Tree Response to Herbivory Is Affected by Endogenous Rhythmic Growth and Attenuated by Cotreatment With a Mycorrhizal Fungus

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Herbivores and mycorrhizal fungi interactively influence growth, resource utilization, and plant defense responses. We studied these interactions in a tritrophic system comprising *Quercus robur*, the herbivore *Lymantria dispar*, and the ectomycorrhizal fungus *Piloderma croceum* under controlled laboratory conditions at the levels of gene expression and carbon and nitrogen (C/N) allocation. Taking advantage of the endogenous rhythmic growth displayed by oak, we thereby compared gene transcript abundances and resource shifts during shoot growth with those during the alternating root growth flushes. During root flush, herbivore feeding on oak leaves led to an increased expression of genes related to plant growth and enriched gene ontology terms related to cell wall, DNA replication, and defense. C/N-allocation analyses indicated an increased export of resources from aboveground plant parts to belowground. Accordingly, the expression of genes related to the transport of carbohydrates increased upon herbivore attack in leaves during the root flush stage. Inoculation with an ectomycorrhizal fungus attenuated these effects but, instead, caused an increased expression of genes related to the production of volatile organic compounds. We conclude that oak defense response against herbivory is strong in root flush at the transcriptomic level but this response is strongly inhibited by inoculation with ectomycorrhizal fungi and it is extremely weak at shoot flush.

Keywords: fungus-plant interactions, genomics, metabolomics, plant defense mechanisms, proteomics.

Plants growing in natural environments interact with an intriguing diversity of organisms. Among the most common interactions of plants are those with herbivorous insects and mycorrhizal fungi, which have important consequences for the performance of individual plants, dynamics of plant populations, as well as for the composition and structure of plant communities (Crawley 1996; Schädler et al. 2004; Smith and Read 2010; van der Heijden and Sanders 2002). 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 belowground 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 and Brandl 2001). Furthermore, the majority of trees in boreal and temperate regions (>95%) are associated with ectomycorrhizal fungi (Baar et al. 1999; Kõljalg et al. 2000; Smith and Read 2010; 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). Thus, the three-partite relationship between insects, trees, and mycorrhizal fungi is a widespread multitrophic interaction with a high ecological and economic relevance.

Furthermore, 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 (Borowicz 1997; Gehring and Whitham 1994; Kempel et al. 2010). This resource supply may also allow plants to invest more into resistance against generalist insect herbivores (Bi et al. 2007; Gange and West 1994;

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Halldórsson et al. 2000; Jones and Last 1991; Kempel et al. 2010; Yao et al. 2007). Several authors, however, have observed that this protective effect cannot be attributed only to the improved nutritional status (Fritz et al. 2006) because belowground mutualists also may affect the plant's defensive phenotype in multiple and interacting ways (Schädler and Ballhorn 2017). Mycorrhizal fungi should enable the plant to express defense responses even under conditions where they do not provide a nutritive benefit. In addition, the premycorrhizal phase of the interaction between ectomycorrhizal fungi and tree roots is already accompanied by the stimulation of plant growth and enhanced photosynthesis (Felten et al. 2009; Herrmann et al. 2004). The impact of this premycorrhizal phase on above- and belowground plant morphology is strong in oak trees (Herrmann et al. 1998), including modifications of gene expression patterns (Frettinger et al. 2007). Because plant defense responses are triggered by modifications in the crosstalk between salicylic and jasmonic acid already in the premycorrhizal stages of the mycorrhizal symbiosis (Pozo and Azcón-Aguilar 2007), corresponding effects on herbivores can be expected.

First analyses of gene expression have demonstrated that herbivores can influence the transcription of hundreds of plant genes (Baldwin et al. 2001; Hermsmeier et al. 2001; Roda and Baldwin 2003; Schmidt et al. 2005). Herbivory can even lead to complete changes in metabolic organization (Hui et al. 2003) and can influence the expression of genes involved in regulation of photosynthesis and pathogen resistance (Hermsmeier et al. 2001). Also, interactions between plants and mycorrhizal fungi lead to important changes in gene expression in the host plant (Frettinger et al. 2007; Herrmann et al. 2015; Tarkka et al. 2013; Volpin et al. 1994; Wiemken and Boller 2002). Many of those genes are important for resource allocation and responses to abiotic and biotic stresses (Herrmann and Buscot 2007; Liu et al. 2007). Thus, not only an overlap in the impact of both types of interaction but also interactive effects on gene expression patterns can be expected.

Quercus robur is characterized by an endogenous rhythmic growth (ERG); in saplings and adult trees, root flushes (RF) and shoot flushes (SF) occur in a rhythmic alternating pattern of stem extension or leaf expansion and root growth (Harmer 1990; Herrmann et al. 2015). These growth flushes are characterized by defined stages which are not only distinguishable by morphological traits but also play an important role in the physiology and ecology of the plant. Most importantly, carbon (C) is allocated to the shoot during SF and to the roots during RF (Herrmann et al. 2015, 2016), which is also reflected by the concentration of nonstructural carbohydrates in the roots (Angay et al. 2014). Such patterns of rhythmic allocation shifts may have a crucial impact on plant response to herbivore feeding. For example, the allocation of resources to above- or belowground parts of plants during the specific growth phases might have consequences for the plant's ability to respond to herbivory. In turn, in response to herbivory, plants can vary the allocation of resources according to conflicting ecological, morphological, and physiological demands within the plant (Schultz et al. 2013). Thus, if there is an increased demand for the production of defense-related compounds in leaves, the amount of C deposited in fine roots of trees can be reduced, whereas especially herbaceous plants suffering high biomass losses due to herbivory often tend to allocate resources belowground (Frost and Hunter 2008) which, in turn, should influence the interaction between mycorrhizal fungi and host plants. This ERG is regulated by plant internal clocks and accompanied by changes in the expression patterns of numerous genes related to a multitude of metabolic and physiological processes (Herrmann et al. 2015). Thus, differing gene expression patterns during the single growth stages can be expected to have

consequences for interactions with other biota (Herrmann et al. 2016). For instance, Kurth et al. (2015) and Maboreke et al. (2016) showed that more genes involved in defense and cell wall remodeling were differentially expressed in oak roots during SF in response to belowground interactors. This implies that the nongrowing roots at this stage are able to invest more into defense responses rather than growth processes (Kurth et al. 2015). Therefore, it might be hypothesized that the pattern is reversed for leaf herbivory, with stronger expression of defense-related genes during the RF. The mediation of the tripartite relationship between plants, mycorrhizal fungi, and insect herbivores by plant growth stages is, furthermore, an unexplored possible source of variability in the outcomes of this multitrophic interaction. To understand the underlying effects of the ectomycorrhizal fungus on the host plant, an analysis of ongoing transcriptional and nutritional changes in this type of multitrophic interaction has to be performed.

Here, we report a controlled laboratory study of the interactions of the pedunculate oak clone DF159 microcuttings (Herrmann et al. 1998), the larva of the herbivore *Lymantria dispar*, and inoculation with the ectomycorrhizal fungus *Piloderma croceum* at the levels of gene expression in leaves and whole-plant C and nitrogen (C/N) allocation. We studied gene expression in *Q. robur* DF159 leaves using the DF159-specific developed reference transcriptome OakContigDF159.1 of the TrophinOak project (Tarkka et al. 2013). We tested the following hypotheses:

- i) We expect that oak trees differentially express defense-related genes in leaves when they are attacked by insect herbivores, particularly when the trees are interacting with mycorrhizal fungi.
- ii) We expect that the gene expression pattern in response to leave herbivory is stronger in RF, when the leaves are not growing.
- iii) We expect that insect herbivory negatively affects C allocation to roots, and this will be more pronounced in RF when roots are the main C sink.
- iv) We expect that modification in the gene expression patterns related to the ERG reflects the modifications in allocation patterns.

RESULTS

Plant biomass.

Dry biomass of oak microcuttings during RF was higher than during SF (Table 1) in all plant fractions, except for lateral roots and source leaves. All microcuttings exposed to herbivory were damaged by the caterpillars. However, herbivory by *L. dispar* alone had no significant effect on plant biomass when compared with the control (Table 1), which indicated that biomass removal by the herbivore was not substantial. Biomass changes of oak in response to feeding by *L. dispar* were further not altered by *P. croceum* inoculation or plant developmental stage (Supplementary Figs. S2 and S3; Table 1). The duration of the experiment was too short to allow the development of visible signs of mycorrhiza formation. However, already during the premycorrhizal phase, *P. croceum* treatment increased both the root and shoot biomass significantly (three-way analysis of variance [ANOVA]) (Table 1). The biomass of source leaves and lateral roots of microcuttings in RF were significantly higher than those in SF, and the biomass of sink leaves of these microcuttings were significantly lower than that in SF (three-way ANOVA; factor *stage*, RF versus SF) (Table 1).

¹³C and ¹⁵N in plant tissue.

Sink leaves of microcuttings exposed to herbivory had significantly lower levels of both ¹³C and ¹⁵N, 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 , with significantly more ^{13}C and ^{15}N incorporated in principal roots during RF. Herbivory by *L. dispar* reduced ^{13}C and ^{15}N accumulation in sink leaves and stems but increased ^{15}N accumulation in source leaves. *P. croceum* inoculation and *L. dispar* herbivory had a combined effect on the incorporation of ^{13}C and ^{15}N only in stems with significantly more ^{13}C and ^{15}N incorporated compared with the effect of each 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 developmental stage.

Gene expression.

Twenty-four RNA templates were used to prepare cDNA libraries, which were subjected to paired-end RNA sequencing

Table 1. Effects of the factors stage (root flush versus shoot flush), inoculation with *Piloderma croceum*, *Lymantria dispar* (herbivory by larvae of *Lymantria dispar*), and interaction of *P. croceum* and *L. dispar* on biomass, analyzed in a three-way analysis of variance^a

Plant fraction (dry mass)	<i>t</i> values				<i>n</i>
	Stage	<i>P. croceum</i>	<i>L. dispar</i>	<i>P. croceum</i> / <i>L. dispar</i>	
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	-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	-2.17*	2.83**	0.39	1.11	71

^a Interactions with the factor stage were not significant and are not presented. The *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 (***, **, and * indicate $P < 0.001$, 0.01, and 0.05, respectively).

Table 2. Effects of the factors stage (root flush versus shoot flush), inoculation with *Piloderma croceum*, *Lymantria dispar* (herbivory by third instar larvae of *Lymantria dispar*), and interaction of *P. croceum* and *L. dispar* on incorporation of ^{13}C and ^{15}N ^a

Plant fraction	Stage	<i>t</i> values			<i>n</i>
		<i>P. croceum</i>	<i>L. dispar</i>	<i>P. croceum</i> / <i>L. dispar</i>	
¹³ C excess					
Sink leaves	0.44	-1.01	-2.91**	1.82	39
Source leaves	-0.90	1.68	1.40	-1.38	44
Stem	-1.15	-0.40	-2.61**	1.95(*)	40
Lateral roots	-0.28	4.27***	1.67	-0.50	45
Principal roots	-2.92*	-1.60	0.44	-0.95	45
¹⁵ N excess					
Sink leaves	1.94(*)	-0.63	-2.65*	1.04	39
Source leaves	-1.28	3.19**	1.99(*)	-1.25	44
Stem	-1.31	-6.01***	-4.21***	4.75***	41
Lateral roots	-1.02	3.80***	1.08	0.20	44
Principal roots	-2.05*	0.04	0.49	-0.93	44

^a Interactions with the factor stage were not significant and are not presented. 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 (***, **, *, and (*) indicate $P < 0.001$, 0.01, 0.05, and 0.1, respectively).

(RNA-Seq). On average, 17,196,808 clean reads were obtained and 14,392,437 (84%) of these reads mapped against the *OakContigDF159.1* reference transcriptome (Supplementary Table S1).

The highest number of contigs representing differentially expressed genes (DEG) ($n = 4,447$) was observed in source leaves of RF plants subjected to herbivory only, with 1,082 upregulated and 3,365 downregulated contigs (Fig. 3A; Supplementary Table S2). In contrast, lower levels of DEG were found with the inoculation with *P. croceum* only ($n = 396$) and for plants subjected to both *P. croceum* and *L. dispar* ($n = 512$). In microcuttings in sink leaves at SF (Fig. 3B), significantly lower numbers of contigs were detected, with 80 DEG at *L. dispar*, 56 at *P. croceum*, and 50 at cotreatment. Differential expression of 10 contigs was confirmed by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis (Supplementary Table S4; Supplementary Fig. S4).

Both the gene ontology (GO) term enrichment analysis (Fig. 2) and analysis of KEGG pathways (Supplementary Fig. S5) of samples in RF revealed that a strong enrichment of DEG related to growth and development, DNA replication, transcription, and translation. Furthermore, induced DEG involved in cell wall components and other aspects of growth were enriched in RF in response 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 sink leaves of plants in SF compared with plants in RF. In leaves of SF plants subjected to herbivory, the GO term enrichments indicated a focusing 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 plants subjected to inoculation with *P. croceum* and plants subjected to *L. dispar*, the GO term for apoplast (a cell component) was depleted. Along with the ^{13}C and ^{15}N allocation results, for RF plants, we found enrichments in GO terms related to starch biosynthesis in oak inoculated with *P. croceum* and exposed to herbivory, which was depleted in oak plants exposed to herbivore feeding alone. Furthermore, the term nitrate reductase complex was depleted in SF plants but enriched in RF 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. Most of the enriched pathways induced by herbivory were found during RF when subjected to *L. dispar*, and included pathways of amino acid metabolism and nucleotide metabolism. The depleted pathways in RF plants included many biosynthetic pathways of secondary metabolism, carbohydrate metabolism, and energy metabolism. In leaves of SF plants, only a few pathways were significantly enriched (N and C metabolism) and none were depleted.

A deeper insight into the actual gene expression levels can be provided by determining the DEG compared with the respective controls (Fig. 5; Supplementary Table S3). From the contigs related to the defense system against herbivory (Fig. 5), in leaves of microcuttings in RF subjected to *L. dispar*, two upregulated *chitinase* contigs were identified, as well as four *peroxidase* and *laccase* contigs (*thaumatin* and *pathogenesis-related protein 1* and *10* contigs). In contrast, three *catalase* contigs were downregulated. In RF plants treated with *P. croceum*, *chitinase IV* and *serine endopeptidase inhibitor* contigs were downregulated and three *laccase* contigs upregulated; however, the plants subjected to *P. croceum* did not show differential expression of these contigs. In the source leaves at RF subjected to *P. croceum* and *L. dispar*, two up- and

downregulated *thaumatin*, a downregulated *endopeptidase inhibitor*, and three upregulated *laccase* contigs were detected. In leaves of SF plants subjected to herbivory, transcripts of two *thaumatin* and a *chitinase* contig were much more abundant than in the untreated plants but the *P. croceum*-treated plants showed no significant differences. Also, in sink leaves with cotreatment, *thaumatin*, *chitinase*, and *subtilisin inhibitor* contigs were upregulated. Contigs encoding phenylalanine ammonia or tyrosine alanine ammonia lyase or lipoxygenase were stably expressed in all interaction types. Encoding an essential component of salicylic acid-dependent signaling, *enhanced disease susceptibility* contig was upregulated in all

interaction types, and *MYB transcription factor* at *L. dispar* and cotreatment. Three *callose synthase* contigs were upregulated in *L. dispar* and one at cotreatment; however, *polygalacturonase* and *hexose transporter* were upregulated in *L. dispar* but downregulated in cotreatment. Some contigs were differentially expressed only upon interaction with *L. dispar*. One contig encoding soluble and two granule-bound starch synthases, as well as three encoding starch branching enzyme, were downregulated in *L. dispar* treatment in source leaves at RF but in none of the other treatments. Only a few secondary metabolite biosynthesis-related contigs were differentially expressed. Putatively related to the production of volatile

Fig. 1. Effect of inoculation with *Piloderma croceum*, herbivory by caterpillars of *Lymantria dispar*, and inoculation with *P. croceum/L. dispar* 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* (Pilo), fed to larvae of *Lymantria dispar* (Lym), inoculated with *P. croceum* and fed to larvae of *Lymantria dispar* (Pilo/Lym), or not inoculated with the fungus and not fed to the herbivore (control). 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.

organic compounds (VOC), *geranylgeranyl-diphosphate reductase* was regulated exclusively by *L. dispar* and *germacrene-D synthase* was downregulated by *L. dispar* but upregulated in cotreatment in source leaves at RF. From phenylpropanoid biosynthesis-related contigs, three *caffeoyl-CoA 3-O-methyltransferase* transcripts were upregulated by *L. dispar* in RF and one in SF.

DISCUSSION

The results of our multitrophic experiments with an endogenous rhythmically growing plant show that a specific growth

stage, RF, displays a strong defense response during attack by a generalist herbivorous caterpillar. It has been previously shown that, in our study system, morphological (Herrmann et al. 1998), physiological (Herrmann et al. 2004), and genetic effects of inoculation in the premycorrhizal phase mirror the plant responses with a developed mycorrhiza. By avoiding the side effects of the variability of mycorrhization rates between plants and the confounding effects of the developed mycorrhizal structure, inoculated plants in the premycorrhizal stage represent an ideal tool for focusing on the genetic basis of plant responses on mycorrhization. Our results suggest that the developmental stage of the plant is an important factor

Fig. 2. Effect of inoculation with *Piloderma croceum*, herbivory by caterpillars of *Lymantria dispar*, and inoculation with *P. croceum/L. dispar* 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* (Pilo), fed to larvae of *Lymantria dispar* (Lym), inoculated with *P. croceum* and fed to larvae of *Lymantria dispar* (Pilo/Lym), or not inoculated with the fungus and not fed to the herbivore (control). Bars in the boxplots indicate the upper and lower quartile with median; whiskers indicate minimum and maximum values. Note that the y-axes are log-transformed.

influencing the response of rhythmically growing plants to herbivory. In addition, the interaction with mycorrhizal fungi even before mycorrhizas are formed can strongly influence plant defense gene expression responses against herbivory.

Impact on plant biomass and C and N incorporation.

The significant effects of inoculation with *P. croceum* on the biomass of both shoots and roots showed the impact of the fungus during the premycorrhizal phase confirming previous observations by Herrmann et al. (1998). In contrast, the short period of feeding by the herbivore did not significantly affect plant biomass at the levels of sink leaves, source leaves, lateral roots, or principal roots; thus, the effects of the herbivory treatment on genetic and physiological traits must not be interpreted as a consequence of biomass loss. This is in line with the aim of the experiment, to study the consequences of herbivory not as a result of biomass removal but as a trigger for plant responses as affected by inoculation with *P. croceum*. Moreover, plant responses to inoculation with *P. croceum* in terms of biomass changes were not affected by herbivore feeding.

We also found that the allocation of C and N was strongly affected by herbivory in a growth-stage-specific way, and that these effects were partly attenuated by the inoculation with the mycorrhizal fungus. Our findings of an enhanced incorporation of C and N into growing roots during RF confirm the allocation shifts related to the ERG described for oak microcuttings DF159 by Herrmann et al. (2015). Babst et al. (2008) reported an increased C export from leaves of *Populus nigra* leaves after exposition to *L. dispar* caterpillars. In accordance with this study, we showed that changes in allocation patterns upon herbivory may occur in a few hours after damage even at low levels of herbivory. Such quick responses can be suggested to be important for the plant's ability to tolerate herbivory. Accordingly, our findings show that oak in RF respond to herbivory with a reduced accumulation of carbohydrates into leaves. According to transcriptional changes (see below) and

¹³C-excess data, this effect is attenuated or even reversed in *Piloderma croceum*-inoculated oak.

Effects on gene expression patterns.

In our study system, changes in gene expression patterns in oak leaves in response to herbivory were prominent only in source leaves during RF but not in the sink leaves during SF. Our results indicate that oak trees use different strategies against leaf herbivory depending not only on ectomycorrhizal interaction even during the premycorrhizal phase but also on the developmental stage of the tree. The lower number of differently regulated pathways in leaves of oak in SF compared with RF underlines the strong impact of growth stage and the resulting differences in responses to herbivore attack, confirming our first hypothesis. We are also aware that the feeding pattern may explain these differences to a certain degree. Because caterpillars preferred feeding on source leaves, weak transcriptional changes in the sink leaves of SF seem to be plausible. However, systemic defense responses are common in trees (Eyles et al. 2010) and have also been repeatedly demonstrated for oak in response to feeding by *Lymantria* spp. (Roden and Mattson 2008; Rossiter et al. 1988; Schultz and Baldwin 1982). Faeth (1992) showed that leaf tissue removal increased the level of allelochemicals in the regrowing new foliage. Also, transcriptional changes in trees following herbivory have been shown to be systemic (Babst et al. 2009) and may be even faster and stronger in sink than source leaves (Philippe et al. 2010). Therefore, it is rather unlikely that the very weak response during the SF can be related to the feeding pattern. In accordance with our results, the stage in which roots are not growing (SF) has been found to be also accompanied by stronger changes in gene expression patterns in response to root-associated biota such as the mycorrhiza helper bacterium *Streptomyces* sp. AcH 505 (Kurth et al. 2015) and the plant-parasitic nematode *Pratylenchus penetrans* (Maboreke et al. 2016). Both growth and defense responses involve strong differences in plant gene expression patterns and physiological

Fig. 3. Venn diagrams of contigs representing differentially expressed genes in leaves of oak microcuttings in **A**, source leaves during root flush (RF) and **B**, sink leaves during shoot flush (SF) compared with the control microcuttings. Differential expression was calculated for source leaves in RF and sink leaves in SF by pairwise comparisons of treatments: control versus inoculation with *Piloderma croceum*, control versus herbivory by *Lymantria dispar*, and control versus inoculation with *P. croceum* and herbivory by *L. dispar*. Significance was tested using false discovery rate $P < 0.01$.

processes and, due to metabolic costs, growth-defense trade-offs exist (Karasov et al. 2017). One important way that plants mitigate such costs is by inducible defense gene expression, and our data indicate that, in oak, this is achieved even more precisely, through strong inducible expression of defense genes at the site of interaction in the rest phase only.

Like chemical defense, compensatory growth of plants is a common but costly direct response to herbivory (Haukioja and

Koricheva 2000; McNaughton 1983; Stowe et al. 2000). We could show that many GO terms and pathways related to growth, DNA replication, and starch biosynthesis were enriched in leaves of plants in RF only upon induction by herbivory alone whereas these responses to herbivory were weaker for plants subjected to inoculation with *Piloderma croceum*. Furthermore, our RNA-Seq data suggest that, during RF (when leaf development otherwise rests), herbivory leads to

Fig. 4. Heatmap of gene ontology (GO) term enrichment analysis for leaves of oak in root flush and shoot flush. 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.

the activation of leaf molecular machinery for cell division and expansion. Again, this reaction is alleviated in the presence of the mycorrhizal fungus, suggesting that fungal carbohydrate drain might direct the resources to the roots. Similarly, we found an upregulation of genes responsible for the production of *protease inhibitors* and *peroxidases* which act as effective antinutrients (Howe and Jander 2008) only in plants with herbivores alone. In the herbivory treatment with *L. dispar*, we found a gene encoding hexose transporter upregulated and genes related to starch synthesis downregulated compared with the control. Furthermore, terpene synthesis-related *geranylgeranyl-diphosphate reductase* and tannin production-related *caffeoyl-CoA 3-O-methyltransferase* genes were induced upon *L. dispar* treatment.

These genes are known to play a role for plants upon stress (Kiyosue et al. 1998; Seki et al. 2002) and their regulation patterns may indicate the need of saving valuable C resources from herbivory (Babst et al. 2009; Ferrieri et al. 2013), and reconfiguration of primary and secondary metabolism due to herbivory (Schwachtje and Baldwin 2008).

GO terms of growth, metabolism, and direct defense (e.g., chitin catabolism and response to wounding) were also enriched in leaves of plants in SF subjected to herbivory but no enriched or depleted terms were identified in leaves of plants in SF subjected to both inoculation and herbivory. Our finding of several enriched GO terms related to the cell wall in leaves of oak in RF subjected to herbivory feeding indicates an increasing rigidity in cell walls of plants exposed to herbivory (Onkokesung et al. 2010). Kersten et al. (2013) found a similar pattern for oak as well and interpreted the plant's cell wall as the first barrier in the line of herbivory defense. Moreover, genes encoding pathogenesis-related proteins are also known to be induced by arthropod feeding (Shafique et al. 2014) and were also upregulated in our study only when plants were fed upon by the herbivore.

Similarly, in the study of Kurth et al. (2015), the gene expression response of oak to *Streptomyces* sp. AcH 505 was also attenuated by the coinoculation with this mycorrhizal fungus. This suggests that *P. croceum* treatment buffers extensive gene expression responses of the oak (Herrmann et al. 2015). Interestingly, in our study, the alleviation of gene expression responses to herbivory by the inoculation with *P. croceum* is comparable with the effect of being in SF. Thus, increased plant vigor which may result from an increased availability of resources caused by either SF or the early phase of symbiosis may redundantize strong responses of the plant to the attack by herbivores.

However, several contigs were also differentially expressed in leaves both exposed to *L. dispar* and in the cotreatment with *P. croceum*. For example, contigs representing genes encoding chitinases were upregulated in leaves of all treatments (herbivore and fungus separately and in combination) compared with the control, which might be explained by the diverse functions of these enzymes, including nonspecific defense reactions which may also occur in response to symbionts (Brunner et al. 1998; Hartl et al. 2012; Kasprzewska 2003; Veluthakkal et al. 2012). Other genes described as related to defense against herbivores and pathogens such as *thaumatin like proteins* genes (Safavi et al. 2012; Shafique et al. 2014; Wang et al. 2010) have been found to be upregulated in all treatments involving the herbivore regardless of inoculation with *P. croceum*.

Indirect defense of plants often involves the release of VOC 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 (Havill and Raffa 2000; Kessler and Baldwin 2001; Mondor and Roland 1997, 1998), which are known to efficiently control

populations of herbivorous insects such as *L. dispar* (Alalouni et al. 2013). Terpene synthase and nerolidol synthase are involved in the synthesis of the VOC sesquiterpenes and mono-terpenes (Arimura et al. 2004; Baer et al. 2014; Pichersky and Gershenzon 2002). Terpenoid metabolism-related genes

Contig	Log2 FC	Annotation
<i>Control vs. P. croceum Source Leaf RF</i>		
comp39593_0_1	-4.37	Chitinase IV
comp19846_0_1	10.29	Endopept. inhibitor
comp41268_0_2	8.05	Laccase 15
comp43452_1_1	4.83	Laccase 14
comp38061_0_1	-2.83	Laccase 10
<i>Control vs. L. dispar Source Leaf RF</i>		
comp21061_0_1	6.6	PRP 10
comp37808_0_1	4.41	Thaumatococin
comp40152_0_1	1.91	PRP 1
comp42425_1_11	4.48	Chitinase I
comp34683_0_1	2.24	Chitinase I
comp43452_0_8	8.15	Laccase 14
comp38061_1_2	8.92	Laccase 15
comp37403_0_1	9.37	Laccase 6
comp41268_0_1	6.07	Laccase 15
comp40997_1_1	-2.73	Laccase 3
comp39773_1_1	-3.93	Laccase 14
comp32167_0_1	8.14	Peroxidase 53
comp37126_0_2	6.94	Peroxidase
comp38831_0_5	4.47	Peroxidase 37
comp30270_1_1	4.16	Peroxidase
comp29279_1_1	2.65	Peroxidase 31
comp43534_1_1	-2.81	Peroxidase 5
comp40472_0_1	-4.34	Peroxidase 5
comp42954_1_26	-7.03	Peroxidase 48
comp32087_0_2	-1.21	Catalase
comp32087_0_3	-8.33	Catalase 2
comp32087_0_2	-1.21	Catalase
comp23177_1_1	-1.7	Catalase
comp32087_0_3	-8.33	Catalase
comp37985_0_1	1.34	SOD
<i>Control vs. L. dispar Sink Leaf SF</i>		
comp36076_0_1	9.15	Thaumatococin
comp27895_0_1	9.1	Thaumatococin
comp40096_0_1	8.63	Chitinase IV
<i>Control vs. P. croceum and L. dispar Source Leaf RF</i>		
comp43430_0_3	5.65	Thaumatococin
comp32144_0_1	3.08	Thaumatococin
comp39324_1_4	-2.13	Thaumatococin
comp27895_0_1	-3.64	Thaumatococin
comp39593_0_2	-3.75	Chitinase IV
comp19846_0_1	-3.88	Endopept. inhibitor
comp43452_0_8	6.71	Laccase
comp41268_0_3	6.09	Laccase 14
comp38061_0_1	5.43	Laccase
<i>Control vs. P. croceum and L. dispar Sink Leaf SF</i>		
comp36076_0_1	6.03	Thaumatococin
comp40096_0_1	5.93	Chitinase IV
comp43013_0_1	7.17	Subtilisin inhibitor

Fig. 5. Differentially expressed genes related to herbivory response in leaves of oak. Pairwise comparisons of control versus inoculation with *Piloderma croceum*, control versus herbivory by *Lymantria dispar*, and control versus inoculation with *P. croceum* and herbivory by *L. dispar*. Annotations mark sequence descriptions derived from BLAST2GO contig assignment, addressing putative functions. RF = root flush and SF = shoot flush. Colors indicate direction in regulation: blue = downregulation and red = upregulation. Significance was tested using false discovery rate (FDR) and set for all contigs shown at FDR < 0.01.

represented by contigs were exclusively upregulated in plants subjected to both *P. croceum* and *L. dispar*, which indicates the strong influence of *P. croceum* on the defense strategy of oak. Furthermore, Kaling et al. (2018) reported that feeding damage and oviposition by poplar leaf beetle were reduced by ectomycorrhiza development between *Laccaria bicolor* in the hybrid poplar *Populus × canescens*, and that this was associated with increased production of N-containing compounds, volatile aldoximes, and phenylacetone nitrile during herbivory in ectomycorrhizal plants. The production and secretion of inducible compounds clearly warrants further investigation.

Conclusions.

The results of our study indicate that there are separate and interacting effects of inoculation with an ectomycorrhizal fungus and insect herbivory on gene expression patterns in oak dependent on the developmental stage during the endogenous rhythmic growth. Specifically, herbivory-induced changes in resource allocation and regulation of genes related to growth and defense are stronger during phases of root growth and for plants growing without the mycorrhizal fungus, indicating that additional resource supply or allocation of resources to the site of interaction tone down plant responses. The gene expression patterns of oak facing herbivory suggest a change from direct defense mechanisms and compensatory growth to indirect defense via volatile production during the premycorrhizal phase. Taken together, our results indicate a mediating effect of the fungus, especially during a developmental stage when leaf loss hits hardest because only roots are growing. Interestingly, our results suggest that the symbiosis with mycorrhizal fungi does not only support plants in their ability to respond to herbivory but may also induce a change in defense strategies during the RF. Costs and benefits of different strategies to deal with herbivory in a multitrophic context involving beneficial soil microbiota such as mycorrhizal fungi are worth further investigation.

MATERIALS AND METHODS

Oak, fungus, and herbivorous insect.

Our experiment was based on oak microcuttings in a microcosm system (TrophinOak joint experimental platform) (Herrmann et al. 2016). We used genetically identical micropropagated pedunculate oak trees of the clone DF159 (*Q. robur* L.) to exclude genetic variability as a confounding factor in our analyses (Ruhnke et al. 2006, 2009; Schädler et al. 2010). In contrast to seedlings, the obtained microcuttings showed a typical ERG with alternating RF and SF, and resembled mature trees physiologically (Herrmann and Buscot 2008; Herrmann et al. 1998). These flushes can be clearly identified using morphological traits following Herrmann et al. (1998). Furthermore, microcuttings are small enough to use in experiments in the laboratory under controlled conditions.

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 γ -sterilized soil, as previously described by Herrmann et al. (2016) and Tarkka et al. (2013). To investigate the effects of a mycorrhizal fungus on a host tree during an early phase of the symbiosis, we used the ectomycorrhizal fungus *P. croceum* Hjortst. strain 729 (DSM-4824). *P. croceum* is a common ectomycorrhizal fungus of both coniferous and hardwood tree species and an established model for both ecological and physiological studies, as described by Tarkka et al. (2013). In brief, half of the oak microcuttings were inoculated with the fungus at the beginning of the experiment by mixing a substrate mixture of vermiculite and sphagnum

peat containing a 2-week-old liquid fungal mixture (1:1 [vol/vol]) with the sterilized soil (Supplementary Fig. S1). For the other half of the microcuttings, soil was mixed with the same but sterile noninoculated substrate mixture.

To test the effect of herbivory, we used larvae of the polyphagous moth *Lymantria dispar* (Lepidoptera: Noctuidae). 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.

The Petri dish microcosm experiments were conducted in growth chambers with a photosynthetic photon flux density of $180 \mu\text{mol m}^{-2} \text{s}^{-1}$, a long-day setting (16 h of light and 8 h of darkness), a constant temperature of 23°C, and a relative air humidity of 75% (Ton et al. 2002). After 4 weeks, 5 ml of sterilized tap water was added to each microcosm with a sterile syringe.

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

Sixty days after setting up the microcosms, all microcosms were briefly opened under sterile conditions and $^{15}\text{NH}_4^{15}\text{NO}_3$ (5 ml at $0.02 \text{ g liter}^{-1}$; 98 atom% ^{15}N ; Sigma) was added to the soil containing roots. One day after the addition of ^{15}N -labeled ammonium nitrate, the “aboveground” parts of each plant chosen for herbivory exposure (*L. dispar*: $n = 12$; *L. dispar/P. croceum*: $n = 15$) were exposed to one-third instar larva of *L. dispar*. Each plant was covered with a bag with a mesh size of $100 \mu\text{m}$ to contain the larva. The feeding progress was checked twice an hour, and nonfeeding larvae were exchanged with fresh ones. Larvae were allowed to feed on all leaves. However, they nearly exclusively fed on source leaves and avoided sink leaves. 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 $^{13}\text{CO}_2$ (10 atom%) in the dark. The CO_2 concentration was adjusted to $401 \pm 3 \mu\text{l liter}^{-1}$ (mean $\pm$ standard deviation [SD]), yielding $9.2 \pm 0.1 \text{ atm\% } ^{13}\text{C}$ (mean $\pm$ SD). The ^{13}C labeling of the microcuttings took place in the subsequent 16 h, starting with the first light.

Plants were considered according to morphological traits either during the RF stage B (RF; 35) or to the SF stage D (SF; 36), as defined by Herrmann et al. (2015), which form the levels of the factor stage in our analysis (control: RF, $n = 15$ and SF, $n = 12$; inoculation: RF, $n = 8$ and SF, $n = 9$; herbivory: RF, $n = 6$ and SF, $n = 6$; and inoculation/herbivory: RF, $n = 6$ and SF, $n = 9$). The following plant compartments were separated according to Angay et al. (2014): (i) sink leaves (developing leaves that continue to import photoassimilates from source leaves) (i.e., terminal leaves, not yet fully developed or buds), (ii) source leaves (fully developed leaves that export their own products of photosynthesis) (i.e., subterminal leaves), (iii) stems, (iv) principal roots (i.e., main roots originating from stem), and (v) lateral roots (i.e., fine roots originating from principal roots). After determining the fresh weights, all samples were immediately frozen in liquid N and stored at -80°C . Dry weights used in the statistical analyses were calculated using reference values of each plant organ of RF and SF oak clone DF159 microcuttings given by 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 $\delta^{13}\text{C} < 0.1\text{‰}$ (SD, $n = 10$). ^{13}C and ^{15}N excess were calculated per plant compartment and on a whole-plant level over unlabeled microcuttings.

Transcriptomics.

Despite the drawbacks of GO analysis (Ashburner et al. 2000; Rhee et al. 2008; Young et al. 2010), together with pairwise comparisons of gene expression levels, GO enrichment analysis is a useful method for extracting patterns from masses of transcriptomic data and annotating the oak transcriptome according to the OakContigDF159.1 reference library (Tarkka et al. 2013).

Source and sink leaves of plants during RF and SF, respectively, were used for transcriptomic analysis according to Tarkka et al. (2013). The treatments included control, herbivory by *L. dispar*, inoculation with *P. croceum*, and treatment with *L. dispar* as well as *P. croceum*. First, the plants of each stage of growth and treatment type were shared in three pools. At this stage, each pool, which represents a biological replicate, consisted of at least two plants. The leaves of the pooled plants were used for RNA extraction.

In total, the 24 leaf RNA samples consisted of three biological replicates each of control/RF, control/SF, *L. dispar*/RF, *L. dispar*/SF, *P. croceum*/RF, *P. croceum*/SF, *L. dispar*/*P. croceum*/RF, and *L. dispar*/*P. croceum*/SF. Total RNA was extracted using the MasterPure Plant RNA Purification Kit (Epicentre Technologies Corporation, Madison, WI, U.S.A.), according to the manufacturer's protocol, from 50 mg of leaf material. The quality and quantity of RNA were checked using a Nanodrop spectrophotometer (Thermo Scientific) and a Nano Chip and Bioanalyzer 2100 (Agilent). 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. Illumina reads were processed as described by Tarkka et al. (2013). Briefly, poly (A) tails and low-complexity and low-quality sequences were removed with SeqClean. 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 preprocessing.

Gene expression analysis by real-time qRT-PCR. The reliability of RNA-Seq analyses derived from microcuttings of oak clone DF159 in controlled trophic interactions with *P. croceum* has been demonstrated earlier by Herrmann et al. (2015), Kurth et al. (2014), and Tarkka et al. (2013). To obtain independent plant material to confirm the RNA-Seq results, we performed a second experiment with an identical experimental setup. According to morphological traits, 28 plants were at RF and 28 plants at SF (control: RF, $n = 7$ and SF, $n = 7$; inoculated: RF, $n = 7$ and SF, $n = 7$; herbivory: RF, $n = 7$ and SF, $n = 7$; and inoculated plus herbivory: RF, $n = 7$ and SF, $n = 7$). After determining the fresh weights, all samples were immediately frozen in liquid N and stored at -80°C . Total RNA from oak source leaves at RF was extracted using the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol, from 50 mg of leaf material. The total RNA was treated with RNase-free DNase set (Qiagen). The quality and quantity of RNA were checked using a Nanodrop spectrophotometer (Thermo Scientific), and a Nano Chip and Bioanalyzer 2100 (Agilent). First-strand cDNA was synthesized from 1 μg of purified total RNA using the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's instructions. Real-time qRT-PCR was performed using the iQ 5 real-time PCR

detection system (Bio-Rad) and the gene-specific primers described in Supplementary Table S5. Amplification conditions were 10 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The specificity of each PCR amplification procedure was verified by melt-curve analysis of the PCR product with a heat dissociation protocol (from 60 to 95°C). Relative quantification of specific mRNA levels was performed using the comparative method of Livak and Schmittgen (2001). Expression values were normalized using the oak 18S ribosomal RNA gene (Tarkka et al. 2013).

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 and Dewey 2011). The significance of differences in gene expression was measured via pairwise comparisons between contigs of control versus *L. dispar*, control versus *P. croceum*, and control versus *P. croceum*/*L. dispar*, separately for plants in RF and for plants in SF, using the edgeR function (Robinson et al. 2010) of the Bioconductor package (Gentleman et al. 2004) in R (R Core Group). To ensure that RNA-Seq data are solid, contigs were called to be differentially expressed in a treatment compared with the corresponding control condition when exhibiting a Benjamini-Hochberg adjusted $P < 0.01$ after adjustment for multiple testing (Bluthgen et al. 2005; Conesa et al. 2005; Kanehisa et al. 2014). This false discovery rate is strict compared with the usually used level of $P < 0.05$. Resulting gene lists were analyzed for enriched GO terms and KEGG pathways using the Bioconductor package Goseq (Langmead et al. 2009). Biomass and C/N allocation were statistically analyzed in R using the lm function, implementing a three-way ANOVA. ^{13}C and ^{15}N values were $\log_{10}$ -transformed prior analyses to achieve normal distribution of residuals.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

TrophinOak project: <https://www.ufz.de/trophinoak/>
SeqClean: <https://sourceforge.net/projects/seqclean>
R Core Group: <http://www.r-project.org>