiosis and its beneficial effects on the plant.

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5.12 Imani, J., Li, L., **Schäfer, P.***, Kogel, K.H.* STARTS – a stable root transformation system that accelerates the functional analyses of proteins in roots of the monocot model plant barley. *Shared senior authorship. (Intended to be submitted by the end of November 2010).

STARTS – a stable root transformation system that accelerates the functional analyses of proteins in roots of the monocot model plant barley

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Abstract

Large data sets are generated by the various “omics” platforms defining transcriptomes, proteomes, and metabolomes. Currently, the limited step is the functional analysis of proteins and thus translation of the data into a biological and physiological context. The unavailability of robust and fast transformation systems for monocotyledonous plants is a major restriction in functional analyses. Here we present a stable root transformation system for barley, designated STARTS, that allows the fast overexpression and silencing of genes. By applying STARTS, functional analyses can be reduced to 6-8 weeks, which makes the system incomparably faster than conventional transformation approaches. Thus, STARTS represents a highly efficient method for functional analyses of proteins. We demonstrate that STARTS can be reliably applied for non-invasive protein localization, for the analyses of root developmental processes as well as the examination of mutualistic and pathogenic barley root-microbe interactions. We propose that STARTS can be established in other cereals (e.g. wheat, rice).

Introduction

Roots are the central organ for supplying plants with nutrients and water and are suggested to improve plant adaptation and thus productivity under changing environmental conditions (White and Brown, 2010). As it is pivotal for a sessile organism like plants, this adaptive potential has also considerable relevance for crop production. Food security is a global concern and the central task of agricultural production systems to provide food for a growing world population (FAO, 2009). Abiotic stresses such as low nitrogen supply, drought and salt stress and heavy metal toxicity (Witcombe et al., 2008) as well as biotic stress as a result of pathogen (fungi, bacteria) and insect invasion affect crop production (Cook, 2007; Fuller et al., 2008). Furthermore, roots are also entry point for leaf pathogens (Sesma and Osbourn, 2004). Root disease represent a serious threat as protective cultivation methods, resistant germplasms or chemical control strategies are missing. Therefore, enhancement of crop production menas to improve cropping systems and to increase plant productivity. Support is given by the interaction of plant roots with mutualistic microbes, such as mycorrhizal fungi or N-fixing bacteria, which considerably improve nutrient and water uptake of roots and enhance plant growth. Importantly, plants are also protected against abiotic and biotic stresses (Parniske, 2008; Oldroyd and Downie, 2008; Schäfer and Kogel, 2009). However, the molecular base of root colonization and of the benficial effects are just partly understood.

In addition to classical breeding strategies, biotechnological approaches bear the potential to improve stress adaptation of crop plants (Mittler and Blumwald, 2010). The post-genome era provides a multiplicity of “omics” information including transcriptome,

proteome or metabolome data sets whose translation into biological significant phenotypes is a major limitation. The function of plant proteins during plant development or under stress condition is commonly determined by silencing (via RNA interfernce, RNAi) or overexpression of respective genes. Various transient and stable transformation systems have been developed to transfer genes or RNAi constructs into leaf tissue in monocotyledonous (e.g. barley) and dicotyledonous plants (e.g. tomato, tobacco, *Arabidopsis*) in order to study protein functions (Newell, 2000). Transient transformation is mostly done by virus-based techniques or by agroinfiltration, which is a *Agrobacterium tumefaciens*-based transfection, have been used to overexpress or silence plant genes (Kumagai et al., 1995; MacFarlane and Popovich, 2000; Mallory et al., 2001). In addition, biolistic approaches are widely used in which small plasmid coated particles (tungsten, gold; diameter of ~ 1 µm) harboring the gene of interest are bombarded into host cells unsing a gene gun (Kikkert et al., 2004). Although these approaches are unquestionable faster than stable transformation, transferred genes are not inserted into the plant genome and the plant tissue is just partially transformed. Consequently, the synthesis of recombinant protein is mostly lower as compared to stable transformation (Chung et al., 2006). This is partly overcome by combining an improved TMV vector with *Agrobacterium tumefaciens*-based transfection (agroinfiltration) thereby enhancing transient transformation efficiencies in plant tissues (Marillonet et al., 2005).

The predominant method for stable plant transformation is *Agrobacterium tumefaciens*-mediated gene transfer (Herrera-Estrella et al., 1983). *A. tumefaciens* has a broad host spectrum (Newell, 2000) and naturally incorporates the well-characterized transferred DNA (T-DNA), which is part of the bacterial tumor inducing (Ti)-Plasmid, into the plant

genome (Tzfira and Citovsky, 2006). By this means, *A. tumefaciens* replicates in plants. By replacing the T-DNA with a gene of interest, *A. tumefaciens* can be used to achieve the expression of recombinant proteins in plants and study their effect on plant developmental aspects, plant reproduction or biotic and abiotic stress. Various *A. tumefaciens*-mediated transformation procedures have been developed. For instance, *Arabidopsis* is transformed by the floral dip method (Clough and Bent, 1998). In barley, cells of the scutellum of immature embryos are transformed by co-cultivating bacteria with *A. tumefaciens*. The partially transformed scutella are used to regenerate calli prior to induce shoot growth from single transformed cells and to regenerate whole plants (Tingay et al., 1997). However, this procedure is laborious and time-consuming and functional studies can be started after obtaining homozygous T2 plants, which takes about 12 months. It makes this system highly inefficient for root-related functional studies but alternative transformation system are not available. An efficient barley root transformation system would require the robust and fast introduction of candidate genes into roots and each cell should be transformed.

We present here a stable root transformation system (STARTS) for barley, which combines the robustness of conventional stable transformation approaches with a highly time-efficient generation of transformed root. We demonstrate here that STARTS is applicable to generate roots in which barley genes can be specifically targeted for silencing and overexpression. We provide evidence that STARTS is suitable to robustly silence genes irrespective of their transcription level and to perform non-invasive protein localization studies. Importantly, STARTS can be efficiently applied to for functional

studies in root developmental as well as mutualistic and pathogenic root-microbe interaction.

Results

Development of the stable root transformation system (STARTS)

We aimed to establish a fast and robust root transformation system for the functional characterization of proteins in roots. In a first experiment, we set up conditions that allowed the fast and efficient generation of roots from barley calli. As summarized in Fig. 1, the scutellum of immature barley embryos were isolated and transferred to callus induction medium allowing rapid cell division to obtain calli. These calli were transferred to root induction medium and multiples several roots differentiated from single cells of calli. This procedure reduced the time for the generation of roots to about 6 weeks compared to approximately 12 months required for root generation homozygous T2 transformants by classical techniques. By contrast, our new approach would allow the direct usage of regenerated roots. To test the usage of the system to generate stably transformed roots, we performed *Agrobacterium tumefaciens*-mediated transformation. *A. tumefaciens* was carrying the green fluorescent protein (GFP) under control of constitutive promoter of maize *ubiquitin*. The isolated scutella of immature embryos (Fig. 2A, B) were co-cultivated for two days with this *A. tumefaciens* to allow transformation of scutella cells with GFP. GFP expression was observed in scutella cells at 1-2 days after co-cultivation (Fig. 2B) and in dividing scutella cells at 1-2 weeks after *A. tumefaciens* transformation (Fig. 2C). Later expression of GFP was also was observed in calli regenerated from transformed scutella at 2-3 weeks after co-cultivation (Fig 2D). The calli were subsequently transferred into root induction medium and roots were developing from single callus cells within 2-3 weeks. GFP expression was observed in all root cells (Fig. 2E, F). This experiment confirmed the accessibility of the system for

stable genetic transformation and the generation of stably transformed roots within 6 weeks. The system was defined as stable root transformation system (STARTS).

Next, we addressed the question whether STARTS could be applied for gene silencing. Therefore, we monitored silencing of the endogenous barley gene Hv*EXPANSIN B1* (Hv*EXPB1*) which is required for root hair development (Kwasniewski and Szarejko, 2006). Scutella were co-cultured with A. tumefaciens carrying a silencing construct for Hv*EXPB1*. Six weeks after transformation, the number of root hairs were counted in three different section of the root: (I) Elongation zone, (II) young maturation zone, and (III) old maturation zone (Fig. 3A). Roots generated from scutella of wild type cultivar Golden Promise as well as scutella co-cultured with an empty vector construct developed root hairs. By contrast, Hv*EXPB1*-silenced roots exhibited a significantly reduced number of root hairs in all root zones (Fig. 3B). In addition, quantitative real time PCR (qRT-PCR) was performed to determine the degree of Hv*EXPB1* silencing. The levels of Hv*EXPB1* transcripts were normalized to barley *ubiquitin*. The amount of Hv*EXPB1* transcript was strongly reduced in silenced roots, which correlated with the reduction of root hairs (Fig.3C).

Although these analyses proved the ability of STARTS to silence genes, we were interested to determine the efficiency and stability of STARTS-mediated silencing. Therefore, scutella derived from immature barley embryos stably overexpressing GFP by the Cauliflower Mosaic Virus 35S promoter (35S) were co-cultured with *A. tumefaciens* carrying a GFP-RNA interference (RNAi) construct. GFP silencing was observed in generated barley calli (Fig. 4A-F). Furthermore, GFP silencing was stable and was observed in shoots generated from GFP-silenced calli (Fig. 4G, H). As expected, different

degrees of silencing were obtained in different transformed calli. However, in 80% of all cases, >50% of calli cells were silenced as indicated by the absence of GFP (Fig. 5A). In addition, the extent of GFP silencing in roots generated from GFP-silenced calli was determined. About 50% of all roots dispayed an intermediate or complete reduction in GFP expression (Fig. 5B). Subsequent qRT-PCR analysis revealed a drastically reduced level of *GFP* transcripts even in those roots that exhibited an intermediate silencing phenotype (Fig. 5C). This might be explained by the brightness of GFP under fluorescent light that results in an overestimation of GFP accumulation. According to the reduced transcript levels in GFP-silenced roots, GFP protein accumulation was strongly reduced in all samples (Fig. 5D). In sum, the analyses revealed the efficiency of STARTS to overexpress and silence genes. STARTS can be applied to silence genes that are strongly regulated at the transcript level. Further, STARTS-mediated gene silencing is stable and transferred to shoots.

STARTS is suitable for protein localization studies

The subcellular localization of proteins helps to predict their function. In order to investigate the suitability of STARTS for this kind of analysis, we transformed scutella with a modified version of Green Fluorescent Protein (*mGFP4-ER*), which is provided mit a 5´-terminal signal peptide sequence and a 3´-terminal HDEL sequence to ensure Endoplasmic Reticulum (ER) localization (Haseloff et al., 1997). For our analysis, *mGFP4-ER* was under control of the constitutive 35S promoter. As an independent control, we generated barley plants that stably expressed *mGFP4-ER*. In root tissue derived from roots transformed and generated by STARTS (Fig. 6A, right panels) as well

as in roots germinated from seeds of stably transformed (Fig. 6A, left panels) *mGFP4-ER* plants, the GFP was clearly detected in the ER and in the nuclear periphery by confocal laser-scanning microscopy (Fig. 6A). In transformed cell GFP appeared in the ER. These results demonstrated the suitability of STARTS for protein localization studies.

STARTS is an efficient method to analyze protein function in pathogenic and mutualistic barley root-microbe interactions

We were interested whether STARTS is applicable to study the effect of barley proteins in barley root-microbe interactions. More precisely, we studied the impact of BAX INHIBITOR-1 overexpression on barley root colonization by the mutualistic fungus *Piriformospora indica* and the pathogenic fungus *Fusarium graminearum*. Both microbes follow a cell death-dependent root colonization strategy (Deshmukh et al., 2006; Babaeizad et al., 2009). BI-1 is a negative cell death regulator and the roots of barley plants stably overexpressing BI-1 exhibited reduced colonization by both fungi (Deshmukh et al., 2006; Babaeizad et al., 2009). For the analyses, two types of barley roots were generated by STARTS. In one case, scutella were transformed with *Agrobacteria* that carry a construct with Ubi::*BI-1* while another set of scutella were co-cultured with *Agrobacteria* carrying a construct without gene (empty vector, EV) and thus served as control. Thereafter, roots stably expressing either *BI-1* or EV were generated. The roots generated by STARTS expressing either *BI-1* or EV were morphological indistinguishable. Both sets of roots were inoculated either with spores of *P. indica* or *F. graminearum* and harvested at two different time points in order to follow fungal developments. For the quantification of fungal colonization, roots were separately

harvested and subjected to DNA extraction. By using primers specific for *P. indica internal transcribed spacer* (Pi*ITS*) (Deshmukh et al., 2006) or for *F. graminearum tubulin* (Fg*TUB*) (Reischer et al., 2004), fungal DNA was eventually determined by quantitative real time-PCR (qRT-PCR). In addition, primers specific for barley *ubiquitin* (Hv*UBI*) (Deshmukh et al., 2006) were used to quantify the amount of plant DNA by qRT-PCR, which served as internal standard in the quantification of fungal DNA in roots expressing *BI-1* or EV. *F. graminearum* (Fig. 6B) as well as *P. indica* (Fig. 6C) showed a significantly reduced ability to colonize STARTS-generated roots overexpressing *BI-1* at different time points after inoculation. These results were highly comparable to previous studies with barley plants stably overexpressing BI-1 (Deshmukh et al., 2006; Babaeizad et al., 2009) and indicated the applicability of STARTS to analyze the impact of proteins on barley root colonization by microbes.

DISCUSSION

Stable transformation of barley plants is a robust method for functional studies or for biotechnological approaches (Langen et al., 2006, Babaeizad et al., 2009; Kogel et al., 2010). However, the generation of transgenic barley is time-consuming and laborious and thus too inefficient for high-troughput functionally examinations of proteins. Therefore, transient system have been developed for cereal leaves (Schweizer et al., 1999; 2000) but respective system are not existing for barley roots. Those transient root transformation systems that have been established, e.g. Tobacco Rattle Virus (TRV)-based transformation of roots of *Nicotiana benthamiana* (MacFarlane and Popovich, 2000) or *Agrobacterium rhizogenes*-mediated transformation (Limpens et al., 2004) cannot be transferred to barley due to host specificities of the virus or microbe. In addition, those systems bear certain disadvantages. *A. rhizogenes*-mediated gene transfer is mostly not achieved in all cells of a root resulting in patchy transformation genotypes (Limpens et al., 2004). In addition, progenies of transformed plants are discussed to be morphologically abnormal (Newell, 2000).

We describe here the establishment of a stable root transformation system for barley, which we called STARTS. We demonstrate that STARTS has certain advantages compared to above mentioned systems. As an *Agrobacterium tumefaciens*-based transformation technique, scutella of immature embryos were transformed by co-cultivation. The transfer of obtained scutella to selction media allowed generation of calli. The novelty of the approach was the transfer of these calli to a root inducing medium (Fig. 1). As a principle of the method, regenerated roots were originating from one cell thereby obtaining roots in which all cells are homogenous in a given trait. Therefore, we

observed variation in the efficiency of silencing (Fig. 4, 5). Notably, STARTS is obviously capable to suppress genes under strong transcriptional regulation as we observed silencing of GFP even under control of the strong constitutive ubiquitin promoter of maize (Fig. 5). Most importantly, STARTS accelerates functional studies in roots. Currently, about 52 weeks are required for the generation of transgenic roots by conventional methods. STARTS allowed the functional analyses of proteins in about 6 weeks (Fig. 1, 2). The applicability of the system for functional studies might be indicated by a series of experiments. We were able to perform non-invasive protein localization studies using roots overexpressing GFP equipped with a signal peptide and an endoplasmic reticulum retention sequences (*mGFP4-ER*) (Haseloff et al., 1997) (Fig. 6A). Interestingly, a root hairless phenotype was obtained by silencing of *EXPANSIN B* (Fig. 3). As this experiment visualized gene silencing by STARTS, it further suggests that the system might represent a powerful tool for the identification and functional characterization of plant factors influencing root organization, root architecture, root development, cell differentiation, root-leaf communication, as well as nutrient and water uptake. Particularly, nutrient (e.g. phosphorus) and water availability in soils represent limiting factors in modern crop production. It is further believed that global climate changes will elevate these constricts in future crop production (FAO, 2009; Gilbert, 2009; Smit et al., 2009). Current strategies are focused on supplying plants with more efficient root systems thereby optimizing water uptake (White and Kirkegaard, 2010). In this field major achievements have been made in model plants such as *Arabidopsis*. In addition to directly improve barley traits, STARTS might facilitate the translation of these findings to barley and other cereals. In this respect, mutualistic root-colonizaing microbes, such as

the fungus *Piriformospora indica*, have been shown to enhance nutrient-/ water recruitment and to increase abiotic stress tolerance of plants. conveys various beneficial effects to colonized host plants (Waller et al., 2005; Sherameti et al., 2008; Schäfer and Kogel, 2009; Yadav et al., 2010). The potential of these mutualistic symbioses might be accessible if we will understand the molecular base of these interactions. For instance, which plant factors stop root pathogen inavsion and which processes support mutualistic root symbioses. STARTS might accelerate the identification of respective traits (proteins) and the timely introduction into crop plants. We previously reported that stable overexpression of *BAX INHIBITOR-1* (*BI-1*) in barley plants exhibited enhanced resistance to the pathogenic fungus *Fusarium graminearum* as well as *P. indica* (Deshmukh et al., 2006; Babaeizad et al., 2009). As a proof of concept, we confirmed the reduced susceptibility of STARTS-generated roots overexpresssing *BAX INHIBITOR-1* (*BI-1*) to both microbes (Fig. 6B, C).

An inherent drawback of STARTS might be seen in the unpredictability of inserted gene copy numbers in single cells from which roots are regenerated. However, the system allows the simultaneous and fast generation of dozens of roots originating from independent transformation events. Therefore, protein-specific phenotypes should be consistently detectable in almost all roots thereby even reducing the occurrence of misinterpretations or transformation artefacts due to genome insertion effects. Our data suggest that STARTS allows to pre-screen the effect of candidate genes in a given trait. Importantly, calli can be immediately transferred to respective media to regenerate stably transformed plants in which observed phenotypes can be independently confirmed.

In sum, STARTS is suggested to elucidate the effect and function of proteins in root development, stress tolerance/resistance or other aspects. With the fast development of the next generation sequencing technology, more plant genomes will be sequenced in near future. Efficient determination of the function of identified genes/proteins on a large scale is a major challenge to improve the productivity and quality traits of crop plants. In addition, roots are the organs most subject to beneficial and microbial interactions, STARTS might provide an efficient method for analyzing the function of proteins in mutualistic and pathogenic interactions.

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Author contributions

L.L. and J.I.performed the experiments. J.I., P.S, and K.H.K. designed the research. P.S. and K.H.K. jointly wrote the paper.

Figure legends

Figure 1. Comparision of the conventional transformation method and the stable root transformation system (STARTS). Scutella are isolated from immature embryos (A) and co-cultured with Agrobacterium tumefaciens for transformation. Calli are regenrated from scutella (B). For conventional transformation approaches, calli are subjected to shoot and thereafter to root induction medium to obtain T0 plants. After two generations, homozygous T2 plant are obtained, which takes 52 weeks in total. For STARTS, calli (B) are subjected to root induction medium to regenerate root from individual, transformed cells. Transformed roots are obatined within 6 weeks.

Figure 2. Overexpression of green fluorescent protein in barley roots by STARTS. (A) scutellum separated without the embryo axis 2 days after transformation by co-cultivation with *Agrobacterium tumefaciens*. (B) Same as A. GFP expression in cells of the scutellum. (C) 1-2 weeks after co-cultivation expression of GFP is observed in dividing scutellum cells. (D) 2-3 weeks after co-cultivation, callus is formed from transformed scutellum cells. (E, F) GFP expressing roots that were regenerated from single GFP-transformed callus cells. GFP was visualized by excitation at 450-490 nm and emission was detected at 510-530 nm using a stereofluoresence microscope (Leica, Germany). Bars = 2 mm (a, b, c, d) and 2 cm (e, f).

Figure 3. Silencing of Hv*EXPANSIN B* by STARTS. (A) Root hairless phenotype was observed at the elongation- (I), young maturation- (II), and old maturation zone (III) using a binocular microscope. A, C, E: Root hair phenotype in wild type Golden Promise.

B, D, F: Root hair phenotype in roots transformed with the *HvEXPB* RNAi construct. Bars= 2 mm. (B) Number of root in the root zones (defined in A) of roots obtained from wild type, after empty vector transformation (control), and after Hv*EXPB* RNAi (silencing). Displayed are means of three independent experiments with standard errors. Asterisks indicate significance at $p < 0.001$ using two-tailed Student's *t*-test. (C) Amount of Hv*EXPB1* transcript determined by quantitative Real Time-PCR in Golden Promise roots (GP), empty vector transformed roots (EV), and various roots silenced in Hv*EXPB1* (S1-S6). Displayed are means of three independent experiments with standard errors.

Figure 4. Silencing of GFP in callus, regenerated roots, and regenerated leaves by STARTS. (A, B) Partial silencing of GFP in calli. (C, D) GFP silencing in roots regenerated from GFP-silenced area of a callus (E, F) complete silencing of *GFP* in a callus. (A, C, E) Bright field images. (B, D, F) Fluoresnce immage showing GFP expression. (G) Leaf of transgenic plant expressing *GFP* under control of the Cauliflower Mosaic Virus 35S. (H) *GFP* silencing in shoot regenerated from a *GFP*-silenced callus. GFP was visualized by excitation at 450-490 nm and emission was detected at 510-530 nm using a stereofluoresence microscope (Leica, Germany). Bars = 2 mm.

Figure 5. GFP silencing by STARTS. For the experiments scutella were isolated from immature embryos constitutively overexpressing GFP. (A) Degree of GFP-silencing in calli regenerated from scutella overexpressing GFP and co-cultivation with A. tumefaciens carrying an GFP-RNAi construct. The number of calli were determined that were not silenced (a), partially silenced (b), or completely silenced (c). Data are means of

60 independent calli obtained from two independent experiments. Error bars represent standard deviations. (B) Degree of GFP-silencing in roots regenerated from calli. Three different silencing phenotypes were determined. Non silenced roots (no), partially silenced roots (partial) and completely silenced roots (complete). Data displayed are means of 120 independently regenerated roots obtained from two independent experiments. Error bars represent standard deviations. Bar = 2 cm. (C) Degree of GFP-silencing in Golden Promise (GP), roots of GFP overexpressing plants (GFP), and roots displaying various degrees of GFP silencing (as described in B) using quantitative Real Time-PCR. (D) Immunodetection of GFP using monoclonal anti GFP antibody. Samples were the same as described in C.

Figure 6. Functional analyses of proteins using STARTS. Non-invasive mGFP4-ER localization using confocal laser-scanning microscopy. (A, left panels) mGFP-ER localization in the ER (upper left) and nuclear periphery (lower left) in roots of stably transformed plants. (A, right panels) mGFP-ER localization in the ER (upper right) and nuclear periphery (lower right) in roots derived from STARTS. GFP emmission was detected at 510-530 nm after excitation with a 488 nm laser line using a TCS-SP2 CLSM (Leica, Germany). Bars = 20 μm.

(B) *Fusarium graminearum* infection was reduced in barley roots overexpressing *GFP*-fused *BAX INHIBITOR-1* (*GFP-BI-1*) as compared to roots overexpressing GFP (GFP) at 5 days after inoculation (dai). Fungal structures are visualized by WGA-AF488 (excitation: 485 nm, emmission: 510-530 nm) using an Axioplan epifluorescence microscope (Zeiss, Germany). *F. graminearum* colonization was determined in barley

roots at 2 and 5 dai by quantitative Real Time-PCR. Root colonization was significantly reduced in the *GFP-BI-1* expressing roots as compared to GFP expressing roots. Displayed are means with standard errors of two independent biological experiments. $p<0.05$, two-tailed student's *t*-test.

(C) *Piriformospora indica* colonization was reduced in barley roots overexpressing *GFP*-fused *BAX INHIBITOR-1* (*GFP-BI-1*) as compared to roots overexpressing GFP (GFP) at 3 and 7 days after inoculation (dai). *P. indica* colonization was determined in barley roots by quantitative Real Time-PCR. Root colonization was significantly reduced in the *GFP-BI-1* expressing roots as compared to GFP expressing roots. Displayed are means with standard errors of three independent biological experiments. $p<0.05$, two-tailed student's *t*-test.

Materials and Methods

Generation of transgenic barley root by STARTS

Transformation was carried out with spring barley (*Hordeum vulgare* L.) cv. Golden Promise grown in a climate chamber at 18°C/14°C (light/dark) with 65% relative humidity, a 16 h photoperiod and a photon flux density of 240 µmol m^{-2} s^{-1}. For barley transformation, the below mentioned vectors were introduced into the *Agrobacterium tumefaciens* AGL-1 strain through electroporation (*E. coli* Pulser, Bio-Rad). Two weeks post anthesis, spikes from barley plants were harvested. After removing awns, kernels were put in a bottle and placed on ice. For each transformation 100-200 kernels were surface sterilized in 70% ethanol for 5 min and subsequently incubated in sodium hypochlorite (3% active chlorine) for further 20 min. The kernels were washed once with sterilized water (pH 3) and then rinse 3 times with sterile distilled water under sterile conditions. Immature embryos were taken from the caryopses, and the embryonic axis was removed with a sharp scalpel using a binocular microscope. Immediately, the obtained scutella were placed upside down onto callus induction medium (BCID, Tab. 1) (Tingay et al., 1997). 25-30 scutella were collected in the middle of a petri dish on callus induction medium. 200 µl overnight *Agrobacterium tumefaciens* culture (OD=0.6) was added drop wise onto the scutella. Thereafter, scutellum were turned downside-up and co-cultivated at 24°C for 40-60 minutes in the dark. Thereafter, 10-12 scutella were transferred to a new plate and co-cultivated under the same conditions for 48 h. To support the preferential formation of transgenic callus and to remove the persisting *Agrobacteria*, the scutella were cultured on BCID medium supplemented with 50 mg L^{-1} hygromycin and 150 mgL^{-1} ticarcillin/clavulanate (1:15). The calli were subcultured at an

interval of 2 weeks under the same conditions until root were harvested. For root induction transgenic calli were transferred into modified root induction medium (Tab. 2) (Jensen et al., 1983). Roots developed from calli within two weeks.

Transformation vectors

For overexpression of GFP, GFP BI-1, and for localization studies in barley roots, the binary vectors pLH6000-Ubi-GFP; pLH6000-Ubi-GFP-BI-1 (Deshmukh et al., 2006), and pLH6000-35S-mGFP-ER were used, respectively. mGFP4-ER was previously published (Haseloff et al., 1997). For silencing of barley *Expansin B* (Hv*EXPANSIN B*) a 155 bp sequence was cloned into the entry pENTR-D-TOPO vector (Invitrogen). The Hv*EXPANSIN B* segment was then inserted into the destination vector pIPKb007 (Himmelbach et al., 2007) substituting for the *Ccdb* gene using the LR reaction (Gateway system). For GFP-silencing a 280 bp of the coding sequence of *GFP* was cloned into the p7U-RNAi vector (DNA-Clonig service Hamburg).

Preparation of fungal inoculum and root inoculation

Fusarium graminearum wild-type (strain 1003) was used throughout this investigation. The fungus was routinely cultured on SNA (synthetic nutrient poor agar) plates containing 0.1% KH_2PO_4, 0.1% KNO_3, 0.1% MgSO4.7H_2O, 0.05% KCL, 0.02% glucose, 0.02% sucrose, and 2% agar. Plates were incubated at room temperature and under constant illumination from one near-UV tube (Phillips TLD 36 W/08) and one white light tube (Phillips TLD 36 W/830HF). Conidial suspension was scratched from one-week-old plates by using sterile water and filtered through mira-cloth (Calbiochem) prior to the

adjustment of conidia concentrations to 1.2×10^4 spores mL^{-1}. STARTS-generated roots were inoculated with 1.2×10^4 spores mL^{-1} in 0.02% tween 20 (v/v) + 0.5% gelatine (w/v) for 2 h. Thereafter, inoculated roots were transferred to agar plates. Root samples were harvested at 2 and 5 days after inoculation (dai) and subjected to DNA isolation, which was used for determining the amount of fungal DNA by quantitative Real Time-PCR. *P. indica* isolate was propagated as described (Deshmukh et al., 2006). For inoculation, STARTS-generated roots were immersed in an aqueous solution of 0.05% Tween-20 containing 5×10^5 spores ml^{-1} for 2 h. Root samples were harvested at 3 and 7 dai and subjected to DNA isolation, which was used for determining the amount of fungal DNA by quantitative Real Time-PCR.

Extraction of genomic DNA and quantitative Real Time PCR

Transcript levels of *GFP*, Hv*EXPANSIN B, P. indica* internal transcribed spacer (Pi*ITS*), and *F. graminearum* beta-tubulin (Fg*TUB*) were determined via the $2^{-\Delta\Delta Ct}$ method by relating the amount of target transcript to plant ubiquitin (Schmittgen and Livak, 2008). Genomic DNA was isolated from 100 mg root tissue by using the DNeasy plant Mini Kit (Qiagen) according to the manufacturer's instructions. For quantitative Real Time-PCR, 10 ng of total DNA were used. Amplifications were performed in 7.5 µl of SYBR green JumpStart Taq ReadyMix (Sigma-Aldrich) with 0.7 pmol oligonucleotides, using an 7500 Fast cycler (Applied Biosystems). After an initial activation step at 95°C for 5 min, 40 cycles (95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 65°C for 15 s) were performed. Respective melting curves were determined at the end of each cycle to ensure amplification of only one PCR product. Ct values were determined with the 7500 Fast

software supplied with the instrument. The primers used for all anlyses are listed in Tab. 3.

Immunoblotting

Proteins were isolated from 3-week-old roots by grinding 100 mg of root tissue in liquid nitrogen, prior to resuspension in extraction buffer (50 mM TRIS-acetate, pH 7.4, 10 mM potassium-acetate, 1 mM EDTA, 5 mM DTT, 0.5 mM PMSF) followed by two subsequent centrifugation steps at 1000 rpm for 15 min and at 15 000 rpm for 30 min. The supernatant was used for analysis. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad). 20 ug proteins were separated on a 12.5% SDS-PAGE gel. After electrophoresis, proteins were blotted onto nitrocellulose and subjected to 1:4000 diluted mice anti-GFP antibody (Molecular Probes) followed by 1:10000 diluted mice anti-HRP antibody. Signals were detected using an ECL Plus Western Blotting Detection Kit for HRP (Amersham Biosciences). Total protein was stained by Coomassie Brilliant Blue (Bio-Rad) to check equal sample loading.

Staining of *F. graminearum* in root tissue

For visualization of root coloniazion, hyphae of *F. graminearum* were stained with the chitin-specific dye WGA-AF 488 (Molecular Probes) and prepared for Confocal laser-scanning microscopy (CLSM) as described (Deshmukh et al., 2006).

Confocal laser-scanning microscopy and non invasive mGFP4-ER localization

Sunbcellular localization of mGFP4-ER and WGA-AF 488-stained *F. graminearum* was performed by CLSM. In both cases, root and fungal cells were excited with a 488 nm laser line to detect emmission of mGFP-ER and WGA-AF 488 at 505–530 nm.

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Figure 1

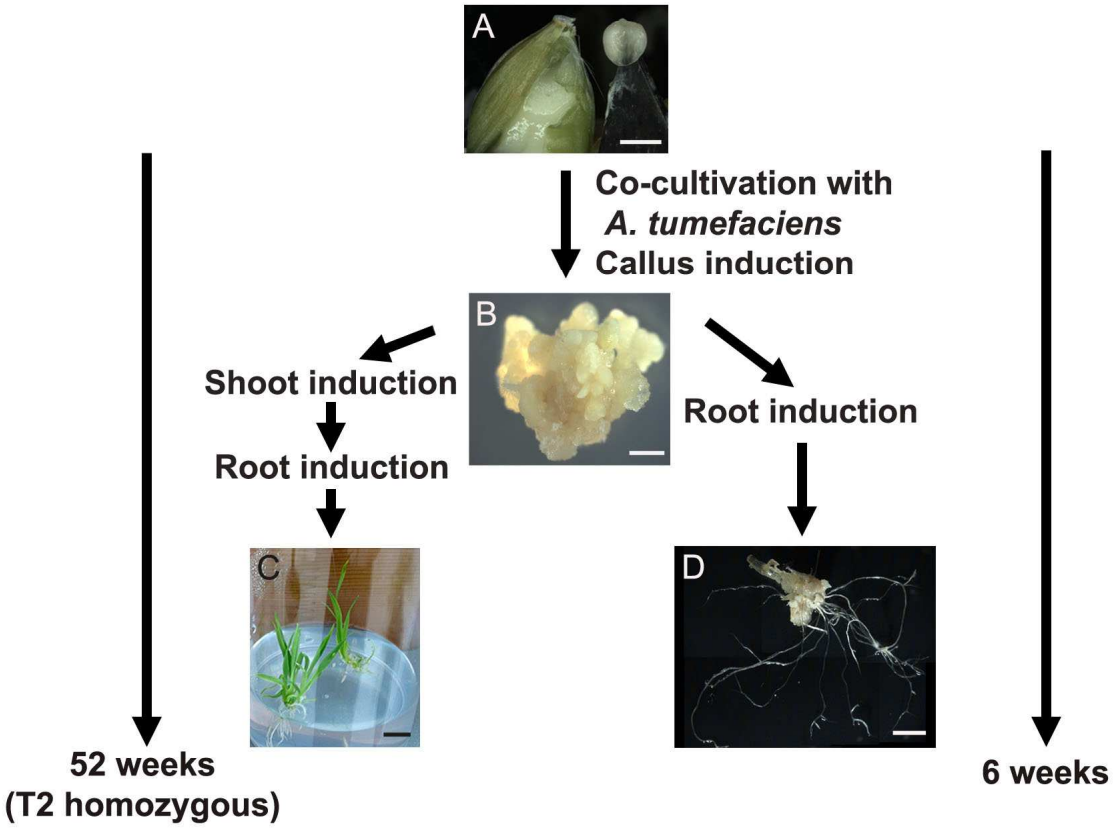
A
Co-cultivation with
A. tumefaciens
Callus induction
B
Shoot induction
Root induction
Root induction
C
D
52 weeks
(T2 homozygous)
6 weeks

Figure 2

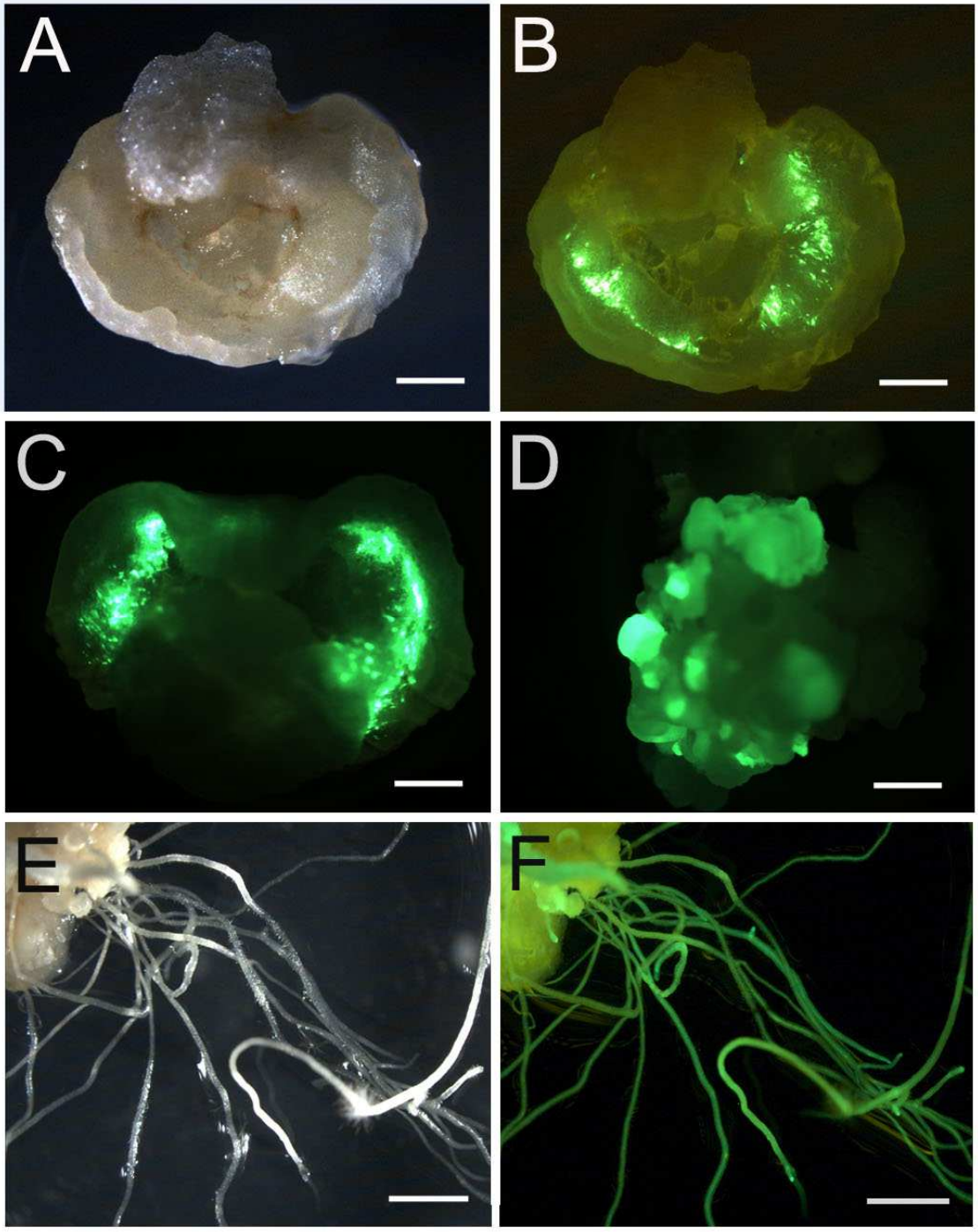

Figure 3

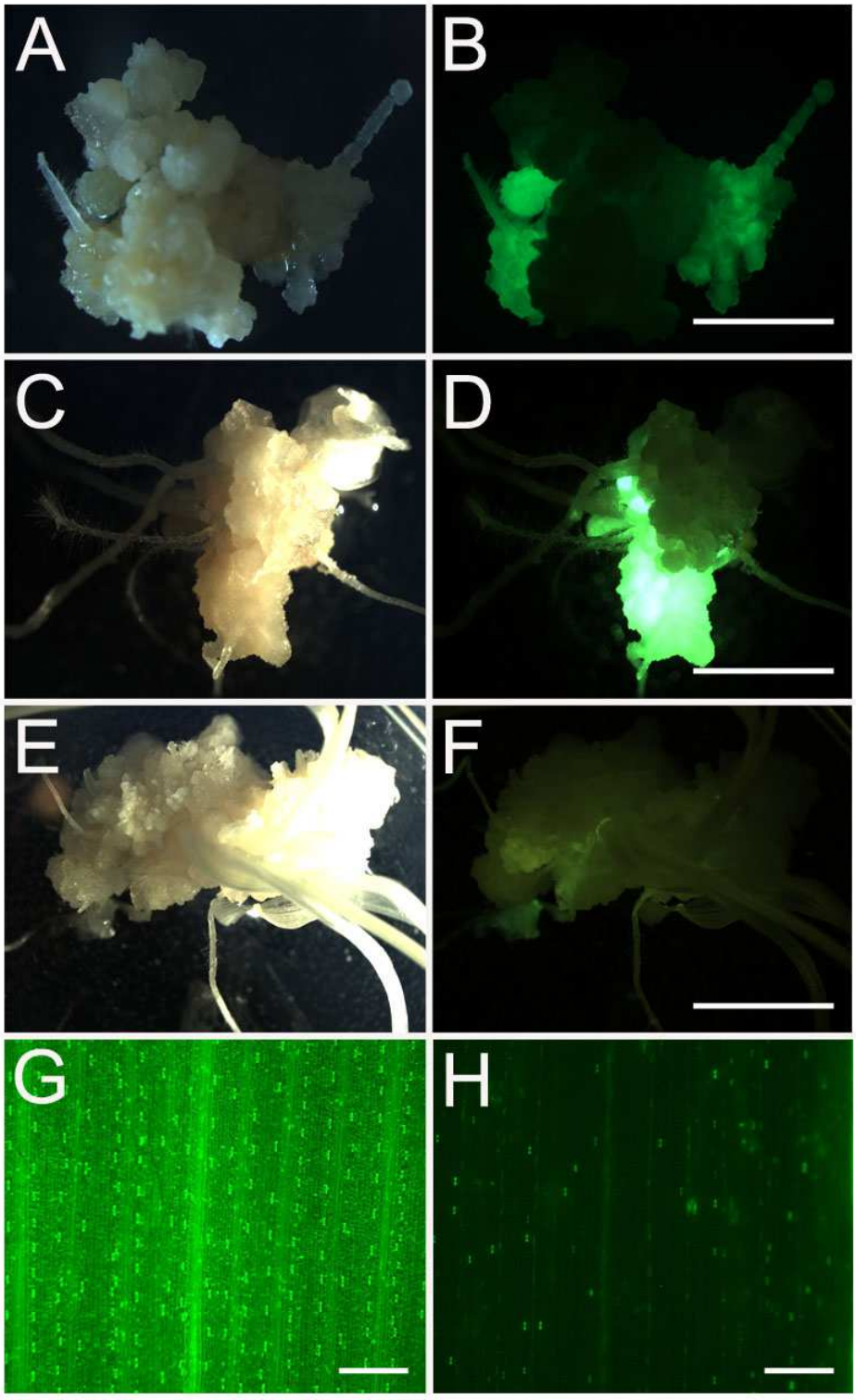

Figure 4

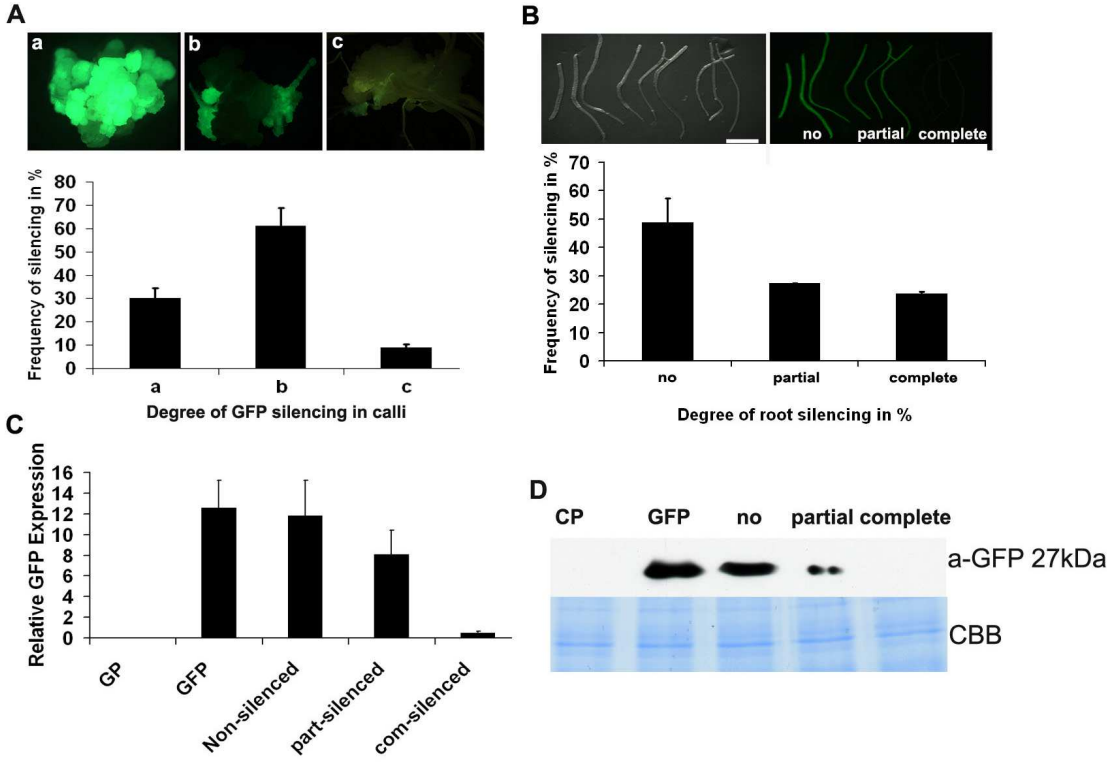
A
a
b
c
Frequency of silencing in %
80
70
60
50
40
30
20
10
0
a
b
c
Degree of GFP silencing in calli
B
no
partial
complete
Frequency of silencing in %
70
60
50
40
30
20
10
0
no
partial
complete
Degree of root silencing in %
C
Relative GFP Expression
16
14
12
10
8
6
4
2
0
GP
GFP
Non-silenced
part-silenced
com-silenced
D
CP
GFP
no
partial complete
a-GFP 27kDa
CBB

Figure 5

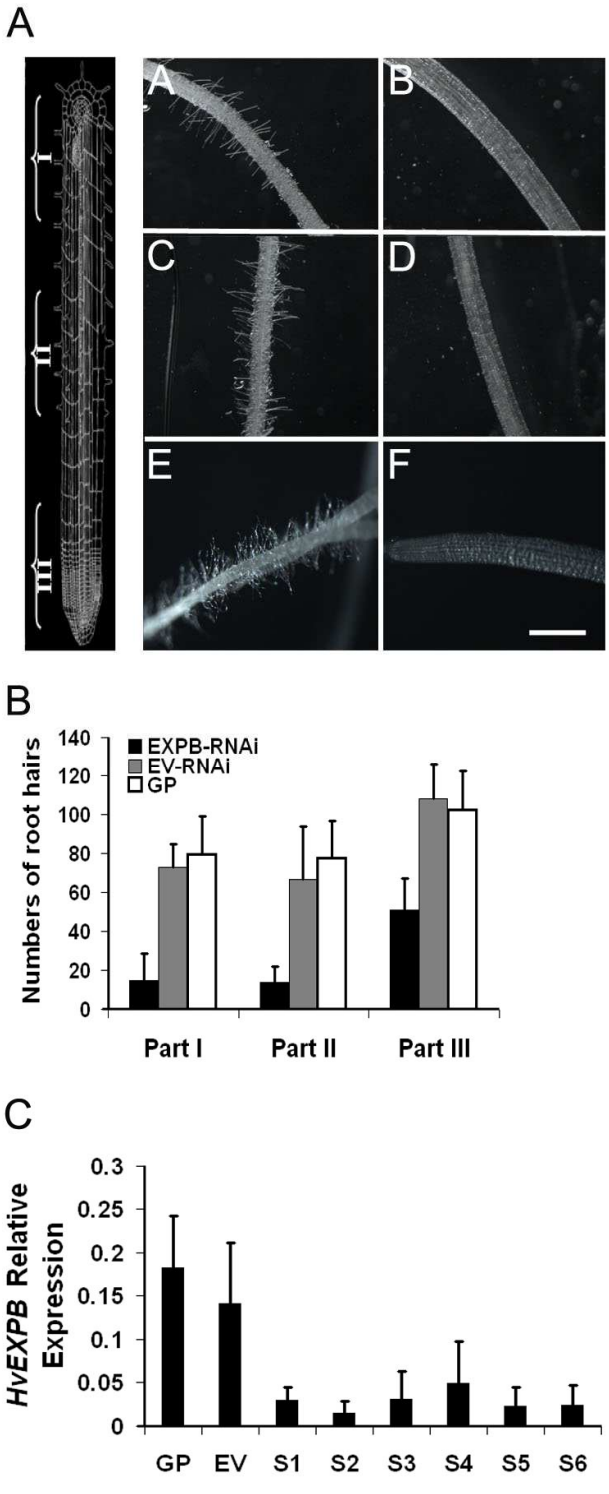
A
I
II
III
A
B
C
D
E
F
B
Numbers of root hairs
EXPB-RNAi
EV-RNAi
GP
140
120
100
80
60
40
20
0
Part I
Part II
Part III
C
HvEXPB Relative Expression
0.3
0.25
0.2
0.15
0.1
0.05
0
GP
EV
S1
S2
S3
S4
S5
S6

Figure 6

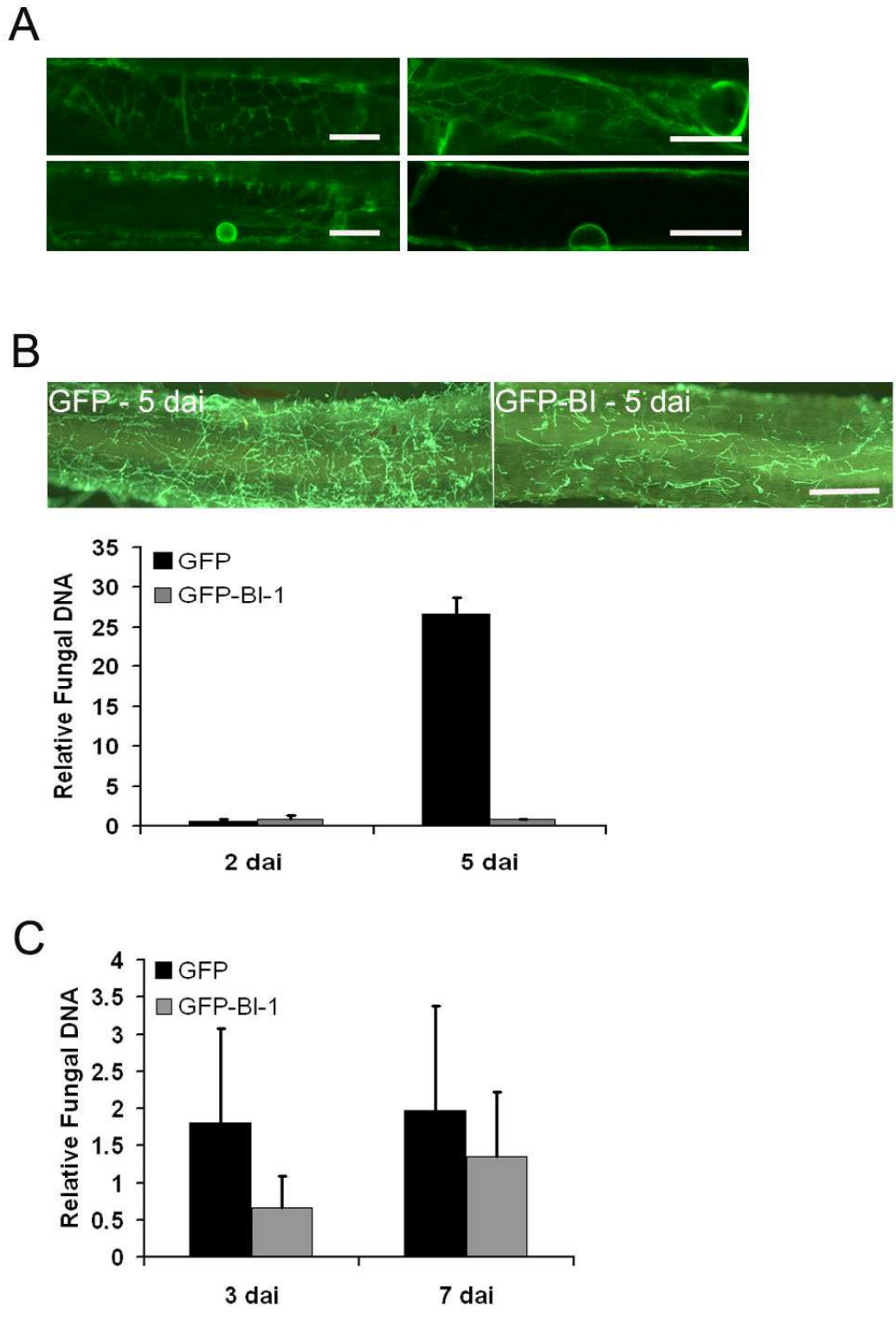
A
B
GFP - 5 dai
GFP-BI - 5 dai
Relative Fungal DNA
GFP
GFP-BI-1
2 dai
5 dai
C
Relative Fungal DNA
GFP
GFP-BI-1
3 dai
7 dai

Table 1. Barley callus induction medium (BCID)

MS-stock (Duchefa M0221)	4.3 g
$CuSO_4.5H_2O$	1.2 mg
Maltose	30 g
Thiamin HCl	1 mg
Myo-inositol	250 mg
Caseinhydrosylate	1 g
L-Proline	690 mg
Dicamba	2.5 mg
Phytoagar (Duchefa)	5 g
H_2O	1000 ml

Table 2. Barley root induction medium

$Cacl_2.2H_2o$	295 mg
KH_2PO_4	170 mg
KNO_3	2200 mg
$MgSo4.7H_2O$	310 mg
$NaH_2Po_4.H_2O$	75 mg
$(NH_4)_2SO_4$	67 mg
NH_4NO_3	600 mg
$COCL_2.6H_2O$	0.025 mg
$CUSO_4.5H_2O$	0.025 mg
H_3BO_3	3 mg
$MnSO_4.H_2O$	5 mg
$Na_2MoO_4.2\ H_2O$	0.25 mg
$ZnSO_4\ 7\ H_2O$	5 mg
Fe-citrat 5 H_2O	20 mg
Fe-EDTA***	28 mg
Nicotinamid	1 mg
Pyridoxine-HCL	1 mg
Thinmin-HCL	10 mg
Arginin	25 mg
Asparagin	50 mg
Asparaginsaure	30 mg
Glutamin	120 mg
Proline	50 mg
Threonin	25 mg
Pepton from Casein	125 mg
Myo-inosit	100 mg
Coconutmilk*	25 ml
Glucose	7 g
Saccharose	20 g
Charcoal**	1 g
Phytoagar (Duchefa)	5 g
H_2O	1000 ml

* Filter sterilization required

** Filter sterilized and added after media has been adjusted to pH 5,3

Table 3 List of primers used for qRT-PCR-based studies

Hv*Ubiquitin* (GenBank accession no. M60175)	Forward 5'-ACCCTCGCCGACTACAACAT-3' Reverse 5'- CAGTAGTGGCGGTCGAAGTG-3'
Fg*Tubulin* (GenBank accession no. AY635186	Forward 5'-GGTCTCGACAGCAATGGTGTT-3' Reverse 5'-GCTTGTGTTTTTCGTGGCAGT-3'
Hv*Expansin B* (genbak accession no. AY351786)	Forward 5'- CTGGTTCTGCAATTTGTGAG-3' Reverse 5'-CTTTGCTGTGACTACAACTG-3'
Synthetic GFP (GenBank accession no. AM261415)	forward 5'-ACCATCTTCTTCAAGGACGA-3' Reverse 5'- GGCTGTTGTAGTTGTACTCC-3'
Pi*ITS* (GenBank accession no. AF 019636)	forward 5'-CAA CAC ATG TGC ACG TCG AT-3' Reverse5'- CCA ATG TGC ATT CAG AAC GA-3'

5.13 Kogel, K.H., Voll, L.M., **Schäfer, P.**, Jansen, C., Wu, Y., Langen, G., Imani, J., Hofmann, J., Schmiedl, A., Sonnewald, S., von Wettstein, D., Cook, R.J., Sonnewald, U. (2010) Transcriptome and metabolome profiling of field-grown transgenic barley lack induced differences but show cultivar-specific variations. *Proceeding of the National Academy of Sciences of the United States of America* 107: 6198-6203.

Transcriptome and metabolome profiling of field-grown transgenic barley lack induced differences but show cultivar-specific variances

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The aim of the present study was to assess possible adverse effects of transgene expression in leaves of field-grown barley relative to the influence of genetic background and the effect of plant interaction with arbuscular mycorrhizal fungi. We conducted transcript profiling, metabolome profiling, and metabolic fingerprinting of wild-type accessions and barley transgenics with seed-specific expression of (1,3-1, 4)-β-glucanase (GluB) in Baronesse (B) as well as of transgenics in Golden Promise (GP) background with ubiquitous expression of codon-optimized *Trichoderma harzianum* endochitinase (ChGP). We found more than 1,600 differential transcripts between varieties GP and B, with defense genes being strongly overrepresented in B, indicating a divergent response to subclinical pathogen challenge in the field. In contrast, no statistically significant differences between ChGP and GP could be detected based on transcriptome or metabolome analysis, although 22 genes and 4 metabolites were differentially abundant when comparing GluB and B, leading to the distinction of these two genotypes in principle component analysis. The coregulation of most of these genes in GluB and GP, as well as simple sequence repeat-marker analysis, suggests that the distinctive alleles in GluB are inherited from GP. Thus, the effect of the two investigated transgenes on the global transcript profile is substantially lower than the effect of a minor number of alleles that differ as a consequence of crop breeding. Exposing roots to the spores of the mycorrhizal *Glomus* sp. had little effect on the leaf transcriptome, but central leaf metabolism was consistently altered in all genotypes.

food safety | glucanase | chitinase | sustainability

Breeding for improved grain weight, higher grain yield, disease resistance, and climatic adaptation by selection of spontaneous mutations shaped the modern barley (*Hordeum vulgare* L.) crop plant beginning as early as 10,000 years ago. With the technical advance to generate transgenic crops with improved agronomic performance, it has become necessary to assess the substantial equivalence of transgenic crop plants; that is, validate that no undesired side effect of the genetic modification has occurred relative to their parental lines (see ref. 1 for review). The availability of the "omics" techniques opens the possibility to probe substantial equivalence in nontargeted global analyses, providing unbiased results.

We have recently developed a 44-K barley microarray based on the assembly of 444,652 barley ESTs into 28,001 contigs and 22,937 singletons, of which 13,265 are represented on the array (2). In contrast, a comprehensive analysis of the metabolome (i.e., all metabolites in a specimen) is not possible because of the immense diversity of primary and secondary plant metabolites (3, 4). Thus, investigating the metabolome requires the prioritization of metabolite subsets as defined by their physicochemical properties or abundance. Although approaches to metabolite profiling are fueled by a multitude of individual targeted metabolite assays of high specificity and accuracy, metabolite fingerprinting aims at obtaining global metabolite patterns by NMR- or MS-based applications, only allowing for suboptimal recovery of individual metabolites (3).

When applied to pathway-engineered transgenic plants, global transcriptome and metabolome analyses could not reveal substantial differences between genetically modified (GM) and non-GM plants. No significant alterations in transcriptome were exhibited in wheat plants expressing *Aspergillus fumigatus* phytase compared with the corresponding non-GM variety, except for changes associated with seed development (5). Similarly, GC-MS-analyzed fructan-producing transgenic potato tubers did not exhibit significant changes, except for metabolites directly connected to the introduced pathway (6), and *Arabidopsis* expressing up to three *Sorghum bicolor* genes involved in the biosynthesis of the cyanogenic glucoside dhurrin did not exhibit any robust transcriptional changes compared to the parental lines (7).

Assessing the influence of natural genotypic variation and environmental factors on multiparallel datasets is of paramount importance to better evaluate the impact of transgene expression. To avoid unnecessary bias, the regarded transgene should not directly influence metabolic pathways in the target plant. An NMR comparison of wheat-flour metabolome derived from field-grown transgenic wheat expressing high molecular weight glutenin and the corresponding parental line revealed that, despite some differences in central free amino acid and sugar metabolism between GM and non-GM varieties, year and field site had a stronger effect on the dataset than expression of the transgene (8). Metabolome analysis of *Bt*-maize by NMR also revealed significant differences in free amino acid contents of the parental line; however, other likely-influential factors were not assessed in this study (9). Comparative transcript profiling of different maize cultivars harboring an identical *Bt* transgene insertion event revealed that the variability between cultivars was much greater than the influence of the transgene (10, 11). Independently, comparison of the potato tuber proteome of 21

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tetraploid cultivars with eight potato landraces and five transgenic potato lines led to the same conclusion (12).

In the present study, we investigated two transgenic barley cultivars. The first, hereafter termed ChGP, was designed for ubiquitous expression of a secreted, codon-optimized 42-kDa endochitinase *cThEn(GC)* from *Trichoderma harzianum* (13) in the variety Golden Promise (GP). *Trichoderma* chitinases can degrade rigid fungal cell walls of mature hyphae, conidia, chlamydospores, and sclerotia, in addition to the soft structure of hyphal tips (14–16). Recombinant *cThEn(GC)* conferred growth inhibition to the necrotrophic fungal root pathogens *Rhizoctonia oryzae* and *Rhizoctonia solani* AG8 in vitro (13). Overexpression of *cThEn(GC)* in tobacco and potato yielded high levels of transgene expression, and the transgenics displayed medium-level to complete-resistance phenotypes toward the necrotrophic fungal pathogens *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea*, and *R. solani* (17).

The second transgenic line employed in the study was pJH271 Beta-Glu-307 (271.06 × Baronesse), hereafter termed GluB, that exhibits hordein-D-promoter-driven, endosperm-specific expression of the chimeric heat-stable (1,3-1,4)-β-glucanase from *Bacillus amyloliquefaciens* and *Bacillus macerans* (18). The GluB transgenics were generated in the GP background, outcrossed to the elite cultivar Baronesse (B), and selected for high yield and good field performance by the single-seed descent method. GluB plants accumulate the recombinant enzyme in storage protein vacuoles and lack β-glucan in endosperm cell walls (19, 18). Expression of (1,3-1,4)-β-glucanase in the endosperm improves the nutritional value of barley for poultry (20, 21).

Making use of comparative, parallel transcriptome profiling, targeted metabolome profiling, and nontargeted metabolite fingerprinting, the present study assesses substantial equivalence in leaves of field-grown transgenic barley relative to the variation between cultivars and to the effects caused by the interaction with mycorrhizal fungi.

Results

Generation and Analysis of Transgenic Barley Plants Expressing Recombinant *Trichoderma* Endochitinase. We constitutively expressed the codon-optimized recombinant *T. harzianum* endochitinase ThEn42(GC) (13) in barley *cv.* Golden Promise either (*i*) fused to the barley chitinase 26 (HvChi26) secretion signal peptide driven by the Cauliflower mosaic virus 35S (CaMV 35S) promoter (Fig. S1*A*) or (*ii*) fused to the chitinase 33 (HvChi33) secretion signal peptide and driven by the maize ubiquitin promoter (Fig. S1*B*). After identification of primary transformants with expression of recombinant endochitinase by immunological detection and subsequent selection for homozygous T_1 transformants (*SI Materials and Methods*), we chose for further study two Ubi::ChGP (Ubi::ChGP-9 and -19) transgenic lines and one 35S::ChGP transgenic line that exhibited the strongest endochitinase expression. We assayed tissue specificity of chitinase activity (Fig. 1*A* and *SI Materials and Methods*, with a quantitative method using the fluorogenic substrate methylumbelliferyl-chitotrioside (Fig. S2*A*). Chitinase activity was highest in coleoptiles, reaching up to 320 $\mu g \cdot g^{-1}$ FW in both Ubi::ChGP lines and 280 $\mu g \cdot g^{-1}$ FW in 35S::ChGP-36 (Fig. 1*A*). Although CaMV 35S-driven chitinase expression in roots was close to that in leaves, ubiquitin-promoter-driven chitinase expression was 6- and 3-fold lower in roots compared with primary leaves or coleoptiles, respectively. Both Ubi::ChGP lines yielded very similar results.

To assess, whether chitinase expression confers antifungal activity, we checked if resistance to *R. solani* AG8 was increased in the transgenics, being quantified as number of wilted leaves per plant (Fig. 1*B* and *SI Materials and Methods*). Compared with the GP wild-type, only 35S::ChGP plants exhibited significantly milder disease symptoms after 1 week of cocultivation with *R. solani* AG8. This finding suggests that, despite the proof of concept obtained from the 35S::ChGP plants, endochitinase amounts might be too low in roots of the two Ubi::ChGP lines to diminish susceptibility toward the highly virulent *R. solani* AG8. Nevertheless, we selected line Ubi::ChGP-9 for further experiments to minimize effects of chitinase expression on the growth of challenging fungi in the field, which could influence both the transcriptome and the metabolome.

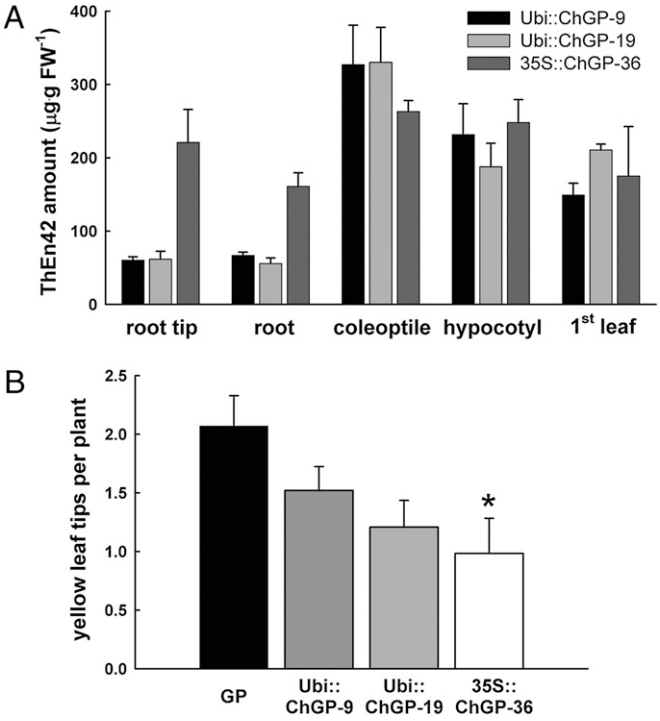

Fig. 1. Characterization of ChGP transformants tissue-specific accumulation of endochitinase ThEn42 in ChGP transformants and its effect on root infections by *R. solani* AG8. (*A*) Amounts of endochitinase in tissues of ChGP transformants. Seedlings of the transgenic barley lines Ubi::ChGP-9 (black bars), Ubi::ChGP-19 (light gray bars), and 35S::ChGP-36 (dark gray bars). Endochitinase content in root tips, upper parts of the roots, coleoptiles, hypocotyls, and first leaves (*Left* to *Right*) were determined with a fluorometric assay (Fig. S2*A* and *SI Materials and Methods*). Data are the mean of five replicate samples ± SEM (*B*) Reduced disease symptoms on ChGP transformants after root inoculation with *R. solani* AG8 (*SI Materials and Methods*). Significant differences to GP with $P < 0.05$ are indicated by an asterisk above the bars and were calculated with a Welch's modified *t* test (29).

Metabolome Analysis of Field-Grown Barley Leaves. We conducted both a targeted metabolite profiling and a metabolite fingerprinting approach with field-grown plants of the four barley genotypes GP, ChGP, B, and GluB that were cultivated in the field at the Giessen Experimental Station (Giessen, Germany; *SI Materials and Methods*). The plants were grown with and without amendment of soil in the plots with Amykor (Amykor Wurzel-Vital), a mixture of the mycorrhizal fungi *Glomus mosseae* and *Glomus intraradices*. Targeted analysis of 72 metabolites, including major carbohydrates, free amino acids, carboxylates, phosphorylated intermediates, major antioxidants (such as ascorbate, glutathione, and tocopherol), as well as carotenoids (for complete dataset, see Table S1), revealed only three differences associated with endochitinase expression in the GP background. In contrast, the contents of sucrose, starch, the amino acids Gln, Ala, and Leu, as well as of the carboxylate oxoglutarate were significantly reduced in GluB compared to B (Table S1). Comparisons of the two unmodified varieties B and GP revealed more consistent differences (e.g., UDPGlc and the amino acids, Tyr, Phe, Ala, Leu, and Cys) (Fig. 2*A*). Furthermore, several consistent changes in central leaf metabolism

in response to Amykor treatment were revealed (Fig. 2*B*): although the amounts of free hexoses and central phosphorylated intermediates (3PGA, PEP, RuBP, Glc1P), free inorganic phosphate and the carboxylates isocitrate and malate increased upon treatment, the contents of sucrose, the two major amino acids Glu and Asp, as well as chlorophyll, lutein, and glutathione all decreased in response to mycorrhizal inoculation (Fig. 2*B*).

The elevated pool sizes of several phosphorylated intermediates indicate improved phosphate availability as a possible consequence of successful mycorrhizal root colonization. Thus, it was instructive to determine the extent of mycorrhizal root colonization in plots with and without treatment with Amykor. Quantification of fungal genomic DNA by qPCR based on the *G. mossae* ITS sequence relative to host ubiquitin revealed increased fungal abundance in roots from treated compared with untreated plots (Fig. S3), despite considerable amounts of fungal DNA in plants (e.g., in GP) from untreated plots. Furthermore, microscopic analysis of plants grown in plots treated with Amykor confirmed arbuscle formation in all specimens (*SI Materials and Methods*), demonstrating that mycorrhiza were intact and functional. To validate that the characteristic changes in the leaf metabolome were brought about by improved mycorrhizal colonization of plants in the Amykor-treated plots, we determined the same targeted metabolome profile of ChGP and GP plants grown under controlled conditions in the greenhouse with soil that was either devoid of mycorrhizal inoculum or fortified with the same dosage of Amykor as in the field experiment. The contents of phosphorylated intermediates and hexoses were altered in a similar fashion between mycorrhizal and non-mycorrhizal plants of both examined genotypes in the field and in the greenhouse experiment (Table S2), providing strong indication for successful symbiotic interactions in all genotypes in the field experiment (*SI Materials and Methods*).

In a principal component analysis (PCA), the targeted metabolome data were able to distinguish both the effect of mycorrhizal infection and cultivar-specific differences by principal components 1 (PC1) and PC2, respectively (Fig. 3*A*; see Fig. S4*A* for the corresponding hierarchical cluster analysis). Interestingly, the metabolite profile in GluB transgenics was less affected by mycorrhizal infections compared to the other genotypes, and GluB was more distant to non-GM B plants than the ChGP transgenics was from their wild-type counterpart. As indicated by individual metabolite contents (mentioned in the previous paragraph), sugars, major amino acids, and phosphorylated intermediates strongly loaded on PC1 in response to mycorrhizal infections. Likewise, sugar and free amino acid contents contributed strongly to PC2, distinguishing cultivar-specific differences. Nearly identical results were obtained when data from the 307 most significant mass signals of a metabolite fingerprinting approach were fed into the PC analysis (Fig. 3*B*), except that GP and ChGP were more distant to each other in the Amykor treatment compared to untreated samples.

Transcriptome Analysis of Field-Grown Barley Leaves. Our next goal was to compare the discriminatory power of the metabolome analysis to that of the corresponding transcriptome dataset obtained from identical sample pools (*SI Materials and Methods*). PCA resulted in a similar discrimination of genotypes as reported for the metabolome analyses, with GluB again being distant from B (Fig. 3*C*; see Fig. S4*B* for the corresponding hierarchical cluster analysis). In contrast to the metabolome analysis, treatment with Amykor could not be clearly resolved in the PC analysis; indeed, no statistically significant differentially transcribed genes were detected in three of the four examined genotypes in response to the Amykor treatment. Only in GP, 4 out of 31,198 features detected on the array were differentially expressed in response to the mycorrhizal fungi (Table S3). However, 1,660 genes (697 up

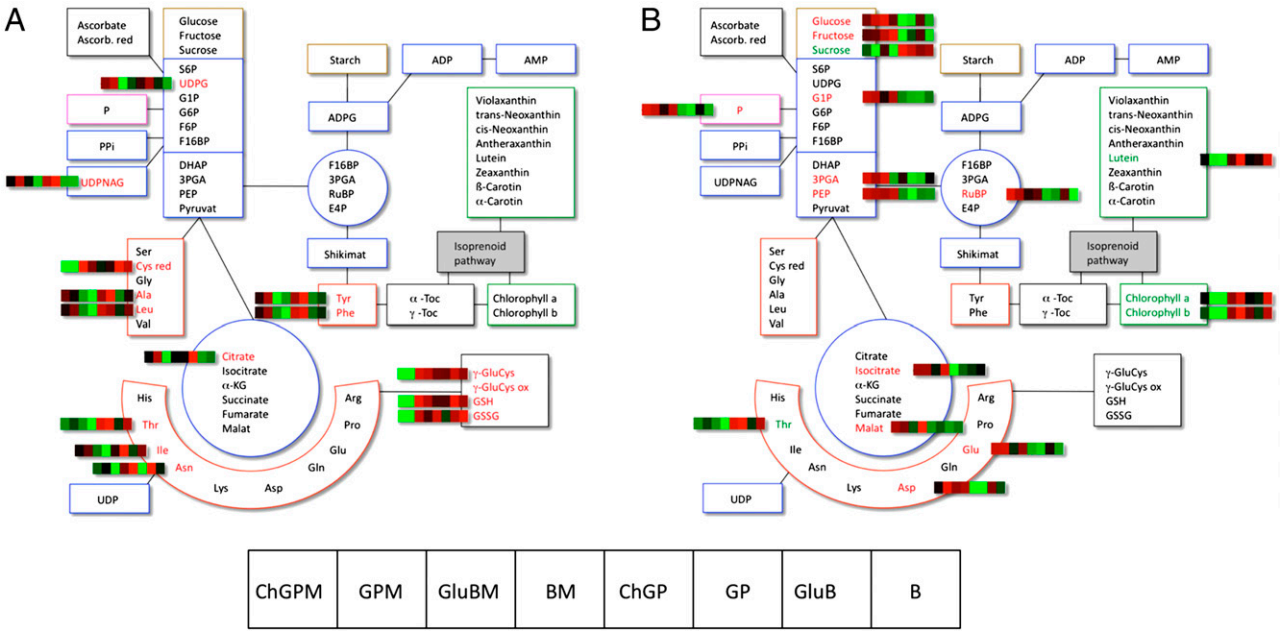

Fig. 2. Differentially abundant metabolites in barley leaves. Overview of differentially abundant metabolites from the targeted profiling approach with leaf material from 4-month-old, field-grown barley plants representing the treatments (*A*) cultivar or (*B*) Amykor. The schematic metabolic diagrams in (*A*) and (*B*) represent a map of all analyzed metabolites. The heat map strips next to the metabolite names were taken from the hierarchical cluster analysis (Fig. S4*A*) conducted with the program Cluster v2.11 (30), with red signals denoting an increased metabolite content relative to average and green signals indicating decreased metabolite contents relative to average. The consistent sample order in these strips is indicated at the bottom of the figure using the genotype and treatment abbreviations used throughout the text and as explained below. The entire dataset, including the results of the significance tests, are given in Table S1. Please note that the color pattern has no implications on statistically significant differences in pairwise comparisons, which were calculated with a Welch-Satterthwaite test embedded in the VANTED software v1.7 (31) and are displayed in Table S1. GP, Golden Promise; B, Baronesse; ChGP, Chitinase GP; GluB, Glucanase B; M, Amykor treatment.

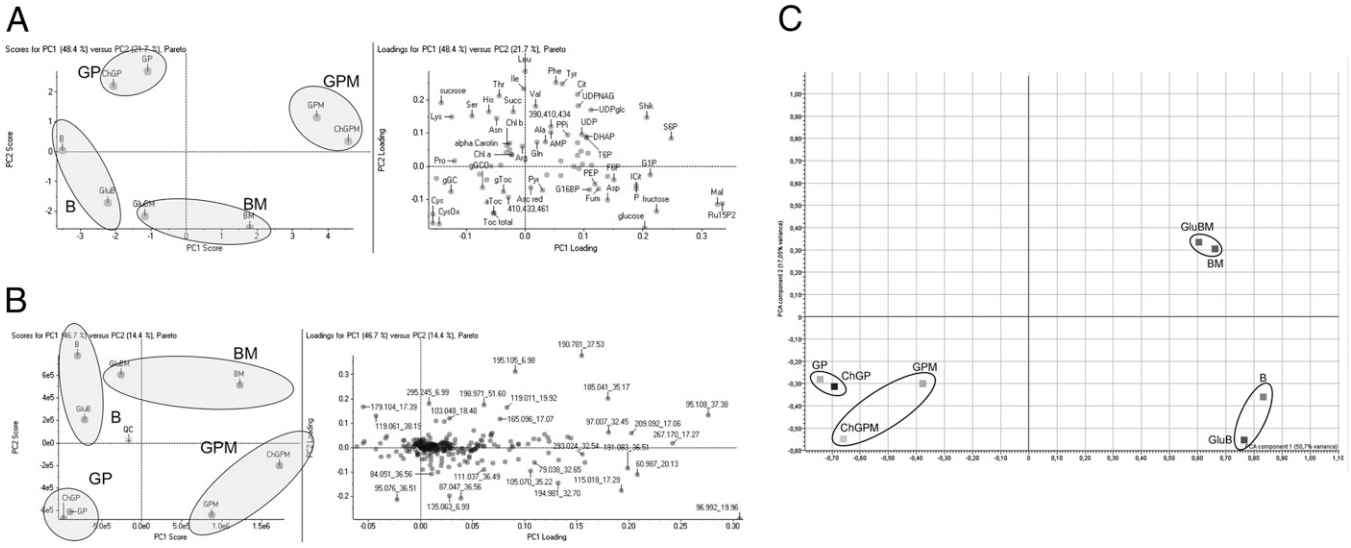

Fig. 3. Principal component analysis (PCA) of multiparallel datasets obtained from field-grown barley leaves. (*A*) PCA based on 72 metabolites that were analyzed in a targeted fashion (complete dataset displayed in Table S1): For PCA, mean values of four replicate samples per genotype and treatment were taken and the resulting data points labeled as described below. (*Left*) PCA plot of principal component 1 (PC1) versus principal component 2 (PC2). Circles are drawn around spots derived from the genotype of identical cultivar or treatment and are labeled by letters as indicated below. (*Right*) loadings plot for PC1 and PC2. The 72 metabolites are individually labeled. (*B*) PCA of metabolite fingerprinting data. The analysis was computed for the 307 most significant mass signals obtained by metabolite fingerprinting and is based on mean values from four replicate samples (see *Materials and Methods*). Compounds are labeled according to the quantified transition. Data arrangement and labeling are as described in *A*. (*C*) PCA of transcriptome data. PCA was performed based on data from two replicate hybridizations per genotype and treatment. RNA was extracted from aliquots of pooled sample material also used for metabolome analysis. From the 1,660 genes differentially expressed between cultivars B and GP (Table S3), five of the most significant ones were confirmed by qRT-PCR analysis of independent sample aliquots (Fig. S2*B*). GP, Golden Promise; B, Baronesse; ChGP, Chitinase GP; GluB, Glucanase B; M, Amykor treatment.

and 863 down) were differentially transcribed between the cultivars GP and B (Tables S3 and Dataset S1), indicating strong cultivar-specific expression patterns. The result of the microarray data analysis was confirmed in independent sample pools by qRT-PCR, picking five genes with cultivar-specific transcript abundance (Fig. S2*B*). Along with genes involved in carbohydrate metabolism and genes coding for storage proteins, defense-associated genes were strongly overrepresented among the differentially regulated genes in the GP to B comparison (Fig. S2*C*). This result likely reflects a greater level of disease resistance obtained deliberately or fortuitously over years of breeding and selection for ever better-adapted and higher-yielding modern barley varieties. Of particular interest, 22 differentially transcribed genes were found between GluB and its non-GM counterpart B (Table S3), corresponding with the distance of these two genotypes in the PCA. Sixteen of these 22 differential genes, (i.e., approx. 73%) were also discriminated in the GP to B comparison. Although surprising at first glance, this finding can be explained by the pedigree of GluB. GluB was produced by transformation of GP with glucanase transgene, which was later introduced into the cultivar B by outcrossing and selection of single-seed descendants. Thus, differential gene expression between GluB and B could be caused by retention of a few GP alleles in the GluB genotype.

To obtain data in support of this hypothesis, we attempted to refine the chromosomal location of the 16 genes that were differentially transcribed in both the GluB to B and the GP to B comparisons on the current genetic map of barley (http://www.harvest-web.org/hweb/bin/gmap.wc?wsize=1263 × 854). Although 14 of the unigenes had no assigned map position, 2 could be located between 142 cM and 167 cM on the lower arm of chromosome 7H. Analysis of two simple sequence repeat (SSR) markers located in the region of interest revealed that both carried the GP allele (Fig. 4 and Fig. S5).

Discussion

The comprehensive dataset generated in the present study provides a comparison of the alterations in leaf transcriptome and metabolome caused by (*i*) the presence of transgenes, (*ii*) cultivar, and (*iii*) biotic interactions in the root. This dataset leads us to four major conclusions.

First, the effect of recombinant *Trichoderma* chitinase on both the metabolome and transcriptome was negligible compared to the differences between the wild-type cultivars GP and B.

Second, the metabolome analysis has proven to be as sensitive as the survey of the corresponding transcriptome as a means to detect minor differences between B and the out-bred transgenic GluB. In addition, both targeted and untargeted metabolome analyses discerned an influence of mycorrhizal infection on leaf

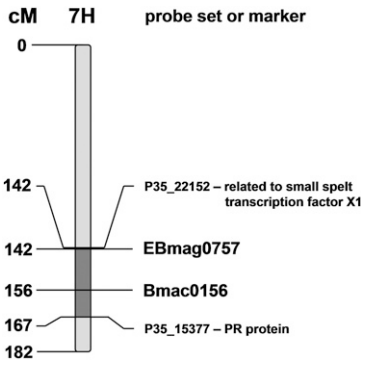

Fig. 4. Inheritance of GP alleles in GluB on the lower arm of barley chromosome 7H. Two of the 16 unigenes differentially expressed in both the B vs. GP and the B vs. GluB comparisons could be mapped to the current physical barley map (www.harvest-web.org) and were located at 142 cM and 167 cM on chromosome 7H, respectively. Analysis of the two polymorphic SSR markers EBmag0757 (142 cM) and Bmac0156 (156 cM) revealed retention of the respective GP alleles in GluB that had been generated by introgressing the *GluB* transgene from GP into B. Locations of the employed markers and the two unigenes of interest on the genetic map are given on the left, their names and annotations are to the right to the chromosomal sketch.

metabolism that was not achieved by transcriptome analysis alone. In targeted experiments, on the other hand, differential display was used successfully to identify transcripts of five differentially expressed genes from leaves of mycorrhiza-colonized tomato (22). Based on our results, metabolome analysis represents a more immediate probe of physiological status of the plant than the transcriptome. We have found that a subset of phosphorylated intermediates of central metabolism was more abundant in leaves of Amykor-treated than in control plants, reflecting improved phosphate availability in treated plants.

Third, comparisons of the metabolome and transcriptome between the transgenic GluB and wild-type B revealed four differentially abundant metabolites and 22 deregulated transcripts, suggesting a more distant positioning in the PCA between these two lines, as compared to ChGP and GP. Although the reason for the deviation in the metabolome of GluB and B remain elusive, about 73% of the differentially transcribed genes between GluB and B were similarly deregulated between GP and B. The evidence for genetic linkage of 2 out of the 16 coregulated genes between GluB and GP by SSR marker analysis indicates that the differences in the transcript profiles of GluB and B could be attributed to retention of introgressed GP traits in the GluB background. This finding could also hold true for the differences in the GluB and B metabolome profiles. Our finding suggests that introgression of a few alleles can convey a stronger effect on substantial equivalence than the introduction of the two regarded transgenes.

Fourth, compared with the previously addressed slight changes, the data compiled for GP and B revealed 1,660 differentially regulated genes and a considerable, albeit minor, number of steady-state metabolite pools that were substantially different. Targeted qRT-PCR analysis of five genes that strongly differed in expression between GP and B disclosed that, for most of them, transcripts were only specifically abundant in one of the two cultivars. Defense-associated genes such as pathogenesis-related gene-4 (*PR-4*) were overrepresented in the 1,660 genes. Because we did not include a substantial number of defense-related metabolites in our targeted metabolome analysis, the difference in defense priming between GP and B remained obscure in the metabolite dataset. As no symptoms of infection were visible on any genotype at sampling date, our data indicates that subclinical or latent infections at the field site had triggered defense-gene expression. Thus, our results suggest that B, unlike GP, was in an alert state with basal expression of pathogenesis-related genes. The variety GP lacks most resistance genes (23) and exhibits a much weaker defense response compared to bred high-end varieties. We can thus estimate that past breeding of elite lines, such as B for putative disease resistance, represent the strongest effect on global gene expression between cultivars in the field, where plants are subjected to perpetual challenge by microbial pathogens and pests. Such large differences are not expected to be caused by single transgenes, although evidence on pathogen-challenged disease-resistant GM crops is thus far unavailable. Although resistance toward pathogen challenge in the field should be increased in ChGP because of the presence of endochitinase, transcript profiles of ChGP, and because GP did not exhibit significant differences, unlike the B to GP comparison described above. This result means that endochitinase expression did not affect the transcriptome in challenged plants. In comparison, strong differences in transcript profiles of Bt maize compared with non-GM cultivars were to be expected upon corn borer infestation, representing sick and healthy plants, respectively.

Conclusion

In summary, our results substantially extend observations that cultivar-specific differences in transcriptome and metabolome greatly exceed effects caused by transgene expression. Furthermore, we provide evidence that, (*i*) the impact of a low number of alleles on the global transcript and metabolite profile is stronger than transgene expression and that, more specifically, (*ii*) breeding for better adaptation and higher yields has coordinately selected for improved resistance to background levels of root and leaf diseases, and this selection appears to have an extensive effect on substantial equivalence in the field during latent pathogen challenge.

Materials and Methods

Barley Seed. The seed used in this study represented barley lines pYW210-9-(4001-4360), pYW210-19-(4701-6100), pYW300-36-(7121-7187), pJH271-Beta Glu-307 and the cultivars Golden Promise and Baronesse. Line pYW210-9-(4001-4360), termed ChGP, and lines pYW210-19-(4701-6100) and pYW300-36-(7121-7187), which are constitutively expressing endochitinase ThEn42 (GC) from *T. harzianum* (13), were produced for this study (see below). Line pJH271-Beta Glu-307, termed GluB, constitutively expresses a thermostable (1,3-1,4)-β-glucanase and was described earlier (18).

Double-Cassette Vector Construction with the Ubiquitin Promoter and Barley Transformation. For construction of plasmid pYW300 (Fig. S1*A*), the Cauliflower mosaic virus 35S promoter was amplified from plasmid *pBI221* (Clontech Inc.), digested with *Hin*dIII and *Pst*I, and inserted into *Hin*dIII- and *Pst*I-digested plasmid pAM110-cTHEn42(GC) (Fig. S1*C*). The *Hin*dIII-*Not*I fragment of this plasmid was moved into pAM300b (Fig. S1*D*), and the *Hin*dIII-*Eco*RI fragment from this intermediate vector was then inserted into the pJH 260 binary vector as described for the vectors with the ubiquitin promoter (see below). The sequence of the plasmid pYW300 has been assigned GenBank Accession number DQ469639.

Plasmid pYW210 (Fig. S1*B*) was constructed in the binary cloning vector pJH260 derived from pBIN19, as follows: The fragment containing *cThEn42(GC)* provided with the *pUbi 1* promoter and the *SP(HvChi33)* signal sequence was excised from plasmid pAM110-cThEn42(GC) (Fig. S1*C*) with *Hin*dIII and *Not*I. The resulting fragment was inserted into *Hin*dIII-/*Not*I-digested plasmid pAM300b (Fig. S1*D*), yielding plasmid pAM300. A *Hin*dIII / *Eco*RI fragment of the insert was cloned into pJH260 to produce plasmid pYW210 (GenBank Accession Number DQ469636). For barley transformation, see *SI Materials and Methods*.

Metabolome Profiling and Metabolite Fingerprinting. Targeted analysis of free amino acids (24), major leaf carbohydrates (25), ascorbic acid, tocopherols and glutathione (26), carotenoids (2), phosphorylated intermediates and carboxylates (27) was conducted as previously described.

Untargeted metabolome profiling by ESI-MS was carried out on a QTrap3200 mass spectrometer (Applied Biosystems) after metabolite extraction and ion exchange chromatography as described (26). Negative Ions were generated at −4.5 kV and a declustering potential of −20 V. The entrance potential was from −6 to −4 V, and gas pressures were 20 psi (curtain), 30 psi (nebulizer), and 20 psi (turbogas). A mass range of *m/z* 60–610 was recorded with one scan per second over 80 min. Peak alignment was performed after import into Marker View (Applied Biosystems) with a retention time tolerance of 0.75 min and a mass tolerance of 1.0 amu. Maximal number of peaks was set to 500. Retention time corrections and normalization was done with the internal standard pipes (*m/z* 301.1; RT 16.6 min). For PC analysis of fingerprinting data, quality control samples were generated as described (28) by pooling equal-volume amounts from all analyzed samples. Artifact peaks were removed before the analysis was conducted with MarkerView.

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Supporting Information

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SI Materials and Methods

Barley Transformation and Analysis of Primary Transformants. Barley cultivar Golden Promise (GP) was transformed by cocultivation of immature zygotic embryos with *Agrobacterium tumefaciens* strain AGL1 carrying a disarmed Ti plasmid and plasmid pYW210 or pYW300 (Fig. S1 *A* and *B*) as previously described (1). Genomic DNA of bialaphos-resistant regenerated T_0 plantlets was extracted from 3-cm long segments and tested for the presence of *ThEn42(GC)*. Primers SPThEnUP 5′-CTTCTGCAGATGAG ATCGCTCGCGGTGG-3′ and SPThEnDOWN 5′-GCGAGCT CCTAGTTGAGGCCGCTGC-3′ were used to amplify a 1,170-bp fragment of *cThEn42(GC)*.

Field Experiments for Global Transcriptome- and Metabolome-Profiling Analyses. The field trial from which the samples for joint transcriptome and metabolome analyses were taken was carried out at the Giessen experimental station in 2007 in a replicated randomized block design. Each block consisted of eight 0.8- × 0.5-m plots in which genotypes were randomly distributed and half of the plots were pretreated with Amykor (see below). Plots were separated by a 0.5-m wide strip of fallow and the entire field was isolated by a guard strip consisting of a 1-m wide fallow and 5-m wide corridor planted with summer barley variety cv. Scarlett after plot preparation. Plots were prepared by conventional tillage. Subsequently arbuscular mycorrhiza inoculum using Amykor Wurzel-Vital (Amykor) was shallowly incorporated into the soil of half of the plots one day before planting. Amykor consists of expanded clay-based inoculum mainly containing *Glomus intraradices* and *Glomus mosseae*. The other part of the plots was treated with expanded clay lacking fungal inoculum. On the following day, barley grains (300 grains per square meter) were planted with a dedicated Wintersteiger Precision Space Planter. Roots were harvested 10 weeks after planting to assess mycorrhizal colonization; ten pools of leaf material of at least ten leaves per pool were sampled in the middle of the light period 4 months after planting for combined transcriptome and metabolome analysis as described (2).

Challenge with *Rhizoctonia solani*. Mycelium of *Rhizoctonia solani* AG8 grown for 4 to 5 d in potato dextrose broth was vacuum filtrated and chopped in a Waring blender in de-ionized water. Barley seeds of the lines Ubi::ChGP-19, Ubi::ChGP-9, and 35S::ChGP-36 were surface-sterilized in a solution of 10% sodium hypochlorite and germinated for 2 d on wet filter paper at room temperature. Seedlings were planted in a Turface:Oldri Mix (2:1 vol/vol) and incubated in a growth chamber (70% relative humidity, 16-h photoperiod for 7 d). After removal from the substrate, the roots of the plants were rinsed with tap water and immediately dipped into the mycelial suspension. Plants were then wrapped in wet filter paper, placed in 50-mL microfuge tubes, and incubated in a growth chamber for 1 week. For analysis, seedlings were unwrapped and the number of yellow leaf tips per seedling was determined.

Quantitative Determination of Endochitinase Activity in Plant Tissues. Tissues were ground after adding 400 μL of extraction buffer (50 mM Na-acetate with 100 μg/mL BSA, pH 5.5), vortexed, and centrifuged for 10 min at 18,000 × *g*. The supernatant was transferred into a fresh tube and stored at 4 °C. For the quantitative assay, 5 μL of protein solution were mixed with 45 μL Na-acetate buffer. Five microliters of 1:20 diluted sample were mixed with 45 μL Na-acetate buffer and 0.5 μg methylumbelliferyl-chitotrioside (Sigma). Samples were incubated at room temperature for 10 min with gentle shaking. Fifty microliters of 0.3 M glycine/NaOH buffer (pH 10.6) were added to stop the reaction. Fluorescence was measured using a Safire spectrophotometer (Tecan) (excitation/emission 455 nm/360 nm). The amount of enzyme was determined with a standard curve using ThEn-42 enzyme expressed in *Pichia pastoris*.

Western blot analysis of ChGP T_0 transformants. PCR-positive T_0 plantlets were tested for the production of the recombinant enzyme by Western blot. Ten milligrams of young leave or root material was harvested and ground in 100 μL (leaves) or 300 μL (roots) of protein extraction buffer [50 mM $NaPO_4$ pH7.0, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 0.1% polyvinylpyrolidone (40,000)] using a homogenizer. This suspension was then centrifuged at 18,000 × *g* for 10 min, and 50 μg of total protein was separated by SDS/PAGE, transferred to nitrocellulose membranes, and probed with antibodies raised against the purified recombinant endochitinase expressed in *P. pastoris* (3).

Isolation of ChGP T_1 transformants. The T_0 plants that showed presence of the *ThEn-42* gene by PCR in the developing seedling leaves and the endochitinase protein in leaves and roots were tested for inheritance of the transgene in 20 seeds from each of the harvested T_0 plants. Leaf pieces of each T_1 plant were tested by PCR for the presence of the *ThEn-42* gene. The forward primer Y 28 5′-CTACGCCGACTACCAGAAGC-3′and the reverse primer Y 29 5′-TAGTCCTTGATGGCCTGCTC-3′ were used for amplification.

Selection of homozygous ChGP T_1 transformants. For determination of the presence of *Trichoderma* endochitinase in single seeds, a high throughput qualitative screening test was developed (Fig. S2*A*). Single seeds were cut and incubated in 200 μL sodium acetate buffer (pH 5.5) containing 2 μg methylumbelliferyl-chitotrioside (Sigma) for 10 min at room temperature in the dark. Placement on an UV transilluminator shows intense fluorescence of the methylumbelliferon after cleavage from the chitotrioside. Grains lacking the *Trichoderma* endochitinase do not elicit the fluorescence. Grain of T_1 plants carrying the *ThEn-42* gene as identified by PCR were propagated in the greenhouse or field. T_2 seeds of these plants were screened for homozygosity.

Southern blots of ChGP T_1 plants. Because Southern blots of the T_0 plants could not be made, these were simulated by pooling the leaf pieces of 20 seedlings from each T_1 progeny, extracting the DNA, and performing the blotting as previously described (4). Ten micrograms of *Sst*I-digested genomic barley DNA was separated by agarose gel electrophoresis and blotted onto nylon membranes. The 4.8-kb plasmid pPICZα-Sp-FSP-FAP-ThEnCh(GC) harboring the 1.3-kb codon-optimized *ThEn-42* was linearized and served as the positive control. The hybridization probe was obtained by amplifying a 800-bp product with the primer Y28 5′-CTACGCC-GACTACCAGAAGC-3′ and Y29 5′–TAGTCCTTGATGGC-CTGCTC-3′ and labeling it with [α-^{32}P]dCTP using the all All-in-One random labeling system (Sigma). The transformants giving single-insertion bands were analyzed further.

Cytological Analysis of Mycorrhizal Colonization. Intracellular fungal structures (arbuscules) of the root samples were microscopically determined (Axioplan 2 microscope; Zeiss). After clearing of the roots in 10% KOH and subsequent Trypan blue staining, 100 randomly selected 1-cm root segments were examined per sample.

Quantitation of *G. mosseae* by Determination of the Large Subunit Ribosomal RNA Gene from Genomic Fungal DNA. Barley roots of six to eight plants per plot were harvested in liquid nitrogen and total

DNA then isolated using Qiagen DNeasy Plant Mini Kit (Qiagen). For detection of endomycorrhizal fungal DNA in barley roots, real-time PCR was used. The 28S rDNA (GenBank accession no. AY541918) was amplified, with the specific primer pair Mos-F 5′-GAAGTCAGTCATACCAACGGGAA-3′ and Mos-R 5′-CTCGCGAATCCGAAGGC-3′ (5). The reactions were set up with SYBR Green Jump Start Taq Ready Mix (Sigma-Aldrich). The procedure was performed as described (6). The threshold cycle (Ct) was calculated by the MxPro-Mx3000P Stratagene software. The Ct values obtained were calculated relative to barley ubiquitin content (2).

Transcript Profiling. All transcript analyses were performed with the Gene Spring software (Agilent) using data generated with the custom-designed Agilent microarrays, as described (2).

Transcript Quantitation of Barley Genes Differentially Transcribed Between GP and Baroness by qRT-PCR. Total RNA was isolated as described in the materials and methods section and treated with DNase I (Fermentas). cDNA was synthesized from 2.5 μg total RNA using the RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) and transcript amounts of the indicated genes relative to barley ubiquitin were analyzed in 1:100 dilutions of the reverse transcriptase reactions by qRT-PCR on a Mx3000P qPCR system (Stratagene) using the Brilliant II SYBR Green QPCR Master Mix (Stratagene) following the manufacturer's instructions. Cycling conditions were 10 min 95 °C, followed by 40 cycles of 15 s 95 °C, 20 s 60 °C, and 15 s 72 °C. Primers for three genes with significantly higher transcription level in Golden Promise (GP) and two in Baronesse (B) were derived from the consensus sequence of the respective unigenes and are listed below along with the best BLASTX hit; barley ubiquitin primers were taken from Schäfer et al. (2).

p35_17228 (PR4, *Triticum monococcum*):
Hv_17228_F 5′-CAGCCCGCAGAAGAACAAC-3′
Hv_17228_R 5′-GGTCCAGCCGTACTTGGAG-3′
p35_22781 (uncharacterized protein, rice):
Hv_22781_F 5′- CCGTCATTTCCATGTTGACC-3′
Hv_22781_R 5′- ATCAGACGTTGGCTCACTCC-3′
p35_14133 (Thaumatin-like protein TLP5, barley):
Hv_14133_F 5′- GACCAGACCAGCACCTTCAC-3′
Hv_14133_R 5′- GTCCTTATTCCTTATTGACCCAAG-3′
p35_9462 (ent-kaurene synthase, rice):
Hv_9462_F 5′- TTACTCGGCTCATTGCTCAC-3′
Hv_9462_R 5′- AGGGTTCTTCCTAAATCACATCC-3′
p35_27344 (putative cytochrome P450 protein, rice):
Hv_27344_F 5′- AGGTGCTGGGATACGATGTG-3′
Hv_27344_R 5′- GTCGGGCCAGTAGAGCTTG-3′

Simple Sequence Repeat Marker Analysis. Seven simple sequence repeat and simple sequence-length polymorphism markers of the region between 142 and 167 cM on barley chromosome 7H were tested for being polymorphic between B and GP. The two simple sequence repeat markers, EBmag0757 and Emac0156, were amplified from pooled B, GluB, and GP leaf material in standard PCR reactions as described by Ramsay et al. (7).

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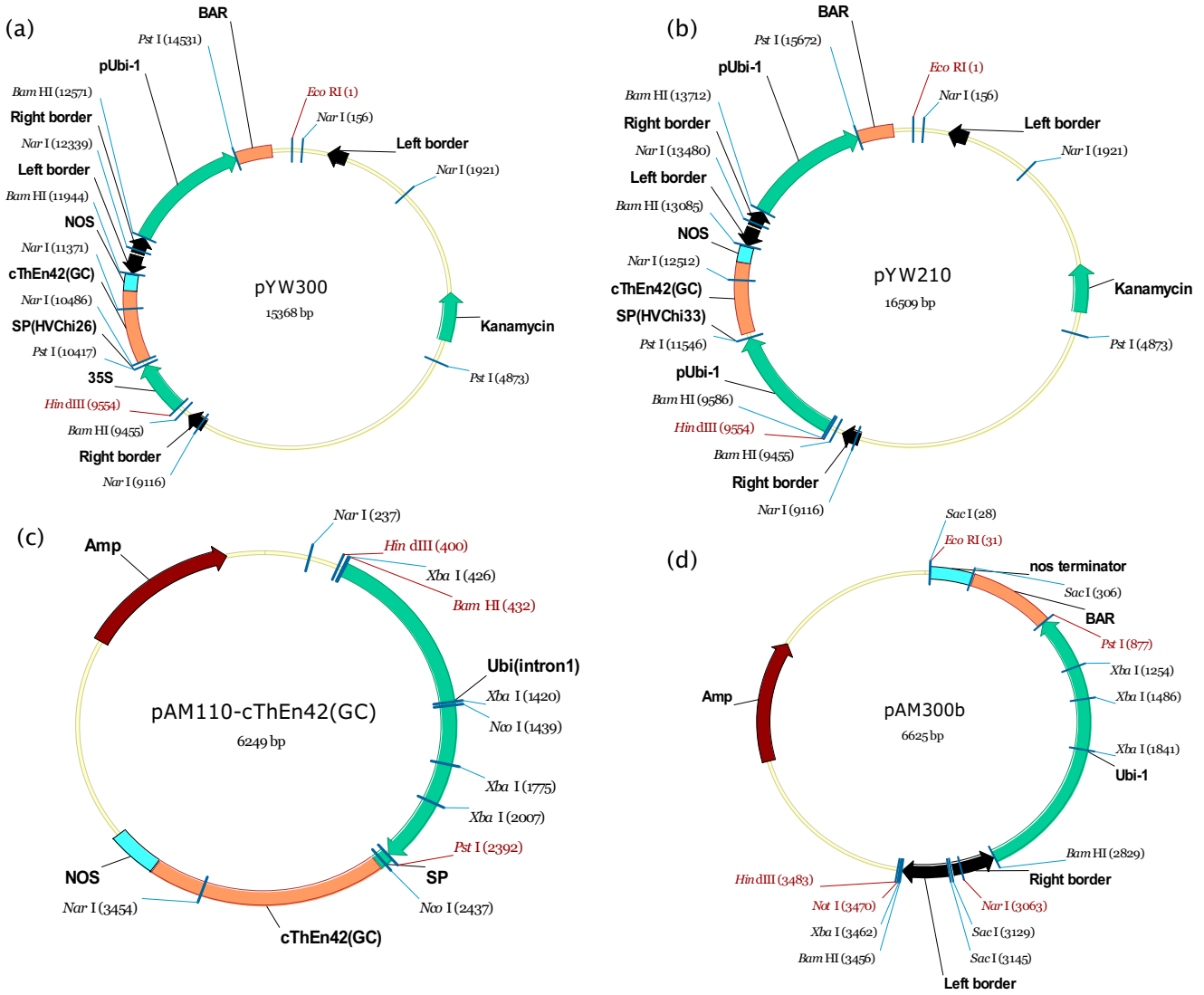

Fig. S1. (*A–D*) Maps of vectors used in this study.

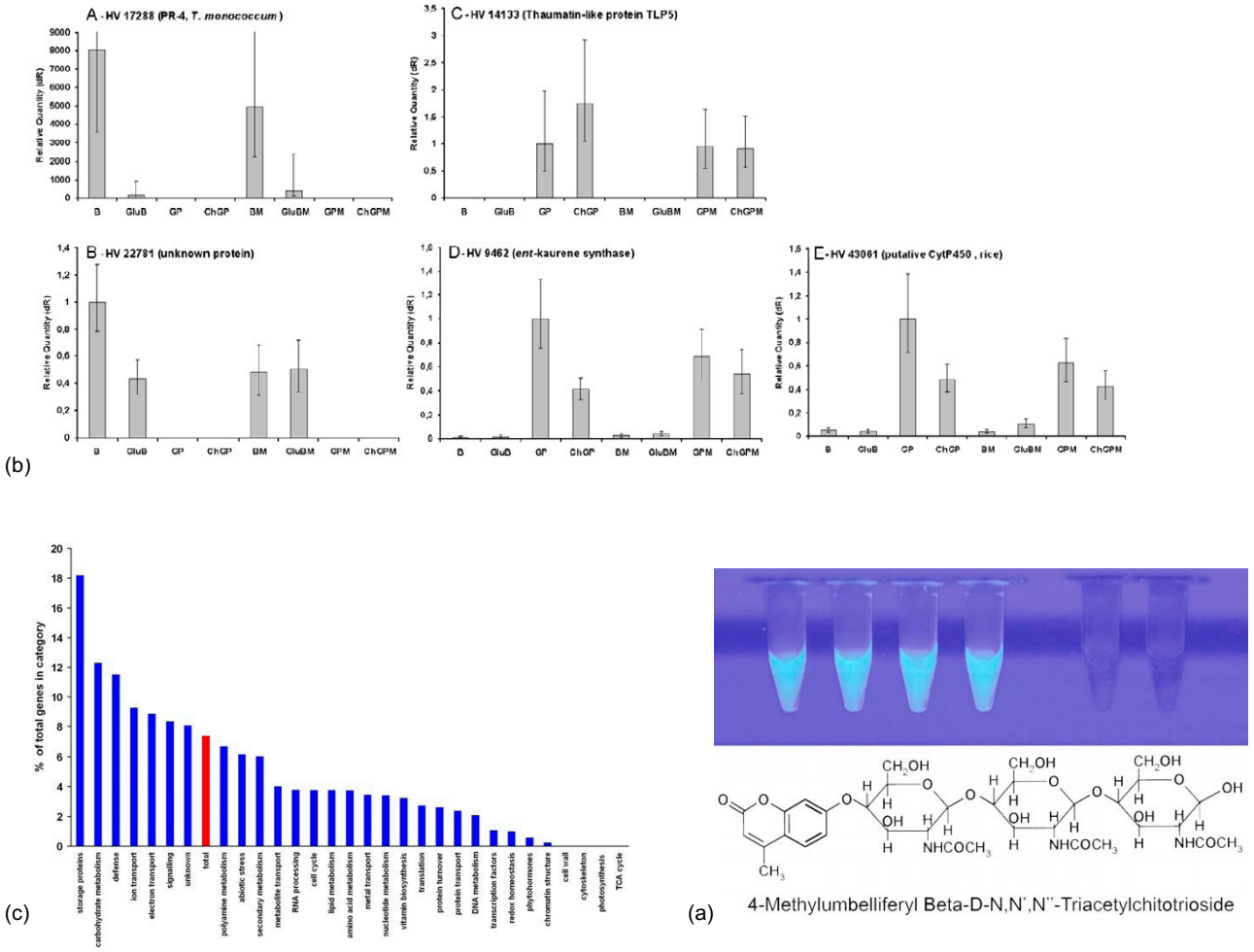

Fig. S2. (*A*) Fluorometric assay for endochitinase quantitation. (*B*) Analysis of genes differentially transcribed between Baronesse (B) and Golden Promise (GP) by qRT-PCR. (*C*) Functional classification of differentially transcribed genes between the cultivars B and GP.

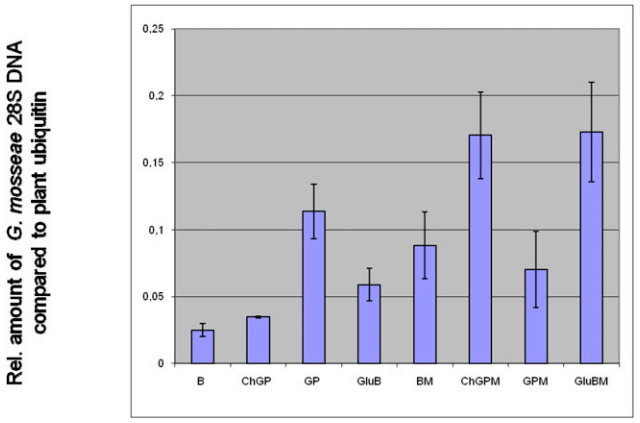

Fig. S3. Quantitation of barley root colonization by mycorrhizal fungi.

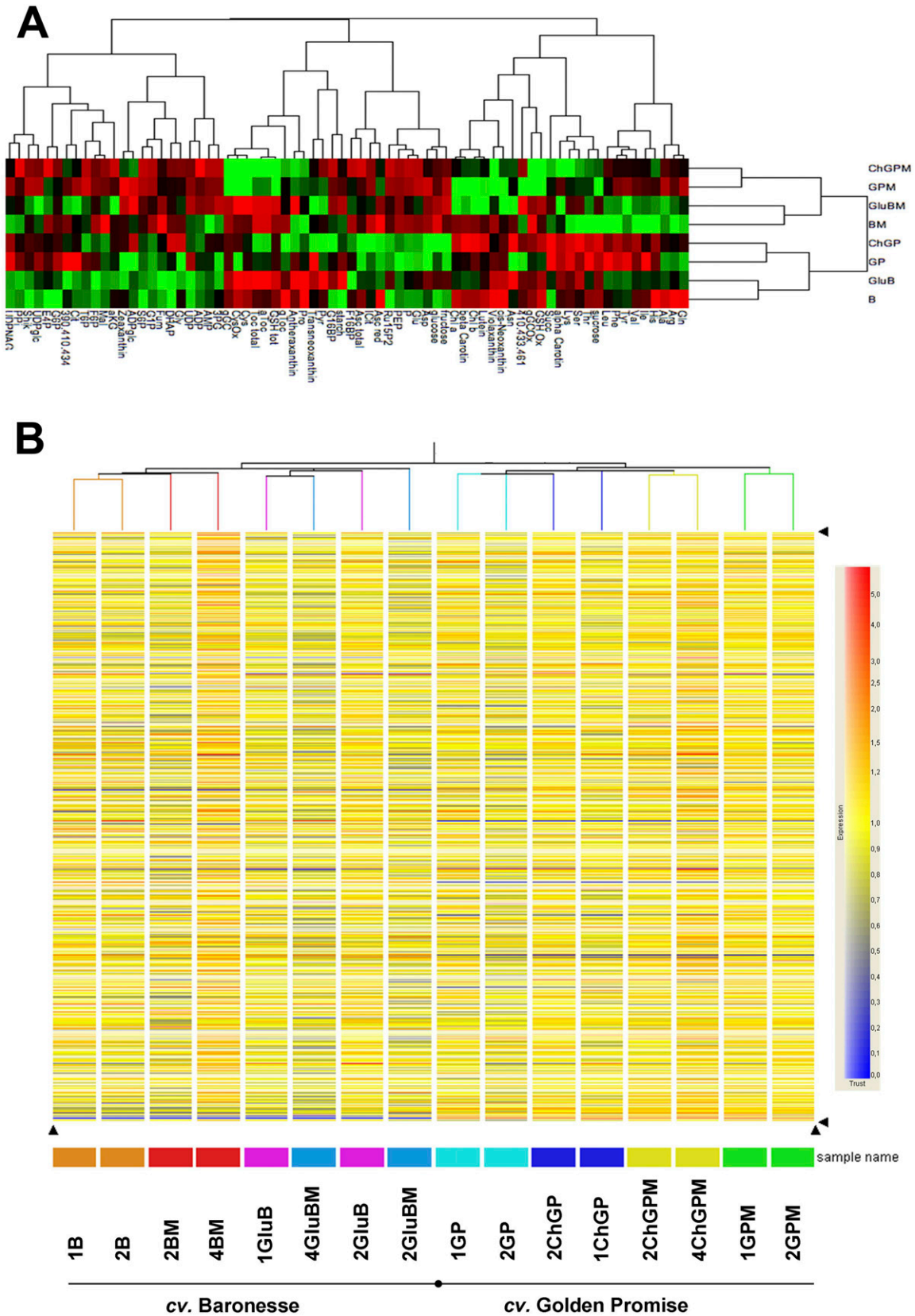

Fig. S4. (*A* and *B*) Hierarchical cluster analysis of metabolome and transcriptome data.

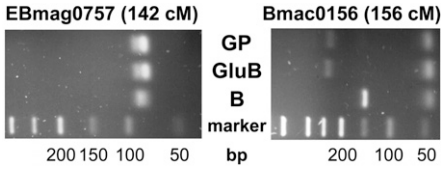

Fig. S5. Simple sequence repeat marker analysis of chromosomal regions that exhibit similar gene expression in (1,3-1, 4)-β-glucanase (GluB) and GP.

Table S1. Compilation of metabolite data of the targeted metabolite analysis

Metabolites	ChGPM		GPM		GluBM		BM		ChGP		GP		Glu-B		B
Sugars (μmol/g FW)															
Glucose	56.9 ± 18.4	m	54.2 ± 3.6		47.8 ± 2.4		62.5 ± 14.8		22.5 ± 2.6		36.4 ± 7.9		40.1 ± 6.3		26.8 ± 6.9
Fructose	33.3 ± 2.4	m	35.6 ± 1.8	m	28.6 ± 3.4		27.4 ± 2.4		14.9 ± 10.2		16.7 ± 3.2		23.4 ± 2.9		17.6 ± 1.9
Sucrose	42.4 ± 20.6		21.1 ± 7.4		44.5 ± 7.5		24.7 ± 17.2	m	88.4 ± 3.7		79.5 ± 8.2		50.0 ± 11.4		75.4 ± 11.9
Starch	6.4 ± 1.0		6.6 ± 0.8	c	6.1 ± 0.6		4.5 ± 0.8		4.9 ± 1.4		6.5 ± 1.3	c	6.6 ± 0.3	g	2.8 ± 0.2
Amino acids (nmol/g FW)															
Asp	50.1 ± 11.0		58.3 ± 9.0	m	46.2 ± 9.0		51.5 ± 7.6		32.6 ± 3.1		34.0 ± 6.6		45.1 ± 1.4		40.6 ± 11.4
Glu	203.1 ± 15.3	m	197.0 ± 13.3	m	145.4 ± 28.5		177.2 ± 17.2		140.0 ± 10.1		137.2 ± 16.7		154.5 ± 14.8		140.1 ± 28.8
Asn	4.8 ± 0.8	m	5.5 ± 1.5		5.2 ± 0.1	m	3.1 ± 1.6		6.6 ± 1.1		4.7 ± 1.9		3.2 ± 0.8		7.0 ± 2.5
Ser	55.2 ± 8.9	m	62.5 ± 7.7		63.9 ± 16.6		38.7 ± 10.6	m	84.3 ± 5.7		75.1 ± 22.3		69.5 ± 7.1		66.7 ± 10.8
Gln	33.9 ± 11.0		39.6 ± 14.3		27.0 ± 6.3		23.1 ± 11.2	m	31.6 ± 5.9		24.2 ± 6.2	c	24.8 ± 4.0	g	46.2 ± 6.8
Gly	30.1 ± 8.7		27.4 ± 8.6		23.9 ± 8.1		27.4 ± 11.1		29.4 ± 5.6		20.9 ± 5.2		22.2 ± 7.8		26.3 ± 5.4
His	1.3 ± 0.3		1.7 ± 0.5	c	1.0 ± 0.4		0.8 ± 0.3		1.4 ± 0.3		1.5 ± 0.2		1.2 ± 0.4		2.4 ± 1.0
Thr	4.4 ± 0.6		4.6 ± 1.1		3.7 ± 0.5		3.4 ± 0.5	m	5.9 ± 1.0		6.6 ± 1.2		4.1 ± 0.7		5.7 ± 1.4
Arg	46.6 ± 9.9		47.0 ± 9.6		39.4 ± 4.2		36.9 ± 7.2	m	43.8 ± 2.7		40.7 ± 6.5		41.6 ± 4.8		52.5 ± 7.8
Ala	70.4 ± 8.6		72.7 ± 12.1		52.8 ± 14.8		55.3 ± 7.4	m	63.8 ± 9.2		56.1 ± 12.3		56.2 ± 9.1	g	73.1 ± 4.8
Pro	7.5 ± 1.2		9.3 ± 2.5	c	8.8 ± 2.4		5.2 ± 0.4		8.3 ± 0.9		10.2 ± 1.0		20.0 ± 11.4		26.9 ± 15.5
Tyr	3.7 ± 0.9		4.1 ± 0.7	c	2.1 ± 0.2	m	2.5 ± 0.9		3.7 ± 0.7		4.3 ± 0.6	c	2.5 ± 0.2		2.9 ± 0.3
Val	17.9 ± 2.5		18.8 ± 2.6	c	13.0 ± 1.3	g	10.5 ± 0.5	m	18.9 ± 5.1		18.3 ± 4.2		13.9 ± 1.5		17.7 ± 3.9
Ile	5.2 ± 1.5		5.5 ± 1.0	c	3.6 ± 0.9		2.7 ± 0.1	m	4.8 ± 0.5		6.5 ± 1.5		3.8 ± 0.7		5.3 ± 1.3
Lys	0.9 ± 0.2	m	1.7 ± 0.6	c	1.3 ± 0.1		1.0 ± 0.2		1.8 ± 0.1		2.4 ± 0.6		1.6 ± 0.5		1.9 ± 0.6
Leu	8.1 ± 0.9		6.7 ± 1.7		4.0 ± 1.0		3.4 ± 0.3	m	7.3 ± 1.1		9.5 ± 1.8	c	4.2 ± 1.0	g	6.3 ± 0.9
Phe	11.6 ± 2.7		12.8 ± 2.0	c	7.3 ± 1.4		6.2 ± 0.5	m	12.6 ± 1.9		13.2 ± 1.5	c	7.7 ± 0.6		9.3 ± 1.6
Oxidative protection (nmol/g FW)															
Asc red (μmol/g FW)	3.6 ± 0.1		3.5 ± 0.3		3.6 ± 0.3		3.9 ± 0.3		3.3 ± 0.2		3.4 ± 0.1		3.4 ± 0.2		3.8 ± 0.4
Asc total (μmol/g FW)	4.5 ± 0.7		3.4 ± 0.8		3.8 ± 0.4		4.0 ± 0.2		3.5 ± 0.1		3.6 ± 0.1	c	3.7 ± 0.4		4.1 ± 0.2
GSH Ox	2.0 ± 1.4	m	2.0 ± 1.3		5.4 ± 0.4		5.1 ± 1.1	m	7.1 ± 0.9	g	3.7 ± 2.0	c	5.1 ± 1.2	g	8.2 ± 1.6
GSH	2.7 ± 3.0	m	2.8 ± 4.7	c	23.3 ± 3.0		26.2 ± 5.9		17.3 ± 6.6		17.7 ± 8.5		21.4 ± 1.6		26.2 ± 1.9
aToc (ng/mg)	79.1 ± 5.7		94.5 ± 16.6	m	111.9 ± 4.1		119.2 ± 8.3		86.5 ± 5.5		98.1 ± 10.4		118.9 ± 3.8		107.2 ± 21.3
gToc (ng/mg)	1.5 ± 0.6		2.1 ± 0.1		1.7 ± 0.0		2.4 ± 1.1		1.1 ± 0.1	g	1.6 ± 0.1		1.9 ± 0.4		3.5 ± 1.7
Cys	0.3 ± 0.0	g	0.2 ± 0.0		2.4 ± 0.2	m,g	1.6 ± 0.2	m	0.9 ± 0.9		1.2 ± 1.1		1.9 ± 0.2	g	2.4 ± 0.2
gGC	0.8 ± 0.3		0.6 ± 0.5	c	1.4 ± 0.2		1.6 ± 0.1		1.4 ± 0.4		1.4 ± 0.5		1.4 ± 0.2		1.5 ± 0.3
Phosphorus (mg/g DW)															
P	6.6 ± 0.2	m,g	6.0 ± 0.1	m	4.2 ± 0.7		5.4 ± 1.3		3.6 ± 0.5		3.4 ± 0.7		4.0 ± 0.5		3.6 ± 0.3
Pigments (AU/g FW)															
cis-Neoxanthin	9.4 ± 1.8		8.4 ± 0.8		7.9 ± 0.9		9.5 ± 1.8		9.8 ± 1.9		9.8 ± 1.7		9.8 ± 1.9		10.8 ± 1.4
Violaxanthin	16.0 ± 2.7		14.9 ± 0.5	m	17.0 ± 1.4	m	20.1 ± 3.6		18.4 ± 1.5		19.4 ± 0.3		19.2 ± 0.6		21.6 ± 3.8
Antheraxanthin	4.4 ± 0.9		4.7 ± 0.5		6.0 ± 1.0		4.2 ± 0.6		4.3 ± 0.1		5.3 ± 0.5		5.3 ± 0.4		5.3 ± 0.7
Lutein	42.2 ± 2.5		36.3 ± 4.3		34.0 ± 2.1	g	42.2 ± 1.5		44.9 ± 4.1		41.0 ± 0.7		39.6 ± 3.5		45.1 ± 5.3
Zeaxanthin	4.0 ± 0.9		6.3 ± 2.0		4.7 ± 1.5		4.3 ± 0.8		4.4 ± 1.4		2.7 ± 0.2	c	2.7 ± 0.3		4.2 ± 0.7
β-Carotin	24.5 ± 0.7		22.0 ± 2.9		22.3 ± 1.6	g	26.0 ± 0.7		27.4 ± 2.7		25.0 ± 2.0		23.0 ± 1.1		26.5 ± 2.0
Chl b	52.3 ± 5.6		46.3 ± 4.4	m	44.5 ± 0.4	m,g	55.3 ± 2.2		58.7 ± 6.1		55.0 ± 2.0		48.6 ± 1.6	g	57.2 ± 2.9
Chl a	49.9 ± 1.2		43.2 ± 4.4	c	42.8 ± 2.1	g	50.8 ± 1.3		52.3 ± 5.8		48.8 ± 2.7		46.2 ± 2.1	g	52.8 ± 3.2
α-Carotin	0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0
410,433,461	1.0 ± 0.2		0.8 ± 0.0		1.0 ± 0.2		1.0 ± 0.3		1.0 ± 0.1		1.0 ± 0.0		1.1 ± 0.1		1.2 ± 0.2

Table S1. Cont.

Metabolites	ChGPM		GPM		GluBM		BM		ChGP		GP		Glu-B		B
Transneoxanthin	1.1 ± 0.3		0.9 ± 0.3		0.9 ± 0.3		0.7 ± 0.2		1.0 ± 0.2		1.1 ± 0.3		1.0 ± 0.3		1.0 ± 0.3
390,410,434	0.2 ± 0.0		0.2 ± 0.1		0.2 ± 0.0		0.2 ± 0.1		0.2 ± 0.1		0.3 ± 0.0	c	0.2 ± 0.0		0.3 ± 0.0
Glycolysis and Citric Cycle (nmol/g FW)															
3PG (μmol/g)	1.2 ± 0.3		1.1 ± 0.1	m	1.0 ± 0.2		0.7 ± 0.2		0.9 ± 0.1		0.7 ± 0.1		0.7 ± 0.1		0.9 ± 0.2
Cit (μmol/g)	4.2 ± 0.6		5.1 ± 0.7		2.6 ± 0.4		3.8 ± 1.0		3.9 ± 1.1		5.6 ± 0.7	c	2.8 ± 0.6		3.2 ± 0.4
Icit (μmol/g)	10.2 ± 0.8	m	9.4 ± 1.1	m	5.2 ± 1.2	g	9.2 ± 0.8	m	3.4 ± 0.7		5.4 ± 0.6		5.3 ± 1.2		6.1 ± 1.2
Mal (μmol/g)	18.5 ± 2.6	m	14.3 ± 2.4	m,c	5.0 ± 0.8	g	19.4 ± 0.4	m	5.1 ± 0.7		6.2 ± 1.0		5.3 ± 0.5		5.4 ± 0.9
Ru15P2 (μmol/g)	6.2 ± 0.4	m	4.8 ± 0.9	m	2.6 ± 0.3		3.4 ± 0.8	m	1.8 ± 0.2		2.0 ± 0.3		2.4 ± 0.7		1.5 ± 0.4
Pyr	62.1 ± 18.3		41.2 ± 18.5		31.8 ± 18.7		47.1 ± 14.7		32.9 ± 6.4		40.7 ± 23.8		66.4 ± 28.2		50.5 ± 24.4
UDPglc	86.9 ± 17.9		88.4 ± 19.7		52.2 ± 5.7		63.1 ± 11.5		73.3 ± 9.4		80.4 ± 20.1		61.2 ± 17.4		58.4 ± 5.2
PEP	111.4 ± 16.1		106.5 ± 9.3	m	95.6 ± 13.7		102.6 ± 21.3		84.6 ± 11.4		77.5 ± 8.1		81.2 ± 4.4		83.6 ± 17.3
ADP	53.0 ± 5.5	m	46.8 ± 3.9		44.4 ± 10.7		40.7 ± 8.7		40.9 ± 2.7		42.9 ± 3.0		39.1 ± 2.3		40.7 ± 8.2
aKG	97.6 ± 7.3		80.9 ± 8.7		52.2 ± 4.7	g	73.6 ± 1.2		74.7 ± 19.3		70.3 ± 3.2		57.8 ± 5.6	g	73.8 ± 6.1
Succ	66.6 ± 12.5		77.5 ± 13.5		75.6 ± 9.3		67.0 ± 11.6		99.7 ± 23.2		98.3 ± 20.1	c	68.0 ± 17.3		60.8 ± 13.4
Fum	46.5 ± 9.8		43.0 ± 8.6		42.2 ± 17.2		56.9 ± 30.6		39.5 ± 6.7		36.0 ± 3.1		27.0 ± 5.6		31.6 ± 11.4
Shik	96.5 ± 28.1		97.2 ± 11.7	c	46.2 ± 9.5	g	66.3 ± 6.7	m	72.9 ± 11.6		71.2 ± 16.6		45.3 ± 10.9		41.3 ± 6.5
G6P	150.7 ± 39.3		120.6 ± 9.6	m	109.5 ± 25.5		125.4 ± 34.3		123.7 ± 21.1		141.5 ± 10.0	c	96.0 ± 16.3		71.7 ± 14.6
F6P	88.1 ± 29.3		86.1 ± 3.8		66.8 ± 23.7		83.0 ± 6.2		63.2 ± 9.2		75.4 ± 6.8		46.7 ± 21.2		55.1 ± 23.4
F16BP	107.6 ± 40.3		81.4 ± 25.4		69.2 ± 19.9		94.0 ± 15.7		69.1 ± 18.8		83.0 ± 27.3		57.8 ± 20.9		84.4 ± 22.5
ADPglc	14.7 ± 2.5		24.2 ± 8.7		21.1 ± 4.6		14.7 ± 4.1		20.9 ± 7.0		11.0 ± 3.8		13.1 ± 2.8		13.9 ± 5.5
G16BP	17.8 ± 5.1		16.4 ± 3.2		15.4 ± 3.0		14.1 ± 3.1		12.3 ± 1.2		15.0 ± 2.3		15.2 ± 2.6		12.9 ± 1.7
AMP	26.0 ± 3.7		23.4 ± 1.6		20.3 ± 3.2		13.4 ± 2.2		19.9 ± 0.7		18.5 ± 3.2		16.3 ± 1.8		17.8 ± 1.4
E4P	34.7 ± 6.6		25.6 ± 1.6		22.0 ± 4.1		25.6 ± 6.0		27.2 ± 6.0		28.8 ± 2.0	c	19.8 ± 4.8		20.0 ± 2.3
PPi	14.4 ± 2.0		12.9 ± 1.8		11.8 ± 2.7		11.0 ± 2.4		12.2 ± 0.9		12.8 ± 1.7		10.7 ± 0.5		11.3 ± 2.7
G1P	4.8 ± 0.8	m	4.3 ± 0.6	m	2.8 ± 0.5		3.8 ± 1.3		2.4 ± 0.8		2.3 ± 0.2		2.0 ± 0.4		2.4 ± 0.6
UDP	13.1 ± 3.4		13.0 ± 1.1		11.6 ± 2.9		9.6 ± 2.7		9.9 ± 2.0		12.5 ± 2.4		8.0 ± 1.3		8.4 ± 1.3
S6P	5.2 ± 1.1	m	4.5 ± 0.6	m	2.8 ± 1.0		2.9 ± 0.6		2.4 ± 0.4		2.2 ± 0.4		1.7 ± 0.2		1.5 ± 0.5
T6P	0.8 ± 0.2	m	0.8 ± 0.2		0.5 ± 0.1		0.6 ± 0.2		0.5 ± 0.1	g	0.7 ± 0.0		0.5 ± 0.0		0.5 ± 0.0
DHAP	18.2 ± 2.2		16.8 ± 3.7		15.7 ± 4.2		16.9 ± 2.5		18.1 ± 4.5		16.1 ± 2.2		13.6 ± 2.1		13.7 ± 3.5
UDPNAG	10.9 ± 1.7		12.6 ± 1.7	c	8.8 ± 1.7		7.5 ± 1.1		11.6 ± 2.0		12.7 ± 2.9		7.5 ± 1.7		7.8 ± 1.9

Data from 72 metabolites analyzed in targeted assays are given as mean values of four independent replicates ± SEM. Significantly different metabolite contents between genotypes or treatments with $P < 0.05$ according to a Welch-Satterthwaite test [Junker BH, Klukas C, Schreiber F (2006) VANTED: A system for advanced data analysis and visualization in the context of biological networks. *BMC Bioinformatics* 7:109.] are indicated by lowercase letters in parentheses, reflecting groups of the same significance class. B, Baronesse; ChGP, Chitinase GP; GluB, Glucanase B; GP, Golden Promise; M, Amykor treated. Significant differences in pairwise comparisons with $P < 0.05$ are indicated by the following letters: m for comparison of Amykor vs. not treated plants; c for differences between non-GM genotypes of the same treatment and g for differences between GM and non-GM genotypes. m, mycorrhiza effect; c, cultivar effect; g, transgene effect. ChGP, GPM > GP, GluBM > GluB, and BM > B are indicated by letter m in the respective first mentioned column.

Table S2. Consistent effects of Amykor treatment of soil on the barley metabolome in field and greenhouse experiments

	Field experiment					Greenhouse experiment		
Mean ratio	GPM/GP	ChGPM/ChGP	BM/B	GluBM/Glu	field +M/−M	GPM/GP	ChGPM/ChGP	greenhouse +M/−M
Phosphate	1.8	1.9	1.5	1.1	1.58 ± 0.18	2.1	1.3	1.7 ± 0.57
G1P	1.9	2.3	1.7	1.5	1.85 ± 0.17	6	2.3	4.15 ± 2.62
3PGA	1.6	1.4	1.1	1.5	1.4 ± 0.11	2.6	2.6	2.6 ± 0
PEP	1.4	1.3	1.3	1.2	1.3 ± 0.04	2.3	2.4	2.53 ± 0.07
Ru1,5bP	2.5	3.2	3.4	1.4	2.63 ± 0.45	2	1.7	1.85 ± 0.21
Hexoses	1.8	2.9	2.2	1.3	2.05 ± 0.34	1.6	0.7	1.15 ± 0.64

Differences of leaf metabolite contents between Amykor treated an untreated barley plants in the field experiment with all four barley genotypes and a greenhouse experiment with lines in the Golden Promise background. Columns with metabolite ratios of treated over nontreated barley plants (+M/−M) were calculated based on the average of all examined genotypes. B, Baronesse; ChGP, Chitinase GP; GluB, Glucanase B; GP, Golden Promise; +, Amykor treated; +M/−M, metabolite ratios treated vs. untreated.

Table S3. Differentially transcribed genes between field grown barley varieties

Genotype comparisons	No. of differentially transcribed genes
Indicated barley types	
GP vs.ChGP	0
B vs. GluB	22
B vs. GP	1,660
B vs. GP ∩ B vs. GluB	16
± Amykor (*Glomus* sp.) treatment	
GP vs. GPM	4
ChGP vs. ChGPM	0
B vs. BM	0
GluB vs. GluBM	0

Field grown barley plants were cultivated as described in *SI Materials and Methods* and pooled leaf samples were harvested after 10 weeks. The number of differentially transcribed genes between the indicated barley genotypes (*Top*) and between Amykor treated and untreated plots of the same genotype (*Bottom*) is displayed. The microarray flag pass list was filtered for fold-change > 2 and $P > 0.05$ after Benjamini-Hochberg multiple test correction (see spreadsheets in Dataset S1).

Other Supporting Information Files

Dataset S1 (XLS)

6 Summary

The sebacinoid fungus *Piriformospora indica* colonizes roots of an extraordinary broad range of monocotyledonous and dicotyledonous plants. Root colonization is associated with the transfer of muliple beneficial effects to the host plants. This includes abioitic stress tolerance, biotic stress resistance against root and leaf pathogens as well as growth promotion and yield increase. The studies presented here indicated that root colonization by *P. indica* did not result in strong transcriptomic changes in barley leaves that might explain the systemic resistance in leaves against *Blumeria graminis* f.sp. *hordei*. The fungus might rather alter the metabolic status of leaves as root colonization significantly reduced lipid peroxidation, fatty acid desaturation, and metabolic heat emission but strongly elevated the antioxidative capacity in leaves. These metabolic alterations were also observed in salt tolerant barley cultivar California Mariout and thus might reflect salt tolerance mediated by *P. indica*. Interestingly, *P. indica* and closely related *Sebacina* species were found to be colonized by endosymbiotic bacteria in a species-specific manner. Moreover, these bacteria were able to transfer beneficial effects to plants as reported for sebacinoid fungi (e.g. *P. indica*). Although plant benefits mediated by bacteria were weaker as compared to their fungal hosts, this adds more complexity to the mutualistic interaction of plants with sebacinoid fungi.

Plant root colonization by *P. indica* is a sophisticated process and the fungus evolved effective strategies to suppress root innate immunity. Based on molecular and genetic data, immune suppression is a prerequisite for successful root colonization. As effective immune suppression was observed in *Arabidopsis* and barley roots, it might explain the ability of *P. indica* to colonize a broad spectrum of plants. In addition, the manipulation of hormone metabolism by *P. indica* might essentially contribute to its colonization success. For instance, gibberellic acid (GA), ethylene, and jasmonate were required for root compatibility. Among these hormones, GA might be recruited by *P*. *indica* for defense suppression. Reduced root colonization of barley mutants impaired in GA signaling and synthesis by *P. indica* was associated with an elevated root immune reponse. Cell biological studies revealed a high adaptation of *P. indica* to *Arabidopsis* roots. The fungus initially colonized living root cells as indicated by the ultrastructural intactness of cell organelles such as the endoplasmic reticulum (ER), the nucleus or mitochondria. This biotrophic colonization phase was followed by a cell death-dependent colonization phase. *P. indica* initiated this cell death by inducing ER stress that resulted in the activation of a vacuole-mediated and caspase-dependent cell death.

Comparable results in the barley root-*P. indica* interaction implicate that root cell death is a common colonization strategy by the fungus. Moreover, as cell death-dependent colonization was reduced in barley plants overexpressing the negative cell death regulator BAX INHIBITOR-1 (BI-1). BI-1 is a known integrator of ER stress that supports cell viability under unfavorable environmental conditions.

A stable barley root transformation system was developed, which allowed studying the effect of barley proteins on the mutualistic interaction of roots with *P. indica* as well as with the pathogen *Fusarium graminearum*. The system was suitable for efficient overexpression and silencing of candidates and drastically accelerated molecular as well as genetic studies in barley roots.

In order to investigate the effect of transgenes on the barley metabolome and transcriptome, investigation were performed under field conditions. These studies revealed that the stable expression of neither a fungal chitinase nor a chimeric bacterial β-glucanase in barley significantly altered the plant transcrptome or metabolome in comparison to the respective parent lines. Moreover, the studies indicated that alterations in the genome of cultivars generated by classical breeding strategies result in more pronounced changes of the metabolome and transcriptome.

7 Zusammenfassung

Der zu den *Sebacinales* gehörende Pilz *Piriformospora indica* besiedelt die Wurzeln eines ungewöhnlich breiten Spektrums an monokotylen und dikotylen Pflanzen. Die Wurzelbesiedlung ist dabei für die Wirtspflanze mit mehreren nützlichen Effekten verbunden. Diese umfassen Toleranz gegenüber abiotischen Stressfaktoren, Resistenz gegenüber biotischen Stressfaktoren wie Blatt- und Wurzelpathogenen sowie Wachstumsförderung und Verbesserung des Ertrags. Die hier präsentierten Studien zeigen, dass die Wurzelbesiedlung durch *P. indica* nicht zu starken Veränderungen im Transkriptom von Gerstenblättern führen, die die systemische Resistenz gegenüber *Blumeria graminis* f.sp. *hordei* erklären könnten. Vielmehr scheint der Pilz den metabolischen Status von Blättern zu beeinflussen, da die Besiedlung von Wurzeln zu einer signifikant reduzierten Lipidperoxidation, Fettsäuredesaturation und metabolischen Wärmeemission führt, die antioxidative Kapazität in Blättern jedoch stark erhöht. Diese Veränderungen im Metabolismus konnten ebenfalls in dem salztoleranten Gerstenkultivar Mariout beobachtet werden und könnten daher die von *P. indica* vermittelte Salztoleranz erklären. Interessanterweise werden *P. indica* und nahe verwandte *Sebacina* Arten selbst von endosymbiotisch lebenden Bakterien in einer Species-spezifischen Weise besiedelt. Diese Bakterien vermitteln dieselben nützlichen Effekte für Pflanzen, wie sie für die sebacinoiden Pilze (z.B. *P. indica*) beschrieben wurden. Allerdings sind die von den Bakterien hervorgerufenen nützlichen Effekte schwächer als die ihrer pilzlichen Wirte. Dies zeigt jedoch eine zusätzliche Komplexität der mutualistischen Interaktion von Pflanzen mit sebacinoiden Pilzen.

Die Besiedlung von Pflanzenwurzeln durch *P. indica* ist ein vielschichtiger Prozess. Der Pilz hat offenbar effektive Strategien entwickelt, um das Abwehr-/Immunsystem der Wurzel zu supprimieren. Molekulare und genetische Daten zeigen, dass diese Immunsuppression eine Voraussetzung für eine erfolgreiche Wurzelbesiedlung darstellt. Die effektive Abwehrsuppression konnte sowohl in *Arabidopsis*- als auch in Gerstenwurzeln beobachtet werden, und könnte die Erklärung für die Fähigkeit von *P. indica* sein, dieses breite Spektrum an Wirtspflanzen besiedeln zu können. Darüber hinaus könnte auch die Beeinflussung des pflanzlichen Hormonstoffwechsels wesentlich zum Besiedlungserfolg von *P. indica* beitragen. Beispielsweise tragen Gibberellinsäure (GA), Ethylen und Jasmonsäure zur Wurzelkompatibilität bei. GA könnte von *P. indica* sogar für die Abwehrsuppression benutzt werden. Wurzeln von Gerstenmutanten, die entweder in der GA Signalweiterleitung oder GA Synthese gestört

sind, werden schlechter durch *P. indica* besiedelt, was mit einer erhöhten Immunantwort in den Wurzeln in Zusammenhang zu stehen scheint. Zellbiologische Studien zeigen ein hohes Maß an Anpassung von *P. indica* an *Arabidopsis* Wurzeln. Der Pilz besiedelt zunächst lebende Wurzelzellen, was anhand der ultrastrukturellen Integrität des Endoplasmatischen Retikulums (ER), des Zellkerns und der Mitochondrien gezeigt werden kann. Diese biotrophe Phase der Besiedlung wird von einer Zelltod-abhängigen Phase gefolgt. *P. indica* scheint diesen Zelltod zu initiieren, indem er ER-Stress verursacht, der in der Aktivierung eines Vakuolen-vermittelten, Caspase-abhängigen Zelltods mündet. Ähnliche Ergebnisse zur Gersten-*P. indica* Interaktion implizieren, dass der Wurzelzelltod generell zur Besiedlungsstrategie des Pilzes gehört. Die Tatsache, dass Gerstenpflanzen, die den negativen Zelltodregulator BAX INHIBITOR-1 (BI-1) überexprimieren, schlechter besiedelt werden, unterstützt ebenfalls die These einer Abhängigkeit der Besiedlung von Zelltod. BI-1 ist ein bekannter Integrator von ER-Stressreaktionen, der das Überleben von Zellen unter ungünstigen Umweltbedingungen sicherstellt.

Im Rahmen dieser Arbeit wurde außerdem ein stabiles Wurzeltransformationssystem für Gerste entwickelt, das helfen soll, die Funktion von Gerstenproteinen in der mutualistischen Interaktion mit *P. indica* oder mit Pathogenen wie *Fusarium graminearum* zu überprüfen. Das System ist sowohl für die Überexpression als auch das *Silencing* von Kandidatengenen geeignet und wird die Möglichkeit der molekularen und genetischen Untersuchungen in Gerstenwurzeln stark verbessern.

Um mögliche Effekte von Transgenen auf das Gerstenmetabolom und –transkriptom zu untersuchen, wurden Freisetzungsversuche durchgeführt. Diese Studien belegen, dass weder die stabile Expression einer pilzlichen Chitinase noch einer chimären bakteriellen β-Glucanase das Gerstentranskriptom oder Metabolom signifikant im Vergleich zu den jeweiligen nicht-transgenen Elternlinien veränderte. Diese Studien zeigen zudem, dass Veränderungen im Genom von Kultivaren, die durch klassische Züchtung erstellt wurden, sehr viel größere Auswirkungen auf das Metabolom und Transkriptom haben.

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