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

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

The rhizosphere, the narrow zone of soil influenced by the root system, is a nutrient-rich habitat providing niches for numerous microorganisms (Van der Ent et al. 2008). Among these, there are many fungi and bacteria with properties beneficial to plants (Berg and Smalla 2009). Some plant-beneficial bacteria (e.g., *Bacillus*, *Pseudomonas*, and *Streptomyces* spp. [Kloepper et al. 2004; Weller and Thomashow 2007; Schrey

and Tarkka 2008]) have been reported to protect plants against pathogenic microorganisms through a range of mechanisms, including competition for nutrients, secretion of antibiotics and lytic enzymes, and stimulation of plant defenses (Berg and Smalla 2009).

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

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

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

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

RESULTS

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

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

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

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

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

Although three times more contigs with DE were observed in co-inoculated than in singly inoculated plants, the results of GO (gene ontology) term and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis showed numbers in the same range for all three treatments, i.e., 50, 48 and 70 enriched GO terms and 21, 13, and 19 enriched KEGG pathways for AcH 505 alone, *M. alphitoides* alone, and co-inoculation, respectively. Inoculation with AcH 505 led to enrichment for GO terms ethylene biosynthesis, calmodulin binding, chitin binding and catabolism, shikimate biosynthesis, and phenylpropanoid metabolism (Fig. 3), and enrichment for KEGG biochemical pathway associations

phenylalanine, tyrosine, tryptophan and flavonoid biosynthesis, amino sugar and nucleotide sugar metabolism, carbon fixation, and nitrogen metabolism (Fig. 4). GO terms associated with photosynthesis and light harvesting and with chlorophyll binding were depleted. *M. alphitoides* inoculation led to a similar pattern of GO term enrichment to that with AcH 505 inoculation, with the exceptions of an increase in GO term defense response to bacterium and depletion in GO term isoprene biosynthesis, in the case of *M. alphitoides* (Fig. 3). Neither the KEGG-association phenylalanine metabolism nor flavonoid biosynthesis was increased. The depletion of the photosynthesis-related GO terms in response to AcH 505 and *M. alphitoides* was consistent with the observation that chlorophyll fluorescence measurements showed significantly reduced photosynthetic yield at a light intensity of 180 PAR ($P < 0.001$). In co-inoculated plants, in addition to the GO terms enriched following single inoculations, the terms regulation of hydrogen peroxide metabolism, receptor activity, salicylic acid biosynthesis, response to chitin, and aromatic compound metabolism were increased.

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

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

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

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

DISCUSSION

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

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

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

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

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

terium *Pseudomonas fluorescens* SS101. The central importance of the amino acid tryptophan as a precursor of camalexin and phenylalanine as an entry point to the phenylpropanoid pathway was also indicated in the analysis of *Pseudomonas fluorescens*-induced responses in *Arabidopsis* (Weston et al. 2012). The RNA-Seq data presented here showed enrichment in transcripts related to tryptophan, phenylalanine, and phenylpropanoid biosynthesis, suggesting that priming by *Streptomyces* spp. in pedunculate oak has determinants in common with the *Pseudomonas-Arabidopsis* system.

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

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

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

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

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

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

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

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

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

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

Plant growth and physiological responses.

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

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

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

Conclusions.

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

MATERIALS AND METHODS

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

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

Streptomyces sp. strain AcH 505 was quantified based on the work of Kurth and associates (2013), which focused on the colonization of soil and oak rhizosphere by the bacterium and a mycorrhizal fungus. Kurth and associates (2013) described the construction of *Streptomyces* sp. strain AcH 505-specific primers for real-time qPCR analysis and their use for its relative quantification in the soil and in the rhizosphere of oak. AcH 505 was also visualized in the rhizosphere of oak

by scanning electron microscopy (Kurth et al. 2013). In the context of an experiment investigating the course of the bioassay, we observed that the level of the *Streptomyces* strain did not differ significantly at weeks 7 and 9, suggesting a stable concentration at the second application and the harvest time point. Our data also suggest that the *Streptomyces* strain did not enter the leaves. Strain AcH 505-specific qPCR signal was not detected from leaf DNA, which indicates that the *Streptomyces* strain is physically separated from the leaf pathogen.

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

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

RNA extraction and Illumina sequencing.

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

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

Read processing and analysis of differential expression.

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

Primer design and validation for qRT-PCR.

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

Real-time PCR assays.

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

Enzyme activity analysis.

PAL and POD activities were measured from leaf tissue extracts of noninoculated, AcH 505-inoculated, *M. alphitoides*-infected, and dual (bacterium and fungus)-inoculated oak microcuttings from the same experimental material as were used for Illumina sequencing. Leaf tissue was homogenized under liquid nitrogen. Four individual measurements were carried out per treatment. For PAL measurement, 10 mg of the powder was suspended in 500 µl of 100-mM Na-borate buffer, pH 8.8, containing 5 mM beta-mercaptoethanol (Zucker 1968). The extract was filtered through 15-µm nylon tissue, and 10 µl

of the extract were added to 290 µl of the reaction mixture containing 2 mM L-phenylalanine as the substrate in Na-borate buffer at pH 8.8. PAL activity was measured in a spectrophotometer at 290 nm each minute for up to 15 min after the addition of the extract to the reaction mix. POD activity was measured based on Mensen and associates (1998). Ground leaf tissue (10 mg) was suspended in 500 µl of 100 mM Na-acetate buffer, pH 5.6. The extract was filtered through 15-µm nylon tissue and 10 µl of the filtered extract was transferred to the reaction mixture, containing 990 µl of 50 mM Na-acetate, 4 mM guaiacol, and 2 mM of the POD substrate, H₂O₂. POD activity was measured every minute at 470 nm in a spectrophotometer for up to 30 min. Increase in absorbance at 290 nm during PAL measurements was linear up to 10 min and, during POD measurements, at 470 nm up to 30 min after the start of the reaction. All data reported correspond to the linear phase of the reaction. Relative PAL and POD enzyme activities were determined as the change in the absorbance in 1 min, converted by the extract from 1 mg of fresh weight of leaf tissue.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

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