Dual RNA-seq of the plant pathogen Phytophthora ramorum *and its tanoak host*

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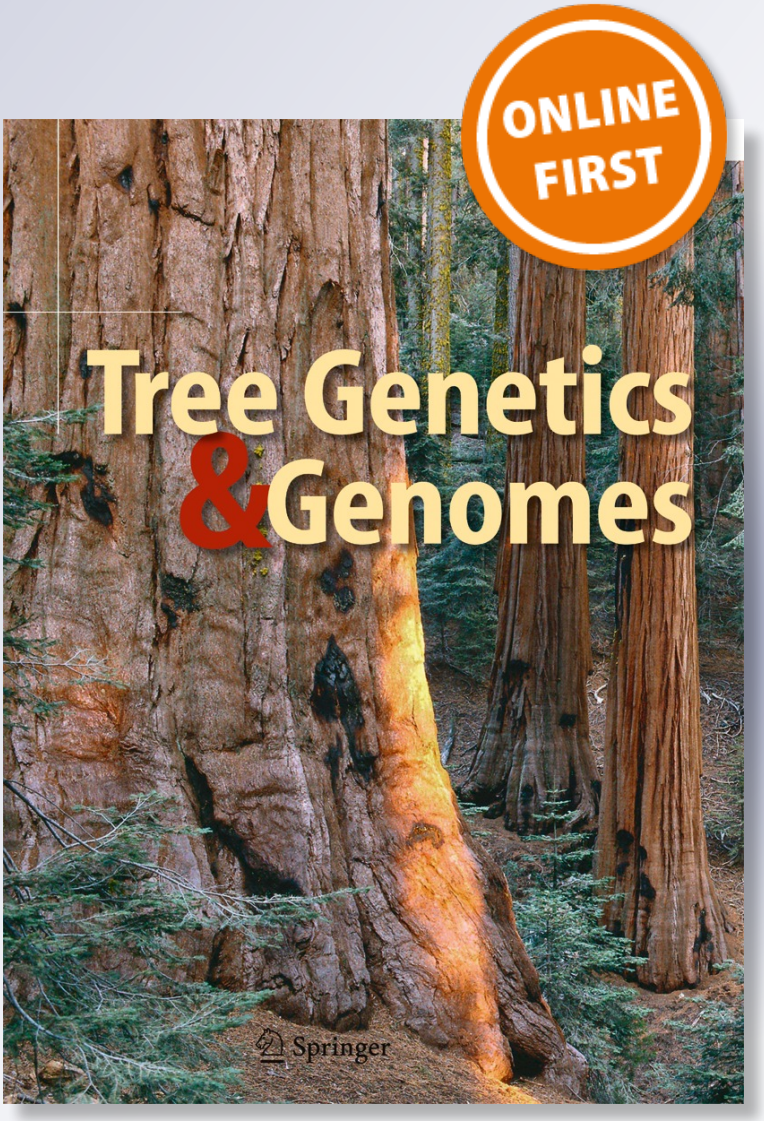

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ORIGINAL PAPER

Dual RNA-seq of the plant pathogen *Phytophthora ramorum* and its tanoak host

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Abstract Emergent diseases are an ever-increasing threat to forests and forest ecosystems and necessitate the development of research tools for species that often may have few pre-existing resources. We sequenced the mRNA expressed by the sudden oak death pathogen *Phytophthora ramorum* and its most susceptible forest host, tanoak, within the same tissue at two time points after inoculation, and in uninfected tanoak controls. Using the *P. ramorum* genome to differentiate host and pathogen transcripts, we detected more than 850 *P. ramorum* transcripts at 5 days post-inoculation and a concurrent upregulation of host genes usually associated with pathogenicity. At 1 day, in contrast, we did not detect pathogen expression or significant enrichment of functional categories of host transcripts relative to controls, highlighting the importance of sequencing depth for *in planta* studies of host–pathogen interactions. This study highlights processes in molecular host–pathogen interactions in forest trees and provides a first reference transcriptome for tanoak, allowing the preliminary identification of disease-related genes in this study and facilitating future work for this and other members of the family Fagaceae.

Keywords Hemibiotroph · Host–pathogen interactions · Oomycete · Pathogenesis · Sudden oak death

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Introduction

Emergent diseases are an increasing threat to forests worldwide (Brasier 2008; Burdon et al. 2006; Loo 2009). Mass die-offs of foundation species have cascading ecosystem effects stemming from irreversible changes in community structure (Ellison et al. 2005), including a global impact due to the atmospheric carbon emitted by dying trees (Breshears and Allen 2002; Kurz et al. 2008). Sudden oak death (SOD, causal agent *Phytophthora ramorum*) is one disease with such far-reaching impacts. Tanoak (*Notholithocarpus densiflorus*, formerly *Lithocarpus densiflorus*), a monotypic genus in the family Fagaceae (Manos et al. 2009), is the only species in US forests that has been found to be susceptible to both disease forms: sudden oak death, a fatal canker known for the destruction it has caused in US forests but which does not support sporulation, and ramorum blight, a leaf and twig blight which has minimal fitness effects on its primary hosts, yet is responsible for the pathogen's spread (Davidson et al. 2003, 2008). Tanoak is endemic to California and coastal

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Oregon and holds approximately 68 Tg of carbon across its range, with highest densities in areas that are at high risk for sudden oak death (Lamsal et al. 2011). As the primary masting tree in many coastal evergreen forests, tanoak is truly a foundational species, providing food for humans and wildlife (Bowcutt 2011; Meyers et al. 2006), and being one of the primary ectomycorrhizal hosts in redwood and Douglas-fir forest systems (Bergemann and Garbelotto 2006). Tanoaks are highly susceptible to SOD, and the decline or extirpation of large size classes is predicted in areas that favor disease (Cobb et al. 2012; Ramage et al. 2011; Waring and O'Hara 2008). However, individual trees have been reported to survive 10 years or longer in heavily infested sites (McPherson et al. 2010), and there are indications that resistance may play a role in tanoak disease dynamics (Hayden et al. 2011, 2013).

The understanding of plant host–pathogen relationships at a genomic scale has been largely limited to herbaceous hosts and particularly to model systems. Nonetheless, a long history of research in forest species has done much to advance the understanding of the molecular genetics of disease resistance in trees. This has included the discovery of resistance genes (reviewed by Ersoz et al. 2010), and genomic studies of chestnut (*Castanea* spp.) including those focused on disease resistance (Barakat et al. 2009; Wheeler and Sederoff 2009). Genome-wide association studies have lent insights to adaptive traits in *Pinus* species (Eckert et al. 2009; Lepoittevin et al. 2011), but due to the stature, long generation times, and large genomes of forest trees, the genomic study of non-commercial species—for which populations of clonal replicates or defined phenotypic lines are unavailable—has been especially limited (Parchman et al. 2010; Rampant et al. 2011).

The advent of high-throughput sequencing coupled with advances in bioinformatic technologies has paved the way for genomic studies in systems that were previously intractable to research (Ekblom and Galindo 2011; Neale and Kremer 2011). One such approach is RNA-seq, the sequencing of cDNA libraries from individuals experiencing various physiological challenges, with no prior knowledge of the genes' identity or sequence (Mortazavi et al. 2008). Typically, methods employing RNA-seq have relied on reference genomes to assemble and quantify the millions of small sequence fragments produced by high-throughput methods, but bioinformatic advances now allow the de novo assembly of an entire transcriptome (Grabherr et al. 2011; Robertson et al. 2010).

The complete genome of *P. ramorum* has been sequenced and annotated (Tyler et al. 2006), and this has added much to the understanding of oomycete evolution and pathogenicity (e.g., Jiang et al. 2008; Kasuga et al. 2012; Seidl et al. 2011; Win et al. 2012). As we report here, the genome sequence also enables bioinformatic tools to separate pathogen transcripts from the host when both have been concurrently sequenced in infected plant tissue. Subtraction of human-genomic sequences from pathogens has already been used to separate and even diagnose microbial sequences from infected tissue (Isakov et al. 2011; Weber et al. 2002; Xu et al. 2003). These methods have recently been applied to tomatoes (Li et al. 2012), for which a reference genome is also available. Simultaneous transcriptional profiling of a plant host–pathogen interaction in systems with both the host and pathogen references available has been accomplished, first using SuperSAGE (serial analysis of gene expression) (Matsumura et al. 2003) and more recently using RNA-seq (Kawahara et al. 2012; Petre et al. 2012). Expression analysis after the generation of de novo reference transcriptomes for both host and pathogen has been reported in a turfgrass pathosystem (Orshinsky et al. 2012). The work we report here combines approaches, using reference-based mapping to subtract pathogen sequences, followed by de novo assembly of a the transcriptome of the non-model forest-tree host.

The precise mode of infection of tanoaks by *P. ramorum* in nature has not been characterized, but it has been observed that disease in large trees follows a pattern of aerial spread of sporangia in rain splash or rare wind events, and frequently begins as leaf and twig infections in the crown, with trunk cankers following (Hansen et al. 2008). Tanoak mortality likely results from girdling by cambial cankers (Rizzo et al. 2002), but mycelia and extensive tylosis formation have also been observed in the trees' xylem, suggesting that vascular obstruction also plays an important role in disease etiology (Parke et al. 2007; Stamm and Parke 2013). In laboratory studies of *P. ramorum*, infection most often occurs within 48 h from the release of swimming zoospores from the asexual sporangia (Moralejo and Descals 2011). *Phytophthora* spp. are hemibiotrophic, living in necrotic tissue following the first stages of infection. Effectors, large families of secreted proteins (Hardham and Cahill 2010; Kamoun 2006), are thought to have a dual role in hemibiotrophs, initially helping to evade plant defenses, but in later stages inducing programmed plant cell death and subsequent necrosis (Jiang et al. 2006; Kleemann et al. 2012). The chemical application of members of the elicitin family of effectors has been shown to independently cause photosynthetic declines in tanoak (Manter et al. 2007).

We undertook a first transcriptomic study of tanoak infected with *P. ramorum*, with the aim to provide a reference transcriptome for tanoak. We sequenced tissue from infected and non-inoculated control leaves at two times post-inoculation, in order to provide guidance for future experimental methods while making preliminary identification of disease-related genes in this pathosystem.

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Materials and methods

Experimental treatments

Pathogen isolate

Within North American forests, *P. ramorum* populations appear to be comprised of relatively few genotypes, which all were clonally derived from a single founder individual (Croucher et al. 2013; Mascheretti et al. 2008) within the North American 1 (NA1) lineage (Ivors et al. 2006). NA1 isolate Pr52 (CBS110537; ATCC MYA-2436, taken from the Garbelotto Lab culture collection and originally obtained from D. Rizzo, University of California, Davis) has been frequently used in laboratory inoculations (Anacker et al. 2008; Garbelotto et al. 2009; Hayden et al. 2011; Tooley et al. 2009; Tooley et al. 2004). Comparative studies have reported this strain to be highly pathogenic on leaf and canker hosts (Hüberli and Garbelotto 2011).

To induce sporangia formation, 1×1 cm pieces V8 agar (Erwin and Ribeiro 1996) colonized by the Pr52 were flooded with sterile 1 % soil extract and incubated at 18 °C for 2 days, followed by a 20-min cold shock at 4 °C to induce zoospore release. To prevent premature encystment by zoospores, all plastic and glassware to come in contact with zoospores were soaked in 10 % hydrochloric acid and rinsed in deionized water prior to use.

All pathogen propagation and plant inoculations were completed under permit and according to conditions set by the United States Department of Agriculture Animal and Plant Health Inspection Service, and the California Department of Food and Agriculture.

Tanoak cuttings

Because tanoak is a non-commercial species, there are no established stocks of clonal propagates or breeding lines. A 4-year-old potted *N. densiflorus* sapling grown in a lath house in Berkeley, CA from wild-collected seed was selected for this first study, based on its moderate susceptibility to detached leaf inoculation assays (Lundquist and Hayden, unpublished data). Twelve cut branches, approximately 20–30 cm long with at least three leaf nodes, were moved from a lath house to randomized positions in a growth chamber with a 12 h, 20 °C day, and 13 °C night, in individual sterilized 500 mL bottles of deionized water, and were misted with tap water in 15-min intervals twice daily.

After equilibrating for 24 h in the growth chamber, a 1-cm length of petiole and midrib on each of three leaves per cutting was wounded by lightly scraping with a sterile scalpel. Immediately thereafter, 100 μL of zoospore suspension averaging 10^5 spores per milliliter (inoculation treatments) or sterile 1 % soil extract (controls) was dropped on the leaf axil, which was surrounded by a cup made from Parafilm (SPI Supplies) to prevent runoff of the suspension.

Leaves were harvested two times after inoculation. Plants' transcriptional responses to inoculation with pathogens have been documented as early as 1 h post-inoculation (Norelli et al. 2009; Orłowska et al. 2011), but disease progression is generally at first quite slow in tanoaks, and visible lesions usually do not develop earlier than 3–5 days post-inoculation (personal observation, K. Hayden). The accumulation of *P. ramorum* DNA in inoculated *Umbellularia californica* leaf tissue has been shown to increase after 48 h post-inoculation, corresponding with the appearance of lesions (Hayden et al. 2006). At 24 h (1 day) and 120 h (5 days) after inoculation, a 3 cm by 1 cm strip of tissue, including both petiole/midrib and leaf blade, was excised and flash-frozen from each leaf on three cuttings per inoculation treatment and time period. There were thus four sample units: 1 day inoculated (I1), 1 day control (C1), 5 days inoculated (I5), and 5 days control (C5) (Fig. 1). At 1 day no lesions were visible, and at 5 days faint, continuous lesions were visible in inoculated leaves.

RNA isolation, library preparation, and high-throughput sequencing

Prior to extraction, all of the excised, frozen tissue corresponding to a treatment group was pooled into a single, 75–100-mg sample. Bulk RNA was extracted using a chloroform–isoamyl alcohol extraction and lithium chloride precipitation (Chang et al. 2007; Le Provost et al. 2007). The terminal DNAse treatment and column cleanup steps described by Le Provost et al. were omitted to save time and costs because the Illumina RNA-seq protocol includes purification of poly-adenylated RNA, removing DNA. The purity and concentration of RNA extracts were characterized with the Bioanalyzer 2100 platform (Agilent Technologies, Santa Clara, CA).

Samples were diluted to contain a total of 5 μg ribonucleic acids in 50 μL of nuclease-free water. Poly-adenylated RNA was purified and enriched, and unstranded libraries were prepared as described by Illumina (San Diego, CA) GAII protocols (part no. 1004898 Rev. A), except that Agencourt AmPure XP kits (Beckman Coulter, Brea, CA) were used for PCR purification steps.

Single-end, non-indexed 80-bp fragment sequencing was performed at the Oregon State University Center for Genome Research and Biocomputing (CGRB), using Illumina GAII equipment and software pipeline version 1.2. Each flowcell included a PhiX control lane.

There was a single sample and flowcell lane for each treatment group. Groups I1, I5, and C1 were sequenced in the same flowcell. Sequencing failed in the C5 lane in the

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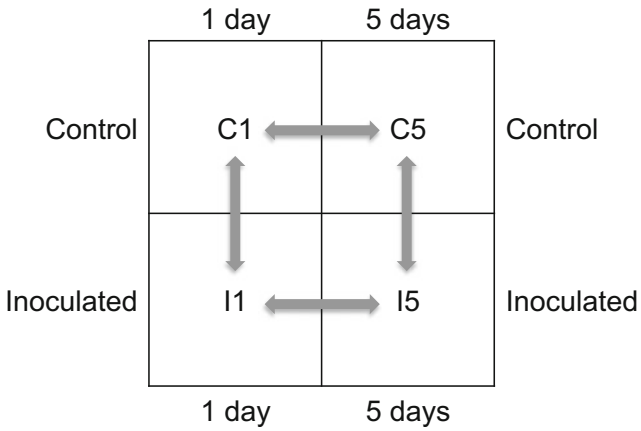

Fig 1 Experimental scheme. Pairwise comparisons for differential expression analysis are indicated by *gray arrows*; *horizontal arrows* represent comparisons within treatment, and *vertical arrows* represent those within time

shared flowcell, so C5 data were generated on a different flowcell, in the same facility and with the same protocols.

Sequence data were initially processed using the Illumina pipeline, followed by filtering for primer dimers using custom scripts (brianknaus.com). The reads were further filtered for cumulative quality scores, with an error probability limit P=0.05, using CLC Genomics software (www.clcbio.com), and for a minimum 40-bp fragment size after quality filtering.

Differentiation of host and pathogen sequences

Filtered reads were mapped to the *P. ramorum* genome (Tyler et al. 2006) using two degrees of stringency: first to the unfiltered genome model ramorum1 using CLC Genomics software with permissive mapping parameters of 50 % length fraction and 80 % minimum similarity, in order to conservatively remove pathogen sequence, and secondly, to characterize the observed *P. ramorum* transcription, sequences were mapped to the genome and transcriptome together using the combined programs Bowtie2 and TopHat2 (Langmead et al. 2009; Trapnell et al. 2009), with the call: tophat2 –solexa-quals –library-type fr-unstranded -i 30 -I 5000 -G [path to Phyra1_1.gff] [path to ramorum1 indices] [data file in fastq format].

The option –i 30 sets the minimum intron length to 30 bp, and -I sets the maximum distance between splice junctions to 5,000. These lengths are smaller than defaults for mammalian systems, in accordance with the relative paucity and smaller size of oomycete introns (Kamoun 2003; Shen et al. 2011). After mapping, isoforms were finalized and compared to the reference transcripts using the program Cufflinks (Trapnell et al. 2010, 2012).

P. ramorum functional annotation

Mappings to the *P. ramorum* Phyra1.1 model were first annotated using the reference transcript model annotation (Tyler et al. 2006). Secondly, we performed a local blastx search of translated nucleotides against custom databases of *Phytophthora* species RXLR avirulence homologs (Jiang et al. 2008), cellulose-binding and elicitin-like proteins (Sierra et al. 2010), and crinkler and necrosis-inducing (CRN) proteins (Haas et al. 2009; Tyler et al. 2006), as well as against the PHI-base pathogen–host interaction database release 3.2 (Winnenburg et al. 2006). All searches were implemented with a minimum e-value cutoff of 10^{-10}.

Assembly and validation of de novo tanoak transcriptome

The reads that were not mapped to the *P. ramorum* genome model ramorum1 by the least stringent mapping procedure were pooled together to create a de novo transcriptome. The pooled reads were assembled using the program Trinity (v20110519, Grabherr et al. 2011), which uses three software modules to refine an initial transcriptome assembly. All three steps were performed on the CGRB compute cluster, with the third "Butterfly" step using a highly parallel step via a custom script and the Sun Grid Engine queuing system; contigs with fewer than 300 bp were excluded from the data.

To confirm that *P. ramorum* sequences had been fully removed from the dataset and to identify sequences corresponding to endophytes or other microbial associates, the tanoak transcriptome was queried against the NCBI comprehensive non-redundant (nr) database using a translated-nucleotide-to-translated-nucleotide (tblastx) search (Altschul 1990). An additional blastn search was performed against the mitochondrial (mt) databases (www.ncbi.nlm.nih.gov). In both cases, an expected value of less than 10^{-10} was used to call matches.

Tanoak mapping, normalization, and statistical testing

Quantification of sequenced mRNA poses a special problem: alternative splicing can cause reads from even unique gene regions to map equally well to multiple transcripts (reviewed by Pachter 2011). For this reason, we first mapped each lane individually to the reference using the short-read aligner Bowtie (Langmead et al. 2009) v0.12.7 and converted the output in Sequence Alignment/Map (SAM) format to the binary BAM format via SAMtools (Li et al. 2009). We then used eXpress (v1.0.0; Roberts and Pachter 2013) to resolve multiple and other ambiguous mappings, using parameters as recommended. This program uses an expectation-maximization algorithm to assign mappings probabilistically,

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but unlike other programs with similar functionality, eXpress does not require an annotated genome.

Analysis of RNA-seq expression data is confounded by differences in sequence counts among lanes, as well as by biases for sequence length and GC-content (Bullard et al. 2010; Hansen et al. 2010; Oshlack and Wakefield 2009). We therefore performed three normalizations on the sets of transcript counts. First, initial transcript-length and GC-content normalization was performed in eXpress, generating effective counts rather than total or unique counts. Second, full-quantile normalization for transcript-level GC-content and length biases was performed within lanes using the R package EDASeq (Risso et al. 2011). Finally, full-quantile normalization in EDASeq was again used to correct for among-lane differences in GC-content and library size.

Differences in normalized counts among treatment groups were tested in 4 pairwise comparisons (Fig. 1). Within time, we compaired Inoculated vs Control at 1 day (I1 vs C1); and Inoculated vs Control at 5 days (I5 vs C5). Within treatment, comparisons were 5 days vs 1 day, Inoculated (I5 vs I1); and 5 days vs 1 day, Control (C5 vs C1).

Seven thousand seven hundred thirty-two transcripts with an experiment-wide mean effective count less than ten across lanes were assumed to be non-informative and were excluded prior to pair-wise comparisons, leaving 40,656 transcripts for analysis.

Pairwise differences in normalized counts were tested using the negative binomial test for experiments in the R package DESeq (v1.8.3; Anders and Huber 2010), using pooled dispersion estimates with only the fitted values shared across genes as recommended by the authors for samples without replication. The significance threshold was set at FDR$<$0.05 after correction for multiple comparisons (Benjamini and Hochberg 1995).

Tanoak functional annotation

Similar to those mapping to *P. ramorum*, tanoak transcripts were annotated by a Blast2GO search of the NCBI nr database, with a minimum e-value of 1×10^{-10}, followed by searches against custom databases. The Plant Resistance Genes database (Sanseverino et al. 2010), which consists of resistance genes derived from reference genes, from NCBI Unigene and from predictions based on domain functional prediction, and the PHI-base interaction database (Winnenburg et al. 2006) were used to annotate resistance genes and other pathogen–host interaction genes within the tanoak transcripts. Finally, the transcriptome sequences were searched against a set of sequences related to disease response in *Castanea mollissima* and *Castanea dentata* infected with the chestnut blight fungus, *Cryphonectria parasitica* (Barakat et al. 2009).

We tested for over-representation of protein categories within each pairwise comparison of time and treatment (Fig. 1) using Fisher's exact test within the Blast2GO platform (Conesa et al. 2005). Sequences with greatest similarity to taxa other than plants were excluded from the data before testing.

Results

Concurrent sequencing of *P. ramorum* and tanoak transcriptomes

We used short-read high-throughput sequencing of tanoak cuttings, with and without inoculation by the sudden oak death pathogen *P. ramorum*, to (1) provide the first concurrent *in planta* transcriptomes of *P. ramorum* and a forest host and (2) to get a first portrait of the gene sets involved in the interaction. A total of 52 Gbp of sequence data were produced, ranging from 331 to 2,186 Mbp per sequence lane (Table 1).

Differentiation of host and pathogen sequences

P. ramorum was convincingly detected in the I5 sample by both the most permissive mapping method (CLC Genomics for removal of putative pathogen sequences), with 4.5 % mapped by the least stringent criteria, and the most stringent (Bowtie/Tophat/Cufflinks for *P. ramorum* transcript characterization, with 1.1 % mapped) (Table 2). The percentages of reads mapped to *P. ramorum* were not distinguishable among I1, C1, and C5 groups and ranged from 0.1 to 1.4 %, depending on the mapping method (Table 2). The GC-content of mapped reads was highest in lane I5, and at 0.58 was equivalent to the average GC-content of transcribed sequences described in *P. ramorum* (Jiang and Govers 2006) and in other *Phytophthora* species (Kamoun 2003).

Sequential mapping of the I5 sample to the *P. ramorum* genome and transcriptome model references identified 858 unique transcripts, from 729 unique reference transcripts (Supplementary Table 1). The majority of these, 522, either completely matched or were contained within an annotated intron chain. Two hundred twenty-five overlapped annotated exons and an additional 21 shared at least one splice junction with the reference. This last group may represent improvements to current gene models or may be novel isoforms.

P. ramorum functional annotation

Only sample I5 returned more than negligible blast hits to pathogenicity-related databases. In the control and 1-day samples, none of the sequences that were mapped to the *P. ramorum* genome were matched to genes in the *Phytopthora*-specific pathogenicity databases and no more

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Table 1 Summary statistics for RNA-seq of tanoak and *P. ramorum* (I), and mock-inoculated tanoak controls (C), including mean read length after trimming for quality

Identifier	Treatment	Time post-inoculation	Raw reads	Mean read length (bp)
I1	Inoculated	1 day	4,420,578	75
I5	Inoculated	5 days	10,353,182	70
C1	Control	1 day	20,238,150	75
C5	Control	5 day	28,324,515	77

than three were matched to the more general PHI-base pathogen proteins. The very few transcripts mapped to *P. ramorum* in sample I1, however, were significantly longer than those mapped from the control lanes (P=0.007, $F_{2,22}$=20.6), while there was no significant difference in transcript length within treatment groups. In contrast, in *P. ramorum*-mapped transcripts in sample I5, there were matches to at least 30 CBEL-type proteins (Tyler et al. 2006; Sierra et al. 2010) and 6 Avr-homologs (Jiang et al. 2008) and to a single CRN (Tyler et al. 2006; Haas et al. 2009) (Supplementary Table 2).

Assembly and validation of de novo tanoak transcriptome

The Trinity assembly of all reads that did not map to the *P. ramorum* reference produced a de novo transcriptome consisting of 48,388 unique sequences in 34,642 isogroups, with $N50$=1,289. The vast majority (more than 29,000) of the 31,894 that were matched to sequences in the NCBI nr database with taxonomic information were best matched to a sequence within the plants, Viridiplantae (Fig. 2). The next-most represented group was fungi (primarily Ascomycota). The third most abundant taxonomic category was Arthropoda, of which 607 out of 644 transcripts matched aphid sequences. These aphid sequences were likely due to the presence of aphids in the growth chamber. A total of six transcripts matched mitochondrial sequences. Very few (three transcripts) included Stramenopiles among the best matches, and only a single transcript returned a best hit to a *Phytophthora*: a *Phytophthora* sojae hypothetical protein. The dearth of matches to close relatives of *P. ramorum* suggests that differences in gene expression among inoculated and non-inoculated samples are not due to presence of perhaps novel *P. ramorum* transcripts, which failed to map to the reference, but rather a response of the plant itself. It is possible that there may be systematic differences in the presence or magnitude of expression of transcripts originating from microbial or insect associates in inoculated tissue relative to controls. However, if such systematic differences exist, most are likely to be erased with the exclusion of transcripts which were best matched to taxa outside plants.

Tanoak mapping, normalization, and statistical testing

Sequenced library sizes were dramatically different among treatments/lanes, both in raw reads and in transcript-level counts (Table 1, Supplementary Fig. 1). Post-normalization transcript-level count and GC-content distributions were comparable among lanes (Supplementary Figs. 1 and 2).

The largest differences in expression were observed in inoculated samples at 5 days post-inoculation compared to 1 day post-inoculation (within treatment: I5 vs. I1, Fig. 3), in which 1,040 transcripts were significantly differentially expressed. This finding contrasts with 169 differentially expressed transcripts in the control (samples C5 vs. C1). Within sampling time, there were 245 transcripts significantly differentially expressed between inoculated and control at 1 day post-inoculation and 271 at 5 days post-inoculation (Fig. 3). Results from unreplicated comparisons should be viewed as preliminary identification of differentially expressed sequences (Bullard et al. 2010; Pachter 2011) to be used in guiding further study, but in need of confirmation through replicated experiments.

Tanoak functional annotation

From the de novo tanoak transcriptome, consisting of the 48,388 unique sequences assembled from all reads that were

Table 2 Summary statistics for mapping to the *P. ramorum* reference via different protocols, and to the de novo tanoak transcriptome

Identifier	*P. ramorum* genome[a] (lax mapping)	*P. ramorum* genome and transcript model[a] (stringent mapping)			De novo tanoak transcriptome
	Mapped (%)	Mapped (%)	Transcripts	GC-content	Mapped reads
I1	1.2	0.1	8	0.51	0.5×10^7
I5	4.5	1.1	858	0.58	1.4×10^7
C1	1.4	0.1	12	0.51	2.5×10^7
C5	0.9	0.1	13	0.51	3.5×10^7

[a] Tyler et al. 2006

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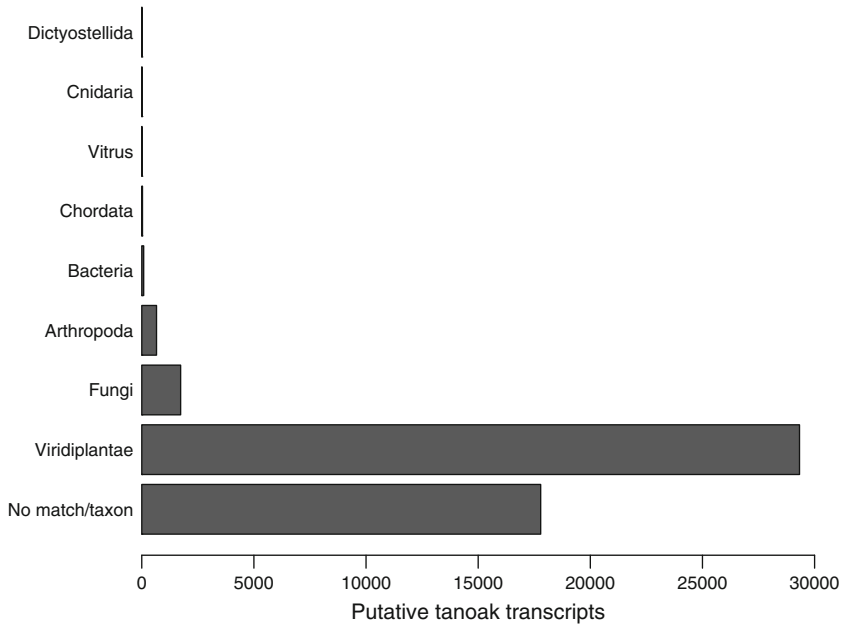

Fig. 2 Taxonomic characterization of the 48,388 contigs in the de novo transcriptome by matching to the NCBI nr database; broad taxonomic group name is followed by the number of transcripts with a top match to this group. Seven taxa with fewer than ten matching transcripts are not labeled; these include stramenopiles, to which three transcripts matched

not mapped to *P. ramorum*, 34,378 were matched to the NCBI nr database. Of these matches, 24,351 were assigned protein functional annotations. Relatively few matches to LRR-domain and other curated plant resistance genes (Sanseverino et al. 2010) were observed, 1,059 within the entire tanoak assembly, and not more than 6 were represented in any differentially expressed subset. There was more similarity to *C. mollissima* and *C. dentata* disease-related transcripts, with some 7,051 sequence matches overall.

Enrichment tests of Gene Ontology (Ashburner et al. 2000) terms among sequences differentially expressed in pairwise comparisons (Fig. 1) lend preliminary insight to changes in

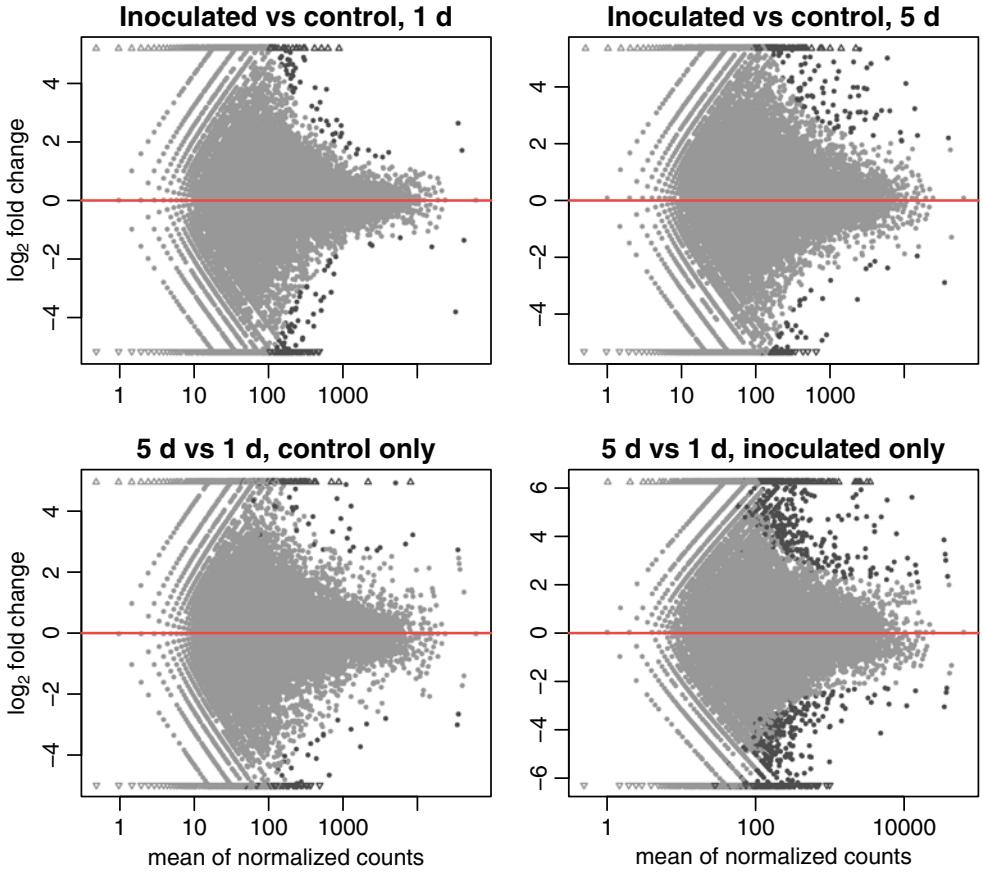

Fig. 3 Pairwise comparisons of expression of tanoak transcripts. Transcripts with statistically significant differential expression at an FDR-adjusted $P<0.05$ are represented with *dark gray dots*. Transcripts with zero expression in one condition are represented by *open triangles*

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branch physiology over time (Table 3, C5 vs C1, I5 vs I1) and inoculation status (I5 vs C5, I1 vs C1). The need for verification with further replicates is highlighted by low sequence yield in sample I1 (Table 1, Supplementary Fig 1). Housekeeping functions were included among enriched terms in comparisons to I1 (Table 3), rendering these comparisons useful only as supporting results of the others and as a starting point for investigations of this pathosystem. Regardless of inoculation status and consistent with senescence, there was a decrease in functions, processes, and cellular components related to photosynthesis and thylakoid membranes, and an increase in ion binding and myrcene and terpene synthase functions in the cut branches over time. At 5 days, there were significant increases in protein functions associated with disease and defense response in inoculated branches relative to control (Table 3). Specifically, processes and functions involving the binding, metabolism, and catabolism of chitins, aminoglycans, and polysaccharides were increased relative to both the control and to sample I1. There was also marginally significant enrichment of monooxygenase and oxidoreductase activity in inoculated tissue relative to control at 5 days.

Discussion

We provide the first transcriptome for tanoak, a species that is unique and valuable in its own right, and has relationships to economically important Fagaceae, and identify sequences that may be important in the tanoak-*P. ramorum* pathosystem. Emergences of newly discovered (Brasier et al. 2005; Donahoo et al. 2006; Duran et al. 2008; Hansen et al. 2003; Reeser et al. 2007; Scott et al. 2009) and newly hybridized (Brasier et al. 1999) *Phytophthora* spp. in forests have accelerated in recent years. The techniques we present may be applied to these systems for genomic study of new hosts and pathogens simultaneously—a similar technique could be applied to use homology to *P. ramorum*, *P. sojae*, and *Phytophthora infestans* to sort host and pathogen sequences even for *Phytophthora* spp. for which genomes have not been sequenced.

We assembled the first transcriptome for tanoak; this will now be available for use for future research on this species, or use for comparative genomics in *Quercus* and *Castanea*, or other hardwoods. The assembly of more than 48,000 contigs is reasonably in line with the 69,000 contigs in an assembly of multiple *Quercus robur* EST libraries (Ueno et al. 2010), and the 28,000 and 40,000 assembled from 454 sequences from the *C. dentata* and *C. mollissima* transcriptomes (Barakat et al. 2009). Although there is no doubt that more sequencing at greater depths and of different tissues will reveal new sequences and isoforms, this transcriptome provides a base upon which further research may be built.

P. ramorum transcripts were directly sequenced and subtracted from host transcripts in inoculated leaves, 5 days post-inoculation. For concurrent sequencing of host and pathogen together, the 5 days time proved to be better, as after only 1 day, the amount of mapped *P. ramorum* sequence was not different than that of uninoculated controls. Some amount of mapping is expected due to chance alone even when no *P. ramorum* is present, due to highly conserved regions or to similarity with transcripts of endophytes or of other microbial associates that are inevitably sequenced along with plant tissues.

It was not clear what true proportion of transcripts in infected tanoak tissue should be expected to have been of pathogen origin. Approximately 5 % of the DNA in leaf lesions on California bay laurel (*U. californica*) leaves has been reported to be from *P. ramorum* (Hayden et al. 2006). The 4.5 % of transcripts in I5 mapped to the *P. ramorum* with permissive mapping parameters approached this figure, but may have included some false matches. More stringent mapping found considerably less pathogen, but this result likely included false negatives. The true proportional representation of pathogen transcripts in tanoak leaves 5 days post-inoculation was likely somewhat less than that observed in genomic DNA in bay laurel. Differences in pathogen growth rates in the host substrates, relative differences in transcriptome and genome sizes, and the relative difficulty of extracting of *P. ramorum* nucleic acids from tanoak (Hayden et al. 2006) might all contribute to the detection of fewer pathogen transcripts. Nonetheless, the GC-content of sequences mapped to the *P. ramorum* genome in I5 was identical to that reported in the literature for *Phytophthora* coding sequences (Jiang and Govers 2006; Kamoun 2003), further evidence that these sequences were of pathogen origin. The de novo transcriptome of tanoak was assembled from reads that were unmapped under permissive settings, in order to err on the side of removing *P. ramorum* from the dataset.

The pathogenicity-related transcripts attributed to *P. ramorum* included elicitins, lectins, pectinesterases, and cellulose-binding proteins, all of which are associated with necrosis or breakdown of host cells, as well as 6 Avr-homologs, which are associated both with infection and with the switch from biotrophy to necrotrophy (Hein et al. 2009; Lee and Rose 2010). Dual RNA-seq of host and pathogen by nature is challenged by the very small percentage of pathogen mRNA within the total RNA in infected plant tissue (Westermann et al. 2012). The number of transcripts mapped to *P. ramorum* was slightly more than 5 % of the 15,743 gene models in the *P. ramorum* reference; deeper sequencing by spreading sequencing across flow cell lanes and/or altering inoculation and RNA extraction methods to increase representation of pathogen mRNA will surely identify more pathogen transcripts.

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Table 3 Gene ontology annotations that were significantly enriched (Fisher's exact test, within-comparison FDR<0.10) in sets of differentially expressed of transcripts

	GO term	ID	%Ref	I5 vs C5	I5 vs I1	C5 vs C1	I1 vs C1
MF	(R)-limonene synthase activity	GO:0034002	0.00	–	0.7 (6.38E−02)	–	–
	Carbon-oxygen lyase activity	GO:0016835	0.74	–	3.5 (1.74E−02)	13.3 (1.67E−03)	–
	Carbon-oxygen lyase activity, acting on phosphates	GO:0016838	0.13	3.8 (9.83E−02)	2.8 (4.30E−06)	8.9 (1.67E−03)	–
	Carbon-oxygen lyase activity, acting on polysaccharides	GO:0016837	0.03	–	–	4.4 (5.33E−02)	-
	Cation binding	GO:0043169	14.32	–	–	35.6 (7.84E−02)	–
	Chitin binding	GO:0008061	0.07	3.8 (3.02E−02)	1.8 (6.78E−04)	–	–
	Chitinase activity	GO:0004568	0.10	6.4 (7.90E−05)	2.5 (1.59E−05)	–	–
	Electron carrier activity	GO:0009055	1.66	10.3 (3.95E−02)	–	*−18.5 (3.71E−02)*	–
	Endopeptidase inhibitor activity	GO:0004866	0.03	–	1.8 (9.71E−05)	–	–
	Endopeptidase regulator activity	GO:0061135	0.03	–	2.1 (4.65E−06)	–	–
	Enzyme inhibitor activity	GO:0004857	0.19	–	2.1 (8.70E−03)	–	–
	Heme binding	GO:0020037	1.36	–	–	11.1 (8.71E−02)	–
	Hydrolase activity, hydrolyzing O-glycosyl compounds	GO:0004553	1.70	–	4.9 (7.49E−02)	–	–
	Ion binding	GO:0043167	14.33	–	–	35.6 (7.84E−02)	–
	Lyase activity	GO:0016829	2.04	–	6.7 (2.41E−03)	17.8 (3.09E−03)	–
	Magnesium ion binding	GO:0000287	0.72	–	3.5 (1.29E−02)	8.9 (7.84E−02)	–
	Monooxygenase activity	GO:0004497	1.20	9.0 (3.95E−02)	–	–	–
	Myrcene synthase activity	GO:0050551	0.05	–	2.1 (9.35E−06)	6.7 (3.09E−03)	–
	NADH dehydrogenase (quinone) activity	GO:0050136	0.35	–	–	8.9 (1.32E−02)	11.5 (5.51E−02)
	NADH dehydrogenase (ubiquinone) activity	GO:0008137	0.35	–	–	8.9 (1.32E−02)	11.5 (5.51E−02)
	NADH dehydrogenase activity	GO:0003954	0.39	–	–	8.9 (1.86E−02)	11.5 (7.09E−02)
	Oxidoreductase activity	GO:0016491	10.89	–	–	33.3 (2.61E−02)	42.3 (2.72E−02)
	Oxidoreductase activity, acting on NADH or NADPH	GO:0016651	0.77	–	–	8.9 (9.70E−02)	–
	Oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	GO:0016655	0.47	–	–	8.9 (3.00E−02)	–
	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	GO:0016705	1.86	10.3 (7.32E−02)	–	–	–
	Pattern binding	GO:0001871	0.16	–	1.8 (2.98E−02)	–	–
	Pectate lyase activity	GO:0030570	0.03	–	–	4.4 (5.33E−02)	–
	Peptidase inhibitor activity	GO:0030414	0.05	–	1.8 (2.74E−04)	–	–
	Peptidase regulator activity	GO:0061134	0.05	–	2.1 (1.33E−05)	–	–
	Polysaccharide binding	GO:0030247	0.16	–	1.8 (2.98E−02)	–	–
	Secologanin synthase activity	GO:0050616	0.05	–	–	4.4 (7.36E−02)	–
	Serine-type endopeptidase inhibitor activity	GO:0004867	0.02	–	1.1 (1.39E−02)	–	–
	Structural constituent of cuticle	GO:0042302	0.03	–	1.1 (4.63E−02)	–	–
	Structural constituent of ribosome	GO:0003735	2.38	–	12.7 (2.69E−12)	–	–
	Structural molecule activity	GO:0005198	2.98	–	14.5 (1.69E−12)	–	–
	Terpene synthase activity	GO:0010333	0.09	–	2.1 (1.23E−04)	6.7 (9.44E−03)	–
	Tetrapyrrole binding	GO:0046906	1.42	9.0 (7.84E−02)	–	11.1 (9.86E−02)	–
BP	Aminoglycan catabolic process	GO:0006026	0.09	6.4 (6.61E−05)	2.5 (8.64E−06)	–	–
	Aminoglycan metabolic process	GO:0006022	0.14	6.4 (2.24E−04)	2.5 (1.12E−04)	–	–
	ATP synthesis coupled electron transport	GO:0042773	0.23	–	–	6.7 (5.84E−02)	–
	Carbohydrate catabolic process	GO:0016052	0.98	–	3.5 (9.24E−02)	–	–
	Cell killing	GO:0001906	0.02	2.6 (8.30E−02)	–	–	–

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Table 3 (continued)

	GO term	ID	%Ref	I5 vs C5	I5 vs I1	C5 vs C1	I1 vs C1
BP	Cell wall macromolecule catabolic process	GO:0016998	0.16	–	1.8 (3.23E−02)	–	–
	Cell wall organization or biogenesis	GO:0071554	1.13	–	3.9 (7.55E−02)	–	–
	Cell–cell signaling	GO:0007267	0.08	–	1.4 (3.23E−02)	–	–
	Cellular biosynthetic process	GO:0044249	17.68	–	26.1 (5.07E−02)	–	–
	Cellular macromolecule biosynthetic process	GO:0034645	11.89	–	19.1 (6.00E−02)	–	–
	Cellular respiration	GO:0045333	0.68	–	–	13.3 (1.67E−03)	19.2 (2.48E−03)
	Chitin catabolic process	GO:0006032	0.09	6.4 (6.61E−05)	2.5 (8.64E−06)	–	–
	Chitin metabolic process	GO:0006030	0.12	6.4 (1.37E−04)	2.5 (4.61E−05)	–	–
	Electron transport chain	GO:0022900	0.74	–	–	13.3 (1.67E−03)	19.2 (2.48E−03)
	Energy derivation by oxidation of organic compounds	GO:0015980	0.73	–	–	13.3 (1.67E−03)	19.2 (2.48E−03)
	Gene expression	GO:0010467	11.58	–	19.4 (2.24E−02)	–	–
	Generation of precursor metabolites and energy	GO:0006091	1.86	–	–	*−18.5 (5.56E−02)*	19.2 (5.51E−02)
	Killing of cells of other organism	GO:0031640	0.01	2.6 (7.21E−02)	–	–	–
	Lateral inhibition	GO:0046331	0.00	–	0.7 (6.38E−02)	–	–
	Macromolecule biosynthetic process	GO:0009059	11.92	–	19.1 (6.21E−02)	–	–
	Nervous system development	GO:0007399	0.03	–	1.1 (4.63E−02)	–	–
	Neurogenesis	GO:0022008	0.02	–	1.1 (1.99E−02)	–	–
	Oxidation-reduction process	GO:0055114	9.65	–	–	31.1 (2.71E−02)	42.3 (1.37E−02)
	Oxidative phosphorylation	GO:0006119	0.23	–	–	6.7 (5.84E−02)	–
	Photosynthesis	GO:0015979	0.83	–	–	*−22.2 (6.41E−05)*	*−13.6 (1.36E−02)*
	Photosynthesis, light reaction	GO:0019684	0.47	–	–	*−14.8 (5.49E−03)*	–
	Polysaccharide catabolic process	GO:0000272	0.25	6.4 (2.51E−03)	2.5 (3.10E−03)	–	–
	Regulation of endopeptidase activity	GO:0052548	0.00	–	0.7 (6.38E−02)	–	–
	Regulation of peptidase activity	GO:0052547	0.02	–	1.1 (1.99E−02)	–	–
	Respiratory electron transport chain	GO:0022904	0.29	–	–	8.9 (8.94E−03)	15.4 (2.48E−03)
	Translation	GO:0006412	4.17	–	16.3 (1.37E−11)	–	–
	Translational elongation	GO:0006414	0.43	–	2.5 (5.60E−02)	–	–
CC	Chloroplast	GO:0009507	9.56	–	–	*−37.0 (5.28E−02)*	–
	Chloroplast part	GO:0044434	4.67	–	–	*−29.6 (1.39E−02)*	–
	Chloroplast thylakoid	GO:0009534	1.63	–	–	*−29.6 (7.90E−06)*	*−13.6 (6.38E−02)*
	Chloroplast thylakoid membrane	GO:0009535	1.25	–	–	*−29.6 (1.58E−06)*	*−13.6 (3.35E−02)*
	Eukaryotic translation elongation factor 1 complex	GO:0005853	0.05	–	1.1 (7.67E−02)	–	–
	Extracellular region	GO:0005576	1.14	–	6.7 (1.65E−06)	–	–
	Intracellular non-membrane-bounded organelle	GO:0043232	5.68	–	17.7 (1.78E−09)	–	–
	Light-harvesting complex	GO:0030076	0.03	–	–	*−7.4 (2.17E−02)*	–
	Lipid particle	GO:0005811	0.05	–	1.1 (9.24E−02)	–	–
	Macromolecular complex	GO:0032991	10.43	–	21.6 (2.00E−05)	–	–
	Mitochondrial envelope	GO:0005740	1.32	–	–	11.1 (7.84E−02)	19.2 (1.56E−02)
	Mitochondrial inner membrane	GO:0005743	1.03	–	–	11.1 (4.26E−02)	19.2 (8.09E−03)
	Mitochondrial membrane	GO:0031966	1.23	–	–	11.1 (7.10E−02)	19.2 (1.39E−02)
	Mitochondrial part	GO:0044429	1.61	–	–	–	19.2 (3.32E−02)
	Non-membrane-bounded organelle	GO:0043228	5.68	–	17.7 (1.78E−09)	–	–
	Organelle inner membrane	GO:0019866	1.27	–	–	11.1 (7.61E−02)	19.2 (1.45E−02)
	Organelle subcompartment	GO:0031984	1.66	–	–	*−29.6 (7.90E−06)*	*−13.6 (6.38E−02)*
	Photosynthetic membrane	GO:0034357	1.46	–	–	*−33.3 (4.05E−07)*	*−13.6 (5.25E−02)*
	Photosystem	GO:0009521	0.23	–	–	–	*−9.1 (1.40E−02)*

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Table 3 (continued)

	GO term	ID	%Ref	I5 vs C5	I5 vs I1	C5 vs C1	I1 vs C1
CC	Plastid part	GO:0044435	4.79	–	–	*–29.6 (1.53E–02)*	–
	Plastid thylakoid	GO:0031976	1.63	–	–	*−29.6 (7.90E−06)*	*−13.6 (6.38E−02)*
	Plastid thylakoid membrane	GO:0055035	1.25	–	–	*–29.6 (1.58E–06)*	*−13.6 (3.35E−02)*
	Proton-transporting two-sector ATPase complex	GO:0016469	0.43	–	2.5 (5.77E−02)	–	–
	Respiratory chain	GO:0070469	0.44	–	–	11.1 (2.17E−03)	15.4 (7.82E−03)
	Ribonucleoprotein complex	GO:0030529	3.47	–	14.1 (2.05E−10)	–	–
	Ribosome	GO:0005840	2.75	–	13.8 (1.76E−12)	–	–
	Thylakoid	GO:0009579	2.02	–	–	*−33.3 (2.43E−06)*	–
	Thylakoid membrane	GO:0042651	1.35	–	–	*−33.3 (4.05E−07)*	*−13.6 (4.08E−02)*
	Thylakoid part	GO:0044436	1.62	–	–	*−33.3 (6.86E−07)*	*−13.6 (6.38E−02)*

The percentage of sequences with the given annotation is given for the entire de novo reference and for each differentially expressed set, with FDR in parentheses. Enrichment tests were performed separately for sets with increased or decreased expression; negative entries, in italics, indicate pairs where expression was lower in the first term

MF molecular function, *BP* biological processes, *CC* cellular component

P. ramorum transcripts matching cellulose-binding, elecitin, and lectin-like proteins were detected in greater numbers, with searches revealing 30 transcripts matching genes with those annotations in *P. ramorum* (Tyler et al. 2006) and *P. infestans* databases (Sierra et al. 2010). However, only one matched to CRN (crinkler and necrosis-inducing) genes, matching a pseudogene originally identified in *P. infestans* (Haas et al. 2009).

Tanoak disease response transcripts were better characterized than *Phytophthora* transcripts. More than 1,000 R-gene homologs were discovered within the transcriptome, presenting a starting point to examine tanoak–*P. ramorum* molecular interactions in greater detail. The 160 tanoak transcripts that were differentially expressed after inoculation and were matched to chestnut genes associated with disease response to a fungal canker (Barakat et al. 2009) likewise point to regions for further study. The pathogenesis-related families of chitinases, glycosyl hydrolases, and thaumatin proteins (Van Loon and Van Strien 1999) were especially well represented. Expression of these gene families has been shown to increase over the course of infection of grape vine in susceptible interactions with powdery mildew, but not in resistant interactions (Fung et al. 2008). Nonetheless, in early infection stages they are more highly expressed in incompatible interactions of rice with rice blast fungus than compatible interactions (Kawahara et al. 2012). Among the matches to chestnut sequences were two dirigent-like disease resistance genes with increased expression after inoculation. Dirigent-like genes are associated with suberization and the synthesis of lignin and phytoalexins (Davin and Lewis 2000). Xylem obstructions caused by tyloses, suberized bubbles of parenchymal tissue, have been implicated in the loss of hydrolic conductivity and rapid wilting response that can accompany *P. ramorum* infection in tanoaks (Collins et al. 2009; Parke et al. 2007) and have been shown to increase in proportion to the relative production of pathogen elicitins (Manter et al. 2007). Rapid tylosis production may present a barrier to pathogen movement through vessels and, like other defensive responses, is also associated with pathogenicity. In either case, these genes may present useful targets of study as biomarkers for infection, disease response, or disease tolerance.

Preliminary analysis of differential expression over time after inoculation identified sequences involved in tanoak disease response; these sequences can help target research of disease interactions in forest trees. Functions for polysaccharide and aminoglycan binding and metabolis/catabolis were enriched in tanoak sequences with increased expression 5 days post-inoculation, as were monooxygenase and electron carrying functions, consistent with signal transduction and cell killing activity. These stand in comparison to the reduced expression in terms related to photosynthesis in non-inoculated branches over time, perhaps demonstrating that the observed disease response was in spite of overall stress and reduction in activity in cut branches. Despite the problems inherent with comparisons of single technical replicates—and in particular, the confounding of sequencing depth with treatment effects—there was demonstrable enrichment of plant expression of proteins associated with necrosis and disease response in susceptible hosts 5 days post-inoculation, at the same time that *P. ramorum* was expressing a variety of cellulose-binding and lectin-like proteins and a few avirulence homologs. Together, they portray an interaction of a susceptible host and hemibiotrophic pathogen during its necrotrophic phase.

With sufficient pathogen growth and sequencing depth, it is possible to identify interacting pathogenicity and resistance

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processes for pathosystems with a distinct paucity of host genomic resources—and the same could be accomplished for interactions of a well-annotated host with a new pathogen. As there is no expectation that disease introductions will decelerate in coming years, this type of research will become increasingly important.

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