Multitrophic interactions in oak

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Erstgutachter: Dr. habil. Martin Schädler
Zweitgutachter: Prof. Dr. Roland Brandl

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Chapter I

General introduction

Plant-insect interactions are played out in an ecological arena that is larger than the plant itself.

Dicke and Takken 2006

In natural ecosystems species interaction play an important role for the dynamics and structure of communities. Species are continually exposed to a variety of interacting species, both above- and belowground (Ehrlich & Raven, 1964; Thomson 2009). These interactions may have both detrimental (e.g. herbivores, pathogens) as well as beneficial (e.g. symbionts) for plant performance. The separate effects of single interacting organisms on plant physiology and performance are known to have important consequences on the performance of plants as well as the dynamics of populations and communities of plants (for root symbionts see Smith & Read 2007, van de Heijden & Sanders 2002). However, several types of interaction usually simultaneously affect plant growth and physiology under natural conditions. By altering the plant's quality and quantity these interactors further affect each other, resulting in potentially complex feedback loops (Wardle *et al.,* 2004). Recent evidence accumulated that the underlying genetic and physiological pathways induced by and involved in the different above- and below-ground interactions overlap substantially (Schenk *et al.* 2008). However, our knowledge of such interacting effects and the underlying genetic and physiological mechanisms within plants as the central partner and the ecological consequences is still very sketchy (Gehring & Whitham 2002). Moreover, these interacting effects can not be expected to be predictable from the separate effects of single interactors. Consequently, an understanding of the separate and simultaneous influences of these effects on the plant and their reciprocal interactions is needed.

Interactions between mycorrhizal fungi and herbivores

Amongst the most ubiquitous partners are mycorrhizal fungi and herbivorous insects which are associated with virtually all trees. The separate effects of these two groups of organisms on plant physiology and performance are known to have important consequences for the performance of plants as well as the dynamics of populations and communities of plants (e.g., mycorrhiza: Smith & Read 2007, van de Heijden & Sanders 2002; herbivores: Crawley 1997). Moreover, insect herbivores and mycorrhizal fungi are known to

affect each other, resulting in feedback effects on plant performance. Firstly, by providing nutrients mycorrhizal fungi may increase the vigor and nutritional quality of host plants with positive effects on the performance of insect herbivores as well as positive effects on the ability of the plant to tolerate herbivory (Gehring & Whitham 1994a, Borowicz 1997, Halldorsson *et al.* 2000). Secondly, the resource supply by mycorrhizal fungi may allow plants to increase the investment into resistance against generalist insect herbivores (Jones & Last 1991, Gange & West, 1994; Bi *et al.,* 2007; Yao *et al.,* 2007). In turn, herbivory may importantly feed back on the interactions between plants and mycorrhiza. Herbivory may have a negative effect on mycorrhizal fungi because defoliation by herbivores decreases photosynthesis and therefore the allocation of resources to the mycorrhizal fungi (Smith & Read, 1997; Gehring & Whitham, 2002). Ectomycorrhizal fungi may use between 10 to 50 % of the photosynthesis products of host plants (Simard *et al.,* 2002; Hobbie & Hobbie, 2006) and therefore EM fungi may react very sensitively to herbivory induced changes in C-allocation (Markkola *et al*., 2004; Stark & Kytöviita, 2005). After feeding by herbivores, Frost & Hunter (2008) investigated a by 63% decreased allocation of carbon belowground in red oak and an increased allocation to new foliage. Similarly, herbivory by caterpillars of the moth *Lymantria dispar* decreased C-allocation to the root system (Babst *et al*. 2008). Further, herbivory affects the balance between the important C-pools starch and soluble sugars in leaf tissue (Babst *et al*., 2005) and roots of trees (Kosola *et al*., 2002). Therefore, herbivory is an important trigger of the C-allocation patterns within plants with possibly important consequences on feedbacks with other associated organisms. However, the importance of herbivory induced changes in allocation patterns for plant-mycorrhiza symbioses remained unexplored until now. However, evidence accumulated that this stimulation of plant resistance is not only due to the well-known effects of the mycorrhiza on plant nutrition, but also to changes in the pathways of signals that trigger the defence systems (Bi *et al.,* 2007; Yao *et al*., 2007; Pozo & Azcón- Aguilar, 2007). Thus, the ability of the plant to use these additional resources made available by mycorrhization for anti-herbivore defence determines the direction of the effects on herbivore performance. Kempel *et al*. (2009) demonstrated a central role of defence induction for the outcome of mycorrhiza-herbivore interactions what highlights the importance of signalling pathways within the plant for this tri-partite relationship.

In addition, soil detritivores alter plant nutrient supply and therefore plant growth performance and vitality. For instance Collembola recycle plant litter thereby increasing nutrient availability of plants, but they also alter plant growth via changing plant – microbial interactions, in particular that between plants and fungi (Chamberlain *et al.*, 2006). Furthermore, Collembola affect plant performance not only through provisioning of nutrients but also influence their growth and survivability by a number of other indirect mechanisms like grazing on rhizosphere microorganisms and modifying the soil structure (Scheu, 2001; Gormsen *et al.*, 2004; Friberg *et al.*, 2005).

Mycorrhization and plant defenses

Coevolutions of plants and insects has brought to light a magnitude of strategies to cope with herbivory. Undoubtedly, physiological and ecological constraints play key roles in the evolution of plant growth patterns, especially in relation to defenses against herbivory (Herms and Mattson, 1992). Direct defenses, in contrast to indirect defenses, are plant traits (e.g. secondary metabolites, silica, thorns, trichomes etc.) that by themselves affect herbivore performance and are generally categorized by their mode of action (Baldwin *et al.*, 2001). Morandi (1996) reviewed the stimulation of secondary compounds like e.g. flavonoide and isoflavonoide by arbuscular mycorrhizal fungi. Further, the impact of secondary metabolites on insect herbivores has been shown by Gange and West (1994), who found increased levels of aucubin and catalpol in plants of *Plantago laureolata* inoculated with an arbuscular mycorrhizal fungus. Furthermore, mycorrhizal colonisation also increases phenolic contents in *Cynara cardunculus* (Ceccarelli *et al.*, 2010)

Further, indirect defense mechanisms are an effective way of reducing herbivory (Baldwin *et al.*, 2001). Often this involves the induced release of volatile organic compounds (VOCs) in response to herbivore attack. These volatiles can serve in tri-trophic systems to attract enemies of herbivores, such as parasitic wasps and flies or predatory mites, which can protect the signalling plant from further damage (Kessler & Baldwin, 2001; Rosta´ s & Turlings, 2008). Amongst the most common volatile signals in indirect defense are metabolites of the lipoxygenase (LOX) pathway, metabolites of the shikimic acid pathway, and products of the terpenoid pathway (monoterpenes, sesquiterpenes, homoterpenes; Pichersky & Gershenzon, 2002). There are a few studies suggesting a connection of mycorrhizal symbiosis and the production of VOCs as indirect defense. For instance, Gange *et al*.

(2003) found lower infection rates of a leaf mining insect in *Leucanthemum vulgare* by a parasitoid wasp when the host plant was in symbiosis with arbuscular mycorrhizal fungi, which might be caused by an enhanced VOC emission in mycorrhizal plants. Further evidence for other forms of symbiosis was raised by Ballhorn *et al*. (2012). The authors measured the release of jasmonic acid induced volatiles of rhizobia-colonized and rhizobia-free lima bean plants and found differences in the composition of the volatile blend of plants.

Recent developments in genomics allow for the first time to analyse the genetic basis of biotic interactions. The use of DNA microarrays, for instance, provided information on the patterns of gene expression caused by biotic interactions and their consequences for plant metabolism and defense (Colebatch *et al.,* 2002, Michel *et al.,* 2006; Cartieaux *et al.,* 2008). These patterns are the first step for the identification of genes involved in biotic interactions of e.g. mycorrhizal fungi and herbivores (Wullschleger *et al.,* 2007). The first generation of microarray experiments demonstrated that herbivores influence the transcription of hundreds of genes (Baldwin *et al.*, 2001; Hermsmeier *et al*., 2001; Roda & Baldwin 2003; Schmidt *et al*., 2005), perhaps leading to an entire metabolic reorganization of the plant (Hui *et al.*, 2003). Of course, interactions between plants and mycorrhizal fungi lead also to changes in the expression of genes in the host plant (Wiemken & Boller, 2002). Some of these genes are relevant for the allocation of resources as well as reactions to stress and defense (Herrmann & Buscot, 2007).

The role of plant growth stages

Major trees of boreal, temperate regions, e.g. *Quercus robur*, *Castanea sativa*, *Picea abies, Fraxinus excelsior* and *Abies alba*, grow with mycorrhiza and show a determinate pattern of rhythmic growth (Herrmann, 1998). Further, each growth unit is characterized by an acrotonic lateral branching, in which the most apical of the axillary buds give rise to vigorous ramifications (Harmer, 1990). Variations in seasonal climate in close connection with the tree age allows up to 5 growth units within a vegetation period (Gruber, 1987). In trees older than 10 years, the number of growth flushes is mostly reduced to the regular spring flush and one additional lammas shoot ("Johannistrieb") in July (Adams & Bastien, 1994). Those trees develop this rhythmicity in nature, as well as under controlled culture conditions (i.e. 25° C and long day illumination), uninfluenced by changes in nutrient supply which indicates a endogenous control of plant growth (Lavarenne, 1966; Champagnat *et al*., 1986).

These growth flushes, accompanied by rhythmic allocation of nutrients, play an important role in the physiology and ecology of the plant. Such patterns of rhythmic growth could have a crucial impact on plant response to herbivore feeding. For example, plants attacked during root flush might be more vulnerable to herbivores than those attacked during shoot flush because nutrients are allocated towards the root system during root flush. On the other hand, as the leaf surface area is reduced by herbivory, the amount of C deposited in fine roots can be reduced (Frost, 2008), which in turn might influence the interaction between mycorrhizal fungi and host plants.

Harmer (1990) showed that the alternating growth flushes in shoot and roots, typical for the oak development, only appear in seedlings older than two years. A number of experiments demonstrated further that there is considerable intraspecific variability of herbivory related traits in trees (Ruhnke *et al.*, 2006, Ruhnke *et al.*, 2009). For *Quercus robur* it was shown that the intensity of herbivory for different polyphagous species and performance parameters of insect herbivores differed between single oak individuals (Ruhnke *et al*., 2006). This genetic variability can be further expected to have influences on interactions with other associated organisms (Schädler *et al.,* 2010). Microcuttings provide genetically uniform material and therefore prevent confounding effects of oak genotypes on herbivore performance. The use of micropropagated oaks (*Quercus robur*; further on referred to as microcuttings) therefore suits the needs for an experimental platform with a considerable size, expression of rhythmic growth, genetically uniformity and are produceable in great number to satisfy the needs of collaborating working groups of the TrophinOak project in which also the present studies of this dissertation participates (for further information see: http://www.ufz.de/trophinoak/)

Aims of the thesis

The present dissertation is devoted to shed light on the interactions between ectomycorrhizal fungi and leaf consuming insects in oak with a focus on changes in allocation patterns and the underling genetic basis. Although the genetics of oak have attracted increased attention in recent years, no whole genome, nor transcriptome sequence is available today (Barreneche *et al.*, 1998; Ueno *et al*., 2010; Kremer *et al.*, 2012).

Accordingly, the second chapter of this dissertation is devoted to the technical preparation of a comprehensive reference transcriptome. We treated *Q. robur* microcuttings with seven trophic interactions (mycorrhizal fungus, leaf herbivore, leaf pathogen,

mycorrhizal helper bacterium, rhizosphere consumer, root pathogen and root feeding nematode to obtain as big as possible numbers of expressed genes. In preparation of the sequencing using two technically different approaches (454 pyrosequencing and Illumina RNA-Seq) and resulted in the OakContigDF159.1 reference transcriptome.

In the third chapter of the present dissertation I study the effects of leaf herbivory caused by a generalist herbivore caterpillar, mycorrhizal symbiosis with an ectomycorrhizal fungus and of endogenous rhythmic growth in a multifactorial experiment. Aim of this experiment is the investigation of the genetic and physiological basis of mediating effects of a mycorrhizal fungus on oak-herbivore interaction. Using the reference library OakContigDF159.1 I measured gene expression of single genes, as well of “gene families” using Gene Ontology categories and physiological pathways using the approach of the Kyoto Encyclopedia of Genes and Genomes. Further I apply a nutrient tracer analyses using stable isotopes of carbon and nitrogen.

The fourth chapter aims to shed more light on more complex interaction patterns of above-ground and below-ground trophic partners. This is the first study of a combined investigation the collembolan species *Protaphorura armata* to the well established system of oak, mycorrhiza and leaf herbivore, I investigate changes in nutrient allocation in reaction to the trophic interaction.

The overall aim of this thesis is to use *Quercus robur* and the mycorrhizal fungus *Piloderma croceum* to quantify the interacting effects of mycorrhization and insect herbivory on plant performance. Besides the initial construction of a reference transcriptome, I performed several experiments to explore (1) the separate and joint effects of insect herbivory and mycorrhiza on patterns of gene expression and plant growth, (2) changes in patterns of carbon and nitrogen allocation within the plant and triggered by the interplay of mycorrhization and insect herbivory and its consequences for herbivore and plant performance, and (3) the separate and joint effects of mycorrhiza and Collembola on plants and herbivorous insects.

Chapter II

OakContigDF159.1, a reference library for studying differential gene expression in *Quercus robur* during controlled biotic interactions: use for quantitative transcriptomic profiling of oak roots in ectomycorrhizal symbiosis

with:

Mika T. Tarkka, Sylvie Herrmann, Tesfaye Wubet, Lasse Feldhahn, Sabine Recht, Florence Kurth, Sarah Mailänder, Markus Bönn, Maren Neef, Oguzhan Angay, Marcel Graf, Hazel Marboreke, Frank Fleischmann, Thorsten E. E. Grams, Liliane Ruess, Martin Schädler, Roland Brandl, Stefan Scheu, Silvia D. Schrey, Ivo Grosse, François Buscot

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SUMMARY

Oaks (*Quercus* spp.), which are major forest trees in the northern hemisphere, host many biotic interactions, but molecular investigation of these interactions is limited by fragmentary genome data. To date, only 75 oak expressed sequence tags (ESTs) have been characterized in ectomycorrhizal (EM) symbioses. We synthesized seven beneficial and detrimental biotic interactions between microorganisms and animals and a clone (DF159) of *Quercus robur*. Sixteen 454 and eight Illumina cDNA libraries from leaves and roots were prepared and merged to establish a reference for RNA-Seq transcriptomic analysis of oak EMs with *Piloderma croceum*. Using the Mimicking Intelligent Read Assembly (MIRA) and Trinity assembler, the OakContigDF159.1 hybrid assembly, containing 65 712 contigs with a mean length of 1003 bp, was constructed, giving broad coverage of metabolic pathways. This allowed us to identify 3018 oak contigs that were differentially expressed in EMs, with genes encoding proline-rich cell wall proteins and ethylene signalling-related transcription factors showing up-regulation while auxin and defence-related genes were down-regulated. In addition to the first report of remorin expression in EMs, the extensive coverage provided by the study permitted detection of differential regulation within large gene families (nitrogen, phosphorus and sugar transporters, aquaporins). This might indicate specific mechanisms of genome regulation in oak EMs compared with other trees.

INTRODUCTION

Oaks (*Quercus* spp.) are key trees in many of the vegetation types found in the temperate and Mediterranean biomes of the Holarctic (Iverson & Prasad, 2001). The oak genus includes species and lineages with specific adaptations to a wide range of climates and habitats (Ellenberg, 2010). For instance, the pedunculate oak, *Quercus robur* L., is widely distributed across Europe in predominantly humid areas, and prefers compact, calcareous and hydromorphic grounds (Levy *et al*., 1992).

Being long-lived and widely distributed trees, oaks harbour large communities of microorganisms and invertebrates, which interact with their host and with each other (Brändle & Brandl, 2001; Jumpponen & Jones, 2009). Most of the fine roots of oaks form ectomycorrhizas (EMs) with soil fungi, a form of mutualistic symbiosis which facilitates nutrient acquisition (Richard *et al*., 2005; Herrmann & Buscot, 2007). Oaks are also often infected by a series of parasites which are believed to be partly responsible for the decline of this tree species during recent decades (Thomas et al., 2002). For instance, the pathogen species *Phytophthora ramorum* is the causative agent of sudden oak death in North America and Europe (Grünwald *et al*., 2012), and infection by the epiphytic pathogenic fungus *Microsphaera alphitoides* leads to a decrease in the total leaf Chl content and net carbon assimilation rate (Brüggemann & Schnitzler, 2001; Hajji *et al.*, 2009). Oaks also host species-rich assemblages of herbivores and mites (Brändle & Brandl, 2001), which may decrease their growth rate and even cause mortality (Marquis & Whelan, 1994).

The genetics of the pedunculate oak and of the closely related sessile oak (*Quercus petraea*) have attracted increased attention during recent years (Barreneche *et al*., 1998; Ueno *et al*., 2010; Kremer *et al*., 2012). These two sympatric oak species have become model systems for comparative analyses of physiological differentiation and speciation in forest trees (Epron & Dreyer, 1993; Abadie *et al*., 2012). As a first step towards genomic analyses of both pedunculate and sessile oak, Ueno et al. (2010) developed a combined Q. robur and *Q. petraea* cDNA contig assembly, based on large collections of expressed sequence tags (ESTs). These collections, however, consisted mainly of leaf ESTs from the sessile oak, and included only a limited number of ESTs from oak tissues involved in biotic interactions.

Based on an experimental system using genetically identical microcuttings from pedunculate oak clone DF159 (Herrmann *et al.*, 1998), the joint experimental platform TrophinOak, 'Multitrophic Interactions with Oaks', has recently been established in order to study interactions among *Q. robur,* microorganisms and invertebrates in a soil-based culture system under controlled conditions (www.trophinoak.de). Seven representative interacting organisms are part of the platform and were used in the experiments presented in this paper (see Table 1):

– The ectomycorrhizal fungus *Piloderma croceum* J. Erikss. & Hjortst. strain 729 (DSM-4924) was selected for mycorrhizal syntheses. Mycorrhizal interaction between *Q. robur* and *P. croceum* has been intensively studied (Krüger *et al.*, 2004; Herrmann & Buscot, 2007).

– Formation of mycorrhiza is promoted by mycorrhization helper bacteria, and the strain *Streptomyces* sp. AcH 505, which promotes ectomycorrhiza formation and root branching (Maier *et al.*, 2004; Schrey *et al*., 2005), was selected.

– Leaves of oak seedlings are particularly vulnerable to powdery mildew infections (Edwards & Ayres, 1981), and *Microsphaera alphitoides* (syn. *Erysiphe alphitoides*), the causal organism of the majority of powdery mildew infections in *Q. robur*, is the representative powdery mildew species in the project.

– The involvement of *Phytophthora quercina* in the decline of oaks in Europe has been well documented in the last two decades (Jung & Blaschke, 1996; Jönsson *et al*., 2003), and this root pathogen was selected.

– Caterpillars of the phytophagous moth Lymantria dispar, which are known to feed preferentially on oaks, were selected for experimentation. Herbivory by *L. dispar* has been related to a shift in carbon

allocation towards the below-ground parts of trees (Babst *et al*., 2008).

– The nonspecific plant parasitic nematode *Pratylenchus penetrans*, which produces root lesions in broadleaved trees, was chosen as a representative root feeder (Viggars & Tarjan, 1949; Jaffee *et al*., 1982).

– Plant rhizospheres are colonized by species of the extremely widespread collembolan genus *Protaphorura* (springtails). The chosen representative, *Protaphorura armata,* lives predominately on plant resources, presumably fine roots or root hairs (Endlweber *et al*., 2009).

Currently, there is no full genome sequence available for any oak species. Therefore, one key objective of the TrophinOak project is to generate a reference transcriptome library, specific to the pedunculate oak clone DF159, which is comprehensive enough to enable RNA sequencing (RNA-Seq) analyses of all seven biotic interactions under investigation. To meet this objective, we performed a series of 454 sequencing runs on transcripts from roots and leaves of DF159 microcuttings interacting with the seven biotrophic organisms listed, and from noninfected control tissues (Table 1). Particular care was taken to obtain the most diverse possible collections of reads, and for this purpose, normalized cDNA libraries were prepared for 454 pyrosequencing from roots and leaves for each interaction type. In addition, to obtain a high amount of coverage of each transcript, sequences with a read length of 100 bp were obtained from paired-end libraries (average insert size 400 bp) of root and shoot tissues using Illumina sequencing technology. Both types of reads were combined to create a hybrid transcriptome assembly. After evaluating the coverage of this library by in silico comparisons with genome-sequenced plant species, the effect of mycorrhiza formation with *P. croceum* on the expression levels of oak genes was quantified by RNA-Seq analysis. Our objective was to gain an in-depth insight into the regulation of gene expression in EM oak roots, by greatly increasing the number of transcripts known to be differentially expressed. Previous studies on the oak clone DF159 had identified only 51 differentially expressed transcripts in premycorrhizal roots and 75 in EM, using subtractive suppressive hybridization (SSH; Krüger *et al*., 2004) and macroarrays (Frettinger *et al*., 2007), respectively.

Bruns & Shefferson (2004) have pointed out that the EM symbiosis habit was acquired independently by diverse plant lineages, and that these independent acquisitions may have relied on parallel gains of morphologies and behaviours in plants and fungi.

Whether the genetic background of these changes relies on gains and losses of genes, as has been shown for EM fungi (Plett & Martin, 2011), has not been analysed in plants, and gene diversification and changes in gene expression patterns could matter as well. On this basis, we hypothesized that with the help of a large reference library we might be able to detect that EM formation in oak leads to specific patterns of up- and down-regulation among different members of gene families, and that the plant genes induced in other EM associations may not be induced in oak Ems.

MATERIALS AND METHODS

The experimental culture system

To obtain a homogeneous soil substrate for the experiments, 3 m 3 of the upper soil were collected from an oak forest stand at the Dölauer Heide close to Halle/Saale, Saxony Anhalt, Germany (51.51016°N, 11.91291°E). The A0 (humus, –10 cm) and A1A2 (organic, –30 cm) horizons were gathered, air-dried, sieved at 5 mm, mixed 1 : 1 (v/v), separated into 500 ml aliquots, and sterilized at 50 kGy by BGS Beta-Gamma-Service (Wiehe, Germany). The soil aliquots were stored at 8°C and their sterility was tested before use by plating on LB agar.

Micropropagation and rooting of the pedunculate oak (*Q. robur* L.) clone DF159 was done according to Herrmann *et al.* (2004), reviewed in Herrmann & Buscot (2008). To ensure the maximum possible production of microcuttings, the plant hormones indole acetic acid and 6-benzylaminopurine were continuously supplied to the cultures. The root part of each microcutting was placed into square Petri dishes

Table 1 Treatments of pedunculate DF159 oak (*Quercus robur* L.) microcuttings with seven different interacting organisms. The microcuttings were grown in Petri dish soil microcosms for 6 weeks to produce the material for the contig assembly and for 8 weeks for the study of differential gene expression in ectomycorrhizas. Day 0 in the Table indicates the date on which the oak microcuttings were placed in the soil microcosms.

Sample	Treatment type
No treatment *None applied*	No inoculation
Ectomycorrhizal fungus *Piloderma croceum*	Fungal inoculum was mixed with the soil substrate once, at day 0
Mycorrhization helper bacterium *Streptomyces* sp. AcH 505	2.5 x 10^7 spores were applied to the soil twice, at 3 and 4.5 weeks
Leaf pathogen *Microsphaera alphitoides*	1.5 x 10^6 spores were applied to leaves once, at 4 weeks
Leaf herbivore *Lymantria dispar*	One caterpillar per plant was applied once, at the last day before harvest
Root pathogen *Phytophthora quercina*	1.0 x 10^6 zoospores per plant were applied to the soil once, at 5 weeks
Root feeding nematode *Pratylenchus penetrans*	≈1.0 x 10^4 nematodes per plant were applied to the soil once; at 5 weeks
Rhizosphere consumer *Protaphorura armata*	Ninety individuals per plant were applied to the soil once, at 5 weeks

(12 x 12 cm^2) filled with c-sterilized soil, which is an adaptation of the initial cultivation system described in Herrmann *et al*. (1998). Shoots were grown outside the Petri dishes. Seven interacting organisms were introduced to the culture system either at the time of establishment (mycorrhizal fungus) or later; procedures used are listed in Table 1 and detailed in Supporting Information, Methods S1, except for the interaction with *P. croceum*, which was used for quantitative RNA-Seq analysis, and is detailed later in this paper. For all interactions, the oak microcuttings were grown at 23°C with a 16 : 8 h day : night (photosynthetic photon flux density of 180 µ mol m^{-2} s^{-1}). After transfer into the Petri dish system, the plants were cultivated for 6 wk, before the tissues were harvested for RNA extraction. While the shoot tissues consisted of a mixture of leaves at different developmental stages (buds, sink and source leaves), the root tissues were exclusively lateral roots. After harvest, tissues were immediately submerged in liquid nitrogen. Material was ground in a mortar with a pestle under liquid nitrogen, divided into aliquots, and stored at –80°C.

Piloderma croceum J. Erikss. & Hjortst. Strain 729 (DSM- 4924) was cultivated in Petri dishes on Melin Norkrans Modified by Marx (1969) agar medium supplemented with 0.1% (w/v) casein hydrolysate in

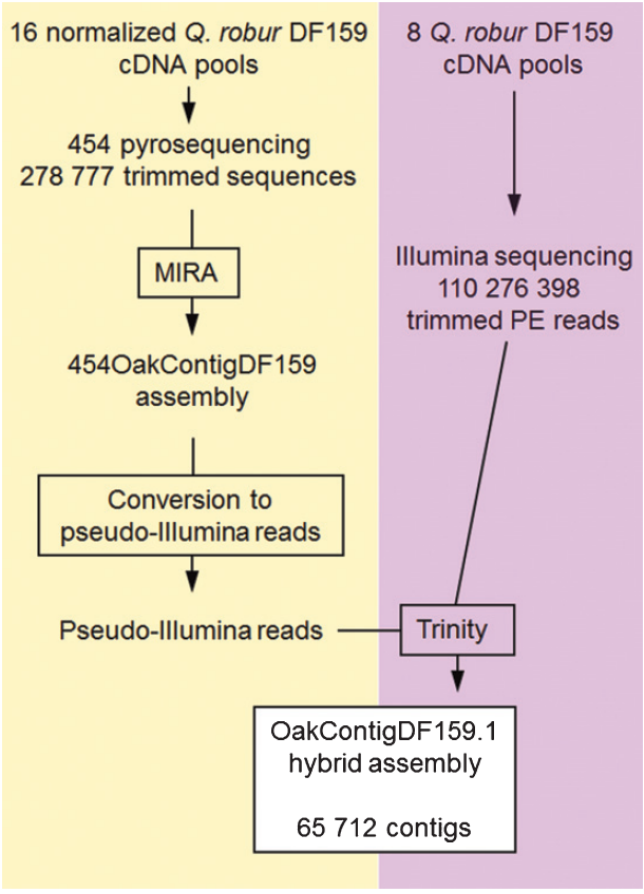

Fig. 1 Pedunculate oak DF159 (*Quercus robur*) hybrid assembly pipeline for Roche 454 and Illumina reads. 454 reads are assembled by Mimicking Intelligent Read Assembly (MIRA) and converted into overlapping 100 bp single-end reads. Single-end and 100 bp paired-end (PE) Illumina reads are assembled by Trinity.

darkness at 20°C (Herrmann *et al.*, 1998). Fungal inoculum was produced by inoculating a substrate mixture of vermiculite (675 ml), sphagnum peat (75 ml) and 300 ml Melin Norkrans modified by Marx (1969) liquid medium without carbohydrates and with 1/10 strength for P and N as described in Herrmann et al. (1998) with a 2-wk-old liquid fungal culture previously grown in 100 ml glass flasks at 20°C in the dark with shaking at 100 rpm. After 4 wk incubation at 20°C in the dark, the inoculum was used for mycorrhizal

(a)

	MIRA 454	Trinity Illumina	Trinity 454/Illumina
Contigs and single reads	73,151*	57,737	65,712
Amount of nt	36,349,699	58,854,648	65,913,455
Mean length (bp)	496	1019	1003
Number of contigs with complete CDS	20,040	29,755	33,871

*31,607 contigs and 41,544 un-assembled reads (singletons)

(b)

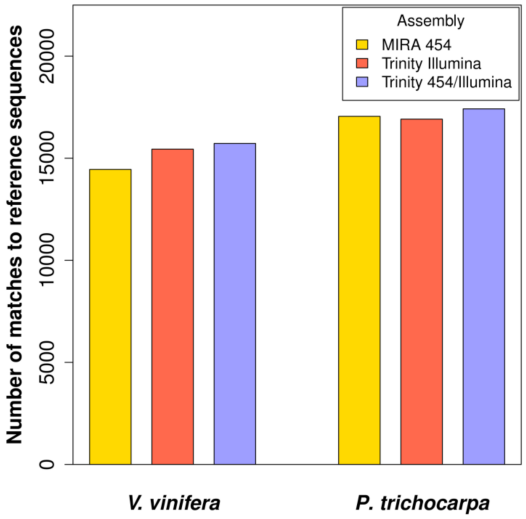

Fig. 2 Characteristics of the assemblies generated by the Mimicking Intelligent Read Assembly (MIRA) and Trinity assembly programs. (a) Basic assembly metrics. Values are shown from MIRA assembly of 454 reads, Trinity assembly of Illumina reads only, and Trinity assembly of Illumina reads and MIRA contigs, as well as unassembled single reads converted into overlapping 100 bp single-end reads. (b) Numbers of BLASTx matches of the contigs against *Vitis vinifera* and *Populus trichocarpa* RefSeq protein databases at an e-value cut-off of 1.0e–20. MIRA 454, yellow bars; Trinity Illumina, red bars; Trinity 454/Illumina, purple bars. CDS, polypeptide coding sequence.

synthesis, mixing it 1 : 1 (v/v) with the gamma-sterilized soil. The first yellow mycorrhizal root tips were visible after 5 wk of coculture. Two sets of plants were produced with *P. croceum*. One set was harvested at 6 wk at the onset of EM formation. To obtain a larger amount of EM for quantifying differential gene expression, a second set of plants was harvested 8 wk after inoculation with *P. croceum*.

RNA extractions

Based on preliminary experiments comparing the performance of different RNA extraction methods with oak roots, the MasterPure Plant RNA Purification Kit (Epicentre, Hessisch Oldendorf, Germany) was selected for RNA extractions. Fifty milligrams of leaf or 100 mg of root material were used for each extraction. The extracted RNA was treated with DNase I (Fermentas, St Leon-Rot, Germany), and RNA quantification was carried out using NanoDrop (Thermo Scientific, Passau, Germany) and a Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Darmstadt, Germany). RNA quality was checked on a Nano Chip with a Bioanalyzer 2100 (Agilent, Böblingen, Germany).

Preparation and normalisation of cDNA pools for 454 pyrosequencing and Illumina RNA-Seq

Eight leaf and eight root samples were prepared for 454 pyrosequencing, corresponding to above- and below-ground tissues of plants interacting with each of the seven organisms plus noninoculated control plants; each sample was prepared from four plants. Each of the 16 cDNA samples was prepared from 1 lg

total RNA with a SMARTer PCR cDNA Synthesis Kit and amplified with Advantage DNA Polymerase (Clontech, Saint-Germain-en- Laye, France). To reduce the prevalence of high-abundance transcripts and to equalize transcript concentrations in the cDNA samples, the SMARTer amplification products (5 lg) were subjected to TRIMMER cDNA normalization (Evrogen, Heidelberg, Germany). The normalized cDNA pools were then used to prepare 454 sequencing libraries and sequenced in-house by means of a titration run followed by two picotitre plates with eight lanes each on a Roche 454 GS-FLX Titanium platform.

One sample from the total root system and another from leaves were used to produce sequences with a read length of100 bp from paired-end libraries (average insert size 198 bp), which were sequenced using an Illumina HiSeq 2000 at the Beijing Genomics Institute, Hong Kong, China. In addition, for the transcriptome assembly as well as the transcriptomic study of EM plants, three individually selected samples of EMs and three samples of noninoculated fine roots were used to prepare sequences with a read length of 100 bp from paired-end libraries (average insert size, 400 bp), which were sequenced by Illumina HiSeq 2000 at IGA Technologies, Udine, Italy. The latter two steps resulted in eight Illumina libraries in total.

Read processing and construction of the OakContigDF159.1 hybrid assembly

The 454 reads were screened for primers and adaptors with crossmatch (P. Green, http://bozeman.mbt.washington.edu/phredphrap/phrap.html). The following steps were implemented using custom Java scripts. The 454 reads were masked, and for each read, the longest nonmasked region was extracted. Remaining primer and adaptor artefacts were also eliminated. For both 454 and Illumina reads, poly(A) tails, low complexity and low quality sequences were removed with SeqClean (http://compbio.dfci.harvard.edu/tgi/software/). Nucleotides with quality score < 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. In order to minimize the number of contaminating reads, a decontamination procedure was introduced for both the 454 and the Illumina reads, as described (Fig. S1). A hybrid assembly approach was selected to combine 454 and Illumina reads to produce an OakContigDF159.1 reference transcriptome. This process is described in Methods S1 (see the 'Construction of OakContigDF159.1 hybrid assembly' section) and illustrated in Fig. 1.

Analysis of differential expression in EMs by Illumina RNA-Seq

Illumina libraries from EMs and from fine roots were used to quantify gene expression. The Illumina reads were aligned against the OakContigDF159.1 hybrid assembly by bowtie (Langmead et al., 2009) and quantified by RSEM (Li & Dewey, 2011) and the significance of differences in gene expression was measured using the DESeq (Anders & Huber, 2010) function of the Bioconductor package (Gentleman et al., 2004) in R (R core group, http://www.r-project.org/). The tools used for transcript annotation and for metabolic pathway analyses, and the quantitative reverse transcription polymerase chain reaction (qRT-PCR) methodology, are described (Table S1).

RESULTS

Generation of a hybrid OakContigDF159.1 reference transcriptome

Root and shoot material from successfully established interactions between oak microcuttings and seven representative organisms, and from control plants, were used to generate a total of 821 534 reads from TRIMMER-normalized cDNA pools using a Roche 454 FLX instrument with Titanium chemistry (Table S2). Most 454 reads were either unique or present in low numbers in the normalized cDNA pools. The 454 reads with homology to genes known to be expressed at a low level were differentially represented in the individual 454 libraries (Fig. S2). For instance, only two cDNA pools included reads homologous to the transcriptional suppressor gene LHP1 of *Arabidopsis thaliana*.

Additional Illumina RNA-Seq of eight cDNA pools, four from roots, three from EMs and one from leaves, allowed a greater depth of sequencing for the pedunculate oak clone DF159 transcriptome assembly. Depending on the sample, the libraries yielded 21–62 mio 100 bp paired-end reads with a Q20 percentage (percentage of sequences with predicted sequencing error rate lower than 1%) of over 93% (Table S3).

Contaminating reads originating from oak-interacting organisms were, as far as possible, eliminated from all sequence libraries by BLASTx searching against reference datasets (see Methods S1 and Fig. S1 for details). A pedunculate oak DF159 reference transcriptome was produced from the decontaminated reads using a combination of overlap layout consensus (OLC) and short read assemblers (Fig. 1). In the first step, the Mimicking Intelligent Read Assembly (MIRA) OLC assembler was

implemented to generate contigs from 454 reads. MIRA contigs and singletons (reads which were not incorporated into MIRA contigs) were then converted into overlapping 100 bp single-end reads and assembled with the Illumina reads using the Trinity short read assembler. The 454/Illumina hybrid assembly generated more contigs, which encoded a larger number of predicted full-length polypeptide coding sequences than the 454 or Illumina read assemblies alone (Fig. 2a). BLASTx searches against *Vitis vinifera* and *Populus trichocarpa* protein indices showed that the numbers of matches to the reference sequences were highest for 454/Illumina hybrid assembly contigs at e-values > 1e 50 and highest for sequences in Illumina-only assemblies at e-values < 1e–50 (Fig. S3). Comparable numbers of matches in the two Trinity assemblies occurred at 1e–50 (Fig. 2b). Crosscomparison of the MIRA and Trinity assemblies by BLASTn with the threshold 1e–50 showed that 71 305 of 73 161 (97%) MIRA contigs and single reads are homologous to Trinity 454/ Illumina contigs, and 69 057 (94%) are homologous to Trinity Illumina contigs. On the basis of the slightly higher number of matches to reference sequences, the 454/Illumina hybrid assembly was selected as being the most comprehensive.

The OakContigDF159.1 reference transcriptome comprises 65 712 contigs with a mean length of 1003 bp, totalling 65 913 455 bp. Contig lengths in this transcriptome range from 200 to 15 438 bp. More than 57% of the contigs have a length of over 500 bp and > 36% are over 1000 bp. As expected, the Trinity contigs of the OakContigDF159.1 reference transcriptome show the highest degree of homology with sequences from higher plants (Fig. S4). The contigs were classified using the Gene Ontology (GO) terminology with Blast2GO and a range of diverse functions could be assigned to them (Fig. S5; Table S4). On the basis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) global metabolic pathway annotation, the distributions of metabolic pathway-related accessions in the OakContigDF159.1 assembly and in the *A. thaliana* proteome were highly comparable (Fig. S6). The results of these analyses demonstrated that the OakContigDF159.1 assembly is comprehensive and adequate for the analysis of oak gene expression at the transcriptome level.

Differential oak gene expression induced by mycorrhiza formation

In total, 3018 contigs of the OakContigDF159.1 reference transcriptome were differentially expressed, of which 1399 were up-regulated and 1619 down-regulated

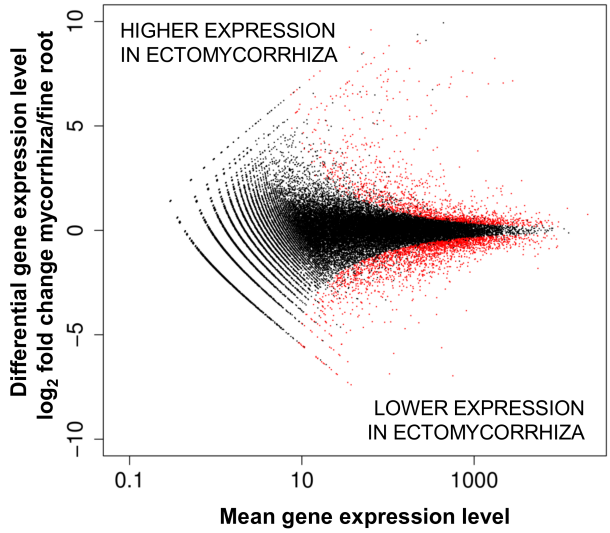

Fig. 3 RNA-Seq based comparison of gene expression levels in fine roots and ectomycorrhizas (EMs) of pedunculate oak DF159 (Quercus robur) with Piloderma croceum. Raw read counts were generated by quantification using RSEM, and differentially expressed contigs were detected by DESeq. Red dots mark contigs detected as being significantly differentially expressed at a 10% false discovery rate with Benjamini–Hochberg multiple testing adjustments ($P < 0.01$). In EMs, 3018 contigs were differentially expressed, of which 1399 were up-regulated and 1619 were downregulated.

in oak EMs with *P. croceum* (Fig. 3). Differential expression levels of 14 contigs were confirmed by qRT-PCR analysis (Fig. 4). On the one hand, GO enrichment analysis using DAVID detected significantly enriched GO terms containing the words ribosome, vacuole, response to stimulus, generation of precursor metabolites and energy, starch metabolic process and transporter activity among genes up-regulated in EMs, and enriched KEGG terms included ribosome and spliceosome (Table S5). On the other hand, GO terms that were depleted in EMs included root growth, cytoskeleton, auxin-mediated signalling pathway and auxin polar transport, laccase activity and phenylpropanoid metabolism (Table S5).

Highly significant up-regulation of gene expression was observed for contigs encoding, for example, galactinol synthase, inositol transporter, and remorin (Table 2). Other up-regulated contigs encoded sucrose and SWEET1 sugar transporters. The RNA-Seq analysis also revealed a general up-regulation of contig family members. For instance, seven predicted ethylene response transcription factors, eight predicted proline-rich proteins, and six predicted that aquaporin

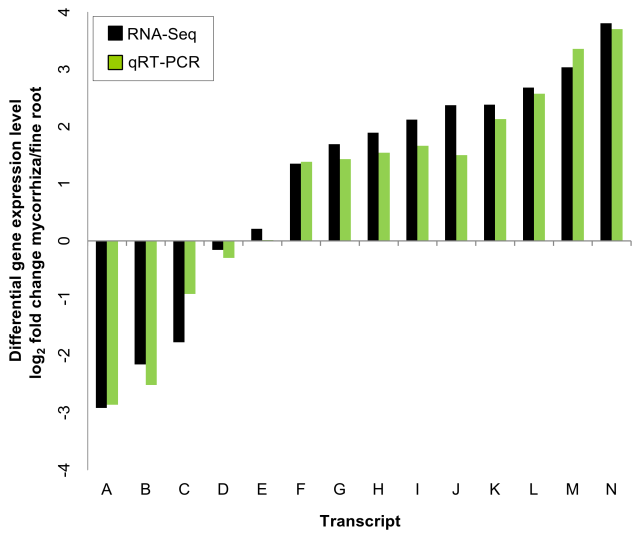

Fig. 4 Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) confirmation of 14 differentially expressed genes in ectomycorrhizas (EMs) synthesized between oak microcuttings DF159 and *Piloderma croceum* in comparison to noninfected lateral fine roots. RNASeq results (black bars) represent means of three biological replicates. qRTPCR results (green bars) represent means of three biological and two technical replicates, normalized with respect to an 18S rRNA gene. The coefficient of variation was < 6.0 for all qRT-PCR reactions. The transcripts analysed were predicted to encode the following proteins by BLASTx searches against the nr database at an e-value cut-off of 1.0e–20: A, extensin; B, sieve element occlusion protein; C, plasma membrane H+-ATPase; D, endo-1,4-beta-glucanase; E, endomembrane transport protein; F, 1 aminocyclopropane-1-carboxylate oxidase; G, glucose-1- phosphate adenylyltransferase; H, nucleoredoxin; I late embryogenesis abundant protein 5; J, proline-rich protein PRP1; K, inositol transporter; L, calcium-binding protein; M, aspartic proteinase; N, galactinol synthase.

contigs had higher expression levels in EMs (Table S6). The expression levels of contigs associated with the starch metabolic pathway also increased in EMs (Fig. S7).

Contigs encoding pumilio RNA binding protein and sieve element occlusion protein were the two most strongly downregulated in EMs (Table 3). In agreement with the results of GO enrichment analysis, nine auxin-related contigs were downregulated in EMs (Table S6). Cell wall protein, ammonium and phosphate transporter contig families included contigs that were both up- and down-regulated in EMs (Table S6). Overall, the high resolution of RNA-Seq enabled the identification of numerous EM-regulated genes and the visualization of coregulated contig families.

DISCUSSION

In this study, RNA-Seq enabled the generation of the first specific reference transcriptome for the pedunculate oak clone DF159 under a range of biotic interactions; the study of global transcriptional responses in *P. croceum* ectomycorrhizal roots despite the lack of reference genome sequence or array platform.

Hybrid assembly of 454 and Illumina reads to produce a reference transcriptome

Mimicking Intelligent Read Assembly (MIRA) was chosen for the preassembly of 454 reads from cDNA of leaves and roots of pedunculate oaks involved in seven types of interactions plus a noninfected control, since it proved to be the most robust of the assemblers tested. By contrast, the Illumina reads generated from EMs and noninfected roots and leaves were assembled well by Trinity. Numerous studies suggest that hybrid 454/Illumina assembly is superior in quality to assemblies from 454 or Illumina reads alone (Blythe *et al.*, 2010; Sandmann *et al*., 2011; Hornett & Wheat, 2012). Following this advice, we constructed a hybrid assembly pipeline for the pedunculate oak reads. The hybrid assembly approach generated more contigs than the Illumina- only assembly, and included sequence information from the majority of MIRA contigs and singletons. Furthermore, the number of unique contigs was noticeably larger in the hybrid assembly than in the Illumina-only assembly. High representation of global KEGG biochemical pathways among the contigs indicates that the OakContigDF159.1 reference transcriptome provides extensive coverage, even though it does not cover the whole-genome sequence.

Differential gene expression in pedunculate oak EMs

Developmental reprogramming has been observed previously in both roots and fungal hyphae upon formation of EMs (Johansson *et al*., 2004; Duplessis *et*

Table 2 The 20 most significantly up-regulated contigs in ectomycorrhizas synthesized between oak DF159 (*Quercus robur*) microcuttings and *Piloderma croceum*

GENES UP-REGULATED IN MYCORRHIZA

Contig no.	Raw read counts in Mycorrhiza RNA-Seq	Raw read counts in Fine Roots RNA-Seq	*P*-value RNA-Seq	Myc/FR (log2 fold change) RNA-Seq	Myc/FR (log2 fold change) qRT-PCR	Predicted function	Alignment e-value, organism giving the best blastx match
43090_0_2	2058.39	147.45	4.60e-182	3.80	3.70***	Galactinol synthase	1e-93, *Populus trichocarpa*
36915_0_2	5973.13	1146.97	4.71e-150	2.38	2.13***	Inositol transporter 1	3e-114, *Glycine max*
35872_0_1	4171.80	1229.97	1.01e-149	1.76		No match	
42280_0_1	3225.65	884.76	1.98e-147	1.86		Hypothetical protein	0, *Populus trichocarpa*
29157_0_2	639.69	13.27	4.76e-139	5.59		Protein phosphatase 2c	1e-65, *Populus trichocarpa*
36374_0_1	3219.85	992.25	6.17e-124	1.69	1.43	Glucose-1-phosphate adenylyl-transferase	0, *Populus trichocarpa*
29927_0_1	1204.42	147.21	7.95e-123	3.03	3.35***	Aspartyl protease	6e-174, *Ricinus communis*
550515_0_1	1736.25	11.49	2.50e-108	8.23		No match	
38461_0_5	534.23	18.19	5.85e-106	4.87		Pantothenate kinase 2	0, *Vitis vinifera*
43090_0_1	701.43	44.69	4.27e-101	3.97		Galactinol synthase	4e-157, *Populus trichocarpa*
36915_0_1	2941.45	725.17	4.06e-96	2.02		Inositol transporter 1	0, *Glycine max*
28563_0_1	2521.02	802.43	2.31e-94	1.65		Remorin	3e-76, *Jatropha curcas*
40696_0_2	2352.19	575.33	2.26e-91	1.09		Expansin b1	4e-103, *Ricinus communis*
36836_0_1	422.87	9.68	4.78e-88	5.44		Farnesylated protein	1e-51, *Vitis vinifera*
21193_0_1	2043.99	817.43	9.54e-83	2.00		Lipid binding protein	1e-23, *Ricinus communis*
42096_3_1	730.62	80.61	2.27e-81	3.17		Hypothetical protein	1e-71, *Populus trichocarpa*
32514_0_1	1503.68	402.99	2.47e-75	1.89		Nucleoredoxin 2	2e-174, *Vitis vinifera*
42599_0_1	7358.51	4082.83	1.31e-70	0.84		Granule-bound starch synthase	0, *Prunus persica*
33802_0_1	3493.05	801.10	5.66e-62	2.12	1.66*	Late embryogenesis abundant protein	1e-20, *Citrus sinensis*
33859_0_1	3548.20	1705.6	1.40e-59	1.05		Formate dehydrogenase	0, *Quercus robur*

The contigs most significantly up-regulated according to the test statistic implemented in DESeq are listed. The RNA-Seq-based gene expression levels in mycorrhiza (Myc) and in fine roots (FR) are means of three biological replicates. The mean number of reads that map to the respective contigs is given. P values represent the probability of no difference between treatments with Benjamini–Hochberg multiple testing adjustment. Putative gene functions were predicted by BLASTx searching against the nr database. The expected value of the sequence with the best BLASTx hit and its source organism are given in each case. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) results represent means of three biological and two technical replicates, normalized with respect to an 18S rRNA gene. The coefficient of variation was < 6.0 for all qRT-PCR reactions. Asterisks indicate significant differences according to a randomization test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

al., 2005; Martin *et al.*, 2007). However, the authors of these papers noted a much greater magnitude of change in gene expression in the mycelium (up to 20% of the analysed transcripts) than in the root cells (2% of the transcripts). Our RNA-Seq analysis of plant gene expression in mature pedunculate oak EMs found a > twofold change (4.6% of the plant contigs were differentially expressed in EMs at a significance level of $P < 0.01$). In total we found 3018 differentially expressed plant genes in oak EMs, which increases

Table 3 The 20 most significantly down-regulated contigs in ectomycorrhizas synthesized between oak DF159 (*Quercus robur*) microcuttings and *Piloderma croceum*

GENES DOWN-REGULATED IN MYCORRHIZA

Contig no.	Raw read counts in Mycorrhiza RNA-Seq	Raw read counts in Fine Roots RNA-Seq	*P*-value RNA-Seq	Myc/FR (log2 fold change) RNA-Seq	Myc/FR (log2 fold change) qRT-PCR	Predicted function	Alignment e-value, organism with the best blastx match
42518_1_2	8.99	1123.58	5.77e-237	-6.96		Pumilio RNA binding protein	0, *Vitis vinifera*
40371_0_1	2289.14	5126.08	4.36e-95	-1.16		Sieve element-occlusion protein	0, *Malus* x domestica
42634_0_1	3033.65	7169.74	1.13e-96	-1.24	-0.66	MDR type ABC transporter	0, *Vitis vinifera*
43602_1_1	5226.82	9598.42	4.15e-90	-0.87		Beta-glucosidase 24	5e-180, *Sorghum bicolor*
32110_0_1	6552.97	11553.25	1.25e-87	-0.81		Sucrose synthase	0, *Manihot esculenta*
39154_0_1	6915.03	11639.13	9.58e-77	-0.75		Ent-kaurenoic acid oxidase	4e-135, *Medicago truncatula*
43332_0_2	5906.27	7538.81	8.46e-68	-0.81		Cytochrome p450	3e-159, *Populus trichocarpa*
43934_0_1	2585.79	4894.94	9.36e-61	-0.92		U-box domain protein 20	2e-162, *Populus trichocarpa*
42460_1_2	392.97	1290.31	1.76e-54	-1.71		Metal transporter	1e-156, *Vitis vinifera*
23289_0_1	653.02	1731.4	2.13e-51	-1.40		Serine threonine protein kinase	0, *Populus trichocarpa*
35773_0_2	318.11	1063.76	2.34e-46	-1.74		Trehalose phosphate synthase	0, *Vitis vinifera*
44296_0_1	563.66	1469.16	2.28e-43	-1.38		Hypothetical protein	3e-164, *Populus trichocarpa*
36775_0_1	287.34	982.41	1.20e-41	-1.77	-0.93	Plasma membrane H+ ATPase	0, *Cucumis sativus*
43667_2_1	201.98	788.65	1.30e-41	-1.96		Hypothetical protein	0, *Populus trichocarpa*
42185_1_1	1885.13	3395.16	1.63e-39	-0.84		ATP binding protein	0, *Ricinus communis*
42363_1_1	4379.53	6831.20	1.65e-39	-0.64		Phenylalanine ammonia-lyase	0, *Quercus suber*
37455_0_1	33.92	348.41	2.48e-39	-3.36		Hypothetical protein	2e-95, *Ricinus communis*
38461_0_1	339.44	1059.08	1.10e-38	-1.64		Pantothenate kinase 2-like	0, *Ricinus communis*
40709_0_2	56.13	489.57	7.93e-37	-3.12		Translation initiation factor eif-4f	1e-152, *Carica papaya*
41450_0_1	1575.09	2892.91	2.43e-36	-0.87		Cytochrome P450	0, *Ricinus communis*

The contigs most significantly down-regulated according to the test statistic implemented in DESeq are listed. The RNA-Seq-based gene expression levels in mycorrhiza (Myc) and in fine roots (FR) are means of three biological replicates. The mean number of reads which map to the respective contigs is given. P values represent the probability of no difference between treatments with Benjamini–Hochberg multiple testing adjustment. Putative gene functions were predicted by BLASTx searching against the nr database. The expected value of the sequence with the best BLASTx hit and its source organism are given in each case. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) results represent means of three biological and two technical replicates, normalized with respect to an 18S rRNA gene. The coefficient of variation was < 6.0 for all RT-qPCR reactions. Asterisks indicate significant differences according to a randomization test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the number identified by previous SSH (Krüger *et al*., 2004) or macroarray (Frettinger *et al.*, 2007) approaches applied to the same experimental system by a factor of 40. In addition, the quantification was confirmed by qRT-PCR analyses for selected genes. This indicates that the strategy adopted in the present study provided comprehensive and accurate coverage of gene expression changes.

Previous analyses of *Eucalyptus–Pisolithus*, *Betula–Paxillus* and *Quercus–Piloderma* symbioses did not indicate expression of Emspecific plant genes, but showed rather subtle changes in the level of gene

expression (Voiblet *et al*., 2001; Johansson *et al.*, 2004; Duplessis *et al*., 2005; Le Quere *et al.*, 2005; Frettinger *et al*., 2007). This suggests that the development and metabolism of plant EM tissues are driven by differential regulation of transcriptional regulators, signal transduction, and metabolic pathways, rather than by expression of symbiosis-specific genes (Duplessis *et al.*, 2005; Martin *et al.,* 2007). Our data confirm these findings.

Down-regulation of plant defence-related genes

Early plant response to mycorrhizal fungi involves nonspecific, broad-spectrum defences, including increased chitinase and peroxidase activities during hyphal penetration into the apoplastic space of the root cortex. However, this pattern of overexpression is only transient and it is attenuated in mature EMs (Sauter & Hager, 1989; Albrecht *et al.*, 1994; Münzenberger *et al.*, 1997). In agreement with these observations, we found chitinase and laccase contig families, as well as phenylalanine ammonia lyase contigs, to be down regulated in oak mycorrhizal roots (Table S6), and GO enrichment analysis identified genes related to phenylpropanoid metabolism as being depleted (Table S5). This confirms that plant defences were attenuated in the mature oak EMs examined here, while roots of oak clone DF159 at the premycorrhizal stage of association with *P. croceum* (Frettinger *et al*., 2006) overexpressed one class III chitinase. The down regulation of chitinase that we found in the mature EM confirms the transitory nature of induction of defence related genes during EM formation on oak.

Plants experiencing abiotic environmental stresses produce elevated concentrations of the phytohormone ABA and generate stress resistance responses through ABA signal transduction. As EM formation attenuates plant stress (Smith & Read, 2008), down-regulation of ABA-induced genes is to be expected in Ems. In accordance with this hypothesis, we detected the down regulation of two contigs encoding putative ABA receptors in mature oak EMs, confirming the previous analysis of Voiblet *et al*. (2001), who were the first to show the down-regulation of a gene encoding an ABA induced protein in EMs of eucalyptus.

Enhanced expression of ethylene-related contigs

In the *Quercus–Piloderma* symbiosis, we detected enhanced ethylene signalling (Table S6), which has not been previously reported (Voiblet *et al.*, 2001; Johansson *et al*., 2004; Duplessis *et al.*, 2005; Frettinger *et al.*, 2007). The gaseous phytohormone ethylene inhibits root elongation and regulates transcription of numerous cell wall-related genes

(Sanchez-Rodriguez *et al*., 2010). When *Arabidopsis* roots engage in symbiosis with the generalist endophytic fungus *Piriformospora indica*, ethylene biosynthesis is induced. Moreover, if ethylene signalling is impaired in *Arabidopsis*, this results in reduced root colonization by the fungus. This suggests that the hormone has a role in symbiotic root colonization by *P. indica* (Khatabi *et al.*, 2012). EM fungi produce ethylene in pure culture, and ethylene production is enhanced in symbiosis with tree roots (Graham & Linderman, 1980). Moreover, a role for ethylene in the dichotomous branching of the short root tips in pines has been established (Kaska *et al*., 1999). The up-regulation of the ethylene-related transcription factor family in oak EMs indicates that the ethylene signalling may play a role in suppressing root elongation and regulating the morphogenetic program of the symbiotic roots.

Differential expression of auxin-related contigs

Previous research has shown that auxin signalling is central to the regulation of EM root development (Tagu *et al.*, 2002). Herrmann *et al*. (2004) showed that addition of IAA to the *Quercus–Piloderma* culture system stimulates EM formation. In the *Hebeloma cylindrosporum–Pinus pinaster symbiosis*, an auxin overproducing mutant strain of *H. cylindrosporum* developed EMs with a thicker fungal mantle and multilayered Hartig net (Gea *et al.*, 1994), suggesting that this phytohormone controls EM morphogenesis. Felten *et al*. (2009) observed that before EM development, exudates of *Laccaria bicolor* stimulate lateral root formation in poplar, concomitantly with an up-regulation of multiple auxin-related genes, for example, components of polar auxin transport and auxin signalling. The present study revealed that, in the mature oak EM, auxin signalling genes are differentially expressed (Table S6) and that the expression levels of most putative auxin transporters, and, in particular, of many contigs encoding transcription factors, decreases. As many auxin-related genes are down-regulated in mature oak EMs, this indicates that auxin signalling is central to the early mycorrhizal phase, and less important in regulating processes in the mature symbiotic roots.

Overexpressed remorin contig

One of the most significantly up-regulated contigs showed homology to remorins, and, to our knowledge, this is the first report of EM-induced remorin expression. Remorins act as scaffolding proteins in signalling complexes, and they have crucial functions in plant–microbe interactions. For instance, a remorin protein interacts with symbiotic receptors and regulates

bacterial infection in legume root nodule symbiosis (Lefebvre *et al*., 2010), and induction of a remorin gene takes place upon the establishment of arbuscular mycorrhizal symbiosis (Kistner *et al*., 2005). In the present study, only one of the 15 remorin contigs contained in our OakContigDF159.1 library was up-regulated in EMs, suggesting that this member of the family may play a specific role in the oak–*Piloderma* interaction.

Specific up-regulation of proline-rich protein contigs

In EMs of broadleaved trees such as pedunculate oaks, fungal colonization induces dramatic changes in root epidermal cells, which are stimulated to enlarge radially and to loosen their cell wall structure (Peterson & Farquhar, 1994). Our results suggest that a network of plant cell wall proteins, particularly prolinerich proteins (Table S6), participates in the remodelling of cell walls of symbiotic roots. The proline-rich protein (PRP) and extensin subfamilies belong to the ubiquitous plant protein family commonly known as hydroxyproline-rich glycoproteins (Newman & Cooper, 2011). The PRPs have been related to plant development, biotic interactions and environmental stresses (van de Wiel *et al.*, 1990; Newman & Cooper, 2011). Previous analysis performed on the oak clone DF159 detected one PRP transcript which was up-regulated in both premycorrhizal roots and mature EMs (Frettinger *et al.*, 2007). Using the oak contig assembly, the expression pattern of the PRP family in EM oak roots was shown to be tightly regulated, confirming the crucial role played by these proteins in determining the extracellular matrix of EM root cells. Extensins are joined to each other and to cell wall components by cell wall peroxidases (Schnabelrauch *et al.*, 1996), increasing the tensile strength of the primary cell wall (Lamport *et al.*, 2011). Two extensin contigs and several peroxidase-encoding contigs were downregulated in EMs, indicating that there is reduced potential for cross-linking of cell wall components in EM roots. This hypothesis was supported by the up-regulation of an expansin-encoding contig, since expansins have the capacity to induce extensibility and stress relaxation in plant cell walls (Sanchez-Rodriguez *et al.*, 2010). Cell wall extensibility is further modulated by the xyloglucan endotransglucosylase/ hydrolases (XTH) (Sanchez-Rodriguez *et al.*, 2010), but most of the XTH contigs were down-regulated in the oak EMs (Table S6). Overall, the striking and specific up-regulation of the PRP contig family in oak indicates the importance of these proteins in mycorrhiza related cell wall reorganization.

Expression of genes associated with metabolic pathways

Oak GO terms related to metabolic pathways were altered upon EM formation. The oak data corroborate those from previous analyses of aspen EM (Larsen *et al.*, 2011), as, in both cases, enrichment for GO terms related to starch metabolism and transporter activity was detected. These changes are central to the physiology of EM tissue, as it acts as a strong carbon sink and is the site of intensive sugar and nutrient transport (Nehls, 2008). In the poplar-fly agaric symbiosis, the host plant supplies the fungal partner with hexoses by converting apoplastic sucrose to glucose and fructose by means of plant invertase (Nehls, 2008). In the present study, up-regulation of a sucrose transporter contig was observed, but the invertase encoding contig family was constitutively expressed. Whereas enhanced expression of three monosaccharide transporter genes takes place in poplar Ems (Nehls, 2008), from the oak transcriptome, none of the 12 contigs similar to the poplar monosaccharide transporter genes was up-regulated. This confirms our second hypothesis, that some of the EM-related genes of other systems are not affected in oak. More recently, plant SWEET genes have been shown to be implicated in sugar efflux targeted to plant pathogens and symbionts, and the SWEET1 protein of *Arabidopsis* expresses glucose transporter activity (Chen *et al*., 2010). In the present oak EM study, one putative bidirectional glucose transporter of the SWEET1 family was shown to be up-regulated. Although transporter activity has yet to be confirmed for the predicted oak SWEET1 protein, the result could indicate direct export of hexose into the plant apoplast to support the fungus and may suggest the existence of a complementary sugar exchange mechanism in oak Ems.

External EM fungal hyphae transport nutrients, particularly ammonium and phosphorus, to plant roots (Selle *et al*., 2005; Loth-Pereda *et al*., 2011). EM formation with Amanita muscaria results in up regulation of three poplar ammonium transporter (AMT) genes (Selle *et al*., 2005). In oak EMs, one AMT contig was up-regulated and three were down-regulated, indicating a lower induction of plant AMT expression in oak EM than in poplar EM. This result is in accordance with our first hypothesis, which postulates that EM formation in oak leads to specific patterns of up- and down-regulation among the different members of gene families. In line with our first hypothesis, we also observed the up-regulation of one, and down-regulation of two, pedunculate oak phosphate transporter family 1 genes. In poplar, two phosphate transporters of the same family were up-regulated and two were down-regulated

in EMs (Loth-Pereda *et al*., 2011), suggesting that specific EM-related phosphate transporting proteins exist in both systems.

Marjanovic *et al*. (2005) reported that four poplar aquaporin genes encoding members of the plasma membrane intrinsic protein family were up-regulated in the poplar-fly agaric symbiosis, and here, six oak aquaporin contigs of the same family were found to be up-regulated. These proteins are potentially involved in cell turgor regulation in EM tissues.

Philippe *et al*. (2010) observed the induction of poplar galactinol and raffinose synthase contigs and increased raffinose concentrations as a systemic response to herbivory, and suggested that raffinose might be involved in plant responses to biotic interactions. In oak EMs, genes of the raffinose pathway were up-regulated, and a galactinol synthase contig was one of those that were most significantly overexpressed in EMs. However, induction of raffinose during EM formation has not yet been confirmed by metabolite analysis.

Taken together, our data support and confirm the view that instead of a general reprogramming of metabolic networks or transporter families, gene families are precisely regulated to adjust the plant metabolism to mycorrhizal symbiosis. The greatly increased capacity offered by our reference transcriptome for identification of differential gene expression in oak EMs enabled us not only to identify single genes but also to analyse regulation within whole gene families. This degree of precision enabled us to reveal several traits important for the function of EM symbiosis in oaks (regulation of ethylene or remorin encoding genes), which had not been detected in other host plants investigated to date, such as poplar, eucalypt or birch. In the oak model system, different up- and down-regulation patterns were found in genes and gene families already observed to be involved in EM symbiosis on other host plant models (invertase, transporters of monosaccharides, ammonium and phosphorus, and aquaporins). Confirmation that these traits are really oak-specific, however, requires analysing at a similar depth the gene expression of these host plants when inoculated with *P. croceum*. In addition, elucidation of the pedunculate oak whole-genome sequence and supporting functional analysis will further facilitate comparisons between host responses in different EM systems.

CONCLUSIONS

Deep next-generation sequencing was successfully implemented to generate a more complete oak transcriptome. The reference transcriptome of the

pedunculate oak clone DF159 thus produced is a valuable addition to previously existing oak genomic resources, including the sessile and pedunculate oak contig transcriptome assembly (Ueno *et al.*, 2010). It is also supporting an ongoing pedunculate oak genome sequencing project (Faivre-Rampant *et al*., 2011; Kremer *et al*., 2012), as the reference transcriptome will help in achieving a better understanding of interactions between host and associated organisms, allow development of new reagents sets for 'omic approaches, and assist the experimental annotation of the pedunculate oak genome. Of immediate significance is the ability to use the assembly for RNA-Seq analyses to look at global changes in oak gene expression. Here we have shown the power of this strategy by identifying an extensive transcriptional program associated with EMs on oak roots. In the context of the TrophinOak project, we will use this resource to analyse the responses of the oak clone DF159 to a wide range of beneficial and detrimental interacting organisms, in relation to plant development and under variable environmental conditions.

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Chapter III

Ectomycorrhizal fungus changes defense strategies against leaf herbivory − shedding fight on multitrophic interactions in oak

with:

Mika T. Tarkka, Markus Bönn, Roland Brandl, François Buscot, Lasse Feldhahn, Thorsten E. E. Grams, Sylvie Herrmann, Martin Schädler

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SUMMARY

Mycorrhizal fungi and herbivores interactively influence the growth, resource utilization, and herbivore defense of plants. The largely unexplored genetic and physiological basis of these interactions may differ in the different plant growth phases, thereby contributing to the complexity of plant responses. We studied these interactions in a tri-trophic system comprising oak (*Quercus robur* clone DF159), larvae of the moth *Lymantria dispar,* and the ectomycorrhizal fungus *Piloderma croceum* under controlled laboratory conditions at the level of gene expression using transcriptomics and at the level of carbon/nitrogen allocation for plants from different growth stages. Herbivore feeding on oak leaves led to increased expression of genes related to compensatory growth and to enriched GO terms described with cell wall, cell division and DNA replication and other direct defense mechanisms of oak, like an enhanced expression of e.g. chitinases in root flush and protease inhibitors in shoot flush. C/N-allocation analyses indicated an increased export of resources from aboveground plant parts and accordingly genes associated with the transport of sugars were increased upon herbivore attack. Inoculation with an ectomycorrhizal fungus attenuated these effects in the pre-mycorrhizal stage but caused an increased expression of genes related to the production of volatile organic compounds. We conclude that the inoculation with ectomycorrhizal fungi mediates the plant's defense strategy and that this effect is moreover dependent on the growth stage of plants. These results from a rather simple tri-trophic lab system give insights into the complexity of plant responses to a multi-trophic world.

INTRODUCTION

Plants growing in natural environments interact with an intriguing diversity of organisms. These biotic interactions, both above- and below-ground, have important consequences for the performance of individual plants, dynamics of plant populations, and composition and structure of plant communities (herbivores: Crawley, 1996; mycorrhizal fungi: Heijden & Sanders, 2002; Smith & Read, 2010). Amongst the most common interactions of plants are those with mycorrhizal fungi and herbivorous insects. Under natural conditions, both types of interaction simultaneously affect the physiology and growth of the host plant. The underlying genetic and physiological pathways induced by and involved in the above- and below-ground interactions seem to overlap (Schenk *et al*., 2008). However, the simultaneous and concerted influence of these interactions on plant performance is still poorly understood.

Most research on interactions between plants, herbivores, and root symbionts has concentrated on herbaceous plants and arbuscular mycorrhizal fungi. Trees have been mostly neglected in such studies, even though they generally harbor a high density and diversity of insect herbivores (Brändle & Brandl, 2001), and the majority of trees in boreal and temperate regions (>95%) are associated with ectomycorrhizal fungi (Sanders, 1997; Baar *et al*., 1999; Kõljalg *et al*., 2000; Taylor, 2002), which are key elements of forest nutrient cycles and therefore important drivers of processes and services of forest ecosystems (Read *et al*., 2004).

Insect herbivores and mycorrhizal fungi have

indirect plant-mediated effects on each other. Mycorrhizal fungi provide nutrients to plants, thereby increasing host plant vigor and nutritional quality, which in turn can increase the performance of insect herbivores (Gehring & Whitham, 1994; Borowicz, 1997; Kempel *et al*., 2010). This resource supply may also allow plants to invest more into resistance against generalist insect herbivores (Jones & Last, 1991b; Gange & West, 1994; Halldórsson *et al*., 2000; Bi *et al*., 2007; Yao *et al*., 2007; Kempel *et al*., 2010). Several authors, however, have observed that this protective effect cannot attributed to improved nutritional status alone (Fritz *et al*., 2006).

Furthermore, recent research on arbuscular mycorrhizhal fungi has brought evidence of enhanced resistance against shoot pathogens induced by these fungi (Pozo & Azcón-Aguilar, 2007; Koricheva *et al*., 2009; Campos-Soriano *et al*., 2012). In herbaceous plants associated with mycorrhizal fungi, qualitative and quantitative changes in flavonoid contents have been observed (Vierheilig & Piche, 2002; Akiyama *et al*., 2002). Further changes in phenolic compounds, defense-related phytohormones have also been reported, indicating an induction of defense against pathogens and herbivores (Fester & Hause, 2005; Lopez-Raez *et al*., 2010). Such interactions between fungi and herbivores are based on the differential expression of genes in the host plant, with consequences for resistance, performance, and co-evolution of plants and fungi as well as plants and insects (Gange *et al*., 2002).

First analyses of gene expression have demonstrated that herbivores can influence the transcription of hundreds of plant genes (Hermsmeier *et al*., 2001; Baldwin *et al*., *2001*; Roda & Baldwin, 2003; Schmidt *et al*., 2005). Herbivory can even lead to complete changes in metabolic reorganization (Hui *et al*., 2003) and can influence the expression of genes involved in regulation of photosynthesis and pathogen resistance (Hermsmeier *et al.*, 2001). In poplar trees, herbivory leads to an increase in the expression of genes regulating the production of proteinase inhibitors, which act against insect herbivores (Hui *et al.*, 2003); oxidative enzymes that act as “anti-nutrients” (Major & Constabel, 2006); and many enzymes that are involved in the synthesis of secondary metabolites (Babst *et al*., 2009); and when poplar trees are stressed or wounded, chitinase-related genes are often expressed (reviewed in Christopher *et al*., 2014). Also interactions between plants and mycorrhizal fungi lead to changes in gene expression in the host plant (Wiemken & Boller, 2002). Some of these genes are relevant for resource allocation and for reactions to stress and defense (Herrmann *et al*.,

1998; Frettinger *et al*., 2006a). This stimulation of plant resistance is not only due to the well-known effects of mycorrhiza on plant nutrition, but also to changes in signal pathways that trigger direct and indirect defense systems (Arimura *et al*., 2000; Bi *et al*., 2007; Yao *et al*., 2007; Pozo & Azcón-Aguilar, 2007), yet can even effects pre-mycorrhizal stages of symbiosis be similar to the effects of mature mycorrhiza formations (Volpin *et al*., 1994; Herrmann *et al*., 1998, 2004; Frettinger *et al*., 2006a). Furthermore, plant defense against pathogens and herbivores seems to be regulated by a network of interconnecting pathways (Ton *et al*., 2002; Glazebrook, 2005; Koornneef & Pieterse, 2008; Schenk *et al*., 2008).

Plant growth is usually characterized by different growth phases that differ in resource allocation within the plants. Temporal growth patterns may interfere with plant responses to associated organisms and might contribute to the complexity of observed effects. *Quercus robur* is characterized by an endogenous rhythmic growth; in saplings and adult trees, root and shoot growth flushes occur in a rhythmic alternating pattern of stem extension/leaf expansion and root growth (Harmer, 1990). These growth flushes play an important role in the physiology and ecology of the plant, as carbon (further on abbreviated with C) is allocated to the shoot during shoot flush (Le Hir *et al*., 2005). Such patterns of rhythmic allocation could have a crucial impact on plant response to herbivore feeding. For example, plants attacked during root flush might be more vulnerable to herbivores than those attacked during shoot flush because nutrients are allocated towards the root system during root flush. On the other hand, as the leaf surface area is reduced by herbivory, the amount of C deposited in fine roots can be reduced (Frost & Hunter, 2008), which in turn might influence the interaction between mycorrhizal fungi and host plants.

Oaks represent the tree genus harboring the highest number of herbivore species in Central Europe (Brändle & Brandl, 2001); therefore, oaks are a promising model for studying interactions with herbivores. Here we report a controlled laboratory study of the interactions of oak, the larva of *Lymantria dispar,* and the mycorrhizal fungus *Piloderma croceum* at the levels of gene expression, and carbon/nitrogen allocation. As the oak genome sequence is not available, we studied gene expression using the recently developed transcriptome OakContigDF159.1 of the TrophinOak project (http://www.trophinoak.de) (Tarkka *et al.*, 2013). We tested the following hypotheses:

1) as defense is costly, we expect oak to express defense-related genes when attacked by insect

herbivores particularly when the tree interacts with mycorrhizal fungi; and

2) we expect insect herbivory to negatively affect C allocation to roots, and this will be more pronounced in root flush when roots are the main C sink; such an effect would highlight the potential role of insect herbivory as a modulator of resource exchange dynamics between oak and fungus.

MATERIALS AND METHODS

Oak, fungus, and herbivorous insect

To suppress effects of variability between host individuals (Ruhnke *et al*., 2006, 2009; Schädler *et al*., 2010), we used genetically identical microcuttings of pedunculate oak clone DF159 (*Quercus robur* L.). In contrast to seedlings, microcuttings have a physiology that resembles that of mature trees, including the typical endogenous rhythmic growth of root flush and shoot flush (Herrmann *et al*., 1998; Herrmann & Buscot, 2008). Microcuttings are comparable in size to seedlings; thus, making it possible to perform experiments in the laboratory, which would be difficult with saplings and impossible with mature trees.

The oak clone DF159 was micropropagated and rooted according to Herrmann, *et al*. (1998), then cultivated in soil-based microcosms by placing rooted microcuttings in Petri dishes filled with gamma-sterilized soil as previously described Tarkka, *et al*. (2013). Fungal cultivation with the ectomycorrhizal fungus *Piloderma croceum* Hjortst. strain 729 (DSM-4924) (Basidiomycota, Atheliales, Atheliaceae), Atheliaceae), which is a common mutualist of both coniferous and hardwood tree species and an established model for both ecological and physiological studies, were as described by Tarkka, *et al*. (2013). The fungus was added to the oak microcuttings at the beginning of the experiment (Fig S1).

To test the effect of herbivory, we used larvae of the moth *Lymantra dispar* (Lepidoptera: Noctuidae). *L. dispar* is polyphagous, with a strong preference for oak leaves (Alalouni *et al*., 2013). When mass outbreaks of this species occur, the insect causes severe damages, especially in its invasive ranges, e.g., North America (Hannemann, 1979; Aukema *et al*., 2011; Alalouni *et al*., 2013). Egg masses of the New Jersey lab strain were raised under the same environmental conditions as the oak microcuttings and reared on artificial gypsy moth diet (based on wheat germ). Third instar larvae were used in the experiments.

Experimental design and C/N allocation analysis

Microcuttings were produced as described by (Herrmann *et al*., 1998, 2004). These microcuttings

were grown in square Petri dishes (12 cm × 12 cm; further on called microcosm) filled with γ-sterilized soil; the above-ground plant parts protruded through a hole on the side of the petri dish. The microcosm experiments were conducted in growth chambers with a photosynthetic photon flux density of 180 µmol m^{-2} s^{-1}, a long day setting (16 h/8 h), a constant temperature of 23 °C, and a relative air humidity of 75% (Ton *et al*., 2002). After four weeks, sterilized tap water was added to each microcosm with a sterile syringe.

We used a full factorial experiment consisting of control (oak microcuttings alone), inoculation (oak microcuttings plus *P. croceum*), herbivory (oak microcuttings plus *L. dispar*), and combined inoculation /herbivory (oak microcuttings plus *P. croceum* plus *L. dispar*).

Sixty days after setting up the microcosms, all microcosms were briefly opened under sterile conditions and $^{15}NH_4{}^{15}NO_3$ (5 mL of 0.02 g L^{-1}; 98 atom % ^{15}N; Sigma, Germany) was added to the soil containing roots. One day after the addition of ^{15}N-labeled ammonium nitrate, the "above-ground" parts of each plant chosen for herbivory exposure (herbivory: n = 12; inoculation with *P. croceum*/herbivory: n = 15; Table S1) was exposed to one third instar larva of *L. dispar*. Each plant was covered with a bag with a mesh size of 100 µm to contain the larva. The feeding progress was checked twice an hour, and non-feeding larvae were exchanged with fresh ones. After 6 h, all larvae were removed. Immediately thereafter, all plants were transferred to a labeling chamber. That night, the ambient air CO_2 in the labeling chamber was completely exchanged with $^{13}CO_2$ (10 atom%) in the dark. The CO_2 concentration was adjusted to 401 ± 3 µL L^{-1} (mean ± SD) yielding 9.2 ± 0.1 atm% ^{13}C (mean ± SD). The ^{13}C-labeling of the microcuttings took place in the subsequent 16 h starting with the first light.

Plants were assigned to either root flush (35) or shoot flush (36), which form the levels of the factor *stage* in our analysis (see Angay *et al.* (2014); control: root flush, n = 15; shoot flush, n = 12; inoculation with *P. croceum* root flush, n = 8; shoot flush, n = 9; herbivory: root flush, n = 6; shoot flush, n = 6; inoculation with *P. croceum*/herbivory: root flush, n = 6; shoot flush, n = 9). Microcuttings were harvested by cutting at ground level. The following plant fractions were separated according to Angay *et al.* (2014): (1) sink leaves, i.e. terminal leaves, not yet fully developed or buds; (2) source leaves, i.e. sub-terminal, fully developed leaves; (3) stems; (4) principal roots, i.e. main roots originating from stem; and (5) lateral roots, i.e. fine roots originating from principal roots. After

Table 1 Effects of the factors *stage* (root flush vs. shoot flush), inoculation with *Piloderma croceum*, herbivory (feeding by *Lymantria dispar* larvae), and inoculation/herbivory (*P. croceum* and *L. dispar*) on biomass, analyzed in a three-way ANOVA. Interactions with the factor stage were not significant and are not presented. Boxplots of each plant fraction can be found in the supplementary material. t-values were calculated using the lm function implemented in R; significant t-values are in bold, signs indicate direction of the effects, and signs for the factor *stage* indicate the direction of shoot flush ($p < 0.001$: *** ; $p < 0.01$: ** ; $p < 0.05$: *).

	t- values				*n*
Plant fraction (dry mass)	*Stage*	Inoculation	Herbivory	Inoculation/ herbivory	
Sink leaves	**2.19 ***	1.46	−1.48	0.55	71
Source leaves	**−4.68 *****	**3.27 ****	1.18	−1.9	71
Total above ground biomass	**−4.06 *****	**3.86 *****	0.48	−1.76	71
Lateral roots	-1.28	**3.45 *****	0.11	−0.9	71
Principal roots	**−2.17 ***	1.09	0.51	−0.87	71
Total below ground biomass	**−2.17 ***	**2.83 ****	0.39	−1.11	71

determining the fresh weights, all samples were immediately frozen in liquid nitrogen and stored at −80 °C. Dry weights used in the statistical analyses were calculated using reference values of each plant organ of root flush and shoot flush oak clone DF159 microcuttings given in Angay *et al.* (2014).

For analyses of C and N allocation, parts of plant organs of each treatment and control were separately pooled (n = 6 biological replicates), dried, and ground using a ball mill (Type MM2, Retsch, Hahn, Germany). Stable C and N isotopes in the ground tissues were quantified using an isotope-ratio mass spectrometry (GVI-Isoprime, Elementar, Hanau, Germany) coupled to an elemental analyzer (EA3000, Euro Vector, Milan, Italy). Repeated measurement of a laboratory working standard gave a precision of $\delta^{13}C < 0.1‰$ (SD, n = 10). ^{13}C and ^{15}N excess were calculated per plant fraction and on whole-plant level over unlabeled microcuttings.

Transcriptomics

Total RNA of each pooled sample (see below) was extracted using the MasterPure Plant RNA Purification Kit (Epicentre Technologies Corporation, Madison, WI), according to the manufacturer's protocol from 50 mg leaf material and up to 100 mg root material of each sample. The quality and quantity of RNA separated on formaldehyde-agarose gels were checked using a Nanodrop spectrophotometer (Thermo Scientific), and a

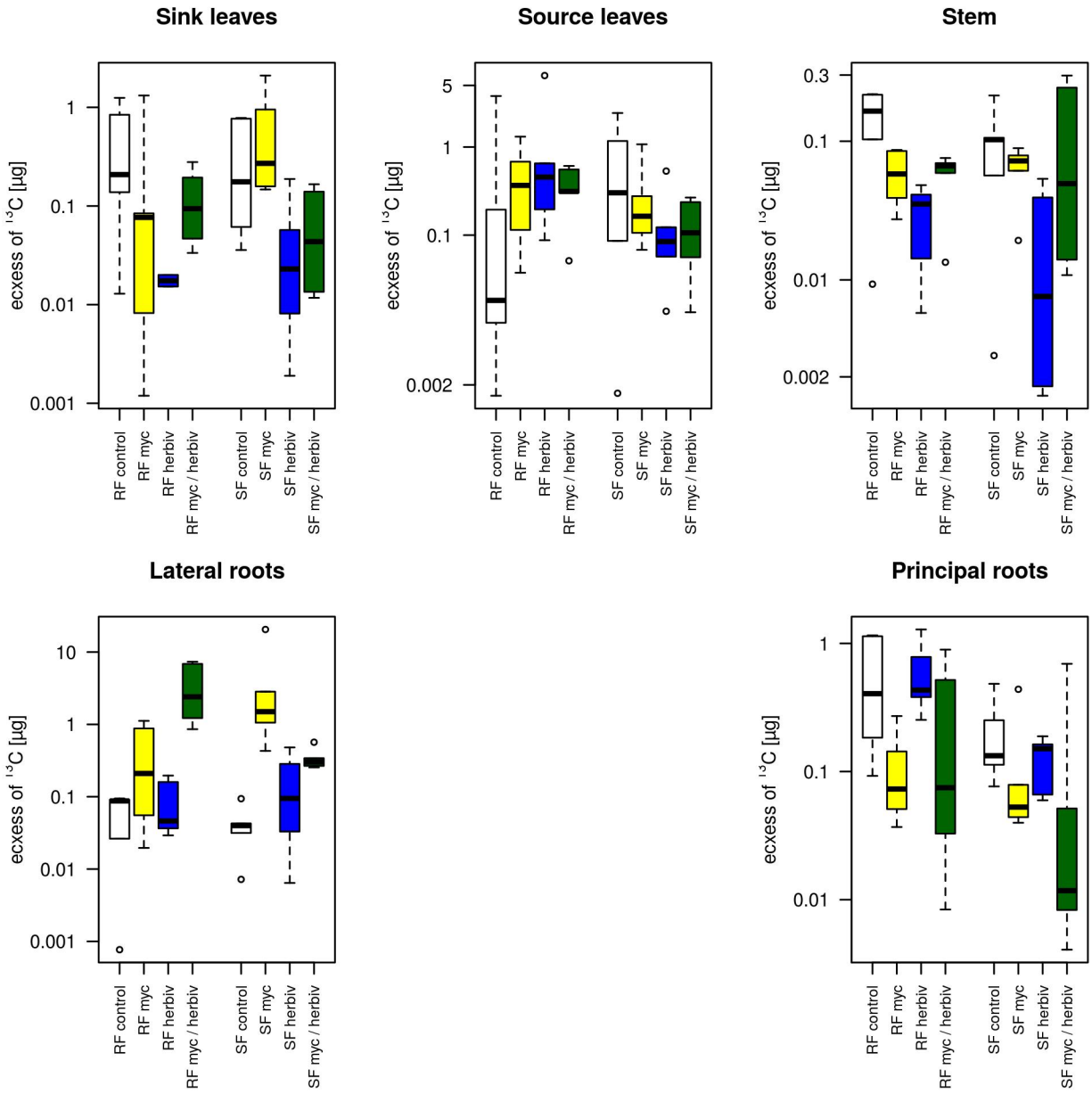

Figure 1 Effect of inoculation with *P. croceum*, herbivory, and inoculation with *P. croceum*/herbivory on the incorporation of ^{13}C into individual plant organs of oak microcuttings in root flush (RF) and shoot flush (SF). Oak microcuttings were inoculated with *P. croceum* (myc; yellow bars), fed to larvae of *Lymantria dispar* (herbivory; blue bars), inoculated with *P. croceum* and fed to larvae of *Lymantria dispar* (myc/herbiv; green bars), or not inoculated with the fungus and not fed to the herbivore (control; colorless bars). Bars in the boxplots indicate the upper and lower quartile with median; whiskers indicate minimum and maximum values; dots are outliers. Note that the y-axes are log-transformed.

Nano Chip and Bioanalyzer 2100 (Agilent).

Samples of terminal leaves and of lateral roots of plants in root flush or in shoot flush from the control and each of the three treatments (inoculation with *P. croceum*, herbivory, inoculation with *P. croceum*/herbivory) were pooled separately. The leaf samples were pooled as follows: three pools of at least two biological replicates each of control/root flush; control/shoot flush, inoculation with *P. croceum*/root flush, inoculation with *P. croceum*/shoot flush,

herbivory/root flush, herbivory/shoot flush, inoculation with *P. croceum*/herbivory/root flush, and inoculation with *P. croceum*/herbivory/shoot flush. Due to the low quantity of RNA in root samples, we were not able to analyze the effects of root flush and shoot flush separately. Therefore, in this case, the factor *stage* was discarded and all root samples of the control and each of the three treatments were pooled separately as follows: three pools each of inoculation with *P. croceum* (root flush plus shoot flush; three biological replicates each),

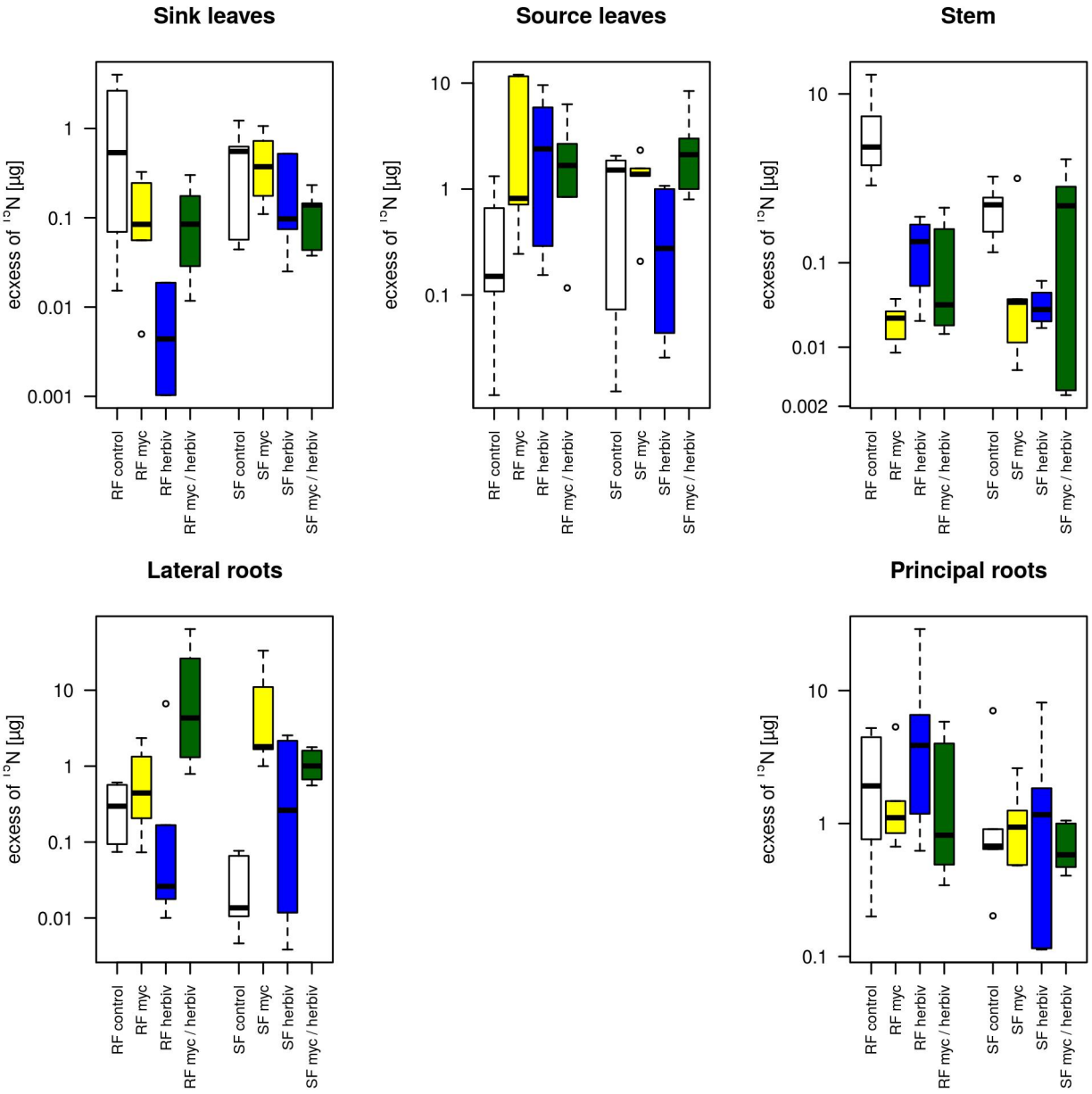

Figure 2 Effect of inoculation with *P. croceum*, herbivory, and inoculation with *P. croceum*/herbivory on the incorporation of ^{15}N into individual plant organs of oak microcuttings in root flush (RF) and shoot flush (SF). Oak microcuttings were inoculated with *P. croceum* (myc; yellow bars), fed to larvae of *Lymantria dispar* (herbiv; blue bars), inoculated with *P. croceum* and fed to larvae of *Lymantria dispar* (myc/herbiv; green bars), or not inoculated with the fungus and not fed to the herbivore (control; colorless bars). Bars in the boxplots indicate the upper and lower quartile with median; whiskers indicate minimum and maximum values; dots are. Note that the y-axes are log-transformed.

herbivory (root flush plus shoot flush; three biological replicates each), and inoculation with *P. croceum*/herbivory (root flush plus shoot flush; three biological replicates each), and two pools of control (root flush plus shoot flush; three biological replicates each).

RNA samples were used to produce 100-bp paired-end libraries which were sequenced using an Illumina HiSeq 2000 at the Beijing Genomics Institute, Hong Kong, China. The reliability of RNA-Seq analyses derived from microcuttings of oak clone DF159 in controlled trophic interactions with *P. croceum* has been demonstrated recently by Tarkka *et al.* (2013) and Kurth *et al.* (2014). Illumina reads were processed as described in Tarkka *et al.* (2013). Briefly, poly(A) tails, low complexity, and low quality sequences were removed with SeqClean (http://compbio.dfci.harvard.edu/tgi/software/). Nucleotides with a quality score < 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 pre-processing.

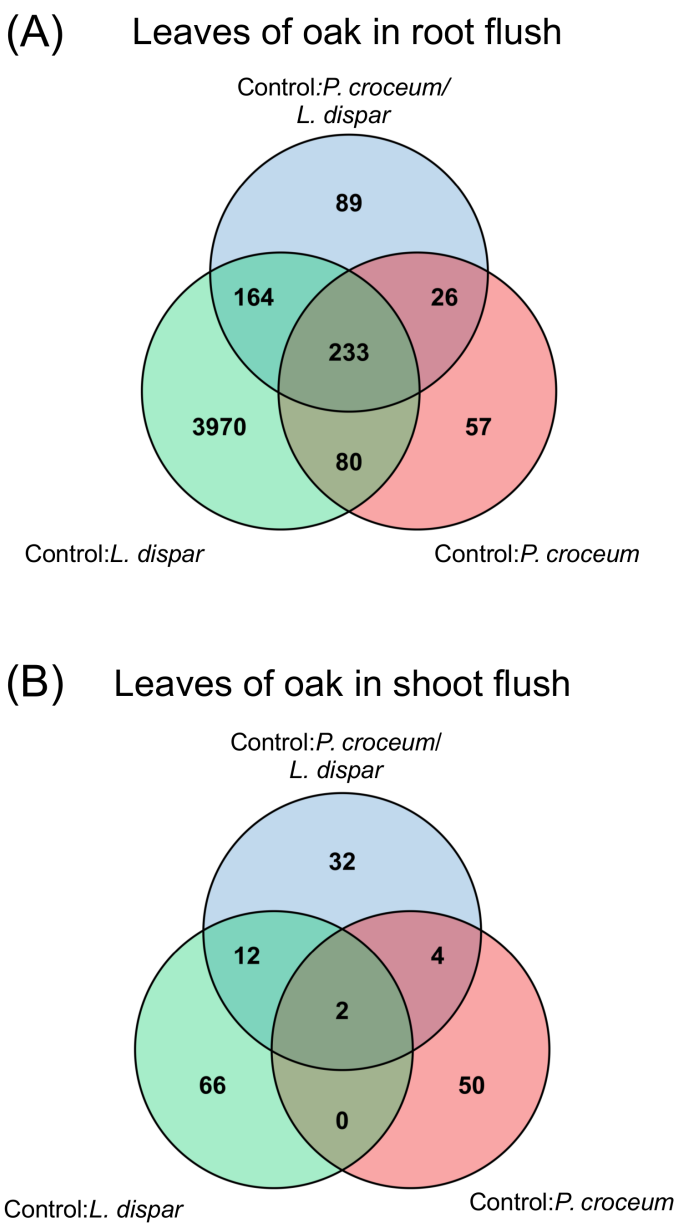

Figure 3 Venn diagrams of contigs representing differentially expressed genes in (A) leaves during root flush, (B) leaves during shoot flush These genes were expressed at higher levels when oak microcuttings were inoculated with *P. croceum*, herbivory, or inoculation with *P. croceum*/herbivory compared to the control microcuttings.

Statistical analyses

The processed Illumina reads were aligned against the OakContigDF159.1 reference transcriptome (Tarkka et al., 2013) using bowtie (Langmead et al., 2009) and quantified using RSEM (Li & Dewey, 2011). The significance of differences in gene expression was measured via pairwise comparisons between contigs of control vs. herbivory, control vs. inoculation with *P. croceum*, control vs. inoculation with *P.*

croceum/herbivory, separately for plants in root flush and for plants in shoot flush, using the edgeR function (Robinson et al., 2010) of the Bioconductor package (Gentleman et al., 2004) in R (R Core Group, http://www.r-project.org/). If the genes represented by these contigs were differentially expressed, we refer to them as differentially expressed genes. When contigs are compared with a reference group, the gene ontology (GO) term and KEGG pathway enrichments are usually statistically assessed (Bluthgen *et al.*, 2005; Conesa *et al.*, 2005; Kanehisa *et al.*, 2014). Using the Bioconductor package GOseq (Langmead et al., 2009), we made pairwise comparisons between treatments and their respective controls to evaluate enrichments or depletions of GO terms and KEGG pathways. The pairwise comparisons between control and treatments were considered to be significant when Benjamini-Hochberg adjusted *P*-values were below 0.01. Biomass and C/N allocation were statistically analyzed in R using the lm function, implementing a three-way analysis of variance (ANOVA).

RESULTS

In the presence of *Piloderma croceum,* both the root and shoot biomass significantly increased (three-way ANOVA; Fig S2 and S3; Table 1). The biomass of source leaves and lateral roots of microcuttings in root flush were significantly higher than those in shoot flush, and the biomass of sink leaves of these microcuttings were significantly lower than that in shoot flush (three-way ANOVA; factor *stage*, root flush vs. shoot flush; Table 1).

All microcuttings exposed to herbivory were damaged by the caterpillars. However, herbivory alone had no significant effect on biomass when compared to the control (Table 1), which indicated that that biomass removal by the herbivore was not substantial. The effects of herbivory and *P. croceum* inoculation did not interact and were not significantly influenced by the growth stage (Fig S2 and S3; Table 1).

Excess of C and N

Sink leaves of microcuttings exposed to herbivory had significantly lower levels of both ^{13}C and ^{15}N, and lateral roots of microcuttings exposed to *P. croceum* inoculation had significantly higher levels of both ^{13}C and ^{15}N (Table 2; Figs 1 and 2). The growth stage had a significant effect on the incorporation of ^{13}C and ^{15}N only in principal roots during shoot flush, when less ^{13}C and ^{15}N was incorporated. *P. croceum* inoculation and *L. dispar* had a combined effect on the incorporation of ^{13}C and ^{15}N only in stems, where significantly more ^{13}C and ^{15}N was incorporated compared to the effect of each

GO term	description	Control vs. inoculation	Control vs. herbivory	Control vs. inoculation/ herbivory
Leaves of oak in root flush				
	Biological Process			
GO:0000724	regulation of DNA replication			
GO:0000911	cell proliferation			
GO:0006260	DNA replication			
GO:0006261	regulation of DNA replication			
GO:0006306	DNA methylation			
GO:0006996	organelle organization			
GO:0010075	regulation of DNA replication			
GO:0051301	cell division			
GO:0006412	translation			
GO:0006032	chitin catabolism			
GO:0055114	starch biosynthesis			
GO:0006633	fatty acid biosynthetic process			
GO:0008643	carbohydrate transport			
GO:0009765	photosynthesis, light harvesting			
GO:0019252	starch biosynthetic process			
GO:0019310	inositol catabolic process			
	Cellular Component			
GO:0000786	microtubule			
GO:0005576	extracellular region			
GO:0005634	nucleus			
GO:0005874	microtubule			
GO:0005886	cell wall			
GO:0009505	cell wall			
GO:0009506	plasmodesma			
GO:0009344	nitrite reductase complex [NAD(P)H]			
GO:0009535	chloroplast thylakoid membrane			
	Molecular Function			
GO:0003677	DNA binding			
GO:0003777	microtubule motor activity			
GO:0005506	iron ion binding			
GO:0005507	copper ion binding			
GO:0005515	protein binding			
GO:0005524	ATP binding			
GO:0009011	starch synthase activity			
GO:0005358	high-affinity hydrogen:glucose symporter activity			
GO:0009055	electron carrier activity			
GO:0016168	chlorophyll binding			
GO:0050113	inositol oxygenase activity			
GO:0003735	structural constituent of ribosome			
Leaves of oak in shoot flush				
	Biological Process			
GO:0006032	chitin catabolism			
GO:0009611	response to wounding			
GO:0010109	regulation of photosynthesis			
GO:0015760	glucose-6-phosphate transport			
GO:0009415	response to water stimulus			
GO:0010037	response to carbon dioxide			
GO:0031640	killing of cells of other organism			
GO:0009643	photosynthetic acclimation			
GO:0006882	cellular zinc ion homeostasis			
GO:0070588	calcium ion transmembrane transport			
GO:0010262	somatic embryogenesis			
	Cellular Component			
GO:0030126	COPI vesicle coat			
	Molecular Function			
GO:0004568	chitinase activity			
GO:0004867	serine-type endopeptidase inhibitor activity			
GO:0005198	structural molecule activity			
GO:0008061	chitin binding			
GO:0016905	myosin heavy chain kinase activity			
GO:0003955	NAD(P)H dehydrogenase (quinone) activity			
GO:0004089	carbonate dehydratase activity			

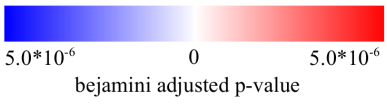

Figure 4 Heatmap of GO term enrichment analysis. Colors represent the p-value adjusted by the Benjamini-Hochberg procedure for the term enrichment, with green representing enrichment and red representing depletion of the corresponding GO terms.

factor alone. *P. croceum* also had a negative effect on the incorporation of ^{15}N in stems and a positive effect on the incorporation of ^{15}N in source leaves (Table 2). The effects of herbivory and fungal inoculation were not significantly mediated by the growth stage. Data derived from molecular analysis, supported the C/N analysis.

Transcriptomics

Thirty-five RNA templates were used to prepare cDNA libraries, which were sequenced (Table S3), resulting in a mean sequence length of 1,713,403,922 bp across templates. After processing, 1,690,701,038 bp remained for further analysis.

In microcuttings in root flush (Fig 3A), the highest number of contigs representing differentially expressed genes (3,970) was in leaves of oaks subjected to herbivory, compared to 57 in leaves of *P. croceum* inoculated oaks and 89 in leaves in oaks subjected to both *P. croceum* inoculation and herbivory. In microcuttings in shoot flush (Fig 3B), a similar pattern was found, but the number of contigs representing differentially expressed genes in leaves of oaks was lower than the number in leaves of oaks in root flush subjected to herbivory. In roots of oaks, the highest numbers of contigs representing differentially expressed

Table 2 Effects of the factors *stage* (root flush vs. shoot flush), inoculation with *Piloderma croceum*, herbivory (feeding by *Lymantria dispar* larvae), and inoculation/herbivory (*P. croceum* and *L. dispar*) on incorporation of ^{13}C and ^{15}N. Interactions with the factor stage were not significant and are not presented. Boxplots of each plant fraction for excess of ^{13}C and ^{15}N can be found in the supplementary material. Values were log-transformed prior to analyses; significant t-values are in bold, signs indicate direction of the effects, and signs for the factor *stage* indicate the direction of shoot flush ($p < 0.001$: *** ; $p < 0.01$: ** ; $p < 0.05$: * ; $p < 0.1$: (*)).

	t- values				*n*
Plant fraction	*Stage*	Inoculation	Herbivory	Inoculation/ herbivory	
^{13}C excess					
Sink leaves	0.7	−0.82	**−3.06 ****	1.65	37
Source leaves	−0.87	1.52	1.35	−1.33	44
Stem	−0.73	−0.46	**−2.9 ****	**2.13 ***	39
Lateral roots	0.23	**4.42 *****	1.37	−0.47	44
Principal roots	**−2.69 ***	**−2.06 ***	−0.08	−0.68	43
^{15}N excess					
Sink leaves	**1.73 (*)**	−1.07	**−2.66 ***	1.27	37
Source leaves	−0.69	**2.47 ****	1.32	−0.7	44
Stem	−0.83	**−5.12 *****	**−3.64 *****	**3.87 *****	39
Lateral roots	−0.57	**3.34 ****	0.68	0.08	44
Principal roots	**−2.24 ***	−0.04	0.8	−0.94	44

	Control vs. Inoculation		Control vs. herbivory		Control vs. inoculation/ herbivory		
Leaves of oak in root flush							
Contig	**log2fold change**	**FDR**	**log2fold change**	**FDR**	**log2fold change**	**FDR**	**Sequence description**
42425_1_11	4.30	n.s.	4.48	***	3.82	n.s.	class I chitinase
104300_0_1	5.80	n.s.	6.30	**	4.97	n.s.	class V chitinase
39593_0_1	-2.83	**	-1.21	n.s.	-4.48	***	class IV chitinase
42016_0_2	4.33	**	5.70	***	4.64	***	enhanced disease susceptibility 5
39773_5_6	1.02	n.s.	1.32	*	1.74	*	multicopper oxidase
42229_0_1	1.54	n.s.	1.86	***	1.42	n.s.	inter-alpha-trypsin inhibitor heavy
39011_2_2	8.25	*	9.72	***	8.53	**	tannin-related r2r3 myb transcription partial
32167_0_1	0.08	n.s.	8.14	***	7.29	*	lignin forming peroxidase
34500_1_1	6.88	**	7.80	**	6.51	**	s-adenosyl-l-methionine:salicylic acid carboxyl methyltransferase-like protein
6591_0_1	-2.32	n.s.	0.68	n.s.	6.84	***	terpene synthase
33448_0_1	0.02	n.s.	-0.12	n.s.	2.25	*	nerolidol synthase
31427_0_5	0.18	n.s.	-10.49	**	-0.23	n.s.	fructose- 1,6-bisphosphatase
35851_1_1	-0,43	n.s.	-5.58	***	0.44	n.s.	erd6-like sugar transporter
26867_0_1	-1.57	n.s.	-8.31	**	2.86	n.s.	beta-galactosidase 16
41058_0_2	-5.84	**	-1.73	n.s.	-5.01	**	nitrate and chloride transporter
40206_0_1	-3.24	*	-0.28	n.s.	-0.41	n.s.	nitrate transporter -like
43364_1_1	6.72	n.s.	8.41	***	5.70	n.s.	callose synthase 10
35609_0_1	1.89	n.s.	2.49	***	2.01	*	glycoside hydrolase family 28 protein
Leaves of oak in root flush							
32814_0_1	-0.05	n.s.	3.05	*	1.16	n.s.	class I chitinase
46973_c_1	-3.24	n.s.	6.74	*	3.68	n.s.	class II chitinase
39593_0_1	0.64	n.s.	7.85	**	4.25	*	class IV chitinase
43013_0_1	0.82	n.s.	11.45	***	7.17	***	alpha-amylase subtilisin inhibitor
33631_0_2	0.00	n.s.	10.71	***	-5.16	n.s.	putative protease inhibitor
40378_0_1	0.10	n.s.	4.41	*	2.84	n.s.	hexose transporter
38297_0_6	-0.09	n.s.	-8.37	*	-1,07	n.s.	chlorophyll a/b-binding protein
40206_0_1	1.37	n.s.	8.0	**	5.45	***	nitrate transporter -like
43364_1_1	6.63	n.s.	5.84	n.s.	5.70	***	callose synthase 10

Figure 5 Heatmap of contigs representing differentially expressed genes, showing pairwise comparisons of control vs. inoculation with a mycorrhizal fungus, control vs. herbivory, and control vs. inoculation/herbivory. Sequence description derived from BLAST2GO contic assignment, addressing putative functions. Colors indicate direction in regulation: blue, down regulation; and red, up regulation. Significance was tested using false discovery rate (FDR): $p < 0.001$: *** ; $p < 0.01$: ** ; $p < 0.05$: * ; n.s.: not significant.

genes were from oaks subjected to herbivory (100) and in oaks inoculated with the mycorrhizal fungus (93).

Both the GO term enrichment analysis (Fig 4) and analysis of KEGG pathways (Fig S4; Li & Dewey, 2011; Kurth *et al.*, 2014) of all contigs representing differentially expressed genes reflected the high number of such contigs from leaves of plants in root flush. Pairwise comparisons between control vs. inoculation with *P. croceum*, control vs. herbivory, and control vs. inoculation with *P. croceum*/herbivory revealed that GO terms and KEGG pathways assigned to growth and development, DNA replication, transcription, and translation were highly enriched and those assigned to cell wall components and other growth related contigs

were enriched in leaves of oak microcuttings in root flush and subjected to herbivory, whereas those assigned to catabolism, photosynthesis, chlorophyll binding, starch biosynthesis, and cell components were depleted (Fig 4).

GO term enrichment was not as strong for leaves of oak in shoot flush as for leaves of oak in root flush. In leaves of oak microcuttings in shoot flush subjected to herbivory, the enrichment was focused more on direct defense mechanisms, e.g., chitin catabolism and killing cells of other organisms, i.e., pathogenesis related, such as thaumatin-like protein. In leaves of microcuttings subjected to inoculation with *P. croceum* and in leaves of oaks microcuttings subjected to *L. dispar*, the GO term for apoplast (a cell component) was depleted. Along to the ^{13}C and ^{15}N allocation results, we found in leaves of oak in root flush enrichments in terms related to starch biosynthesis in oak inoculated with *P. croceum* and exposed to herbivory, which was depleted in oaks exposed to herbivore feeding alone. Further was the term nitrate reductase complex found depleted in shoot flush plants, whereas enriched in root flush plants. We found terms related to physiological functions as photosynthesis mainly depleted in leaves of oak exposed to herbivory, which was in line with a significant reduction of ^{13}C incorporation.

Analysis of the KEGG pathways (Kanehisa *et al*., 2014) revealed a pattern similar to that obtained in the GO enrichment analysis (Fig S4). Most of the enriched pathways were in leaves of oaks in root flush that were subjected to *L. dispar*, and included pathways of amino acid metabolism and nucleotide metabolism. The depleted pathways in these leaves included many biosynthetic pathways of secondary metabolism, carbohydrate metabolism, and energy metabolism. In leaves of oak in shoot flush, only few pathways were significantly enriched (nitrogen and carbon metabolism), and none were depleted.

A deeper insight into the actual gene expression levels can be provided by determining the contigs that represent genes whose expression levels changed compared to the respective controls (Fig 5). In leaves of microcuttings in root flush subjected to *L. dispar*, contigs representing chitinase genes that are up-regulated were identified, e.g., the gene encoding class I chitinase was up-regulated with a log2-fold change of 4. In contrast, this gene was not significantly up-regulated in microcuttings in root flush inoculated with *P. croceum* or inoculation/herbivory. In leaves of oak in shoot flush, a contig representing a putative protease inhibitor was up-regulated with a log2-fold change of 10

only in samples subjected to herbivory. Contigs representing lignin-coding genes that were up-regulated only in samples subjected to herbivory or to *P. croceum* inoculation/*L. dispar*. Several contigs representing genes encoding a terpene synthase ((-)-germacrene-D synthase) and nerolidol synthase (production of volatile organic compounds) were identified exclusively in leaves of microcuttings in root flush with *P. croceum* inoculation/herbivory, showing a up-regulation with a log2-fold change of 6.8 and 2.3 respectively. Contigs representing genes putatively related to direct defense responses, such as inter-alpha-trypsin inhibitor heavy, were identified only in microcuttings subjected to herbivory in both root flush and shoot flush. Several contigs, representing genes involved in carbohydrate transport, production and incorporation were found differentially expressed in pairwise comparisons between oak exposed to herbivory and those inoculated with *P. croceum* and exposed to herbivory, e.g. erd-like sugar transporter with a log2fold-change of 6.1 and beta-galactosidase 16 with a log-fold change of 11.27. Oaks in shoot flush were not as rich on differential expressed genes as oak in root flush. A contig representing a nitrate transporter gene was differential expressed between control and the herbivory treatment as well as the combined inoculation/herbivory treatment displaying a up-regulation. Furthermore was a contig representing genes encoding proteins involved in photosynthesis (chlorophyll a/b binding protein) strongly down-regulated in oaks exposed to herbivory, but not differential expressed in the combined inoculation/herbivory treatment.

DISCUSSION

The results of our multi-trophic experiments under controlled laboratory conditions revealed the impact of inoculation with a mycorrhizal fungus on microcuttings of a deciduous tree during attack by a generalist herbivorous caterpillar. We found that biomass, C and N allocation, and expression of genes was not only affected by inoculation and herbivory but also differed between plants in different growth stages (root flush or shoot flush).

The significant effects of *Piloderma croceum* on the biomass of both shoots and roots indicated a successful inoculation and confirm the previously described pre-mycorrhizal effect of *P. croceum* on the host plant [40]. In contrast, the short period of feeding by the herbivore did not affect plant biomass and thus the effects of the herbivory treatment have not to be interpreted as a consequence of biomass loss. A comparison of the number of contigs representing

differentially expressed genes in the different treatments and in plants in both growth flushes revealed extensive changes in gene expression in leaves of plants in root flush fed upon by the herbivore. In this growth stage, the number of such contigs was 40 times higher due to herbivory in leaves of non-inoculated plants than on leaves of plants inoculated with *P. croceum*. The effect was smaller in leaves of oak in shoot flush, where the number of such contigs was twofold higher in plants subjected to herbivory than in plants with inoculation and herbivory. Our results therefore show for the first time that the effects of this tri-trophic interactions strongly depend on plant growth stage.

Effects on genes related to defence

Although we are aware of the drawbacks of gene ontology (Ashburner *et al*., 2000; Yon Rhee *et al*., 2008; Young *et al*., 2010), it provides a means of annotating the oak transcriptome according to the reference library. Furthermore, GO term enrichment analysis is a useful method for extracting patterns from masses of data.

Compensatory growth of herbs and trees is a common but costly direct reaction to herbivory (McNaughton, 1983; Stowe *et al*., 2000; Haukioja & Koricheva, 2000). The results of GO term enrichment and KEGG pathway analyses of contigs from leaves of plants in root flush revealed that many GO terms and pathways related to growth, DNA replication, cell proliferation, photosynthesis, and starch biosynthesis were enriched in plants subjected to herbivory. Enrichments and depletions were also identified in leaves of plants in root flush subjected to both inoculation with the mycorrhizal fungus and herbivory, but at a much lower level than when plants were subjected only to inoculation or herbivory alone. GO terms of growth, metabolism, and direct defense (e.g., chitin catabolism and response to wounding) were enriched in leaves of plants in shoot flush subjected to herbivory, but no enriched or depleted terms were identified in leaves of plants in shoot flush subjected to both inoculation and herbivory. The expression of genes encoding pathogenesis-related proteins induced by arthropod feeding has been reported by Shafique et al. (2014), and these genes were also up-regulated in our study only when plants were fed upon by the herbivore.

Improved nutrient uptake owing to mycorrhization leads to a better condition of the host plant (e.g., Read, 1991; Pearson & Stewart, 1993; Nehls *et al*., 2007). In keeping with this, we found GO term enrichments mainly related to growth and photosynthesis in plants inoculated with *P. croceum*, which indicated improved nutrient uptake even in the pre-mycorrhizal stage of symbiosis (Volpin *et al*., 1994;

Herrmann *et al*., 1998, 2004; Frettinger *et al*., 2006a).

The finding of a depletion of the same GO terms in plants inoculated with *P. croceum* and feeding by *L. dispar* is consistent with results of KEGG pathway analysis. Presence of the mycorrhizal fungus seems to "cool down" the strong and costly reaction of the plant to herbivory. Our finding of a lower number of regulated pathways in leaves of oak in shoot flush compared to that in root flush underlines the strong impact of growth stage and the resulting differences in responses to herbivore attack. Furthermore, this shoot flush effect is similar to the "cooling down" effect of the ectomycorrhizal fungi on the reaction to herbivory. This suggests that increased plant vigor results from an increased availability of resources caused by either shoot flush or association with ectomycorrhizal fungi. We conclude that plants growing without the support of the fungus are forced to respond more strongly to attack by herbivores than plants with the mycorrhizal symbiont. This indicates that the presence of a mycorrhizal symbiont soothes the direct defense reactions of plants. In our study, this seems to apply e.g. for protease inhibitors, which act as effective anti-nutrients, negatively affect the growth and development of herbivores by inhibiting gut proteases (Howe & Jander, 2008). Our finding of an up-regulation of a putative protease inhibitor protein in plants attacked by the herbivore but not in plants inoculated with the mycorrhizal symbiont may serve as a further indication of the "cooling down" effect.

Several genes represented by contigs were expressed in all plants exposed to *P. croceum* and/or *L. dispar*. For example, contigs representing genes encoding chitinases were up-regulated in plants of all treatments compared to the control, which might be explained by the diverse functions of these enzymes (Brunner *et al*., 1998; Kasprzewska, 2003; Hartl *et al*., 2012; Veluthakkal *et al*., 2012). Besides beneficial effects of pre-mycorrhizal stages of symbiosis, it is thought that early stages of mycorrhiza formation on roots can be as stressful to plants as other factors, and triggers systemic, nonspecific defense reactions, including increased chitinase activities. Our finding of up-regulation of a class I chitinase gene in leaves of plants in root flush or shoot flush, and in roots (root flush and shoot flush combined) only in plants subjected to herbivory underlines that attenuation in protruding and mature mycorrhiza formations (Sauter & Hager, 1989; Albrecht *et al*., 1994; Smith & Read, 2010).

Indirect defense of plants often involves the release of volatile organic compounds triggered by herbivore feeding (Arimura *et al*., 2004). In tri-trophic

systems, these compounds might attract natural enemies of the arthropods, such as parasites and parasitoids (Kessler & Baldwin, 2001; Alalouni *et al*., 2013; for trees see Mondor & Roland, 1997, 1998; Havill & Raffa, 2000). Terpene synthase and nerolidol synthase are involved in the synthesis of the volatile organic compounds sesquiterpenes and monoterpenes (Pichersky & Gershenzon, 2002; Arimura *et al*., 2004; Baer *et al*., 2014). The encoding genes represented by contigs were exclusively up-regulated in plants subjected to both *P. croceum* and *L. dispar*, which indicates the strong influence of the mycorrhizal fungus *P. croceum* on the defence strategy of oak. However the production and secretion of these compounds warrants further investigation. Nevertheless this indicates that mycorrhizal inoculation may lead to a switch from compensatory growth and direct defense strategies to indirect defense by attracting the natural enemies of the herbivore with these compounds. Thus, our first hypothesis can only be partially confirmed, since the inoculation with a mycorrhizal fungus did not generally increased the expression of defence related genes but seems to alter the defence strategy of oak.

Nutrient allocation patterns

The results of the C-N allocation analysis correspond with the patterns derived from GO term – and DEG comparisons. Babst et al. (2008) reported an increased C export from leaves of *Populus nigra* leaves after leaf chewing by *L. dispar* caterpillars. Accordingly, our findings show that oaks in root flush respond to herbivory with a reduced incorporation of carbohydrates into leaves. According to transcriptional changes and C-excess data, this effect is attenuated or even reversed in inoculated oak. In the herbivory treatment we found genes encoding erd6-like sugar transporters and beta galactosidase 16 down-regulated compared to the control, the inoculation and the herbivory/inoculation treatments. Up-regulation of both genes was shown in *Arabidopsis thaliana* induced by cold stress, and other abiotic stess factors as high salinity (Kiyosue *et al*., 1998; Seki *et al*., 2002). As defense induction caused by herbivore feeding can reconfigure primary metabolism (Schwachtje & Baldwin, 2008), our finding of an up-regulation of these genes indicates the need of saving valuable C resources from herbivorous insects supports former results derived from, e.g. *Arabidopsis thaliana* and poplar surveys (Babst *et al*., 2009; Ferrieri *et al*., 2013).

Differences in gene expression patterns to herbivory between plants with root flush or shoot flush growth

When we compared the trophic interactions of plants with root flush growth and plants with shoot flush

growth, profound effects of this rhythmic growth on the response patterns triggered by herbivory were revealed. Our results indicated that oaks use different strategies to deal with herbivory depending not only on the presence of a mycorrhizal fungus, but also on the growth stage. Our RNA-Seq data suggested that during root flush, when roots elongate and leaf development rests, herbivory leads to an initiation of compensatory growth. This reaction is alleviated in the presence of the mycorrhizal fungus, which emphasizes once more the soothing effect of mycorrhiza on its host. However, these mediating effects of growth stage are not reflected in responses of biomass and resource allocation within the plant.

CONCLUSIONS

Our results indicated that gene expression patterns in oaks are modulated by effects of inoculation with a mycorrhizal fungus and insect herbivory alone and together. Specifically, the expression of defense-related genes in oak upon attack by an insect herbivore depends on the presence or absence of the mycorrhizal symbiont. The strategy of dealing with herbivory changed from direct defense without the fungus to indirect defense with the fungus, and the compensatory growth reaction was lower in presence of the mycorrhizal symbiont. In addition, we shed more light on the consequences of endogenous rhythmic growth in the ability of the plant to fight insect pests with indirect defense mechanisms with the support of a mycorrhizal partner. Nutrient allocation is clearly altered by the mycorrhizal fungus, but our results did not support our hypothesis, as the amount of ^{13}C and ^{15}N incorporated into sink leaves in plants subjected to herbivory decreased, but that incorporated into roots did not. The method we used here to study the ecology of trophic interactions has been extended to various types of interactions.

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Chapter IV

Nutrient allocation in oak as affected by detritivore – herbivore – mycorrhiza interactions

with:

Marcel Graf, Stefan Scheu, Roland Brandl, Thorsten E. E. Grams and Martin Schädler

(Manuscript in preparation)

SUMMARY

Mycorrhizal fungi and herbivores interactively influence the growth, resource utilization, and herbivore defense of plants. The largely unexplored genetic and physiological basis of these interactions may differ in the different plant growth phases, thereby contributing to the complexity of plant responses. We studied these interactions in a tri-trophic system comprising oak (*Quercus robur* clone DF159), larvae of the moth *Lymantria dispar,* and the ectomycorrhizal fungus *Piloderma croceum* under controlled laboratory conditions at the level of gene expression using transcriptomics and at the level of carbon/nitrogen allocation for plants from different growth stages. Herbivore feeding on oak leaves led to increased expression of genes related to compensatory growth and to enriched GO terms described with cell wall, cell division and DNA replication and other direct defense mechanisms of oak, like an enhanced expression of e.g. chitinases in root flush and protease inhibitors in shoot flush. C/N-allocation analyses indicated an increased export of resources from aboveground plant parts and accordingly genes associated with the transport of sugars were increased upon herbivore attack. Inoculation with an ectomycorrhizal fungus attenuated these effects in the pre-mycorrhizal stage but caused an increased expression of genes related to the production of volatile organic compounds. We conclude that the inoculation with ectomycorrhizal fungi mediates the plant's defense strategy and that this effect is moreover dependent on the growth stage of plants. These results from a rather simple tri-trophic lab system give insights into the complexity of plant responses to a multi-trophic world.

INTRODUCTION

Plants encounter numerous above- and below-ground interactions with a bewildering variety of organisms (Ehrlich & Raven, 1964; Thompson, 2009). As sessile life forms they have developed strategies to handle both, beneficial and antagonistic interactions with species, above- and belowground, including direct attacks such as leaf herbivores but also by root herbivores and pathogens below the ground (Crawley, 1996; Freiberg et al., 1997; Heijden & Sanders, 2002). The different strategies to handle attacks range from physical barriers like thick secondary cell walls and trichomes to secondary metabolites which are toxic to hostile organisms and referred to as direct defense (Kessler & Balwin, 2001, 2002). In addition to contributing to defense mechanisms, rapid changes in metabolite allocation mitigates detrimental effects of herbivory (Schwachtje *et al*., 2006).

However, plants also form mutual relationships with other organisms. Most plant species are in close association with symbiotic fungi by forming mycorrhiza-symbiosis, which are beneficial to both partners (Herrmann *et al.*, 1998; Smith & Read, 2010). This relationship fosters water and nutrient uptake especially nitrogen and phosphorus by increasing nutrient exploition of the rhizosphere via extraradical

hyphae. Notably, in addition to fostering plant nutrient exploiuation, the association of plants with mycorrhizal fungi also improve plant defense against antagonists via priming defense (Pozo & Azcón-Aguilar, 2007; Koricheva *et al.*, 2009; Campos-Soriano *et al*., 2012). Furthermore, decomposers including dung beetles and earthworms impact secondary metabolite synthesis and defense array of plants against herbivory (Wurst et al., 2004; Blouin et al., 2006). In addition, soil detritivores such as Collembola and earthworms alter plant nutrient supply and therefore plant growth performance and vitality. For instance Collembola recycle plant litter thereby increasing nutrient availability of plants, but they also alter plant growth via changing plant – microbial interactions, in particular that between plants and fungi (Chamberlain *et al.*, 2006). Furthermore, Collembola affect plant performance not only through provisioning of nutrients but also influence their growth and survivability by a number of other indirect mechanisms like grazing on rhizosphere microorganisms and modifying the soil structure (Scheu, 2001; Gormsen *et al.*, 2004; Friberg *et al.*, 2005). Further, Collembolan driven increase of nutrient uptake impacts plant growth and plant nutrient concentration (Lussenhop & Bassirirad, 2005; Mitschunas et al., 2006; Ladygina *et al*., 2010) which may affects above ground herbivore performance (Schütz *et al.*, 2008). Surprisingly, until now there are no investigations regarding to interactions of ectomycorrhizal plants like oaks and decomposer animals (e.g. Collembola) which are most notably existing in forest soils. However there is a rising number of studies dealing with interactive effects of above-ground herbivory and mycorrhizal fungi (e.g. Bacht *et al.* unpblished), there is no knowledge about multitrophic interactions of above-ground herbivory and below-ground communities of Collembolans and mycorrhizal fungi.

They are among the most widespread and abundant soil arthropods and reach densities of > 100,000 individuals per square meter in forests. Therefore, it is likely to assume they have an impact on

plant – mycorrhiza interactions (Gange, 2000; Kaneda & Kaneko, 2004; Jonas *et al.*, 2007). However, plants have been shown to respond to the presence of Collembola in the rhizosphere due to root elongation and branching even though total biomass and nutrient concentration remained unaffected (Endlweber *et al*., 2006; Endlweber & Scheu, 2007)

Recent research on arbuscular mycorrhizhal fungi has brought evidence of enhanced resistance against shoot pathogens induced by these fungi (Pozo & Azcón-Aguilar, 2007; Koricheva et al., 2009; Campos-Soriano et al., 2012). Further, qualitative and quantitative changes in flavonoid contents have been observed, in plants associated with mycorrhizal fungi (Vierheilig & Piche, 2002; Akiyama et al., 2002). Fester and Hause (2005) have also reported of profound changes in phenolic compounds, defense-related phytohormones, indicating a induction of defense against pathogens and herbivores (Fester & Hause, 2005; Lopez-Raez *et al*., 2010).

Most research dealing with interaction of mycorrhiza and herbivory has concentrated on herbaceous plants, and arbuscular mycorrhizal fungi. However trees, in boreal and temperate regions mostly in symbiosis with ectomycorrhizal fungi, harbor a generally high number of interacting organisms, with an intriguing density and diversity of herbivores (Brändle & Brandl, 2001). The ecological importance of trees is accounted by the longer life span of tree, compared to herbaceous plants. For several reasons this might cause pronounced effects on biodiversity: first, the long life span allows trees to implement advanced defense mechanisms against herbivory, second, changes in the apparency and light environment in tree stands might alter levels of herbivory and third, old trees may change the abundances and efficiency of natural enemies (Moore & Francis, 1991; Tylianakis *et al*., 2004; Boege & Marquis, 2005).

In order to improve our understanding of multitrophic above- and below-ground interactions we established a laboratory experiment centered on an ectomycorrhizal plant (*Quercus robur*). one of the most

common European tree species of significant economic importance. The ectomycorrhizal fungal species (*Piloderma croceum*), widespread in deciduous forests and the Collembolan species *Protaphorura armata* representing euedaphic Collembola, common in the rhizosphere of plants were added. To add above-ground interaction, caterpillars of the generalist leaf herbivore *Lymantria dispar* were included. To unravel morphological and nutritional changes in *Q. robur* due to the presence of *P. croceum*, *P. armata* and *L. dispar* we established a full factorial experiment investigating the impact of multitrophic interactions of *Q. robur* with biological interactors including mutualists and antagonists. Using ^{13}C and ^{15}N labelling changes in carbon and nitrogen allocation of *Q. robur* in presence of *P. armata* and *P. croceum* and *L. dispar* was investigated. Specifically, we addressed the following hypotheses:

1. Enhanced nutrient supply by mycorrhizal fungi enhance plant growth, but in particular the allocation of carbon to roots and the allocation of nitrogen to leaves.
2. Defense against herbivores varies with plant nutrient supply. Therefore, we expect direct defense mechanisms to be more pronounced in mycorrhizal oaks, supported by enhanced nutrient supply. In Contrast, positive effects of the mycorrhizal fungus on the oaks defense mechanisms will be attenuated by Collembolan feeding on fungal hyphae.
3. Collembola interaction will lead oaks to enhanced N incorporation due to enhanced mineralization in the soil. Collembola feeding on hyphae ectomycorrhizal oak will affect this,
4. by reducing nutrient supply by the fungus.

MATERIALS AND METHODS

Seeds and soil

In order to obtain a homogeneous soil substrate for the experiments, 1 m^3 of the upper soil were collected from an oak forest stand at the Dölauer Heide close to Halle/Saale, Saxony Anhalt in Germany (51.51016 N, 11.91291 E). The A0 (humus, -10 cm) and A1A2 (organic, -30 cm) horizons were sampled, air dried, sieved at 5 mm, mixed 1:1 (v/v), further sterilised with a steam sterilisation system and washed thrice with dH2O in order to dissolved nutrients. Seedlings were raised from acorns applied from a German federal seed dealer (Staatsklenge Nagold). Prior to planting, the acorns were peeled and washed with water. After an initial planting in sterile sand, acorns were submerged for 30 min. in hydrogen peroxide (10 v/v). After thoroughly washing with water, acorns were planted in the substrate. Position of oak individuals in the climate chamber was randomised weekly, oaks were rinsed with sterile tap water (30ml) twice a week. After six weeks the oaks were inoculated with *Piloderma croceum*. Seedlings not designated for mycorrhizal infection were treated the same way as the inoculated ones, but with a vermiculite/peat mixture without *P. croceum*. During the inoculation procedure all seedlings were measured for stem length. The whole time the acorns and later on, the seedling were kept under controlled conditions of 70% rel. humidity, 25°C and long days (16/8 h; day/night).

Experimental design

63 *Quercus robur* seedlings were raised under controlled conditions and analysed. The experiment was set up in a full factorial design with the following factors: *Piloderma croceum* as a inoculation treatment with a mycorrhizal fungus, *Lymantria dispar* as leaf herbivore, as well as *Protarophura armata* (Colembola), each with its respective control, resulting in 8 treatment combination.

Piloderma croceum

P. croceum inoculum was produced at the Helmholtz Centre for Environmental Research UFZ Halle. The production of the *P. croceum* inoculum comprises two steps. First, mycelium is grown in the modified Melin-

Norkans (MMN) liquid medium with 10 g/L glucose over 10 days and added to solid sterilized vermiculite/perlite substrates according to the procedure described in Herrmann et al. (1992). After three to five weeks incubation, the substrate was rinsed with sterile and purified water in order to suppress rest of carbohydrates and reduce the risks of contamination.

Lymantria dispar

Egg masses of the New Jersey lab strain were raised under the same environmental conditions as the Oak seedlings and fed with artificial gypsy moth died (based on wheat germ). Caterpillars of the third larval stage were chosen for the experiment, weight measured before and after feeding.

Protaphorura armata

The Collembola species *Protaphorura armata* (Collembola, Tullberg) was taken from laboratory cultures established from field populations close to Darmstadt (Germany). Cultures were kept on a mixture of sterilized potting soil and clay pellets (3:1) at 14°C in darkness.

Nine weeks after inoculation with *P. croceum* all seedlings were equipped with a bag of 100µm mesh, strapped to one mature leaf per oak. After weighing the caterpillars, half of the bags were equipped with one third instar larvae. Every source-leaf of the *Lymantria* treatment was photographed in 90° angle with a scale, in order to measure the fed leaf material afterwards. After 5 days of feeding, the caterpillars were removed and weighted, the removed leaf area was documented and devoured leaf mass was calculated. For harvest, the seedling were cut at ground level and separated into root and shoot. The following plant fractions were separated according to Angay *et al.* (2014): (1) sink leaves, i.e. terminal leaves, not yet fully developed or buds; (2) source leaves, i.e. sub-terminal, fully developed leaves; (3) roots, i.e. main roots originating from stem and lateral roots, i.e. fine roots originating from principal roots. After determining the fresh weight of the single plant organs, half of the harvested replicates was frozen in liquid nitrogen immediately, the other half was dried for one week at 60°C and weighted.

C-/N labelling and allocation analyses

Together with the inoculation process, the substrate of every seedling was mixed with ground ^{15}N labeled *Lolium perenne* material. Nine weeks after inoculation and C labeling, every plant chosen for herbivory exposure was exposed to previously weighted third instar larvae of *L. dispar*. To cage larvae, one source leaf per plant was covered by a bag with a mesh size of 100µm. After six hours all caterpillars were removed. Directly after the feeding, about 36 hours before harvest, all microcuttings were transferred to the ^{13}C labeling chamber. During the night before the actual C labeling procedure, the CO_2 was completely exchanged with labeled CO_2 with 10 Atom Percent (atom%) $^{13}CO_2$. According to the previous light period and with unchanged climatic conditions, the labeling procedure took place in the subsequent 16 hours starting with the first light. CO_2 concentration was adjusted to 400 ± 2 µL L^{-1} (mean ± SD) yielding in 7.9 ± 0.3 atm% ^{13}C (mean ± SD).

For the allocation analyses parts of every plant fraction were pooled to a total of six biological replicates which were dried and ground using a ball mill (Type MM2, Retsch, Hahn, Germany). An isotope-ratio mass spectrometry (IRMS) (GVI-Isoprime, Elementar, Hanau, Germany) coupled to an element analyzer (EA3000, Euro Vector, Milan, Italy) was used to quantify the stable C and N isotopes. Repeated measurements of a laboratory working standard gave a precision of $\delta^{13}C < 0.1$ ‰ (SD, n = 10). Incorporation of ^{13}C and ^{15}N were calculated as excess per organ and on whole-plant level over unlabeled microcuttings serving as controls. Allocation patterns were calculated as percentage of plant fractions share of excess on whole-plant levels excess.

Statistical analyses

Statistical analyses of biomass and C-/N- allocation were done in R (R core group, http://www.r-project.org/), using the lm function for realizing a three factorial ANOVA. Data were transformed (ln transformation) to obtain normal distribution wherever necessary. The significance level was set

Table 1 Effects of the factors mycorrhization (inoculation with *Piloderma croceum*), collembola (presence of *Protaphorura armata*), herbivory (feeding by *Lymantria dispar* larvae), mycorrhization/collembola (*P. croceum* and *P. armata*) mycorrhization/herbivory (*P. croceum* and *L. dispar*) and all factors combined (*P. croceum, P. armata* and *L. dispar*) on biomass, analysed in a three-way ANOVA. t-values were calculated using the lm function implemented in R; significant t-values are in bold, signs indicate direction of the effects ($p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: ***).

	t- values							n
Plant fraction (dry mass)	*P. croceum*	*P. armata*	*L.dispar*	P. croceum * P. armata	*P. croceum* * *L. dispar*	*P. armata* * *L. dispar*	*P. croceum* * *P. armata* * *L. dispar*	
Sink leaves	–0.89	–0.85	–0.06	**2.05 ***	–0.06	–0.16	–0.7	63
Source leaves	**2.65 ***	–0.83	**3.01 ****	1.21	**–1.94 (*)**	–0.16	–0.34	63
Total above ground biomass	**2.94 ****	–0.53	**2.38 ***	1.19	1.32	0.05	–0.76	63
Total below ground biomass	**2.28 ***	–0.31	**3.24 ****	–0.29	–1.05	–0.84	0.58	63
^{13}C excess								
Sink leaves	0.11	–1.17	0.57	–1.19	**2.02 ***	–0.07	–0.46	55
Source leaves	1.47	**2.88 ****	1.53	–0.77	–0.66	–1.47	0.53	53
Roots	**2.82 ****	0.68	1.39	–0.57	–0.92	0.55	0.21	52
^{15}N excess								
Sink leaves	–1.21	0.31	–0.98	**1.68 (*)**	0.44	–0.63	–0.21	55
Source leaves	0.34	1.43	–0.77	–0.06	–0.88	–0.65	0.38	53
Roots	0.26	-0.06	0.17	1.32	0.89	0.09	–0.15	52

to $P < 0.05$; values for $0.05 \leq P < 0.10$ were defined as marginally significant. To quantify caterpillar performance, we calculated according to Waldbauer (1968), relative consumption rate [RCR = leaf area consumed [cm^2]·(initial larval weight [mg])$^{-1}$·(time)$^{-1}$); relative growth rate: [RGR = (larval biomass gained [mg]·(initial larval weight [mg])$^{-1}$·(time)$^{-1}$)]; relative consumption efficiency: [RCE = final larval weight [mg]−(initial larval weight) [mg]·(leaf area consumed) [cm^2]$^{-1}$. Performance of the Collembola population was calculated as fitness rate (final number of individuals at harvest, divided by initial number; n=50 per seedling). These parameters were used as dependent variables in a three factorial ANOVA with the factors inoculation and Collembola.

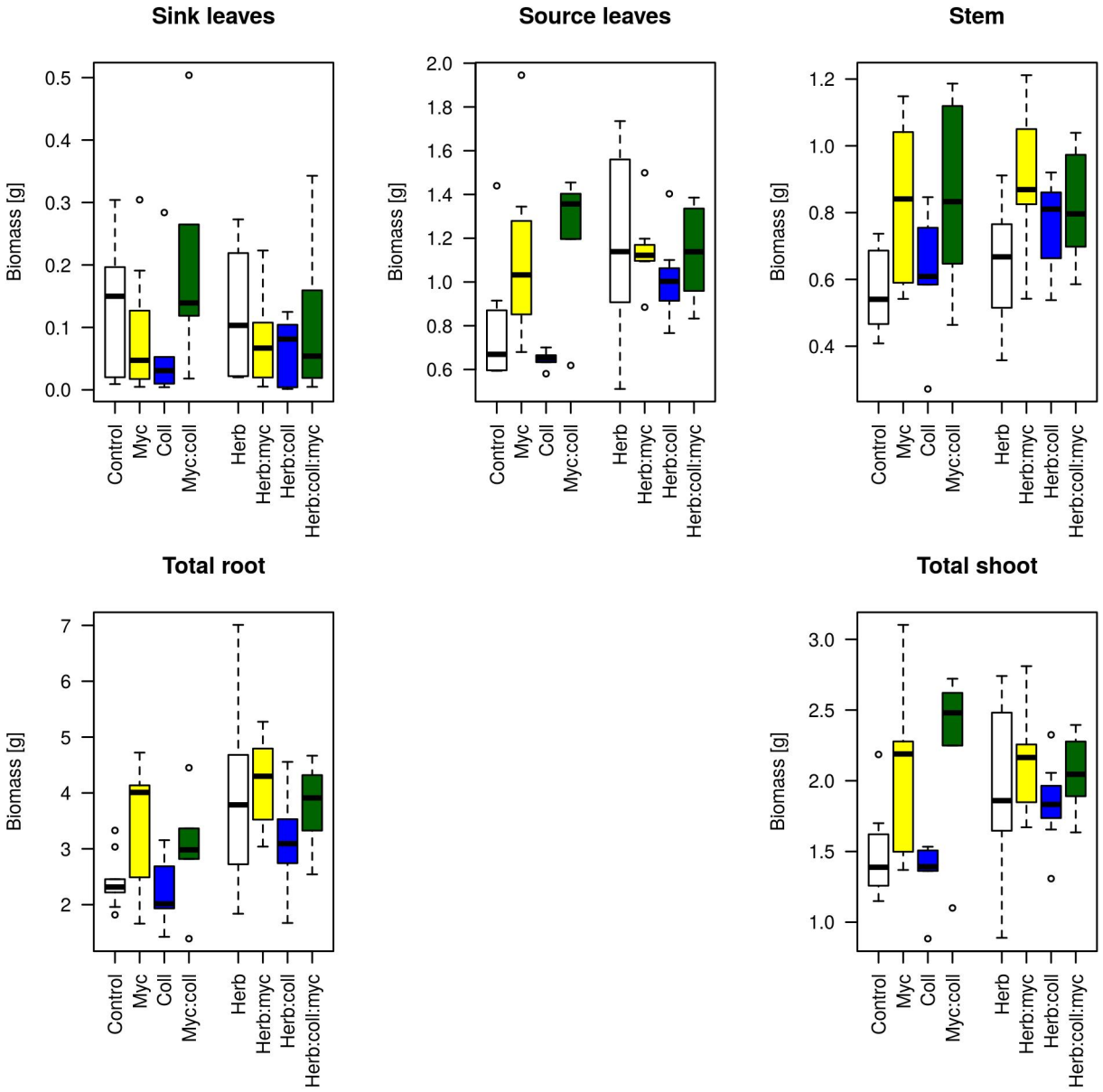

Figure 1 Effect of mycorrhization on the biomass (dry weight) of *Quercus robur* seedlings. Oaks were fed to larvae of *Lymantria dispar* (herb; right side of each boxplot), inoculated with *Piloderma croceum* (myc; yellow boxes), accompanied by *Protaphorura armata* (coll; blue boxes), inoculated with *Piloderma croceum* and *Protaphorura armata* (myc/coll; green boxes), or not inoculated with the fungus and (control; colorless boxes). Bars in the boxplots indicate the upper and lower quartile with median; whiskers indicate minimum and maximum values; dots are outliers.

Results

A multi-factorial ANOVA revealed a significant increase in root and shoot biomass in presence of the mycorrhizal fungi, showing that the inoculation was successful (Fig 1; Table 1). Presence of Collembola increased the mass of sink leaves, but only in the combined treatment with mycorrhiza. Biomass of source leaves, as well as roots was increased by the leaf

herbivore. An interacting effect of inoculation with *P. croceum* in combination with herbivory caused a marginally significant decrease in source leaf mass.

Bioassay of *Lymantria dispar* and *Protaphorura armata*

Caterpillar mortality was not affected by inoculation with the mycorrhizal fungus or the presence of Collembola, all caterpillars survived the feeding experiment. The bioassay variables acquired for *L. dispar,* RGR, RCR and RCE were not influenced by the factors inoculation or Collembola, nor was there any significant interacting effect. Survival rate of *P. armata* ranged from 4.49 to 0.96; enhanced marginally significant by herbivore feeding only on the leaves (t=1.88; p=0.07).

Incorporation and allocation of C/N

On whole-plant level, excess of whether ^{13}C nor ^{15}N was altered.

Analysing the plant fractions of oak seedlings (source leaves, sink leaves and roots) we found increases of both ^{13}C and ^{15}N excess while not ^{13}C nor ^{15}N was decreased significantly (Table 1). Roots of oak

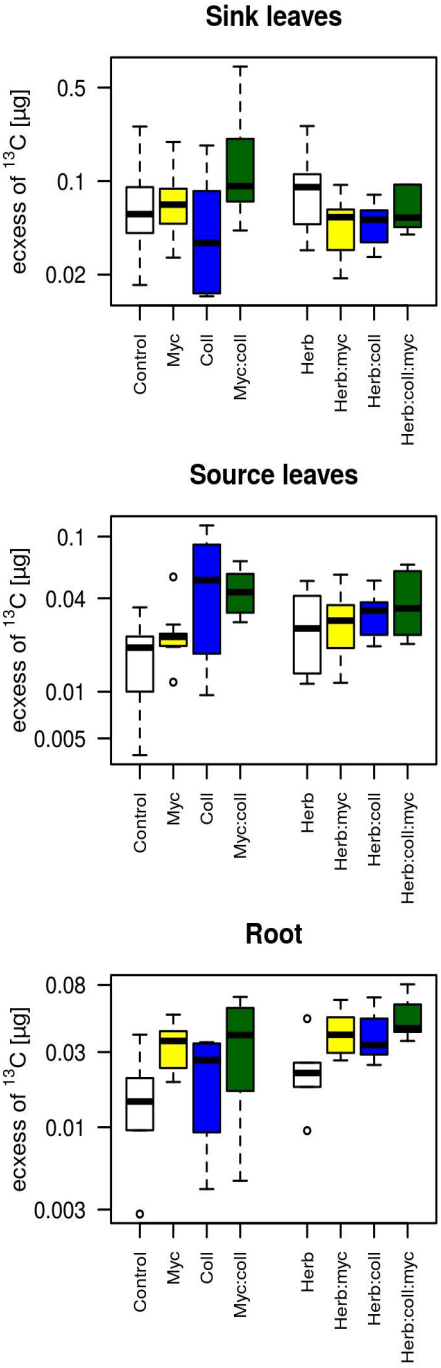

Figure 2 Effect of mycorrhization, collembola, herbivory, mycorrhization/collembola, mycorrhization/herbivory and mycorrhization/collembola/herbivory on the excess of ^{13}C of plant fractions of oak seedlings, calculated over unlabeled oak samples. Seedlings were fed to larvae of *Lymantria dispar* (herb; right side of each boxplot), inoculated with *Piloderma croceum* (myc; yellow boxes), accompanied by *Protaphorura armata* (coll; blue boxes), inoculated with *Piloderma croceum* and *Protaphorura armata* (myc/coll; green boxes), or not inoculated with the fungus and (control; colorless boxes). Bars in the boxplots indicate the upper and lower quartile with median; whiskers indicate minimum and maximum values; dots are outliers. Note that the y-axes are log-transformed.

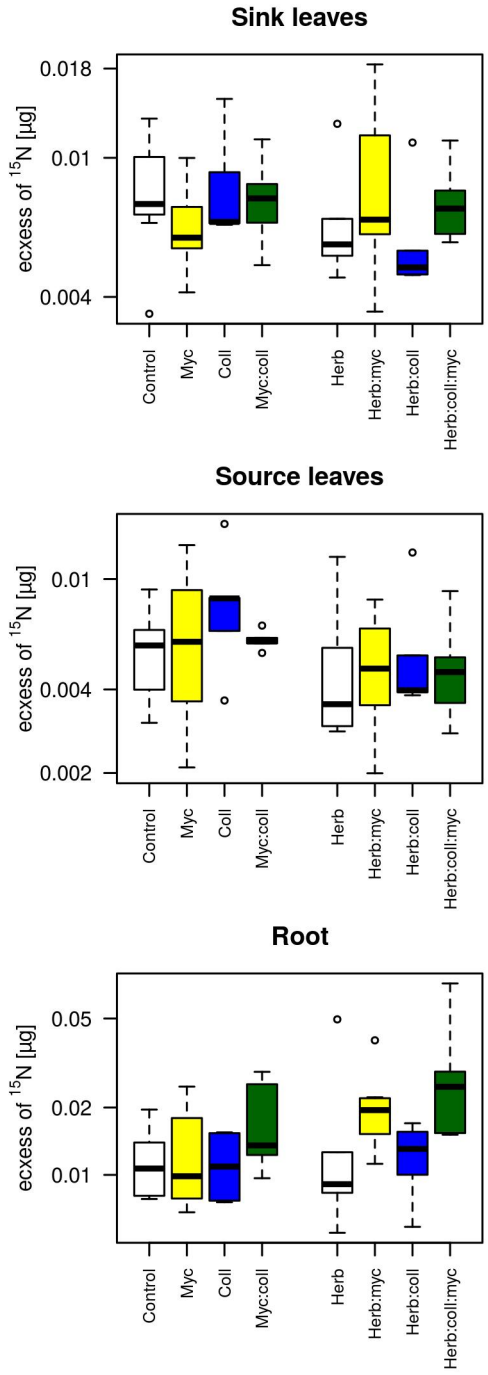

Figure 3 Effect of mycorrhization, collembola, herbivory, mycorrhization/collembola, mycorrhization/herbivory and mycorrhization/collembola/herbivory on the excess of ^{15}N of plant fractions of oak seedlings, calculated over unlabeled oak samples. Seedlings were fed to larvae of *Lymantria dispar* (herb; right side of each boxplot), inoculated with *Piloderma croceum* (myc; yellow boxes), accompanied by *Protaphorura armata* (coll; blue boxes), inoculated with *Piloderma croceum* and *Protaphorura armata* (myc/coll; green boxes), or not inoculated with the fungus and (control; colorless boxes). Bars in the boxplots indicate the upper and lower quartile with median; whiskers indicate minimum and maximum values; dots are outliers. Note that the y-axes are log-transformed.

seedlings inoculated with *P. croceum* had significant higher levels of ^{13}C compared to the controls. Presence of Collembola in the soil had a positive effect on the incorporation of 13C in source leaves. On sink leaves, only the combination treatment with inoculation and herbivory showed an enhanced incorporation of ^{13}C compared to the control. ^{15}N was solely increased in the combination treatment with the mycorrhizal symbiont and Collembola combines in the soil (Figs 2 and 3).

Differences in plant fractions of excess of ^{13}C and ^{15}N on whole-plant excess were used to assess allocation patterns (Table 2; Figs 4 and 5). Inoculation with *P. croceum* enhanced allocation of C to the roots. Collembolans in the soil enhanced the allocation of C to source leaves, while C allocation to sink leaves was reduced. In contrast, C allocation to sink leaves was enhanced in oak seedlings subjected to mycorrhiza and Collembola in comparison to the control. Allocation of C was not mediated by leaf herbivory, neither alone nor in combination with *P. croceum* or *P. armata*.

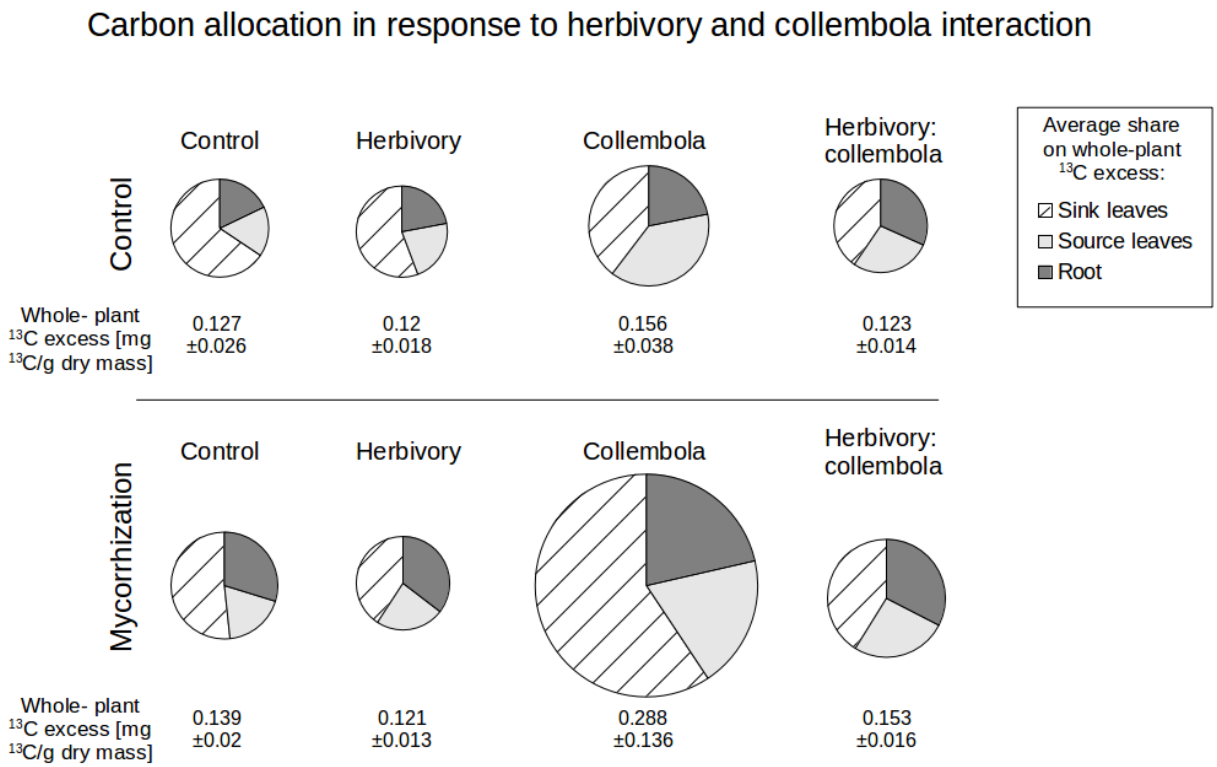

Figure 4 Carbon allocation in response to *Protaphorura armata* (collembola) in the soil, leaf herbivory by feeding of *Lymantria dispar* (herbivory), collembola and leaf herbivory (herbivory:collembola). Upper part shows data without mycorrhization, lower part shows data of seedlings inoculated with *Piloderma croceum*. Pie chart area gives whole-plant ^{13}C excess (see values below), C allocation pattern to plant fractions is reflected by pie slices (means ± SE).

Nitrogen allocation was not significantly mediated by any of the factors (Fig 5).

DISCUSSION

The role of plants as primary producers and resource for consumers, as well as their interaction with other organisms, has been intensively studied and is rather well understood (Gange, 2000; Kessler *et al.*, 2006; Pierik *et al.,* 2013). However, understanding of interrelationships of organisms above- and below-ground, and the role of plants as linkage between these two realms still is in its infancy (Bardgett *et al*., 1998; Van der Putten & Vet, 2001; Eisenhauer *et al*., 2011). From the perspective of plant vitality and plant defense strategies, understanding of plant induced defense by above- and belowground mutualists or antagonists will unravel processes influencing other plant associated organisms, in particular herbivores and pathogens

(Bezemer & Vandam, 2005). Until today knowledge on interactions of mycorrhizal plants with above- and belowground herbivores as well as animals involved in the decomposition process is limited to herbaceous plants, in particular crop species and weeds (Blossey & Hunt-Joshi, 2003; Vos *et al*., 2013).

The results showed that oaks indeed respond to the studied biological interactions, including changes in morphology, C and N concentrations and nutrient and carbon allocation to different plant compartments. The results mirror the complex interactions which oak seedlings are facing during early development and early phases of mycorrhization, meaning the interaction of *P. croceum* and *P. armata* which impacted oak sink leaf biomass and ^{15}N tissue concentration. In addition a *P. croceum* and *L. dispar* interaction impacted source leaf biomass negatively and increased sink leaf 13C tissue concentration. There is already a large body of data

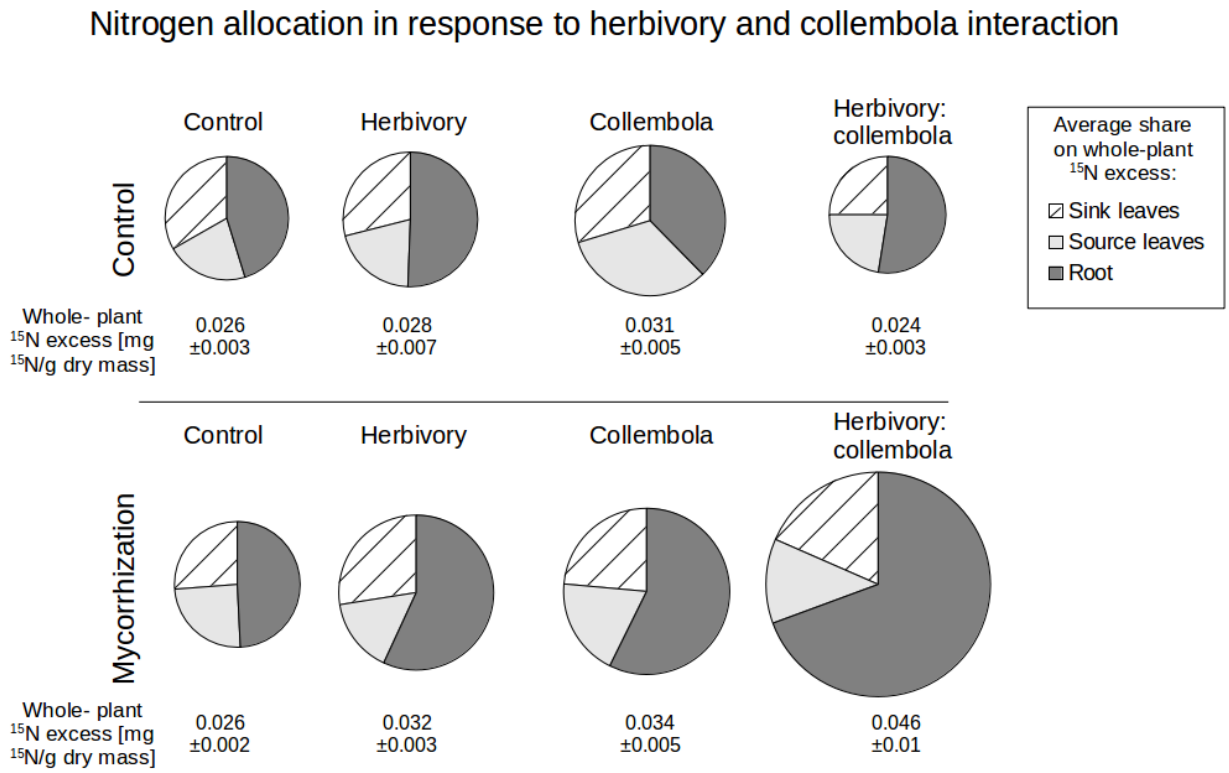

Figure 5 Nitrogen allocation in response to *Protaphorura armata* (collembola) in the soil, leaf herbivory by feeding of *Lymantria dispar* (herbivory), collembola and leaf herbivory (herbivory:collembola). Upper part shows data without mycorrhization, lower part shows data of seedlings inoculated with *Piloderma croceum*. Pie chart area gives whole-plant ^{15}N excess (see values below), C allocation pattern to plant fractions is reflected by pie slices (means ± SE).

describing this kind of premycorrhizal effects (e.g. (Frettinger *et al*., 2006a, 2007; Felten *et al*., 2009). However, we were able to add important knowledge regarding its influence in multitrophic systems.

As expected, the presence of *P. croceum* as ectomycorrhizal symbiont to oak seedlings stimulated plant growth. This was reflected by increased root and shoot biomass but also the increased incorporation of assimilated carbon into roots (see also Sanders, 1997; Herrmann *et al*., 1998; Smith & Read, 2010). Increased incorporation of ^{13}C into the root systems indicates that inoculation with *P. croceum* resulted in the formation of a fully functional oak-mycorrhiza-symbiosis. These findings confirm our first hypothesis in terms of enhanced growth of mycorrhized oak seedlings, as well as enhanced C incorporation. Although we found no increase of N incorporation to sink leaves, we could show an increase of sink leaf biomass in mycorrhized oaks.

Herbivory affects plants in various ways, as well as to other associated, interacting organisms, such as mycorrhizal fungi. Defoliation by herbivores decreases leaf area available for photosynthesis, but can also increase light levels to previously shaded portions of the canopy, thereby increasing photosynthetic capacity in remaining leaves (Mabry & Wayne, 1997). Further, plant sap sucking aphids indirectly interact with AM fungi through plant metabolism. The AM presence increased the attractiveness of host plant to aphids, whereas aphids inhibit the formation of a plant-mycorrhiza-symbiosis between *Vicia faba* and different AM fungi (Babikova *et al.*, 2014). In addition, mycorrhizal hyphae networks are capable of communicating informations about herbivore attack between plants (Babikova *et al.*, 2013). Compensatory growth is a common but costly strategy of plants against herbivores (McNaughton, 1983; Stowe *et al.*, 2000; Haukioja & Koricheva, 2000). Interestingly, in non-inoculated oaks of our experiment, herbivory resulted in an increase in biomass of source leaves and roots suggesting that the plants responded to herbivory with compensatory growth which may also be viewed as

defense reaction (Strauss & Agrawal, 1999). This is in contrast to our second hypothesis, where we suspected that direct defense mechanisms as compensatory growth should be more pronounced in mycorrhizal oaks. Notably, *Piloderma* inoculated oaks responded to herbivory in part oppositely, i.e. with reduced biomass of source leaves, suggesting that plants modify their defense response in presence of multiple interacting partners.

Collembola also affected plant biomass depending on the presence of mycorrhiza. In presence of mycorrhiza, the presence of Collembola increased biomass of sink leaf. This effect was associated with an increased ^{15}N tissue concentration in sink leafs. Collembola are known to increase nutrient mineralization and plant growth (Partsch *et al*., 2006, Ke & Scheu, 2008), further Bardgett & Chan (1999) showed N mineralization and shoot N concentration in *Nardus stricta* to be increased in presence of Collembola, with the effect being more pronounced in presence of nematodes. But Collembola interaction with ectomycorrhizal trees is little studied. In line with these findings, we found increased ^{13}C excess in photosynthetically active source leaves of oaks in presence of Collembola. A preference of some Collembola species for mycorrhiza infected roots, though in arbuscular mycorrhiza, has been discussed in the literature (Thimm & Larink, 1995; Caravaca & Ruess, 2014). Our results indicate a positive interaction effect between the mycorrhizal fungus and Collembolans. The symbiosis with *P. croceum* seems to increase nutrient uptake even more, assumingly due to the extended hyphal network and Collembolan mineralized nutrients. Therefore we reject our third hypothesis.

Further, Collembola presence is known to alter root morphology in *Trifolium repens* and reduce shoot biomass in *Lolium perenne* while enhancing root length and number of root tips (Endleweber & Scheu, 2007). In another study it was shown that Collembola occurrence in the non-mycorrhizal rhizosphere of *Trifolium repens* can have a negative impact on the reproduction of the

aphid *Myzus persicae* (Scheu *et al.*, 1999). Overall it can be assumed that Collembola density is crucial for the out coming impact on plant growth and development (Gange, 2000).

CONCLUSIONS

Our results suggests once more that ectomycorrhizal symbionts, and even its premycorrhizal effects are of crucial importance to trees in natural ecosystems. In this study we didn't found evidence for hyphal consumption of *P. armata* on *P. croceum* and no negative effects on performance of mycorrhized oak. Furthermore, we found none of the expected interactive effects of above-ground herbivory and Collembolan below-ground. However, this study suggests a strong compensatory growth reaction of oak seedlings after exposure to leaf chewing caterpillars with a soothing effect of *P. croceum* on this reaction.

ACKNOWLEDGEMENTS

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Chapter V

Summary and outlook

Several studies, including the present dissertation, have illustrated the ecological importance of trophic interactions between species. Centred on a plants point of view, this includes defense responses, nutrient supply supported by symbionts and decomposers, as well as interacting effects linking different kinds of interaction. Due to the complexity of natural habitats, a suitable way for studying trophic interactions in a multi-factorial way was of urgent need.

The second chapter is devoted to the construction of a reliable reference data base for large scale gene expression analysis. The OakContigDF159.1 provides a reliable reference to study gene expression patterns, e.g. triggered by different trophic or multitrophic interactions.

In order to provide a reliable reference which covers the largest possible number of gene transcripts, we treated micropropagated cuttings of the pedunculate oak (*Quercus robur*) with seven different interacting species plus control treatment. These microcuttings had to cope with an ectomycorrhizal fungus *(Piloderma croceum*), a leaf herbivore (*Lymantria dispar*), a Collembolan species (*Protaphorura armata*), a leaf pathogen (*Microsphaera alphitoides*), a root feeding nematode (*Pratylenchus penetrans*), a root pathogen (*Phytophthora quercina*) and a mycorrhiza helper bacterium (*Streptomyces* AcH 505) in experiments under controlled laboratory conditions. After an initial normalisation of RNA templates, sequencing using the two architectures, 454 pyrosequencing and Illumina sequencing took place. Further, we used the Mimicking Intelligent Read Assembly (MIRA) and the Trinity assembler, to construct the OakContigDF159.1 hybrid assembly, containing 65,712 contigs with a mean length of 1003 bp. Illumina constructed cDNA libraries were used to examine gene expression induced by mycorrhiza formation. After aligning the cDNA libraries of fine roots against the reference, reads were quantified by RSEM, differences in gene expression was measured in R, revealing 3018 differentially expressed contigs, of which 1399 were up-regulated and 1619 down-regulated in oak EMs with *P .croceum*.

Our data support and confirm the view that gene families are precisely regulated to adjust the plants metabolism to mycorrhizal symbiosis, rather than by expression of symbiosis-specific genes. Furthermore, our findings of e.g. down-regulated chitinases confirmes that defense-related genes are attenuated in mature mycorrizal symbiosis. taking account to the mycorrhizal symbiosis, the expression of a SWEET1 glucose transporter could indicate a direct export of

hexose into the plant apoplast to support the fungus and may suggest the existence of a complementary sugar exchange mechanism in mycorrhizal symbiosis in oak.

As mycorrhizal fungi and herbivores interactively influence the growth, resource utilization, and defense of plants, the third chapter shall provide a deeper insight in actual gene expression changes and metabolic adjustments of oak facing herbivory in a system including mycorrhizal fungi.

Again, microcuttings of the *Quercus robur* clone DF159 served as a experimental platform for a full factorial experiment. In order to examine the separate and combined effects of a mycorrhizal fungus and a leaf chewing caterpillar on oak, I analysed changes in gene expression and nutrient allocation patterns on a total of 71 ^{13}C and ^{15}N labelled microcuttings, of which 35 were in root flush and 36 in shoot flush. First, I extracted total RNA templates of pooled samples which were further used to produce 100-bp paired-end libraries and sequenced using an Illumina HiSeq 2000. After alignment against the reference transcriptome OakContigDF159.1, significance of differences in gene expression was measured via pairwise comparisons between contigs of control vs. herbivory, control vs. inoculation with *P. croceum*, control vs. inoculation/herbivory, separately for plants in root flush and for plants in shoot flush. Furthermore, I analysed changes in nutrient incorporation using calculated differences of ^{13}C and ^{15}N excess over unlabelled control plants. Significance of effects was tested using a three-way ANOVA.

I found an increased expression of genes related to compensatory growth and direct defense in oaks in root flush, an effect that was attenuated in presence of *P. croceum*. Further was the expression of genes related to the production of VOC increased in oak inoculated with *P. croceum* and exposed to *L. dispar*. I found diverse effects of the growth stage, inoculation and herbivory on the incorporation of nutrients. Additionally I found, on the one hand an increased incorporation of ^{13}C and ^{15}N in sink leaves of oaks exposed to herbivory, on the other hand the incorporation of ^{13}C decreased in source leaves of oaks in shoot flush and exposed to herbivory. In stems, the incorporation of both, ^{13}C and ^{15}N was decreased in oak exposed to herbivory, an effect that was reversed in inoculated oak exposed to herbivory.

Taken together, these findings indicate that oaks may switches from direct herbivore defense mechanism, including compensatory growth, during root flush to the indirect defense mechanism of producing chemicals attractive to natural enemies of herbivores when the

plant benefits from pre-mycorrhizal effects.

The experiment described in the fourth chapter was designed to investigate above-ground and below-ground interaction of herbivory, collembola and mycorrhization. I used a four-factorial system comprising seedlings of *Quercus robur, Lymantria dispar*, *Protaphorura armata* and *Piloderma croceum*. Under controlled laboratory conditions I applied a ^{13}C and ^{15}N labelling prior to the feeding experiment with the leaf chewing caterpillars.

I found strong pre-mycorrhizal effects, reflecting an increased root and shoot biomass but also an increased incorporation of currently assimilated carbon into the root system. The results showed further, that oaks indeed respond sensitively to the studied biological interactions, including changes in morphology, C and N concentrations and nutrient and carbon allocation to different plant fractions.

I didn't found evidence for hyphal consumtion of *P. aramta* and no negative effects of collembola presence on performance of oak inoculated with a mycorrhizal fungus. Furthermore, I found none of the expected interactive effects of above-ground herbivory and below-ground collembolan activity. However, this study suggests a strong compensatory growth reaction of oak seedlings after exposure to leaf chewing caterpillars with a soothing effect of *P. croceum* on this reaction.

Outlook

The present dissertation offers detailed information about the effects caused by interactions between the partners tree, mycorrhizal fungus and herbivorous insect and elucidates the importance of future research in the multitrophic context. Even though I added a collembolan population to cover also one type of below-ground interaction, the picture drawn is far from being complete.

- Following the example given in chapter four (Nutrient allocation in oak as affected by detritivore – herbivore – mycorrhiza interactions), I advice the combination of further interactions around the oak, e.g.: leaf pathogen, root pathogen, root feeding nematode and mycorrhizal helper bacteria. The construction of the OakContigDF159.1 reference transcriptome and the following gene expression surveys on the suggested interactions allows the detection of target genes. Due to the knowledge gathered by previous work, future experiments examining gene expression patterns will become much cheaper,

by sequencing short fractions of the genom/transciptome instead of the whole transcriptome, or by gene expression measurements using qRT-PCR technology.

- The reaction of the mycorrhizal partner in these trophic interaction remains unclear. Therefore, a change in the point of view could offer a promising avenue for future studies. The genome and the transcriptome of *Piloderma croceum* strain "F 1598" could be used for experimental surveys like those in the present thesis, but with the inclusion of a gene expression survey on the site of the fungus. On the one hand this may lead to promising insights in the establishment of mycorrhizal symbiosis. On the other hand, knowledge about fungal reaction on above-ground herbivory, hyphal consumption, root feeding nematodes ect., could be gathered.
- In preparation of future research, microcuttings of *Quercus robur* clone DF159 have been planted in open field trials, in Kreinitz and Bad Lauchstädt. This allows further studies under natural conditions. The genetic uniform microcuttings are characterised under the conditions of several separate interactions, which makes them an ideal model system to study environmental variability. Furthermore, coupled with experimentations on litter decomposition, including the characterization of the involved microorganisms and enzymatic processes, this release of oaks as living analysis systems could be used as a kind of a "Phytometer". Alongside to research on biotic factors, also the effect of climate change, draught periods etc. could be studied. The planting of microcuttings on the fields of the diversity experiment in Kreinitz, would allow to study even effects of tree diversity on gene expression of oaks, which would be a great opportunity in this field of research.

Deutsche Zusammenfassung

Mit der vorliegenden Dissertation konnte ich die Bedeutung von trophischen Interaktion, als Wechselwirkung zwischen Arten darstellen. Betrachtet man dies von der Seite der Pflanzen aus, zeigen sich sowohl Effekte wie Verteidigungsreaktionen, Änderungen in der Nährstoffverteilung innerhalb der Pflanze als auch interagierende Effekte. Die Komplexität natürlicher Habitate hat es bisher sehr schwierig gemacht solche Interaktionen zu untersuchen. Die Möglichkeit ein komplexes System ins Labor zu bringen und Untersuchungen unter kontrollierten Bedingungen mit mehreren Faktoren durchführen zu können, stellt einen großen Vorteil dar und soll im Folgenden näher beleuchtet werden.

Das erste Kapitel dieser Dissertation ist einer allgemeinen Einleitung in das Thema gewidmet.

Im zweiten Kapitel stelle ich das experimentelle System näher vor. Des Weiteren beschreibe ich die Konstruktion der verlässlichen Referenzdatenbank OakContigDF159.1, welche das Studium von Genexpression auf Gesamttranskriptom-Ebene ermöglicht. Damit das Referenztranskriptom eine möglichst hohe Anzahl an Transkripten aus dem Genom der Stieleiche (*Quercus robur*) beinhaltet, behandelten wir microcuttings der Stieleiche mit insgesamt sieben Arten, welche jeweils eine andere Form der trophischen Interaktion mit der Eiche eingehen. Zusätzlich einer Kontrolle wurden die microcuttings *Piloderma croceum, Lymantria dispar, Protaphorura armata, Microsphaera alphitoides, Pratylenchus penetrans, Phytophthora quercina and* Streptomyces AcH 505 ausgesetzt. Normalisierung und anschließende Sequenzierung mittels Illumina und 454 Pyro-Sequenzierung resultierten, nach Nachbereitung mit MIRA und Trinity assembler, in der OakContigDF159.1 Datenbank.

Im dritten Kapitel untersuche ich mit Hilfe der Referenzdatenbank OakContigDF159.1 differenzielle Genexpression, sowie Änderungen in der Nährstoffverteilung. Hierzu habe ich ein multifaktorielles Experiment mit *Piloderma croceum* und *Lymantria dispar* durchgeführt. Die Versuchspflanzen wurden im Vorfeld mit den stabilen Isotopen ^{13}C und ^{15}N markiert, um nach der Ernte den Isotopen-Überschuss mit einer unmarkierten Kontrolle zu vergleichen und mittels einer 3-faktoriellen ANOVA zu analysieren. Zusätzlich wurde die Gesamt-RNA extrahiert und mittels Illumina sequenziert. Die enhaltenen cDNA-Bibliotheken habe ich an die Referenzdatenbank angepasst und erhielt so 35

Transcriptome welche ich weiterhin zur paarweisen Berechnung von differentieller Genexpression verwendete.

Pflanzen die mit dem Mykorrhizapilz *Piloderma croceum* inokuliert waren, zeigten starke prämykorrhizale Effekte, welche sich z.B. in einer Erhöhung der Biomasse zeigten. Herbivorie verursachte von Genen die mit kompensatorischem Wachstum in Verbindung stehen, sowie eine verstärkte Expression von Primär-Verteidigungs Genen. Dieser Effekt wurde durch inokulierung mit *P. croceum* abgeschwächt. Die Expressionsdaten ließen zudem bei inokulierten und befressenen Eichen auf eine erhöhte produktion von volativen organischen Substanzen schließen, die der Attraktion von Fraßfeinden der Herbivoren dienen. Zusätzlich zeigte sich bei Pflanzen mit Herbivorenfraß eine erhöhte Aufnahme von ^{13}C and ^{15}N in junge Blätter, wohingegen sich die Aufnahme von ^{13}C in reife Blätter durch Herbivory verringert zeigte.

Auf diesen Ergebnissen folgerte ich, dass eine Interaktion mit einem Ekto-Mykorrhiza Pilz, selbst in einem frühen Stadium der Symbiose, deutliche Effekte auf das Abwehrverhalten einer Pflanze haben kann. Hier wurde die heftige Abwehrreaktion, inkl. kompensatorischen Wachstums, durch den Pilz abgeschwächt und sogar verlagert auf Mechanismen der sekundären Abwehr. Die Gegenwart des Pilzes scheint eine "beruhigende" Wirkung auf die Pflanze auszuüben.

Das vierte Kapitel ist der genaueren Untersuchung der ober- und unterirdischen Wechselwirkungen der verschieden trophischen Partner gewidmet. Hierzu führte ich ein multifaktorielles Experiment mit den trophischen Partnern *Piloderma croceum, Lymantria dispar* und *Protaphorura armata* durch. Auch hier fand ich starke prä-mykorrhizale Effekte, sich die in einer Erhöhung der Biomasse, analog zu einer erhöhten Einlagerung von ^{13}C and ^{15}N in *P. croceum* inokulierten Eichen zeigten. Weiterhin zeigte sich eine Erhöhung der Biomasse bei Eichen die *L. dispar* ausgesetzt waren. Obwohl ich keine Anzeichen für eine Interaktion der ober- und unterirdischen Interaktoren der Eiche fand, hatten alle Faktoren einzeln betrachtet deutlichen Einfluss auf die Nährstoffverteilung. Ich konnte keine Hinweise von Hyphenfraß durch Collembolen finden, ebenso konnte ich keine negativen Effekte der Collembolenpopulation auf inokulierte Eichen feststellen. Im Gegenteil zeigte sich eine erhöhte Nährstoffaufnahme, was ich als Hinweis auf gesteigerte Nährstoffmineralisierung durch die Collembolen deutete.

Appendix Chapter II

SUPPORTING METHODS

Interaction of oaks with mycorrhization helper bacterium *Streptomyces* sp. AcH 505

Streptomyces sp. AcH 505, isolated by Andreas Maier from the soil around Norway spruce mycorrhizas in Haigerloch, Germany , was maintained on ISP2 agar medium. For AcH 505 treatment, roots of individual oak microcuttings were inoculated twice (3 weeks and 1.5 weeks before harvest) with 2.5 x 10^7 bacterial spores. In a preliminary experiment, this level of bacterial application was shown to significantly increase mycorrhiza formation on oak microcuttings after eight weeks.

Interaction of oaks with the oak powdery mildew *Microsphaera alphitoides*

Microsphaera alphitoides was obtained from infected oak leaves from a natural woodland, and after verification of its identity by PCR with specific ITS primers , it was maintained on oak leaves. Heavily infested leaves were used to infect oak microcuttings. Mildew developed homogeneously on emerging leaves when fungal spores were inoculated onto swelling and bursting buds. For the experiment, oak buds and young leaves were inoculated with 1.5 x 10^6 *M. alphitoides* spores per plant 14 days before harvest. Spore quantity was calculated by collecting and counting spores from mock inoculations in a Fuchs-Rosenthal counting chamber. Infection became visible after seven to ten days.

Interaction of oaks with the root pathogen *Phytophthora quercina*

The *Phytophthora quercina* T. Jung strain QUE 6 (CBS 789.95) was selected for the present work. The cultures were grown on V8 agar for 14 days in darkness at 20 °C. Production of zoosporangia was induced by flooding the culture first with sterile distilled water for 7 days and subsequently with sterile soil solution for 5 more days, with daily exchange in each case. On the day of pedunculate oak inoculation, soil solution was replaced with 10 ml sterile distilled water and the release of zoospores was induced by chilling the cultures for 1 hour at 4 °C followed by 30 minutes of incubation at room temperature. After gathering the zoospores and placing them on ice, the zoospore concentration was determined using an Abbe-Zeiss cell counting chamber and adjusted to 2.0 x 10^5 zoospores per ml. 5 ml of the suspension were applied in concentric circles onto the pre-flooded (with 50 ml sterile distilled water for one hour) roots and incubated for 5 hours in a horizontal position. After removing the surplus of water, the microcosms were re-sealed and returned to an upright position. Plants were inoculated

with *P. quercina* one week before harvest. At the time of harvest, *P. quercina* was re-isolated from fine roots to monitor the success of the infestation.

Interaction of oaks with the gypsy moth *Lymantria dispar*

Egg masses of gypsy moth (*Lymantria dispar* L.*)* were obtained from a lab colony of the New Jersey Standard Strain. Larvae were reared on an artificial diet based on wheat germ at 25 °C, 60 – 70 % relative humidity and a 16 h day and 8 h night photoperiod. For the oak-gypsy moth system, one third-instar larva of *L. dispar* was added to each microcutting. For this purpose, the whole plant was caged in 200 µm nylon mesh. Plants were inspected each hour and inactive larvae were replaced by active ones. Larvae avoided feeding on very young leaves and on the older leaves from the first shoot flush and generally preferred fully developed mature leaves. Larvae were allowed to consume a leaf area corresponding to 30 - 50 % of the fully developed leaf area of each plant. After 6 hours, all larvae were removed from the microcuttings, and the plants were harvested.

Interaction of oaks with the root nematode *Pratylenchus penetrans*

Axenic cultures of *Pratylenchus penetrans* (Cobb) Philip & Stek**,** kindly provided by Johannes Hallman (Julius Kühn-Institute, Germany)**,** were established and multiplied on carrot disks according to . For the experiment, 5 ml aliquots of nematode suspension in water were evenly applied to the root system, with each plant receiving ≈10,000 nematodes. Plants were incubated with *P. penetrans* for 7 days prior to plant harvest. Roots were stained with acid fuchsine and microscopic observation was used to reveal root penetration by *P. penetrans.*

Interaction of oaks with Collembola *Protaphorura armata*

Protaphorura armata was taken from the culture collection of the Animal Ecology Group of the Georg August University Göttingen. The animals were kept on a mixed substrate of potting compost and clay. For the experiments with oak microcuttings, ninety *P. armata* individuals were introduced into each oak microcosm one week before harvest of microcuttings.

Decontamination: Removal of 454 and Illumina reads from oak-interacting organisms

Blastx bit-scores were used to evaluate homologies between the reads produced in this study and reference sequences (Fig. S1). All reads were first subjected to blastx searching against the Plant RefSeq protein dataset (P). A read was considered to be potentially homologous to a RefSeq plant sequence if the blastx bit-score was >

10. Reads with a lower bit-score (i.e. with less homology to plant sequences) were discarded. The remaining reads were blastx-searched against a common dataset (C) to remove general sequence contaminants. In addition to the RefSeq accessions of taxa listed in Figure S1b, the genome of *Piloderma croceum* (version 1.0, http://genome.jgi.doe.gov/Pilcr1/Pilcr1.home.html, Joint Genome Institute, USA) was included in the common dataset. In order to identify specific sequence contaminants from the organisms used for inoculation, the following 454 libraries were subsequently decontaminated with specific (S) datasets (see Fig. S1b for the taxa included): *Streptomyces* sp. AcH 505*, Phytophthora quercina, Lymantria dispar, Protaphorura armata* and *Pratylenchus penetrans.* The draft genome of *Streptomyces* sp. AcH 505 (T. W., F. B., L. F., M. T., unpublished) was included in the specific dataset for *S.* sp. AcH 505.

A read was considered to be a potential contaminant if it had a blastx bit-score > 10. We observed that the candidate contaminants included some sequences of possible plant origin, and these reads were subjected to a final blastx analysis against plant, common and specific datasets. When the homology of such reads was higher to plant accessions than to the accessions in common or specific datasets, they were regarded as being sequences of plant origin. In Figure S1 these three steps have been indicated as circle-1 indicating the blastx-search plant RefSeq protein dataset, circle-2 the blastx-searches against common and specific decontamination datasets, and circle-3 the retrieval of plant sequences from the candidate contaminant sequences, respectively.

The decontamination of reads was an iterative process. After the first complete decontamination cycle described above, the retained reads were assembled. However, blastx search of these assembled contigs against the nr database of NCBI indicated that this primary contig assembly might still be contaminated with sequences of fungal and bacterial origin. In order to eliminate this residual contamination, the common dataset (C) was subsequently extended to include protein sequences from the contaminating organisms detected, and the decontamination process was repeated. The retained reads were again assembled and the assembly subjected to a blastx search against the nr database. As expected, the best blastx matches were against plant datasets (Fig. S4). Out of 65,712 contigs, 88% (57,940 contigs) showed highest blastx identity to sequences from higher plants, and 1.4% (941 contigs) to sequences from other organisms. The remaining 6,831 contigs showed no significant matches to sequences in the nr

database with an e-value cutoff of 1e-5.

Construction of OakContigDF159.1 hybrid assembly

A 454/Illumina read hybrid assembly approach was selected in order to combine the different read types. The strategy used for hybrid assembly included a pre-assembly of 454 reads and integration of the 454 contigs with Illumina reads as overlapping 100 bp 454 sequences (100-mers or pseudo-Illumina reads). To estimate the quality of the hybrid assembly, an additional assembly of Illumina reads alone was performed.

The pre-assembly of 454 reads was implemented with MIRA 3.4.1.1 . Assembly parameters were chosen based on the following metrics. The number of reads used for the assembly, the number of contigs > 100 bp generated, and the N50 length (smallest contig size in which half the assembly is represented) were estimated. The sequence coverage and accuracy was then analysed by blastx searching against the *Vitis vinifera* and *Populus trichocarpa* RefSeq protein databases at NCBI. For this purpose, a custom script was implemented, which extracted the contig most homologous to each *V. vinifera* and *P. trichocarpa* protein; consequently, each contig was covered by a maximum of one reference transcript. Full length coding regions were predicted by AUGUSTUS 2.5.5 , trained for *Arabidopsis thaliana*. The 454 reads were assembled by MIRA with the parameters mira --job=denovo,est,accurate,454 454_SETTINGS -AL:mo=40:mrs=90.

The short read assembler Trinity 2012_06_08 was used to assemble the eight Illumina RNA-Seq datasets, with and without 454-derived contigs. Trinity assembly was performed with the parameters trinity.pl --min_kmer_cov 3. Trinity produces large numbers of short contigs, which were removed using a threshold of 200 bp. The 454 and the Illumina reads were subsequently mapped against the contigs by bowtie. When none of the reads aligned with a contig, this contig was discarded. The contigs retained were clustered to reduce sequence redundancy using CD-HIT according to with the parameters –c 0.99 and –aS 1.0.

Classification of the contigs by Gene Ontology terms and by KEGG metabolic pathway analysis

The contigs were classified using the gene ontology (GO) terminology with the Blast2GO software portal , and the GO annotations were grouped using GOSlimViewer . GO enrichment analyses were implemented with the functional annotation tool in DAVID Bioinformatics Resources 6.7 . Briefly, the oak contig list was compared to the background of the *Arabidopsis thaliana* genome dataset. *P*-values indicating enrichment were corrected using the Benjamini-Hochberg pro-

cedure.

Interactive Pathways Explorer 2.0 was used to visualize KEGG global metabolism by first subjecting oak contigs to a blastx search against *A. thaliana* polypeptides, with an e-value cutoff of 1e-5. TAIR10 accessions of the matching *A. thaliana* polypeptides were then mapped against the global KEGG metabolism map of *A. thaliana* (ath01100). As a comparison, the analysis was repeated with *A. thaliana* TAIR10 polypeptides. The software Pathway Tools was used to explore the AraCyc 9.0 and PoplarCyc 5.01 pathway collections from BioCyc and PlantCyc (version December 12th 2012; http://www.plantcyc.org/tools/tools_overview.faces), respectively. Based on the results of these analyses, the KEGG software portal was used to construct biochemical pathways and differential gene expression was visualised with the software tool Vanted 2.0 .

Real-time-quantitative reverse transcriptase-PCR (RT-qPCR)

To confirm the differential expression of genes revealed by RNA-Seq, the expression of 14 genes was measured by RT-qPCR. Primer pairs (Table S1) were constructed based on the OakContigDF159.1 assembly. They were designed using Primer3 software at http://frodo.wi.mit.edu/primer3/ taking the following criteria into account: melting temperature of 55 to 65 °C, GC content of 58 to 63 % and primer length of 18-22 bp. The amplified products were between 70-150 bp in length. In addition, the expression levels of *PRP1* and *Lea5* genes were evaluated with primers from . Primer sequences and estimated amplicon sizes are listed in Table S1.

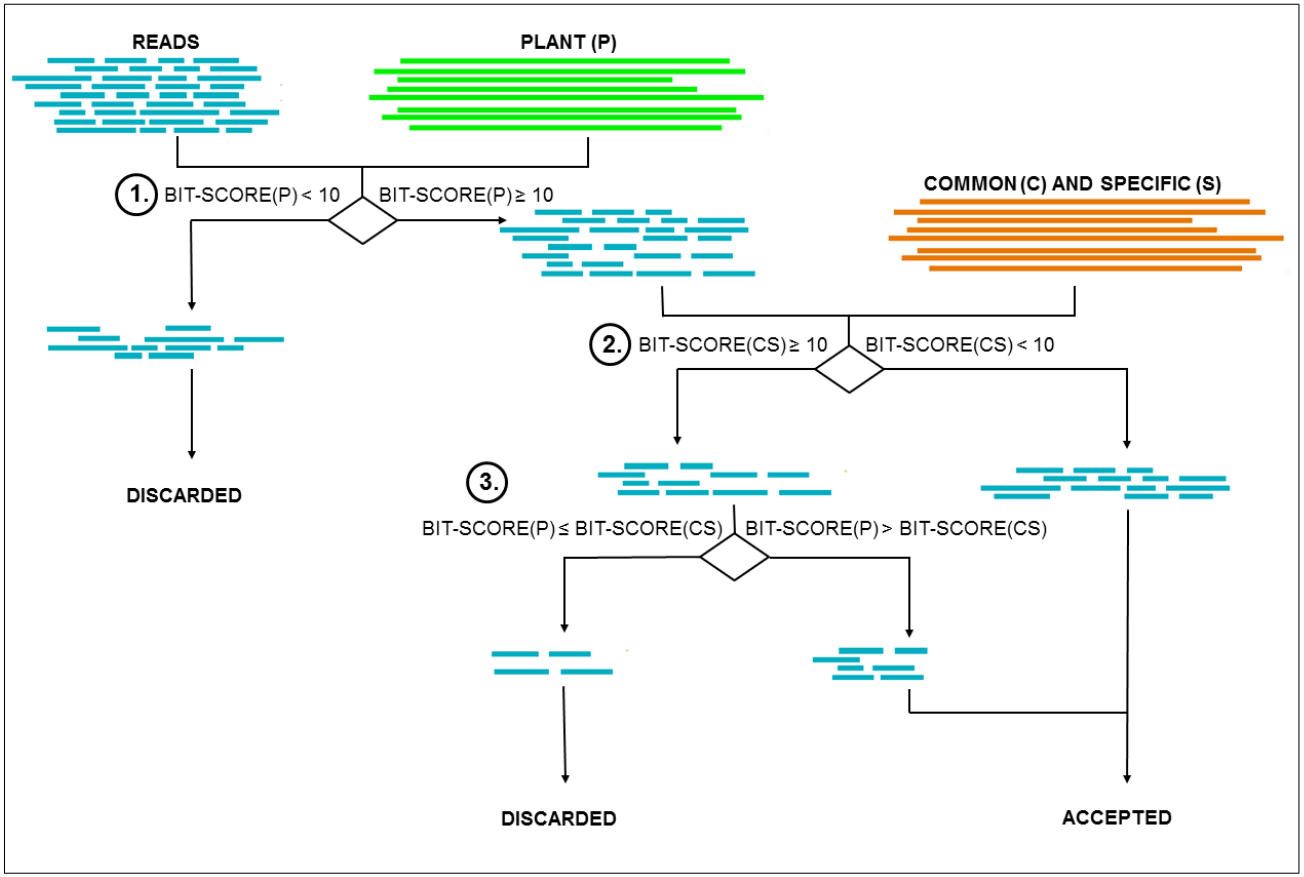
All newly designed primers were first tested for functionality and predicted amplicon size by carrying out PCR with a DNA template. Each primer pair was also tested with *P. croceum* DNA and RNA to rule out the possibility that they might also amplify fungal genes from the mycorrhizas. Before RNA-based analyses, an additional *DNase* I digestion was performed using 1 U *DNase* I (Fermentas) and 500 ng RNA in a 10-20 µl volume. DNA was digested for 30 min, and the reaction was stopped by adding 1 µl 50 mM EDTA. The absence of genomic DNA contamination was confirmed by performing PCR amplification using total RNA as template and 18S_2 primers. The specificity and the efficiency of the primer pairs were estimated by means of melting and standard curves, with a dilution series (32, 16, 8, 4, 2, 1 ng μl^{-1}) of RNA as template.

RT-qPCR was performed using iScript One-Step RT-PCR Kit (containing SYBR Green and fluorescein; Bio-Rad). Reaction mixtures for cDNA synthesis

contained 7.5 µl of One-Step Master Mix, 0.3 µl of iScript Reverse Transcriptase, 1 µl RNA adjusted to 1 and 8 ng, and 400 nmol of each gene-specific primer. The experiments were carried out in 96-well plates with an iQ 5 Multicolor Real-Time PCR Detection System (Bio-Rad). PCR was always performed with three biological and two technical replicates for each reaction, together with reference gene detection. Reverse transcription was carried out for 10 min at 50 °C and cycling conditions were 10 s at 95 °C; 30 s at 55 °C.

The *18S rRNA* gene was selected as a reference for the RT-qPCR analysis. To confirm that its expression level was stable, RNA from leaves of non-inoculated control oak microcuttings, mechanically wounded leaves, *M. alphitoides* infected leaves, leaves from oaks with root inoculation of *Streptomyces* GB 4-2 (Lehr *et al*., 2008; isolated by K. Poralla), tissue with and without mildew infection, and leaves of oaks with root inoculation of *Streptomyces* AcH505 were tested by RT-qPCR with *18S rRNA* primers (primer pair 18S_2; Table S1). Root RNA was extracted from the same plant material. Constant Ct-values for RNAs from differently treated plants were taken as an indication of stable *18S rRNA* gene expression levels. Transcript abundances in ectomycorrhiza and fine root samples were determined by the Ct value or the number of cycles needed to reach a specific threshold level of detection in the exponential phase of the PCR reaction, using the Relative Expression Software Tool .The coefficient of variation (CV) 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 randomisation test within the Relative Expression Software Tool.

(a)



(b)

Plant (P) reference taxa

RefSeq Embryophyta

Common (C) reference taxa

Basidiomycota: *Coprinopsis, Laccaria, Piloderma, Postia, Schizophyllum, Serpula*
Ascomycota: *Ajellomyces, Arthrobotrys, Aspergillus, Botryotinia, Cordyceps, Magnaporthe, Marssonina, Neosartorya, Neurospora, Penicillium, Podospora, Pyrenophora, Sclerotinia*
Mucoromycetales: *Rhizopus*
Nematoda: *Caenorhabditis*
Protista: *Paramecium*
Bacteria: *Candidatus Glomeribacter, Klebsiella, Marssonina, Nocardia, Propionibacterium, Pseudonocardia*

Specific (S) reference taxa

Libraries with *Streptomyces* sp. AcH 505:
Streptomyces
Libraries with *Phytophthora quercina:*
Phytophthora
Libraries with *Lymantria dispar* and *Protaphorura armata:*
Acyrthosiphon, Aedes, Anopheles, Apis, Bombus, Bombyx, Culex, Drosophila, Megachile, Nasonia, Pediculus, Tribolium
Libraries with *Pratylenchus penetrans:*
Brugia, Loa, Trichinella

Fig. S1 Strategy for removing 454 and Illumina reads from oak-interacting organisms. (a) The three stages in the blastx search-based sequence decontamination process (see Supporting Methods for details). Circle-1 marks blastx-search against plant RefSeq protein dataset (P), circle-2 indicates blastx-search against a common dataset (C) to remove general sequence contaminants and a specific (S) dataset to remove sequences from the inoculated organisms, and circle-3 indicates the retrieval of oak sequences from the candidate contaminant sequences. (b) List of taxa at the genus level, from which the NCBI RefSeq or Joint Genome Institute datasets were derived for the removal of reads from oak-interacting organisms.

Gene	Control		+Pilo.		+Strepto.		+Micro.		+Phyto.		+Lyman.		+Praty.		+Prota.	
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	-	+	-	+	-	+	+	+	+	+	-	+	+	+	+
3	+	-	+	+	-	+	-	+	+	+	+	+	+	-	-	+
4	+	-	-	-	-	-	+	+	+	-	+	-	+	+	+	+
5	+	-	+	+	-	-	+	-	-	-	+	+	-	-	+	-
6	+	-	+	+	-	-	+	-	-	-	-	+	-	-	-	+
7	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-
8	+	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-
9	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+
10	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-

Gene	Blastx predicted identity of the Roche 454 read	Functional category
1	Auxin-responsive IAA	Auxin signalling
2	Photosystem II light harvesting complex	Photosynthesis
3	Photolyase/blue-light receptor 2	Photosensor
4	Early-responsive to dehydration stress	Drought stress
5	Carbamoyl phosphate synthetase A	Nitrogen metabolism
6	Phosphotyrosyl phosphatase activator	Signalling
7	Ubiquitin carboxyl-terminal hydrolase 22	Protein degradation
8	Topoisomerase II-associated PAT1	Chromosome structure
9	Transcription factor PIF4	Phytochrome signalling
10	Chromo domain-containing LHP1	Signalling

Fig. S2 Representation of reads with homology to *Arabidopsis* accessions in 16 pedunculate oak Roche 454 transcript libraries. Abbreviations: Control, no inoculation; Pilo, *Piloderma croceum*; Strepto, *Streptomyces sp*. AcH 505; Micro, *Microsphaera alphitoides*; Phyto, *Phytophthora quercina*; Lyman, *Lymantria dispar*; Praty, *Pratylenchus penetrans*; Prota, *Protaphorura armata*; L, leaves; R, roots; +, present in the 454 library; -, not present in the 454 library.

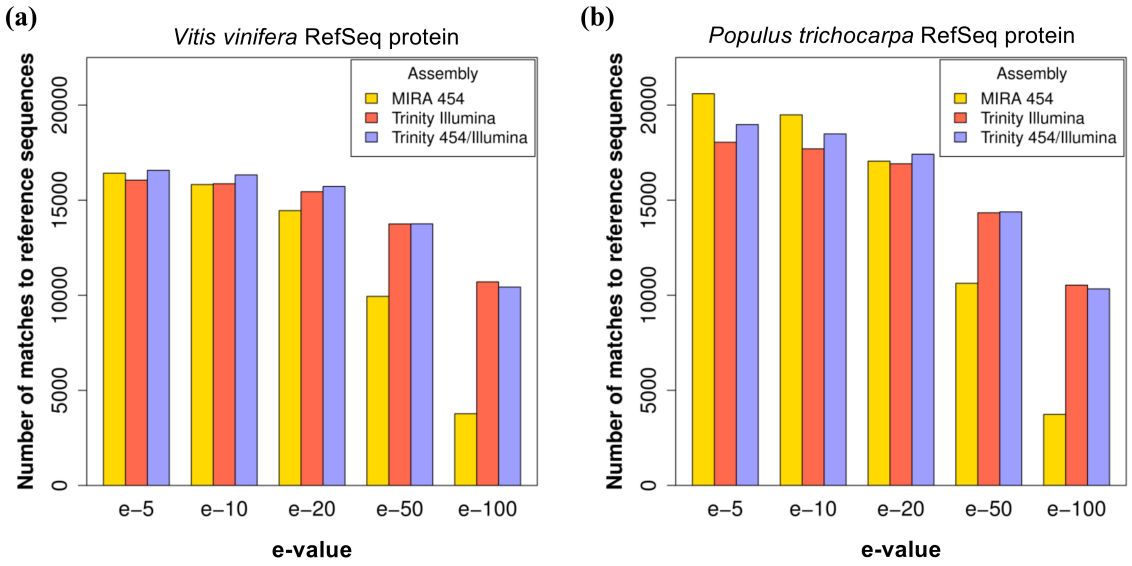


Fig. S3 Comparison of the oak assemblies generated by the MIRA and Trinity assembly programs with reference databases. MIRA assembly of 454 reads, Trinity assemblies of Illumina reads only, and Trinity assembly of Illumina reads, MIRA contigs and single reads converted into overlapping 100 bp single-end reads, were compared by blastx searches. Number of blastx matches given by the contigs against (a) *Vitis vinifera* and (b) *Populus trichocarpa* RefSeq protein databases.

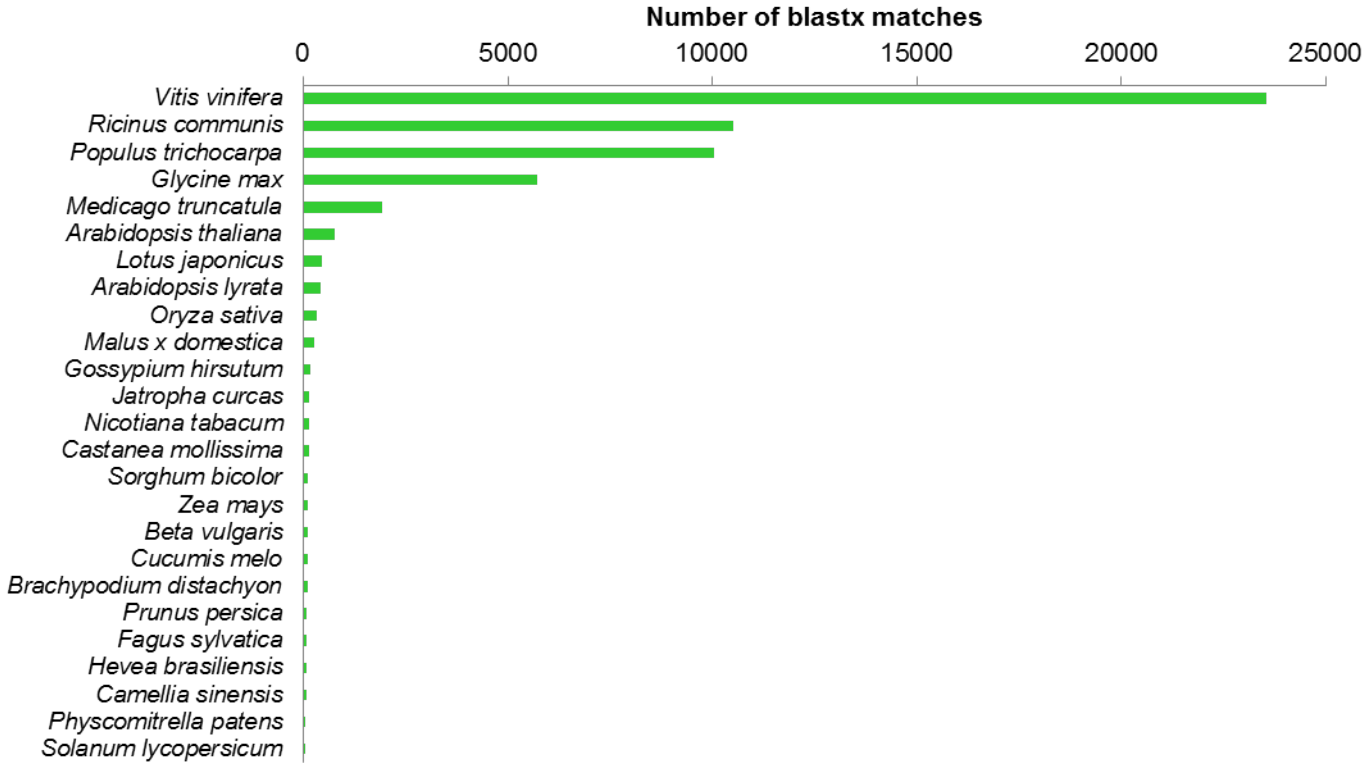


Fig. S4 Relatedness of the contigs in the OakContigDF159.1 reference transcriptome to sequences in the GenBank nr database. The contigs were submitted for blastx searching against the GenBank nr database at the National Center for Biotechnology Information (NCBI). The twenty five plant species that gave the largest numbers of top blastx matches are shown.

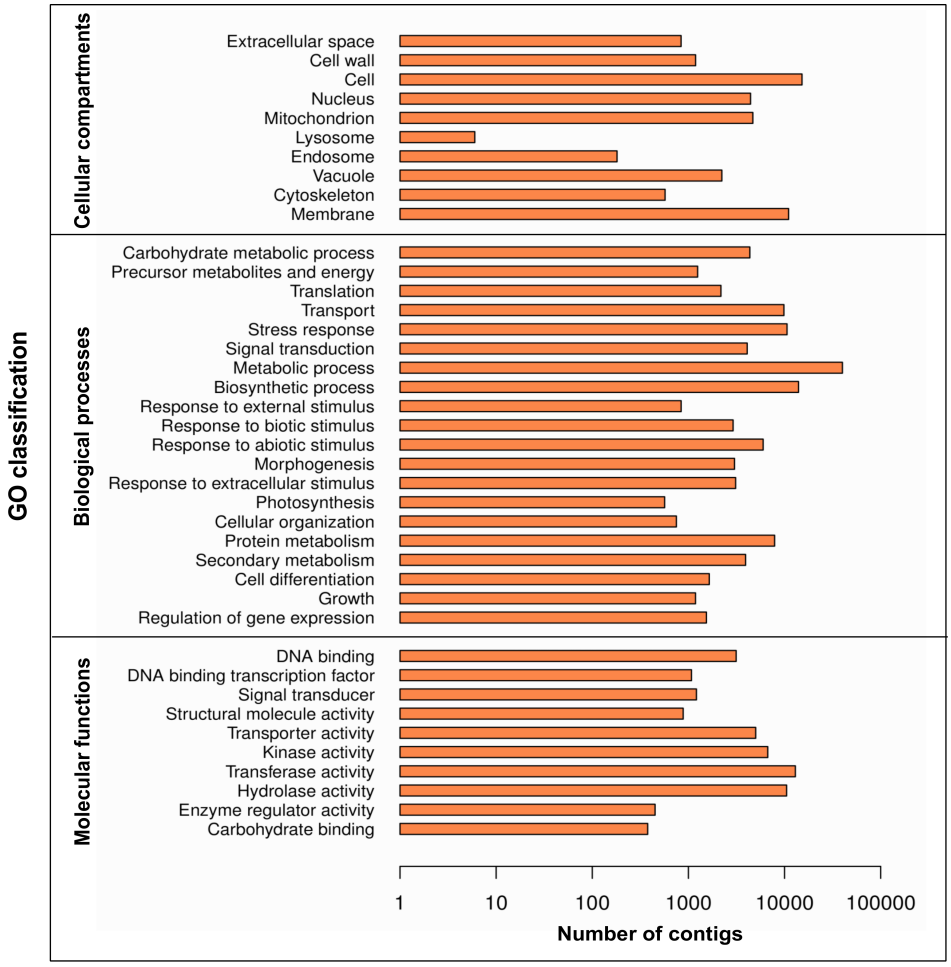


Fig. S5 Classification of contigs in the OakContigDF159.1 reference transcriptome using Gene Ontology terms. Contigs were GO-annotated by Blast2GO and grouped by GOSlimViewer. Examples of GO annotations in the categories molecular functions, cellular compartments, and biological processes are shown.

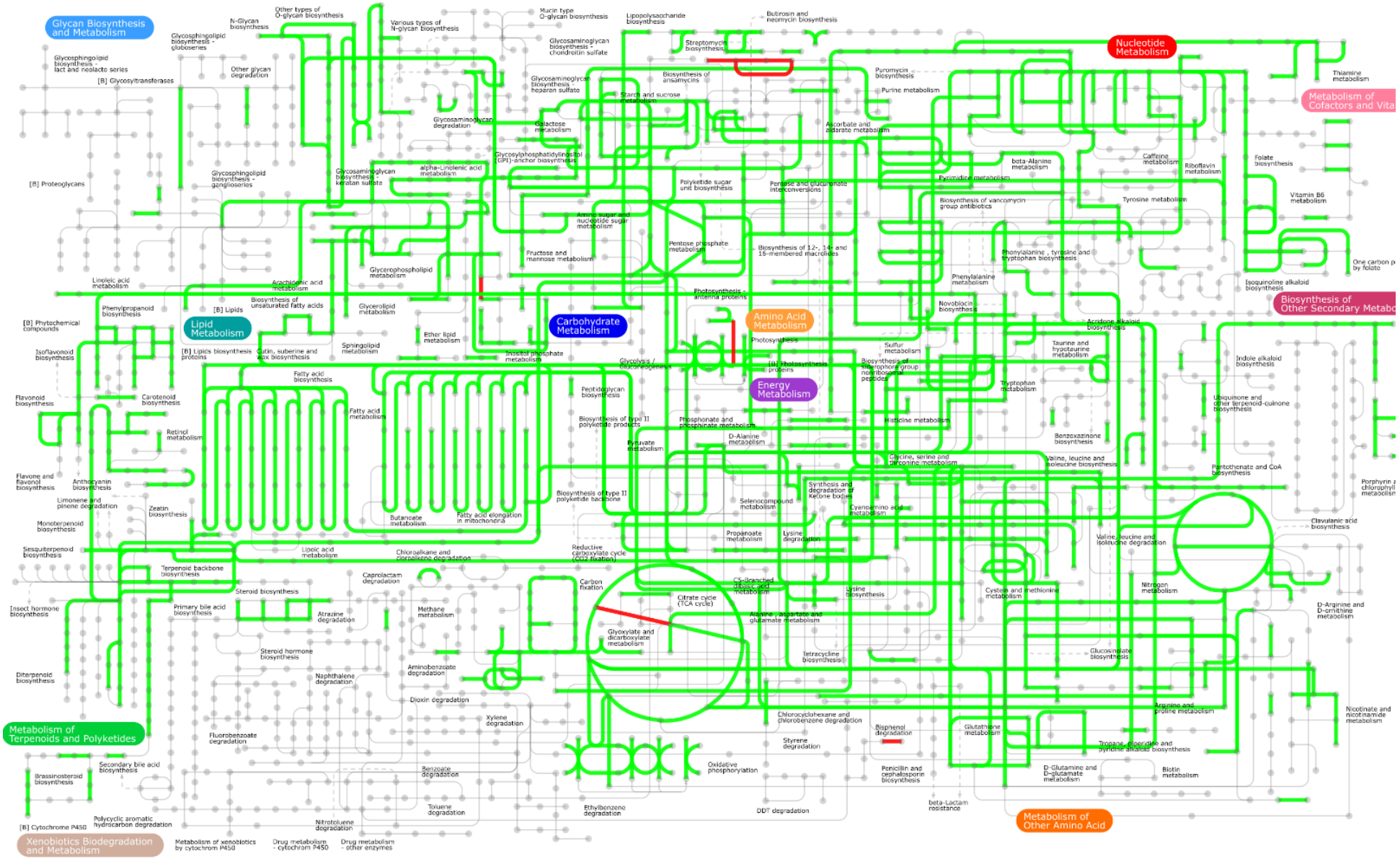
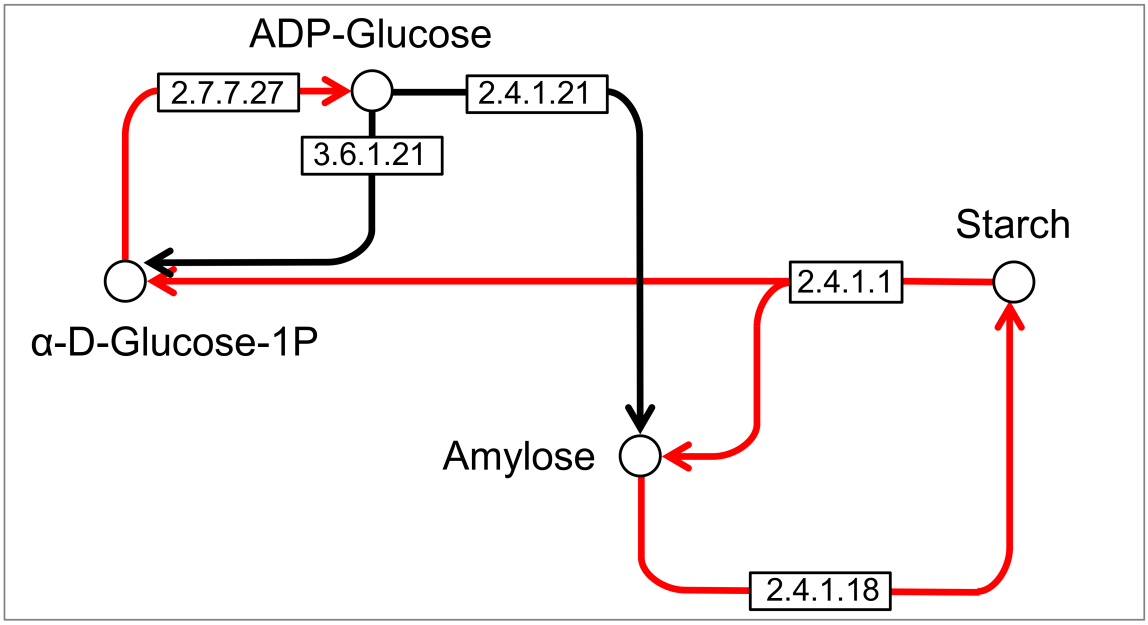


Fig. S6 Comparison of coverage of global KEGG metabolic pathways by the OakContigDF159.1 reference transcriptome relative to the *Arabidopsis thaliana* proteome. *A. thaliana* metabolic pathways also found in OakContigDF159.1 are shown as green lines, while those missing are marked in red.



Contig number	Raw read counts in Mycorrhiza	Raw read counts in Fine Roots	P value	Myc/FR (log_2 fold change)
EC:2.4.1.1, (1->4)-alpha-D-glucan:phosphate alpha-D-glucosyltransferase				
40088_c0_seq1	78	29	0.008	1.399
40088_c0_seq2	320	90	0	1.827
40088_c0_seq8	803	384	0	1.064
40088_c0_seq19	418	87	0	2.255
23137_c0_seq2	295	134	0	1.138
EC:2.4.1.18, 1,4-alpha-glucan branching enzyme				
42131_c0_seq4	15	68	0.008	-2.181
37914_c0_seq1	447	254	0	0.815
EC:2.7.7.27, ATP-alpha-D-glucose-1-phosphate-adenylyltransferase				
41521_c0_seq3	3219	992	0.01	1.698
42599_c0_seq1	789	285	0	1.467

Fig. S7 Mycorrhiza formation on oak roots leads to increased expression levels of transcripts associated with starch metabolism in comparison to levels in fine roots. The contig expression levels were analysed by RNA-Seq. (a) Combined contig expression levels associated with starch metabolism-related Enzyme Commission numbers (EC numbers). Red arrows indicate higher total numbers of mapped reads from mycorrhizas than from fine roots. (b) Expression levels of the differentially expressed contigs.

Table S1 Quantitative polymerase chain reaction primers. Blastx searching against the NCBI nr database was used to predict the identity of the target transcripts. Asterisks indicate primers from.

Primer	Sequence (5'-3')	Blastx predicted transcript identity
CA13661-f	CACCTGGCATCCTTGGAG	Extensin
CA13661-r	GGTCTTTGTCTGGGGCTACACC	
CA16488-f	GAACACAGCCCATCCTCTCT	Sieve element occlusion protein
CA16488-r	CGAAGACGACAGCCTCCTAA	
CA9232-f	GGCTCGGGCTGGTATTAG	Plasma membrane H+-ATPase
CA9232-r	GGGTCAGGATCTGCTCAGGA	
CA325-f	GAGGCTGTGACCATACAAGC	Endo-1,4-beta-glucanase
CA325-r	GCACTTGTGGCTTTGTCAGG	
CA8705-f	AGCCTCTTCCTGGGTCTC	Endomembrane transport protein
CA8705-r	CTCTTCCTCTCGGGCATGGT	
CA4304-f	CACCTTGTCCACAGCCAGAG	1-aminocyclopropane-carboxylate oxidase
CA4304-r	GCAATCCAGGCACATCATCC	
CA9325-f	GCAGGTGTTGCTGTTCTTCTGG	Glucose-1-P-adenylyltransferase
CA9325-r	GAGGCTGTGACCATACAAGC	
CA10387-f	GTGTACCATCCAGGGCATC	Thioredoxin
CA10387-r	GCACCTCATACCCACATTCC	
Lea5-f*	CATTGGACGGGATTGATGAGG	Late embryogenesis abundant 5
Lea5-r*	GTGATCAGTGACCTCCAGGC	
PRP1-f*	CTTGTGCTGGACCTCTGG	Proline-rich protein PRP1
PRP1-r*	GCATAAACAGTAGTCGGATGGG	
CA377-f	CTCAACACCAGCTTTAGCC	Inositol transporter
CA377-r	TGGTTGGGGCTGGACTAC	
CA20973	GGTGCCAAGGGTGTCAAA	Calcium-binding protein
CA2973	CTGGAGCCACTGTCAAAGC	
CA4305-f	GGGACAGGAGAAGGGCTA	Aspartic proteinase
CA4305-r	GACGAAGCGGGTCACAAC	
CA1557-f	GAGGAAGAGAACATGGACAGG	Galactinol synthase
CA1557-r	GAGGAAGAGAACATGGACAGG	
18S_2-f	CAAGGTGGACTCTCTCACGG	18S rRNA
18S_2-r	CCTCGGATGCAGAACACC	

Table S3 Transcripts in roots and leaves of oak DF159 microcuttings and in ectomycorrhizas synthesised with *Piloderma croceum* as revealed by Illumina sequencing. RNAs were 100 bp paired-end sequenced using the HiSeq2000 version of the Illumina system. All reads were used to generate the pedunculate oak reference transcriptome, and the reads FROOT 1 to 3 and MYCO 1 to 3 were used to estimate changes in oak gene expression levels upon mycorrhiza formation in pedunculate oak inoculated with the fungus *Piloderma croceum*. The term trimmed reads indicates reads that have been processed to a higher quality. The trimming steps include the removal of adapter sequences, sequencing artefacts and reads from other organisms.

Library	Dataset	Number of reads (mean length ± SD)	Number of raw bases	Number of trimmed reads (mean length ± SD)	Number of trimmed bases	Number of decontaminated reads (mean length ± SD)	Number of decontaminated bases
LDIIL_R6	*L. dispar* caterpillar, root sample	28,785,780 (100 ± 0)	2,878,578,000	26,742,031 (98 ± 5)	2,629,268,576	17,195,756 (99 ± 1)	1,719,237,046
PCRIL_L6	*P. croceum* mycorrhiza, leaf sample	28,947,224 (100 ± 0)	2,894,722,400	25,083,660 (98 ± 5)	2,463,896,470	17,030,641 (98 ± 5)	1,702,671,255
FROOT1	No treatment fine roots	35,484,706 (100 ± 0)	3,548,470,600	23,374,074 (97 ± 8)	2,274,191,122	14,519,937 (98 ± 6)	1,417,915,913
FROOT2	No treatment fine roots	34,343,015 (100 ± 0)	3,434,301,500	20,842,612 (97 ± 7)	2,035,293,414	13,583,329 (98 ± 6)	1,332,890,970
FROOT3	None, fine roots	62,496,259 (100 ± 0)	6,249,625,900	41,105,168 (97 ± 8)	4,001,988,613	20,073,458 (98 ± 6)	1,968,403,291
MYCO1	Individual *P. croceum* mycorrhizas	21,366,078 (101 ± 0)	2,157,973,878	17,326,725 (99 ± 7)	1,713,355,292	9,296,305 (99 ± 5)	922,262,350
MYCO2	Individual *P. croceum* mycorrhizas	22,510,865 (101 ± 0)	2,273,597,365	18,245,941 (99 ± 7)	1,802,180,568	9,465,172 (99 ± 5)	937,736,287
MYCO3	Individual *P. croceum* mycorrhizas	21,303,775 (101 ± 0)	2,151,681,275	17,365,350 (99 ± 6)	1,718,293,477	9,111,800 (99 ± 5)	901,500,664
IN TOTAL		**255,237,702**	**25,588,950,918**	**190,085,561**	**18,638,467,532**	**110,276,398**	**10,902,617,776**

Table S4 GO annotation of contigs in the OakContigDF159.1 reference transcriptome. The contigs were annotated by Blast2GO with GenBank nr as the reference database. The dataset can be downloaded at https://www.ufz.de/trophinoak/index.php?de=31205.

Table S5 GO enrichment analysis of ectomycorrhizas synthesised between oak DF159 and *Piloderma croceum*. GO enrichment analyses were implemented with the functional annotation tool in DAVID. Myc, mycorrhiza; FR, fine roots.

Gene Ontology term	**GO term description**	**Fold change Myc/FR**	***P*-value after Benjamini procedure**
GO terms for differentially expressed genes enriched in Myc			
GO:0022626	Cytosolic ribosome	3.780	2.75e-20
GO:0005622	Intracellular	1.166	5.85e-10
GO:0005773	Vacuole	2.232	2.29e-09
GO:0050896	Response to stimulus	1.229	0.001
GO:0043227	Membrane-bounded organelle	1.142	3.10e-03
GO:0006091	Generation of precursor metabolites and energy	1.763	0.011
GO:0005982	Starch metabolic process	4.029	0.034
GO:0005215	Transporter activity	1.265	0.036
KEGG pathway associations of differentially expressed genes increased in Myc on the basis of fold change			
ath03010	Ribosome	1.932	0.24
ath03040	Spliceosome	1.767	0.421
GO terms for differentially expressed genes less abundant in Myc			
GO:0000166	Nucleotide binding	-1.799	1.75e-54
GO:0016462	Pyrophosphatase activity	-2.312	2.91e-25
GO:0004871	Signal transducer activity	-1.981	8.38e-13
GO:0004672	Protein kinase activity	-1.781	1.21e-11
GO:0016265	Death	-2.716	1.50e-09
GO:0044430	Cytoskeletal part	-2.824	6.20e-06
GO:0050793	Regulation of developmental process	-1.766	0.003
GO:0008471	Laccase activity	-5.234	0.003
GO:0009698	Phenylpropanoid metabolic process	-2.015	0.007
GO:0009734	Auxin mediated signalling pathway	-2.234	0.007
GO:0006807	Nitrogen compound metabolic process	-1.147	0.012
GO:0005992	Trehalose biosynthetic process	-4.480	0.012
GO:0009926	Auxin polar transport	-2.954	0.02
GO:0022622	Root system development	-1.760	0.032
KEGG pathway associations of differentially expressed genes less abundant in Myc on the basis of fold change			
ath03030:	DNA replication	-3.418	0.018
ath00940:	Phenylpropanoid biosynthesis	-1.825	0.454
ath00970:	Aminoacyl-tRNA biosynthesis	-2.126	0.719
ath00230:	Purine metabolism	-1.587	0.701
ath00360:	Phenylalanine metabolism	-1.624	0.798

Table S6 Differentially expressed transcripts in ectomycorrhizas synthesised between microcuttings of the pedunculate oak clone DF159 and *Piloderma croceum*. The OakContigDF159.1 transcripts were selected based on their predicted biological functions, including transcripts encoding cell wall proteins, signalling polypeptides and chitinases. Blastx searching was used to predict the functions of the contig-encoded polypeptides.

Contig no.	Raw read counts in mycorrhiza	Raw read counts in fine roots	*P*-value	Myc *vs.* FR (log_2 fold change)	Predicted function
Proline-rich protein-related contigs					
28167_c0_seq1	2298	443	3.52e-15	2.37	Proline-rich protein PRP1
27955_c0_seq1	269	62	9.81e-17	2.09	Proline rich protein
28167_c0_seq2	469	135	2.13e-19	1.78	Proline-rich protein PRP1
21940_c0_seq1	1524	498	3.99e-53	1.61	Proline-rich cell wall protein 2
21940_c0_seq2	675	246	6.49e-23	1.45	Proline-rich cell wall protein 2
37383_c0_seq1	150	70	0.0011	1.09	Proline rich protein
41209_c0_seq1	936	629	6.84e-06	0.57	Proline-rich cell wall protein
42760_c0_seq1	1215	805	2.19e-05	0.59	Proline-rich glycoprotein
28257_c0_seq1	2190	1645	2.80e-04	0.41	Hydroxyproline-rich glycoprotein
43327_c1_seq7	337	537	8.17e-05	-0.67	Hydroxyproline rich glycoprotein
36333_c0_seq1	362	578	3.69e-05	-0.67	Hydroxyproline-rich glycoprotein
43233_c1_seq1	619	1118	7.19e-10	-0.85	Hydroxyproline-rich glycoprotein
34894_c1_seq1	88	194	1.60e-04	-1.13	Hydroxyproline-rich glycoprotein
Expansin related contigs					
40696_c0_seq2	2352	575	2.26e-91	2.03	Expansin-like b1
38597_c0_seq1	29	93	0.00079	-1.65	Expansin-like b1
42087_c0_seq2	12	97	2.34e-09	-2.96	Expansin
42087_c0_seq1	4	46	3.35e-05	-3.27	Expansin-b3-like precursor
Xyloglucan endotransglucosylase/hydrolase (XTH)-related contigs					
22994_c1_seq1	997	636	8.24e-08	0.65	XTH
43120_c0_seq11	2068	2471	0.0014	-0.26	XTH
43120_c0_seq8	81	174	0.00064	-1.09	XTH
44218_c0_seq1	103	227	2.20e-05	-1.14	XTH inhibitor
37028_c1_seq2	16	60	0.0038	-1.83	XTH
37028_c2_seq1	12	62	0.00018	-2.37	XTH
Extensin related contigs					
19989_c0_seq1	99	5	7.85e-10	4.08	Extensin family protein
32486_c0_seq1	464	709	2.05e-05	-0.61	Extensin family protein
35925_c0_seq1	57	154	2.47e-05	-1.41	Extensin family protein

Contig no.	Raw read counts in mycorrhiza	Raw read counts in fine roots	*P* value	Myc *vs.* FR (log_2 fold change)	Predicted function
Auxin signalling related contigs					
30241_c1_seq3	816	618	0.0046	0.40	Auxin response factor 3
38228_c2_seq3	69	21	0,0054	1.66	Auxin influx carrier component
19890_c0_seq1	2767	2194	2.98e-06	1.26	Auxin associated protein
39524_c0_seq1	3311	2055	1.92e-26	0.69	Auxin induced mono-oxygenase
43589_c3_seq7	1508	933	1.50e-06	0.69	Auxin induced pcnt115-like protein
43589_c3_seq5	4366	3147	6.35e-16	0.47	Auxin induced pcnt115-like protein
36749_c0_seq1	265	446	0,0089	0.43	Auxin induced protein
44137_c0_seq1	1234	964	0.0032	0.36	Auxin-responsive protein iaa13
19927_c0_seq1	6275	5002	2.82e-10	0.32	Auxin repressed protein
41841_c0_seq1	1144	1424	4.40e-03	-0.31	Auxin response factor 1
33319_c0_seq1	761	997	0,0019	-0.38	Auxin response factor 5
29024_c0_seq1	759	1170	9.37e-09	-0.62	Auxin response factor 6
19485_c0_seq1	416	1009	5.99e-20	-1.27	Auxin response factor 6
30567_c2_seq1	110	456	4.10e-26	-2.05	Auxin response factor 7
42796_c0_seq1	47	113	0.0022	-1.25	Auxin response factor
43565_c0_seq1	1022	1925	3.98e-11	-0.91	Auxin transport protein
37248_c1_seq1	90	186	0,00089	-1.04	Auxin efflux carrier protein
37248_c0_seq1	68	145	0,0029	-1.09	Auxin efflux carrier protein
38167_c0_seq1	265	466	9.90e-06	-0.81	Auxin efflux carrier component
20073_c0_seq1	1230	1565	0.00048	-0.34	Auxin induced 5ng4
36368_c0_seq1	955	1234	0,0012	-0.37	Auxin responsive protein
20100_c1_seq1	512	687	0.0059	-0.42	Auxin responsive protein
32000_c0_seq1	679	960	3.44e-05	-0.50	Auxin responsive protein
32281_c1_seq1	295	439	0,0034	-0.50	Auxin responsive protein

Contig no.	Raw read counts in mycorrhiza	Raw read counts in fine roots	*P* value	Myc *vs.* FR (log_2 fold change)	Predicted function
Ethylene signalling transcription factor-related contigs					
18724_c0_seq1	68	23	6.60e-03	1.55	AP2 ERF domain-containing transcription factor 3
32408_c1_seq2	360	143	1.95e-10	1.33	Ethylene-responsive transcription factor ERF003
33501_c0_seq1	339	211	1.70e-03	0.68	Ethylene-responsive transcriptional coactivator
28737_c0_seq1	937	604	1.72e-07	0.63	AP2 ERF domain-containing transcription factor
27633_c0_seq1	1632	1061	1.14e-07	0.62	Ethylene-responsive transcription factor
36646_c0_seq2	338	225	0.01	0.58	Ethylene-responsive transcription factor RAP2-7
32111_c0_seq1	2837	2196	2.55e-07	0.37	Ethylene-responsive transcription factor RAP2-4
34234_c0_seq1	740	954	8.30e-03	-0.37	AP2 ERF domain-containing transcription factor
34589_c2_seq1	638	953	1.89e-06	-0.58	AP2-like ethylene-responsive transcription factor ant-like
35474_c0_seq2	55	137	0.0005	-1.31	AP2-like ethylene-responsive transcription factor ant-like
Abscisic acid signalling related contigs					
39870_c0_seq2	115	268	9.21e-07	-1.21	Abscisic acid receptor PYL4
41933_c1_seq1	63	246	6.9e-06	-1.95	Abscisic acid receptor PYL4
Chitinase related contigs					
37928_c4_seq3	167	272	0.0068	-0.71	Chitinase
37928_c4_seq1	101	194	0.0026	-0.93	Chitinase
35193_c0_seq2	37	99	0.002	-1.41	Chitinase class 4
Sugar transporter related contigs					
38572_c0_seq1	157	52	1.61e-06	1.57	Sucrose transporter
39582_c1_seq2	211	91	2.72e-05	1.21	Bidirectional sugar transporter SWEET 1
37411_c1_seq1	161	499	5.83e-20	-1.63	Hexose transporter

Contig no.	Raw read counts in mycorrhiza	Raw read counts in fine roots	*P* value	Myc *vs.* FR (log_2 fold change)	Predicted function
Phosphate transporter related contigs					
37520_c0_seq3	142	46	3.49e-06	1.60	Phosphate transporter PHO1
37520_c0_seq1	207	452	8.87e-10	-1.12	Phosphate transporter PHO1
42379_c0_seq4	95	36	0.0032	-1.38	Phosphate transporter PHO1
Ammonium transporter related contigs					
36778_c0_seq1	303	11	2.75e-45	4.67	Ammonium transporter
36737_c0_seq1	613	847	0.00086	-0.46	Ammonium transporter
38574_c0_seq1	145	279	0.00012	-0.93	Ammonium transporter 1
34978_c0_seq1	49	110	0.0099	-1.16	Ammonium transporter AMT2
Aquaporin related contigs					
comp27667_c0_seq1	198	85	2.65e-5	1.22	Aquaporin PIP
comp34309_c0_seq2	82	35	0.0079	1.21	Aquaporin PIP 2-5
comp24553_c0_seq1	769	370	1.16e-14	1.06	Aquaporin PIP 2-7
comp33626_c1_seq1	4404	2545	1.75e-32	0.79	Aquaporin PIP 1-1
comp37527_c0_seq1	423	256	0.00012	0.72	Aquaporin SIP 2-1
comp37244_c0_seq1	1221	785	6.18e-10	0.64	Aquaporin PIP 1-3

Appendix Chapter III

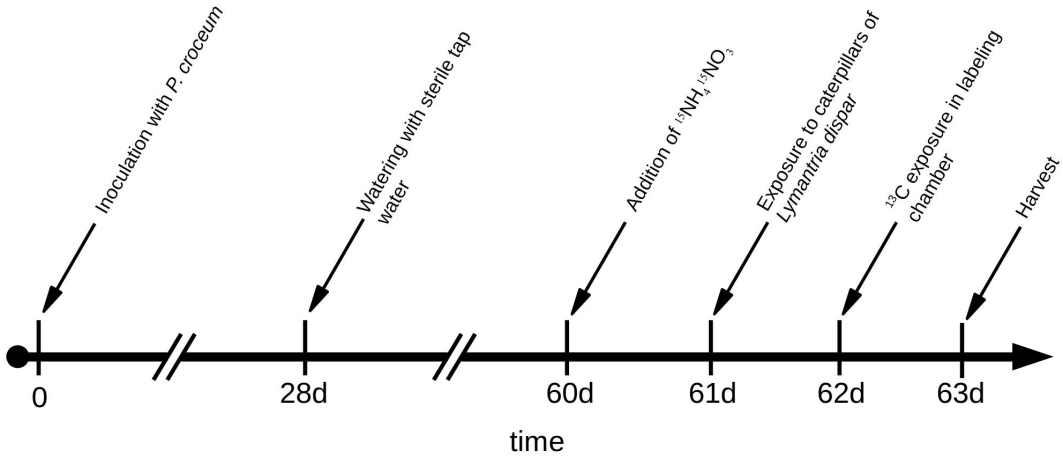


Figure S1 Experimental timeline from the inoculation of the microcuttings with *Piloderma croceum* at day 0 to harvest at day 63.

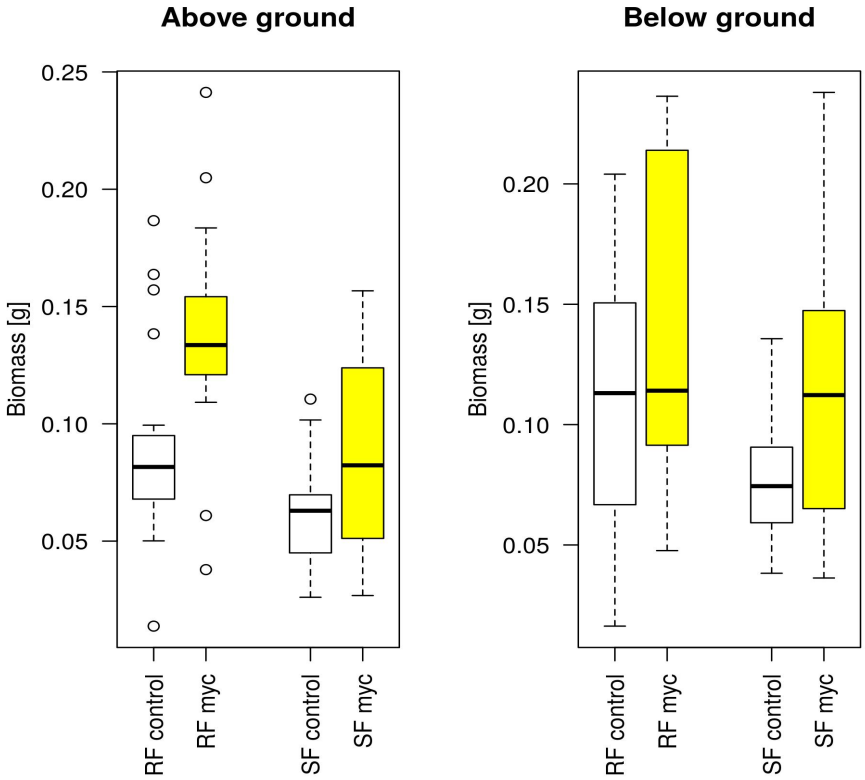


Figure S2 Effect of inoculation with a mycorrhizal fungus on the biomass (dry weight) of oak shoots and oak roots in root flush (RF) and in shoot flush (SF). Oak microcuttings were inoculated with *Piloderma croceum* (myc; yellow bars); control microcuttings were not inoculated with the fungus (control; colorless bars). Bars in the boxplots indicate the upper and lower quartile with median; whiskers indicate minimum and maximum values; dots are outliers.

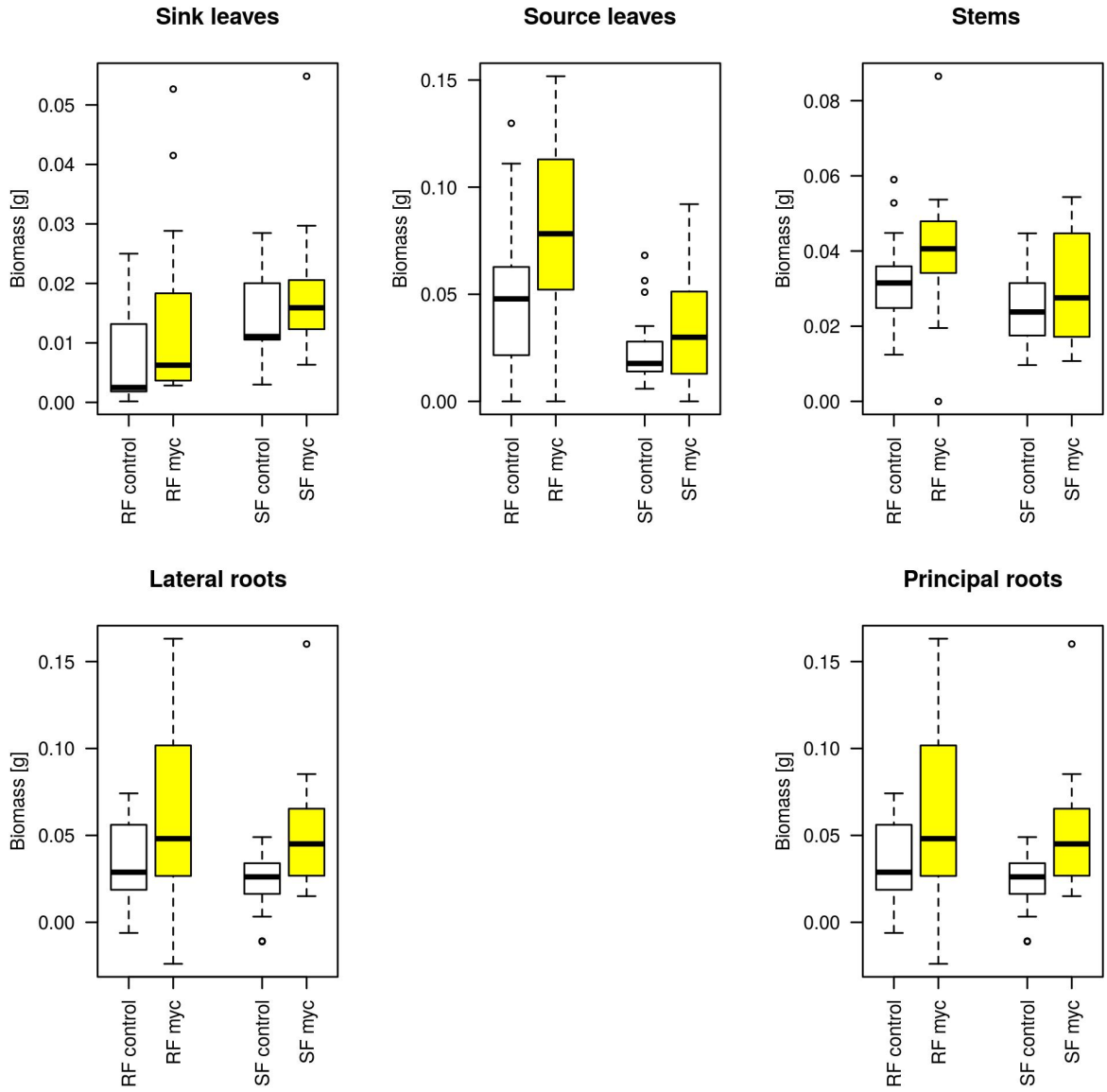


Figure S3 Effect of inoculation with a mycorrhizal fungus on the biomass (dry weight) of separate oak plant organs in root flush (RF) and in shoot flush (SF). Oak microcuttings were inoculated with *Piloderma croceum* (myc; yellow bars); control microcuttings were not inoculated with the fungus (control; colorless bars). Bars in the boxplots indicate the upper and lower quartile with median; whiskers indicate minimum and maximum values; dots are outliers.

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Michael Bacht

Curriculum Vitæ

Zur Person

geboren am 5.5.1983 in Essen

verheiratet, ein drei jähriger Sohn

Wissenschaftliche Laufbahn

seit 04/2011	Wissenschaftlicher Mitarbeiter an der Philipps-Universität Marburg
11/2010 - 04/2011	Freier Wissenschaftlicher Mitarbeiter an der Philipps-Universität

Akademische Bildung

10/2008 - 11/2010	Studium an der Philipps-Universität Marburg Abschluss: Master of Science Studiengang: Organismic Biology Thema der Master-Arbeit: "Genetic divergences in Ring Ouzel (*Turdus torquatus*) of the European low mountain ranges″
10/2005 - 09/2008	Studium an der Philipps-Universität Marburg Abschluss: Bachelor of Science Studiengang: Biology Thema der Bachelor-Arbeit: "DNA-Barcoding: developing a molecular threshold for rapid diversity assessment in termites"

Schulbildung

08/1993 - 06/2002	Besuch der städtischen Gesamtschule Essen-Borbeck Abschluss: Allgemeine Hochschulreife

Erklärung

Hier mit versichere ich, dass ich meine Dissertation mit dem Titel:

“Multitrophic interactions in oak”

selbstständig und ohne unerlaubte Hilfe verfasst habe. Ich habe mich keiner als der in ihr angegeben Quellen oder Hilfsmittel bedient und alle vollständig oder sinngemäß übernommenen Zitate als solche gekennzeichnet. Diese Dissertation wurde in der vorliegenden oder einer ihr ähnlichen Form noch bei keiner anderen in- oder ausländischen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Michael Bacht

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Marburg an der Lahn; Juli 2015