Plant recognition by *Trichoderma harzianum* elicits upregulation of a novel secondary metabolite cluster required for colonization

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Summary

Trichoderma harzianum is a filamentous ascomycete frequently applied as plant beneficial agent in agriculture. While mycoparasitism and antagonism of *Trichoderma* spp. against fungal pathogens are well known, early responses of the fungus to the presence of a plant await broader investigation. In this study we analyzed these early stages of plant-fungus communication at the molecular level. We show that *T. harzianum* B97 is an efficient colonizer of plants and chemotropically responds to a plant extract. Patterns of secreted metabolites revealed that the fungus chemically responds to the presence of the plant and that the plant secrets a fungus specific metabolite as well. Hence we developed a strategy for omics analysis to simulate the conditions of the early plant recognition eliciting a chemotropic response in the fungus and found only 102 genes to be differentially regulated, including nitrate and nitrite reductases. Among them, a so far uncharacterized, presumably silent gene cluster was strongly induced upon recognition of the plant. Gene deletion of two genes of this Plant Communication Associated (PCA) cluster revealed that they are essential for colonization of soybean roots. Moreover, for part of the gene cluster, a DNA motif with palindromic sequence was detected. Phylogenetic analysis indicated that the PCA cluster is only present in the Harzianum clade of *Trichoderma* and was likely acquired by horizontal gene transfer (HGT) from *Metarhizium* spp., with the clustered genes originating from fungi, bacteria and plants.

We conclude that the plant recognition specific PCA cluster mediates early chemical communication between plant and fungus, is required for colonization and it is likely responsible for the high potential of *T. harzianum* and closely related species for biocontrol applications.

Significance statement

Interactions of plants with fungi – beneficial or pathogenic – are crucial for the ecological function of both partners. Yet, the chemical “language” they use and how or when they use it is still insufficiently known. We describe discovery of a novel secondary metabolite cluster, which is transcriptionally induced in the early phase of interaction, even before contact. Presence of this cluster is essential for colonization of the plant, hence reflecting the very start of an intimate plant-fungal interkingdom interaction. Acquisition of the cluster from other organisms highlights the evolutionary adaptation of *T. harzianum* to plant interaction and likely contributes to its success as plant symbiont.

Introduction

Natural environments harbor a complex community of microorganisms, which fulfill crucial tasks in the carbon cycle and can interact with plants as symbionts (Averill et al., 2022; Harman, Lorito, et al., 2004) or pathogens (Doehlemann et al., 2017). Climate change and global warming are bringing increased disease pressure, abiotic stresses and promotes invasion of plant pathogens in new habitats (Bebber, 2015; Raza & Bebber, 2022). Hence, better understanding for knowledge based application of biocontrol agents and biostimulants is required (Del Buono, 2021; Liu et al., 2022). Fungi evolved elaborated mechanisms for dealing with their biotic and abiotic environment in terms of sensing and signaling mechanisms as well as strategies for effective competition and antagonism (Khan et al., 2020; Syed Ab Rahman et al., 2018). Fungal secondary metabolites are thereby of crucial importance for interactions with other microbes, animals and also with plants (Rangel et al., 2021). The abilities of some fungi to antagonize and kill their competitors is applied for protection of plants against pathogens and fungi of the genus *Trichoderma* are among the most broadly applied for this purpose (Sood et al., 2020). Consequently, this genus also dominates research towards mycoparasitism, plant protection and biocontrol of plant pathogens (Ramirez-Valdespino et al., 2019; Tyskiewicz et al., 2022).

Fungi of the genus *Trichoderma* (Schalamun & Schmoll, 2022; Woo et al., 2022) are typical inhabitants of the rhizosphere and are found in soils worldwide (Druzhinina et al., 2011). They belong to the most versatile microorganisms for both agricultural and industrial applications (Mukherjee et al., 2013). Several *Trichoderma* species are known as efficient biocontrol organisms and act as important symbionts with plants (Guzman-Guzman et al., 2019; Harman, Howell, et al., 2004). They are studied in detail for their capabilities in producing antibiotics, parasitizing other fungi – predominantly plant pathogens – and to compete with deleterious plant pathogens (Druzhinina et al., 2011; Tyskiewicz et al., 2022). Beneficial *Trichoderma* strains further induce root branching and can increase shoot biomass and trigger systemic resistance as well as plant nutrient uptake (Contreras-Cornejo et al., 2016). One of the major advantages of *Trichoderma* as biocontrol agents (BCAs) is that they can be found almost ubiquitously, which enables development of regional integrated crop protection strategies, with only minor interference with the natural microbial flora (Averill et al., 2022; He et al., 2021).

Among the most important functions for successful fungal plant interaction – beneficial or pathogenic - is the ability to colonize plant roots (Haueisen & Stukenbrock, 2016; Tyskiewicz et al., 2022). *Trichoderma* spp. are able to efficiently colonize plant roots, although they mostly remain at the outer layers of the plant tissue (Contreras-Cornejo et al., 2016; Tyskiewicz et al., 2022). Nevertheless, also truly endophytic strains of *Trichoderma* (Chaverri et al., 2015; Harman & Uphoff, 2019) are known to associate with plants, including also some *T. harzianum* strains (Bailey & Melnick, 2013; Chaverri et al., 2015). Fungi communicate with their environment using a broad array of signals (Bazafkan et al.,

2015; Leeder et al., 2011; Macias-Rodriguez et al., 2020). Chemical communication between fungi and plants is essential for interaction and diverse secondary metabolites are known to play an important role in this interplay (Macias-Rodriguez et al., 2020). Moreover, adhesion and the function of hydrophobins are required for plant-fungus interaction (Taylor et al., 2022). Intriguingly, stressed plants were found to secrete specific compounds attracting beneficial fungi (Lombardi et al., 2018). Overall, a considerable number of effectors (Ramirez-Valdespino et al., 2019) and secondary metabolites (Contreras-Cornejo et al., 2016) including volatile organic compounds (Joo & Hussein, 2022) are already known to contribute to successful biocontrol. In the plant, recognition of beneficial fungi like *Trichoderma* spp. leads to metabolic changes (Schweiger et al., 2021) and the onset of systemic resistance (Newman et al., 2013). Acquisition of nutrients is supported by plant-fungus interactions, also if the requirements for mycorrhiza are not fulfilled.

One major question of the last decades was how fungi sense the presence of a plant. Several years ago, a seminal study on plant recognition by the fungal pathogen *Fusarium oxysporum* provided groundbreaking insights in this respect (Turra & Di Pietro, 2015; Turra et al., 2015). It was shown that this fungus chemotropically responds to the presence of a plant and that this response is dependent on a pheromone receptor of the fungus, which obviously senses a peroxidase of the plant (Nordzieke et al., 2019; Turra et al., 2015). This research and the developed method opens up new possibilities to study plant fungus as well as other intra- and interkingdom interactions and their determinants (Turra et al., 2016).

Biocontrol of plant pathogens (He et al., 2021) is a complex mechanism involving processes from secretion of enzymes to production of secondary metabolites to mycoparasitism on the fungal pathogen (Karlsson et al., 2017). *Trichoderma* spp. as well as other fungi applied in agriculture as plant beneficial agents produce a broad array of secondary metabolites (Lehner et al., 2013; Zeilinger et al., 2016). Nevertheless, these fungi (except for a few more problematic species like *T. brevicompactum*) have a long history of safe application worldwide and no contamination of treated crops has been observed and hardly any negative effects on plants are known. Thereby, not only the fungi themselves, but also their secondary metabolites can be applied in agriculture (Vinale & Sivasithamparam, 2020). Moreover, investigation of secondary metabolites of *Trichoderma* and their functions bears the opportunity to identify novel, bioactive compounds potentially useful in medicine and industry (Shenouda & Cox, 2021). Interestingly, evolutionary analysis revealed that the core genome of *Trichoderma* species comprises about 7000 genes and that a considerable number of genes crucial to their well-known functions in litter degradation or secondary metabolism was acquired by horizontal gene transfer (Druzhinina et al., 2018; Druzhinina et al., 2016; Schalamun & Schmoll, 2022). This successful sourcing of advantageous genes by *Trichoderma* is assumed to be corroborated by their capability of mycoparasitism, which brings them in contact with foreign DNA (Druzhinina et al., 2018). Acquisition

of secondary metabolite clusters can be particularly beneficial for a biocontrol agent, due to their potential function in communication with the plant and/or fending of competitors or antagonizing pathogens.

Trichoderma harzianum B97 (Compant et al., 2017) was selected for its high efficiency in stimulation of plant growth. Moreover, the strain shows solubilization of phosphate and can alleviate abiotic stresses. Due to its proven efficiency in agricultural applications, it was the ideal isolate for studying its communication with a plant in more detail. We show that *T. harzianum* B97 chemotropically reacts to the presence of a plant and chemically communicates with a living plant as well. Moreover, we found that the ability of B97 to efficiently colonize plant roots depends on a secondary metabolite cluster specifically induced during early plant recognition and likely acquired by horizontal gene transfer.

Results

T. harzianum is an efficient colonizer of plant roots

T. harzianum B97 was isolated from agricultural soil in France (Compant et al., 2017) and selected for its plant beneficial characteristics. Good colonization of plants by the fungus would indicate efficient communication with the plant (Lareen et al., 2016). Therefore we studied, whether *T. harzianum* B97 would be able to efficiently colonize plant roots. We analyzed colonization after co-inoculation of wheat seedlings with *T. harzianum* B97. Staining of roots with wheat germ agglutinin (WGA)-AlexaFluor488 and confocal microscopy showed efficient colonization of roots by *T. harzianum* B97 (Figure 1A).

T. harzianum B97 shows chemotropic response to soybean root exudates

Recognition of the presence of the plant in the vicinity is crucial for initiation of interaction. Moreover, successful interaction with different plant species is a desirable trait for biocontrol agents. Recently, attraction of a *T. harzianum* strain to plant roots was shown (Lombardi et al., 2018). Hence we wanted to test first, whether *T. harzianum* B97 chemotropically reacts to the presence of root of soy plants. Optimization of the assay to *T. harzianum* B97 yielded an optimal working concentration of 0.0025 % peptone from casein to support germination without inducing multipolarity. We tested the response of *T. harzianum* B97 to 1 % (w/v) glucose, which yielded a chemotropic index of 9.66 ±1.15 % and was hence in the range seen previously for fungi (Turra et al., 2015). Subsequent analysis of chemotropic response to soybean root extracts showed a chemotropic index of 8.32% ± 0.15%, representing low but clearly present response (Figure 1B).

Secretion of secondary metabolites changes in the presence of soy bean roots

As *T. harzianum* B97 clearly reacts to the presence of the plant, we wanted to test whether chemical communication is initiated as a consequence of recognition. We used conditions as close as possible to those applied in the chemotropic assay, with a low level of carbon source (0.1 % (w/v) glucose) and minimal medium nutrients to support growth and still allow plant recognition. After 34 hours growth of *T. harzianum* B97, roots of soybean plants were placed in 3 cm distance of the fungal growth front and incubated for 13 hours in the darkness to enable communication. To ensure that communication occurs via the medium or via volatile organic compounds, but not due to direct contact, we only used plates where fungus and plants remained without physical contact at the time of harvesting. Thereafter, agar slices were excised from the area covered by the fungus for assessing changes in fungal metabolite profiles as well as from the area opposite of the plant root to analyze alterations in metabolites secreted by the plant (Figure 1C). Secondary metabolite patterns from the fungus grown without a plant and of the plant in the absence of the fungus under otherwise similar conditions were used as controls.
Indeed, after 13 hours of exposure of the fungus to the plant, we observed additional bands appearing in the high performance thin layer chromatography (HPTLC) analysis, reflecting reaction of the fungus to the plant (Figure 1D,E). Also the plant secreted additional compounds upon detection of the fungus, which were not present in the assay without the fungus (Figure 1D,E). Consequently, *T. harzianum* B97 initiates chemical communication with the soy plant roots within 13 hours of co-cultivation.

Transcriptome analysis of early stages of plant recognition

Having confirmed that plant recognition by *T. harzianum* B97 indeed occurs and elicits a two-way communication, we adapted transcriptome analysis to these conditions by covering the agar surface with cellophane to enable harvesting of the mycelium. Due to the high similarity of *T. harzianum* B97 with the previously sequenced reference strain *T. harzianum* CBS226.95 (Druzhinina et al., 2018) we refer to protein IDs from the respective public genome database at JGI (https://mycocosm.jgi.doe.gov/Triha1/Triha1.home.html) in the following.
Since transcript levels reflect investment of resources for functions important under a certain condition, we checked which functions were represented under the conditions used for secondary metabolite screening and transcriptome analyses. Functional category analysis of the 250 genes with highest transcript levels under the applied conditions showed considerable investments in metabolic functions, energy production and transport among others (Figure 2A.
Besides the expected enrichment in metabolic functions, energy metabolism and carbohydrate metabolism, we also found that the highest levels of transcript abundance were enriched in functions in stress response (p-value 1.96e-03) and unfolded protein response (p-value 7.89e-04). Additionally, also polysaccharide metabolism is significantly enriched in this highly transcribed gene set (p-value 2.49e-

11), with the homologues of the cellobiohydrolases *cbh1* and *cbh2* comprised in this group. These patterns reflect that the chosen condition indeed represents a condition of low nutrient availability inducing cellobiohydrolases likely due to starvation. Consequently, this condition closely resembles the conditions present in the chemotropic assay.

Specific gene regulation in the presence of a plant

Comparison of genes differentially regulated between growth alone on the plate and in the presence of a plant confirmed that our experimental setup captured a very early specific stage of plant recognition by *T. harzianum* B97 and likely represents the onset of communication. In total, only 102 genes were significantly (p-value <0.01) regulated more than 2fold (41 down, 61 up) upon recognition of the plant (supplementary file 1), which share functions in energy production, metabolism and transport (Figure 2B). Genes upregulated in the presence of the plant were enriched in functions of C-compound and carbohydrate metabolism (p-value 1.04e-03), glycolysis and gluconeogenesis (p-value 4.09e-03) and electrochemical potential driven transport (p-value 4.00e-03). Interestingly, the gene set down-regulated in the presence of the plant is enriched in functions in secondary metabolism (p-value 5.03e-04) and drug/toxin transport (p-value 4.26e-03).

Specifically, we detected a nitrate reductase (Triha_507858) and a nitrite reductase gene (Triha_507859) to be up-regulated 17fold or 4fold, respectively upon plant recognition. These genes represent the homologues of the *Aspergillus nidulans* genes *niiA* and *niaD*, which share a bidirectional promotor and play important roles in nitrogen uptake and metabolism (Brownlee & Arst, 1983; Johnstone et al., 1990). However, the putative homologue of *crnA*, the major facilitator superfamily (MFS) transporter gene associated with this cluster in *A. nidulans* (Triha1_142220) and the major transcription factor genes responsible for regulation of nitrogen metabolism, *areA* (Triha1_451) and *areB* (Triha1_70872), are not significantly differentially regulated under these conditions.

Considering fungus-plant interaction, also the more than 5fold up-regulation of Triha_398864, encoding a homologue of Epl1/Sm1 is interesting. These ceratoplatanin-like proteins play a role in colonization of plant roots and as effectors (Gao et al., 2020). In *T. harzianum*, Epl1 regulates virulence of the plant pathogen *Botrytis cinerea*, mycoparasitism as well as plant immunity at early stages of root colonization (Gomes et al., 2015; Gomes et al., 2017), which is in perfect agreement with our hypotheses.

Furthermore, transcript abundance of two predicted protease genes (Triha1_541862; 5.3fold down and Triha1_98848; 2.2fold down) is decreased in the presence of plant roots. As two putative terpene synthase genes, *tps1* (Triha1_497584; 2.8fold up) and *tps11* (Triha1_523651; 3.1fold up) are up-regulated, a role of terpenoid compounds in plant interaction is worth further investigation. Additionally,

an as yet uncharacterized non-ribosomal peptide synthase (NRPS, Triha_155805) and a putative polyketide synthase (PKS, Triha_546993) are more than 2 fold upregulated upon plant sensing.

A further, strongly upregulated gene (32fold) is Triha1_36398, which is still uncharacterized and its encoded protein comprises no known domains. However, analysis of putative protein interaction partners using the homologue of this protein in *T. reesei* using the STRING database ((Szklarczyk et al., 2021); https://string-db.org; version 11.5) suggests a connection to a predicted ferric reductase, which fits to its genomic vicinity next to a putative ferric reductase (Triha1_76871) in *T. harzianum*, which is also strongly upregulated and fits to the general picture of gene regulation in B97 upon plant recognition.

We conclude that the recognition of a plant in the environment causes *T. harzianum* to modulate secondary metabolism, but to also elevate certain metabolic capabilities. Although functions in C-compound and carbohydrate metabolism are enriched among up-regulated genes, this gene set does not include the common plant cell wall degrading enzymes. The high expression level of cellulases detected in all analyzed samples (see above) is not significantly altered upon recognition of a plant.

A secondary metabolite cluster strongly up-regulated upon plant recognition

Despite the low number of regulated genes, we still found a strongly regulated gene cluster (Figure 3A, B), which is silent when the fungus is growing alone, and strongly induced upon recognition of the plant with up to 1000-fold upregulation (Figure 3B; supplementary file 1). This gene cluster is located on scaffold 23: 272 000 – 286 000 in the reference strain *T. harzianum* CBS226.95 and comprises all seven genes present in this area. We termed the cluster *P*lant *C*ommunication *A*ssociated (PCA) cluster which is comprised of TH_323871/*pca1*, TH_513502/*pca2*, TH_513502/*pca3*, TH_99174/*pca4*, TH_513504/*pca5*, TH_513505/*pca6* and TH_513506/*pca7*. None of the genes in the cluster was previously characterized and hence the metabolite produced cannot be predicted.
Interestingly, also a further putative ferric reductase gene related to *pca1* is strongly up-regulated upon plant recognition (Triha1_76871; 21.2fold) as is a copper transporter gene closely related to *pca2* (Triha1_83588; 30.8fold). Both genes are not located in the genomic vicinity of the PCA cluster.

A SNP analysis of differences between *T. harzianum* B97 (Compant et al., 2017) and the publicly available sequence of *T. harzianum* (*sensu stricto*) CBS226.95 (Druzhinina et al., 2018) revealed no intragenic, no intergenic and no nonsynonymous SNPs in the region of the PCA cluster. Only one synonymous SNP was detected in 513506 and one in 513501 in the 5' UTR, hence strengthening the identification of *T. harzianum* B97 as *sensu stricto*. Consequently, we will refer to the *T. harzianum* CBS226.95 protein IDs and sequences hereafter.

In order to gain information on the potential function of the PCA cluster, we performed domain analysis and checked homologous genes in other fungi. PCA1 comprises a NADPH oxidase domain (cd06186) catalyzing the generation of reactive oxygen species (ROS) as well as a ferric reductase like transmembrane component (pfam01794) and is related to *A. fumigatus* FRE7, which is regulated by veA (Lind et al., 2015), upon response to Fe starvation (Kurucz et al., 2018) and during hypoxia (Losada et al., 2014). PCA2 contains a copper transporter domain (pfam04145), which may be involved in oxidative stress protection or pigmentation and is related to putative low affinity copper transporters in *Aspergilli*. PCA3 is a member of the transferase superfamily (cl23789), which comprises enzymes that catalyze the first committed reaction of phytoalexin biosynthesis, but also trichothecene 3-0-acetyltransferase. The *A. fumigatus* homologue, which is not closely related to PCA3, has a predicted role in festuclavine biosynthesis. PCA4 contains an NAD/NADP octopine/nopaline dehydrogenase (pfam02317) domain as well as a glutamate synthase or related oxidoreductase domain (cl28234), which may be involved in amino acid transport and metabolism. PCA4 has no homologues in *Aspergilli*. PCA5 is a major facilitator superfamily transporter (pfam07690), related to a cycloheximide resistance protein. PCA6 belongs to the superfamily of S-adenosylmethionine-dependent methyltransferases, class I (cl17173). PCA7 has a Cytochrome P450 domain (cl12078), which may be involved in the degradation of environmental toxins. The homologue of PCA7 in *A. nidulans*, STCF, is a putative sterigmatocystin biosynthesis P450 monooxygenase with a predicted role in sterigmatocystin/aflatoxin biosynthesis. However, we want to note here, that the genes within the PCA cluster likely originate from horizontal gene transfer from other fungi or even plants (see below) and that therefore these potential functions should be considered preliminary.

The cluster comprises multiple occurrences of a novel DNA motif

Due to the striking up-regulation of the PCA cluster genes upon plant recognition, we were interested whether a common DNA motif might be present in the promotors of the genes. To this end, we analyzed the promotors of all *pca* genes for promotor motifs using MEME. Interestingly, we found a palindromic motif (Figure 3A,C) in the intergenic regions between *pca1* and *pca2* as well as *pca3* and *pca4*. This motif is present twice in each region and was not yet characterized in fungi.

The PCA cluster is required for plant root colonization

We further asked whether the early and strong transcriptional response of the PCA cluster genes upon plant recognition would be predictive of a role in colonization, which is crucial for plant-fungus interaction and plant protection (Macias-Rodriguez et al., 2020). For construction of deletion strains we chose *pca1*, the putative NADPH oxidase, since previous work revealed an involvement of ROS – directly or indirectly – in plant-fungus interaction (Nordzieke et al., 2019; Turra et al., 2015).

Additionally, we prepared a null mutant of *pca5*, encoding a transporter potentially involved in signal compound emission. Both mutant strains were viable and showed no striking growth defects.

We tested the ability to colonize plant roots by inoculating soybean seeds with the mutant strains or the wildtype and evaluated the presence of fungal mycelia on young roots after 8 days (Figure 4). Confocal microscopy was performed from at least three replicate assays and multiple sites per root. Roots grown from uninoculated seeds were used as controls. This analysis showed that while the wildtype *T. harzianum* B97 efficiently colonized the root surface. Neither Δ*pca1* nor Δ*pca5* were able to colonize and the root grown from seeds inoculated with the mutant strains and these samples rather resembled the uninoculated control (Figure 4). We conclude that the requirement of these two genes of the PCA cluster is representative for the importance of this cluster and its upregulation upon plant recognition for efficient colonization of plants by *T. harzianum* B97.

The PCA cluster is specific to Trichoderma and was likely acquired by HGT

Because of the obvious importance of the PCA cluster for plant recognition and colonization, we were interested in its conservation and evolution in fungi. Since the genomic region of the cluster in B97 did not comprise a notable number of SNPs in comparison with *T. harzianum* CBS226.95, we will refer to the region in the latter strain in our further analyses and descriptions.

After identifying the genomic area of the PCA cluster in *T. harzianum*, we performed a blastn analysis with all dikarya genome sequences available at JGI (2140 genomes). These genome sequences cover the group of Sordariomycetes (528 genomes) very well, including numerous strains of the genus *Trichoderma*, which would allow for association to the well-studied clades of the genus (Atanasova et al., 2013). All other groups of fungi in JGI mycocosm outside dikarya (Grigoriev et al., 2014) were tested as well, but did not yield homologous sequences. Surprisingly, the search results did not reflect the expected relationships according to the known phylogeny of ascomycetes. Moreover, the cluster was not present in many *Trichoderma* species. Neither *Trichoderma* spp. outside of the Harzianum clade nor the common ancestor of the genus *Trichoderma*, *Escovopsis weberi* (de Man et al., 2016) or closely related species such as *Fusarium* spp. had this cluster, as revealed by only partial coverage of the cluster sequence in the genomes. Rather it was scattered among some species in the Sordariomycetes. However, good coverage of the cluster area was detected for *Metarhizium* species as well as for *Pestaliopsis fici* (Xylariales (Wang et al., 2015)) and *Talaromyces islandicus* (Eurotiomycetes (Schafhauser et al., 2015). Using the respective nucleotide sequences covering the whole clusters for phylogenetic analysis confirmed the close relationship of the cluster sequences (Figure 5). In case of *Metarhizium* spp. we found coverage of the cluster for *M. anisopliae*, *M. robertii* and *M. brunneum*, representing the generalist species of the genus (Sbaraini et al., 2016), but not in intermediate or specialist species, which is also the case for other secondary metabolite clusters in specialist species of *Metarhizium* (Sbaraini et al.,

2016). Interestingly, the PCA cluster was not detected previously in *Metarhizium* (Donzelli & Krasnoff, 2016) and does not overlap with the well characterized secondary metabolite clusters responsible for production of destruxin, ferricrocin or other known toxins (Donzelli & Krasnoff, 2016; Sbaraini et al., 2016). Furthermore, this finding suggested, that the PCA cluster was acquired by *T. harzianum* via horizontal gene transfer (HGT), likely from *Metarhizium* spp.

Individual genes of the PCA cluster presumably originate from plants and bacteria

Our detailed investigation of genome sequences confirmed that the full PCA cluster is indeed only present in the Harzianum clade (Atanasova et al., 2013) of *Trichoderma*, but not in the more ancestral Trichoderma clade or in the evolutionarily younger Longibrachiatum clade. These findings suggest that the PCA cluster was gathered from other organisms by HGT, likely after the split of the Harzianum clade from the Longibrachiatum clade in *Trichoderma*. We screened the identified genomic locations for the presence of the seven genes of the PCA cluster and found all of them in the same order and orientation in the Harzianum clade as well as in *Metarhizium* spp., which strengthens the hypothesis of HGT. In *P. fici* and *T. islandicus* the cluster is not complete, with *pca1* and *pca2* or *pca3* and *pca5* missing, respectively (Figure 6A). Individual genes of the cluster did show hits with blast searches in *Trichoderma* spp. outside of the Harzianum clade as well as in several other species, but they were not assembled in clusters.

We therefore asked whether the *pca* genes might be present in the genomes of other *Trichoderma* clades, yet not assembled in clusters. We performed phylogenetic analysis using at least three blast hits (if present and e-value <e-10; otherwise all available) for the predicted protein sequences encoded by each *pca* gene in various organisms. This analysis allowed us to delineate, whether in these species the cluster simply lost several components during evolution or if those blast hits merely represent unrelated genes with similar function. We used representatives of the Harzianum species complex (Chaverri et al., 2015) (*T. harzianum, T. guizhouense, T. afroharzianum*) and the Harzianum clade (*T. pleuroticola, T. aggressivum* and *T. virens*) (Chaverri et al., 2015). From the further clades we picked representatives from the Longibrachiatum clade (*T. reesei*), the Helicum clade (*T. helicum*) (Jaklitsch & Voglmayr, 2015), the Brevicompactum clade (*T. brevicompactum*) (Thomas Degenkolb et al., 2008) and the Trichoderma clade (*T. atroviride*) (Kubicek et al., 2019) as well as from *M. anisopliae, P. fici* and *T. islandicus* and other fungi having potential homologues in their genomes. Moreover, we added closely related sequences revealed as potential homologues from plants and bacteria as detected in blastp analysis using NCBI Blastp and excluding ascomycetes.

Interestingly, the latter blastp search on the NCBIdatabase yielded also highly related sequences to bacteria and plants for individual PCA proteins (Figure 6B), with *Quercus suber* (cork oak, plant)

homologues as best hit for PCA1, PCA2 and PCA7, basidiomycete proteins for PCA3 and PCA5 as well as bacterial proteins for PCA4 and PCA6, which was already a strong indication that the components of the cluster were assembled from different donor species.

This first exploratory phylogenetic analysis revealed the PCA proteins presumably connected by evolution and enabled us to distinguish those which clustered with proteins only similar, but not homologous to the PCA proteins and hence unrelated to the cluster. Indeed, the PCA homologues from species comprising the cluster formed a separate clade from those with similarity to the PCA sequences, but not organized in a cluster. In a second step we then used the closest hit of respective protein sequences from this sister clade for repeated phylogenetic analyses (Figure 7 A-G).

This analysis confirmed that only the *pca* genes from species having the cluster form a clade together, while *pca*-related genes scattered over the genome formed separate clades. Interestingly, *T. atroviride* seems to have homologues of PCA1 and PCA3 (Figure 7A, C), since the respective proteins cluster closely with the genes assembled in clusters in *T. harzianum* and not outside this clade. Additionally, we also found that hits from fungi outside ascomycetes, as well as those from plants or bacteria clustered among the fungal sequences or as sister clades hence confirming the blastp results. Consequently, acquisition of individual genes even from different kingdoms is likely, although the highly conserved structure of the cluster in *Metarhizium*, *Trichoderma* and in part *P. fici* and *T. islandicus* suggests assembly of the cluster in one of these genera. While we cannot provide evidence of the evolutionary path and chronological order of the gene transfer, or whether a birth and death evolution as reported for the SOR cluster in *T. reesei* (Druzhinina et al., 2016) has occurred, our results still strongly indicate that HGT contributed to acquisition and formation of the PCA cluster.

Discussion

Recognition of plants by fungi is crucial if the actual cry for help in the form of root exudates (Rolfe et al., 2019) is to be heard. Beneficial fungi of the genus *Trichoderma* positively impact plants at multiple levels, also using chemicals for achieving their effect (Macias-Rodriguez et al., 2020), but most importantly, they trigger the systemic immune response of plants (Guzman-Guzman et al., 2019; Harman et al., 2020). *T. harzianum* strains produce a number of secondary metabolites (Hanson, 2005), most of which are not yet assigned to specific biosynthetic gene clusters.

Although secondary metabolism and the gene clusters involved in biosynthesis of secondary metabolites are well studied in *Trichoderma* species (Keswani et al., 2014; Zeilinger et al., 2016), the plant recognition specific PCA gene cluster we found in this study was not described before. Consequently, it is currently not possible to associate a metabolite that might be produced by this cluster or probably modified. However, the enormous extent of the induction of this cluster, which exceeds the changes in transcript abundance of all other regulated genes, indicates that communication with the plant is

associated with this induction, which we confirmed with investigation of two crucial genes of the cluster (*pca1* and *pca5*). Moreover, the fact that the presence of a plant elicits a response in terms of secondary metabolite production is in agreement with the finding that the secondary metabolite pattern of *T. harzianum* B97 is altered in the presence of a plant.

The striking impact of deletion of members of the PCA cluster on colonization of soybean roots strongly supports a function of the associated secondary metabolite(s) in plant-fungus communication. Such communication is vital for beneficial interkingdom-interactions, which was also shown for *Trichoderma* (Macias-Rodriguez et al., 2020). The beneficial effects of *T. harzianum* B97 on plant health are extensive, facilitating commercial application, and may involve an influence of secondary metabolites – including those of the PCA cluster – on innate immunity as shown previously for *Trichoderma* (Newman et al., 2013). However, as root colonization is a prerequisite for efficient interaction, it can be concluded that the early stage recognition and communication represents the major function of the PCA cluster. This hypothesis is further strengthened by the very early induction of the cluster, within only 13 hours of proximity and without direct contact between plant and fungus.

A relevance of an NADPH oxidase involved in ROS production for plant-fungus interaction was previously shown for *T. atroviride* (Villalobos-Escobedo et al., 2020). For *F. oxysporum*, NADPH oxidase was found to be essential for chemotropic response to the presence of a plant (Nordzieke et al., 2019).

Our analysis of gene regulation patterns specific for early plant recognition by *T. harzianum* B97 revealed a significant enrichment of genes involved in detoxification by export, which is likely achieved by transporters. Therefore, we investigated the role of PCA5 in plant interaction and indeed found that this transporter is crucial for colonization. This finding is in agreement with the hypothesis, that the chemical communication with the plant as initiated by induction of the PCA cluster is dependent on export of secondary metabolites serving as signaling molecules by PCA5.

The PCA cluster comprises also three genes putatively involved in biosynthesis or modification of secondary metabolites. Among the genes regulated in response to the presence of the plant, we found several more putative permeases and transporters, but no gene which might encode a transcription factor. Hence, it remains to be demonstrated, how the coordinated induction of the PCA cluster genes is achieved.

One striking example of horizontal gene transfer (HGT) was described for the sorbicillin biosynthetic cluster in *T. reesei*, which was acquired from other fungi, but also other cases are known from this genus (Druzhinina et al., 2018; Druzhinina et al., 2016; Schalamun & Schmoll, 2022). An unexpectedly high

number of genes, including numerous carbohydrate-active enzymes (CAZymes) and plant cell wall degradation associated proteins in the *Trichoderma* genomes originates from other organisms. However, for plant associated fungi, like those of the genus *Trichoderma*, HGT seems to be a rather common phenomenon, especially concerning genes involved in production of secondary metabolites (Spatafora & Bushley, 2015).

Currently we cannot anticipate for the biosynthesis of which compound the PCA cluster is responsible and if it may be harmful or toxic, because secondary metabolite clusters related to the PCA cluster were not characterized before. However, since the problematic *T. brevicompactum*, which produces harmful toxins (T. Degenkolb et al., 2008), does not comprise the PCA cluster, it is unlikely that this cluster causes production of harmful chemicals during early plant recognition.

Interestingly, *T. harzianum* seems to have acquired the PCA cluster from *Metarhizium* (Sheng et al., 2022), which is a known endophyte (Sasan & Bidochka, 2012; Wyrebek et al., 2011) and an insect pathogen infecting hundreds of species (Roberts & Hajek, 1992; St Leger, 1993). In a tripartite interaction, *M. robertsii*, which comprises the PCA cluster, transfers nitrogen from insects they had infected to their plant hosts (Behie et al., 2012). A comparable situation was shown for *Laccaria bicolor* which associates with pine and spruce and transfers nitrogen from collembola in soil to the roots it colonizes (Klironomos & Hart, 2001). Recently, an impact of a strain belonging to *T. afroharzianum*, a species closely related to *T. harzianum*, on the interaction of a plant with pathogenic insect was shown (Di Lelio et al., 2023; Monte, 2023). Although this interaction was rather indirect via modulation of the gut microbiome, also numerous direct antagonistic effects on insects by *Trichoderma* are known (Poveda, 2021). Consequently, an ecological function of *Trichoderma* comparable to that of *Metarhizium* shown by Behie and colleagues (Behie et al., 2012) seems likely.
Since availability and uptake of different nitrogen sources considerably influences secondary metabolism (Tudzynski, 2014), the upregulation of the *niiA* and *niaD* homologues upon plant recognition in *T. harzianum* B97 may not only reflect nitrogen transport to the plant, but could also be a sign for increased efforts for production of specific secondary metabolites for plant communication. Notably, although several genes associated with secondary metabolism are down-regulated, besides the strong induction of PCA cluster genes, also an NRPS encoding gene and a PKS encoding gene are slightly upregulated. These obvious shifts in secondary metabolism are in agreement with the altered pattern we observed due to the presence of the plant.

Species of the Harzianum complex are supposed to be the most common endophytic species in tropical trees (Chaverri et al., 2011), with speciation leading to habitat preferences of soil or endophytism (Chaverri et al., 2015). Among the *Trichoderma* spp of the Harzianum clade, which comprise the PCA cluster, there are also endophytically growing ones like *T. endophyticum* and *T. afrasin* (Chaverri et al.,

2015). The upregulation of the *niiA* and *niaD* homologues, which are involved in nitrogen uptake upon recognition of the plant (*nit3* and *nit6*) indicates that nitrogen metabolism also may play a role in the interaction of *T. harzianum* with the plant, although potentially also other sources of nitrogen are used than by *Metarhizium*, which degrades killed insects to deliver nitrogen to the plant (Behie et al., 2012). Consequently, the PCA cluster is likely to support beneficial communication to the plant at an early stage of colonization, which may involve pretending to be an arriving endophyte delivering additional soil/organic nitrogen. Since the PCA cluster is crucial for colonization by *T. harzianum*, presence of this cluster in the fungus is highly likely to contribute to the high efficiency of members of this clade in biocontrol applications.

Accordingly, microbiome analysis revealed co-occurrence of *Trichoderma* and *Metarhizium* species in the rhizospere at high yielding field sites (Bandara et al., 2021) or with banana plants (Ciancio et al., 2022). Hence the mycoparasitism of *Trichoderma* may well have contributed to acquisition of the PCA cluster as was proposed for acquisition of the SOR cluster in *T. reesei* previously (Druzhinina et al., 2016).

In summary, we developed a strategy to simulate conditions of chemotropic response, which allowed us to detect a secondary metabolite cluster essential for communication with a plant enabling efficient colonization of the root surface (Figure 8). While revealing an intriguing new aspect of plant-fungus interaction, this finding can also be applied to evaluate the specificity of this regulation for prediction of high efficiency biocontrol capacity during strain screening. Thereby, the presence of the cluster as well as its high induction level represent promising features for diagnostic tests in strain screening programs. Additionally, identification of the compound produced by the PCA cluster, which facilitates colonization may support and enhance plant-fungus interaction of diverse biocontrol agents and enable plant-protection of plant varieties with otherwise insufficient response to *Trichoderma*-based biocontrol agents.

Materials and Methods

Strains and cultivation conditions

T. harzianum B97 (Compant et al., 2017) was used throughout the study. For RNA analysis, the strain was revived from long term storage on malt extract agar (3 % w/v). Plates containing modified Mandels Andreotti minimal medium (Mandels & Andreotti, 1978) with 0.1 % (w/v) glucose as carbon source were inoculated with 10 µl of spore solution (10^8 spores/ml) at 28 °C in constant darkness for 34 hours. The modified Mandels-Andreotti medium was prepared as follows: The mineral salt solution contained 2.8 g/l $(NH_4)_2SO_4$ (21.19 mM) (ROTH, Karlsruhe, Germany), 4.0 g/l KH_2PO_4 (29.39 mM) (Sigma-Aldrich, St. Louis, USA), 0.6 g/l $MgSO_4 \cdot 7H_2O$ (2.43 mM) (ROTH, Karlsruhe, Germany) and 0.8 g/l

$CaCl_2 \cdot 2H_2O$ (5.44 mM) (Merck, Darmstadt, Germany). The trace element solution contained 0.250 g/l $FeSO_4 \cdot 7H_2O$ (0.899 mM) (Sigma-Aldrich, St. Louis, USA), 0.085 g/l $MnSO_4 \cdot H_2O$ (0.503 mM) (Merck, Darmstadt, Germany), 0.070 g/l $ZnSO_4 \cdot 7H_2O$ (0.243 mM) (Riedel-de Haen, Seelze, Germany) and 0.143 g/l $CoCl_2 \cdot 6H_2O$ (0.603 mM) (Sigma-Aldrich, St. Louis, USA) and the pH was adjusted to 2.0 with concentrated sulfuric acid. Instead of phosphate buffer, milliQ water was used. The culture medium was prepared combining 500 ml mineral salt solution, 480 ml milliQ water, 20 ml trace element solution, 0.0025 % (w/v) peptone from casein (Merck, Darmstadt, Germany) for inducing germination, 0.1 % (w/v) D-glucose (ROTH, Karlsruhe, Germany) as carbon source (see pre-test for determination of glucose concentration) and 1.5 % (w/v) agar-agar (ROTH, Karlsruhe, Germany).

Plates were covered with cellophane in order to facilitate harvesting of mycelia. For recognition analysis, the roots of soy plants, which arrived second leaf stage, (19 days old, see below) were washed 4 times with sterile distilled water and were placed on the plates with *T. harzianum* B97 in 3 cm distance from the growth front. After further incubation for 13 hours (corresponding to the time for recognition in the chemotropic assay) in darkness, fungal mycelia were harvested for RNA isolation and agar slices from the same area were excised for evaluation of secreted metabolite production. As controls, plates with fungus but no plant and plates with plant but no fungus were used. Five plates each were pooled per sample and three biological replicates were used.

Construction of T. harzianum B97 deletion strains

Vectors for deletion of *pca1* and *pca5* were constructed by yeast recombination cloning using *hph* marker constructs with 1kb flanking regions as described previously (Schuster et al., 2012). Protoplast transformation was used for deletions in *T. harzianum* B97 parental strain with 10 mg/ml lysing enzymes (*Trichoderma harzianum*, Sigma # L-1412) and 150 µg/ml hygromycin B (Roth, Karlsruhe, Germany) for selection. Absence of the gene of interest was confirmed by PCR with primers binding inside the deleted region. A list of primers used is shown in Table 1.

Surface sterilization of seeds and in-vitro culture of soybean plants

The soybeans (*Glycine max* (L) Merr., variety ES TENOR, Die Saat, Austria) were obtained from RWA Austria. For the surface sterilization, the soybeans were soaked in 70 % ethanol for 1 minute and then rinsed 3 times with sterile distilled water. Afterwards, the soybeans were transferred into a sterile beaker containing Danklorix (2.8 % sodium hypochlorite (w/w), Colgate-Palmolive, Vienna, Austria) and Tween 20 (Roth, Karlsruhe, Germany) and stirred for 3 minutes. The soybeans were then washed five times with sterile distilled water. The excess water was removed by placing soybeans on sterile paper tissue.

For the pre-germination, the surface sterilized soybeans were placed on sterile paper tissue soaked with tap water and incubated at 26 °C and 16 hours light:8 h darkness for 5 days. The seedlings were transferred to the *in-vitro* culture, which contained diluted Murashige&Skoog (to 0.5 concentration; Duchefa Biochemie, Haarlem, The Netherlands) and 0.8 % (w/v) Daishin Agar (Duchefa Biochemie, Haarlem, The Netherlands) at pH 5.8. The *in-vitro* cultures of soybean plants were further incubated at the same condition for two more weeks.

Preparation of plant root exudates

After surface sterilization the soybeans were planted in sterilized perlite (premium perlite 2-6, Gramoflor GmbH, Germany). The soybeans were kept in a plant culture room at 26 °C with 16 hours light and 8 hours darkness. After approximately 3 days of emergence, the plantlets were then allowed to grow for further 2 weeks till the second leaf stage arrived. The plantlets were recovered from the perlite carefully and washed gently under running water to remove the perlite. Afterwards, at least 300 plantlet roots were submerged in 500 ml sterile milliQ water and kept for 2 days at room temperature. The obtained root exudates were filter sterilized through Thermo Scientific Nalgene Syringe Filter with 0.2 µM pore size and stored at -80 °C.

Isolation of total RNA

For isolation of total RNA, mycelium of the growth front from 5 replicate plates was pooled and frozen in liquid nitrogen. Three biological replicates were used with 5 pooled plates each. Samples were then treated as described previously (Tisch et al., 2011) using the QIAGEN plant RNA kit (QIAGEN, Hilden, Germany). RNA quality and integrity were checked using Bioanalyzer 2100 (Agilent). Only high quality RNA was used for further analyses.

Transcriptome analysis and bioinformatics

Sequencing of samples along with cDNA preparation was done at VetCORE (Vienna, Austria). The software BWA (Keel & Snelling, 2018) was used for mapping to the genome data of *T. harzianum* (JGI mycocosm; https://genome.jgi.doe.gov/Triha1/Triha1.home.html) (Druzhinina et al., 2018). The software samtools was used for data processing (Li et al., 2009) and the limma package as implemented in R (Ritchie et al., 2015) was used for determination of statistically significant differential expression (>2fold, p-value threshold 0.01). Besides manual annotation of differentially expressed genes, annotations of bidirectional best hits with *T. reesei* and/or *T. atroviride* (Schmoll et al., 2016) were used. Comparison of gene expression patterns between biological replicates yielded significance scores of

≥0.979 for both sample sets. Sequence data are available at NCBI GEO (Gene expression omnibus) under the accession number GSE229209.

HCE3.5 (Seo et al., 2006) was applied to perform hierarchical clustering with default settings and the Poisson correlation coefficient as the similarity/distance measure. FunCat (Functional category) analysis was done with the FungiFun2 online tool (Priebe et al., 2015) based on bidirectional best hit analysis with *T. atroviride*.

Analysis of chemotropic responses

The freshly grown spores from a 4 days old culture were recovered and dissolved in 1 ml spore-solution (0.8 % NaCl and 0.05 % Tween 80). After separation of mycelia by centrifugation through glass wool, the spore solution was centrifuged at 8000 rpm for 2 minutes, the supernatant was discarded, and the spore pellet was resuspended in 1 ml sterile milliQ water. For the chemotropism assay, the spore solution was adjusted to 10^8 spores per ml with sterile milliQ water. The peptone from casein (Merck, Darmstadt, Germany) was used as germination stimulator in 0.5 % water agar. The concentration of peptone from casein was optimized to 0.0025 % (w/v). After 13 hours of incubation at 28°C in darkness, germling orientation was monitored and chemotropic index was calculated as described earlier (Turra et al., 2015).

Analysis of patterns of secreted metabolites

Analysis for alteration of secondary metabolite patterns in the presence of a soy plant was essentially done as described previously (Bazafkan et al., 2015; Hinterdobler et al., 2019). Therefore the same conditions as applied for transcriptome analysis as outlined above were used. Application of high performance thin layer chromatography (HPTLC) and data visualization was performed as described in (Bazafkan et al., 2015) except that separation was done with chloroform and 1 mM trifluoroacetic acid in methanol.

Analysis of colonization by T. harzianum B97 and recombinant strains

Seeds were surface sterilized in 70% ethanol for 7 minutes and rinsed for 3 minutes with sterile milliQ water. Seeds were then put onto MEX (malt extract) plates containing either *T. harzianum* B97, B97Δ*pca1*, B97Δ*pca5* or only MEX without fungus as negative control. Seeds were then placed in sterile magenta boxes containing soil mixture (1:1:1 perlite, sand, potting soil and 25 ml of sterilized tap water), which was autoclaved twice. After 8 days at 22°C under 12 hours light:12 hours darkness conditions, plants were harvested, and roots stained in 15ml phosphate buffer saline (PBS, pH 7.2) containing 5µg/ml wheat germ agglutinin (WGA)-AlexaFluor488 conjugate (Life Technologies, USA) and incubated for 2 hours at 37 °C before rinsing three times with PBS.

All observations were carried out using a confocal microscope (Olympus Fluoview FV1000 with multi-line laser FV5-LAMAR-2 and HeNe(G)laser FV10-LAHEG230-2, Japan). Observations with the confocal microscope were done at objectives of 10x, 20x and 40x. Between 20 and 40 X, Y, Z pictures containing 20 to 60 scans were separately taken at 405, 488, 549 nm wavelengths in blue/green/orange-red channels respectively, with the same settings each time and normal light. The image analysis software Imaris software used at the confocal microscope to visualize 3D reconstructions. X, Y, Z pictures from different channels were then merged using the Image J software (version 1.47v), and Z project stacks were then used to create the pictures as described earlier (Pierron et al., 2015).

Phylogenetic analysis

For phylogenetic analysis, protein and nucleotide sequences were obtained from the NCBI nr database or the genome sequences available at JGI mycocosm. Sequences were aligned using Clustal X or MEGA7 (Kumar et al., 2016; Thompson et al., 1997) with default parameters. MEGA7 was used for phylogenetic analysis using standard parameters and the Maximum likelihood method and 1000 bootstrap cycles.

Author contributions

MiS performed gene deletion and confocal microscopy, GL performed chemotropism analysis and RNA isolation, WH performed secondary metabolite analysis, SC supervised confocal microscopy, DG supported colonization analysis, ADZ contributed to editing of the manuscript, MoS conceived the study, performed transcriptome analysis and wrote the final version of the manuscript. All authors read the manuscript and agreed to publication.

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Conflicts of interest

The study was in part funded by Greencell, France.

Availability of data

All data used for this study are available in the manuscript, its supplementary file and at the NCBI GEO online repository under accession number GSE229209.

Tables

Table 1. Oligonucleotides used in this study

Name	**Function**	**Sequence 5' - 3'**	**Protein ID**	**targtet gene**
Bpca1del5F	creation of 5' flank of deletion cassette	5’GTAACGCCAGGGTTTTCCCAGTCACGACGATGGTGGTGATTGTTGTG 3‘	323871	*pca1*
Bpca5del5R	creation of 5' flank of deletion cassette	5’ATCCACTTAACGTTACTGAAATCTCCAACGGTAAATGCGTTTCAAAG 3‘	323871	*pca1*
Bpca1del3F	creation of 3' flank of deletion cassette	5’CTCCTTCAATATCATCTTCTGTCTCCGACATTAAATGATACACAGGCTG 3‘	323871	*pca1*
Bpca1del3R	creation of 3' flank of deletion cassette	5’GCGGATAACAATTTCACACAGGAAACAGCCATTGTCATCTGCAGTAGAC 3‘	323871	*pca1*
Bpca1screF	confirmation of deletion	5’GGATGGACCTTACCCTTTATCG 3‘	323871	*pca1*
Bpca1screR	confirmation of deletion	5’ACCACAAACGAGTGCTGAAATC 3‘	323871	*pca1*
Bpca5del5F	creation of 5' flank of deletion cassette	5’GTAACGCCAGGGTTTTCCCAGTCACGACGTTGTCCGTTGTCCTATGGC 3‘	513504	*pca5*
Bpca5del5R	creation of 5' flank of deletion cassette	5’ATCCACTTAACGTTACTGAAATCTCCAACATCGCTTATTCGTTCGCAG 3‘	513504	*pca5*
Bpca5del3F	creation of 3' flank of deletion cassette	5’CTCCTTCAATATCATCTTCTGTCTCCGACGGTCCATTTGATAATAGAGAAG 3‘	513504	*pca5*

Bpca5del3R	creation of 3' flank of deletion cassette	5’GCGGATAACAATTTCACACAGGAAACAGCCTATTCACACCCAGAGCAC 3‘	513504	*pca5*
Bpca5screF	confirmation of deletion	5’CCGACGCAGGAAAGAAAC 3‘	513504	*pca5*
Bpca5screR	confirmation of deletion	5’ACAGATGTAGACGCAGCTGG 3‘	513504	*pca5*

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Figures

Figure 1

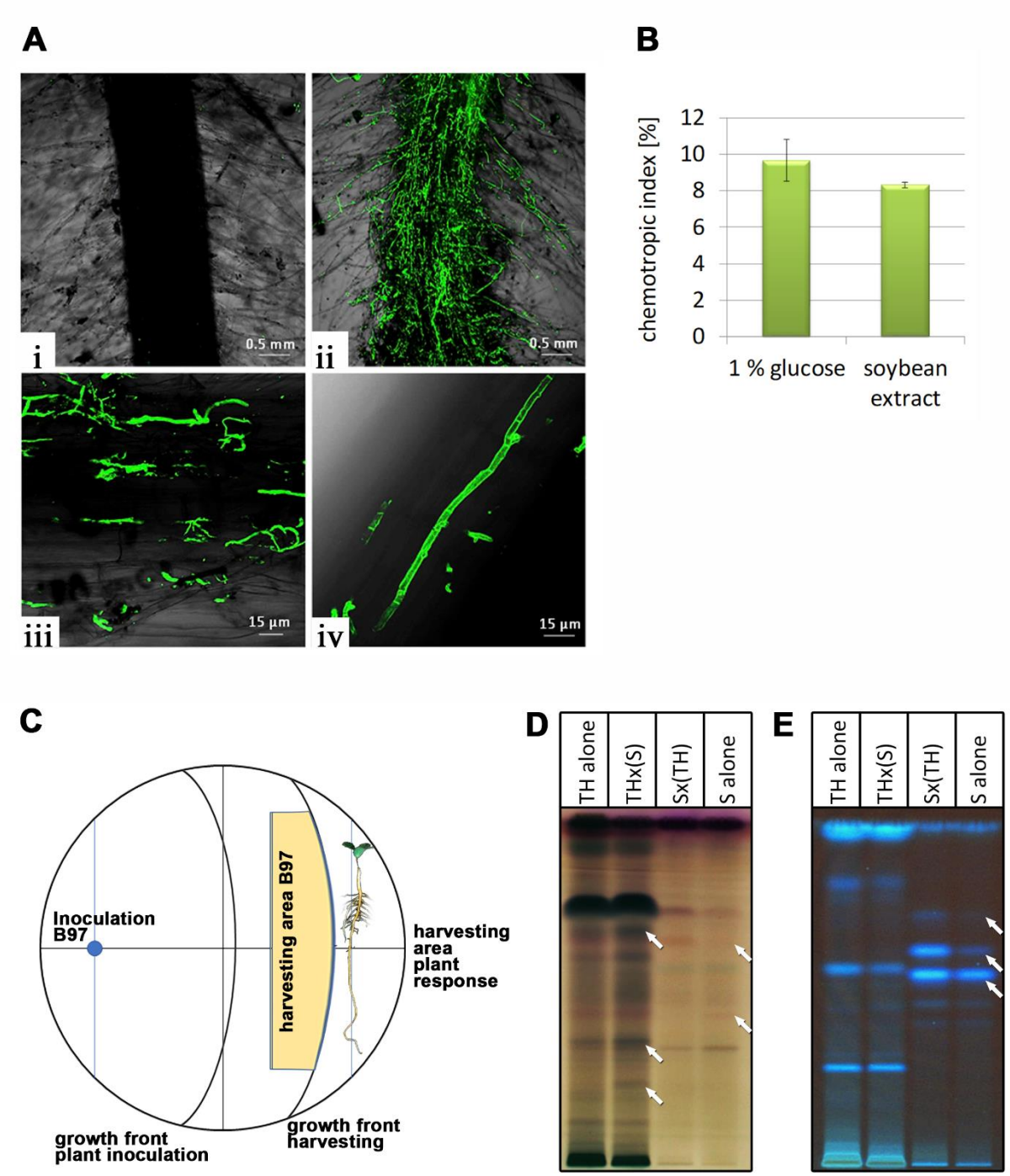
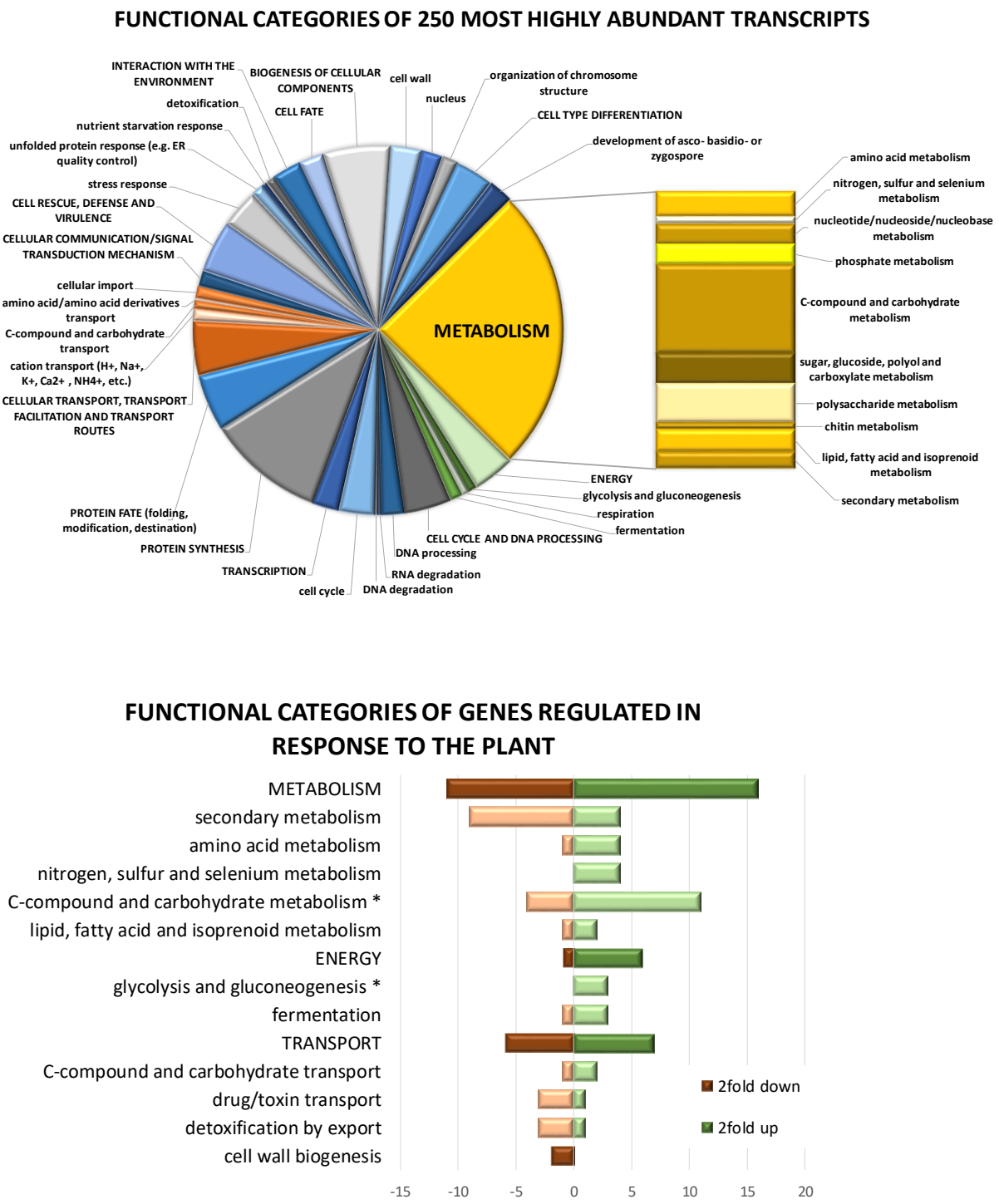


Figure 1. **Assessment of *T. harzianum* B97 – plant interaction**. (A) CSLM Microphotograph of uninoculated control roots (i) and roots with *T. harzianum* B97 (ii-iv) at the root hair zone and stained with WGA-Alexa Fluor488® showing B97 as green fluorescent colonizing root hairs (iii) or the root surface (iv). (B) Chemotropic indices of *T. harzianum* B97 to the presence of 1 % (w/v) glucose or root exudates of soy plant. Analyses were done in biological duplicates, at least 400 germlings were counted

per experiment. (C) Schematic representation of the experimental setup for analysis of plant-fungus communication. Plants were allowed to interact with the fungus for 13 hours and harvesting was done before contact. Mycelia for investigation of the transcriptome was isolated from the mycelial growth front ("harvesting area B97", yellow). For secondary metabolite analysis by HPTLC the agar slice including cellophane overlay from exactly the same area was excised. For analysis of the response of the plant an agar slice on the other side of the root was excised in order to avoid interference with fungal metabolites. For control plates the setup and positioning of harvesting areas was exactly the same. (D, E) HPTLC analysis of *T. harzianum* B97 alone on the plate (TH alone), *T. harzianum* B97 in the presence of the root of soy plant (THx(S)), the root of soy plant in the presence of the fungus (Sx(TH)) and the root of soy plant alone (S alone). Two different visualizations are provided (D: Visible light after anisaldehyde derivatization and E: Remission at 366 nm) and show differentially secreted metabolites between interaction partners alone and in combination.

1053 **Figure 2**



1054

1055 Figure 2 **Functional analysis of gene expression in *T. harzianum* B97**. (A) Functional categories
1056 represented among the 250 most abundant transcripts under the conditions of simulated chemotropic
1057 response. (B) Major functional categories assigned to genes differentially regulated in the presence of
1058 a soy plant. Significantly enriched categories are marked with an asterisk.

1059

Figure 3

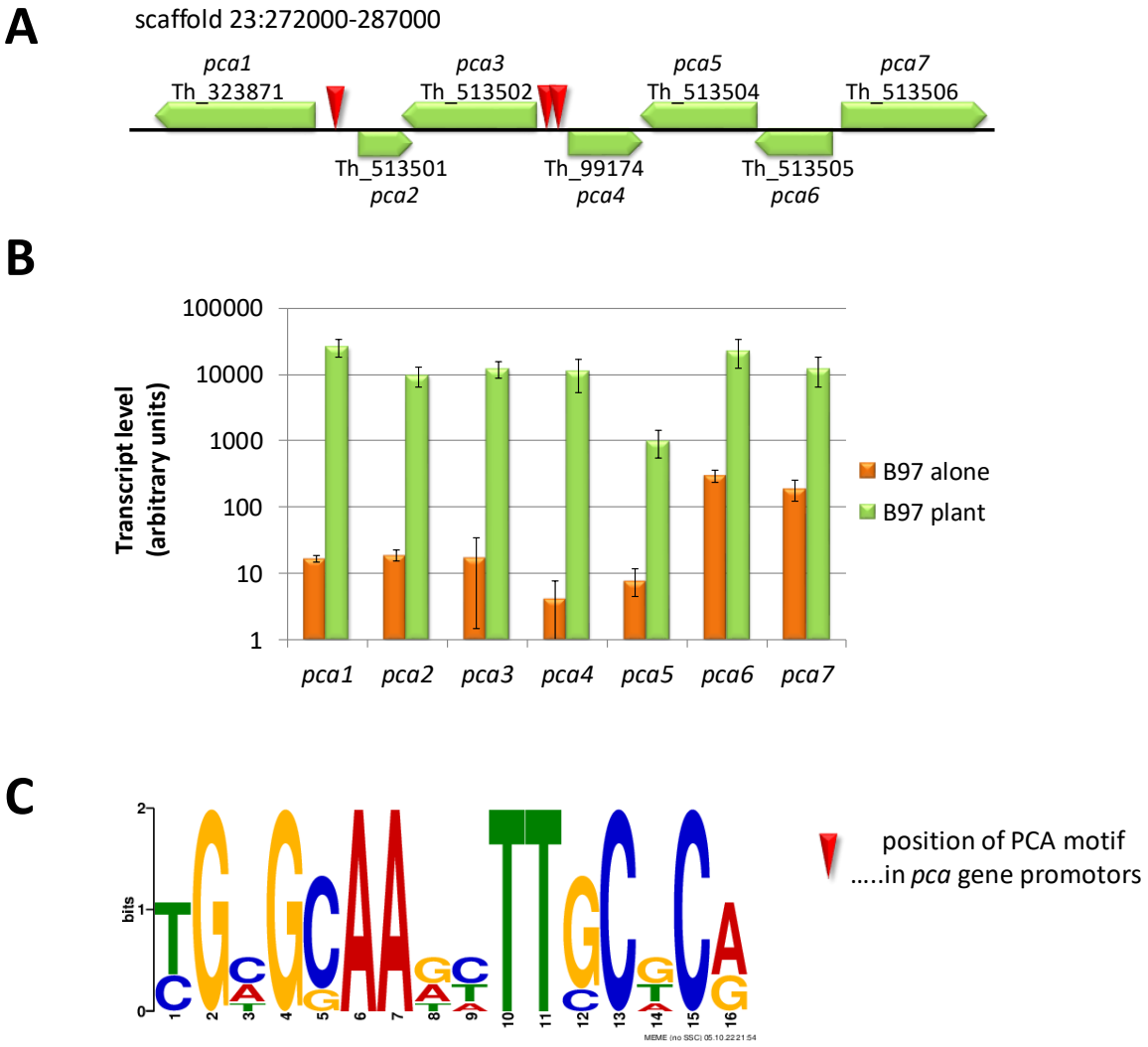


Figure 3. **Schematic representation of the PCA cluster (A) and its regulation upon recognition of the plant (B).** (A) Localization of *pca*-genes in the *T. harzianum* genome (JGI mycocosm; https://mycocosm.jgi.doe.gov/mycocosm/home). Approximate position of the PCA-DNA motif is shown with red triangles. (B) RPKM values of transcript levels of *pca* genes upon growth alone on the plate (orange bars), where transcripts were at very low basal levels (logarithmic scale is shown). Green bars represent transcript abundance upon recognition of the plant. Values represent means of three biological replicates and error bards show standard deviations. In all cases differential regulation is statistically significant (p-value <0.01) (C) PCA motif as found in the potentially bi-directional promotors of *pca1*/*pca2* and *pca3*/*pca4*.

Figure 4

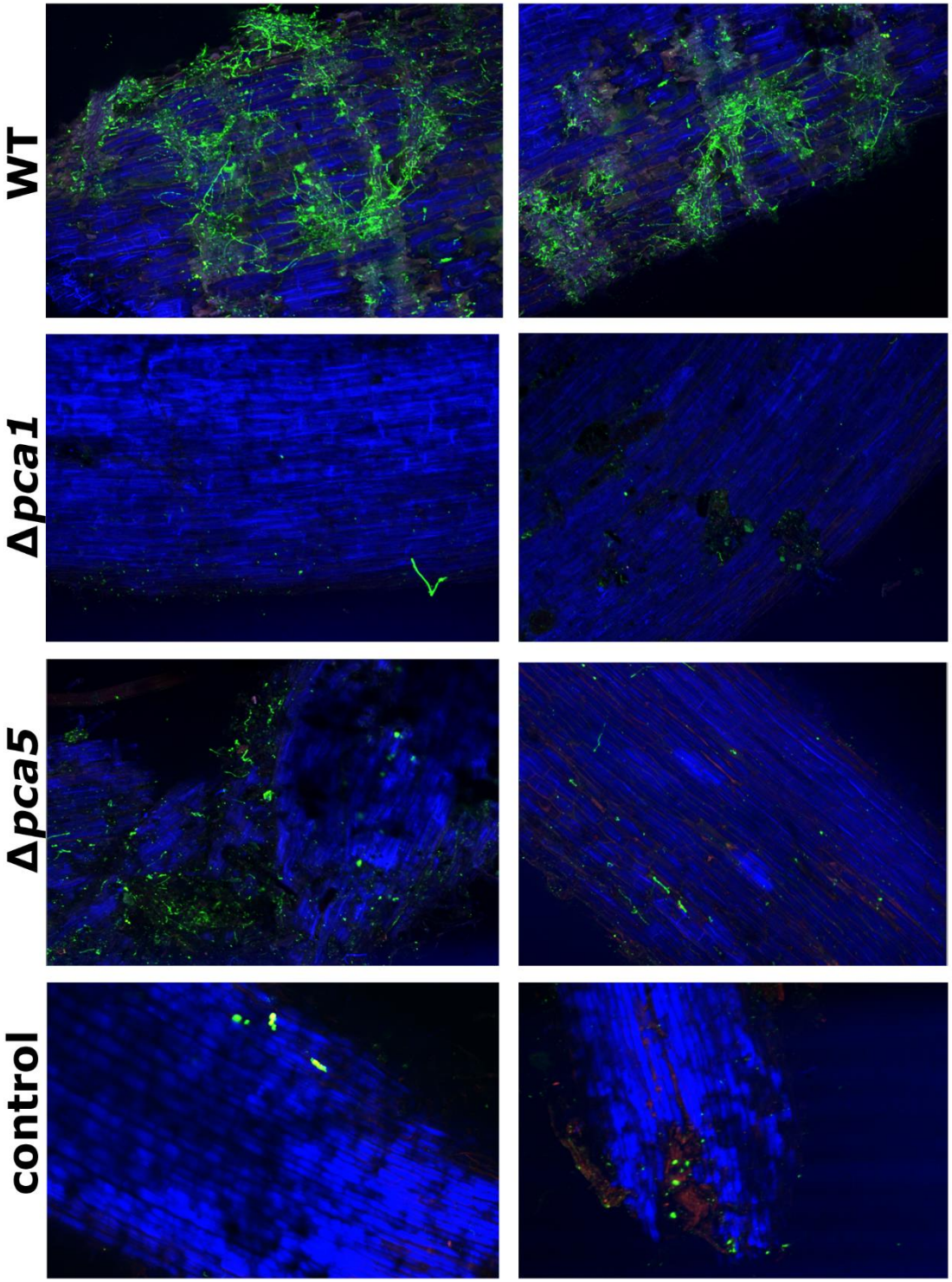


Figure 4. **Colonization of soybean roots by *T. harzianum* B97 wildtype and mutant strains Δ*pca1* and Δ*pca5***. Uninoculated roots were used as control. Fungal mycelia on the soybean roots were stained

with WGA-Alexa Fluor488®. CLSM micrographs are showing B97 hyphae as green fluorescent colonizing the roots of wildtype but hardly detectable with both mutant strains.

Figure 5

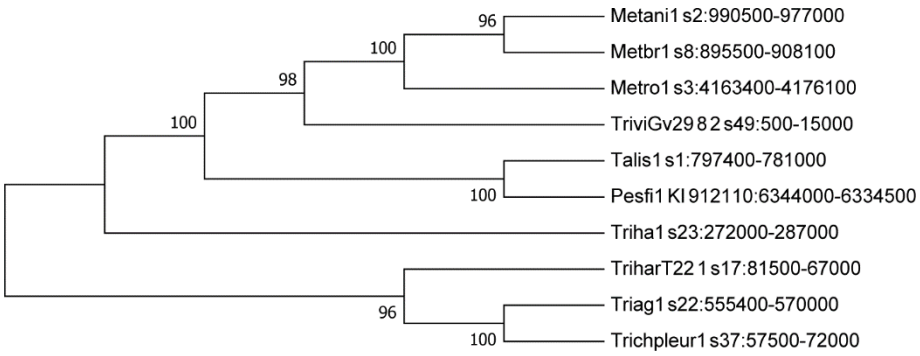
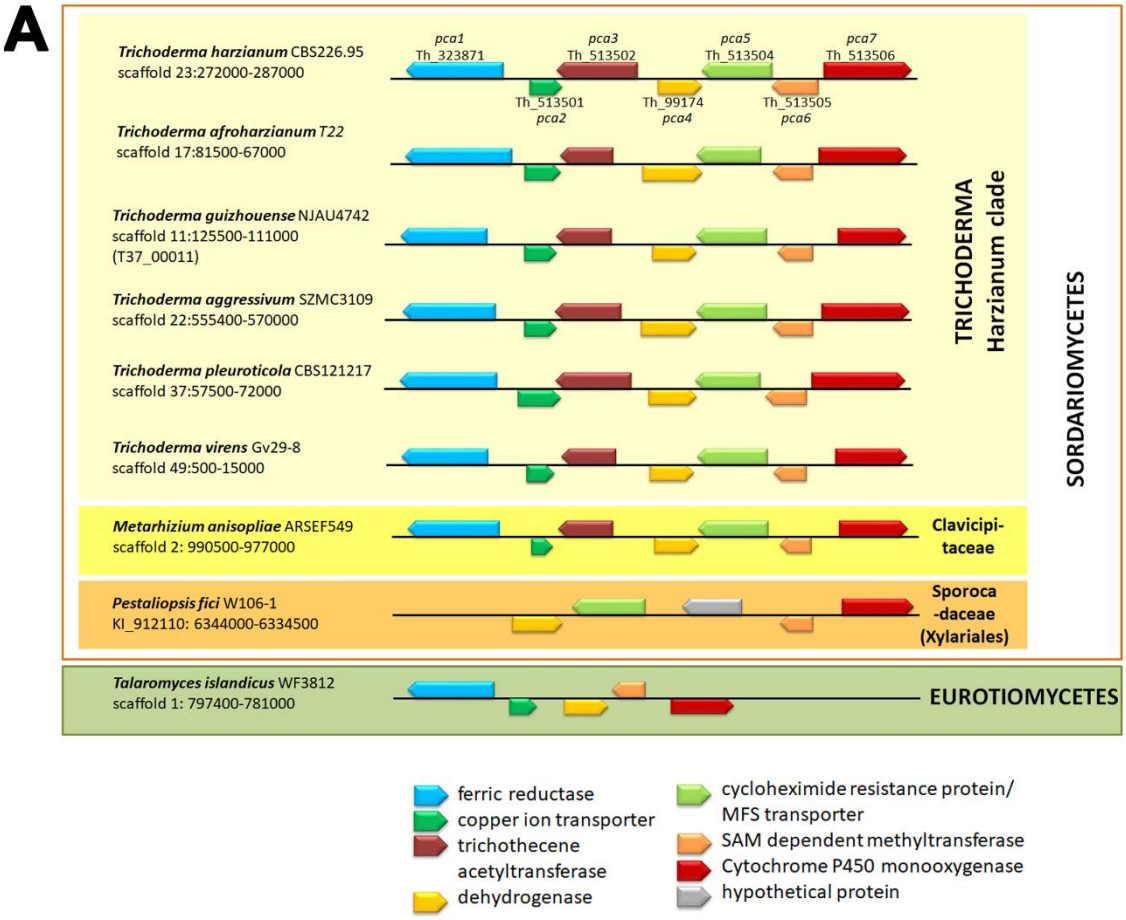


Figure 5 **Molecular phylogenetic analysis of the cluster sequences by Maximum Likelihood method.**

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 7697 positions in the final dataset.

Figure 6



B

organism	PCA1			PCA2			PCA3			PCA4			PCA5			PCA6			PCA7			
	e-value	id [%]	cov [%]	e-value	id [%]	cov [%]	e-value	id [%]	cov [%]	e-value	id [%]	cov [%]	e-value	id [%]	cov [%]	e-value	id [%]	cov [%]	e-value	id [%]	cov [%]	
Quercus suber	**0.0**	**55.45**	**99**	**5,E-40**	**54.48**	**74**													**1,E-81**	**36.89**	**88**	plants
Carpinus fangiana				2,E-20	40.86	81													5,E-65	34.72	78	plants
Daedalea quercina	5,E-159	43.28	98																			basidiomycetes
Flammula alnicola							3,E-179	57.55	99													basidiomycetes
Fomes fomentarium	9,E-106	34.27	99																			basidiomycetes
Fomitiporia mediterranea													6,E-82	35.86	97				2,E-58	31.16	90	basidiomycetes
Galerina marginata							1,E-169	53.55	100										4,E-56	31.61	92	basidiomycetes
Laccaria bicolor													2,E-85	37.59	97							basidiomycetes
Mycena venus	6,E-90	33.03	99										**2,E-133**	**51.44**	**94**							basidiomycetes
Pleurotus eryngii													5,E-82	37.16	97				2,E-57	28.51	91	basidiomycetes
Puccinia graminis				6,E-14	31.15	93																basidiomycetes
Ramaria rubella							**0.0**	**57.08**	**100**	6,E-48	31.73	96										basidiomycetes
Volvariella volvacea	2,E-120	36.86	99																			basidiomycetes
Acinetobacter baumannii				9,E-19	38.46	72																bacteria
Bradyrhizobiom sp.										**4,E-92**	**42.37**	**97**										bacteria
Deltaproteobacteria bacterium	5,E-132	50.36	62	1,E-18	49.02	54										**4,E-58**	**44.02**	**98**				bacteria
Methylocaldum marinum																2,E-53	40.15	98				bacteria
Paraburkholderia sprentiae										3,E-84	39.37	95										bacteria
Sorangium cellulosum																4,E-50	39.77	98				bacteria

Figure 6 **The PCA cluster and proteins related to its components**. (A) Schematic representation of the clusters detected within selected representatives of *Trichoderma* spp. and outside the genus *Trichoderma*. (B) Blastp results of the respective protein sequences from *T. harzianum* B97 against the NCBI nr database with ascomycetes excluded. The top 100 hits for PCA1-7 were screened for most interesting similarities for this table.

Figure 7

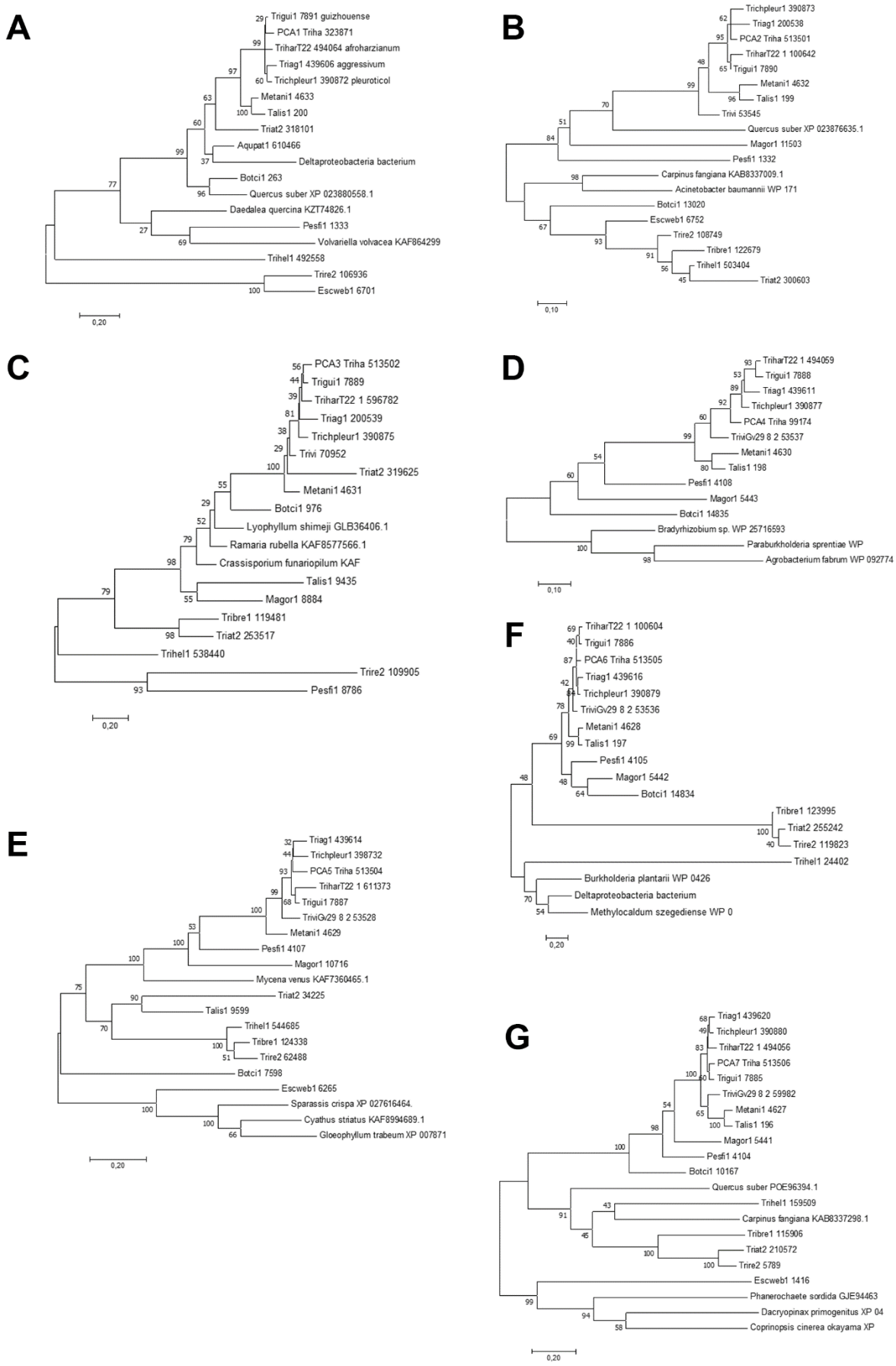

A
Trigui1 7891 guizhouense
PCA1 Triha 323871
TriharT22 494064 afroharzianum
Triag1 439606 aggressivum
Trichpleur1 390872 pleuroticol
Metani1 4633
Talis1 200
Triat2 318101
Aqupat1 610466
Deltaproteobacteria bacterium
Botci1 263
Quercus suber XP 023880558.1
Daedalea quercina KZT74826.1
Pesfi1 1333
Volvariella volvacea KAF864299
Trihel1 492558
Trire2 106936
Escweb1 6701
0,20
B
Trichpleur1 390873
Triag1 200538
PCA2 Triha 513501
TriharT22 1 100642
Trigui1 7890
Metani1 4632
Talis1 199
Trivi 53545
Quercus suber XP 023876635.1
Magor1 11503
Pesfi1 1332
Carpinus fangiana KAB8337009.1
Acinetobacter baumannii WP 171
Botci1 13020
Escweb1 6752
Trire2 108749
Tribre1 122679
Trihel1 503404
Triat2 300603
0,10
C
PCA3 Triha 513502
Trigui1 7889
TriharT22 1 596782
Triag1 200539
Trichpleur1 390875
Trivi 70952
Triat2 319625
Metani1 4631
Botci1 976
Lyophyllum shimeji GLB36406.1
Ramaria rubella KAF8577566.1
Crassisporium funariopilum KAF
Talis1 9435
Magor1 8884
Tribre1 119481
Triat2 253517
Trihel1 538440
Trire2 109905
Pesfi1 8786
0,20
D
TriharT22 1 494059
Trigui1 7888
Triag1 439611
Trichpleur1 390877
PCA4 Triha 99174
TriviGv29 8 2 53537
Metani1 4630
Talis1 198
Pesfi1 4108
Magor1 5443
Botci1 14835
Bradyrhizobium sp. WP 25716593
Paraburkholderia sprentiae WP
Agrobacterium fabrum WP 092774
0,10
E
Triag1 439614
Trichpleur1 398732
PCA5 Triha 513504
TriharT22 1 611373
Trigui1 7887
TriviGv29 8 2 53528
Metani1 4629
Pesfi1 4107
Magor1 10716
Mycena venus KAF7360465.1
Triat2 34225
Talis1 9599
Trihel1 544685
Tribre1 124338
Trire2 62488
Botci1 7598
Escweb1 6265
Sparassis crispa XP 027616464.
Cyathus striatus KAF8994689.1
Gloeophyllum trabeum XP 007871
0,20
F
TriharT22 1 100604
Trigui1 7886
PCA6 Triha 513505
Triag1 439616
Trichpleur1 390879
TriviGv29 8 2 53536
Metani1 4628
Talis1 197
Pesfi1 4105
Magor1 5442
Botci1 14834
Tribre1 123995
Triat2 255242
Trire2 119823
Trihel1 24402
Burkholderia plantarii WP 0426
Deltaproteobacteria bacterium
Methylocaldum szegediense WP 0
0,20
G
Triag1 439620
Trichpleur1 390880
TriharT22 1 494056
PCA7 Triha 513506
Trigui1 7885
TriviGv29 8 2 59982
Metani1 4627
Talis1 196
Magor1 5441
Pesfi1 4104
Botci1 10167
Quercus suber POE96394.1
Trihel1 159509
Carpinus fangiana KAB8337298.1
Tribre1 115906
Triat2 210572
Trire2 5789
Escweb1 1416
Phanerochaete sordida GJE94463
Dacryopinax primogenitus XP 04
Coprinopsis cinerea okayama XP
0,20

Figure 7 **Molecular phylogenetic analysis of the PCA cluster genes by the Maximum Likelihood method.** Maximum likelihood trees of homologues of (A) PCA1, (B) PCA2, (C) PCA3, (D) PCA4, (E) PCA5, (F) PCA6 and (G) PCA7 are shown. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Figure 8

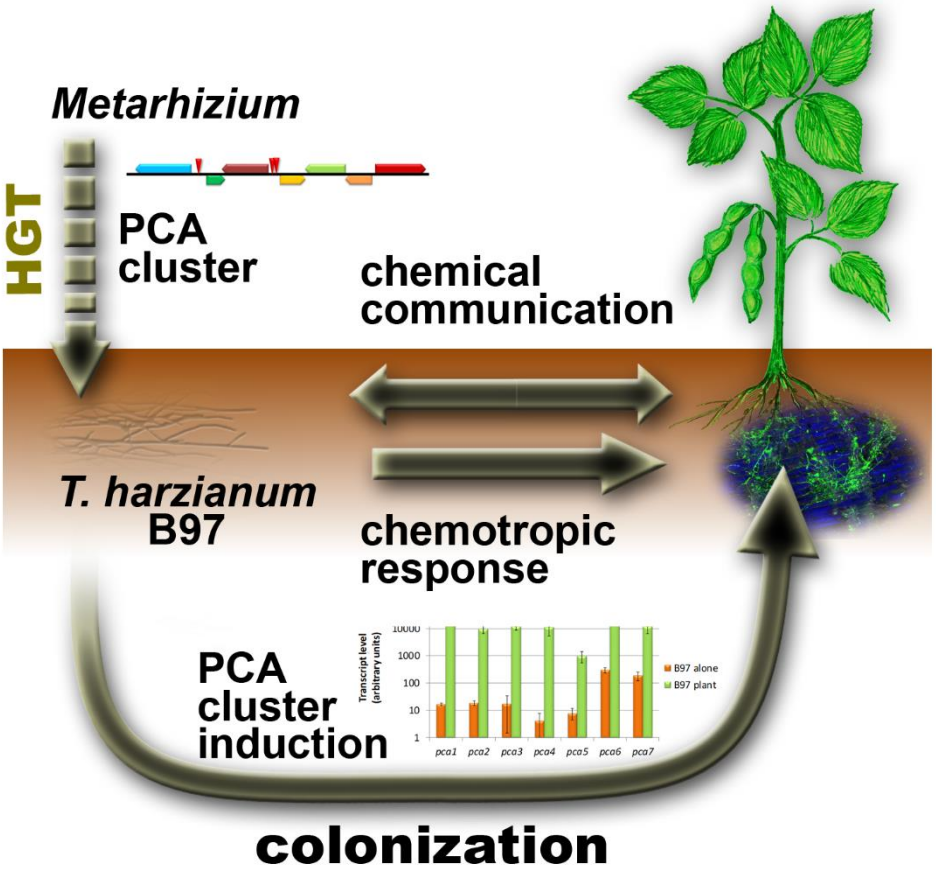


Figure 8 **Schematic representation of the reaction of *T. harzianum* B97 to soy bean.** *T. harzianum* chemotropically responds to the presence of a soy plant. Chemical communication occurs both ways due to alteration of the secondary metabolite pattern of both *T. harzianum* B97 and the soy plant. The PCA gene cluster, which was likely acquired by HGT from *Metarhizium*, is strongly induced upon plant recognition and essential for effective colonization of plant roots.