Impact of mycorrhiza helper bacterium *Streptomyces sp.* AcH 505 on the genetic and physiological regulation in oaks associated to pathogenic and symbiotic fungi

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This thesis was performed within the research project "TrophinOak", which addresses the impact of multitrophic interactions on the pedunculate oak (*Quercus robur*) clone DF159. In this frame, the present work focuses on the genetic and physiological mechanisms ruling the interaction of the mycorrhiza helper bacterium (MHB) *Streptomyces* sp. AcH 505 with microcuttings of DF159 either alone or in presence of the ectomycorrhizal fungus *Piloderma croceum* or the fungal leaf pathogen oak powdery mildew *Microsphaera alphitoides*. The work consists of 3 chapters.

Chapter 1 characterises the growth of AcH 505 and *P. croceum* in a soil-based culture system used within the TrophinOak project. Besides the establishment and evaluation of quantification methods of these microorganisms by quantitative real-time PCR, the impact of the soil microbial community and the oak on the bacterium-fungus interaction was investigated, and AcH 505 and *P. croceum* were visualized by scanning electron microscopy. It was observed that the presence of the soil microorganisms and the oak both affect the bacterium-fungus interaction, and that *P. croceum* enhances the growth of AcH 505.

Chapter 2 presents a study with the oak, AcH 505 and the EM fungus *P. croceum*, enabling to disentangle the direct effect of the MHB on the oak from the indirect one via the EM symbiosis. The used approach was transcriptomic based on RNA sequencing. It was shown that i) differential gene expression occurred between root and the distant leaf tissues (local vs. systemic effects), different developmental stages and treatments, suggesting that oak specifically coordinates its gene expression patterns, and ii) that genes related to plant growth, defence and DNA modification were dominant among the differential expressed genes, suggesting that these processes play essential roles in both symbiotic interactions investigated.

Chapter 3 represents a second transcriptome study, addressing how AcH 505 suppresses powdery mildew infection in oak by analysing RNA Sequencing data from singly- and co-inoculated oaks. This study combined the systemic impact of the root associated bacterium with local effects of the leaf pathogen, thereby linking belowground and aboveground interactions. Systemic defence response is induced by the bacterium and further enhanced upon pathogen challenge, suggesting that on the leaf level, some bacterial effectors are recognized as harmful for the plant.

INTRODUCTION

1. Ecological background

The roots of most trees of temperate ecosystems live in a mutualistic association with ectomycorrhizal (EM) fungi. The resulting symbiosis affects the plant in many different ways. The most important aspect is the improved nutrition of the plant, but various other influences, such as increased resistance to pathogens and to abiotic stresses, have been listed as well (Smith and Read, 2008). Although the symbiosis is often considered a binary plant-fungus interaction, further organisms are also associated (Frey-Klett and Garbaye, 2005). These associated organisms are known to interact within the mycorrhizosphere (Foster and Marks, 1967), the soil compartment including the mycorrhizal roots and the external fungal mycelium. One important function of mycorrhizosphere bacteria is the stimulation of mycorrhiza formation. Bacterial strains exhibiting this property have been termed Mycorrhiza Helper Bacteria (MHB; Garbaye, 1994). MHB and mycorrhizal fungi have been studied with respect to their combined beneficial impacts on plants, in particular related to the enhancement of phosphorus and nitrogen acquisition (Frey-Klett et al., 2007).

1.1. Common oak *Quercus robur* and ectomycorrhizal fungus *Piloderma croceum*

Oaks belong to the family of the *Fagaceae* and are a source of raw materials such as wood, fibres and cork. Besides this economic importance, oaks display a major ecological role. Due to their longevity and their wide geographic distribution, they interact with many organisms below and above ground and therefore represent key drivers of terrestrial biodiversity (Brandle and Brandl, 2001). As dominant primary producer in certain forest ecosystems in terms of abundance and influence on the system, the oak can be called a *foundation species* (Plomion and Fievet, 2013). From the oaks, the pedunculate oak *Quercus robur* is an important deciduous forest tree species in Europe.

Oaks present an endogenous rhythmic growth, which is reflected by alternating root and shoot development phases and growth cease (Lavarenne, 1966). In addition, they are obligatory ectomycorrhizal under natural conditions, and host several basidio- and ascomycete species as symbiotic fungi including *Piloderma croceum*. Belonging to the family of the *Atheliaceae*, *P. croceum* is a frequent symbiont of oak root systems in temperate forests. Mycorrhization with *P. croceum* enhances oak growth and photosynthesis rates by enhancing nutrient uptake, modifying mineral transformation in rock and soil systems and acquisition of phosphorus and nitrogen from organic matter. *P. croceum* is described as a late-stage fungus (Dighton and

Mason, 1985), expressing a high demand for carbohydrates (Gibson and Deacon, 1990) and being a strong sink for assimilates (Herrmann et al., 1998). Symbiotic interactions between *P. croceum* and oak are not restricted to an already established symbiosis but are observed at morphogenetic and physiological levels already during the pre-mycorrhizal stage (Herrmann and Buscot, 2007).

1.2. Mycorrhiza helper bacteria

Mycorrhiza helper bacteria (MHB) promote mycorrhiza formation and functioning (Frey-Klett et al., 2007). First introduced by Duponnois and Garbaye (1991), the MHB effect on arbuscular as well as on ectomycorrhizal symbiosis has been described for various bacterial strains, covering major bacterial clades for different plant models. In most cases the positive effect of MHB is expressed as faster extension rate of the fungal mycelium, but mechanisms for helper activities are diverse. Mycorrhiza formation can be enhanced by stimulating spore germination (Xavier and Germida, 2003) and mycelial growth, promoted by bacterial secondary metabolites such as auxofuran (Riedlinger et al., 2006). Other mechanisms are the reduction of soil-mediated stress (Brulé et al., 2001; Vivas et al., 2005) and the augmentation of plant-fungus contacts by stimulation of lateral root formation (Duponnois, 1992). Helper activities can also improve mycorrhizal functioning. Diverse mechanisms, such as nutrient mobilisation from soil minerals (Toro et al., 1997), fixation of atmospheric nitrogen (Requena et al., 1997) and protection of plants against root pathogens (Frey-Klett and Garbaye, 2005) have been reported to support the symbiosis. The MHB-fungus-plant interactions may thus be of crucial importance within sustainable agriculture and forestry, which relies on biological processes to maintain soil fertility and plant health (Frey-Klett and Garbaye, 2005).

MHB do not only have positive effects on mycorrhiza, but may also exude antifungal products. In such a case and depending on the fungus mycorrhization is promoted or suppressed by the MHB (Garbaye, 1994). For instance, it has been observed that co-cultivation of the MHB *Streptomyces* sp. AcH 505 with EM fungi *Amanita muscaria* and *Suillus bovinus* increased the mycorrhization rate whereas that of EM fungus *Hebeloma cylindrosporum* decreased (Schrey et al., 2005). The reason for this was that *H. cylindrosporum* was more sensitive to the antibiotics produced by AcH 505 than the other two fungi (Riedlinger et al., 2006). This shows that the outcome of the interactions occurring in the rhizospheres of plants between MHB, plant roots and mycorrhizal fungi is species specific. The discovery of MHB led to a number of publications describing experiments on MHB-fungus interactions in various systems with different interacting partners (e.g. Schrey et

al., 2005; Riedlinger et al., 2006; Deveau et al., 2007). However, those studies were mainly limited to simple agar culture-based systems and much remains to be learned about how interactions work under natural conditions and how they are affected by the host plant (Frey-Klett et al., 2007).

Actinomycetes are frequent colonisers of mycorrhizospheres, rhizospheres and plant roots (Coombs and Franco, 2003; Schrey and Tarkka, 2008). The actinomycete and MHB *Streptomyces* sp. AcH 505 has drawn particular attention due to its various modes of interaction with fungi and plants. It promotes mycorrhiza formation by enhancing the growth of fungal mycelium by the growth factor auxofuran (Riedlinger et al., 2006), by the stimulation of fine root formation, by inducing differential fungal gene expression (Schrey et al., 2005) and by facilitating root colonisation due to the decrease of plant defence responses in the roots (Lehr et al., 2007). In contrast to the situation in roots, the resistance of plant leaves against the pathogen *Botrytis cinerea* is increased after root treatment with AcH 505 and the plants maximal photosystem II efficiency is increased (Lehr et al., 2007). Furthermore, AcH 505 exerts a strong impact on the structure of microbial communities, which was recently revealed by fatty acid analysis (Caravaca et al., 2015).

1.3. Oak powdery mildew *Microsphaera alphitoides*

Microsphaera alphitoides which causes the majority of powdery mildew infections in oaks is an ascomycetous fungus belonging to the order of the *Erysiphales*. Various species of this order cause powdery mildew, a disease that affects a wide range of plants. As biotrophic pathogens, these fungi establish long-lasting interactions with the host plant tissues without killing them. Infected plants display white powdery spots on leaves and stems, and the mycelium penetrates the host leaf's epidermal cells and forms haustorial complexes for nutrient scavenging. The haustoria are strong carbon sinks that draw off assimilates of the host, thus severely affecting the normal source-sink system of the plant. Infection leads to enhanced glucose uptake and invertase activity in the infected leaves (Sutton et al., 2007), and to reduced total leaf chlorophyll content, photosynthesis and level of carbon assimilation. The infection associates with an induction of a number of basal defence-associated genes, but fails to elicit the jasmonate/ethylene signal transduction pathway, and renders the plant more susceptible towards other phytopathogens (Zimmerli et al., 2004).

2. Studying multitrophic interactions in plants – application of molecular and physiological approaches

The impact of an interacting organism is manifested in the plant at different levels. Whereas plant morphology is often affected after a certain amount of interaction time, detectable changes at the physiological or molecular levels occur immediately after the perception. Within the work presented here, the interactions of the oak with bacteria and the additional fungi were investigated, with the focus being at differential gene expression.

Genome scale dissection of gene expression levels termed transcriptome analysis has gained increasing attention in various fields of biology in the past years. The transcriptomic methods have evolved from studies based on the expression of a few genes of interest using for instance Northern blots and quantitative PCR over array analysis to next-generation sequencing (Morozova et al., 2009). So far, larger scale gene expression studies of organismic interactions have mainly been performed by microarrays (e.g. Verhagen et al., 2004; Wang et al., 2005). They represent collections of microscopic DNA fragments, probes, attached to a solid surface, and hybridization on the probes can be used to measure the relative expression levels of thousands of genes simultaneously. The microarray approach comes with one important restriction: the probe sequences chosen for the array restrict the spectrum of transcripts that can be analysed (Schenk et al., 2012). In contrast, next-generation sequencing (NGS) does not involve probes, since the sequencing of a transcriptome by NGS - termed RNA-Seq (Wang et al., 2009)- is based on the simultaneous non-selective sequencing of millions of cDNAs. Currently, the Genome Sequencer FLX system (454/Roche) and the Illumina HiSeq2000 are the most frequently used platforms, with the Illumina platform providing more but shorter reads compared to the 454 platform. Due to the great sequencing depth by HiSeq2000, differential gene expression levels between tissues, time points or treatments can be analysed. Compared to microarrays, RNA-Seq bears some other advantages: i) the possibility to detect rare transcripts, ii) higher dynamic range than microarrays, iii) practicability in non-model species and iv) greater accuracy and sensitivity when measuring levels of transcripts and their isoforms, alleviating for instance the microarray-problem of background signals (Strickler et al., 2012). At the moment, the main restrictions of RNA-Seq for gene expression analysis are the costs, the high computational power required and the challenge of data analysis. This often leads to a comparably low depth in sequencing per sample and low amounts of replicates. The costs of RNA-Seq are however declining and the bioinformatic tools are under intensive development, leading to the

prediction that RNA-Seq will replace other transcription profiling techniques (Anderson and Mitchell-Olds, 2011).

3. TrophinOak – background, objectives and design

Forest ecosystems need to be managed at a long-term scale, especially in view of recent climate change. Common assumption is that the future environments will be different from the present ones but that we cannot be certain about the specifics of change (Millar et al., 2007; Bonan, 2008). In Germany, an increase in average temperature and more heterogeneous distribution of precipitation over space and time may lead to shifts in species distribution and present a threat to biodiversity (Parmesan, 2006). In order to improve long-term forest management, the understanding of how multitrophic interactions shape plant communities and how they are affected by global change, is essential. All plants interact with a complex network of other organisms, such as mycorrhizal fungi, beneficial rhizosphere bacteria, pathogens and herbivores. These interactions have an important impact on the energy and nutrient element budget of the plant, by interfering in photosynthesis and nutrient exudation and -uptake into and from the soil. Thus, the plant needs to find a subtle balance for resource and energy management. Pedunculate oak *Quercus robur* represents an important woody plant species which is known to replace other species and to thus diversify forest ecosystems by acting as a foundation species for interaction networks (Müller, 2000; Plomion and Fievet, 2013). It displays an endogenous rhythmic growth with alternating developmental flushes of root and shoot (Lavarenne, 1966), making it particularly interesting with regard to the partitioning of resources.

Even though the importance of understanding multitrophic interactions on plants has been recognised (Tscharntke and Hawkins, 2002), there is a lack of approaches to tackle this question. Moreover, the few existing studies focusing on biotrophic tree interaction synthesized the interactions on artificial substrates (e.g. Frettinger et al., 2007; Felten et al., 2009; Courty et al., 2011), although it is known that soil microbial communities play important roles in the functioning of plants (reviewed in Mendes et al., 2013). Therefore, the aim of the project TrophinOak (Gene Regulation and Resource Allocation in Oaks during Multitrophic Interactions, http://www.trophinoak.de) was to establish an approach for investigating and studying the impact of multitrophic interactions on oak development using close-to-natural soil conditions. The project was realised by seven working groups studying different functional groups of organisms, i.e. an ectomycorrhizal fungus, a root pathogenic microorganism, a root-feeding invertebrate, a rhizospheric invertebrate consumer, a

mycorrhization helper bacteria, a leaf pathogenic fungus, a leaf invertebrate herbivore (Figure 1). In order to compare the impact of each biotrophic partner under standardized conditions, a Joined Experimental Platform (JEP) was established. The JEP allows comparisons between different biotrophic interactions due to the common implementation of i) an experimental model system of mycorrhizal oak microcuttings, ii) a common RNA-Seq protocol for transcriptomic analysis and iii) a mobile labelling infrastructure to quantify resource allocation. The micropropagated oak system consists of the *Quercus robur* L. clone DF159 (Favre and Juncker, 1987) inoculated in a Petri dish system with the basidiomycete *Piloderma croceum* (based on Herrmann et al., 1998). A soil filtrate is added to the soil microcosms to establish a natural microbial community thereby making the system closer to natural conditions. This experimental system displays various advantages to regularly used greenhouse cultures of oak seedlings. Constant temperature and moisture can be held at the Petri dish scale in growth chambers (Herrmann and Buscot, 2007). In contrast to seedlings, micropropagated oaks are genetically identical, and possess some characteristics of older plants. Microcuttings do not produce cotyledons but leaves, and show a developmental pattern with alternating root and shoot flushes. It is to be noted, that root flushing is not shown by young seedlings but by three-year-old oak trees in the field. Together with the implementation of a common RNA-Seq protocol and a mobile labelling infrastructure, the use of a micropropagated oak system allowed detailed analysis of the interrelationships between rhythmic growth and multitrophic interactions, which is unique for forest trees.

Forest tree transcriptomic has recently gained increased attention (e.g. Neale and Kremer, 2011). Nevertheless, the understanding of gene expression in trees is still at its beginning and since even the oaks lack a published reference genome, the OakContigDF159.1 reference transcriptome was constructed in the context of the TrophinOak project (Tarkka et al., 2013). All seven beneficial and detrimental biotic interactions with oak were synthesized and 454 and Illumina cDNA libraries from leaves and roots were prepared. Merging of these libraries yielded a hybrid assembly. The suitability of OakContig DF159 reference as template for transcriptome analysis was confirmed by gene expression profiling of fine roots and ectomycorrhizas with *P. croceum*.

Within TrophinOak two categories of aims were followed. For the first aim, a common experimentation was implemented within each project part to identify regulation processes in oaks during the interactions with the ectomycorrhizal fungus *P. croceum* and another organism. Investigations were performed at two levels: i) the level of gene expression by analysing RNA-Seq data and ii) the level of carbon and nitrogen allocation by analysing

stable isotope labelling data. Connecting molecular and physiological data allowed identifying key processes in plant development during the single and dual interactions. The second aim of the project was to perform experiments that are more specific to each project part. Within these, either different plant developmental stages, or different interacting organisms were considered, dealing with issues such as rhizospheric effects and below aboveground interactions.

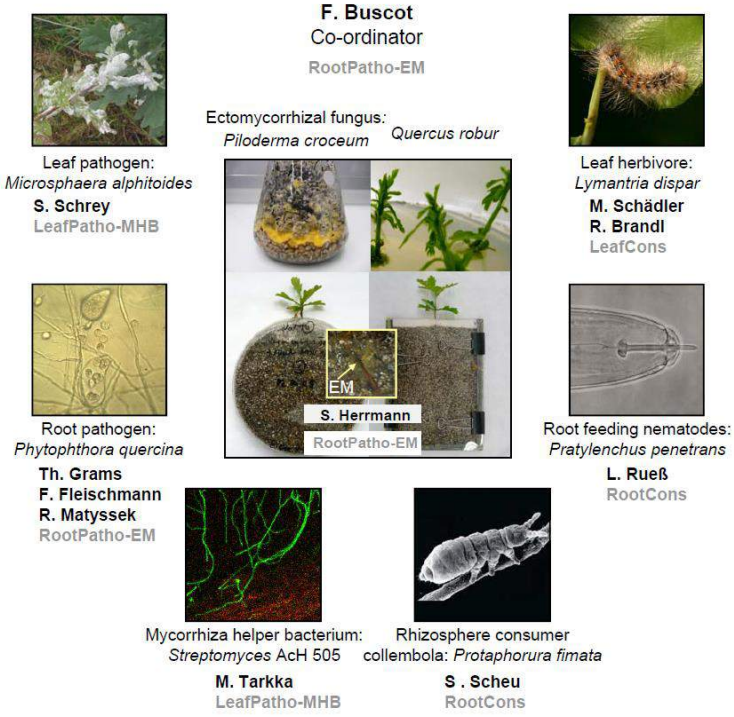

Figure 1 TrophinOak project – the framework for this doctoral thesis. Micropropagated oak system inoculated with the EM fungus *P. croceum* formed the template of the project. Six additional groups were working with a further organism interacting at the leaf-, root- or soil level. Identical methodologies in ^{13}C and ^{15}N labelling as well as in transcriptome analyses between the working groups enabled cross-comparisons of the data. Combination of the oak system and these methodologies was termed Joined Experimental Platform (figure by Sylvie Herrmann).

4. Objectives and structure of this thesis

The thesis presented is implemented into the frame of TrophinOak within the mycorrhiza helper bacterium (MHB) project part. The major goal of this project part is to understand how the oak coordinates its molecular responses during the interactions with the mycorrhiza helper bacterium *Streptomyces* sp. AcH 505. In addition, as being mycorrhizal is the common status of plants in nature, and as MHB have a direct effect on EM fungi, treatments with the two microbial partners were run to tackle their combined effects on the tree and to disentangle the direct effects of AcH 505 on the oak from the indirect ones via the mycobiont. Last the thesis also combined treatments with AcH 505 and the oak powdery mildew *Microsphaera alphitoides*, which, apart from the study of protective effects on the oak against

leaf pathogens via the MHB, bears the potential to unravel systemic and localized gene regulation.

In *chapter 1*, a quantification study of the MHB AcH 505 and the EM-fungus *P. croceum* is presented. The TrophinOak project provides a suitable platform to study the AcH 505-fungus-plant interactions in a soil-based microcosm that is closer to natural conditions, in comparison to other bacterium-fungus interaction studies (e.g. Schrey et al., 2007). The importance of interaction studies is underlined by the fact that in addition to the impact of the soil substrate and the presence of microbial communities (e.g. Schlatter et al., 2010), plants affect the interactions in soil due to the exudation of metabolites, an effect termed "rhizosphere effect" (Berg and Smalla, 2009). The following aims are formulated for this part of the study presented*:* i) the establishment of a quantification method adapted to the analysed microorganisms in the soil-based culture system used, ii) the investigation of the impact of a soil microbial community, added as a soil filtrate to the microcosm, on the bacterium-fungus interaction and iii) the quantification of the bacterium and the fungus in the absence and the presence of the oak. It is expected that this study may provide valuable knowledge about the soil-microcosm used within the TrophinOak project.

C*hapter 2* handles the impact of MHB AcH 505 on oak development and gene expression and it also tackles the tuning of this effect when the EM-fungus *P. croceum* is present. MHB and EM formation both promote plant growth and suppress plant defence responses in root (Lehr et al., 2007; Tarkka et al., 2013). In this context it is investigated whether a synergy exists between the impacts of both microorganisms on oak defence and development in roots. Furthermore, the local root and systemic leaf responses of oak microcuttings during two developmental stages - root and shoot flush - are compared. This design aims at providing gene expression information that could help the understanding of the molecular mechanisms underlying on-site and distal aspects of mutualistic relationships during different developmental stages of the plant.

In c*hapter 3* it is investigated how AcH 505 inhibits leaf infection by oak powdery mildew *M. alphitoides*. It has been reported for numerous rhizobacteria that they are able to induce systemic resistance (ISR) in plants, thereby provoking a faster and stronger defence response upon pathogen attack (e.g. Verhagen et al., 2004; Van der Ent et al., 2008). AcH 505 reduces oak powdery mildew infection, which leads to the formulation of the following aims: i) the elucidation of the molecular mechanisms of the bacterial plant protection observed and ii) the first linkage of belowground and aboveground interactions in oaks, combining a systemic impact of the root associated bacterium to the local effects of the fungus.

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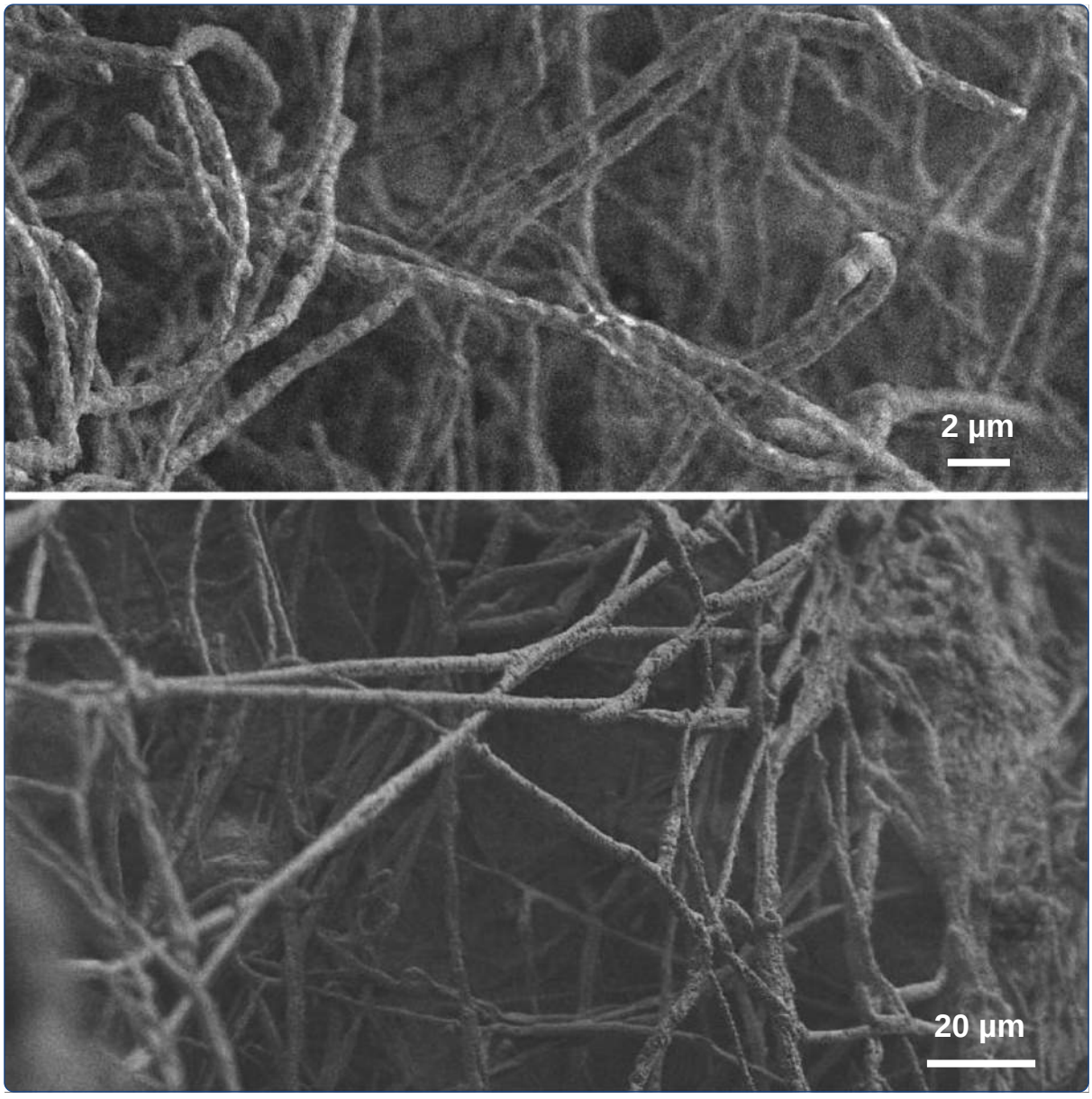

Detection and quantification of a mycorrhization helper bacterium and a mycorrhizal fungus in plant-soil microcosms at different levels of complexity

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Detection and quantification of a mycorrhization helper bacterium and a mycorrhizal fungus in plant-soil microcosms at different levels of complexity

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Abstract

Background: Host plant roots, mycorrhizal mycelium and microbes are important and potentially interacting factors shaping the performance of mycorrhization helper bacteria (MHB). We investigated the impact of a soil microbial community on the interaction between the extraradical mycelium of the ectomycorrhizal fungus *Piloderma croceum* and the MHB *Streptomyces* sp. AcH 505 in both the presence and the absence of pedunculate oak microcuttings.

Results: Specific primers were designed to target the internal transcribed spacer of the rDNA and an intergenic region between two protein encoding genes of *P. croceum* and the intergenic region between the *gyrA* and *gyrB* genes of AcH 505. These primers were used to perform real-time PCR with DNA extracted from soil samples. With a sensitivity of 10 genome copies and a linear range of 6 orders of magnitude, these real-time PCR assays enabled the quantification of purified DNA from *P. croceum* and AcH 505, respectively. In soil microcosms, the fungal PCR signal was not affected by AcH 505 in the absence of the host plant. However, the fungal signal became weaker in the presence of the plant. This decrease was only observed in microbial filtrate amended microcosms. In contrast, the PCR signal of AcH 505 increased in the presence of *P. croceum*. The increase was not significant in sterile microcosms that contained plant roots.

Conclusions: Real-time quantitative PCR assays provide a method for directly detecting and quantifying MHB and mycorrhizal fungi in plant microcosms. Our study indicates that the presence of microorganisms and plant roots can both affect the nature of MHB-fungus interactions, and that mycorrhizal fungi may enhance MHB growth.

Keywords: Streptomycetes, Oak, Real-time PCR, Rhizosphere, Microbial community

Background

Forests soils are highly complex ecosystems and soil microbes are known to have significant effects on plant diversity and productivity [1]. Most trees form a range of mutualistic associations with various filamentous fungi, these root-fungus associations are known as mycorrhizas. Mycorrhizal symbiosis improves plant nutrient acquisition and confers increased resistance to pathogens, while the fungus gains carbohydrates from its host plant [2]. The formation of mycorrhizas affects several aspects of plant physiology and also changes the nutritional and physical properties of the soil. The mycorrhizas and the external mycelia of symbiotic fungi (which together define the mycorrhizosphere) are colonised by bacteria, which may actively influence the growth of external fungal mycelia and mycorrhizal root colonisation. For instance, a group of bacteria known as Mycorrhization Helper Bacteria; MHB [3] stimulate the formation of mycorrhizas. At the time of writing, numerous bacterial strains from a wide range of major clades have been shown to have MHB-type

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functions in both arbuscular and ectomycorrhizal symbioses [4].

Bacteria can facilitate mycorrhization in various ways. In many cases, the positive effects stem from their ability to induce rapid expansion of the fungal mycelium e.g. [5]. Other important mechanisms include the alleviation of soil-mediated stress e.g. [6,7] and the formation of more extensive plant-fungus contacts by stimulating lateral root formation [8]. However, MHB do not always have positive effects on mycorrhiza formation and can exhibit fungus specificity in promoting symbioses [3]. While the effects of MHB on mycorrhizal fungi have been investigated extensively *in vitro*, the effects of the fungi on the MHB have largely been neglected. In their seminal work, Frey-Klett et al. [9] reported that the life span of the *Pseudomonas fluorescens* strain BBc6R8 was significantly prolonged by exposure to the EM-fungus *L. bicolor* S238N. This effect was attributed to the fungus because the survival of the bacterial strain was not affected by the presence of non-mycorrhizal roots.

Actinomycetes are frequent colonisers of mycorrhizospheres, rhizospheres and plant roots [10,11]. They are known for their antagonism against other microbial species [12,13] and are especially rich sources of antifungal compounds [14]. Depending on the circumstances, they can either inhibit or promote the formation of mycorrhizas reviewed in [11], and several actinomycete species exhibit MHB activity, Rhodococcus sp. [15], Streptomyces sp., [16-18]. Among the actinomycete MHB, the strain *Streptomyces* sp. AcH 505 has drawn most attention, since it forms unique interactions with fungi and plants. The extension of the fungal mycelium is promoted by the AcH 505 metabolite auxofuran [5], but the fungal biomass is simultaneously reduced due to the thinning of mycelium [19]. Schrey et al. [20] observed that co-cultivation of MHB *Streptomyces* sp. AcH 505 with *Amanita muscaria* and *Suillus bovinus* increased their rates of mycorrhization. However, co-cultivation with the same strain reduced the *in vitro* growth of *Hebeloma cylindrosporum*. This fungus-specificity is due to the differential sensitivity of the ectomycorrhizal fungi to the naphthoquinone antibiotic WS-5995 B, which is produced by AcH 505 [5] in addition to auxofuran. In the host plant, AcH 505 stimulated fine root formation [20] and facilitated root colonisation by suppressing the plant's defensive responses [21]. However, while exposure to AcH 505 suppressed defensive responses at the root level, it increased the resistance to the causative agent of grey mould *Botrytis cinerea* at the leaf level. While previous studies on AcH 505 provided valuable information on its interactions with the host plant and ectomycorrhizal fungi, they were all based on *in vitro* experiments; to date, no studies on its effects in soil have been conducted.

The discovery of bacteria that promote the establishment and maintenance of mycorrhizas triggered a search for their mechanisms of actions, and a number of publications have described *in vitro* experiments on MHB-fungus interactions, e.g. [5,20,22]. However, much remains to be learned about how MHB-fungus interactions work under natural conditions and how they are affected by the host plant [4]. We therefore investigated the growth responses of AcH 505 and the mycorrhizal fungus *Piloderma croceum* using a soil-based culture system that was established for studying multitrophic interactions in oaks as part of the TrophinOak collaborative project [23], see also www.trophinoak.de. The pedunculate oak *Quercus robur* belongs to the *Fagaceae* family and is obligately ectomycorrhizal under natural conditions. It is host to several symbiotic fungi, including both basidio- and ascomycete species [24]. One of its notable symbiont is *Piloderma croceum*, which has become a model fungus for studying the formation of oak mycorrhizas [25]. In a preliminary investigation, we observed that AcH 505 promotes the formation of mycorrhizas in oak microcosms. The number of mycorrhizas per microcosm was counted prior to harvesting and was found to be slightly increased by inoculation with AcH 505 according to the test of equal proportions ($p = 0.05$).

The study conducted herein was conducted to assess i) whether the effects of Streptomyces sp. AcH 505 and the ectomycorrhizal fungus *Piloderma croceum* on one-another depend on the presence of a host plant, ii) the possible influence of the microbial community on both micro-organisms and iii) how the two micro-organisms influence each other.

For this purpose, AcH 505 and *P. croceum* were cultivated alone and together under four different culture conditions: in the presence of both the host plant (*Q. robur*) and soil microbes (represented by a microbial filtrate), in the presence of the host but not soil microbes, in the presence of soil microbes but no host plant, and in the presence of neither soil microbes nor the host. In microcosms including the plant rhizosphere as well as bulk soil samples were taken for quantification analysis. The experimental setup is summarised in Additional file 1.

The abundances of AcH 505 and *P. croceum* mycelia were estimated by quantitative real-time PCR [26]. Primers were designed to target an intergenic region of the AcH 505 genome, between the *gyrA* and *gyrB* genes. The abundance of eukaryotes in environmental samples can be determined using qPCR experiments targeting the highly variable internal transcribed spacer (ITS) regions of rDNA operons [27,28]. However, fungal genomes contain multiple copies of the ITS-region and the ITS copy number varies between fungal strains [29]. For *P. croceum* Raidl et al. [30] estimated about 150 ITS copies per dikaryotic cell. Thus, it can be beneficial to target single

copy genes or intergenic regions rather than the ITS when quantifying fungi [29]. To compare the performance of these two approaches in fungal quantification, we designed novel ITS primers, as well as a primer pair that targets an intergenic region between two open reading frames (ORFs) in the *P. croceum* genome.

Results

Primer selection for real-time PCR and DNA extraction

Multiple templates were used to design specific primers for *Streptomyces* sp. AcH 505 including rRNA intergenic spacers, gene coding sequences, and regions between adjacent gene coding sequences. The specificity of each primer pair was evaluated by using them in real-time PCR experiments and analysing the melting curve of the resulting amplification products. The primer pair targeting the region between *gyrA* and *gyrB* genes exhibited specificity for AcH 505 sequences (i.e. it did not amplify sequences from *Piloderma croceum*, the soil microbe filtrate, or pedunculate oak DNA) as demonstrated by analysis of the melting curve for the PCR product it yielded. This primer pair had an efficiency of 76% as determined using a standard curve based on a serial two-fold dilution (see Additional file 2). The real-time PCR primers developed by Schubert et al. [31] for use with *P. croceum* samples were also tested but showed lower efficiency (Additional file 3). In addition, a novel ITS-specific primer pair was constructed based on the internal transcribed spacer region of *P. croceum* and primers were constructed to target the intergenic region between two ORFs based on the available genomic data for this species. Both primer pairs exhibited good efficiency and specificity for their respective amplification products (Additional files 4 and 5). The target regions for primer pairs AcH107 and Pilo127 are shown in Figure 1. Standard initial plasmid copy number versus cycle threshold (Ct) curves was used to estimate the frequencies of the target sequences in the DNA samples (Figure 2). The PCR fragments obtained using each primer pair were then cloned into plasmids. Serial plasmid dilutions were applied in each run to define the sensitivity of the method. As few as 10 copies per reaction were detected for each target sequence, and the initial copy numbers were linearly related to signal intensity over a range of 10^6 to 10 copies of standard plasmid DNA. The limits of detection for real-time PCR with the AcH107-, ITSP1- and Pilo127 primers were determined by creating dilution series (in which the concentrations ranged from no dilution to dilution by a factor of 10^{-5}) of bacterial and fungal DNA. All three primers yielded successful amplification at all dilutions above 10^{-5}, corresponding to bacterial and fungal biomasses of approximately 15 and 2.5 ng, respectively (Additional file 6).

AcH 505 and *P. croceum* DNA from the microcosm soil were successfully amplified in all processed samples. The standard curves for the DNA preparations obtained for the different experimental treatments were all very similar, indicating that the samples did not differ in their contents of PCR-inhibiting substances.

Quantification of *Streptomyces* sp. AcH 505 and *Piloderma croceum*

P. croceum significantly promoted the growth of AcH 505 in a culture system without oak microcuttings and in bulk soil samples in a culture system with oak (Figure 3a and c; see Additional file 7 for p-values). In the rhizosphere, *P. croceum* had no impact on AcH 505 in the sterile system, and the negative effects of the filtrate on AcH 505 that were only observed when the oak was present – in the rhizosphere as well as in the bulk soil -, could be released by the fungus (Figure 3b and c).

Treatment with the soil microbe filtrate following the initial application of the mycorrhizal fungus had a significant negative impact on the extraradical mycelium biomass of *P. croceum* in the culture system without pedunculate oak and in bulk soil in the presence of oak (Figure 4a,c,d and f). Co-inoculation with AcH 505 partially relieved this filtrate-based inhibition. In the presence of pedunculate oak, the filtrate's inhibition of *P. croceum* was less pronounced (Figure 4b and e). However, AcH 505 inhibited *P. croceum* in the rhizosphere when the filtrate was applied to the microcosms. In conclusion, the presence of both soil microbes and oak microcuttings had significant effects on the interactions between AcH 505 and *P. croceum* in soil. Highly similar

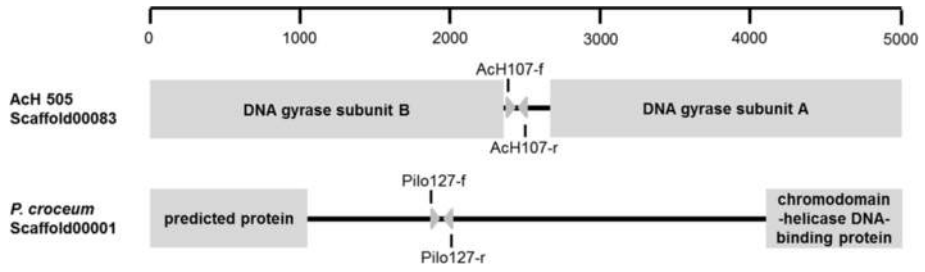

Figure 1 The target regions for the AcH107 and Pilo127 primer pairs.

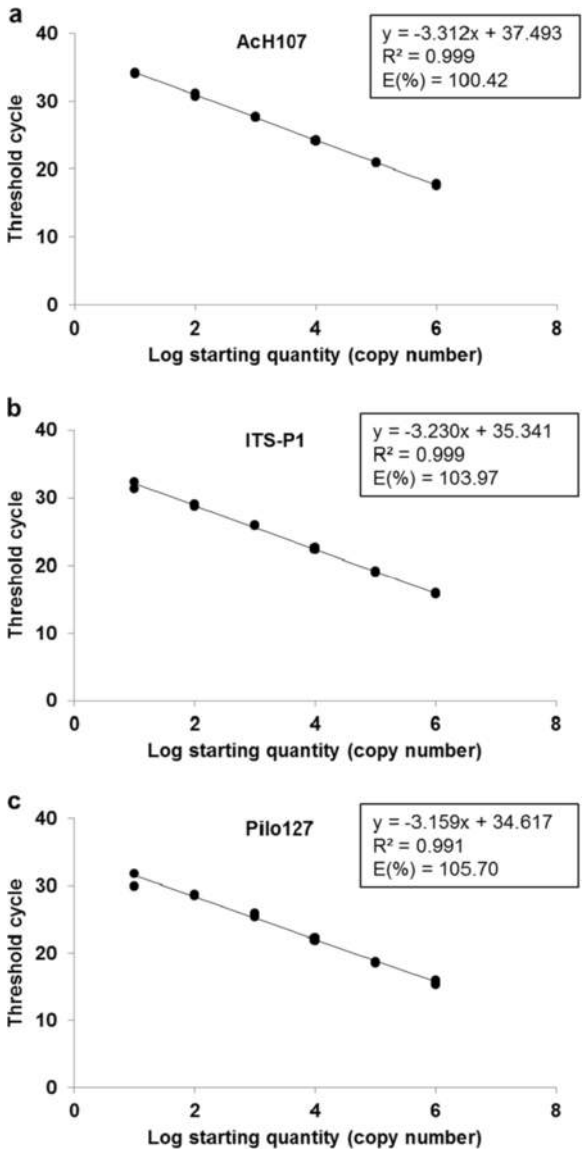

Figure 2 Standard curves for the intergenic *gyrA/gyrB* region (a) and the ITS- (b) and intergenic region (c) in AcH 505 and *P. croceum* respectively. Serial dilutions of plasmids with the target DNA insert were used in individual qRT-PCR assays to generate the standard curves. The R^2 values, slopes and efficiencies are shown for each reaction.

results were obtained using primer pairs that targeted the ITS region (ITSP1f/r) and the intergenic region (Pilo127f/r).

Microscopic analysis of AcH 505 and *Piloderma croceum*

AcH 505 and *P. croceum* were visualised within the soil microcosms using cryo-field emission scanning electron microscopy (Figure 5a and b; see Additional file 8 for a description of the method used). The bacterial filaments (Figure 5a) were easily distinguished by their small diameters (< 1 μm), branching and curvature, and segmentation

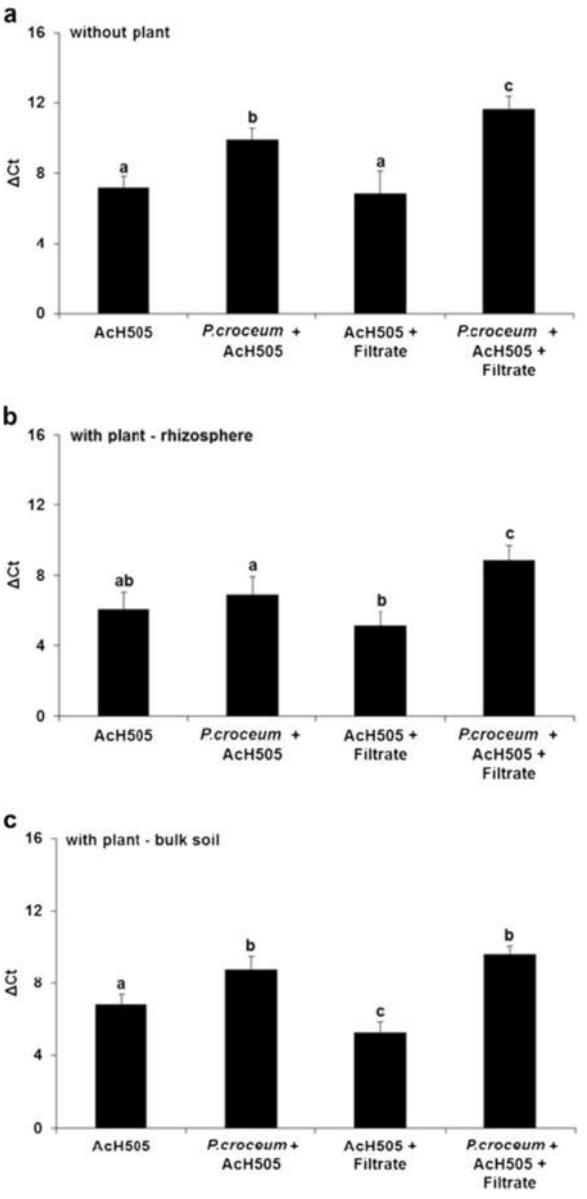

Figure 3 Quantification of the mycorrhization helper bacterium *Streptomyces* sp. AcH 505 in soil microcosms. The relative amounts of AcH 505 were measured by real-time quantitative PCR (qPCR) in the presence or absence of the mycorrhizal fungus *Piloderma croceum*, the soil microbial filtrate, and pedunculate oak microcuttings. In the presence of microcuttings quantification was performed with bulk soil as well as rhizosphere samples. The bars indicate the qPCR abundance of AcH 505 in the absence **(a)** and presence (rhizosphere **(b)** and bulk soil **(c)**) of the host plant. qPCR abundances are reported in terms of delta Ct values, which indicate the number of cycles at which the fluorescent signal exceeds the background level and surpasses the threshold established in the exponential section of the amplification plot. Error bars denote standard errors; bars with different letters are significantly different according to one-way ANOVA and the Tukey HSD test ($P < 0.05$). Note that co-inoculation with *P. croceum* stimulates the growth of AcH 505.

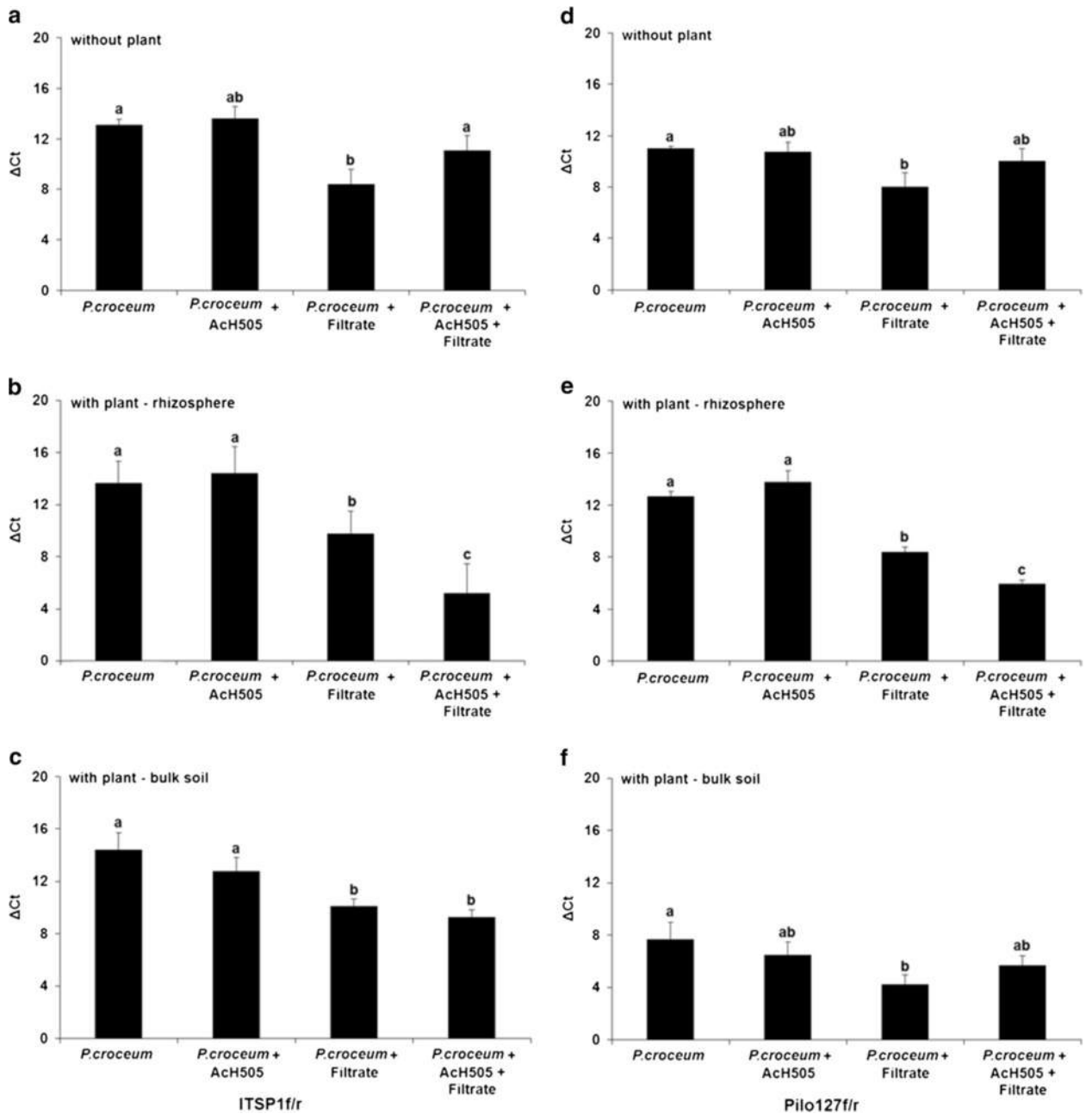

Figure 4 Quantification of the ectomycorrhizal fungus *Piloderma croceum* in soil microcosms. The relative amount of *P. croceum* mycelium was measured by real-time quantitative PCR (qPCR) in the presence or absence of *Streptomyces sp.* AcH 505, the soil microbial filtrate, and pedunculate oak microcuttings. In the presence of microcuttings quantification was performed with bulk soil as well as rhizosphere samples. The bars indicate qPCR abundances of *P. croceum* in the absence **(a,d)** and presence (rhizosphere **(b,e)** and bulk soil **(c,f)** of the host plant. Quantification was performed with the ITSP1f/r **(a,b,c)** and Pilo127f/r **(d,e,f)** primer pairs. The qPCR abundances are reported in terms of delta Ct values, which indicate the number of cycles at which the fluorescent signal exceeds the background level and surpasses the threshold established in the exponential region of the amplification plot. Error bars denote standard errors; bars with different letters are significantly different according to one-way ANOVA and the Tukey HSD test ($P < 0.05$). Note that the presence of the host plant modulates the responses of the microorganisms to one-another.

by occasional septa. Fungal hyphae (Figure 5b) by contrast had an average diameter of 3 μm and were characterised by extensive branching. To visualise the interactions between the micro-organisms, *Streptomyces* sp. AcH 505 was labelled with green fluorescence protein, co-cultured with *P. croceum* on agar, and visualised by confocal laser scanning microscopy (see Additional files 9 and 10 for more details of these methods). The diameter of the AcH

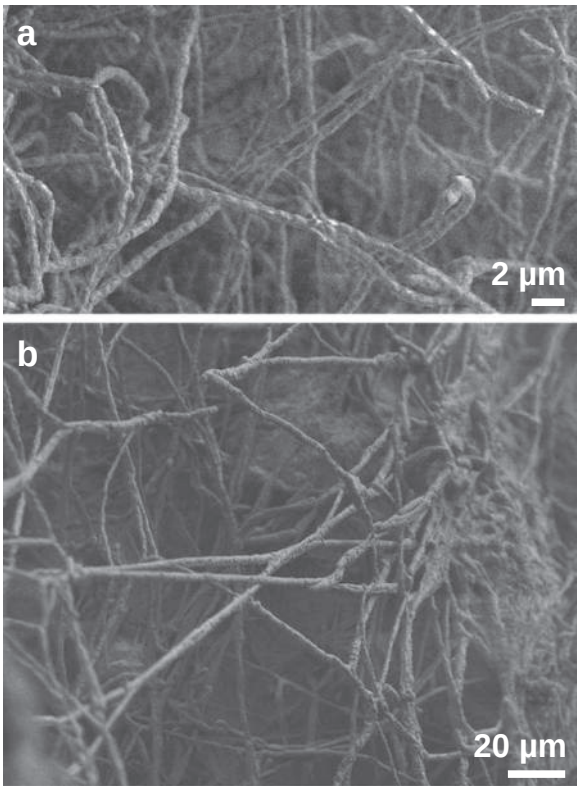

Figure 5 Visualisation of *Streptomyces* sp. AcH 505 (a) and the *Piloderma croceum* (b) mycelium by scanning electron microscopy.

505 filaments in the co-cultures was comparable to that observed by scanning electron microscopy in soil microcosms, and individual AcH 505 filaments often combined to form star-like bundles (Additional file 11). In addition, the AcH 505 filaments aligned on the surfaces of *P. croceum* hyphae. We did not detect adherence of AcH 505 on *P. croceum* in microcosms. The microscopic analyses demonstrate that both organisms can be visualised in soil microcosms.

Discussion

Various hypotheses concerning the mechanisms that underpin the associations between mycorrhization helper bacteria (MHB), fungi and plants have been put forward based on the results of *in vitro* bioassays and cultures [20,22]. We have previously shown [5,19,21] that *Streptomyces* sp. AcH 505 is a fungus-specific MHB that produces fungus growth regulators and affects plant health and development. When tree roots were inoculated with a suspension of AcH 505 mycelia, significant stimulation of mycorrhiza formation was observed [19]. In the oak system, we also could find a slight increase in the number of mycorrhizas when the microcosm soil was inoculated with AcH 505. This was the first time when the mycorrhization helper effect was observed for AcH 505 in a soil based culture system. The present study further demonstrates the potential of this strain by casting light on its performance in a soil-vermiculate formulation, and shows that AcH 505 benefits from the presence of the mycorrhizal fungus.

Specific detection of *Streptomyces* sp. AcH 505

Our initial experiments with AcH 505 were conducted using primers designed against the 16S-23S ribosomal DNA intergenic spacers and single copy genes. However, only the primers targeting the intergenic regions between protein-coding genes yielded specific amplification; the other tested primers were not suitable due to non-specific background amplification when used with samples that included soil microbe DNA. The ribosomal operon is present in multiple copies in streptomycetes [32], and different species within this genus can have different rDNA copy numbers. Moreover, the rate of rDNA sequence variation between the genomes of different *Streptomyces* strains is unknown. According to our preliminary analysis of the AcH 505 genome, the intergenic region between the *gyrA* and *gyrB* genes exists in a single copy and is thus an excellent target for specific quantification. The number of available genome data for different *Streptomyces* strains is increasing [33] and will enable the application of this simple and specific qPCR method for streptomycete quantification for even more bacterial isolates in the future.

Comparable detection and quantification of *Piloderma croceum* by qPCR using two primer pairs

In basidiomycete fungi, the ribosomal genes are also present in multiple copies, and changes in the numbers of rRNA genes occur throughout the fungal life cycle [34]. Regions of rDNA are distributed as large tandem arrays, and intra-genomic variation in the length and the base distribution of rDNA sequences has been described [35]. Most qPCR quantification approaches in fungi are based on the internal transcribed spacer regions (ITS1 and ITS2) of the rDNA, since these are easily accessible by PCR and with their high copy number they allow a sensitive detection [27,28,31]. Due to the methodological constraints listed above, it can be argued that the use of single copy genes or intergenic regions between protein coding genes could allow for more accurate quantification of basidiomycete fungi. Our observations with *P. croceum* indicate, that at least this basidiomycete fungus is as effectively and quantitatively detected with primers targeting the ITS as with those constructed against an intergenic region between protein coding genes. Following the approach of Schubert et al. [31] we detected comparable ratios of ITS signal/mycelial biomass at different levels of fungal mycelium. In contrast, with another approach Raidl et al. [30] quantified the ITS copy number of *P. croceum* by using Taqman PCRs and by measuring the extent of

mycelium from thin layers of sterile mycelium. To conclude, we could here clearly demonstrate how specific qPCR assays can be a powerful tool for elucidating the relative fungal and bacterial biomass in microcosm samples of varying complexity.

Promotion of AcH 505 growth by *P. croceum* and response to soil microbial community

P. croceum promotes AcH 505 growth, which may indicate that the MHB feeds on fungal exudates. These include proteins, amino acids, and organic acids [36]; *P. croceum* is known to exude compounds such as oxalic and malic acid [37]. In ectomycorrhizal fungi such as *P. croceum*, trehalose is the primary storage sugar [38,39], and this disaccharide may be partially responsible for the selection of specific bacterial communities in mycorrhizospheres [4]. The positive impact of *P. croceum* on AcH 505 was more significant in microcosms amended with a microbe filtrate. This shows that competition by microbial community may influence the outcome of microbial interactions. Schlatter et al. [40] also reported, that the microbial community has an impact: *Streptomyces scabiei* DL87 promoted *Streptomyces lavendulae* DL93 in autoclaved, but not in field soil. In general, streptomycetes are competitive because they can derive nutrients from recalcitrant substrates, possess diverse resistance genes and are prolific producers of antagonistic secondary metabolites that inhibit the growth of their competitors [33,41]. It can also be concluded, that AcH 505 is a competitive streptomycete, as the strain was not affected by the microbe filtrate in the rhizospheres of plants.

Fungal responses to soil microbial community and to AcH 505

The soil microbe filtrate inhibited *P. croceum*, and this inhibition could be due to competition for resources or space, or to antagonism [42]. The first of these possibilities, i.e. competitive inhibition, is perhaps more likely: Schrey et al. [43] obtained evidence that *P. croceum* may be particularly tolerant of antagonistic metabolites of Streptomycete isolates from Norway spruce – in an experiment conducted to determine the *in vitro* activity of *Piloderma* sp. mycorrhizas against seven fungi, *P. croceum* was the least severely affected fungus. In this study, *Streptomyces* affected the growth of *Piloderma* only under the influence of the microbial filtrate. This indicates that communities of soil microbes carry out a multitude of small-scale processes that can impact bacterium-fungus interactions [1,36].

Plant rhizosphere reverses the outcome of AcH 505 - *P. croceum* interaction

Our observations of filtrate-amended microcosms demonstrated that the host plant has a strong effect on the MHB-fungus interaction: With the ITS primers it was observed that AcH 505 promoted *P. croceum* growth in the host plant's absence, showed no significant impact in bulk soil, but inhibited the fungus in the rhizosphere. The numbers of ectomycorrhizal fine roots/seedling were not estimated. Thus, we cannot exclude local reductions in the numbers of ectomycorrhizal roots due to the AcH 505 treatment in the presence of soil microbe filtrate. Plants influence the composition and quantity of soil microbes by secreting products into the rhizosphere [44]. Root exudates contain compounds that can exert both stimulatory and inhibitory influences on the rhizosphere microbial community, changing its structure and composition [45]. Conversely, microbial products can induce plant root exudation [46]. AcH 505 influences its environment by the production of growth regulators [5]. In this work, the presence of oak rhizosphere might have led to increased production of antibiotics by AcH 505 which could perhaps cause the inhibition of *P. croceum* in the rhizosphere.

Conclusions

Fungi and bacteria have established specific strategies for interacting with one-another with significant ecological consequences, as reviewed in [42]. Since one of the priorities in this context is to demonstrate the impact of particular organisms on each other, the development of methods for quantifying the abundance of bacteria and fungi in the presence of one-another and other potentially interfering microbes is essential. Our data suggest that significant interactions occur between AcH 505 and *P. croceum*. The competitive abilities of both species differ in sterile and filtrate-amended gamma-sterilised soils, and are also affected by the presence or absence of the host plant. Thus, it would be desirable to investigate fungus-bacterium interactions using model systems that enable step-wise increases in complexity. The ability to discriminate between different MHB and mycorrhizal fungi will make it possible to obtain a deeper understanding of their interactions when investigating microbial consortia rather than individual species. In the context of the TrophinOak project, we will use the methods presented herein to analyse the responses of AcH 505 and *P. croceum* to soil invertebrates and to investigate how the induction of plant defences affects their abundance.

Methods

The soil-based culture system

A soil-based culture system for the quantification of *Streptomyces* sp. AcH 505 and *Piloderma croceum* (DSMZ 4824, ATCC MYA-4870) was established as described by Tarkka et al. [23]. Briefly, micropropagation and rooting of the pedunculate oak clone DF159 (*Quercus robur* L.) were conducted according to Herrmann et al.

[47]. Rooted microcuttings were placed in Petri dishes filled with a 1:1 (vol/vol) mixture of fungal inoculum and gamma sterilised soil. Soil filtrates were prepared as described by Rosenberg et al. [48]. At 4 weeks, 5 ml of filtrate was added to the culture system. *Streptomyces* sp. AcH 505, originally isolated from the soil around Norway spruce mycorrhizas in Haigerloch, Germany [18], was maintained on ISP2 agar medium [49]. For AcH 505 treatment, the culture system was inoculated with 2.5×10^7 bacterial spores at 3 and 7 weeks. The material was grown for eight weeks after which bulk soil were harvested from microcosms without plants and bulk as well as rhizosphere samples from microcosms with plants. Rhizosphere samples were taken by harvesting the soil attached to the root. Samples were submerged in liquid nitrogen and stored at −80°C. The experimental design required the analysis of 72 samples in total: 3 (+ oak (rhizosphere/bulk soil)/- oak) × 2 (+/− *P. croceum*) × 2 (+/− AcH 505) × 2 (+/− soil filtrate) × 3 biological replicates.

DNA extraction

Total DNA was extracted from soil and rhizosphere samples using the PowerSoil DNA Isolation Kit (Mo Bio) according to the manufacturer's recommendations. The quantity and quality of the DNA were estimated using a Nanodrop spectrophotometer (Thermo Scientific) and agarose gel electrophoresis. For AcH 505 and *P. croceum* pure culture DNA, biological material harvested from liquid culture was immediately frozen in liquid nitrogen (N) and homogenised. DNA extraction was then carried out with the PowerSoil DNA Isolation Kit (Mo Bio) for AcH 505 using a protocol based on those described by P. Spanu (Imperial College, London) and Fulton et al. [50] (detailed protocol acquired from A. Kohler and F. Martin (INRA Nancy) at "http://1000.fungalgenomes.org/home/wp-content/uploads/2012/03/Martin_genomicDNAextraction_AK051010.pdf") for *P. croceum*.

Primer design and validation for qRT-PCR

Primers for the quantification of AcH 505 and *P. croceum* were designed using the Primer3 software package [51] http://frodo.wi.mit.edu/primer3/. The designed primer pairs were required to have: a melting temperature of 55–65°C, a GC content of 58 to 63%, primer lengths of 18–22 bp, and amplified product lengths of 70–150 bp. The AcH 505 primers were designed based on genome sequence data (T. Wu., F. B., L. F., M. T. T., unpublished). The ITS region of *P. croceum* (NCBI, JX174048), as well as genomic data for *P. croceum* (Fungal Genomics program, DOE Joint Genome Institute), were used as templates for fungal primer design. The amplicon sizes and sequences for the primers used in this work are listed in Table 1. The identities of the amplified products were verified by Sanger-sequencing.

The constructed primers were initially used in PCR amplifications to test their functionality and to verify the predicted size of the amplicons. The specificity and the efficiency of the primer pairs was verified by melting curves and the construction of standard curves based on a serial two-fold dilution (2^0 - 2^{-5}) using soil DNA as the template. Template plasmids were used to generate a standard curve that was used as an external standard. The target DNA sequence was cloned into the pGEM-T vector (Promega) and the resulting plasmids were purified. All plasmids were quantified by spectrometry using a Nanodrop ND-1000 instrument (Thermo Scientific) and copy numbers were estimated based on the molecular weight of the template. The number of copies of the cloned target DNA in the dilution series ranged from 10^6 to 10^1.

Real-Time PCR assays

Real-time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad). The reaction mixtures contained 7.5 μl of iQ SYBR Green Supermix, 1 μl of DNA solution (corresponding to 1 ng of DNA), and 350 nmol of each gene-specific primer. The experiments were conducted in 96-well plates with an iQ 5 Multicolour Real-Time PCR Detection System (Bio-Rad). PCR was always performed with three biological and three technical replicates. The cycling conditions were 10 s at 95°C, 30 s at 55°C or 62°C. Template abundances were determined based on the Ct values (which measure the number of cycles at which the fluorescent signal exceeds the background level and surpasses the threshold established based on the exponential phase of the amplification plot). The significance of differences between the Ct values of different treatments were

Table 1 Sequence, expected amplicon sizes, and annealing temperature for the AcH 505 and *P. croceum* primers

Target	Amplicon size (bp)	Primer sequence (5′ → 3′)	Annealing temp. (°C)
AcH 505, intergenic region between *gyrA/gyrB* genes	107	AcH107-f (GGCAAGCAGAACGGTAAGCGG) AcH107-r (TGGTCGGTGTCCATCGTGGT)	55
P. croceum, ITS	121	ITSP1-f (GGATTTGGAGCGTGCTGGCGT) ITSP1-r (TTGTGAGCGGGCTTTTCGGACC)	55
P. croceum, intergenic region between two ORFs	127	Pilo127-f (GTCAGAGACGGACGCAGTTG) Pilo127-r (CCAGTCAGCGGAGGAGAA)	62

determined by one way analyses of variance ($p < 0.05$) and grouped according to the Tukey HSD test in R (R Core team, 2012).

Additional files

Additional file 1: Experimental setup for quantification of AcH 505 and *P. croceum* under different culture conditions.

Additional file 2: qRT-PCR melting and standard curves obtained using the AcH107 primer pair.

Additional file 3: qRT-PCR melting and standard curves obtained with the ITS-P primer pair.

Additional file 4: qRT-PCR melting and standard curves obtained with the ITSP1 primer pair.

Additional file 5: qRT-PCR melting and standard curves obtained with the Pilo127 primer pair.

Additional file 6: Correlation of AcH 505 and *P. croceum* biomass with qRT-PCR data.

Additional file 7: Statistical analysis relating to the quantification of the mycorrhization helper bacterium *Streptomyces* sp. AcH 505 and the mycorrhizal fungus *Piloderma croceum* in soil microcosms.

Additional file 8: Cryo-field emission scanning electron microscopy (cryo-FESEM) images.

Additional file 9: Confocal laser scanning microscopy (CLSM) images.

Additional file 10: eGFP labelling of *Streptomyces sp.* AcH 505.

Additional file 11: Visualisation of the *Streptomyces sp.* AcH 505 – *Piloderma croceum* interaction using confocal laser scanning microscopy.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
FK conducted the molecular studies and drafted the manuscript. KZ participated in the quantification experiments. LF performed the AcH 505 genome assembly. TRN helped with the confocal laser scanning microscopy. TWe did the GFP labelling of AcH 505. VK participated in the electron scanning microscopy studies. TWu carried out the AcH 505 genome sequencing. SH coordinated the establishment of microcosms with oak microcuttings within the TrophinOak platform. FB is the lead scientist of the TrophinOak project. MT conceived of the study, participated in its design and coordination, assisted in the sequencing of the AcH 505 genome and helped to draft the manuscript. All authors read and approved the final manuscript.

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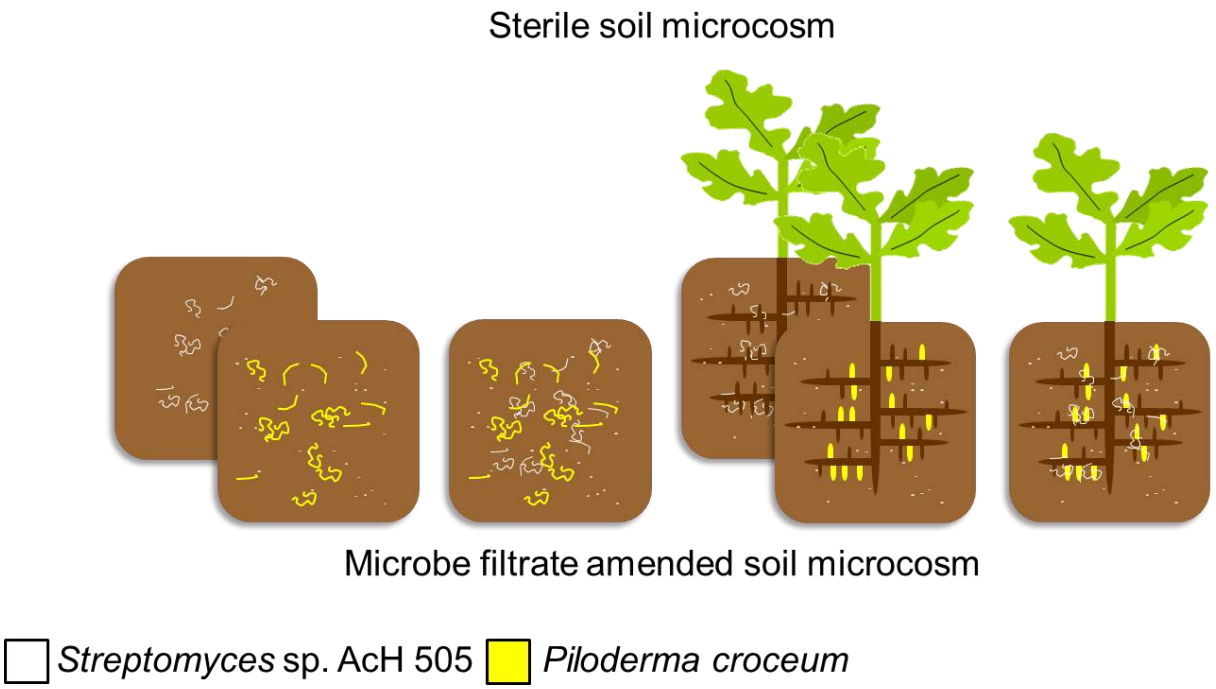
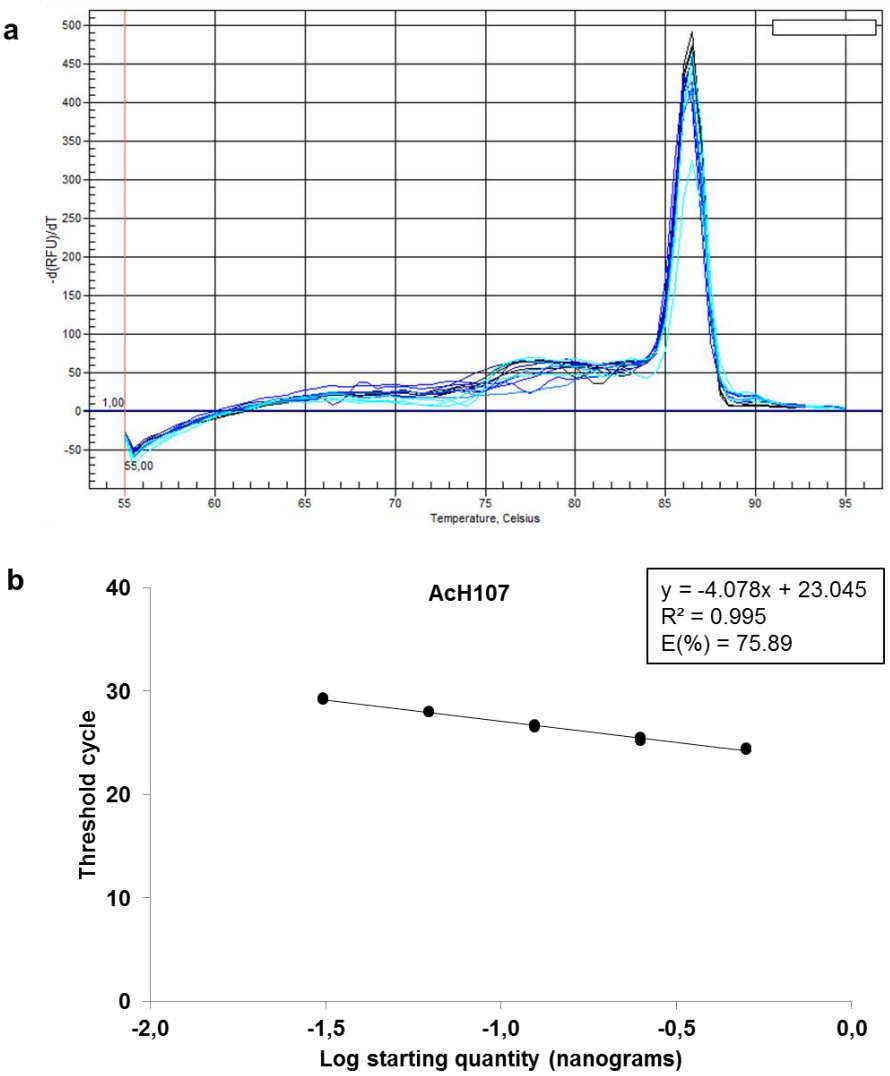
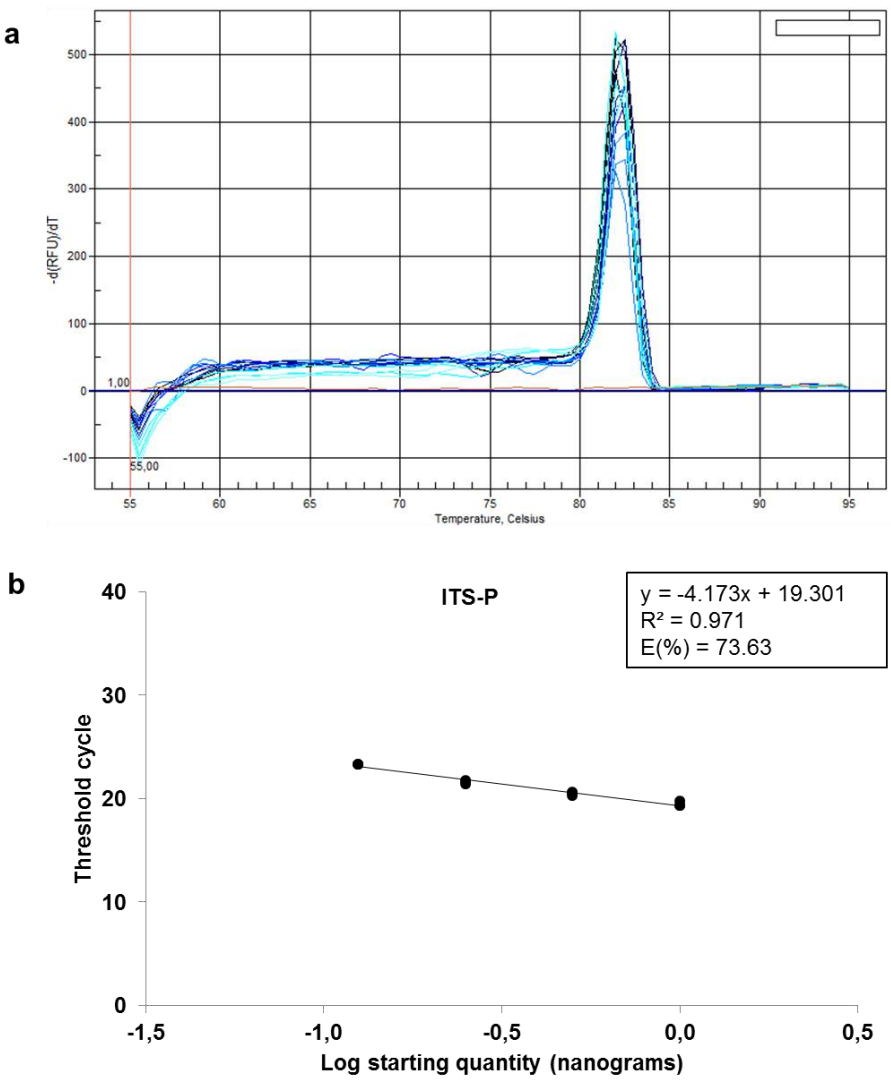
Supporting information



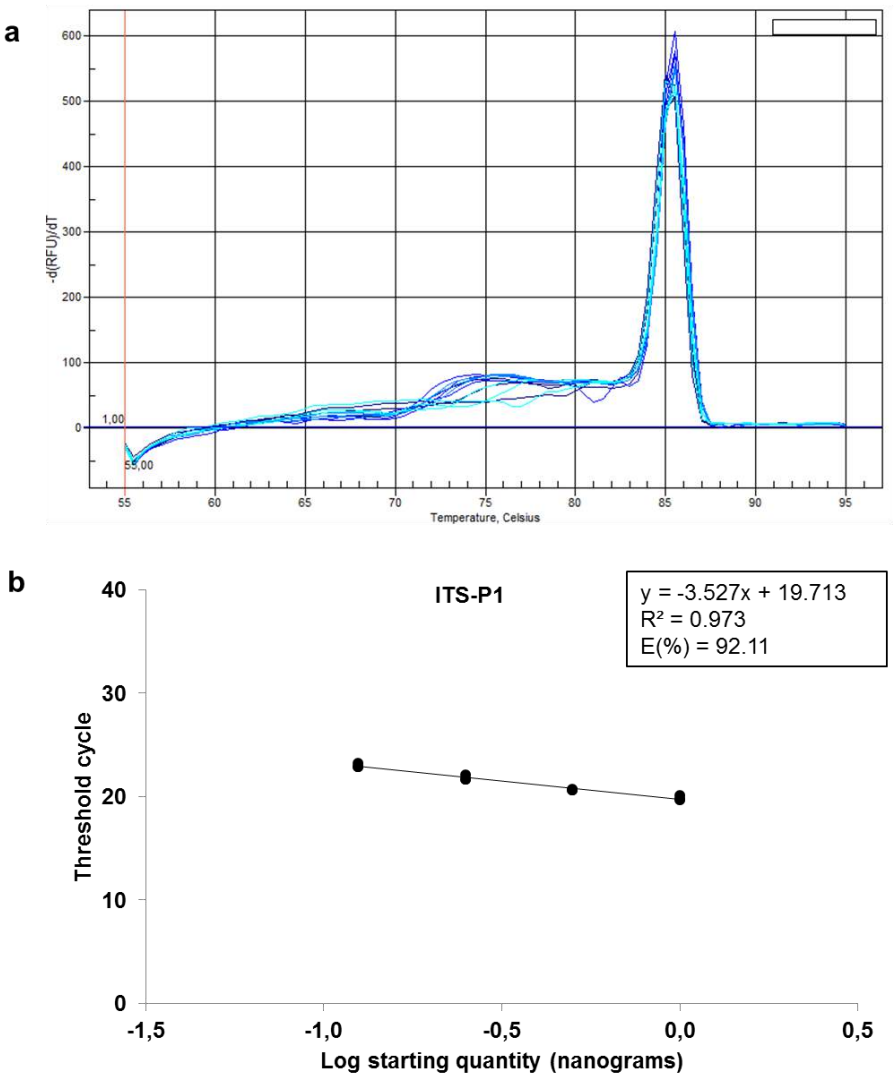
Additional file 1 Experimental setup for quantification of AcH 505 and *P. croceum* under different culture conditions.



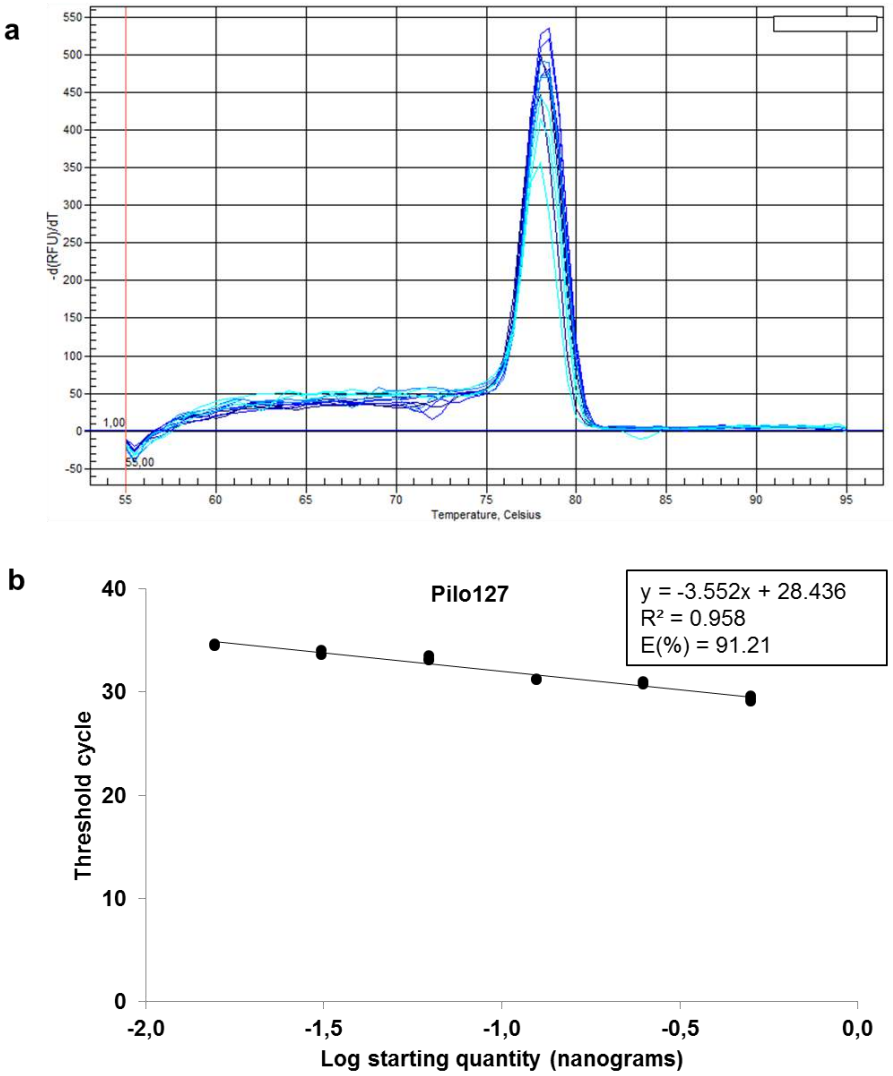
Additional file 2 Standard curves obtained from quantification of *Streptomyces* sp. AcH 505 from non-sterile soil microcosm by the primer pair AcH107. (a) Melting curve of the qPCR amplicon generated from soil DNA. Specific amplification of AcH 505 is indicated by the single sharp peak. (b) Real-time PCR standard curve obtained by amplification of a serial dilution of soil DNA. The curve was generated by plotting the Ct values against the dilutions. The high R^2 value suggests linear amplification.



Additional file 3 Standard curves obtained from quantification of *Piloderma croceum* from non-sterile soil microcosm by the primer pair ITSP. (a) Melting curve of the qPCR amplicon generated from soil DNA. Specific amplification of *P. croceum* is indicated by the single sharp peak. (b) Real-time PCR standard curve obtained by amplification of a serial dilution of soil DNA. The curve was generated by plotting the Ct values against the dilutions. The high R^2 value suggests linear amplification.



Additional file 4 Standard curves obtained from quantification of *Piloderma croceum* from non-sterile soil microcosm by the primer pair ITSP1. (a) Melting curve of the qPCR amplicon generated from soil DNA. Specific amplification of *P. croceum* is indicated by the single sharp peak. (b) Real-time PCR standard curve obtained by amplification of a serial dilution of soil DNA. The curve was generated by plotting the Ct values against the dilutions. The high R^2 value suggests linear amplification.



Additional file 5 Standard curves obtained from quantification of *Piloderma croceum* from non-sterile soil microcosm by the primer pair Pilo127. (a) Melting curve of the qPCR amplicon generated from soil DNA. Specific amplification of *P. croceum* is indicated by the single sharp peak. (b) Real-time PCR standard curve obtained by amplification of a serial dilution of soil DNA. The curve was generated by plotting the Ct values against the dilutions. The high R^2 value suggests linear amplification.

Additional file 6 Correlation of AcH 505 and *P. croceum* biomass with qRT-PCR data. 150 and 250 mg mycelium were extracted in a final volume of 100 and 1000 µl for AcH 505 and *P. croceum*, respectively. For qRT-PCR reactions with 1 µl of undiluted to a dilution of 10^{-5} yielded good amplification of the product. The initial copy number was derived from the plasmid standard curve (Figure 2).

	Mycelium (mg)	Initial copy number	Mycelium (mg) / copy number
AcH505 - 107f/r	1.50E+00	8.97E+05	1.67E-06
	1.50E-01	1.17E+05	1.28E-06
	1.50E-02	1.18E+04	1.27E-06
	1.50E-03	1.35E+03	1.11E-06
	1.50E-04	2.69E+02	5.57E-07
	1.50E-05	1.32E+01	1.14E-06
P.croceum – ITS-P1	2.50E-01	7.58E+06	3.30E-08
	2.50E-02	7.74E+05	3.23E-08
	2.50E-03	7.89E+04	3.17E-08
	2.50E-04	5.24E+03	4.77E-08
	2.50E-05	5.77E+02	4.34E-08
	2.50E-06	4.54E+01	5.51E-08
P.croceum – 127f/r	2.50E-01	8.33E+05	3.00E-07
	2.50E-02	8.85E+04	2.83E-07
	2.50E-03	9.35E+03	2.67E-07
	2.50E-04	5.50E+02	4.54E-07
	2.50E-05	7.45E+01	3.36E-07
	2.50E-06	9.99E+00	2.50E-07

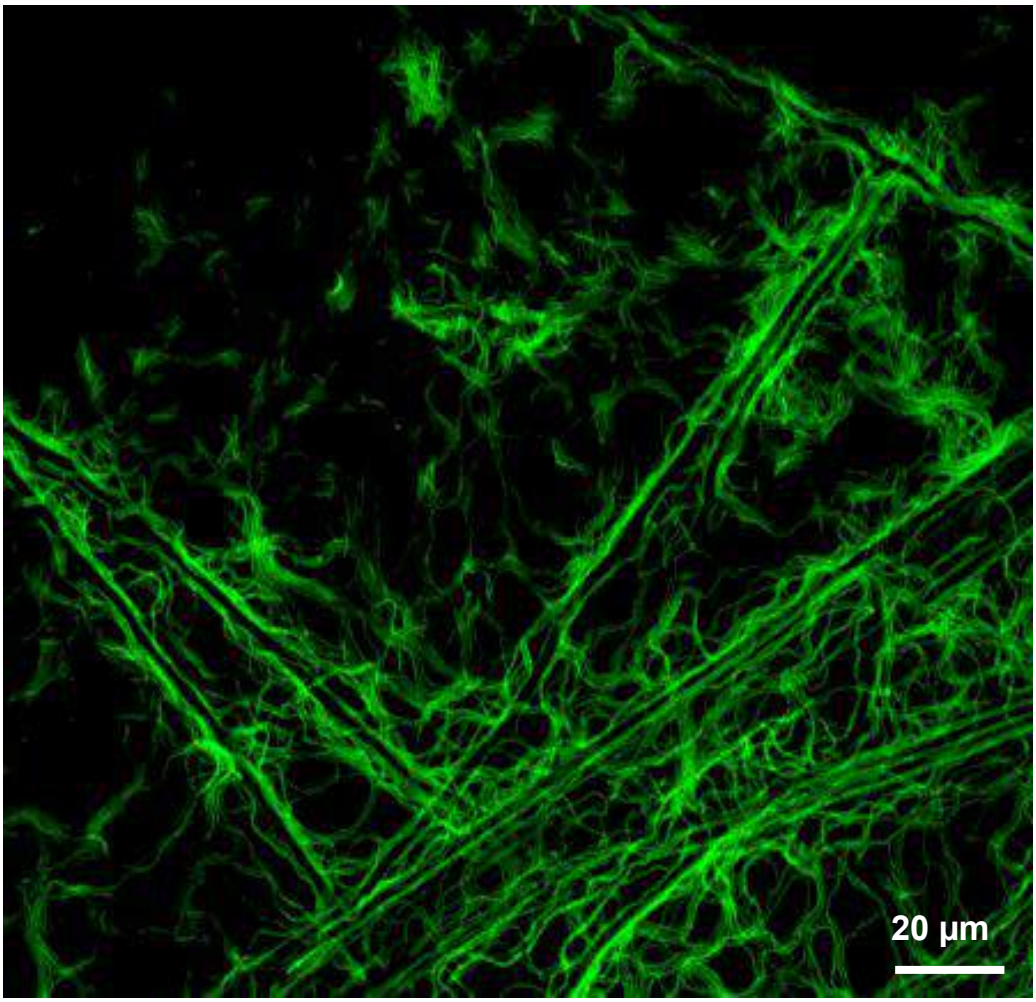
Additional file 7 Statistical analysis of quantification of mycorrhization helper bacterium *Streptomyces* sp. AcH 505 and the mycorrhizal fungus *Piloderma croceum* in soil microcosms. P-values determined by Tukey HSD test for statistical analyses of quantification results (n.s. = not significant).

Primer	Treatment	p-value
AcH107 without plant	AcH505 - AcH505 + Filtrate	n.s.
	AcH505 - AcH505 + *P.croceum*	$p < 0.001$
	AcH505 - AcH505 + Filtrate + *P.croceum*	$p < 0.001$
	AcH505 + Filtrate - AcH505 + *P.croceum*	$p < 0.001$
	AcH505 + Filtrate - AcH505 + Filtrate + *P.croceum*	$p < 0.001$
	AcH505 + *P.croceum* - AcH505 + Filtrate + *P.croceum*	$p < 0.05$
AcH107 with plant rhizosphere	AcH505 - AcH505 + Filtrate	n.s.
	AcH505 - AcH505 + *P.croceum*	n.s.
	AcH505 - AcH505 + Filtrate + *P.croceum*	$p < 0.001$
	AcH505 + Filtrate - AcH505 + *P.croceum*	$p < 0.01$
	AcH505 + Filtrate - AcH505 + Filtrate + *P.croceum*	$p < 0.001$
	AcH505 + *P.croceum* - AcH505 + Filtrate + *P.croceum*	$p < 0.01$
AcH107 with plant - bulk soil	AcH505 - AcH505 + Filtrate	$p < 0.01$
	AcH505 - AcH505 + *P.croceum*	$p < 0.001$
	AcH505 - AcH505 + Filtrate + *P.croceum*	$p < 0.001$
	AcH505 + Filtrate - AcH505 + *P.croceum*	$p < 0.001$
	AcH505 + Filtrate - AcH505 + Filtrate + *P.croceum*	$p < 0.001$
	AcH505 + *P.croceum* - AcH505 + Filtrate + *P.croceum*	n.s.
PiloITS without plant	*P.croceum* - *P.croceum* + Filtrate	$p < 0.05$
	P.croceum - *P.croceum* + AcH505	n.s.
	P.croceum - *P.croceum* + Filtrate + AcH505	n.s.
	P.croceum + Filtrate - *P.croceum* + AcH505	$p < 0.01$
	P.croceum + Filtrate - *P.croceum* + Filtrate + AcH505	n.s.
	P.croceum + AcH505 - *P.croceum* + Filtrate + AcH505	n.s.
PiloITS with plant - rhizosphere	*P.croceum* - *P.croceum* + Filtrate	$p < 0.001$
	P.croceum - *P.croceum* + AcH505	n.s.
	P.croceum - *P.croceum* + Filtrate + AcH505	$p < 0.001$
	P.croceum + Filtrate - *P.croceum* + AcH505	$p < 0.001$
	P.croceum + Filtrate - *P.croceum* + Filtrate + AcH505	$p < 0.001$
	P.croceum + AcH505 - *P.croceum* + Filtrate + AcH505	$p < 0.001$
PiloITS with plant - bulk soil	*P.croceum* - *P.croceum* + Filtrate	$p < 0.01$
	P.croceum - *P.croceum* + AcH505	n.s.
	P.croceum - *P.croceum* + Filtrate + AcH505	$p < 0.001$
	P.croceum + Filtrate - *P.croceum* + AcH505	$p < 0.05$
	P.croceum + Filtrate - *P.croceum* + Filtrate + AcH505	n.s.
	P.croceum + AcH505 - *P.croceum* + Filtrate + AcH505	$p < 0.01$
Pilo127 without plant	*P.croceum* - *P.croceum* + Filtrate	$p < 0.05$
	P.croceum - *P.croceum* + AcH505	n.s.
	P.croceum - *P.croceum* + Filtrate + AcH505	n.s.

	P.croceum + Filtrate - *P.croceum* + AcH505	n.s.
	P.croceum + Filtrate - *P.croceum* + Filtrate + AcH505	n.s.
	P.croceum + AcH505 - *P.croceum* + Filtrate + AcH505	n.s.
Pilo127 with plant - rhizosphere	*P.croceum* - *P.croceum* + Filtrate	$p < 0.001$
	P.croceum - *P.croceum* + AcH505	n.s.
	P.croceum - *P.croceum* + Filtrate + AcH505	$p < 0.001$
	P.croceum + Filtrate - *P.croceum* + AcH505	$p < 0.001$
	P.croceum + Filtrate - *P.croceum* + Filtrate + AcH505	$p < 0.01$
	P.croceum + AcH505 - *P.croceum* + Filtrate + AcH505	$p < 0.001$
Pilo127 with plant - bulk soil	*P.croceum* - *P.croceum* + Filtrate	$p < 0.05$
	P.croceum - *P.croceum* + AcH505	n.s.
	P.croceum - *P.croceum* + Filtrate + AcH505	n.s.
	P.croceum + Filtrate - *P.croceum* + AcH505	n.s.
	P.croceum + Filtrate - *P.croceum* + Filtrate + AcH505	n.s.
	P.croceum + AcH505 - *P.croceum* + Filtrate + AcH505	n.s.

Additional file 8 Cryo-field emission scanning electron microscopy (cryo-FESEM)

Samples were transferred into a special cryo holder, mounted into a drop of glue and clamped. The samples were quickly frozen (>10^3 K/s) in slushy nitrogen. Then, the samples were transferred into the cryo-stage of the preparation chamber (ALTO2500, Gatan, USA) where they were freeze-fractured at -140 °C, freeze-etched at -95 °C for 3 min, and then coated with 3 nm layer of platinum at -135 °C. The coated samples were inserted into the chamber of the JSM-7401F microscope (JEOL, Japan) precooled to -130 °C. Images were obtained by both the secondary and back-scattered electron signal at 3 kV.



Additional file 9 Visualisation of the *Streptomyces sp*. AcH 505 – *Piloderma croceum* interaction using confocal laser scanning microscopy. *Streptomyces sp*. AcH 505 was labelled with Green Fluorescent Protein.

Additional file 10 Confocal laser scanning microscopy (CLSM)

For CLSM imaging of GFP labelled bacteria on roots a SP1 (Leica, Wetzlar) controlled by the confocal software version 2.61 built 1537 was used. The upright microscope was equipped with three lasers (Ar, DPSS561, HeNe). Samples were mounted in a coverwell chamber and examined with a 63x NA 1.2 wi objective lens. For excitation the Ar laser line at 488 nm was used, emission signals were detected from 500-600 nm. Optical sections were recorded at 0.2 µm stepsize with a frame average of eight. Image series are presented as maximum intensity projection using the microscope software.

Additional file 11 eGFP labelling of *Streptomyces sp.* AcH 505

To obtain eGFP labelled *S. sp.* AcH 505, the plasmid pRM4.3, which is a pSET152 derivative containing the *egfp* gene under control of the constitutive ermE* promoter (Chevillotte et al., 2008) was introduced into the strain by interspecific conjugation using a modified protocol according to Kieser et al. (2000): E. coli ET12567 (pUB307) (MacNeil et al., 1992; Flett et al., 1997) was transformed with pRM4.3. Transformants were grown over night in LB supplemented with the antibiotics kanamycin 50 µg x mL^{-1}, chloramphenicol 12.5 µg x mL^{-1}, and apramycin 50 µg x mL^{-1} at 37 °C on a rotary shaker. Cells were harvested, washed twice with LB without antibiotics and resuspended in 1 ml of LB. 100 µL of this cell suspension was mixed with ~ 1x 10^8 spores of *S. sp.* AcH 505 and plated on SFM medium 20 % soybean flour, 20 % mannitol, 1.6 % agar, pH 7.5) supplemented with 10 mM $MgCl_2$. After 16 h cultivation at 29 °C, the plates were overlayed with 1 ml H_2O containing 1.5 mg x mL^{-1}apramycin and 0.75 mg x mL^{-1}nalidixic acid.

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Chapter 2: Large scale transcriptome analysis reveals interplay between development of forest trees and a beneficial mycorrhization helper bacterium

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Abstract

Background

Pedunculate oak, *Quercus robur* is an abundant forest tree species that hosts a large and diverse consortium of beneficial ectomycorrhizal fungi (EMF), whereby EM formation is stimulated by mycorrhization helper bacteria such as *Streptomyces* sp. AcH 505. Oaks typically grow rhythmically, with alternating root flushes (RFs) and shoot flushes (SFs). We explored the poorly understood mechanisms by which oaks integrate signals induced by their beneficial microbes and endogenous rhythmic growth. To this end, we compared transcript profiles of oak microcuttings at RF and SF during interactions with AcH 505 alone and in combination with the basidiomycetous EMF *Piloderma croceum.*

Results

Genes encoding leucine-rich repeat receptor protein kinases (LRR-RPKs) and xyloglucan cell wall transglycolases/hydrolases, as well as genes related to chromatin remodelling and DNA methylation, were up-regulated in both roots and leaves of plants treated with AcH 505. The local root and distal leaf responses differed substantially. For instance, the treatments resulted in more pronounced differential expression of genes involved in the recognition of bacterial and fungal effectors, defence and cell wall remodelling related transcription factors in the roots than in the leaves of oaks. In addition, interaction with AcH 505 and *P. croceum* affected the expression of a higher number of genes during SF than during RF, including AcH 505 elicited defence response, which was attenuated by co-inoculation with *P. croceum* in the roots during SF.

Conclusion

Treatment with AcH 505 induces and maintains signalling through receptor protein kinases and transcription factors and leads to differential expression of cell wall modifying genes in pedunculate oak microcuttings. Local gene expression response to AcH 505 alone and in combination with *P. croceum* are more pronounced when roots are in resting stages, possibly due to the fact that non growing roots re-direct their activity towards plant defence rather than growth.

Background

Soil microbial communities influence numerous physiological processes and traits of plants, including: seed germination, seedling vigour, growth and development, nutrition, the progression of various diseases, and productivity (reviewed in Mendes et al., 2013). Mutualistic fungi form close associations with plants that provide multiple benefits to both sets of organisms. Most importantly, in the context of this study, most temperate forest trees develop a mutualistic root symbiosis, ectomycorrhiza (EM), with fungi. This enhances the tree nutrient acquisition and the fungus ability to extend mycelia through the soil and form fruiting bodies (Smith and Read, 2008). Various other beneficial microorganisms are also frequently associated with EM, including bacteria (Frey-Klett et al., 2007), which may participate in mycorrhiza formation (Poole et al., 2001), mineral solubilisation (Frey-Klett and Garbaye, 2005), and suppression of soil-borne plant pathogens (Schrey et al., 2012). These findings clearly demonstrate the importance of EM-associated bacteria, but the mechanisms by which plants coordinate their responses to bacteria in presence and absence of EM fungi are unclear.

Oaks are a group of major broadleaf forest trees in Europe that are key components of complex networks of biotrophic interactions, involving relationships with endophytic (Faeth and Hammon, 1997), pathogenic (Tack et al., 2012) and EM (Leski et al., 2010) fungi, as well as bacteria that stimulate EM formation (Garbaye et al., 1992) and participate in nutrient solubilisation and acquisition (Różycki et al., 1999; Uroz et al., 2013). Like many tropical and some temperate trees, oaks display an endogenous rhythmic growth pattern, with alternating shoot flushes (SF) and root flushes (RF), throughout their vegetative growth periods (Buscot et al., 2004). Using a microcosm system in which microcuttings of the pedunculate oak *Quercus robur* clone DF159 express these typical alternating flushes (Herrmann et al., 1998), Herrmann et al. (submitted) have found that the rhythmic growth of microcuttings is reflected by oscillations in the resource allocation between the below- and aboveground parts and also by transport-related gene expression patterns. The authors suggested that hormonal signalling pathways and specific cellular developmental changes govern the microcuttings endogenous rhythmic growth patterns, which is not modified by mycorrhizal inoculation although EM plants have an increase resource acquisition. Rhythmic growth of oak trees affects their interactions with associated microorganisms, both beneficial and pathogenic. For instance the distribution pattern on the mother roots and the frequency of mycorrhizal root tips are affected by the endogenous rhythmic growth of *Q. robur* (Buscot et al., 2004). In addition, infestation of roots by the oomycete pathogen *Phytophthora quercina* is reportedly enhanced during RF,

when roots are growing rapidly and the plant below-ground carbon allocation is highest (Angay et al., 2014), and colonisation of leaves by oak powdery mildew is enhanced during SF, when the leaves are growing most rapidly (Mailänder, 2014). It is therefore likely that rhythmic growth generally affects the interactions of the oaks with other organisms.

Previously, we showed that inoculation of oak microcuttings with the mycorrhization helper bacterium *Streptomyces* sp. strain AcH 505 (hereafter AcH 505) enhanced the number of mycorrhizas in microcuttings co-inoculated with *P. croceum* as compared to plants not inoculated with AcH 505 (Kurth et al., 2013). Besides promoting mycorrhiza formation, AcH 505 directly elicits defence responses against the pathogens *Botrytis cinerea* in Norway spruce needles (Lehr et al., 2007). Furthermore, AcH 505 induces systemic defence responses against *Microsphaera* (*Erysiphe*) *alphitoides* in pedunculate oak leaves, involving both jasmonic acid/ethylene and salicylic acid-dependent signalling (Kurth et al., 2014). However, the cited study did not investigate variations in transcriptomic responses to AcH 505 during RF and SF growth stages, effects of AcH 505-treatment in roots, or its interactions with mycorrhizal fungi, which are crucial elements of a MHB's functional role in the oak system.

EM fungi, such as *Piloderma croceum* are essential for optimal development of pedunculate oak microcuttings (Herrmann and Buscot, 2007; Leski et al., 2010). Extensive re-programming of the oak transcriptome has been detected both during pre-symbiotic development and in mature symbiotic EM with *P. croceum* (Krüger et al., 2004; Frettinger et al., 2007; Tarkka et al., 2013). For instance, we reported that EM formation with *P. croceum* leads to defence suppression in oak, embodied by low abundance of defence-related transcripts (Tarkka et al., 2013). But the influence of dual presence of AcH 505 and *P. croceum* on the oak was not investigated.

When microorganisms interact with plants, perception processes play a crucial role. Plants perceive microorganisms by sensing microbe-associated molecular patterns (MAMPs; Newman et al., 2013). MAMP receptors of plants are encoded by *R* genes and they can recognize both pathogenic (Dangl and Jones, 2001) and beneficial microorganisms (Newman et al., 2013). These receptors include transmembrane receptor-like kinases (RLKs) and receptor-like proteins (RLPs), which act in concert with leucine rich repeat-nucleotide binding (LRR-NB) proteins (Lukasik and Takken, 2009). In contrast to those from recognized pathogens, the MAMPs derived from EMF (Salzer et al., 1996), and plant growth promoting rhizobacteria (PGPR) (van Loon et al., 2008), trigger typical immune responses in plants, but these responses are rapidly suppressed after recognition by the host plant receptors (Zamioudis and Pieterse, 2012). Accumulating evidence suggests that transcription factors of

the APETALA 2/ethylene-responsive family play a major role in integrating the response to biotic interactions in concert with plant development. In particular, ERFs are implicated in MAMP-induced systemic resistance signalling (Mizoi et al., 2012). Seven ERF contigs showed up-regulation in *P.croceum*/oak EM (Tarkka et al., 2013), but it has not been investigated how AcH 505 treatment affects their transcription.

In the presented study we examined the responses elicited by AcH 505 in oak roots and leaves during SF and RF, but also the modifications of these responses to AcH 505 in case of co-inoculation with the EM fungus *P. croceum*. Based on our analysis of systemic defence response in leaves to AcH 505 (Kurth et al., 2014) and on the defence suppressing effect of *P. croceum* on oaks (Tarkka et al., 2013) we formulated our first hypothesis stating that AcH 505 induces, while co-inoculation with *P. croceum* attenuates the defence responses in oak. Our second hypothesis states that local molecular responses to the organisms interacting with their roots are stronger during SF, expressed as differential representation of *R* genes, hormonal signalling genes and cell wall proteins. It is based on the observations of Angay et al. (2014) that oak roots express a higher level of root colonisation by *P. quercina* in RF and on the general literature on MAMP perception, hormonal signalling and cell wall modifications occurring during plant-microbe interactions (e.g. Dangl and Jones, 2001; Mizoi et al., 2012). To address these hypotheses we investigated gene expression profiles of pedunculate oak microcuttings.

Results

Oak microcuttings were successfully inoculated with *Streptomyces* sp. AcH 505 (Figure 1). Three replicate RNA-Seq datasets were generated for microcutting lateral roots and leaves, during both RF and SF, following no inoculation, inoculation with AcH 505 and with both AcH 505 and *P. croceum* (n = 36). The numbers of cleaned paired-end reads ranged from 13,539,202 to 15,562,465 per treatment. The validity of the differential expression analyses was confirmed by qRT-PCR analysis (Additional file 1). Numbers of differentially expressed contigs (DEC) among the samples are visualised in the Venn diagrams shown in Figure 2 and tabulated in Additional file 2. AcH 505 treatment induced more DEC during SF than during RF in both roots and leaves. A suppressive effect by AcH 505-*P. croceum* co-inoculation on the numbers of DEC was evident during SF. During RF, proportions of DECs detected following inoculation with AcH 505 and also differentially expressed following dual inoculation were 33 % in roots and 23 % in leaves, but during SF, the values dropped

markedly to 5 % and 3 %, respectively. The sets of DECs that responded to the root microorganisms locally and distally strongly differed.

Compared to non-inoculated controls, a number of GO terms were significantly over-represented for up- or down-regulated contigs in the roots of microcuttings under different inoculation treatments during either or both RF and SF. Affected plant defence-related GO terms are shown in Figure 3. In roots, AcH 505 inoculation led to enrichments of these terms during SF. However, when the oaks were co-inoculated with the EM fungus, these GO-terms were depleted or non-significant, indicating that suppression of plant defence by *P. croceum* (Tarkka et al., 2013) overrules the defence-stimulating effect of AcH 505 in roots.

Protein family (Pfam) enrichment analysis revealed significant changes in Pfam terms related to growth and development, signalling, defence and DNA-modification in both AcH 505- and co-inoculated plants (Figure 4). In roots, expression of the corresponding genes strongly differed between RF and SF. The Pfam terms *Glycosyl hydrolase* 2 and 35, and *Beta-galactosidases*, were under-represented in AcH 505- and co-inoculated plants during RF, but over-represented in plants singly inoculated with AcH 505 during SF. In contrast, the term *Xyloglucan-endo-transglycosylase* (*XET*) was over-represented during RF and under-represented during SF under both inoculation treatments. Leucin rich repeat (LRR) associated Pfam terms were particularly differentially regulated in the roots of plants singly treated with AcH 505 or co-inoculated plants during SF. During RF, under the same treatments, defence related-terms (such as *AP2 domain* and *Thaumatin domain*) were over-represented. Pfam terms related to DNA modification such as *MutS domain V* and *Orotidine 5'-phosphate decarboxylase* were mainly up-regulated during RF in AcH 505- and co-inoculated plants. Major differences in Pfam term representation between RF and SF were also observed in leaves of the plants, depending on the treatment. *Glycosyl hydrolase* 2 and 35, and *Beta-galactosidases*, showed opposite expression patterns in leaves compared to roots. These terms were over-represented in co-inoculated plants during RF and under-represented in AcH 505-inoculated plants during SF. In leaves, the LRR-associated Pfam terms were over-represented during SF. The signalling-related Pfam term *Protein kinase domain* was under-represented in RF. In addition, while most defence-related terms (e.g. *Probable lipid transfer*) were enriched during both RF and SF in inoculated plants, some were depleted in both growth stages (e.g. *Chitinase class I*). Compared to the pattern in roots, fewer terms related to DNA modification were enriched in leaves, exceptions including *Enhancer of rudimentary* in SF and *DNA replication factor CDT1* and *DNA polymerase III* in RF.

To address the AcH 505 driven gene expression in more detail, we searched for those individual contigs that were co-regulated in AcH 505 treatment as well as co-inoculation of both microorganisms (Table 1). We reasoned that these genes regulated in AcH 505 but not influenced by additional presence of *P. croceum*, may be part of the core interactome gene set of AcH 505 on the oak. In the roots of plants during RF, these core contigs were related to plant growth (*XET*) and metabolism (*Kinase-like protein*). During SF the core contigs were related to transcriptional regulation and defence (*Zinc finger protein, Chromatin binding protein, Thaumatin*). Based on the *Arabidopsis thaliana* orthologs, in the leaves of the microcuttings, the contigs with corresponding patterns identified during RF were related to cell wall biosynthesis (*Cellulose synthase*), metabolism (*Serine hydroxymethyltransferase*), and transport (*PHO1 phosphate exporter*), whilst during SF the core contigs were involved in defence and growth-related signalling (*Calcium-binding protein, LRR nucleotide binding sequence [NBS] resistance protein, anthocyanidin synthase*). Furthermore, due to their prevalence in Pfam enrichment accessions, the LRR-associated DECs were treated in more detail and their homology to orthologous transcripts in *A. thaliana* was estimated by Blastx analysis (Additional file 3). Most of them encoded members of the extensive families of LRR receptor-like protein kinases (LRR-RLKs) and receptor like proteins (LRR-RLPs) with roles ranging from recognition of microorganisms to signalling in growth and differentiation. Another abundant group of the R proteins comprised leucine-rich repeat-nucleotide binding site (LRR-NBS) related contigs, which have predicted functions in microorganism recognition and defence signalling. Predicted functions of other identified LRR-encoding DECs are related to auxin responses, microtubule cytoskeleton, cell wall composition and terpenoid biosynthesis. Among these signalling devices and transcription factors, which are most important for the AcH 505 interaction, contigs regulated in both AcH 505- and co-inoculations were detected. From LRR-receptor protein kinase a contig (homologous to *A. thaliana AT3G47110*) was up-regulated in roots, and another (*AT3G14840*), was down-regulated in leaves at RF and SF by both treatments. LRR-NB-ARC domain protein (*AT3G14460*) was up-regulated in both roots and leaves at SF. From transcription factors, AP2/ERF gene homologous to *A. thaliana SHN2* (*AT5G11190*) was up-regulated in roots and leaves at RF by both treatments, and by AcH 505 at SF (Additional file 3).

Discussion

The RNA-Seq approach has been previously used to explore both global transcriptomic responses of oaks to microbes (mutualists and pathogens), and mechanisms underlying their

rhythmic growth (Tarkka et al., 2013; Kurth et al., 2014; Sebastiana et al., 2014; Herrmann et al., submitted). In the study presented here, we applied RNA-Seq analysis to investigate the impact of the MHB AcH 505 on the oak during two rhythmic growth stages and used combined treatment with the EMF *P. croceum* to disentangle the direct impact of AcH 505 from the one that is modified in case of co-inoculation. A strong response of the microcutting roots and leaves to the AcH 505 treatment at the root-resting stage was identified, which was in part attenuated by the *P. croceum* treatment. We began to unravel the gene expression networks linking the microcutting perception of AcH 505 to the plant development, and detected an immune response in the roots following the bacterial inoculation. In this way, our results provide foundation for comparative analyses of interactions between oaks and other microorganisms to elucidate fundamental patterns.

Defence responses and perception of the microorganisms in oak microcuttings

In accordance with our first hypothesis formulated in the introduction, we found that defence gene expression in the oak microcuttings were induced by AcH 505, but attenuated by co-inoculation with *P. croceum* (Figure 3). This suggests that the defence suppression by *P. croceum* dominates over the elicitation of defences by AcH 505. A putative mechanism for EMF-based defence was presented by Plett et al. (2014b), who showed that *Laccaria bicolor* promotes mycorrhiza formation by blocking plant defence-related jasmonic acid signalling. The suppression by *P. croceum* observed in co-inoculated plants seems to be a local effect, as it was only detected in roots. Accordingly, in the frame of the TrophinOak project Mailänder (2014) showed that powdery mildew infection of oak leaves by *Microsphaera* (*Erysiphe*) *alphitoides* was not facilitated by inoculation of plants with *P. croceum* as compared to controls. In contrast, the enhanced defence gene expression in AcH 505-inoculated plants was also observed in the leaves, suggesting a systemic effect. This supports our previous finding that inoculating pedunculate oak roots with AcH 505 elicits a defence response against *M. alphitoides* in the leaves (Kurth et al., 2014).

Detailed analysis of the enriched leucine-rich repeat-containing proteins (LRRs) Pfam terms (Additional file 3) revealed differential expression of transmembrane receptor-like kinases (RLKs) and receptor-like proteins (RLPs). Most RLKs and RLPs have been implicated in recognition of MAMPs, but others relate to developmental processes (Diévart and Clark, 2004), suggesting that they might contribute to signal integration. Some of the LRR-containing receptor transcripts identified in this study were differentially expressed in AcH 505- and co-inoculated oaks, suggesting a function as important core genes and central

response regulators of the MHB. Among them, homologues with implicated roles in defence against pathogens in *A. thaliana* (Hok et al., 2011) were strongly up-regulated, including an RLK (AT2G34930) and a NBS-LRR (AT3G14460). This suggests that they may be involved in the recognition of AcH 505. In roots the expression of RLKs homologous to AT3G47110 and RLP 46 were induced by both treatments. These changes indicate that the AcH 505 induced root-specific signals via these receptors. The diversity of LRR-RLK and RLP proteins in the sets of DECs we identified, suggests that complex signalling mechanisms are involved in oak responses to AcH 505 attachment and concomitant ECM colonisation, which activate or repress various "sensor" proteins. Up-regulated transcripts we identified are probably connected to recognition events and maintenance of the interaction with AcH 505 (Kurth et al., 2014). Down-regulated transcripts could be related to the partial attenuation of defence responses required for the maintenance of the interaction with AcH 505 and *P. croceum*.

Another interesting outcome of our study was the identification of contigs encoding ethylene-responsive element binding factors (ERFs) amongst the core transcriptome affected by both treatments (Additional file 3). This was a novel observation for the AcH 505-interaction, but there are indications that in EM, ethylene signalling may be involved in control of the depth of colonisation of the plant's apoplast by the EMF (Plett et al., 2014a). Our data indicates that as a MHB, AcH 505 may affect this process by the modification of ERF expression. For instance, ERF8 contig was up-regulated in roots by the interaction with AcH 505 as well as by co-inoculation. Interestingly, the homologue in *A. thaliana* (*AtERF8*) is a participant in a signalling node of three interacting ERFs, which appear to negatively regulate chitin signalling in defences against fungi, but positively regulate salicylic acid signalling in plant defences against the bacterial pathogen *Pseudomonas syringae* (Son et al., 2012).

Analysis of AP2/ERF subfamily dehydration-responsive element-binding proteins (DREBs), which have overlapping functions with the ERFs involved in hormone signalling and plant defences (Mizoi et al., 2012), revealed increased expression of oak DREBs after inoculation with AcH 505 (Additional file 3), suggesting that the DREBs might also be involved in signal integration. Another AP2/ERF subfamily, SHINE clade of AP2 domain transcription factors regulate wax biosynthesis, pectin metabolism and cell wall structure (Shi et al., 2011), and the induction of a *SHN2* homolog by both treatments in oak microcuttings suggests that it might have a function in the regulation of cell wall structure, perhaps in combination with the XET proteins of oak. Our identification of AP2/ERF family member gene expression patterns has helped to increase understanding of their functions in oaks and indicates that these

transcription factors could act in concert with RLKs and RLPs in the coordination of fungus- and bacterium-elicited responses during oak development. Clearly, there is now an urgent need to associate these oak signalling genes with hormonal signalling pathways, interactions with insects and pathogens, and with the impact of abiotic environment.

Plant development

Changes in plant growth rates and patterns induced by bacteria and mycorrhizal fungi may be due to associated modifications of the main signalling pathways mediated by hormones (auxin, cytokinins and ethylene) involved in regulating plant development (Vacheron et al., 2013). The expression profiles reported here indicate the role of ethylene and auxin signalling in the response to AcH 505 and co-inoculation. A homologue of BAK1-interacting receptor-like kinase1 gene (BIR1), which stimulates the expression of auxin response regulators in *A. thaliana* (Kim et al., 2013), and a homologue of *A. thaliana* AIR9 gene, an auxin-induced microtubule associated protein which is part of the mechanism positioning the direction of cell division (Buschmann et al., 2006), were induced by both AcH 505 and co-inoculation. Rhythmic growth in oak microcuttings is related to differential expression of genes encoding auxin efflux carriers and auxin signalling proteins (Herrmann et al., submitted), and this process may thus be supported by AcH 505 alone and co-acting with *P. croceum*.

Genomic integrity

It is established that perceptions of, and responses to, environmental stresses may compromise the genomic integrity of plants (Bray and West, 2005), but the impact of biotrophic interactions on genome integrity is less well known. DNA lesion recognition and correction by the DNA mismatch-repair (MMR) systems promote genomic stability. In plants, MMR is initiated by the binding of heterodimeric MutS homologue (MSH) complexes, which recognize and bind mismatched and unpaired nucleotides (Wu et al., 2003). The interaction of the oaks with AcH 505 alone and during co-inoculation with the EMF led to up-regulation of regulation of an oak homolog of *MSH6* in roots during RF. This suggests that interaction with AcH 505 may cause greater stress to the integrity of oak genomes. This deduction is supported by the finding that inoculation of *A. thaliana* with *Pseudomonas putida* induces up-regulation of *RAD51*, another genome integrity-related gene that is required in double-strand break repair, and implicated in transcription of defence genes during immune responses of *A. thaliana* (Srivastava et al., 2012).

Differential representation of cell wall related transcripts

The Pfam term *Xyloglucan endo-transferase* (XET) was respectively over-represented and under-represented in sets of DECs associated with RF and SF in the roots of inoculated plants. XET transcripts (Zhu et al., 2006) and activities (Pritchard et al., 1993) are largely localized in the root elongation zone, and XET activity is required for the maintenance of root elongation (Wu et al., 1994). Thus, since the RF developmental stage coincides with maximal root elongation rates (Herrmann et al., 1998) and SF with pauses in root growth, these associations indicate that AcH 505 may enhance root elongation processes in oaks during RF.

Of the Pfam terms associated with glycosyl hydrolases, *Beta-galactosidase* was over-represented in resting stages, and under-represented in the growing stages of both roots and leaves. The homology of the predicted oak proteins to plant beta galactosidases indicates that they may support tissue differentiation by the modification of cell wall galactans. Of the homologs, *A. thaliana* BGAL12 is localised within cell walls (Gantulga et al., 2009), and associated with root differentiation (Albornos et al., 2012). Homologs of other oak genes have been implicated in the cell wall loosening by degradation of cell-wall galactans during pollen expansion in tobacco (Hrubá et al., 2005) and fruit ripening in tomato (Carey et al., 2001).

Production of secreted peroxidases is a crucial factor for regulation of the flexibility of plant cell walls, and strongly affected by both treatments. For instance, one of the oak peroxidase transcripts induced by AcH 505 alone and together with *P. croceum* is orthologous to the *A. thaliana* gene *AtPER10,* known to be up-regulated after ethylene treatment (Markakis et al., 2012). Increases in ethylene and peroxidase activities are both probably involved in the stimulation of peroxidase-mediated crosslinking in the cell wall, which prevents cell expansion (Markakis et al., 2012). Thus, during SF the up-regulation of peroxidases in oak roots may contribute to cell wall maturation.

An interesting observation in this study regarding cell wall-related transcripts was that both treatments induced a thaumatin gene during SF in roots and leaves, in accordance with previous findings with *P. croceum* (Frettinger et al., 2007). This indicates a thaumatin based defence mechanism in pedunculate oak. Thaumatins are cell wall components that show inducible expression by stresses such as pathogen attack, and are involved in plant defences against microorganisms (Asiegbu et al., 2005), as well as in plant response to mutualists (Verhagen et al., 2004). The importance of thaumatins in biotic interactions of the oak is underlined by our observation that the abundance of fungal *P. croceum* thaumatin like protein transcripts increases in ectomycorrhiza (M. T., S. H., F. B., unpublished).

Together, these results illustrate the interactive relationship between oak cell wall dynamics and plant-AcH 505 interactions. A similar connection has been shown for mycorrhizal symbioses in oaks (Tarkka et al., 2013; Sebastiana et al., 2014) and *Populus* (Plett et al., 2014a), and the suppression of Norway spruce root infection by root pathogenic fungi by *Streptomyces* strain GB 4-2 (Lehr et al., 2008).

Conclusions

The here presented results show a strong local and systemic plant response in oak microcuttings to a mycorrhization helper bacterium under SF, at the resting stage of root growth. The weaker molecular response in RF, in terms of the number of DEC might indicate that the physiology in roots is rather devoted to growth at RF while the processes directed towards interactions are in most part attenuated. Future work will show if the repressed gene expression response consequently interferes with elicitation of defence responses or symbiosis related signalling in RF. At the organismic interactions level, oak root colonisation by AcH 505 and *P. croceum* may be affected, since oak is more susceptible to the root parasite *Phytophthora quercina* during RF than SF (Angay et al., 2014).

In oaks, the innate immune response pathways appear to be coordinated with developmental pathways, leading to changes in numerous cellular and physiological processes. Our results suggest that *R* gene products, receptor proteins and receptor protein kinases detect, while AP2/ERF transcription factors integrate, microbe perception and plant development signals. Our data also indicates a pivotal role of the cell wall as the responsive element in responses to both AcH 505 alone and during co-inoculation with the EM fungus. Future work needs to focus on the candidate genes to verify their involvement in these processes.

Methods

The soil-based culture system

The pedunculate oak clone DF159 (*Quercus robur* L.) was micropropagated according to Herrmann et al. (2004), then cultivated in soil-based microcosms with the ectomycorrhizal fungus *P. croceum* as previously described (Tarkka et al., 2013). Briefly, rooted microcuttings were placed in Petri dishes filled with a 1:1 (v/v) mixture of gamma-sterilized soil and fungal inoculum. Soil filtrates were prepared as described by Rosenberg et al. (2009) and 5 ml of a 1/100 dilution was added to each culture system 4 weeks after culture establishment. The conditions for bacterial and fungal cultivation and plant inoculation were as described by Tarkka et al. (2013). Briefly, *Piloderma croceum* J. Erikss. & Hjortst. Strain 729 (DSM-

4924) inoculum was produced by inoculating a substrate mixture of vermiculite, sphagnum peat and liquid medium with a 2-week-old liquid fungal culture and inoculated at establishment of the microcosm. *Streptomyces* sp. AcH 505 was originally isolated from the soil around Norway spruce mycorrhizas in Haigerloch, Germany (Maier et al., 2004) and maintained on ISP2 agar medium (Shirling and Gottlieb, 1966). For the experiment, the culture system was inoculated with 2.5 x 10^7 AcH 505 spores at 3 and 7 weeks after establishment of the microcosm. With this form of inoculation, AcH 505 stimulates oak-*P. croceum* mycorrhiza formation (Kurth et al., 2013), and elicits plant defences in leaves against the powdery mildew (Kurth et al., 2014). AcH 505 was quantified according to Kurth et al. (2013), who investigated the colonisation of soil and oak rhizosphere by the bacterium and *P. croceum*, and, based on previous experiments described in detail by Kurth et al. (2014), a stable concentration was confirmed at the second application and at the harvest time point.

The oaks were grown for 8 weeks in climate chambers under 23 ± 1 °C (mean ± SD) and long day photoperiods (16 h/8 h), with a photosynthetic photon flux density (PPFD) of 180 µmol m^{-2} s^{-1} and 75 % relative humidity. Harvest times were based on published effects of AcH 505 on EM formation and plant growth (Kurth et al., 2013; Kurth et al., 2014). The use of identical timing of AcH 505 inoculation in different experiments allowed comparative analysis of oak responses to both of the interacting microorganisms.

The experimental design included six treatments: 3 (no inoculation/AcH 505/AcH 505 + *P. croceum*) x 2 (RF/SF). We focused on the treatment with AcH 505 single inoculation, and AcH 505 in co-occurrence with *P. croceum*. The impact of *P. croceum* single inoculation on oak gene expression levels has already been reported (Tarkka et al., 2013; Herrmann et al., submitted). Sink leaves, source leaves, stems, lateral roots and principal roots were weighed at harvest, pictures were taken for leaf surface measurements with WinFolia (Regent Instruments Inc., Quebec, Canada) and samples were submerged in liquid nitrogen. For our investigations we only used plants that were in the developmental root flush (RF) and shoot flush (SF) stages, corresponding respectively to bud swelling stage B and leaf expansion stage D according to Herrmann et al. (1998).

RNA extraction and transcript quantitation by Illumina sequencing

For transcriptomic analyses, lateral roots and source leaves for plants during RF or sink leaves for plants during SF were used. Samples from 2-3 plants subjected to each treatment were pooled and homogenized under liquid nitrogen. Total RNA was isolated from 36 pools (3 treatments x 2 tissues x 2 developmental stages x 3 replicates) using the MasterPure Plant

RNA Purification Kit (Epicentre, Hessisch Oldendorf, Germany) with 100 mg of root and 50 mg of leaf material per extraction. RNA quality and quantity were verified using gel electrophoresis, a NanoDrop 1000 spectrophotometer and an Agilent 2100 Bioanalyzer prior to Illumina sequencing analysis at the Beijing Genomics Institute (Hong Kong, China). 100 bp paired-end libraries were constructed and sequenced (15 million reads per sample) using an Illumina HiSeq2000 sequencing platform.

Read processing and analysis of differential expression

Reads were processed following Tarkka et al. (2013). Briefly, low quality sequences and sequencing artefacts were removed with SeqClean (http://compbio.dfci.harvard.edu/tgi/software/) and low quality sequencing ends were trimmed with a custom Java script. Short sequences (< 50 bp) and sequences lacking paired-end information were discarded. The processed Illumina reads were aligned against the reference transcriptome OakContigDF159.1 (Tarkka et al., 2013) by Bowtie (Langmead et al., 2009) and quantified by RSEM (Li and Dewey, 2011). Fold-changes in gene expression were calculated using the edgeR function (Robinson et al., 2010) implemented in the Bioconductor package (Gentleman et al., 2004). Gene Ontology (Harris et al., 2004) and Pfam (Punta et al., 2012) enrichment analyses were performed with the Bioconductor package GOseq (Young et al., 2010).

Real-time-quantitative reverse transcriptase-PCR (RT-qPCR) primer design and reactions

Differential gene expression data obtained from the Illumina analyses were validated by qRT-PCR analysis of the expression of 10 genes in leaf samples from control and co-inoculated plants. Primer pairs were constructed using the OakContigDF159.1 assembly as a reference and tested for functionality, amplicon size, specificity and efficiency as previously described (Tarkka et al., 2013). Sequences of constructed primer pairs are listed in Additional file 4. The qRT-PCR reactions were performed as described by Tarkka et al. (2013). Briefly, using an iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad) and *18S rRNA* as the reference gene, transcript abundances in the leaf samples were determined based on their Ct values using the Relative Expression Software Tool (REST, Pfaffl et al., 2002). The coefficient of variation (CV) was used as a producibility indicator, with a maximal value of 6.0. Differential gene expression was determined by a randomisation test implemented in REST.

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Figures

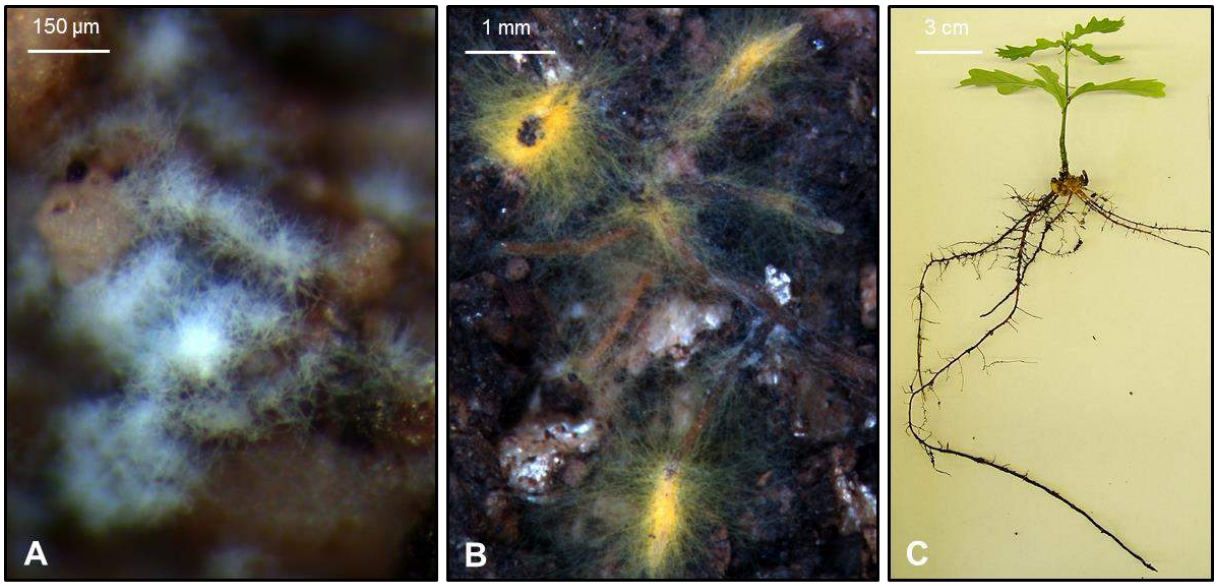

Figure 1 Pedunculate oak microcuttings with interacting microorganisms. *Streptomyces* sp. AcH 505 on soil particles in the microcosm (A), *Piloderma croceum* – oak ectomycorrhizas (B), and a pedunculate oak *Quercus robur* microcutting (C)

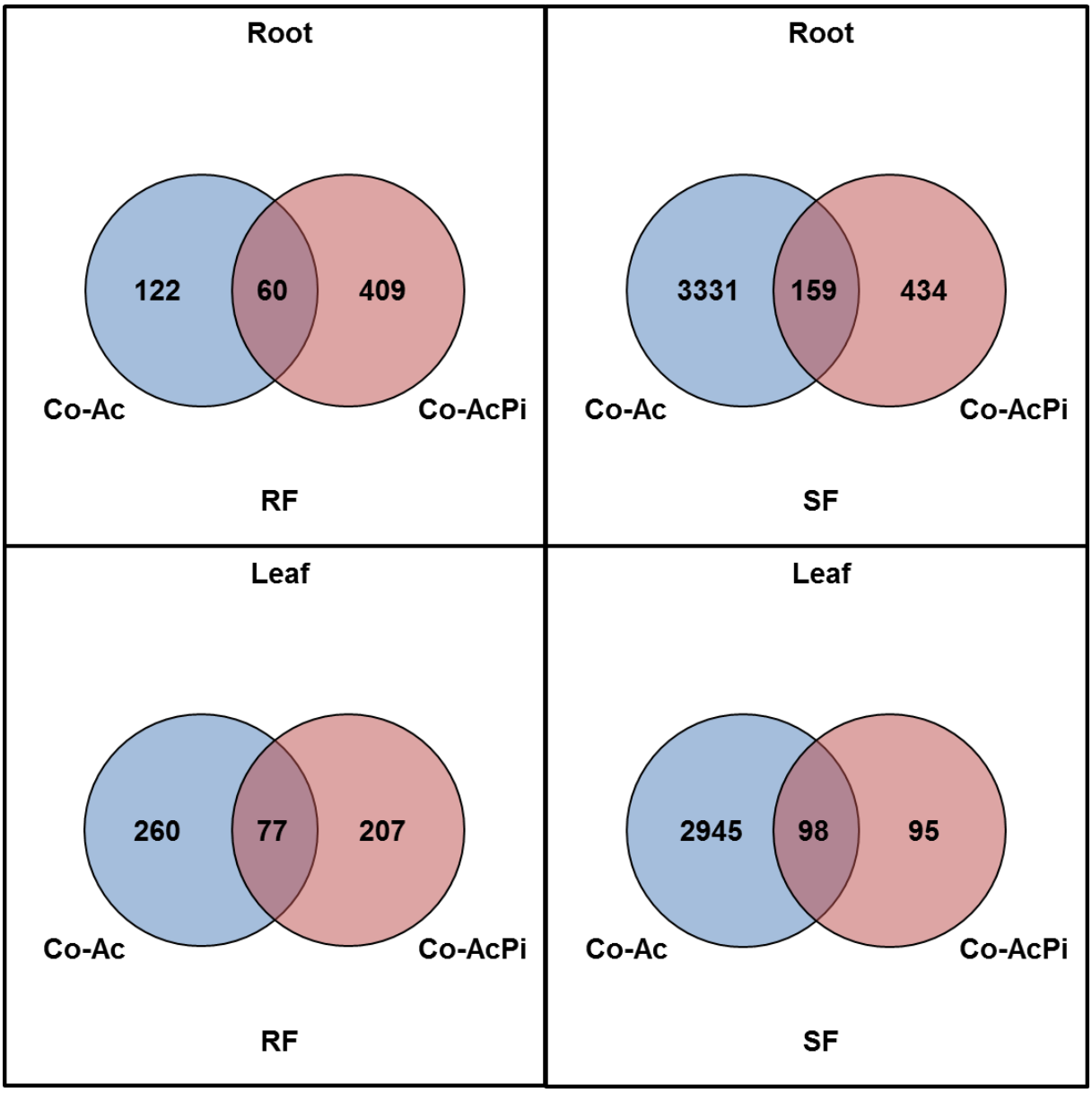

Figure 2 Venn-diagrams illustrating numbers of differentially expressed contigs (DECs: Benjamini-Hochberg adjusted, $p \leq 0.05$). The comparisons depicted are for the following pairs of roots and leaves of microcuttings during root flush (RF) and shoot flush (SF): Control versus AcH 505 inoculated (Co-Ac) and Control- versus AcH 505- and *P. croceum*-inoculated (Co-AcPi).

GO term	Definition	RF		SF	
		CoAc	CoAcPi	CoAc	CoAcPi
GO:0042742	Defence response to bacterium			8.4	-4.1
GO:0009862	Salicylic acid mediated signalling pathway			5.4	
GO:0009867	Jasmonic acid mediated signalling pathway			5.2	-1.6
GO:0010310	Hydrogen peroxide metabolic process		-1.5	3.7	
GO:0000165	MAPK cascade		-1.8	3.5	
GO:0009595	Detection of biotic stimulus		-2.0	3.4	-1.4

Figure 3 Differential representation of plant defence-related Gene Ontology terms in pedunculate oak roots, showing terms over-represented after AcH 505 inoculation specifically during SF and partially depleted in co-inoculated plants. GO enrichment analysis was implemented by GOseq. The intensity of the orange and blue colours indicates degrees of over-representation of GO terms for up- (orange) and down-regulated (blue), respectively. The numbers in the boxes indicate levels of enrichment of the corresponding GO terms, expressed as log10 p-values. The p-value cut-off was set at $p \leq 0.05$, equal to $p \leq 10^{(-1.301)}$.

Root				Leaf				Pfam term description
RF		SF		RF		SF		
Co-Ac	Co-PiAc	Co-Ac	Co-PiAc	Co-Ac	Co-PiAc	Co-Ac	Co-PiAc	
***	***	**	**	***		***		Xyloglucan endo-transglycosylase (XET)
***	**	**			**	**		Glycosyl hydrolases family 2
***	**	*			**	*		Glycosyl hydrolases family 35
***	**	*			**	**		Beta-galactosidase
***	**	*			**	**		Beta-galactosidase jelly roll domain
	**		*	*				Auxin binding protein
	*			*	*		*	Dockerin type I repeat
					*		*	eRF1 domain 3
*	*							Noc2p family
		*		**		**		Cellulose synthase
	***	**	***			***	***	2,3-bisphosphoglycerate-independent phosphoglyceratemutase
			*			*	*	Leucine rich repeat 1
			**				*	Leucine rich repeat 8
		*				**		Leucine rich repeat N-terminal domain
			*				*	Leucine rich repeat 7
*		*	*		*	*	*	Leucine rich repeat 6
*								MIT (microtubule interacting and transport) domain
*		***		***	***	*	**	Protein kinase domain
		***						F-box-like
*	*	***		**		***	*	AP2 domain
				**				Cytoskeletal-regulatory complex EF hand
						*	*	Chitinase class I
	***	**	*	**	***	*		Probable lipid transfer
			*	***		**	**	Terpene synthase, N-terminal domain
				*	*			NADH:flavinoxidoreductase/NADH oxidase family
		*		***			***	tify domain
		***				**		Chitin synthase
			**			***	***	IQ calmodulin-binding motif
	**	***						Thaumatin family
*	**							Est1 DNA/RNA binding domain
**	*							Lamina-associated polypeptide 2 alpha
***	***							MutS domain V
**	*							Orotidine5'-phosphate decarboxylase/HUMPS family
	*							Histone methylation protein DOT1
							**	DNA replication factor CDT1 like
							*	DNA polymerase III subunits tau domain IV
					*	*		Enhancer of rudimentary

Figure 4 Over- and under-represented protein family (Pfam) terms, according to comparisons with non-inoculated controls in roots and leaves harvested from plants during root flush (RF) and shoot flush (SF), treated with AcH 505 (Co-Ac) and both microorganisms (Co-AcPi). Orange colour indicates up-regulated and blue down-regulated enriched Pfam terms. Significance levels are marked by asterisks in the boxes.

Tables

Table 1 Consistently up- or down-regulated contigs in pairwise comparisons of roots and leaves of Control versus AcH 505-inocuclated (Co-Ac) and Control versus AcH 505- and *P. croceum*-inoculated (Co-AcPi) microcuttings during root flush (RF) and shoot flush (SF). Significant up- (orange) and down-regulation (blue) was determined by edgeR with a threshold Benjamini-Hochberg adjusted p-value of 0.05, indicated by "FDR".

	Co-Ac		Co-PiAc		
Contig	fold change	FDR	fold change	FDR	**Sequence description**
			Root - RF		
32163	2.2	1.3E-05	2.9	8.3E-09	Clavaminate synthase
34428	8.8	2.0E-07	7.7	1.3E-04	Ice binding
36512	5.9	1.1E-06	6.0	5.3E-08	Nucleolar complex protein
37308	9.0	1.6E-04	8.9	1.2E-07	Ribulose-phosphate 3
37819	7.9	1.5E-03	8.5	3.6E-10	o-linked c transferase
43120	2.6	7.4E-07	2.2	6.6E-17	XET hydrolase
43557	8.4	7.0E-03	8.6	2.2E-10	tRNA-dihydrouridine synthase
43753	8.6	9.0E-05	8.6	3.9E-08	Kinase-like protein
			Root - SF		
19664	0.8	2.2E-11	1.0	2.3E-03	Thaumatin
24051	-6.4	9.2E-06	-6.5	1.7E-03	40s ribosomal protein
29599	-1.3	6.6E-19	-1.9	9.9E-03	Gibberellin-regulated protein
35114	1.1	1.4E-45	1.4	5.4E-04	Zinc finger protein
41819	-8.5	1.3E-10	-8.7	4.5E-07	F-box family protein
42100	7.2	9.2E-06	6.9	7.8E-04	Chromatin binding
42662	1.6	1.1E-99	1.1	3.1E-05	Cytochrome p450
43426	-7.2	4.8E-07	-7.4	1.9E-04	Sugar transporter
			Leaf - RF		
19833	-0.8	5.0E-03	-0.9	3.6E-04	Serine hydroxymethyltransferase
23339	-1.5	1.6E-06	-1.8	7.6E-10	d-3-phosphoglycerate
33009	8.5	4.5E-09	8.2	9.9E-07	Cop9 signalosome complex subunit
36279	-9.0	1.1E-09	-8.9	1.9E-09	gtp cyclohydrolase
39615	2.2	3.9E-06	1.8	9.3E-04	Multicopper oxidase
40475	-1.1	8.2E-04	-0.9	3.0E-03	Leucine-rich repeat receptor
42008	3.7	3.5E-06	3.1	2.4E-03	Calcium-binding protein
42379	4.3	9.0E-03	4.4	2.1E-03	Pho1-like protein
42669	5.1	1.2E-05	3.8	6.2E-03	Cellulose synthase
			Leaf - SF		
21202	3.4	4.9E-20	1.4	1.7E-04	Anthocyanidin synthase
30731	-0.7	1.9E-20	-0.9	1.3E-03	Cbl-interacting serine threonine-protein
30800	-2.7	1.8E-06	-2.2	3.6E-03	Adenylyl-sulfate reductase
39235	-6.7	1.8E-04	-6.7	5.8E-03	Chaperone protein
42008	2.5	1.6E-41	2.6	6.0E-04	Calcium-binding protein
42290	1.2	3.6E-27	1.8	9.2E-10	Lipoxygenase
42379	8.6	5.4E-11	9.0	1.2E-03	Pho1-like protein
43826	7.8	2.5E-06	7.3	1.1E-04	Disease resistance rpp13
43826	8.8	4.1E-10	7.9	1.1E-05	lrr-nbs resistance protein

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Supporting information

Additional file 1 Results of real-time RT-PCR analysis of 10 contigs with increased or decreased expression levels in leaves of AcH 505 and *P. croceum*-inoculated seedlings relative to levels in Controls according to RNA-Seq.

Contig	RNA-Seq		qRT-PCR		Blastx predicted transcript identity
	log2 fold change	p-value	log2 fold change	p-value	
39043	-1.8	1.0E-02	-1.0	3.5E-03	Inositol oxygenase
43608	-1.7	4.0E-02	-1.1	6.4E-02	Pectinesterase
43258	-1.3	1.4E-03	-1.1	8.0E-02	Glutamate dehydrogenase
43229	-1.0	4.9E-03	-2.0	6.6E-02	Tata box binding protein associated factor
34707	-0.9	0.0E+00	-0.3	4.6E-01	Trehalose-phosphate synthase
42379	-0.9	2.0E-02	-0.3	7.0E-01	Phosphate transporter
32318	0.7	4.0E-02	0.2	2.0E-02	Calcium-binding allergen
32511	1.1	1.6E-03	0.9	1.0E-03	Phosphate transporter
42037	1.2	1.3E-03	1.9	3.4E-02	Peptide nitrate transporter
39751	2.1	1.1E-03	0.3	7.4E-01	Ap2 erf domain-containing transcription factor

Additional file 2 Numbers of differentially expressed contigs detected in the pairwise comparisons of roots and leaves of Controls versus AcH 505-inoculated plants (Co-Ac) and Controls versus AcH 505- and *P. croceum*-inoculated plants during root flush (RF) and shoot flush (SF). Up- and down-regulated indicates up- and down-regulation in the inoculated plants, respectively (Benjamini-Hochberg adjusted $p \leq 0.01$).

Numbers of differentially expressed contigs				
	Root		Leaf	
	Co-Ac	Co-AcPi	Co-Ac	Co-AcPi
RF				
total	182	469	337	284
up-regulated	113	264	226	139
down-regulated	69	205	111	145
SF				
total	3490	593	3043	193
up-regulated	1753	162	1499	115
down-regulated	1737	431	1544	78

Additional file 3 Differentially expressed transcripts in microcuttings of the pedunculate oak clone DF159 inoculated with AcH 505 and *Piloderma croceum*. The OakContigDF159.1 transcripts were selected based on their predicted biological functions, including transcripts encoding receptor kinases and receptor-like proteins, transcription factors and cell wall constituents. Blastx searches against *Arabidopsis thaliana* polypeptides were used to predict the functions of the contig-encoded polypeptides. Treatments: Co, control (no inoculation); Ac, *Streptomyces* sp. AcH 505-inoculated; AcPi, AcH 505- and *Piloderma croceum*-inoculation. L, leaf; R, root; RF, root flush; SF, shoot flush. log_2 indicates log_2 change in gene expression level and FDR false discovery rate (i.e. Benjamini-Hochberg adjusted p-value with a threshold of $p \leq 0.01$)

Treatment	**Contig**	**log_2**	**FDR**	***Arabidopsis* homologue**	**Protein function in *Arabidopsis***	**Biological role or expression pattern in *Arabidopsis***
Signal transduction by LRR containing transmembrane receptor like proteins						
CoAcLSF	comp18677_c0_seq1	-1.72	1.1E-05	AT4G28380	LRR family protein	Signal transduction
CoAcLSF	comp28607_c0_seq1	-0.39	3.9E-03	AT5G53890	LRR receptor-like PK	PSKR2; protein phosphorylation, response to wounding, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcRSF	comp30167_c0_seq1	0.32	3.6E-07	AT3G43740	LRR family protein	Signal transduction
CoAcLSF	comp30167_c0_seq1	-0.37	7.0E-14	AT3G43740		
CoAcLSF	comp30877_c0_seq1	2.11	5.7E-04	AT1G58190	LRR receptor like protein	RLP9; signal transduction
CoAcRSF	comp31038_c0_seq1	-0.62	2.3E-07	AT3G24240	LRR receptor-like PK	Protein phosphorylation, response to molecule of bacterial origin, signal transduction
CoAcLSF	comp31214_c1_seq1	-0.39	1.6E-05	AT5G48940	LRR receptor-like PK	Protein phosphorylation, response to molecule of bacterial origin, signal transduction
CoAcRSF	comp31933_c1_seq1	-0.43	2.4E-05	AT5G56040	LRR receptor-like PK	SKM2; meristem growth, response to molecule of bacterial origin, signal transduction
CoAcLSF	comp31933_c1_seq1	-0.63	2.0E-13	AT5G56040		
CoAcRSF	comp32427_c0_seq1	0.27	3.4E-04	AT3G13380	LRR receptor-like PK	BRL3; protein phosphorylation, signal transduction
CoAcRSF	comp33630_c0_seq1	-0.38	5.1E-03	AT5G01890	LRR receptor-like PK	PXC2; protein phosphorylation, regulation of meristem growth, signal transduction
CoAcLSF	comp34223_c1_seq1	-0.76	1.0E-04	AT5G49660	LRR receptor-like PK	XIP1; regulation of flavonoid biosynthetic process, response to molecule of bacterial origin, signal transduction
CoAcLRF	comp34315_c0_seq1	-1.09	8.3E-03	AT1G74170	LRR receptor like protein	RLP13; signal transduction
CoAcLSF	comp34315_c0_seq1	1.14	5.1E-08	AT1G74170		
CoAcRSF	comp34550_c0_seq1	-0.63	3.0E-03	AT5G51560	LRR receptor-like PK	Protein phosphorylation, regulation of meristem growth
CoAcLSF	comp34550_c0_seq1	0.51	5.3E-03	AT5G51560		
CoAcRSF	comp35210_c0_seq1	-1.45	5.9E-27	AT3G51740	LRR receptor-like PK	IMK2; protein phosphorylation
CoAcLSF	comp35210_c0_seq1	-1.28	1.4E-15	AT3G51740		
CoAcPiRRF	comp35425_c0_seq1	0.72	7.3E-03	AT2G36570	LRR receptor-like PK	PXC1; protein phosphorylation

CoAcRSF	comp35901_c0_seq1	-0.80	2.5E-09	AT3G03770	LRR receptor-like PK	Protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcLRF	comp35901_c0_seq1	1.52	2.2E-05	AT3G03770		
CoAcLSF	comp35901_c0_seq1	0.39	1.2E-03	AT3G03770		
CoAcRSF	comp36126_c0_seq1	-5.26	5.8E-03	AT1G17750	LRR receptor-like PK	PEPR2; abscisic acid and ethylene mediated signalling pathway, response to jasmonic acid stimulus
CoAcRSF	comp36310_c1_seq1	-0.53	3.0E-03	AT4G23740	LRR receptor-like PK	Protein phosphorylation, regulation of meristem growth
CoAcPiLRF	comp36594_c1_seq1	-0.91	4.5E-03	AT5G27060	LRR receptor like protein	RLP53; defence response, signal transduction
CoAcRSF	comp36761_c0_seq1	-0.52	3.1E-03	AT2G15320	LRR family protein	Signal transduction
CoAcLSF	comp37147_c0_seq2	6.60	4.7E-04	AT1G74180	LRR receptor like protein	RLP14; signal transduction
CoAcRSF	comp37569_c0_seq1	-0.55	2.6E-03	AT5G14210	LRR receptor-like PK	Protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcRSF	comp37906_c0_seq1	-0.28	3.9E-05	AT3G23750	LRR receptor-like PK	Protein phosphorylation
CoAcRSF	comp38024_c0_seq1	-0.38	4.7E-03	AT4G20140	LRR receptor-like PK	GSO1; protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcRSF	comp38313_c1_seq1	0.32	1.5E-06	AT5G54080	LRR receptor-like PK	PSKR2; protein phosphorylation, response to wounding, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcRSF	comp38313_c1_seq2	-0.45	5.7E-06	AT5G58300	LRR receptor-like PK	Protein phosphorylation, regulation of meristem growth
CoAcLSF	comp38313_c1_seq2	-0.36	1.5E-03	AT5G58300		
CoAcRSF	comp38911_c1_seq1	0.56	8.2E-08	AT5G61480	LRR receptor-like PK	PXY; organ morphogenesis, protein phosphorylation, regulation of meristem growth
CoAcLSF	comp39268_c0_seq1	-0.54	3.2E-03	AT5G10290	LRR receptor-like PK	Protein phosphorylation
CoAcRSF	comp39335_c2_seq2	-1.78	9.7E-03	AT5G48940	LRR receptor-like PK	Protein phosphorylation, response to molecule of bacterial origin, signal transduction
CoAcRSF	comp39404_c2_seq1	-0.71	3.9E-08	AT2G45340	LRR receptor-like PK	Protein phosphorylation, regulation of meristem growth
CoAcRSF	comp39763_c1_seq1	-0.71	1.4E-05	AT4G36180	LRR receptor-like PK	Cell proliferation, signal transduction
CoAcRSF	comp39927_c0_seq1	0.51	7.6E-04	AT1G79620	LRR receptor-like PK	Cell wall biogenesis, protein phosphorylation
CoAcRSF	comp40559_c0_seq4	-0.37	5.1E-03	AT2G01950	LRR receptor-like PK	VH1; auxin mediated signalling pathway, regulation of meristem growth, signal transduction
CoAcRSF	comp40575_c0_seq1	1.66	8.2E-05	AT2G33060	LRR receptor like protein	RLP27; defence response, signal transduction
CoAcRSF	comp40732_c0_seq1	0.50	8.5E-05	AT3G23750	LRR receptor-like PK	Protein phosphorylation
CoAcPiRSF	comp40766_c2_seq2	0.85	5.5E-03	AT1G58190	LRR receptor like protein	RLP9; signal transduction
CoAcLSF	comp40766_c2_seq6	2.68	2.6E-04	AT5G27060	LRR receptor like protein	RLP53; defence response, signal transduction
CoAcLSF	comp40766_c3_seq2	2.85	9.0E-05	AT2G25470	LRR receptor like protein	RLP21; signal transduction
CoAcRSF	comp40886_c0_seq1	0.66	7.5E-04	AT4G29180	LRR receptor-like PK	Cell proliferation, signal transduction
CoAcLSF	comp41108_c1_seq1	-0.46	1.2E-12	AT1G72180	LRR receptor-like PK	Protein phosphorylation, response to molecule of bacterial origin, signal transduction
CoAcLSF	comp41318_c2_seq1	-0.71	1.2E-14	AT5G58540	LRR receptor-like PK	Plant-type cell wall biogenesis, protein phosphorylation, xylan biosynthetic process

CoAcRRF	comp41334_c0_seq1	5.16	9.0E-03	AT4G04220	LRR receptor like protein	RLP46; defence response, jasmonic acid mediated signalling pathway, salicylic acid mediated signalling pathway
CoAcLSF	comp41334_c0_seq4	4.34	7.5E-05	AT4G04220		
CoAcRSF	comp41504_c0_seq2	0.77	4.4E-04	AT1G74170	LRR receptor like protein	RLP13; signal transduction
CoAcRSF	comp41546_c0_seq1	0.51	1.8E-07	AT1G66150	LRR receptor-like PK	TMK1; protein phosphorylation, signal transduction
CoAcPiLRF	comp41546_c0_seq1	1.01	7.1E-04	AT1G66150		
CoAcLSF	comp41546_c0_seq1	0.34	1.5E-03	AT1G66150		
CoAcPiLRF	comp41659_c0_seq1	-1.00	2.0E-03	AT1G09970	LRR receptor-like PK	RLK7; protein phosphorylation, defence response, signal transduction
CoAcRSF	comp41865_c0_seq1	0.25	1.2E-03	AT5G48380	LRR receptor-like PK	BIR1; defence response, salicylic acid mediated signalling pathway
CoAcRRF	comp41865_c0_seq4	0.99	2.7E-03	AT5G48380	LRR receptor-like PK	BIR1; defence response, salicylic acid mediated signalling pathway
CoAcLSF	comp41865_c0_seq4	0.42	3.9E-04	AT5G48380	LRR receptor-like PK	BIR1; defence response, salicylic acid mediated signalling pathway
CoAcRRF	comp41868_c0_seq2	1.18	1.5E-03	AT1G34420	LRR receptor-like PK	Defence response, protein phosphorylation, salicylic acid mediated signalling pathway
CoAcLSF	comp41868_c0_seq2	0.80	1.9E-16	AT1G34420		
CoAcPiRSF	comp41998_c0_seq1	1.04	1.0E-05	AT1G75820	LRR receptor-like PK	CLV1; cell differentiation, protein phosphorylation
CoAcRSF	comp42008_c1_seq2	0.27	1.4E-03	AT5G01850	LRR receptor-like PK	Protein phosphorylation
CoAcRSF	comp42108_c0_seq1	0.40	1.6E-06	AT5G51350	LRR receptor like protein	MOL1; protein phosphorylation
CoAcLSF	comp42210_c0_seq4	1.40	1.8E-11	AT3G57830	LRR receptor-like PK	Protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcRSF	comp42300_c0_seq1	0.32	3.2E-09	AT5G65700	LRR receptor like protein	BAM1; protein phosphorylation
CoAcRSF	comp42458_c0_seq1	-0.41	7.4E-04	AT1G24650	LRR receptor-like PK	Protein phosphorylation
CoAcLRF	comp42458_c0_seq1	1.23	4.9E-03	AT1G24650		
CoAcLSF	comp42467_c0_seq2	0.40	2.1E-07	AT4G28490		
CoAcRRF	comp42549_c1_seq1	1.75	9.5E-05	AT3G05660	LRR receptor like protein	RLP33; defence response, jasmonic acid mediated signalling pathway, salicylic acid mediated signalling pathway
CoAcRSF	comp42549_c1_seq1	0.57	7.2E-03	AT3G05660		
CoAcRSF	comp42644_c0_seq1	-1.04	4.0E-04	AT1G08590	LRR receptor-like PK	Protein phosphorylation, response to molecule of bacterial origin, signal transduction
CoAcRSF	comp42660_c0_seq2	1.32	1.4E-04	AT4G13920	LRR receptor like protein	RLP50; defence response, signal transduction
CoAcLSF	comp42715_c0_seq1	-1.06	2.5E-22	AT5G49760	LRR receptor-like PK	Protein phosphorylation
CoAcPiLSF	comp42715_c0_seq1	-1.27	3.4E-05	AT5G49760		
CoAcLSF	comp42715_c0_seq2	2.00	1.0E-11	AT5G49760		
CoAcRSF	comp42715_c0_seq3	0.62	2.8E-08	AT5G49760		
CoAcLSF	comp42919_c0_seq1	1.23	1.2E-06	AT5G25930	LRR receptor-like PK	Abscisic acid mediated signalling pathway, defence response
CoAcLSF	comp43093_c0_seq4	-1.15	6.5E-03	AT5G20480	LRR receptor-like PK	EFR; defence response, jasmonic acid mediated signalling pathway, salicylic acid mediated signalling pathway
CoAcLSF	comp43262_c1_seq1	2.12	1.8E-03	AT5G20480		

CoAcLSF	comp43263_c0_seq2	1.72	2.5E-03	AT3G05660	LRR receptor like protein	RLP33; defence response, jasmonic acid mediated signalling pathway, salicylic acid mediated signalling pathway
CoAcLSF	comp43281_c0_seq1	0.55	6.9E-04	AT2G15080	LRR receptor like protein	RLP19; defence response, jasmonic acid mediated signalling pathway, salicylic acid mediated signalling pathway
CoAcRSF	comp43281_c0_seq5	6.46	7.2E-03	AT1G47890	LRR receptor like protein	RLP7; defence response, signal transduction
CoAcPiRSF	comp43281_c0_seq5	6.97	4.1E-04	AT1G47890		
CoAcLSF	comp43340_c1_seq7	-7.09	8.7E-03	AT1G47890		
CoAcRSF	comp43391_c0_seq1	0.58	5.8E-17	AT1G09970	LRR receptor-like PK	RLK7; protein phosphorylation, defence response, signal transduction
CoAcLSF	comp43391_c0_seq1	-0.48	8.8E-08	AT1G09970		
CoAcLSF	comp43403_c0_seq1	-0.46	1.3E-15	AT1G06840	LRR receptor-like PK	Protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcRSF	comp43439_c0_seq1	1.09	1.5E-04	AT5G40170	LRR receptor like protein	RLP54; defence response, jasmonic acid mediated signalling pathway, salicylic acid mediated signalling pathway
CoAcLSF	comp43439_c0_seq6	6.95	8.0E-03	AT5G40170		
CoAcPiLSF	comp43439_c0_seq6	6.91	2.3E-03	AT5G40170		
CoAcRSF	comp43444_c0_seq1	0.51	1.6E-05	AT5G25930	LRR receptor-like PK	Abscisic acid mediated signalling pathway, defence response
CoAcPiLRF	comp43444_c0_seq1	-0.90	4.9E-04	AT5G25930		
CoAcLSF	comp43450_c0_seq1	1.06	6.5E-09	AT3G47110	LRR receptor-like PK	Protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcLSF	comp43450_c0_seq4	1.93	3.8E-03	AT5G20480	LRR receptor-like PK	EFR; defence response, jasmonic acid mediated signalling pathway, salicylic acid mediated signalling pathway
CoAcLSF	comp43450_c0_seq5	1.34	2.7E-07	AT3G47110	LRR receptor-like PK	Protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcRSF	comp43465_c2_seq1	0.89	6.8E-06	AT4G08850	LRR receptor-like PK	Protein phosphorylation
CoAcPiRSF	comp43486_c1_seq1	1.18	7.5E-03	AT1G47890	LRR receptor like protein	RLP7; defence response, signal transduction
CoAcPiLSF	comp43486_c1_seq1	2.24	1.7E-07	AT1G47890		
CoAcPiLSF	comp43486_c1_seq2	2.60	3.9E-03	AT3G28890		
CoAcRSF	comp43489_c1_seq2	-0.69	2.0E-05	AT5G56040	LRR receptor-like PK	SKM2; meristem growth, response to molecule of bacterial origin, signal transduction
CoAcLSF	comp43489_c1_seq2	-0.57	2.1E-10	AT5G56040		
CoAcRSF	comp43491_c1_seq13	7.13	4.7E-04	AT2G33060	LRR receptor like protein	RLP27; defence response, signal transduction
CoAcLRF	comp43518_c1_seq10	-5.55	3.6E-08	AT1G07650	LRR receptor-like PK	Protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcRSF	comp43518_c1_seq22	0.36	6.8E-03	AT1G07650		
CoAcRRF	comp43518_c1_seq35	7.58	3.8E-04	AT1G07650		
CoAcRSF	comp43518_c1_seq39	0.73	2.3E-04	AT3G14840	LRR receptor-like PK	Protein phosphorylation
CoAcLRF	comp43518_c1_seq39	-1.42	1.3E-04	AT3G14840		
CoAcPiLRF	comp43518_c1_seq39	-1.64	8.4E-10	AT3G14840		
CoAcLSF	comp43518_c1_seq39	-1.42	1.5E-24	AT3G14840		

CoAcPiLSF	comp43518_c1_seq39	-2.87	3.2E-18	AT3G14840		
CoAcLSF	comp43549_c0_seq11	1.63	6.3E-07	AT1G47890	LRR receptor like protein	RLP7; defence response, signal transduction
CoAcLSF	comp43549_c0_seq5	1.28	2.9E-10	AT4G13810	LRR receptor like protein	RLP47; defence response, signal transduction
CoAcLSF	comp43660_c0_seq1	0.95	3.0E-03	AT5G25930	LRR receptor-like PK	Abscisic acid mediated signalling pathway, defence response
CoAcLSF	comp43708_c0_seq3	2.63	5.8E-03	AT1G74170	LRR receptor like protein	RLP13; signal transduction
CoAcPiRSF	comp43721_c0_seq1	2.00	1.4E-09	AT3G47110	LRR receptor-like PK	Protein phosphorylation, transmembrane receptor protein tyrosine kinase signalling pathway
CoAcRSF	comp43721_c0_seq2	0.67	8.0E-03	AT3G47110		
CoAcPiRSF	comp43721_c0_seq2	1.13	1.2E-03	AT3G47110		
CoAcRRF	comp43753_c1_seq2	8.62	9.0E-05	AT3G47110		
CoAcPiRRF	comp43753_c1_seq2	8.61	3.9E-08	AT3G47110		
CoAcLSF	comp43781_c0_seq1	0.55	4.5E-04	AT1G47890	LRR receptor like protein	RLP7; defence response, signal transduction
CoAcLSF	comp43788_c1_seq2	0.78	1.5E-11	AT1G35710	LRR receptor-like PK	Defence response, jasmonic acid mediated signalling pathway, salicylic acid mediated signalling pathway
CoAcRSF	comp43788_c1_seq4	0.62	3.7E-05	AT1G35710		
CoAcLSF	comp43788_c1_seq4	0.74	8.8E-04	AT1G35710		
CoAcLSF	comp43794_c0_seq48	3.50	2.9E-03	AT5G48740	LRR receptor-like PK	Protein phosphorylation, xylan biosynthetic process
CoAcRSF	comp43797_c0_seq5	3.31	2.8E-03	AT4G08850	LRR receptor-like PK	Protein phosphorylation
CoAcLSF	comp43799_c1_seq2	1.45	6.4E-04	AT1G79620	LRR receptor-like PK	Cell wall biogenesis, protein phosphorylation
CoAcRRF	comp43804_c0_seq4	-7.01	1.1E-03	AT1G56130	LRR receptor-like PK	Protein phosphorylation
CoAcRSF	comp43804_c0_seq4	7.58	1.1E-04	AT1G56130		
CoAcRSF	comp43804_c0_seq5	6.03	8.5E-03	AT1G56140	LRR receptor-like PK	Protein phosphorylation
CoAcLSF	comp43825_c1_seq1	0.36	1.3E-06	AT1G56140		
LRR containing defence response regulators						
CoAcRSF	comp22700_c0_seq1	0.33	1.8E-07	AT2G25490	F-box protein	EBF1; ethylene mediated signalling pathway
CoAcPiLRF	comp22700_c0_seq1	-0.95	4.3E-04	AT2G25490		
CoAcLSF	comp28580_c0_seq1	0.44	2.5E-07	AT5G21090	LRR family protein	Glycolysis, signal transduction, systemic acquired resistance
CoAcRSF	comp30657_c0_seq1	0.26	1.0E-03	AT5G49980	Auxin F-box protein	AFB5; response to molecule of bacterial origin, signal transduction
CoAcPiRSF	comp35514_c3_seq4	7.86	6.4E-03	AT5G45520	TIR-NB-LRR domain protein	TAO1; defence response, signal transduction, disease resistance
CoAcPiLSF	comp35514_c3_seq4	-6.95	1.6E-03	AT5G45520		
CoAcRSF	comp37347_c0_seq1	-0.51	8.1E-06	AT5G23400	LRR family protein	Defence response, signal transduction
CoAcLSF	comp37347_c0_seq1	-0.79	2.7E-20	AT5G23400		
CoAcPiRRF	comp41428_c0_seq1	0.96	1.6E-03	AT3G20820	LRR family protein	Defence response, signal transduction
CoAcLSF	comp41428_c0_seq1	-0.28	1.3E-03	AT3G20820		
CoAcRSF	comp41588_c0_seq1	0.32	5.3E-03	AT5G23340	LRR family protein	Defence response, signal transduction

CoAcRSF	comp42141_c3_seq1	0.21	1.9E-03	AT3G26810	Auxin F-box protein	AFB2; response to molecule of bacterial origin, signal transduction
CoAcRSF	comp42225_c1_seq1	0.53	5.3E-07	AT2G34930	LRR family protein	Defence response, signal transduction
CoAcRSF	comp42385_c1_seq1	0.70	3.0E-16	AT4G27220	LRR-NB-ARC domain protein	Defence response, disease resistance
CoAcPiLRF	comp43297_c0_seq1	-0.73	7.2E-03	AT2G31880	LRR transmembrane protein	EVR; defence response, protein phosphorylation
CoAcLSF	comp43297_c0_seq1	1.27	1.9E-99	AT2G31880		
CoAcLSF	comp43410_c0_seq2	0.44	4.3E-06	AT5G17680	TIR-NB-LRR domain protein	Defence response, signal transduction, disease resistance
CoAcLSF	comp43491_c1_seq1	-2.67	4.1E-06	AT2G34930	LRR family protein	Defence response, signal transduction
CoAcLSF	comp43491_c1_seq3	7.16	2.2E-04	AT2G34930		
CoAcPiLSF	comp43491_c1_seq3	7.45	5.4E-04	AT2G34930		
CoAcLSF	comp43491_c1_seq9	9.37	3.4E-24	AT2G34930		
CoAcPiLSF	comp43491_c1_seq9	8.62	9.3E-06	AT2G34930		
CoAcRSF	comp43527_c4_seq1	0.45	4.3E-04	AT3G14460	LRR-NB-ARC domain protein	Defence response, disease resistance
CoAcLSF	comp43527_c4_seq1	-0.72	4.3E-11	AT3G14460		
CoAcRSF	comp43569_c0_seq2	0.67	9.2E-06	AT5G44510	TIR-NB-LRR domain protein	TAO1; defence response, signal transduction, disease resistance
CoAcLSF	comp43655_c2_seq2	0.36	7.1E-04	AT3G14470	LRR-NB-ARC domain protein	Defence response, salicylic acid biosynthetic process, systemic acquired resistance, disease resistance
CoAcLSF	comp43696_c0_seq5	1.25	7.4E-03	AT3G14470		
CoAcLSF	comp43817_c0_seq1	0.83	3.6E-04	AT3G14470		
CoAcPiRSF	comp43826_c1_seq36	4.24	1.6E-05	AT3G14460	LRR-NB-ARC domain protein	Defence response, disease resistance
CoAcPiRSF	comp43826_c1_seq5	3.94	5.5E-05	AT3G14460		
CoAcLSF	comp43826_c1_seq6	8.79	4.1E-10	AT3G14460		
CoAcPiLSF	comp43826_c1_seq6	7.93	1.1E-05	AT3G14460		
CoAcLSF	comp43826_c1_seq7	7.77	2.5E-06	AT3G14460		
CoAcPiLSF	comp43826_c1_seq7	7.31	1.1E-04	AT3G14460		
CoAcPiRSF	comp43826_c1_seq9	7.04	2.7E-03	AT3G14460		
LRR containing proteins involved in growth, development and metabolism						
CoAcRSF	comp32486_c0_seq1	0.76	8.3E-12	AT3G22800	LRR family protein	Structural constituent of cell wall
CoAcLSF	comp32486_c0_seq1	-0.94	9.6E-06	AT3G22800		
CoAcLSF	comp34179_c1_seq1	0.77	5.2E-05	AT3G62980	Auxin receptor	TIR1; auxin mediated signalling pathway, signal transduction
CoAcLSF	comp35179_c0_seq1	0.25	3.7E-03	AT1G49750	LRR family protein	Cellular response to nitrogen starvation
CoAcRSF	comp35889_c0_seq1	0.78	6.3E-10	AT3G11330	Plant Intracellular Ras-group-related LRR	PIRL9; brassinosteroid biosynthetic process, microgametogenesis, pollen development
CoAcRSF	comp36931_c0_seq1	0.54	4.0E-08	AT3G11330		
CoAcRSF	comp37661_c0_seq1	0.63	9.2E-05	AT2G19330	Plant Intracellular Ras-group-related LRR	PIRL6; DNA endoreduplication
CoAcRSF	comp38706_c0_seq1	0.45	2.4E-11	AT4G07400	F-box protein	VFB3; ubiquitin-dependent protein catabolic process

CoAcRSF	comp40383_c2_seq1	-0.54	1.7E-04	AT5G16000	NSP-interacting kinase	NIK1; cell wall organization, cytokinin mediated signalling pathway, regulation of meristem growth
CoAcPiRRF	comp40681_c0_seq1	1.44	1.4E-03	AT1G62440	Extensin	LRX2; cell morphogenesis involved in differentiation
CoAcLSF	comp41431_c0_seq1	-0.36	5.5E-04	AT1G60800	NSP-interacting kinase	NIK3; microtubule nucleation, protein phosphorylation, regulation of meristem growth
CoAcRSF	comp41921_c0_seq8	0.50	1.4E-03	AT4G08980	F-box protein	FBW2; posttranscriptional regulation of gene expression, response to abscisic acid stimulus
CoAcRSF	comp43252_c2_seq1	0.46	1.4E-05	AT5G57900	F-box protein	SKIP1; hormone-mediated signalling pathway, signal transduction
CoAcLSF	comp43323_c0_seq1	0.47	3.4E-08	AT3G22330	DEAD-box protein	PMH2; response to cold
CoAcPiRRF	comp43479_c0_seq1	4.45	6.2E-04	AT2G34680	Microtubule associated protein	AIR9; cell differentiation, response to auxin stimulus
CoAcPiRSF	comp43479_c0_seq1	2.51	1.4E-07	AT2G34680		
CoAcRSF	comp43479_c0_seq3	0.74	2.3E-03	AT2G34680		
CoAcRSF	comp43723_c2_seq5	0.49	1.6E-05	AT2G42620	F-box protein	MAX2; auxin polar transport, meristem structural organization
Apetala2 / Ethylene Response Factor transcription factors						
CoAcLSF	comp16570_c0_seq1	-2.56	2.6E-05	AT5G47220	AP2/ERF TF B-3 subfamily	ERF2, ethylene and defence response
CoAcRSF	comp20092_c0_seq1	0.43	5.3E-06	AT2G20880	AP2/ERF TF DREB subfamily	ERF 56, drought stress
CoAcRSF	comp21624_c0_seq1	0.62	3.8E-09	AT5G61590	AP2/ERF TF B-3 subfamily	Growth and development
CoAcLSF	comp21624_c0_seq1	0.31	6.3E-06	AT5G61590		
CoAcRSF	comp22797_c0_seq1	0.29	3.6E-06	AT2G47520	AP2/ERF TF B-2 subfamily	ERF71, Growth and development
CoAcRRF	comp27633_c0_seq1	1.04	5.2E-03	AT1G53170	AP2/ERF TF B-1 subfamily	ERF8, ethylene and defence response
CoAcPiRRF	comp27633_c0_seq1	1.06	4.3E-04	AT1G53170		
CoAcRSF	comp27633_c0_seq1	0.48	3.2E-09	AT1G53170		
CoAcLSF	comp27633_c0_seq1	0.34	6.1E-03	AT1G53170		
CoAcRSF	comp28737_c0_seq1	0.69	2.3E-21	AT2G47520	AP2/ERF TF B-2 subfamily	ERF71, Growth and development
CoAcLSF	comp28737_c0_seq1	0.44	3.1E-04	AT2G47520		
CoAcRSF	comp28930_c0_seq1	1.87	1.5E-05	AT5G50080	AP2/ERF TF B-4 subfamily	ERF110, Growth and development
CoAcLSF	comp30659_c0_seq1	1.77	8.3E-08	AT5G21960	AP2/ERF TF DREB A-5 subfamily	Jasmonate and defence response
CoAcRSF	comp30964_c0_seq2	1.38	2.6E-03	AT4G25490	AP2/ERF TF DREB subfamily	DREB1B, Response to low temperature
CoAcLRF	comp30964_c0_seq2	4.13	2.3E-04	AT4G25490		
CoAcLSF	comp30964_c0_seq2	1.60	1.8E-03	AT4G25490		
CoAcPiLSF	comp30964_c0_seq2	3.28	1.0E-12	AT4G25490		
CoAcRSF	comp30964_c0_seq5	3.43	7.4E-04	AT4G25490		
CoAcRSF	comp32111_c0_seq1	0.53	1.2E-20	AT2G20880	AP2/ERF TF DREB subfamily	ERF 56, drought stress
CoAcLSF	comp32111_c0_seq1	0.38	6.2E-13	AT2G20880		
CoAcRRF	comp32408_c1_seq2	1.17	5.7E-04	AT5G11190	AP2/ERF TF B-6 subfamily	SHN2, wax formation, drought tolerance

CoAcPiRRF	comp32408_c1_seq2	1.26	3.1E-04	AT5G11190		
CoAcRSF	comp32408_c1_seq2	-0.53	3.1E-05	AT5G11190		
CoAcLRF	comp32408_c1_seq2	1.94	2.3E-12	AT5G11190		
CoAcPiLRF	comp32408_c1_seq2	1.25	6.7E-05	AT5G11190		
CoAcLSF	comp32408_c1_seq2	2.63	1.9E-99	AT5G11190		
CoAcLSF	comp32413_c0_seq1	-2.80	4.3E-05	AT5G43410	AP2/ERF TF B-3 subfamily	Ethylene and jasmonate signalling
CoAcLSF	comp32739_c1_seq1	3.83	1.7E-05	AT2G33710	AP2/ERF TF B-4 subfamily	Ethylene and defence response
CoAcPiLSF	comp32739_c1_seq1	4.02	1.5E-03	AT2G33710		
CoAcRSF	comp32772_c3_seq1	0.29	3.4E-03	AT1G12610	AP2/ERF TF DREB A-1 subfamily	DWARF, Growth and development
CoAcPiRRF	comp32966_c0_seq1	2.01	5.7E-03	AT1G33760	AP2/ERF TF DREB A-4 subfamily	Jasmonate and defence response
CoAcLRF	comp32966_c0_seq1	2.58	1.3E-06	AT1G33760		
CoAcLSF	comp32966_c0_seq1	1.63	1.2E-18	AT1G33760		
CoAcRSF	comp37280_c0_seq1	0.83	2.7E-12	AT1G51120	AP2/ERF TF B-3 subfamily	Growth and development
CoAcLSF	comp37280_c0_seq1	-0.49	1.2E-10	AT1G51120		
CoAcRSF	comp37844_c0_seq1	0.61	1.9E-06	AT2G23340	AP2/ERF TF DREB A-5 subfamily	DREB3, Growth and development
CoAcRSF	comp41507_c1_seq1	1.44	5.4E-92	AT2G46870	AP2/ERF TF B-3 subfamily	NGA1, Growth and development
CoAcLSF	comp41507_c1_seq1	-0.56	1.4E-14	AT2G46870		
CoAcRSF	comp42738_c1_seq1	1.12	3.4E-03	AT1G04370	AP2/ERF TF B-3 subfamily	ERF14, ethylene and defence response
CoAcLSF	comp42738_c1_seq1	-1.83	2.1E-07	AT1G04370		
CoAcLSF	comp42830_c1_seq5	0.83	2.2E-03	AT1G50640	AP2/ERF TF B-1 subfamily	ERF3, ethylene and defence response
Xyloglucan Endotransglucosylase Hydrolases						
CoAcLSF	comp19836_c0_seq1	0.27	2.8E-03	AT5G13870	XET / XTH	XTH5, cell wall modifications
CoAcRSF	comp19836_c0_seq1	-0.39	4.7E-03	AT5G13870		
CoAcPiRRF	comp19836_c0_seq1	1.08	4.2E-07	AT5G13870		
CoAcRRF	comp20097_c0_seq1	1.11	2.4E-04	AT4G14130	XET / XTH	XTH15, abiotic stress response
CoAcPiRRF	comp20097_c0_seq1	1.19	8.3E-09	AT4G14130		
CoAcLSF	comp21531_c1_seq1	-0.92	5.1E-03	AT1G11545	XET / XTH	XTH8, cell wall modifications
CoAcLRF	comp22994_c1_seq1	1.61	3.7E-06	AT4G25810	XET / XTH	XTH23, defence response
CoAcLSF	comp22994_c1_seq1	-0.42	8.0E-06	AT4G25810		
CoAcRRF	comp22994_c1_seq1	2.02	2.5E-11	AT4G25810		
CoAcRSF	comp22994_c1_seq1	1.02	4.6E-67	AT4G25810		
CoAcPiRRF	comp22994_c1_seq1	1.19	1.2E-07	AT4G25810		
CoAcLRF	comp28842_c0_seq1	2.50	3.3E-08	AT4G03210	XET / XTH	XTH9, meristem growth
CoAcLSF	comp28842_c0_seq1	-0.65	1.9E-42	AT4G03210		

CoAcPiRSF	comp28842_c0_seq1	-0.93	6.0E-05	AT4G03210		
CoAcLRF	comp33773_c0_seq2	2.97	2.8E-06	AT5G57560	XET / XTH	XTH22, abiotic and biotic stress response
CoAcRSF	comp33773_c0_seq2	1.28	1.2E-05	AT5G57560		
CoAcLSF	comp34821_c0_seq1	-1.25	3.2E-98	AT5G65730	XET / XTH	XTH6, ABA response
CoAcPiRSF	comp34821_c0_seq1	-3.18	4.4E-21	AT5G65730		
CoAcLSF	comp35414_c0_seq1	-0.56	4.2E-06	AT2G36870	XET / XTH	XTH32, cell wall modifications
CoAcRRF	comp35414_c0_seq1	-1.44	2.7E-07	AT2G36870		
CoAcLSF	comp37028_c2_seq1	0.58	9.4E-03	AT4G25810	XET / XTH	XTH23, defence response
CoAcPiRRF	comp37028_c2_seq1	1.00	6.2E-04	AT4G25810		
CoAcLSF	comp39943_c0_seq1	-1.53	1.1E-05	AT4G14130	XET / XTH	XTH15, abiotic stress response
CoAcLSF	comp39943_c0_seq4	-1.98	8.8E-06	AT3G23730	XET / XTH	XTH16, cell wall modifications
CoAcLRF	comp40797_c1_seq7	1.99	3.1E-17	AT1G10550	XET / XTH	XTH33, cell wall modifications
CoAcLSF	comp40797_c1_seq7	1.81	2.1E-99	AT1G10550		
CoAcPiRSF	comp43120_c0_seq1	-8.95	8.8E-12	AT4G25810	XET / XTH	XTH23, defence response
CoAcLRF	comp43120_c0_seq11	3.53	1.8E-06	AT4G25810		
CoAcLSF	comp43120_c0_seq11	-0.57	2.0E-09	AT4G25810		
CoAcRRF	comp43120_c0_seq11	2.55	7.4E-07	AT4G25810		
CoAcRSF	comp43120_c0_seq11	-1.83	3.1E-72	AT4G25810		
CoAcPiRRF	comp43120_c0_seq11	2.24	6.6E-17	AT4G25810		
CoAcRSF	comp43120_c0_seq13	-1.71	3.6E-05	AT5G57560	XET / XTH	XTH22, abiotic and biotic stress response
CoAcRSF	comp43120_c0_seq3	-6.33	9.3E-03	AT4G25810	XET / XTH	XTH23, defence response
CoAcRSF	comp43120_c0_seq5	-2.99	5.7E-03	AT4G25810	XET / XTH	
CoAcRRF	comp43120_c0_seq6	8.80	1.5E-07	AT4G25810	XET / XTH	
CoAcPiRRF	comp43120_c0_seq6	8.66	6.6E-08	AT4G25810	XET / XTH	
CoAcLRF	comp43120_c0_seq7	6.80	2.7E-03	AT4G25810	XET / XTH	

Additional file 4 Quantitative polymerase chain reaction primers. Blastx searches against the NCBI nr database were used to predict the identity of the target transcripts.

Primer	Sequence (5'-3')	Blastx predicted transcript identity
comp32318-f	CGGAAAGATAACGGCAGAGGA	Calcium-binding allergen
comp32318-r	GGAAGCACACGAACCCAT	
comp32511-f	CTTATCTCCAAGCCACCCGA	Phosphate transporter
comp32511-r	GCCTCCCAATGTTTCAGC	
comp34707-f	GCCATCAGTTGTCTCCGTGT	Trehalose-phosphate synthase
comp34707-r	GTGCGAATCTTGGTCTTGCG	
comp39043-f	CAGCACATTTTCCAGTCCAC	Inositol oxygenase
comp39043-r	ACGCATCCTGTTGGTTGT	
comp39751-f	GTCGCCGTTATCTCCTCAC	Ap2 erf domain-containing transcription factor
comp39751-r	GCCTCATCCACTCACACAC	
comp42037-f	TTGGTGGCTTTAGGGAGTGG	Peptide nitrate transporter
comp42037-r	GAGTGTCTTGGATTGCTTGGAG	
comp42379-f	TGGATAACTCCTCCCTTGG	Phosphate transporter
comp42379-r	TCTTTCCCTCATTGCCTTGG	
comp43229-f	CCTCCTTCGCCCTTATCTGC	Tata box binding protein associated factor
comp43229-r	ACTGTGACTTGAGCCCTGTG	
comp43258-f	GCGGGGAGTGTAAGAATAGC	Glutamate dehydrogenase
comp43258-r	AGGGGTTGGGAAGCATAA	
comp43608-f	TGCTTGATGGGGTTGTGGTA	Pectinesterase
comp43608-r	GTGCCTCTTTTTCTGTCTCC	

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e-Xtra*

Streptomyces-Induced Resistance Against Oak Powdery Mildew Involves Host Plant Responses in Defense, Photosynthesis, and Secondary Metabolism Pathways

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Rhizobacteria are known to induce defense responses in plants without causing disease symptoms, resulting in increased resistance to plant pathogens. This study investigated how *Streptomyces* sp. strain AcH 505 suppressed oak powdery mildew infection in pedunculate oak, by analyzing RNA-Seq data from singly- and co-inoculated oaks. We found that this *Streptomyces* strain elicited a systemic defense response in oak that was, in part, enhanced upon pathogen challenge. In addition to induction of the jasmonic acid/ethylene–dependent pathway, the RNA-Seq data suggests the participation of the salicylic acid–dependent pathway. Transcripts related to tryptophan, phenylalanine, and phenylpropanoid biosynthesis were enriched and phenylalanine ammonia lyase activity increased, indicating that priming by *Streptomyces* spp. in pedunculate oak shares some determinants with the *Pseudomonas-Arabidopsis* system. Photosynthesis-related transcripts were depleted in response to powdery mildew infection, but AcH 505 alleviated this inhibition, which suggested there is a fitness benefit for primed plants upon pathogen challenge. This study offers novel insights into the mechanisms of priming by actinobacteria and highlights their capacity to activate plant defense responses in the absence of pathogen challenge.

The rhizosphere, the narrow zone of soil influenced by the root system, is a nutrient-rich habitat providing niches for numerous microorganisms (Van der Ent et al. 2008). Among these, there are many fungi and bacteria with properties beneficial to plants (Berg and Smalla 2009). Some plant-beneficial bacteria (e.g., *Bacillus*, *Pseudomonas*, and *Streptomyces* spp. [Kloepper et al. 2004; Weller and Thomashow 2007; Schrey and Tarkka 2008]) have been reported to protect plants against pathogenic microorganisms through a range of mechanisms, including competition for nutrients, secretion of antibiotics and lytic enzymes, and stimulation of plant defenses (Berg and Smalla 2009).

If plants acquire increased resistance to pathogen attack upon perception of infection by rhizosphere bacteria, the phenomenon has been classified as priming and the process itself is termed induced systemic resistance (ISR). ISR has been demonstrated in a variety of plants, including bean, cucumber, tomato, and it is best characterized in *Arabidopsis thaliana* (Verhagen et al. 2006). Both, ISR and systemic acquired resistance (SAR), which is induced by phytopathogens, result in a stronger defense response against subsequent challenge by a pathogen. Best characterized with rhizosphere pseudomonads, the ISR responses include oxidative burst, cell-wall reinforcements, accumulation of defense-related materials and enzymes, and secondary metabolite production, and two main patterns of ISR signaling have been identified (Conrath et al. 2002).

Beneficial rhizobacteria may trigger one or both the salicylic acid (SA)- or jasmonate/ethylene (JA/ET)-dependent signaling pathways (Ryu et al. 2003; Kloepper et al. 2004; Niu et al. 2011), but *Pseudomonas*-mediated ISR predominantly induces SA-independent signaling (De Vleesschauwer et al. 2008; Pieterse et al. 1998). Actinobacteria can also elicit ISR. By analyzing selected *Arabidopsis* genes, Conn and associates (2008) demonstrated that priming by actinobacteria shares features of both ISR and SAR pathways. Whereas the SAR elicits upregulation of a large number of SAR genes, including genes encoding pathogenesis-related (PR) proteins (Van Loon and Van Strien 1999), ISR does not always involve consistent changes in gene-expression levels. Depending on the bacterial strain in question, the onset of ISR mediated by, for example, pseudomonads is either not associated with any major reprogramming of the transcriptome (Verhagen et al. 2004) or leads to increased levels of expression of genes related to secondary metabolism, photosynthetic light reactions, and hormone metabolism (Weston et al. 2012). In contrast to pseudomonads and *Bacillus* spp., the question of how actinobacteria elicit defense responses at the whole transcriptome level of a plant has not been addressed.

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Most temperate forest trees develop a mutualistic root symbiosis, ectomycorrhiza (EM), with fungi. Fungal hyphae extend from the mycorrhizal structure to the surrounding soil to gather nutrients, forming the mycorrhizosphere. Interactions between bacteria and fungi in mycorrhizospheres can improve plant health (Frey-Klett et al. 2007), for example, by enrichment of bacteria that inhibit fungal pathogens (Frey-Klett and Garbaye 2005; Schrey et al. 2012). Thus far, only two mycorrhizosphere bacteria with the capacity to elicit systemic plant defense responses have been characterized. Root inoculation with *Streptomyces* sp. strain AcH 505 (Lehr et al. 2007) or *Streptomyces* sp. strain GB 4-2 (Lehr et al. 2008) inhibits the infection of spruce needles with *Botrytis cinerea*. The mechanisms behind this priming-like response have not been investigated.

Transcriptome profiling of forest trees is an active area of research (Neale and Kremer 2011; Ueno et al. 2013), but the understanding of the gene expression in these genetically complex organisms is still in its early steps. To date, for instance, even the oaks lack a published reference genome. As an alternative approach, we (Tarkka et al. 2013) and others (Ueno et al. 2010) have capitalized on the recent development of RNA sequencing (RNA-Seq) technology to develop methods for large-scale transcriptome profiling experiments in these important forest trees. In the context of the TrophinOak project of seven research groups working with oak microcuttings to study gene expression and resource allocation in multitrophic interactions (TrophinOak website), the OakContigDF159.1 reference transcriptome was constructed and used for an RNA-Seq analysis of EM-related gene-expression levels (Tarkka et al. 2013). In preliminary experiments, it was observed that inoculation of *Streptomyces* sp. strain AcH 505 on oak roots inhibited infection of the leaves with the oak powdery mildew *Microsphaera alphitoides*. To further analyze the mechanisms underlying this, the accumulation of transcripts in oak leaves during interaction with AcH 505 in the presence or absence of *M. alphitoides* was investigated. Two hypotheses were formulated and tested. i) *Streptomyces* sp. strain AcH 505 provokes plant gene-expression levels with the characters of both ISR and SAR; this hypothesis was based on the observations of Conn and associates (2008) on actinobacteria-mediated priming. ii) Since powdery mildew infection is suppressed by AcH 505, genes related to plant defense response are more upregulated in the co-inoculated plants than in plants inoculated with AcH 505 only.

RESULTS

Oak microcosms were successfully inoculated with *Streptomyces* sp. strain AcH 505 and oak leaves with *Microsphaera alphitoides* (Fig. 1). Inoculation of leaves with *M. alphitoides* had no impact on the quantitative polymerase chain reaction (qPCR) signal from AcH 505 in oak rhizosphere, as quantified by real-time PCR with primers specific for the bacterium (Kurth et al. 2013). Powdery mildew infection was visible as conidia and mycelia covering the adaxial leaf surfaces of the microcuttings, and AcH 505 plants showed significantly reduced powdery mildew infection (P = 0.041) (Fig. 1C). The impact of AcH 505 on the plant was visible not only at the level of leaf infection but also for other morphological parameters, such as total leaf area and root fresh weight, which were both increased by the presence of the bacterium (Supplementary Table S1).

To better understand the suppression of powdery mildew infection by AcH 505, differential expression levels (DE) of transcripts in oak leaf tissue upon single and co-inoculations

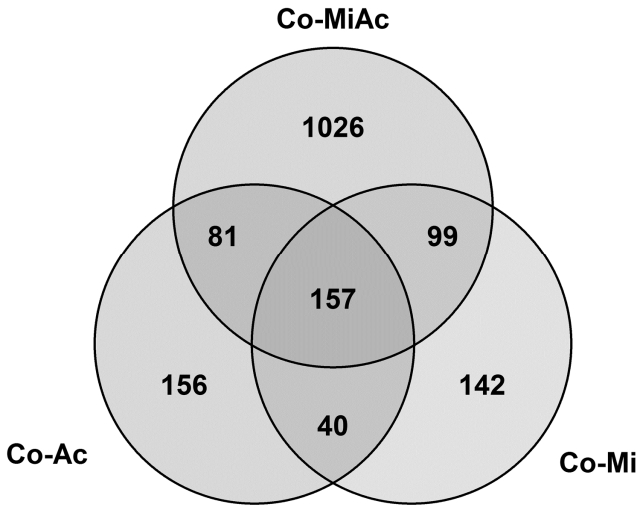

Fig. 2. Venn diagram illustrating the number of differentially expressed contigs in the pairwise comparisons Control-AcH 505, Control-*Microsphaera alphitoides*, and Control-*Microsphaera* + AcH 505 (Benjamini-corrected $P \leq 0.01$).

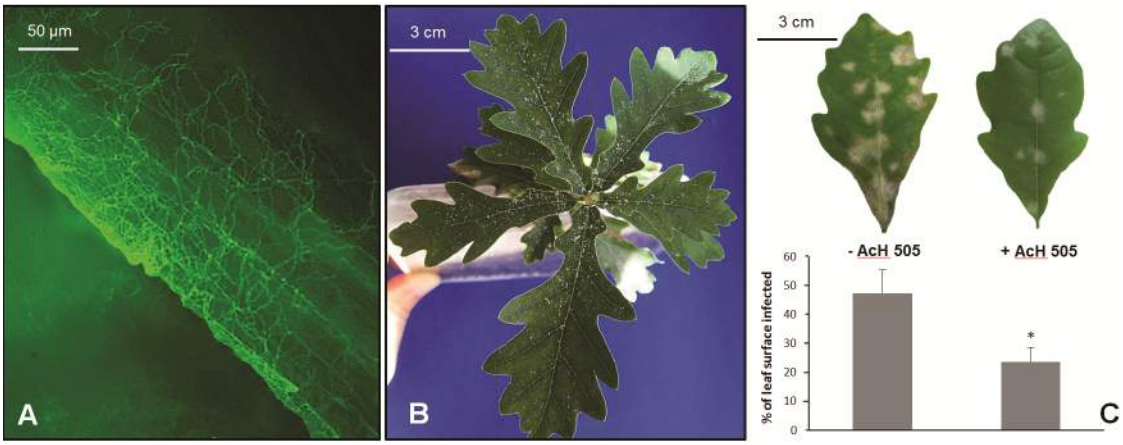

Fig. 1. Pedunculate oak and interacting microorganisms. **A,** Green fluorescent protein–labeled *Streptomyces* sp. strain AcH 505 colonizing the oak root surface, **B,** patchy, early powdery mildew infection on oak leaf surfaces, and **C,** comparison of powdery mildew infection on oak leaves without and preinoculated with *Streptomyces* sp. strain AcH 505. In C, average percentages of leaf surface areas covered by conidia and mycelia resulting from *Microsphaera alphitoides* infection are given. The columns represent an average of 13 individual oak microcuttings with in total of 40 to 60 leaves per treatment, and the error bars mark standard errors. The asterisk indicates a significant difference between the treatments, according to Student's *t*-test (P = 0.041).

were investigated by Illumina RNA-Seq. Inoculation of oaks with AcH 505 alone, inoculation with *M. alphitoides* alone, and co-inoculation with AcH 505 and *M. alphitoides*, resulted in DE of 434, 438, and 1,363 contigs, respectively. Differential expression of 12 contigs was confirmed by quantitative reverse transcription (qRT) PCR analysis (Supplementary Table S2). The distribution of contigs with DE was visualized on a Venn diagram (Fig. 2). Whereas most contigs with DE in AcH 505 and *M. alphitoides* inoculations were also differentially expressed in other treatments, co-inoculation resulted in a large number of unique contigs with DE.

Although three times more contigs with DE were observed in co-inoculated than in singly inoculated plants, the results of GO (gene ontology) term and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis showed numbers in the same range for all three treatments, i.e., 50, 48 and 70 enriched GO terms and 21, 13, and 19 enriched KEGG pathways for AcH 505 alone, *M. alphitoides* alone, and co-inoculation, respectively. Inoculation with AcH 505 led to enrichment for GO terms ethylene biosynthesis, calmodulin binding, chitin binding and catabolism, shikimate biosynthesis, and phenylpropanoid metabolism (Fig. 3), and enrichment for KEGG biochemical pathway associations phenylalanine, tyrosine, tryptophan and flavonoid biosynthesis, amino sugar and nucleotide sugar metabolism, carbon fixation, and nitrogen metabolism (Fig. 4). GO terms associated with photosynthesis and light harvesting and with chlorophyll binding were depleted. *M. alphitoides* inoculation led to a similar pattern of GO term enrichment to that with AcH 505 inoculation, with the exceptions of an increase in GO term defense response to bacterium and depletion in GO term isoprene biosynthesis, in the case of *M. alphitoides* (Fig. 3). Neither the KEGG-association phenylalanine metabolism nor flavonoid biosynthesis was increased. The depletion of the photosynthesis-related GO terms in response to AcH 505 and *M. alphitoides* was consistent with the observation that chlorophyll fluorescence measurements showed significantly reduced photosynthetic yield at a light intensity of 180 PAR ($P < 0.001$). In co-inoculated plants, in addition to the GO terms enriched following single inoculations, the terms regulation of hydrogen peroxide metabolism, receptor activity, salicylic acid biosynthesis, response to chitin, and aromatic compound metabolism were increased.

A detailed look at differential gene expression (Table 1) revealed general upregulation of defense-related contigs. For instance, basic chitinase (*CHI-B*), hevein like protein (*HEL*),

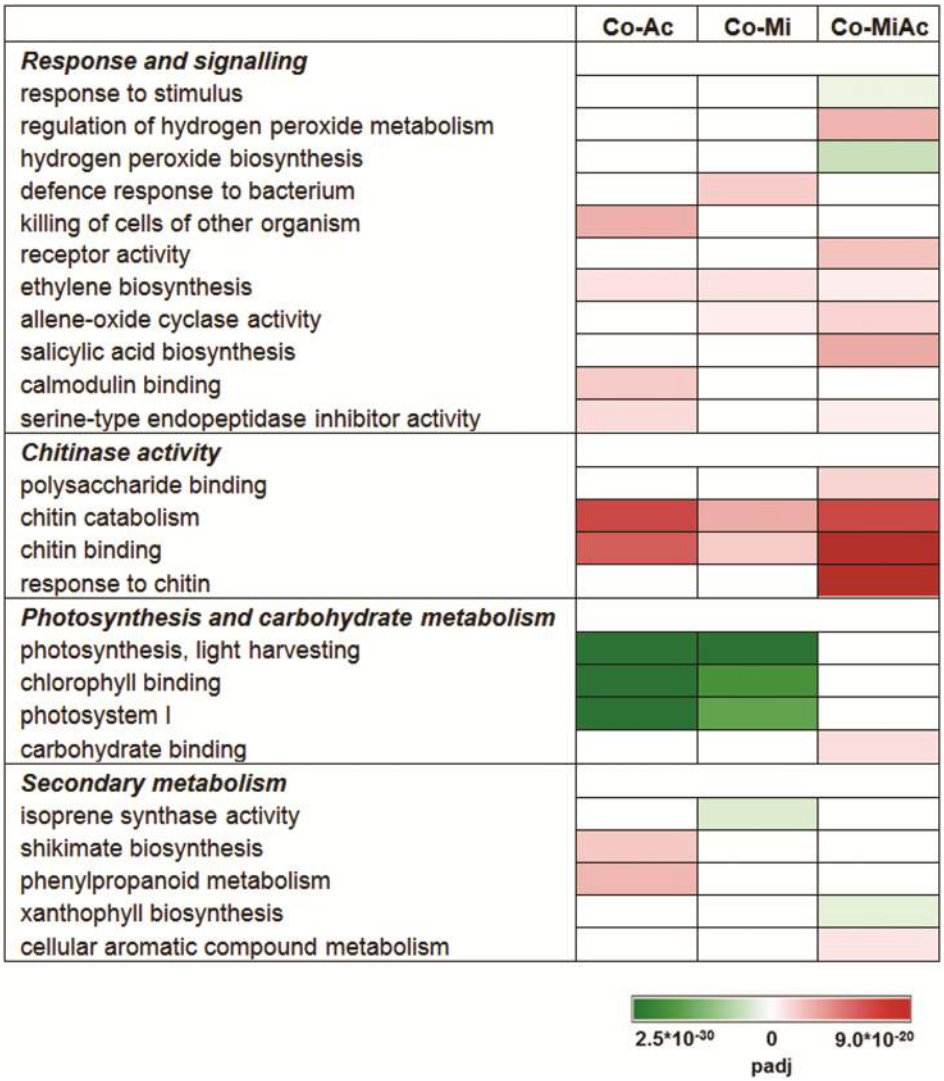

Fig. 3. Gene Ontology (GO) analysis. Enriched up- (red) and downregulated (green) GO categories are shown. The color scale corresponds to Benjamini-corrected *P* values. GO enrichment analyses were implemented by GOseq and GO terms were summarized using REVIGO. Pairwise comparisons of Co-Ac, noninoculated oaks vs. oaks inoculated with AcH 505; Co-Mi, noninoculated oaks vs. oaks inoculated with *Microsphaera alphitoides*; and Co-MiAc, noninoculated oaks vs. oaks co-inoculated with *M. alphitoides* and AcH 505, are shown.

linoleate 13s lipoxygenase (*LOX2*), and phenylalanine ammonia-lyase (*PAL1*) were upregulated in each treatment. Co-inoculation of AcH 505 and *M. alphitoides* did not lead to a general further increase in the expression levels of such genes, but two transcription factor genes (*TGA2* and *MYC2*) and splicing factor genes were more upregulated in co-inoculated than in singly inoculated plants (Table 1).

To quantify the elicited defense response also on a biochemical level, PAL and guaiacol peroxidase (POD) enzyme activities were determined in buffered oak leaf extracts (Fig. 5). PAL activities increased after all inoculations; this change was most significant after the co-inoculation (bacterium and fungus). The activity of POD increased only under co-inoculation.

DISCUSSION

Inoculation of roots with *Streptomyces* sp. strain AcH 505 results in reduced powdery mildew infection of the leaves of pedunculate oak. The RNA-Seq data suggest that AcH 505 i) activates systemic plant defenses and ii) primes the plant to respond more strongly upon pathogen attack. The priming of *Arabidopsis* by *Pseudomonas* rhizobacteria (Conrath et al. 2002; Verhagen et al. 2004) consists of a weak systemic plant response in the absence of the pathogen but an enhancement of systemic disease resistance by systemic defense pathways during subsequent challenge with a pathogen. In contrast, when added alone, *Streptomyces* and *Micromonospora* strains could activate any one or two of the four representative plant defense

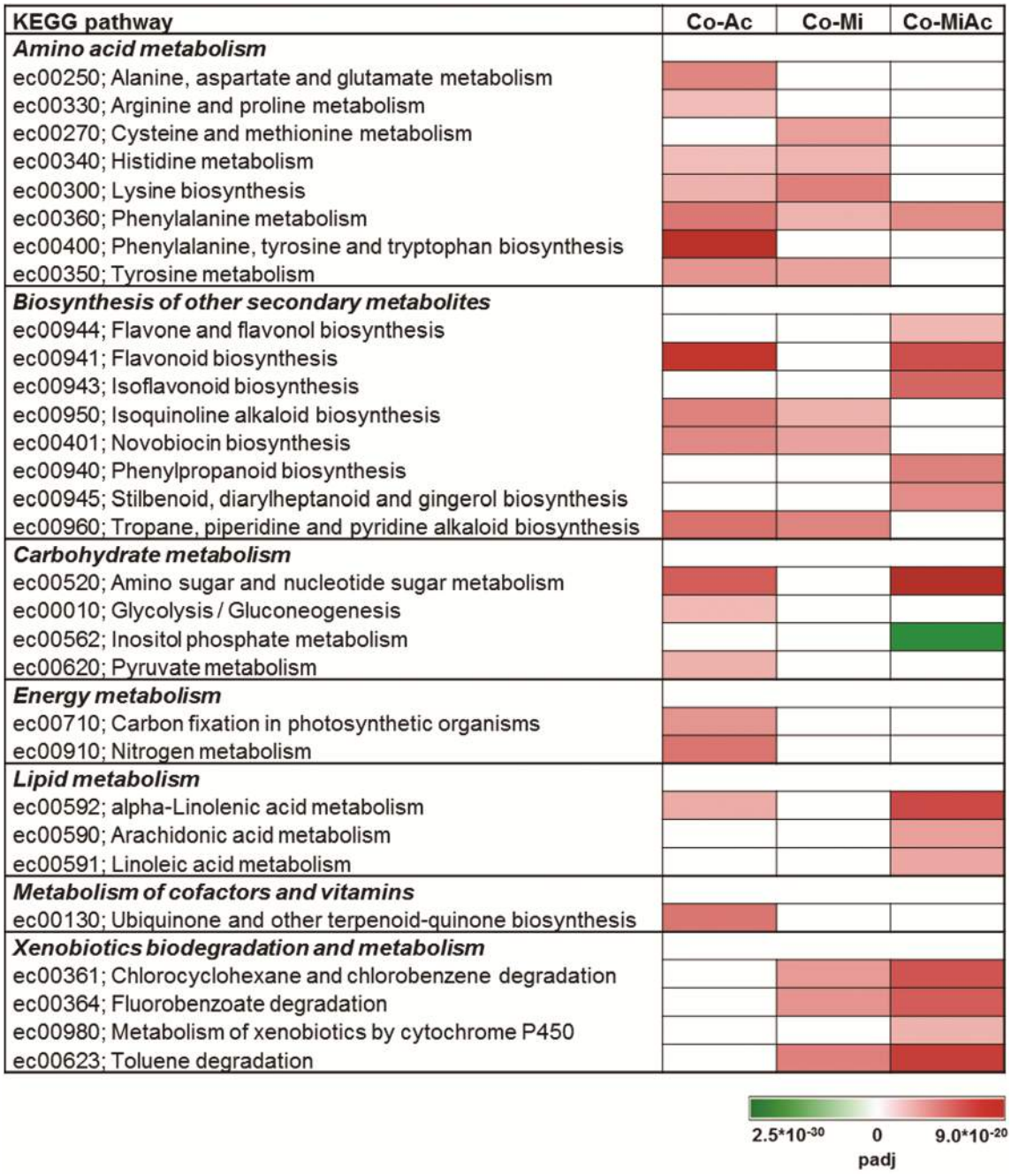

Fig. 4. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis. Increased (red) and depleted (green) KEGG biochemical pathway associations are shown. The color scale corresponds to Benjamini-corrected *P* values. KEGG pathway enrichment analyses were implemented by GOseq. Pairwise comparisons of Co-Ac, noninoculated oaks vs. oaks inoculated with AcH 505; Co-Mi, noninoculated oaks vs. oaks inoculated with *Microsphaera alphitoides*; and Co-MiAc, noninoculated oaks vs. oaks co-inoculated with *M. alphitoides* and AcH 505, are shown.

genes that were analyzed in the absence of a pathogen (Conn et al. 2008). The present transcriptome-scale results support this view. When comparing plants inoculated with AcH 505 with plants inoculated with the pathogen *M. alphitoides*, many similarities in the differential gene-expression profiles were observed, although AcH 505 does not share characteristics of pathogenesis with *M. alphitoides*.

Genes related to the ISR, the SAR, and the JA/ET pathways are induced by AcH 505.

ISR in response to *Pseudomonas* spp. is elicited by a JA/ET-dependent and SA-independent signaling pathway (Verhagen et al. 2004; Van der Ent et al. 2008). In this study, not only were various genes formerly described in the context of ISR- (*CHI-B*, *HEL*, *LOX2*, and *PAL1*) and JA/ET-related genes (allene oxide synthase, cytochrome p450, jasmonate *O*-methyltransferase) upregulated in AcH 505-inoculated oaks also, but the expression of *TGA2*, a gene encoding a transcription factor known to be related to the SAR-pathway (Johnson et al. 2003), was also increased. Thus, the first hypothesis, that AcH 505 provokes plant gene-expression changes with the characteristics of those reported for the ISR, JA/ET pathway, and SAR, could be confirmed. This finding adds weight to the report of Conn and associates (2008), who showed that actinobacteria are able to prime via the SAR and the JA/ET pathways. The phenylpropanoid pathway is known to play an important role in resistance to pathogen attack (Dixon et al. 2002), and numerous amino acids and secondary metabolites have been reported to play prominent roles in ISR induction. In their seminal work, van de Mortel and associates (2012) reported that the secondary metabolites camalexin, glucosinates, and phenylpropanoids are induced in *Arabidopsis* by the rhizobacterium *Pseudomonas fluorescens* SS101. The central importance of the amino acid tryptophan as a precursor of camalexin and phenylalanine as an entry point to the phenylpropanoid pathway was also indicated in the analysis of *Pseudomonas fluorescens*–induced responses in *Arabidopsis* (Weston et al. 2012). The RNA-Seq data presented here showed enrichment in transcripts related to tryptophan, phenylalanine, and phenylpropanoid biosynthesis, suggesting that priming by *Streptomyces* spp. in pedunculate oak has determinants in common with the *Pseudomonas-Arabidopsis* system.

The gene-expression response upon interaction between AcH 505 and pathogen challenge.

The second hypothesis proposed that genes related to plant defense response are more strongly upregulated in the co-inoculated plants than in plants inoculated with AcH 505 only. This hypothesis could be, in part, confirmed. GO term and KEGG pathway analyses as well as the contig expression data support the view that the AcH 505 treatment elicits a significant response in plants, but it is not always enhanced when these plants are challenged with the pathogen. When looking at the number of DE contigs (Fig. 2) the co-inoculated plants displayed a much stronger response than plants inoculated solely with AcH 505 or *M. alphitoides*. In the GO term and KEGG pathway analysis, however, the co-inoculated plants did not display a comparable increase in the number of enriched terms (50 and 70 GO terms and 21 and 19 enriched KEGG pathways for AcH 505 alone and co-inoculation, respectively [Figs. 2 and 3]). It thus seems that, in the context of AcH 505 priming, pathogen challenge leads to a strong plant response with a large number of genes of varying functions being differentially regulated, but there is no distinct pattern of transcripts that

Table 1. Differential gene expression levels of selected up- or downregulated genes[a]

	Co-Ac		Co-Mi		Co-MiAc		
Contig	**log2-fold**	**FDR**	**log2-fold**	**FDR**	**log2-fold**	**FDR**	**Sequence description**
19577	2.1	**	2.0	*	2.4	***	Allene oxide synthase
42988	5.2	***	4.1	*	4.1	**	Cytochrome p450
28996	4.7	***	3.0	**	3.2	***	Jasmonate *O*-methyltransferase
32977	1.2	n.s.	2.6	n.s.	3.9	***	Transcription factor *MYC2*
42675	8.7	***	7.5	***	8.4	***	Glycosyl hydrolase DL4170C
42425	4.7	**	4.3	*	4.9	***	Basic chitinase *CHI-B*
34705	5.3	**	4.5	*	5.1	**	Hevein like protein
43636	1.1	n.s.	1.5	*	1.5	**	Linoleate 13s-lipoxygenase *LOX 2*
42363	3.0	***	2.1	n.s.	1.6	n.s.	Phenylalanine ammonia-lyase
38547	5.7	n.s.	6.5	n.s.	10.9	***	Transcription factor *TGA2*
28813	4.6	***	3.0	**	3.9	***	Glucose-6-phosphate phosphate translocator
29516	3.2	***	3.5	***	3.7	***	Phenylcoumaran benzylic ether reductase
33900	1.6	*	2.0	*	3.2	***	ap2 domain class transcription factor
36040	2.4	***	2.2	*	3.5	***	Farnesene synthase
40378	3.4	***	2.5	***	3.4	***	Hexose transporter
40700	2.4	**	2.6	**	2.5	**	Cinnamyl alcohol dehydrogenase
40819	2.2	***	3.1	***	4.1	***	Glucan endo- -beta-glucosidase
41264	2.0	***	1.8	*	2.1	***	Sulfite exporter family protein
42013	–0.3	n.s.	–1.0	n.s.	–2.9	***	Zeaxanthin epoxidase
42312	–1.5	n.s.	–1.4	n.s.	–5.3	***	CC-NBS-LRR resistance protein
42333	–1.0	n.s.	–1.2	n.s.	–3.5	***	Phytochrome
43577	7.0	***	6.1	***	6.0	***	Laccase
37803	9.3	***	7.7	***	12.6	***	Splicing factor u2af large subunit b
39371	–4.7	**	–2.1	n.s.	–9.0	**	Serine threonine protein kinase
43318	–0.6	n.s.	–11.6	***	–5.9	***	Light-inducible protein
32087	–10.0	***	–16.9	***	–16.8	***	Catalase
43823	–3.9	**	–10.4	**	–1.7	n.s.	DNA topoisomerase

[a] Pairwise comparisons Control-AcH 505 (CO-Ac), Control-*Microsphaera* (Co-Mi), and Control-*Microsphaera* + AcH 05 (Co-AcMi) are shown. Differential gene expression was identified by edgeR with a threshold Benjamini-corrected *P* value of 0.01. Upregulated genes are shown in red and downregulated genes in green. FDR = false discovery rate shown as Benjamini corrected *P* value. Between single and dual inoculations, significant differences between the expression levels of the Ac-MiAc comparison occurred for transcription factors MYC2, TGA2, and AP2-like, glucan endo-β-glucosidase, zeaxanthin epoxidase, light-inducible protein, catalase, and phytochrome, and for both Ac/MiAc and Mi/MiAc comparisons for splicing factor u2af large subunit b and the coiled coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR) resistance protein.

could be associated with changes in specific biochemical processes or compositional changes. Second, many defense-related GO terms such as response to chitin and salicylic acid biosynthesis were enriched only in co-inoculated plants, and several genes related to defense that were already induced by single inoculation with AcH 505 were more strongly upregulated upon challenge with *M. alphitoides* (Table 1). On the other hand, gene-expression level of hevein encoding protein, a well-recognized pathogen-response protein, was lower both with the pathogen and with the co-inoculation in comparison with the AcH 505-treated plants. In conclusion, the changes in gene-expression levels induced by AcH 505 inoculation form a significant part of an effective defense response in oak, and these changes are, in part, amplified upon challenge with the pathogen, although not to the extent described for the *Pseudomonas-Arabidopsis* ISR system (Verhagen et al. 2006).

Plant defense responses that are effective against powdery mildews are often regulated by SA signaling (Glazebrook 2005; Pieterse et al. 2009), and JA has been shown to be involved in the resistance against powdery mildew conferred upon *A. thaliana* by the root-endophytic fungus *Piriformospora indica* (Stein et al. 2008). In our experiments, the transcription factor *MYC2* was up-regulated in co-inoculated oaks. *MYC2* is a key player in the JA-responsive ISR pathway, and in addition, acts as a regulator in JA-abscisic acid (ABA) crosstalk

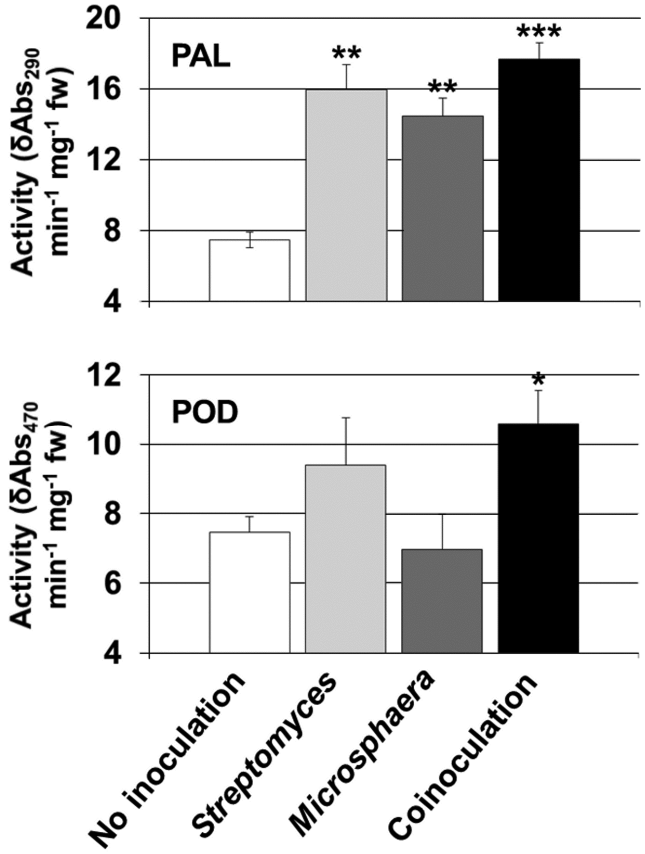

Fig. 5. Phenylalanine ammonia lyase (PAL) and peroxidase (POD) activities in pedunculate oak leaves after interaction with one or both *Streptomyces* sp. strain AcH 505 and *Microsphaera alphitoides*. Control values (open columns) were compared with those after treatment with AcH 505 (pale gray columns), *M. alphitoides* (dark gray columns) or dual (bacterium + fungus) inoculations (black columns). Each column represents four independent measurements from four microcuttings, and the error bars mark standard errors. Asterisks indicate significant difference to the treatment "No inoculation", according to Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

(Kazan and Manners 2013). Involvement of ABA-dependent gene expression in co-inoculated oaks is further indicated in the downregulation of the gene encoding zeaxanthin epoxidase, an enzyme involved in the final steps of ABA biosynthesis (Ton et al. 2009). The role of ABA in resistance against powdery mildews has recently become more evident (Chen et al. 2013; Wawrzynska et al. 2008). Our findings support the observation that cooperative cross-talk between JA, SA, and ABA signaling pathways results in plant protection against powdery mildews by rhizobacteria (Garcia-Gutierrez et al. 2013; Niu et al. 2011).

It is likely that the gene-expression responses of the genes elicited by AcH 505, the pathogen, and the co-inoculation of the actinobacterium with the fungal pathogen depend on the length of the challenge by each organism. The timing for destructive harvest was determined here by the strongest impact of the bacterium on powdery mildew infection. Clearly, a temporal analysis of elicitation and fungal infection would give further information about the process of disease suppression.

Increased PAL and peroxidase activities are important defense-response mechanisms in forests trees, and the enzyme activity measurements of this study supported the observation of elicited defense responses in oak. The most prominent changes were observed with PAL activity, which increased upon all treatments. As the first enzyme of the phenylpropanoid pathway, PAL catalyzes the conversion of phenylalanine to trans-cinnamic acid (Boudet et al. 1971; Camm and Towers 1973). The functions of the phenylpropanoid compounds in plant defense range from preformed or inducible physical and chemical barriers such as lignin and secondary metabolites against infections, and the induction of PAL, thus, plays an important role in plant disease resistance (Dixon et al. 2002). Evidence of the central role of lignin in *Streptomyces*-induced local resistance was presented by Lehr and associates (2008). They observed that, only in the presence of the fungal pathogen *Heterobasidion annosum*, the *Streptomyces* sp. strain GB 4-2 rendered the plant vascular system inaccessible by increased xylem formation and strong lignification. The role of PAL in this process warrants investigation. On the other hand, co-inoculation of oaks stimulated peroxidase activity. The expression and activity of peroxidase may confer plant resistance to pathogens by generating hydrogen peroxide for the oxidative burst (Bindschedler et al. 2006), and the peroxidase-based processes may include the rigidification of plant cell walls (Tarkka et al. 2001). Allison and Schultz (2004) reported that, in the red oak *Quercus rubra*, leaf peroxidase activity increased upon SA treatment and herbivory, suggesting peroxidase-based defense responses in red oak. On the other hand, AcH 505 did not induce peroxidase activity in the current study, which confirmed an earlier observation by Lehr and associates (2007) in Norway spruce seedlings. We suggest that PAL and peroxidase-based defense responses contributed to the increased defense responses after pathogen challenge and the reduction of powdery mildew.

Plant growth and physiological responses.

Plants need to find a subtle balance between the costs and benefits of defense responses in order to maximize their fitness (Bostock 2005). Both the induction of SAR by pathogens and the initiation of ISR by nonpathogenic bacteria come with fitness costs for the plant. For instance, rhizobacteria may decrease plant carbon acquisition and seed production when inducing systemic responses (Cartieaux et al. 2003; Weston et al. 2012). In the present study, the GO terms related to photosynthesis were depleted in AcH 505-inoculated plants, and this observation was underpinned by a decrease in the photosyn-

thetic yield, suggesting a reduction in plant carbon capture. Low levels for the same parameters accompanied powdery mildew infection alone. Interestingly, in the presence of the leaf pathogen, the depletion in photosynthesis-related GO terms was alleviated, pointing to a fitness benefit for primed plants upon pathogen challenge.

Conclusions.

Strains of nonpathogenic bacteria can elicit host defense responses against pathogenic microorganisms, and the current study shows that *Streptomyces* sp. strain AcH 505 protects pedunculate oak from pathogenesis by oak powdery mildew. RNA-Seq analysis of bacterium-primed oaks indicated that priming by AcH 505 does not follow the mechanisms described for strains of nonpathogenic pseudomonad bacteria. Instead, the systemic response to the bacterium includes the induction of expression of a surprisingly large number of defense-related genes, a pattern similar to that elicited by oak powdery mildew. Not only jasmonic acid but also the ET, SA, and ABA defense pathways may play a role in *Streptomyces*-based priming of host responses. This study provides novel insights into the molecular mechanisms of priming leading to enhanced defense and may be instrumental in understanding the defense responses elicited by actinobacteria. In the case of AcH 505, priming acts in concert with antibiotic production and exudation of compounds that stimulate mycorrhiza formation, emphasizing the versatility with which *Streptomyces* bacteria interact with eukaryotes.

MATERIALS AND METHODS

The soil-based culture system and inoculation and quantification of *Streptomyces* sp. strain AcH 505 and *Microsphaera (Erysiphe) alphitoides.*

The pedunculate oak clone DF159 (*Quercus robur* L.) was micropropagated and rooted according to Herrmann and associates (2004). Plants were then grown in a soil-based culture system as previously described (Tarkka et al. 2013). Briefly, rooted microcuttings were placed in petri dishes filled with a 1:1 (vol/vol) mixture of gamma-sterilized soil. Soil filtrates were prepared as described by Rosenberg and associates (2009). At 4 weeks, 5 ml of filtrate was added to each culture system. *Streptomyces* sp. strain AcH 505 was originally isolated from the soil around Norway spruce mycorrhizas in Haigerloch, Germany (Maier et al. 2004) and was maintained on ISP2 agar medium (Shirling and Gottlieb 1966). For the experiment, the culture system was inoculated with 2.5×10^7 AcH 505 spores at 3 and 7 weeks. *Microsphaera alphitoides* was obtained from infected oak leaves from natural woodland and maintained on oak leaves. *M. alphitoides* infection of oak leaves was carried out as described in Tarkka and associates (2013). Heavily infested leaves were used to infect oak microcuttings. Fungal spore quantity was calculated by collecting and counting spores from mock inoculations in a Fuchs-Rosenthal counting chamber, and fungal identity was verified by PCR using specific intergenic transcribed spacer primers based on Cunnington and associates (2003). Each plant was inoculated with 1.5×10^6 *M. alphitoides* spores 14 days prior to harvest.

Streptomyces sp. strain AcH 505 was quantified based on the work of Kurth and associates (2013), which focused on the colonization of soil and oak rhizosphere by the bacterium and a mycorrhizal fungus. Kurth and associates (2013) described the construction of *Streptomyces* sp. strain AcH 505–specific primers for real-time qPCR analysis and their use for its relative quantification in the soil and in the rhizosphere of oak. AcH 505 was also visualized in the rhizosphere of oak by scanning electron microscopy (Kurth et al. 2013). In the context of an experiment investigating the course of the bioassay, we observed that the level of the *Streptomyces* strain did not differ significantly at weeks 7 and 9, suggesting a stable concentration at the second application and the harvest time point. Our data also suggest that the *Streptomyces* strain did not enter the leaves. Strain AcH 505–specific qPCR signal was not detected from leaf DNA, which indicates that the *Streptomyces* strain is physically separated from the leaf pathogen.

The material was grown for 8 weeks, after which plant material was harvested from microcosms. The timing for harvest was decided based on preliminary studies on the impacts of AcH 505 on powdery mildew infection, EM formation, and plant growth. With this form of inoculation, AcH 505 elicits plant defenses against the powdery mildew (the subject of this study) and stimulates oak–*Piloderma croceum* mycorrhiza formation (Kurth et al. 2013). Since *P. croceum* was not inoculated to the oak root system in the present study, no mycorrhizas were detected in the current analysis. Nevertheless, the identical timing of AcH 505 inoculation in different experiments will enable the comparative analysis of oak responses to different interacting organisms.

Visible signs of powdery mildew occurred on oak leaves 5 to 7 days postinoculation with *M. alphitoides*. At this stage, the rating of disease severity started, based on Newsham and associates (2000). For this, the percentages of adaxial leaf-surface area of individual leaves covered by conidia and mycelia were estimated visually. To avoid a subjective bias, the assessment was carried out in parallel by two experimenters. Disease severity assessments were carried out at three individual time points, at days 7, 10, and 13 postinfection. The last measurement was taken to correlate disease severity assessments to Illumina sequencing results, as it took place 1 day prior to harvest. Only expanded leaves were regarded for disease severity assessment and RNA extractions. In total, disease severity was assessed from leaves belonging to 13 individual oak microcuttings per treatment (approximately 40 to 60 leaves per treatment). Statistical significance of average percentages of infected leaf area was evaluated according to student's *t*-test. Two days before harvest, chlorophyll fluorescence measurements were performed using an imaging-PAM (Walz, Effeltrich, Germany). At harvest, leaf samples were submerged in liquid nitrogen and were stored at –80°C. The experimental design resulted in four different treatments: i) with or ii) without *M. alphitoides* and iii) with or iv) without AcH 505.

RNA extraction and Illumina sequencing.

The RNA-Seq analyses were based on two to three biological replicates of each treatment. The presence of infected leaf areas was visually confirmed in the leaves with powdery mildew treatment. Each biological replicate represented a pool of expanded source leaves of two to three plants powdered under liquid nitrogen, and the powder was used for RNA extraction. The different biological replicates (pools) were prepared from different plant individuals. For all samples, complete expanded leaves were ground in a mortar under liquid nitrogen, and the powder was used for RNA extraction. The reasoning behind this approach was to address the average transcriptome of expanded leaves at different treatment types. Sample numbers for RNA-Seq analysis were two for the No inoculation (control) treatment, three for *Streptomyces* inoculation, three for powdery mildew inoculation, and two for *Streptomyces* and powdery mildew inoculation. After homogenization of each pool under liquid nitrogen, total RNA was extracted from eight pools, using the MasterPure plant RNA purification kit (Epicentre, Madison, WI, U.S.A.) with 50 mg of leaf material per

extraction. RNA quantity and quality were estimated using a NanoDrop spectrophotometer (Thermo Scientific, Sugarland, TX, U.S.A.), gel electrophoresis and a Nano Chip with a Bioanalyzer 2100 (Agilent, Santa Clara, CA, U.S.A.). RNA samples were used to produce 100-bp paired-end libraries and were sequenced using an Illumina HiSeq 2000 at the Beijing Genomics Institute, Hong Kong, China.

Read processing and analysis of differential expression.

Illumina reads were processed as described (Tarkka et al. 2013). Briefly, poly(A) tails, low complexity, and low quality sequences were removed with SeqClean (DFCI Gene Indices software tools website). Nucleotides with quality scores <20 were removed from the ends of the reads using a custom Java script. Sequences <50 bp were discarded, as were sequences without paired-end information after preprocessing. The processed Illumina reads were aligned against the OakContigDF159.1 reference transcriptome (Tarkka et al. 2013) by Bowtie (Langmead et al. 2009) and were quantified using RSEM (Li and Dewey 2011), and the significance of differences in gene expression was measured using the edgeR (Robinson et al. 2010) function of the Bioconductor package (Gentleman et al. 2004) in R (R core group). GO term and KEGG pathway enrichment analysis were performed with the Bioconductor package GOseq (Young et al. 2010).

Primer design and validation for qRT-PCR.

To confirm the differential expression of genes revealed by RNA-Seq, the expression of 12 genes was measured by qRT-PCR. Primer pairs were constructed based on the OakContigDF159.1 assembly. They were designed using the Primer3 software package (Rozen and Skaletsky 2000). The primer pairs were required to have a melting temperature of 55 to 65°C, a GC content of 55 to 63%, primer lengths of 18 to 22 bp, and amplified product lengths of 70 to 150 bp. The amplicon sizes and sequences for the primers used in this work are listed in Supplementary Table S3. The primers constructed were tested for functionality, amplicon size, specificity, and efficiency as previously described (Tarkka et al. 2013).

Real-time PCR assays.

Real-time PCR was performed using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA, U.S.A.). The *18S rRNA* gene was selected as a reference for the RT-qPCR analysis (Tarkka et al. 2013). Transcript abundances in leaf samples were determined based on the cycle threshold value (which measures the number of cycles at which the fluorescence signal exceeds the background level and surpasses the threshold established, based on the exponential phase of the amplification plot), using the Relative Expression Software Tool (Pfaffl et al. 2002). The coefficient of variation was calculated in order to assess the reproducibility of the reactions. Values <6.0 were considered to be reproducible. Differential gene expression was determined by a randomization test within the Relative Expression Software Tool.

Enzyme activity analysis.

PAL and POD activities were measured from leaf tissue extracts of noninoculated, AcH 505–inoculated, *M. alphitoides*–infected, and dual (bacterium and fungus)-inoculated oak microcuttings from the same experimental material as were used for Illumina sequencing. Leaf tissue was homogenized under liquid nitrogen. Four individual measurements were carried out per treatment. For PAL measurement, 10 mg of the powder was suspended in 500 µl of 100-mM Na-borate buffer, pH 8.8, containing 5 mM beta-mercaptoethanol (Zucker 1968). The extract was filtered through 15-µm nylon tissue, and 10 µl of the extract were added to 290 µl of the reaction mixture containing 2 mM L-phenylalanine as the substrate in Na-borate buffer at pH 8.8. PAL activity was measured in a spectrophotometer at 290 nm each minute for up to 15 min after the addition of the extract to the reaction mix. POD activity was measured based on Mensen and associates (1998). Ground leaf tissue (10 mg) was suspended in 500 µl of 100 mM Na-acetate buffer, pH 5.6. The extract was filtered through 15-µm nylon tissue and 10 µl of the filtered extract was transferred to the reaction mixture, containing 990 µl of 50 mM Na-acetate, 4 mM guaiacol, and 2 mM of the POD substrate, H_2O_2. POD activity was measured every minute at 470 nm in a spectrophotometer for up to 30 min. Increase in absorption at 290 nm during PAL measurements was linear up to 10 min and, during POD measurements, at 470 nm up to 30 min after the start of the reaction. All data reported correspond to the linear phase of the reaction. Relative PAL and POD enzyme activities were determined as the change in the absorbance in 1 min, converted by the extract from 1 mg of fresh weight of leaf tissue.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

TrophinOak project: www.trophinoak.de
DFCI Gene Indices software tools: compbio.dfci.harvard.edu/tgi/software
R core group: www.r-project.org
Primer3 software package: frodo.wi.mit.edu/primer3

Supporting information

Supplementary Table S1 Morphological analyses of oak plants inoculated with AcH 505 alone, with *M. alphitoides* alone, and co-inoculated. Total leaf area, infected leaf area and root fresh weights are given. Significances were determined by 2-way ANOVA (*= $p \leq 0.05$).

	Total leaf area [cm^2]	2-way Anova	Infected leaf area [%]	2-way Anova	FW total root [g]	2-way Anova
Control	25.79 ± 5.66		-		0.24 ± 0.04	
AcH 505	32.88 ± 4.21	*	-		0.39 ± 0.06	*
Microsphaera	20.67 ± 1.72		28 ± 9		0.28 ± 0.04	
AcH 505 + *Microsphaera*	22.95 ± 2.35		11 ± 2	*	0.32 ± 0.02	

Supplementary Table S2 Real-time RT-PCR analysis of 12 contigs with increased or decreased expression levels according to RNA-Seq in the Control vs. *Microsphaera* + AcH 505 treatment. Contig identities were predicted by blastx searches against the nr database at an e-value cut-off of 1.0e-20.

Contig	**RNA-Seq**		**qRT-PCR**		**Blastx predicted transcript identity**
	log2 fold change	p-value	log2 fold change	p-value	
42312	-5.4	5.8E-08	-0.5	6.0E-03	cc-nbs-lrr resistance protein
42333	-3.6	1.5E-07	-1.3	1.0E-03	Phytochrome
29516	3.7	1.1E-10	4.3	2.0E-03	Phenylcoumaran benzylic ether reductase
41264	2.0	1.6E-03	2.2	1.0E-03	Sulfite exporter family protein
33900	3.2	1.9E-08	1.0	1.0E-03	ap2 domain class transcription factor
40378	3.4	1.4E-09	3.5	1.0E-03	Hexose transporter
36040	3.5	2.5E-10	2.8	1.0E-03	Farnesene synthase
40819	4.1	9.9E-14	4.8	1.0E-03	Glucan endo-beta-glucosidase
43577	6.0	1.0E-06	3.3	1.0E-03	Laccase
28813	3.9	1.3E-10	5.0	1.0E-03	Glucose-6-phosphate phosphate translocator
40700	2.5	1.7E-04	0.7	1.6E-02	Cinnamyl alcohol dehydrogenase
42013	-2.9	3.6E-03	-1.9	1.0E-03	Zeaxanthin epoxidase

Supplementary Table S3 Quantitative polymerase chain reaction primers. Blastx searching against the NCBI nr database was used to predict the identity of the target transcripts.

Primer	Sequence (5´-3´)	Blastx predicted transcript identity
comp42312-f	CGAGTCTGAAGCGAAGGCA	cc-nbs-lrr resistance protein
comp42312-r	TGAAGCAGGACGATAGGAAAC	
comp42333-f	GCGAAGAGGGAATGGAATGC	Phytochrome
comp42333-r	ATGAGGAAGGAGTTGGTGGG	
comp29516-f	AAACTCTTTGTGCTGCCCTA	Phenylcoumaran benzylic ether reductase
comp29516-r	CCAACCTTTGCCTATGTGCG	
comp41264-f	TAAGCCACACCACACAAAGC	Sulfite exporter family protein
comp41264-r	GACCAAACTCTATCCCCCTCC	
comp33900-f	ATCCTTGGTTGACTGCGAC	ap2 domain class transcription factor
comp33900-r	ATGACTGGGGCGGGTTTC	
comp40378-f	ATCCGAGGCACTGACAACG	Hexose transporter
comp40378-r	GCTGGGGACGATTTTTACGC	
comp36040-f	TCAAGTGCTAAATGTGGTGC	Farnesene synthase
comp36040-r	AGAGAGGTGGGTGGAAGAGA	
comp40819-f	CGAGAGAGTGTGCGGTAGTT	Glucan endo-beta-glucosidase
comp40819-r	GGTTCCAGCCATGAAAAGCG	
comp43577-f	GACTACACCAACCCCAACATC	Laccase
comp43577-r	CTCAATCCCTACAAACGCTG	
comp28813-f	GCAGCCCAAAGTGTCTTCTATC	Glucose-6-phosphate phosphate translocator
comp28813-r	ATCCGCTTCATCGTGTTTCCT	
comp40700-f	CGTATTCCAGACAACCTTCCAC	Cinnamyl alcohol dehydrogenase
comp40700-r	GACCAACCACACCCACAT	
comp42013-f	TCGGTATTTCTTTGGGGTTCCT	Zeaxanthin epoxidase
comp42013-r	TGTTGGGTATCGGGTGTTCC	

SUMMARY

Most boreal and temperate forest trees develop root symbiosis, ectomycorrhiza (EM), with fungi. In this symbiosis, the fungal partner derives assimilates from the host plant, and the plant is supported by the fungus with mineral nutrients and enhances defence response against pathogens. Both the establishment and functioning of EM can be stimulated by associated mycorrhiza helper bacteria (MHB). Little is known about the impact of the MHB on the host plant. The thesis presented used high throughput sequencing and quantitative Real-Time PCR to examine the answer mechanisms of pedunculate oak *Quercus robur* to interactions with the MHB *Streptomyces sp.* AcH 505. To reach a higher level of relevance, the studies also considered involvement of two fungi in this interaction: the ectomycorrhizal fungus *Piloderma croceum* and the leaf pathogen powdery mildew *Microsphaera alphitoides*. As MHB and EM fungi (EMF) are part of the symbiotic mycorrhizosphere, their interaction was characterized in a first step. The abundance of the bacterium and the fungus were determined in the culture system used using molecular and fluorescence detection. A central focus of the thesis was to investigate how the oak coordinates its molecular responses to interacting organisms and in which way the rhythmic growth of the oak, characterised by alternating root and shoot flushes, affects the interactions. The studies were conducted within the frame of the research project "TrophinOak".

The study presented in *chapter 1* had a methodological as well as a biological focus. AcH 505 and the basidiomycetous EMF *P. croceum* were quantified in the soil-based culture system with the intention to establish a method for easily and rapidly quantifying both microorganisms within the soil. Another focus lay on investigating how the oak on the one hand, and a soil microbial filtrate (SMF) on the other hand, influence the bacterium-fungus interaction. In this context both microorganisms were visualised in the soil microcosm by scanning electron microscopy and a quantification method using quantitative real-time PCR with specific primers was established. For AcH 505 an intergenic region between two protein-coding genes was chosen and validated as amplification target. For *P. croceum* the ITS region and an intergenic region between two protein-coding genes were chosen as amplification targets. By comparison, it was shown that, in spite of its high copy number in fungi the ITS region allowed a sensitive and specific quantification. Quantification of both microorganisms under different conditions – namely, presence and absence of the oak and a soil microbial filtrate (SMF), respectively – showed that both entities do influence the interaction investigated. *P. croceum* enhanced the signal of AcH 505, pointing to a possible use of fungal

exudates as nutrients by the bacterium. This growth promotion was more pronounced in the presence of SMF, showing that further soil microorganisms can enhance this synergistic effect. In addition, AcH 505 was not affected by the SMF, suggesting that this strain is a strong competitor within the microbial community. *P. croceum,* in contrast, was clearly inhibited by the presence of the SMF, probably due to competition within the microcosm. An impact of AcH 505 on *P. croceum* growth was only observed in the presence of the SMF. In the absence of the oak AcH 505 promoted fungal growth, but in its presence, *P. croceum* was inhibited. In addition, this part of the thesis also implemented a visualization procedure of both microorganisms by scanning electron microscopy in the soil microcosm system used to synthesize biotrophic interactions in TrophinOak. This series of results showed the complexity of the interaction and highlighted the importance of the resident microbial community and host plant as bacterial and fungal growth modulating factors. This study further demonstrated the impact of the use of a "semi-natural" culture system. It can be expected that its use for plant transcriptome analysis during biotrophic interactions could potentially influence the findings compared to the ones in more artificial, e.g. agar-based systems. Semi-natural conditions provide results more in tune with the situation in the soil environment, and the first part of this chapter precisely underlined the role played by more exhaustive soil community in interaction networks. Finally this study reinforced the validity of using microcosms with soil and SMF in the TrophinOak platform.

The plant transcriptome analysis in *chapter 2* examined the impact of AcH 505 on the oak and used co-treatment with the EMF *P. croceum* to disentangle the direct impact of AcH 505 from the impact of co-inoculation. Differential gene expression profiles in oaks upon single and co-inoculations in roots and leaves, upon root and shoot flush were investigated. Gene expression differed between local root and systemic leaf tissues, treatments with the microorganisms and different developmental stages, suggesting that oak specifically coordinates its gene expression patterns. The genes related to plant growth, plant defence and DNA modification were dominant among the differential expressed genes, suggesting that these processes may play essential roles in both the single and double interactions. Especially among co-inoculation, a strong positive impact on plant growth at the morphological level occurred, which was coupled with differential gene expression of transcripts related to growth and metabolism. Plant cell wall modifying enzymes were identified as potential key players of plant-microbe interaction. For instance xyloglucan endo-transglycosylase expression levels were up-regulated in roots and leaves in most treatments. Several leucine rich repeats

containing signalling proteins induced in roots and shoots upon bacterial and fungal inoculation were shown to participate in the recognition of microbes and contribute to plant growth. The treatment with AcH 505 led to an enhanced plant defence gene expression, which was attenuated in roots at SF upon co-inoculation with *P croceum*, suggesting that the defence response of the plant is altered by the interaction of both microorganisms, and that *P croceum* - known to down-regulate plant defence gene expression - overrides the MHB effect. Gene expression patterns strongly differed between developmental stages and between local root and distal leaf tissues. This study contributed to the identification of differentially expressed genes in oak playing important roles for the coordination of interaction with mycorrhizosphere organisms and developmental stage.

The induction of oak defence gene expression by AcH 505 raised the question if the bacterium could affect belowground-aboveground interactions in oak. In the last study (*chapter 3*) of the thesis, we observed that the root interacting AcH 505 suppressed the infection of oak leaves by oak powdery mildew *M. alphitoides*. RNA-Seq data from leaf tissue of oak upon single and co-inoculation were compared and results showed that AcH 505 induced a defence gene expression response in leaves which was partly enhanced by concurrent *M. alphitoides* infection. Besides the increased expression levels of jasmonic acid/ethylene dependent defence response genes, which are common constituents of rhizobacteria induced disease resistance pathways, the involvement of salicylic acid and abscisic acid dependent pathways, rather known for systemic acquired resistance (SAR) pathways that are elicited by pathogens, was suggested. Moreover, the differential gene expression patterns in plants inoculated with AcH 505 shared characteristics with the pattern induced by powdery mildew. The expression profile was similar than previously observed for *Bacillus*-elicited defence response, and indicated an extensive plant response to AcH 505. However, inoculation with AcH 505 came together with a fitness benefit for the plant upon powdery mildew infection: suppression of photosynthesis related gene expression was alleviated by bacterial treatment. Thus, AcH 505 is able to induce a strong plant response in the absence of *M. alphitoides*, thereby eliciting a significant defence response in oaks that is in part enhanced upon powdery mildew infection.

In conclusion, this thesis showed that *Streptomyces sp.* strain AcH 505 interacts with *Piloderma croceum* and oak roots, and also has systemic effects on the leaves. It suggests that the AcH 505 elicited cross talk with oak is affected by the stage of plant growth.

As expected, the impact of AcH 505 was strongly modified by co-inoculation with the EMF *P. croceum*, which targets the same organ as AcH 505, i.e. the root. The main visible effects of AcH 505 on the oak were maintained in case of co-inoculation with the EMF and related gene families were found to be differentially expressed in the oak in both cases. However, numerically, most genes differentially regulated by the MHB alone were not active in presence of the EMF. In total, these results suggest that the two organisms share the symbiotic function for the plant when they play together. Interestingly, the number of genes regulated in plants is higher when one of the two microorganisms is inoculated alone than when they are co-inoculated (compare chapter 2 and Tarkka et al. 2013). This supports a synergistic efficiency in the symbiotic effect. In this frame, it would be of interest to analyse the function of the genes remaining differentially expressed by co-inoculation as these may relate to central direct functions of AcH 505. The second transcriptome study coped with the effect of AcH 505 on a leaf pathogen and enabled to approach systemic effects of MHB on trees. Here, reinforcement of the plant defence targeting leaf enemies, as well as systemic defence functions, was found. This suggests that AcH 505, besides being a MHB, is also a plant helper bacterium. Overall this thesis contributed to the assessment of the impact of microorganisms on plant growth and development. Perspectively, the plant data obtained at the transcriptome level should be associated with analyses at the physiological level (e.g. by analysing plant proteins and metabolites) in order to complete the picture.

Quantitative Real-time PCR quantitation experiments showed that AcH 505 and *P. croceum* have direct interactions, which are modulated by the presence of an integral soil community and plant roots. It was recently described, that AcH 505 affects the structure of microbial communities (Caravaca et al., 2015), which also warrants further investigation. The ensemble of the obtained results fully justified including AcH 505 in the TrophinOak project and, apart from completing the spectrum of dual biotrophic interactions studied on the JEP, this work opened an avenue to study multitrophic effects. Future studies should deal with comparative analyses of oak gene expression to other organisms, both microorganisms and fauna, investigated in the TrophinOak project. The fact that the genomes of AcH 505, *Q. robur* DF159 and *P. croceum* are or will soon be available will enable a new generation of studies.

ZUSAMMENFASSUNG

Die meisten Bäume der borealen und gemäßigten Wälder gehen eine Wurzelsymbiose mit Pilzen ein – die Ektomykorrhiza (EM). Dabei wird der Pilz von der Pflanze mit Kohlenstoff versorgt. Im Gegenzug liefert der Pilz mineralische Nährstoffe und verstärkt die Abwehr der Pflanze gegen Pathogene. Sowohl die Bildung, als auch die Funktionsweise der EM wird durch assoziierte Mykorrhizahelferbakterien (MHB) gefördert. Über den Einfluss der MHB auf die Pflanze ist wenig bekannt. In der vorliegenden Dissertation wurde mit Hilfe von Hochdurchsatz-Sequenzierung und quantitativer real-time PCR die Antwort der Stieleiche *Quercus robur* L. auf die Interaktion mit dem MHB *Streptomyces* sp. AcH 505 untersucht. Um eine höhere Relevanz zu erreichen wurden außerdem zwei pilzliche Interaktionspartner mit einbezogen: der EM Pilz *Piloderma croceum* und der Mehltau *Microsphaera alphitoides.* Da MHB und EM-Pilze Teil der symbiotischen Mykorrhizosphäre sind, wurde ihre Interaktion in einem ersten Schritt charakterisiert. Beide Mikroorganismen wurden dazu in dem verwendeten Kultursystem mittels molekularer und fluoreszenzmikroskopischer Methoden quantifiziert. Der Hauptfokus der Arbeit lag in der Analyse der molekularen Antwort der Eiche auf die interagierenden Organismen. Weiterhin wurde untersucht, inwiefern das rhythmische Wachstum der Eiche, welches durch alternierende Wurzel- und Sprosswachstumsschübe charakterisiert ist, diese Interaktionen beeinflusst. Die Studien wurden im Rahmen des Forschungsprojekts „TrophinOak“ durchgeführt.

Die in *Kapitel 1* vorgestellte Studie wies sowohl einen methodischen als auch einen biologischen Fokus auf. AcH 505 und *P. croceum* wurden in einem bodenbasiertem Kultursystem kultiviert, um eine Methode zur einfachen und schnellen Quantifizierung beider Mikroorganismen im Boden zu entwickeln. Ein weiteres Ziel war es zu untersuchen, auf welche Weise die Eiche einerseits, und ein mikrobielles Bodenfiltrat andererseits, die Bakterium-Pilz Interaktion beeinflussen. In diesem Kontext wurden beide Mikroorganismen mit Hilfe von Rasterelektronenmikroskopie innerhalb des Bodensystems visualisiert und eine Methode unter Verwendung der quantitativen real-time PCR mit dem Einsatz spezifischer Primer etabliert. Für AcH 505 wurde eine Region zwischen zwei proteincodierenden Genen gewählt und als Amplifikationsziel validiert. Für *P. croceum* wurden die ITS-Region und eine Region zwischen zwei proteincodierenden Genen als Amplifikationsziele gewählt. Vergleichend konnte gezeigt werden, dass sich die ITS-Region, trotz ihrer hohen Kopienzahl in Pilzen, für eine spezifische und sensitive Quantifizierung eignete. Die Quantifizierung

beider Mikroorganismen unter den verschiedenen Bedingungen, d. h. die jeweilige An- und Abwesenheit der Eiche und des mikrobiellen Bodenfiltrats, zeigte, dass beide Parameter die Bakterium-Pilz Interaktion beeinflussen. *P. croceum* förderte AcH 505 in seinem Wachstum, was auf eine Verwendung pilzlicher Exudate als Nährstoffe für das Bakterium hindeuten könnte. Diese Wachstumsförderung war bei Zugabe des mikrobiellen Bodenfiltrats stärker ausgeprägt, was dafür spricht, dass das Bodenfiltrat die Bakterium-Pilz Interaktion durch zusätzliche Konkurrenz im Bodensystem beeinflusst. AcH 505 wurde durch das mikrobielle Bodenfiltrat nicht beeinflusst, was darauf hindeutet, dass das Bakterium innerhalb der mikrobiellen Gemeinschaft stark konkurrenzfähig ist. Im Gegensatz dazu, wurde *P. croceum* durch das mikrobielle Bodenfiltrat deutlich gehemmt. Dies ließ sich wahrscheinlich auf Konkurrenz innerhalb des Bodensystems zurückführen. Einen Einfluss von AcH 505 auf *P. croceum* konnte nur in Anwesenheit des mikrobiellen Bodenfiltrats beobachtet werden. In Abwesenheit der Eiche wurde *P. croceum* durch AcH 505 gefördert, in ihrer Anwesenheit allerdings gehemmt. Außerdem wurde in diesem Teil der Dissertation eine Methode zur Visualisierung beider Mikroorganismen innerhalb des Bodensystems mit Hilfe von Rasterelektronenmikroskopie etabliert. Diese Reihe an Ergebnissen zeigte die Komplexität der Interaktionen und unterstrich die Bedeutung der mikrobiellen Gemeinschaft und der Wirtspflanze im Hinblick auf das mikrobielle Wachstum im Bodensystem. Des Weiteren, betonten die Ergebnisse dieser Studie die Bedeutung der Benutzung eines „semi-natürlichen" Kultursystems. Es ist zu erwarten, dass die Nutzung eines solchen Systems im Gegensatz zu artifizielleren Systemen, wie z.B. Agar-basierten Systemen, die Ergebnisse von Transkriptom-Analysen von Pflanzen mit biotrophischen Interaktionen beeinflussen könnte. Semi-natürliche Bedingungen liefern Ergebnisse die in einem höheren Maße mit der Situation im Boden übereinstimmen und *Kapitel 1* betonte gerade die Bedeutung der mikrobiellen Bodengemeinschaft in Interaktionsnetzwerken. Zuletzt bestärkte die Studie die Nutzung von bodenbasierten Mikrokosmen mit mikrobiellem Bodenfiltrat innerhalb des TrophinOak Projekts.

Im Rahmen der Pflanzentranskriptom-basierten Studie in *Kapitel 2* wurde der Einfluss von AcH 505 auf die Eiche untersucht. Weiterhin wurden Eichen zusätzlich mit dem EM-Pilz *P. croceum* co-inokuliert, um den direkten Einfluss von AcH 505 von dem durch die Co-Inokulation entstehendem zu trennen. Dazu wurde die differentielle Genexpression von einzel- und co-inokulierten Eichen in Wurzeln und Blättern während eines Wurzel- und Sprosswachstumsschub analysiert. Das Genexpressionsprofil der Eiche variierte je nach

Interaktion, untersuchtem Gewebe und Entwicklungsstadium, was darauf hindeutete, dass die Genexpression der Eiche spezifisch koordiniert wird. Innerhalb der differentiell exprimierten Gene traten besonders Wachstums-, Verteidigungs- und DNA-modifizierende Gene hervor. Dies deutete darauf hin, dass die jeweiligen Prozesse besonders wichtig für beide Interaktionen sind. Insbesondere in co-inokulierten Eichen konnte ein starker positiver Effekt auf das Pflanzenwachstum beobachtet werden, der mit der differentiellen Expression von wachstumsbezogenen Genen einherging. Enzymen, die die pflanzlichen Zellwände modifizieren, konnte eine mögliche Schlüsselrolle bei der mikrobiell-pflanzlichen Interaktion zugewiesen werden. Zum Beispiel waren Xyloglucan-Endo-Transglycosylasen in Wurzeln und Blättern in den meisten Behandlungen hochreguliert. Mehrere „Leucin rich repeats“, welche Signal-Proteine beinhalten, wurden in Wurzeln und Blättern durch Bakterien- und Pilzinokulation induziert. Ihnen wurde bereits eine Rolle bei der Erkennung von Mikroorganismen und Pflanzenwachstum zugeschrieben. Die Inokulation mit AcH 505 resultierte in einer verstärkten Expression der Abwehrgene der Eiche, welche durch die Co-Inokulation mit *P. croceum* in Wurzeln im Sprosswachstumsschub abgeschwächt wurde. Dies sprach dafür, dass die Abwehr der Pflanze durch die Interaktion mit beiden Mikroorganismen beeinflusst wird und das *P. croceum* - bekannt für die Herabsetzung der pflanzlichen Verteidigungs-Genexpression - den MHB-Effekt überspielt. Die Muster der Genexpression variierten stark zwischen den verschiedenen Entwicklungsstadien der Eiche, sowie zwischen lokalem Wurzel- und distalem Blattgewebe. Diese Studie leistete somit einen Beitrag zur Identifikation von differentiell exprimierten Genen, die eine Schlüsselrolle in der Koordination der Interaktionen mit Mikroorganismen und des Entwicklungsstadiums spielen.

Die in der Eiche durch AcH 505 induzierte Expression der Abwehrgene führte zu der Frage, ob das Bakterium die unter- und oberirdischen Interkationen in der Eiche beeinflusst. Im Rahmen der letzten Studie (*Kapitel 3*) dieser Dissertation, wurde beobachtet, dass das wurzelinteragierende Bakterium AcH 505 die Infektion von Eichenblättern durch den Mehltau *M. alphitoides* unterdrückt. RNA-Seq Datensätze von Eichenblattgewebe von einzel- und co-inokulierten Eichen wurden miteinander verglichen und es konnte gezeigt werden, dass AcH 505 auf Genexpressionsebene eine Verteidigungsantwort in Blättern induziert, welche durch die Co-Inokulation mit *M. alphitoides* verstärkt wird. Es wurde eine verstärkte Expression von Genen beobachtet, die Teil der Jasmonsäure/Ethylen–basierten Verteidigung sind, welche typische Mechanismen für die Rhizobakterien-induzierte Pathogenabwehr darstellen. Es konnte jedoch auch die Beteiligung der Salicylsäure- und Abscisinsäure-

basierten Verteidigungsmechanismen festgestellt werden, die eher für die durch Pathogene induzierte „systemic acquired resistance“ (SAR) bekannt sind. Außerdem, zeigten die Genexpressionsmuster von AcH 505-inokulierten Pflanzen Gemeinsamkeiten mit dem Muster, welches durch *M. alphitoides* induziert wurde. Das Genexpressionsprofil ähnelte der kürzlich beobachteten *Bacillus*-induzierten Abwehr und spricht für eine starke pflanzliche Reaktion auf AcH 505. Trotzdem führte die Inokulation mit AcH 505 zu einem Fitnessvorteil für die Eiche bei Mehltau-Infektion, indem die Hemmung der Photosynthese-bezogenen Gene durch das Bakterium aufgehoben wurde. Folglich ist AcH 505 in der Lage auf eine starke pflanzliche Reaktion in Abwesenheit von *M. alphitoides* zu induzieren, welche zu einer signifikanten Verteidigung der Eiche führt und während der Mehltauinfektion teilweise verstärkt wird.

Insgesamt konnte mit der vorliegenden Dissertation gezeigt werden, dass *Streptomyces sp.* AcH 505 mit *Piloderma croceum* und Eichenwurzeln interagiert, und auch einen systemischen Effekt auf die Blätter hat. Die Arbeit deutet darauf hin, dass die Interaktion von AcH 505 mit der Eiche von dem pflanzlichen Wachstumsstadium beeinflusst wird.
Wie zu erwarten, wurde der Effekt von AcH 505 stark durch die Co-Inokulation mit dem EM-Pilz *P. croceum* beeinflusst, welcher mit dem gleichen Organ interagiert, nämlich der Wurzel. Die Haupteffekte von AcH 505 auf die Eiche wurden auch in co-inokulierten Pflanzen beibehalten und verwandte Genfamilien waren in beiden Fällen differentiell exprimiert. Dennoch war ein Großteil der durch das MHB induzierten Gene in co-inokulierten Pflanzen nicht differentiell exprimiert. Insgesamt, sprechen die Ergebnisse dafür, dass beide Mikroorganismen symbiotische Funktionen für die Pflanze teilen wenn sie zusammen agieren. Interessanterweise war die Anzahl an differentiell regulierten Genen höher wenn einer der beiden Mikroorganismen allein mit der Pflanze interagiert, als bei der Co-Inokulation (siehe *Kapitel 2* und Tarkka et al. 2013). Dies spricht für einen synergistischen Effekt innerhalb der Symbiose. In diesem Rahmen wäre es interessant die Funktion der Gene zu analysieren, die während der Co-Inokulation differentiell exprimiert bleiben, da sie auf zentrale Funktionen von AcH 505 schließen lassen könnten. Die zweite Transkriptomstudie befasste sich mit dem Effekt von AcH 505 auf ein Blattpathogen und erlaubte die Untersuchung des systemischen Effekts von MHB auf Bäume. Hierbei wurden sowohl die Verstärkung der pflanzlichen Abwehr gegen Blattpathogene, als auch systemische Verteidigung gefunden. Dies spricht dafür, dass AcH 505, neben seiner Funktion als MHB auch ein Pflanzen-Helfer-Bakterium ist. Insgesamt trug diese Arbeit zum Verständnis des

Einflusses von Mikroorganismen auf Pflanzenwachstum und -entwicklung bei. In Zukunft sollen die dargestellten Transkriptom-Daten mit physiologischen Daten verbunden werden, indem zum Beispiel pflanzliche Proteine und Metaboliten analysiert werden, um das Bild zu vervollständigen.

Quantitative real-time PCR Experimente zeigten, dass AcH 505 und *P. croceum* direkt miteinander interagieren, was durch die Anwesenheit eines mikrobiellen Bodenfiltrats und Wurzeln moduliert wird. Außerdem sprechen weitere Analysen dafür, dass AcH 505 die Struktur mikrobieller Gemeinschaften beeinflusst (Caravaca et al., 2015), was weitere Untersuchungen rechtfertigt. Die Gesamtheit der Ergebnisse begründete die Einbeziehung von AcH 505 im Rahmen des TrophinOak Projekts. Die Arbeit vervollständigte einerseits das Spektrum der untersuchten dualen biotrophischen Interaktionen innerhalb der Projektplattform und ebnete andererseits den Weg für die Untersuchung multitrophischer Effekte. Zukünftig sollten Studien durchgeführt werden, bei denen vergleichend die Genexpression der Eiche mit anderen Organismen, sowohl Mikroorganismen als auch Tieren, die Teil des TrophinOak-Projekts sind, untersucht wird. Die Tatsache, dass die Genome von AcH 505, *Q. robur* DF159 und *P. croceum* bereits vorliegen bzw. bald vorliegen werden, wird eine neue Generation von Studien ermöglichen.

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Conference proceedings

Presentations

Kurth F, Mailänder S, Zeitler K, Feldhahn L, Grosse I, Grams TEE, Herrmann S, Buscot F, Schrey SD, Tarkka MT - "The plant beneficial bacterium Streptomyces sp. AcH 505 promotes mycorrhiza formation and inhibits powdery mildew infection in pedunculate oak". *Actinobacteria within soils: Capacities for mutualism, symbiosis and pathogenesis, Münster (Germany), October 2012*

Kurth F, Mailänder S, Zeitler K, Feldhahn L, Grosse I, Wubet T, Buscot F, Schrey SD, Tarkka MT - "Plant responses to mycorrhization helper bacterium Streptomyces sp. AcH 505". *7th International Conference on Mycorrhiza (ICOM7), New Delhi (India), January 2013*

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Tarkka M, Feldhahn L, Bönn M, **Kurth F**, Recht S, Mailänder S, Schrey SD, Wubet T, Herrmann S, Grosse I, Buscot F - "Analysis of biotic interactions by RNAseq based on a reference transcriptome". *JCB Workshop "Bioinformatics meets Biodiversity" Jena (Germany), September 2013*

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Tarkka MT, Zeitler K, Feldhahn L, Neu TR, Wubet T, Buscot F, Herrmann S, **Kurth F** - "Mycorrhizal fungus stimulates the growth of Mycorrhization Helper Bacterium in plant-soil-microcosms". *7th International Conference on Mycorrhiza (ICOM7), New Delhi (India), January 2013*

STATUTORY DECLARATION

I, Florence Kurth, hereby affirm that I take note and accept the doctorate regulations of the Faculty of Life Science, Pharmacy and Psychology of the University of Leipzig from January the 20th, 2010.

I further affirm that the presented thesis was prepared autonomously without inadmissible help. All aids used in this thesis as well as scientific ideas which are quoted from or based on other sources were cited at the respective point.

All people who helped me to prepare the conception, to select and analyse the materials of this thesis as well as to improve the manuscript are namely cited in the acknowledgements. With exception of the namely mentioned people no other persons were involved in the intellectual work. No PhD consultant service was employed. Third parties did not get money's worth for benefits that were in conjunction with the content of this dissertation.

I declare that this dissertation has been neither presented nationally nor internationally in its entirety or in parts to any institution for the purpose of dissertation or other official or scientific examination and/or publishing.

Previously unsuccessful dissertations had not taken place.

The original document of the verification of the co-author parts are deposited in the office of the dean.

Leipzig, 11.03.2015

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Florence Kurth

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, Florence Kurth, eidesstattlich, dass mir die Promotionsordnung der Fakultät für Biowissenschaften, Pharmazie und Psychologie der Universität Leipzig vom 20. Januar 2010 bekannt ist und von mir anerkannt wird.

Zudem versichere ich, dass die vorliegende Promotionsarbeit von mir selbstständig und ohne unzulässige Hilfsmittel angefertigt worden ist. Sämtliche von mir verwendete Hilfsmittel sowie die aus fremden Quellen direkt oder indirekt übernommen wissenschaftlichen Gedanken sind als solche in der vorliegenden Arbeit gekennzeichnet.

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Ich versichere, dass die vorgelegte Dissertation weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zwecke der Promotion oder anderer staatlicher oder wissenschaftlicher Prüfungsverfahren vorgelegt und/oder veröffentlicht wurde.

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Die Nachweise über die Anteile der (Co-)Autorenschaften sind im Original im Dekanat der Universität Leipzig hinterlegt.

Leipzig, 11.03.2015

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Florence Kurth

APPENDIX

Verification of the author parts, Florence Kurth
Genetic and physiological responses of oak to mycorrhiza helper bacterium and further mycorrhiza associated bacteria

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Verification of the author parts:

Title:	**Detection and quantification of a mycorrhization helper bacterium and a mycorrhizal fungus in plant-soil microcosms at different levels of complexity**
Journal:	BMC Microbiology
Authors:	Florence Kurth, Katharina Zeitler, Lasse Feldhahn, Thomas R Neu, Tilmann Weber, Vacláv Krištůfek, Tesfaye Wubet, Sylvie Herrmann, François Buscot, Mika T Tarkka

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Rates of Florence Kurth (author 1):
- conducted the molecular studies
- drafted the manuscript

Rates of Katharina Zeitler (author 2):
- participated in quantification experiments

Rates of Lasse Feldhahn (author 3):
- performed the AcH 505 genome assembly

Rates of Thomas R Neu (author 4):
- helped with the confocal laser scanning microscopy

Rates of Tilmann Weber (author 5):
- did the GFP labelling of AcH 505

Rates of Vacláv Krištůfek (author 6):
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Rates of Tesfaye Wubet (author 7):
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Rates of Sylvie Herrmann (author 8):
- coordinated the establishment of microcosms with oak microcuttings within the TrophinOak platform

Rates of François Buscot (author 9):
- is the lead scientist of the TrophinOak project

Rates of Mika T Tarkka (author 10):
- conceived of the study, participated in its design and coordination
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- helped to draft the manuscript

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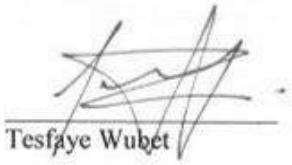
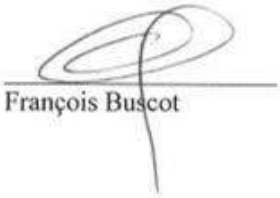


Tesfaye Wubet

Sylvie Herrmann



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Verification of the author parts, Florence Kurth
Genetic and physiological responses of oak to mycorrhiza helper bacterium, symbiotic and pathogenic fungi

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Verification of the author parts:

Title: **Large scale transcriptome analysis reveals interplay between plant development and beneficial host-microbe interactions**

Journal: BMC Genomics

Authors: Florence Kurth, Lasse Feldhahn, Markus Bönn, Sylvie Herrmann, François Buscot, Mika T Tarkka

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Rates of Mika T Tarkka (author 6):
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Verification of the author parts, Florence Kurth
Genetic and physiological responses of oak to mycorrhiza helper bacterium, symbiotic and pathogenic fungi

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Verification of the author parts:

Title:	***Streptomyces* induced resistance against oak powdery mildew involves host plant responses in defence, photosynthesis and secondary metabolism**
Journal:	Molecular Plant Microbe Interaction
Authors:	Florence Kurth, Sarah Mailänder, Markus Bönn, Lasse Feldhahn, Sylvie Herrmann, Ivo Grosse, François Buscot, Silvia D Schrey, Mika T Tarkka

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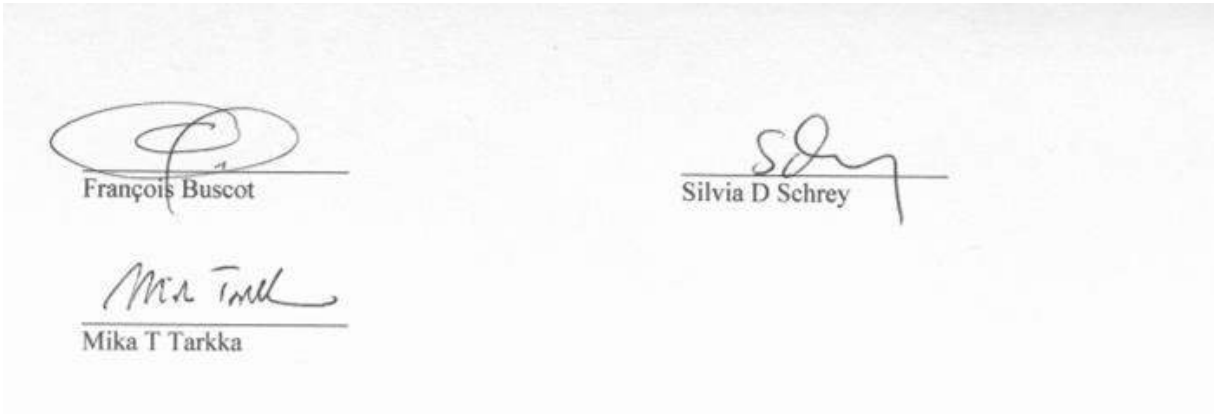
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