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Research paper

Modulation of the leaf transcriptome of *Quercus robur* by specialist and generalist herbivorous insects

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Plants deploy complex transcriptional responses to herbivores yet differences in the responses to generalist versus specialist insects, especially in long-lived tree species, are still poorly understood. Here, we analysed the transcriptional responses in *Quercus robur* L. leaves to infestation by two chewing insect species: the specialist moth *Tortrix viridana* L. and the generalist moth *Lymantria dispar* L. Regardless of insect species, we observed extensive gene induction. Key regulators such as the transcription factors *MYC2*, *JAZ* and *ERF1*, primarily activate defence gene expression via jasmonate and ethylene pathways after feeding by the generalist or the specialist. A total of 1591 genes were differentially expressed between the two herbivore treatments. Feeding by *L. dispar* triggered a broader transcriptional response, stronger activating pathways related to jasmonate, abscisic acid, auxin and ethylene signalling, as well as genes involved in terpene synthesis, monooxygenase activity and phloem development. In contrast, *T. viridana* induced a more specialized profile, including genes associated with serine-type endopeptidase activity, cell wall and cell wall organization, such as those encoding hydroxyproline-rich glycoproteins or pectin esterase inhibitors. This suggests a role of cell wall-related defences in response to specialist herbivores. Network analysis of *Arabidopsis thaliana* (L.) Heynh. homologues highlighted *MYC2* as a central regulatory hub in both responses. Activation of *MYC2* triggers downstream responses, including the induction of secondary metabolism genes, e.g., *QrTPS1* encoding a functional sesquiterpene synthase, with germacrene D as its primary product. Transcriptional differences between resistant and susceptible oak genotypes were more pronounced following specialist than generalist herbivore feeding. These results provide insights into genome-scale herbivore-specific and genotype-mediated defence programmes at the transcriptome level and highlight promising gene targets for future functional genomics and natural variation studies in a keystone forest tree.

Keywords: enzymatic activity, green oak leaf roller, gypsy moth, oaks, recombinant protein expression, sesquiterpene synthase, transcription factors, transcriptomics.

Introduction

Quercus robur L. is one of the predominant oak species in Central Europe alongside *Quercus petraea* (Matt.) Liebl. *Quercus* sp. are the second most common deciduous trees in German forests after beech (National_Forest_Inventory (2022)) and support the highest species diversity at all trophic levels among European trees, providing habitat for over a thousand animal species, including insects, birds and small mammals (ProQuercus). Economically, oak wood is highly valued for its strength and durability, making it essential for construction, furniture, parquet, veneers, railroad sleepers and barrels (Stavi et al. 2022).

Climate change poses increasing threats, exposing trees to intensified biotic and abiotic stressors (Schroeder et al. 2021). Rising temperatures, CO_2 levels and drought exacerbate herbivory by promoting higher insect food consumption and faster development (Hamann et al. 2021). The caterpillars of several herbivorous moth species are summarized as an oak-feeding society. Two of them are the generalist *Lymantria dispar* L. (Erebidae), feeding on a variety of woody and even herbaceous plants (Liebhold et al. 1995), and the specialist *Tortrix viridana* L. (Tortricidae), which feeds almost exclusively on *Q. robur* (and rarely on *Q. petraea*) (Hunter 1990). In severe caterpillar outbreak years, oak trees may experience up to 30% growth loss, premature St John's shoot development and failed fructification. In extreme cases, oak dieback occurs, often exacerbated by secondary stressors such as buprestid beetles, fungal infections or drought (Thomas et al. 2002, Machácová et al. 2022). Frequent insect outbreaks, particularly of the green oak leaf roller (*T. viridana*), have been implicated in oak decline over the last century (reviewed in Kowsari and Karimi 2023). However, resistance to *T. viridana* varies among *Q. robur* individuals. Previous studies (Ghirardo et al. 2012, Bertic et al. 2021) have identified trees that consistently demonstrate high resistance to infestation (T-oaks), while others (S-oaks) suffer severe defoliation. These genotypes have been propagated via grafting for further research (Schroeder 2010).

Tree–insect interactions drive co-evolutionary processes in forest ecosystems (Becerra 2003, Stahl et al. 2018), shaping dynamic defence systems (Han 2019) that include several constitutive and induced defence mechanisms (reviewed in

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War et al. 2012, Mostafa et al. 2022). Constitutive defences are always present and include mechanical barriers and chemical deterrents, such as digestibility reducers as polyphenols and toxins, while inducible defences are activated upon herbivore attack, impairing growth and survival of herbivores.

Induced immunity involves wound-induced signalling, damage-associated molecular patterns and herbivore-associated molecular patterns, such as insect oral secretions (Duran-Flores and Heil 2016, Arimura 2021, Snoeck et al. 2022). Herbivores may also release effectors that suppress plant immunity (Snoeck et al. 2022).

Signal transduction pathways, including calcium ion fluxes, phosphorylation cascades and the jasmonate (JA) pathway, play crucial roles in herbivory resistance (Wu and Baldwin 2009). The JA pathway, in particular, promotes resistance to diverse insect species (Howe and Jander 2008, Arimura et al. 2011, Zebelo and Maffei 2015). A combined transcriptomic and metabolomic study (Kersten et al. 2013) demonstrated strong upregulation of JA-related genes in *Q. robur* leaves following *T. viridana* feeding. The induction of the JA pathway triggers global gene reprogramming, metabolic shifts and activation of defensive proteins (Howe and Jander 2008, Mostafa et al. 2022). Secondary metabolites, including herbivore-induced plant volatiles (HIPVs), accumulate as late defence responses (Dicke et al. 2009). In *Q. robur*, different terpenes, methyl salicylate and green leaf volatile emissions increase after *T. viridana* feeding, with T-oaks producing higher amounts of insect-repelling sesquiterpenes, while S-oaks emit more insect-attracting homoterpenes and monoterpenes (Ghirardo et al. 2012). Transcriptomic data confirm higher sesquiterpene activity in T-oaks (Kersten et al. 2013). Additionally, the levels of polyphenols, particularly flavonoids such as kaempferol, glucosides of kaempferol, quercetin and flavonol, varied between susceptible and resistant oaks (Ghirardo et al. 2012, Bertic et al. 2021, 2023). Secondary metabolites act as feeding deterrents and toxins and reduce the nutritional value of plant food (Mostafa et al. 2022, Bertic et al. 2023), thus inhibiting the growth and development of herbivorous insects in the scope of direct defence responses. T-oaks contain more herbivory-deterrent phytochemicals and appear to be a less nutritional resource for *T. viridana* (Ghirardo et al. 2012, Orgel et al. 2021, Bertic et al. 2023).

Experimental studies comparing plant transcriptional responses to specialist and generalist herbivorous insects are still limited (reviewed in Ali and Agrawal 2012, Volf et al. 2015, Wei et al. 2015), especially in trees (Fabisch et al. 2019). Oaks respond to both generalist and specialist insect herbivores with a combination of shared and distinct defence strategies. This includes producing defensive chemicals and HIPVs (Damestoy et al. 2019, Graham et al. 2024). Oaks increase their production of HIPVs and other chemical defences (such as flavonoids and lignins) in response to insect attack, regardless of whether the herbivore is a generalist or a specialist. The direct differences in response between generalists and specialists are often subtle or context-dependent. For instance, production rates are related to whether the leaves are growing or not, and whether the response of the attacked leaf or subsequent leaves is measured (which, in turn, depends on the duration of the attack), and whether the tree is in symbiosis with ectomycorrhizal fungi (Bacht et al. 2019, Fernández et al. 2025).

This study (i) examines the general transcriptional responses of *Q. robur* to herbivory by chewing insects, (ii) compares the responses of *Q. robur* to infestation by the generalist *L. dispar* with the specialist *T. viridana* and (iii) analyses the differences of the transcriptome responses between oak genotypes with contrasting resistance profiles (S-oaks and T-oaks).

Materials and methods

Plant material

The feeding experiments were performed on grafted *Q. robur* trees, using tree genotypes susceptible (S-) and resistant (T-) to the *T. viridana* infestation (Schroeder 2010, Ghirardo et al. 2012, Bertic et al. 2021). The T-genotypes are the lines ASB2a, ASB14a and ASB17a, and the S-genotypes are the lines ASB5b, ASB13b and ASB47b from the oak stand 'Asbeck' in North Rhine-Westphalia (for details, see Ghirardo et al. 2012 and Kersten et al. 2013). Thus, all S- and T-trees belong to the same oak population (Schroeder and Degen 2008). All ramets are numbered individually.

Feeding experiments and sample collection

The feeding experiments with both insects, *T. viridana* and *L. dispar,* were conducted in a time span of 12 days. This period was necessary due to a variation of the bud break time among T- and S-oak clones and the need to use the trees in a comparable stage of leaf development.

For both feeding experiments, one to two clones were used simultaneously on a day when the leaves were in the fitting stages. *Tortrix viridana* is particularly sensitive to the physiological stage of the leaves. The different larval stages require (i) just opening buds (first instar), (ii) very young leaves (second instar), (iii) unfolding leaves (third instar) and (iv) completely unfolded but not fully mature leaves (fourth and fifth instars) (Hunter 1990, Ivashov et al. 2002, Ghirardo et al. 2012). Therefore, the third and fourth instars were chosen based on this definition. From each clone (ASB trees), 10 individual ramets were used as biological replicates. Either five *T. viridana* larvae or three *L. dispar* larvae were placed on five ramets per clone. The infested trees were covered with thin micro-perforated bags, allowing air to circulate and preventing larvae from escaping ('crispac' bags). The other five ramets per clone served as control trees without larvae but were also covered with 'crispac' bags to simulate the same conditions. Trees with larvae and control trees were maintained in different greenhouse chambers to exclude the effect on control trees of the airborne defence signal generated by the infested trees.

The feeding time was ∼20 h in all cases. After this time, the larvae were removed and three leaves (technical replicates) were collected from each tree (fed and control) and shock-frozen in liquid nitrogen before storing at −80 °C.

RNA extraction and sequencing

Total RNA from 360 leaf samples was extracted individually by applying the Spectrum plant total RNA kit (Sigma, St Louis, MO, USA). To avoid DNA contamination, the On-Column DNase I Digest Set (Sigma, St Louis, MO, USA) was used following the manufacturer's instruction. Quantity of the RNA was determined with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The

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quality was measured with the Bioanalyzer Agilent 2100 (Agilent Technologies, Waldbronn, Germany). Three T-oak and three S-oak clones were included in the RNA-seq study. RNA was prepared from three control trees and three fed trees per clone (three biological replicates per clone and experimental condition each). The RNA of each tree represented the pooled RNA of two leaves from the tree (technical replicates), selected based on RNA quality and quantity parameters. Thus, ultimately 72 RNA-samples (36 from the *T. viridana* and 36 from *L. dispar* feeding experiment) were selected for sequencing (for all samples, see Table S1 available as Supplementary Data at *Tree Physiology* Online).

Strand-specific cDNA libraries with PolyA selection were created from 6 μg of RNA of each sample and sequenced on an Illumina NovaSeq platform to create ~30 million 150 bp paired-end reads per sample (GENEWIZ Germany GmbH).

Clone assignment correction

The clone identity was ascertained by microsatellite markers by genotyping of all still existing ramets of the six used genotypes (in total 234 individuals) with two established multiplex sets of three microsatellite markers each (Scotti-Saintagne et al. 2004, Ghirardo et al. 2012). The PCR-amplified marker fragments were run on a Beckmann capillary sequencer, and the individual genotypes of the samples were analysed using GeneMarker software (version 3.0.0). The initial clone assignment was corrected based on these SSR marker results in the case of six samples (Table S1 available as Supplementary Data at *Tree Physiology* Online).

Transcript quantification, count normalization, differential gene expression analysis and selection of candidate genes

Read mapping and quantification of read counts (read counts available at the Open Science Framework (OSF) were done with the STAR aligner (Dobin et al. 2013) against all chromosomes and unplaced scaffolds of the reference genome dhQueRobu3.1 of *Q. robur* (NCBI_Genome). dhQueRobu3.1 was generated and made available in 2022 by the Darwin_Tree_of_Life_Project (2025), which is a collaboration between biodiversity organizations and genomics institutes that aims to generate high-quality, curated chromosome-level assemblies (Darwin Tree of Life Project Consortium 2022). Due to its highly contiguous chromosome-level nature the assembly dhQueRobu3.1 (GCA_932294415.1) is a major step-up from the earlier assembly (Plomion et al. 2018). dhQueRobu3.1 is available through NCBI/RefSeq (NCBI_Genome) with a high-quality RefSeq annotation release supported by transcriptomic data from different tissues (RefSeq annotation release 100 (NCBI_RefSeq)). dhQueRobu3.1 has already gained recognition as a reliable reference in recent genetic studies (e.g., Mead et al. 2024, Aközbek et al. 2025). Due to the high quality of dhQueRobu3.1 we selected this assembly for our RNA-seq study.

As chromosome IDs of the reference genome did not match between the fasta file and GTF file, the IDs were adapted to be as concise and descriptive as possible (i.e., Chr01, Chr02, etc.). To account for unwanted batch effects, read count correction over all samples has been performed with the bioconductor package SVA (Leek et al. 2012) using the method CombatSeq (Zhang et al. 2020). Batch correction was done with a null model based on clone assignment as a batch variable. Differential gene expression analysis was done in R with the bioconductor package DESeq2 (Love et al. 2014) with the significance cutoff value alpha set to 0.05. Volcano plots were produced with the bioconductor package enhancedVolcano (Blighe et al. 2018).

Statistical and functional analyses

Principal Component Analysis (PCA) and associated plots were done with the bioconductor package PCAtools (Blighe and Lun 2024). Partial Least Square (PLS) and calculation of Variable Importance in Projection (VIP) scores were performed with the bioconductor package mixOmics (Rohart et al. 2017). The PCA and PLS were done using variance stabilising transformed count values provided by DESeq2. Genes meeting the following statistical criteria were defined as differentially expressed genes (DEGs): (i) $|\log_2$ fold change$| > 1$; (ii) adjusted *P*-value < 0.05 (*P*-value corrected for multiple testing) (Benjamini and Hochberg 1995). If DEGs are associated with a VIP score (PLS) > 1, we define them as candidate genes. DEG lists were extended with functional Gene Ontology (GO) terms and names that were extracted, based on the GO term identifier, using the bioconductor Package GO.db (Carlson 2019). To detect the overlap between DEGs, Venn diagrams were created with the R package VennDiagram (Chen and Boutros 2011).

Gene set enrichment analysis (GSEA) (Subramanian et al. 2005) and corresponding visualizations were done with the bioconductor package ClusterProfiler (Wu et al. 2021) and enrichPlot (Yu 2024). All genes with a significant $\log_2$ fold change ($P < 0.05$) were used in GSEA.

The software Mercator4 (Schwacke et al. 2019) was used to map the protein sequences of the reference genome dhQueRobu3.1 to the MapMan ontology version 4.6. (Mercator annotation available at the Open Science Framework (OSF). The software MapMan v. 3.7.1 (Usadel et al. 2005) was used to create visualizations of up- and down-regulated candidate genes in the context of the MapMan ontology. We developed our own MapMan diagram to allow the visualization of the complex nature of the molecular stress responses to herbivorous insects (Supplementary files).

Functional association networks were constructed by STRING (STRING_Consortium_2025; Szklarczyk et al. 2019) on lists of candidate genes using *Q. robur* amino acid sequences (protein annotation of the assembly dhQueRobu3.1). Within this process, these amino acid sequences were mapped to homologous genes in *Arabidopsis thaliana* (L.) Heynh. The following default parameters were used: Network Type: full STRING network; Required score: medium confidence (0.400); false discovery rate (FDR) stringency: medium (5%). Additional options included: hits with the lowest e-value were selected; network edges show interaction confidence only; hide disconnected nodes in the network.

QrTPS1 gene cloning, heterologous expression and in vitro enzymatic activity assay

CDS sequence encoding mature *Q. robur* terpene synthase 1 (*QrTPS1*) was optimized for expression in *Escherichia coli* using GeneArt portal software (Invitrogen-ThermoFisher Scientific). The resulting sequence was synthesized, cloned into the Gateway donor vector pENTR221 (Invitrogen) and

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subsequently subcloned into the Gateway destination vector pDEST17 (Invitrogen) by Life Technologies-ThermoFisher Scientific (Darmstadt, Germany). The *QrTPS1* construct was expressed in the chemically competent *E. coli* cells BL21(DE3) (ThermoFisher Scientific). Crude *E. coli* protein extraction and purification of the His-tagged STS proteins by affinity chromatography on Pure-Cube Ni-NTA agarose (Cube-Biotech, Hessisch-Oldendorf, Germany) were conducted as described in Schnitzler et al. (2005). Analysis of in vitro enzyme activity from the His-tagged *QrTPS1* protein and crude protein extract with the substrate farnesyl pyrophosphate was performed using the stir bar sorptive extraction (SBSE) technique coupled with thermal desorption–gas chromatography–mass spectrometry (TD-GC-MS). An enzymatic activity assay was conducted as described in Nosenko et al. (2023). Sesquiterpenes were annotated by comparing the measured mass spectra with those obtained using commercially available authentic standards or by matching them to NIST20 and Wiley (v.275) spectra libraries. The retention indices of the sesquiterpenes were calculated using non-isothermal Kovats retention indices (van den Dool and Kratz 1963), following the protocol described in Guo et al. (2021).

Results

To study the general transcriptional response of *Q. robur* to herbivory, we included two different insects in the RNA-seq study: a specialist, the green oak leaf roller (*T. viridana*) and a generalist, the gypsy moth (*L. dispar*). Two separate feeding experiments were conducted with each of the insects (20 h feeding) using three resistant (T) and three susceptible (S) oak clones. Resistance or susceptibility of oak individuals to *T. viridana* was established in previous studies (Schroeder 2010, Ghirardo et al. 2012). The inclusion of three biological replicates (ramets) per clone and condition (no feeding/20 h feeding) in the feeding experiments and subsequent sampling of leaves resulted in a total of 36 RNA-samples obtained from each feeding experiment (18 fed samples and 18 control samples without feeding) (Table S1 available as Supplementary Data at *Tree Physiology* Online).

RNA-sequencing of the 36 RNA-samples from the feeding experiments with *T. viridana* yielded an average of ∼50 million Illumina read pairs per sample and the 36 RNA-samples (Table S1 available as Supplementary Data at *Tree Physiology* Online) from the feeding experiments with *L. dispar* yielded an average of ∼36 million Illumina read pairs. On average, 86% and 85% of the paired reads per sample of the two feeding experiments of *T. viridana* and *L. dispar*, respectively, mapped to the *Q. robur* reference genome (NCBI_RefSeq).

Overall transcriptional response of *Quercus robur* to herbivores

Independently of the two herbivore feeding strategies (specialist *T. viridana* and generalist *L. dispar*), larval feeding induced extensive transcriptional changes in all 36 fed leaves, primarily resulting in an upregulation of gene expression compared with 36 control samples (Figure 1a; Figures S1 available as Supplementary Data at *Tree Physiology* Online and S2 available as Supplementary Data at *Tree Physiology* Online). Across all treatments, 1068 DEGs were identified (out of 41,864 analysed genes), with 992 DEGs up-regulated and only 76 DEGs down-regulated (Figure 1a; Tables S2 and S3 available as Supplementary Data at *Tree Physiology* Online), indicating a remarkable transcriptional change. Among the DEGs, 301 have no known function.

A prominent feature of the response was the activation of JA-associated signalling and defence-related pathways. The most significantly up-regulated DEGs include genes annotated as *linoleate 13S-lipoxygenase 3-1*, *TIFY5A* and *CYP94C1* (top DEGs in Table 1). The lipoxygenase-catalysed addition of molecular oxygen to α-linolenic acid initiates JA synthesis. *TIFY5A* (*JAZ8*) is known to be involved in the regulation of the JA-mediated signalling pathway (Zhang et al. 2015). *CYP94C1* gene expression is known to be induced in response to wounding and JA treatment and is therefore involved in defence response in plants (Kandel et al. 2007). LOC126696655, described as protein vapyrin-like, was identified as the most significantly down-regulated DEG (Table S3 available as Supplementary Data at *Tree Physiology* Online).

GSEA identified 26 GO terms enriched with up-regulated genes (normalized enrichment score (NES) between 1.4 and 1.9) and only six GO terms enriched with down-regulated genes (NES between −1.9 and –2.7) (Table S4; Figure 1b; Figure S3 available as Supplementary Data at *Tree Physiology* Online). The top-five enriched GO terms by highest NES include 'monooxygenase activity', 'oxidoreductase activity', 'acting on paired donors', 'with incorporation or reduction of molecular oxygen', 'terpene synthase activity', 'diterpenoid biosynthetic process' and 'magnesium ion binding', all of which include up-regulated genes involved in the key processes of response to stress, such as synthesis of terpenoids, cytochrome P450 proteins and allene oxide synthase proteins. Among others, further GO terms enriched with up-regulated genes are 'ethylene-activated signalling pathway', 'abscisic acid binding', 'S-adenosylmethionine-dependent methyltransferase activity', 'peroxidase activity', 'DNA-binding transcription factor activity' and 'UDP-glycosyltransferase activity' (Figure 1b).

The GO terms enriched with down-regulated genes include the 'SCF ubiquitin ligase complex', which is a multi-protein E3 ubiquitin ligase complex that plays an important role in the gene regulation of the transcription factor MYC2 (reviewed in Gimenez-Ibanez et al. 2015), as well as 'photosynthesis/photosystem II', which indicates a reduction of photosynthesis and growth processes by insect feeding (Figure S3 available as Supplementary Data at *Tree Physiology* Online).

Transcriptome modulation by herbivory in the context of functional interactions

A nonredundant set of *A. thaliana* homologues comprising 294 of the 958 herbivory-induced candidate genes (as defined in Materials and methods) up-regulated in *Q. robur* (Table S3 available as Supplementary Data at *Tree Physiology* Online) was analysed using STRING (STRING_Consortium_2025; Szklarczyk et al. 2019) to construct a STRING functional association network (Figure S4 available as Supplementary Data at *Tree Physiology* Online; list of presented *A. thaliana* homologues and related node degrees in Table S5 available as Supplementary Data at *Tree Physiology* Online). Genes with at least 10 connections were considered as hubs and are presented in the STRING hub network in Figure 2.

The STRING hub network consists of two main clusters connected by the transcription factor *MYC2* in a central position (Figure 2). One cluster (on the right) is dominated by genes assigned to 'transcription regulation' (nodes

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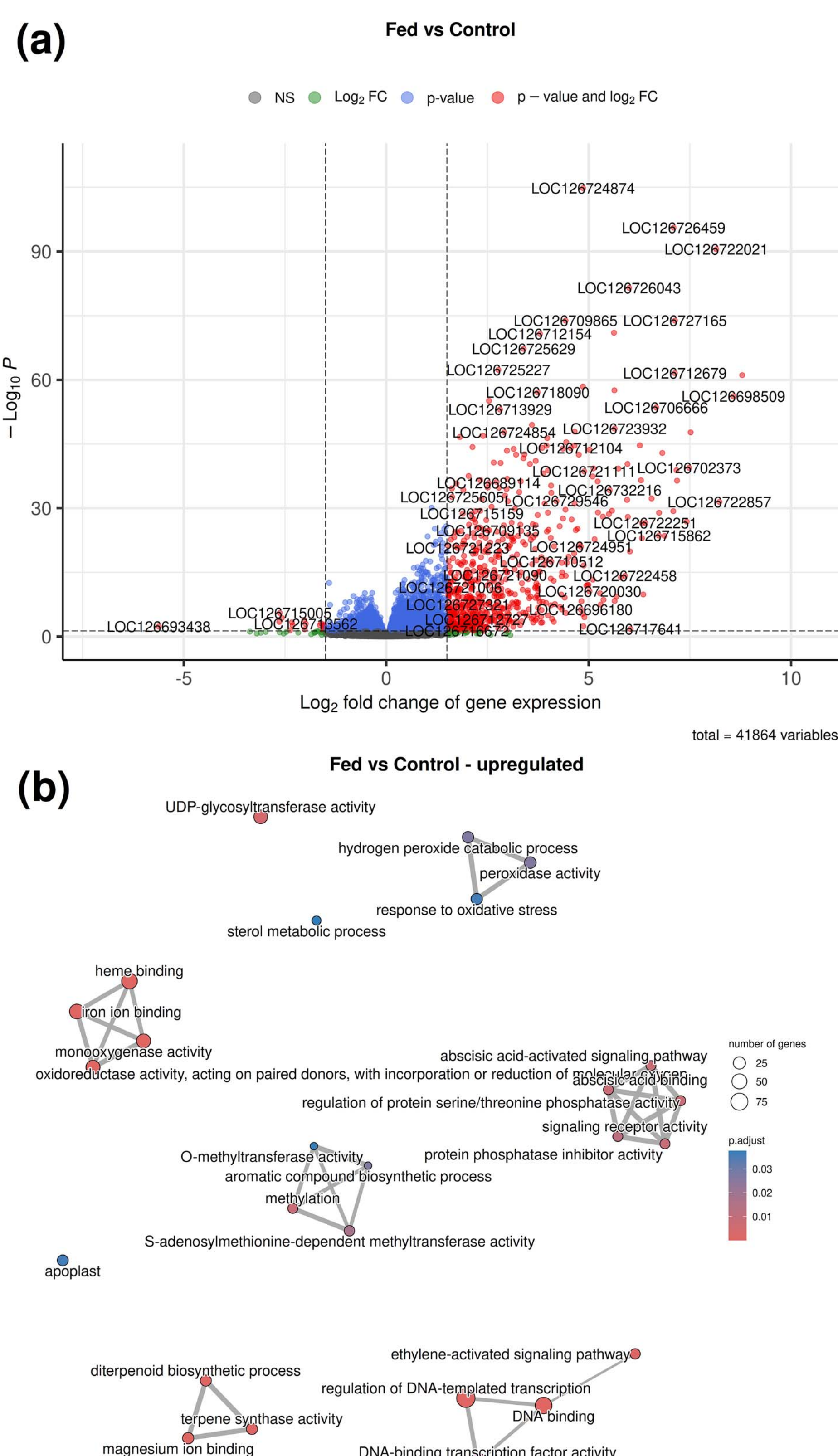

Figure 1. (a) Volcano plot of differentially expressed genes in the comparison of fed and control oak leaf samples (Fed vs Control) independently of the insect species (*T. viridiana* + *L. dispar*). A total of 1068 genes shows $|\log_2$ fold change$| > 1$ and respective adjusted P-value < 0.05 (red dots; differentially expressed genes listed in Table S3 available as Supplementary Data at *Tree Physiology* Online). Blue dots show $\log_2$ fold changes between -1 and $+1$ with adjusted P-value < 0.05. Grey dots denote genes whose expression is not significantly changed. In total, 41,864 genes were analysed. The PCA and PLS plots related to the comparison Fed vs Control are presented in Figures S1 and S2 available as Supplementary Data at *Tree Physiology* Online. (b) The enrichment plot visualizes the results of a gene set enrichment analysis based on the genes differentially expressed between the groups fed and control (DEGs in Table S3 available as Supplementary Data at *Tree Physiology* Online). All overrepresented GO terms with up-regulated genes ($\log_2$ fold change > 1) are presented. Nodes represent GO terms; the size of nodes represents the number of genes associated with a GO term. Edges illustrate overrepresented genes shared between GO terms. The thickness of edges depicts the number of shared genes between GO terms. Overrepresented GO terms with down-regulated genes are presented in Figure S3 available as Supplementary Data at *Tree Physiology* Online.

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Table 1. Top-DEGs in the different comparisons (all DEGs in Table S3 available as Supplementary Data at *Tree Physiology* Online).

Comparison	Up/down	Selection criteria	dhQueRobu3.1 gene ID	LFC	Adjusted *P*-value	VIP score	Gene name
All Fed vs Control	Up	padj	LOC126724874	4,86	1.61E−105	3.79	*Linoleate 13S-lipoxygenase 3–1*
All Fed vs Control	Up	padj	LOC126726459	7,10	2.73E−96	3.68	*TIFY5A*
All Fed vs Control	Up	padj	LOC126722021	8,15	3.00E-91	3.67	*CYP94C1*
Fed *Lymantria* vs *Tortrix*	Up	padj	LOC126727268	2,36	4.15E-130	2.46	Unknown
Fed *Lymantria* vs *Tortrix*	Up	padj	LOC126696935	1,06	7.12E-87	2.43	Unknown
Fed *Lymantria* vs *Tortrix*	Up	padj	LOC126691843	1,65	7.51E-75	2.42	*Ubiquitin-like protein 5*
Fed *Lymantria* vs *Tortrix*	Up	LFC	LOC126717641	23,23	2.47E-26	1.03	*MLP-like protein 28*
Fed *Lymantria* vs *Tortrix*	Up	LFC	LOC126725336	6,71	3.18E-18	1.98	*Acid phosphatase 1-like*
Fed *Lymantria* vs *Tortrix*	Up	LFC	LOC126699053	6,04	1.04E-05	1.25	*Dimethylnonatriene (DMNT) synthase-like protein*
Fed *Lymantria* vs *Tortrix*	Down	padj	HCS81_pgp082	−3,52	9.02E-279	2.49	*atpH*
Fed *Lymantria* vs *Tortrix*	Down	padj	HCS81_pgp080	−3,84	2.04E-263	2.49	*rps2*
Fed *Lymantria* vs *Tortrix*	Down	padj	HCS81_pgp085	−4,10	8.75E-201	2.47	*psbI*
Fed *Lymantria* vs *Tortrix*	Down	LFC	LOC126716419	−6,58	4.06E-26	2.19	*GDSL esterase/lipase At3g48460-like*
Fed *Lymantria* vs *Tortrix*	Down	LFC	LOC126696133	−6,55	1.14E-25	2.18	*GDSL esterase/lipase At3g48460-like*
Fed *Lymantria* vs *Tortrix*	Down	LFC	LOC126728386	−5,81	6.45E-18	2.12	*GDSL esterase/lipase At1g29670-like*
Lymatria S fed vs T fed	Up	padj	LOC126706777	9,04	5.07E-40	2.99	Unknown
Lymatria S fed vs T fed	Up	padj	LOC126719466	9,16	3.97E-28	2.81	Uncharacterized LOC126719466
Lymatria S fed vs T fed	Up	padj	LOC126706676	8,08	1.70E-20	2.82	Unknown
Tortrix S fed vs T fed	Up	padj	LOC126706777	9,67	1.76E-42	2.31	Unknown
Tortrix S fed vs T fed	Up	padj	LOC126706676	8,49	1.15E-27	2.25	Unknown
Tortrix S fed vs T fed	Up	padj	LOC126702699	1,98	2.16E-18	2.15	*Putative calmodulin-like protein 3*
Lymatria S fed vs T fed	Down	padj	LOC126699932	−10,37	4.00E-36	3.01	Unknown
Lymatria S fed vs T fed	Down	padj	LOC126703088	−9,33	2.97E-28	2.99	Unknown
Lymatria S fed vs T fed	Down	padj	LOC126720498	−5,66	3.80E-22	2.85	*Mitochondrial-like glycine dehydrogenase (decarboxylating) 2*
Tortrix S fed vs T fed	Down	padj	LOC126712763	−9,72	2.56E-40	2.19	*Putative glutamate receptor 2.1-like protein*
Tortrix S fed vs T fed	Down	padj	LOC126703088	−10,31	1.25E-40	2.31	Unknown
Tortrix S fed vs T fed	Down	padj	LOC126699932	−10,00	3.40E-39	2.32	Unknown

Selection criteria describe specifically highlighted differentially expressed genes (top-DEGs) by category 'adjusted *P*-value' (padj) or 'log2 fold change' (LFC). up/down, up/downregulated DEG or higher/lower expressed DEG, respectively.

highlighted in blue) and 'cellular response to hypoxia' (nodes in yellow). This cluster includes the WRKY transcription factor *WRKY33* (XP_050244065.1; LOC126692486) in a central position, which was identified as top-1 hub with 48 connections by STRING (Table S5 available as Supplementary Data at *Tree Physiology* Online). The second cluster (Figure 2, on the left) includes mainly genes involved in 'jasmonic acid signalling pathway and

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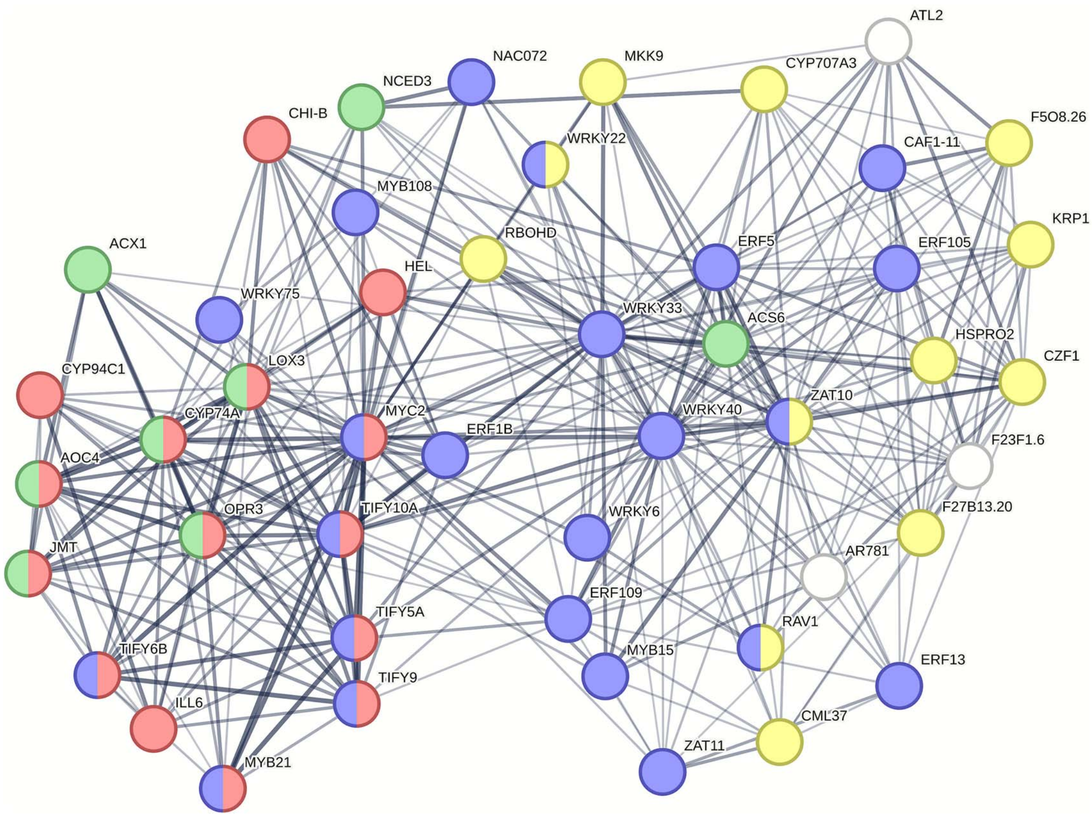

Figure 2. STRING functional association network of the 47 most connected *Arabidopsis thaliana* homologues of all up-regulated *Quercus robur* candidate genes (as defined in Materials and methods) from the comparison Fed vs Control. Genes with at least 10 connections were selected based on a STRING analysis (STRING_Consortium_2025; Szklarczyk et al. 2019) with *A. thaliana* homologues of candidate genes up-regulated in *Quercus robur* (see Table S3 available as Supplementary Data at *Tree Physiology* Online for up-regulated *Q. robur* DEGs and assigned STRING node names and IDs; full network in Figure S4 available as Supplementary Data at *Tree Physiology* Online). Nodes assigned to different gene groups are highlighted as follows: red, local network cluster (STRING) 'Mixed, incl. Jasmonic acid signalling pathway and α-Linolenic acid pathway'; blue, Annotated Keywords (UniProt) 'Transcription regulation'; green, KEGG Pathway 'Biosynthesis of secondary metabolites'; yellow, GO Biological Process 'Cellular response to hypoxia'. All remaining nodes (white) are presented without the assignment of any functional category.

α-Linolenic pathway' or in 'transcription regulation' or in 'biosynthesis of secondary metabolites', such as *MYC2* (XP_050266559.1; LOC126710223), which was identified as the top-2 hub (45 connections) in the network. The transcription factor *WRKY40* (XP_050286796.1; LOC126725856; Figure 2, right cluster) and the gene *TIFY10A* (*JAZ1*; XP_050260428.1; LOC126705448; Figure 2, left cluster) were identified as the top-3 and top-4 hubs, with 43 and 36 connections, respectively (Table S5 available as Supplementary Data at *Tree Physiology* Online). *MYC2* and *JAZ1* are key genes in JA signalling in *A. thaliana*, where *MYC2*-mediated activation of JA signalling is precisely regulated by its repressor, the jasmonate ZIM-domain (JAZ) protein (Song et al. 2022).

Differential responses to generalist and specialist herbivores

Our comparative transcriptomic analysis of *L. dispar*- and *T. viridana*-infested leaves revealed 2142 DEGs, highlighting the distinct responses of the host to herbivory by the generalist and specialist insect (Figure 3a; Tables S2 and S3; Figures S5 and S6 available as Supplementary Data at *Tree Physiology* Online). A total of 1025 *Q. robur* genes showed significantly higher expression after feeding with the generalist compared with the specialist, whereas 1117 genes showed significantly lower expression (Figure 3a; Tables S2 and S3 available as Supplementary Data at *Tree Physiology* Online). Of the 2142 DEGs, 927 have no known function.

Among the top-DEGs higher expressed in leaves fed by *L. dispar* compared with *T. viridana* are genes encoding *ubiquitin-like protein 5*, *major latex-like protein 28* (*MLP28*), *acid phosphatase 1-like* and a *dimethylnonatriene (DMNT) synthase-like protein* (Table 1). MLPs have been identified in many dicots as responding to drought, salt, plant hormones and pathogen infections (Fujita and Inui 2021). Acid phosphatase was shown to be induced by wounding and herbivory (Veljanovski et al. 2010). DMNT synthases are involved in biosynthesis of homoterpene attractants of herbivore parasitoids and predators (Tholl et al. 2011).

The top genes with lower expression in leaves fed by *L. dispar* compared with *T. viridana* based on significance are the plastid DNA genes *atpH*, *rps2* and *psbI* (Table 1). The lower expression of *psbI*, encoding a component of the core complex of photosystem II, may indicate a reduction of photosynthesis in response to the generalist compared with the specialist. The top three genes based on $\log_2$ fold change in this comparison are annotated to encode GDSL esterase/lipases, a class of enzymes with key functions in developmental processes and responses to biotic and abiotic stresses in plants (Cenci et al. 2022).

GSEA identified 23 enriched GO terms with genes showing higher expression values in the feeding experiment with *L.*

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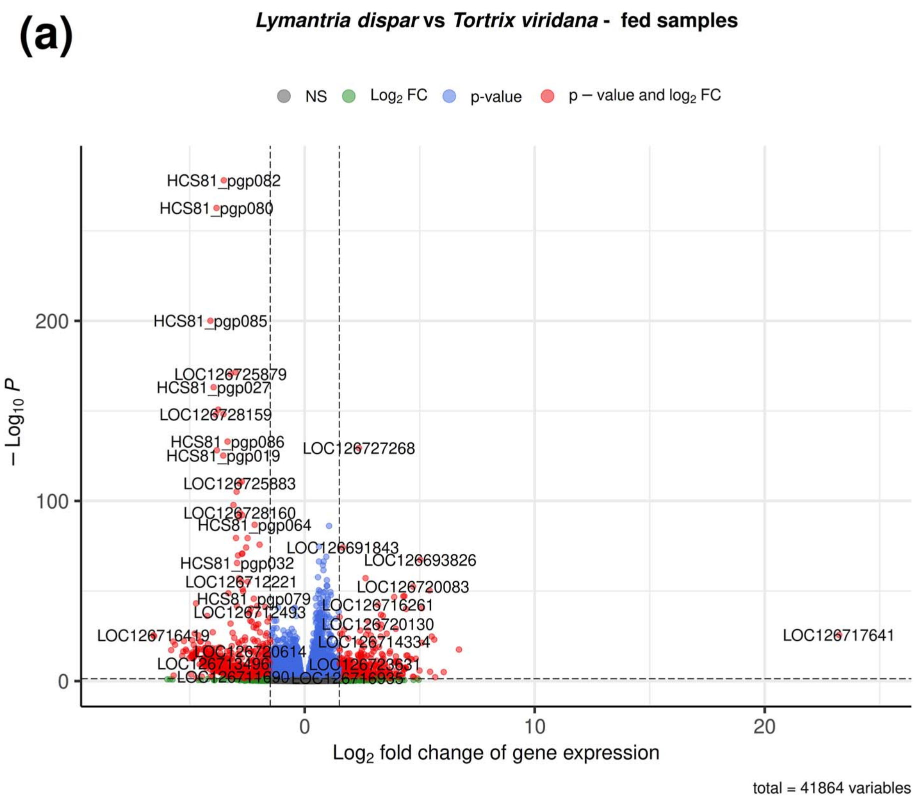

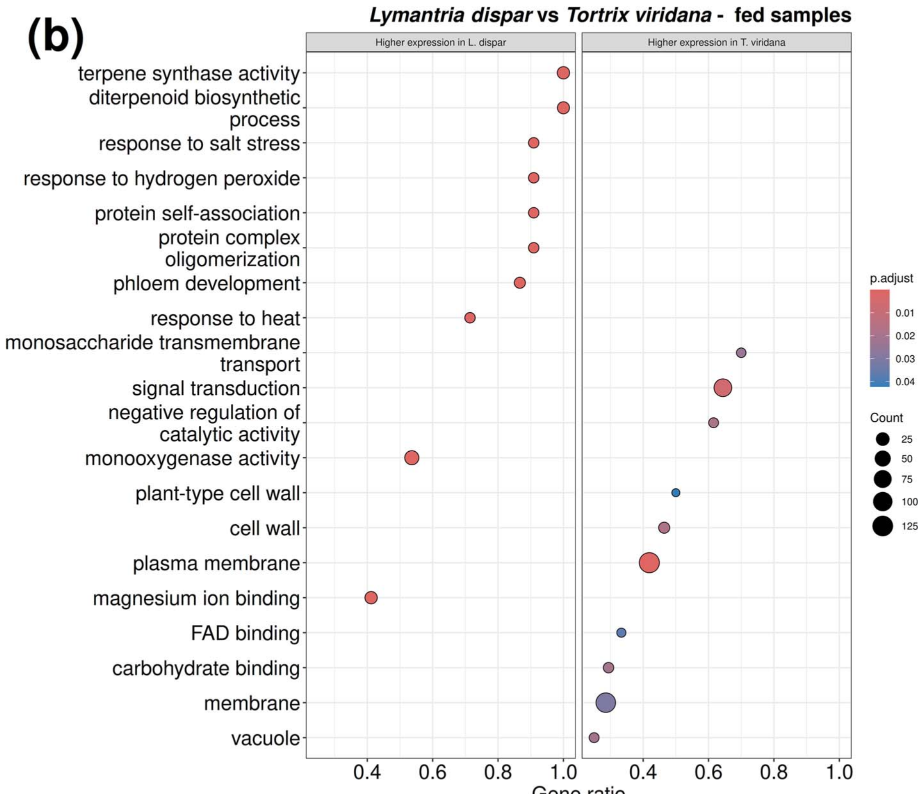

Figure 3. (a) Volcano plot showing $\log_2$ fold changes against *P*-values of differentially expressed genes for the comparison of the insects *Lymantria dispar* and *Tortrix viridana* (*L. dispar* vs *T. viridana*) on fed samples. A total of 2142 genes showed a $|\log_2$ fold change$| > 1$ and respective adjusted *P*-value < 0.05 (red dots; differentially expressed genes listed in Table S3 available as Supplementary Data at *Tree Physiology* Online). Blue dots show $\log_2$ fold changes between -1 and $+1$ with adjusted *P*-value < 0.05. Grey dots denote genes whose expression is not significantly changed. In total, 41,864 genes were analysed. The PCA and PLS plots related to the comparison *L. dispar* vs *T. viridana* are presented in Figures S5 and S6 available as Supplementary Data at *Tree Physiology* Online. (b) Dot plot visualization of gene set enrichment analysis based on the differential gene expression analysis of the groups *L. dispar* vs *T. viridana*. The size of dots corresponds to the number of genes in the core enrichment set. Dot colour encodes the significance of the enrichment. Dots are ordered by gene ratio, which is defined by the number of core enrichment genes divided by all annotated genes associated with the GO term.

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dispar than with *T. viridana* (NES between 1.7 and 2.9) and 34 enriched GO terms with genes showing higher expression values in the feeding experiment with *T. viridana* compared with *L. dispar* (NES between −1.3 and – 2.4) (Figure 3b; Table S6 available as Supplementary Data at *Tree Physiology* Online). The top five GO terms containing genes more prominently expressed due to feeding with *L. dispar* are 'terpene synthase activity', 'diterpenoid biosynthetic process', 'magnesium ion binding', 'response to heat' and 'response to salt stress'. These terms contain genes involved in terpenoid synthesis, and heat shock proteins, respectively. Additional enriched GO terms include 'phloem development', 'monooxygenase activity', 'methylation' and 'ethylene-activated signalling pathway'. The top five GO terms containing genes more prominently expressed due to feeding with *T. viridana* are 'serine-type endopeptidase activity', 'polygalacturonase activity', 'protein phosphorylation', 'protein kinase activity' and 'pectinesterase inhibitor activity'. Further enriched GO terms include 'signal transduction', 'membrane' and three 'cell wall'–related GO terms among others.

Comparing candidate genes (as defined in Materials and methods) in the samples affected by *L. dispar* or *T. viridana* (Table S3 available as Supplementary Data at *Tree Physiology* Online) revealed similar trends towards the differential expression of genes (Figure S7 available as Supplementary Data at *Tree Physiology* Online). Especially, candidate genes assigned to the MapMan sub-bins 'jasmonic acid', 'terpenoid' and 'CAM/C4 photosynthesis' show a higher expression in *Q. robur* fed by *L. dispar* than by *T. viridana*, whereas most of the candidate genes assigned to phosphorylation and about half of those assigned to pectin show higher expression after feeding by *T. viridana*.

The comparison of both feeding experiments (Fed vs Control, each) reveals a broader plant response to the generalist herbivore, as seen from the many more DEGs (especially up-regulated ones) that were detected after feeding by *L. dispar* than by *T. viridana* (*L. dispar*: 1587 DEGs; *T. viridana*: 796 DEGs; Volcano plots in Figures S8 and S9; Table S3 available as Supplementary Data at *Tree Physiology* Online). When comparing the candidate genes from both feeding experiments (each Fed vs Control) with the software MapMan distinct differences in the number of candidates between the two feeding experiments are obvious (Figure 4). More genes related to 'jasmonic acid', 'abscisic acid' and 'auxin' were up-regulated in the *L. dispar* than in the *T. viridana* feeding experiment (Figure 4). In the case of DNA-binding transcription factors, more 'ethylene response factors' (ERF) but fewer WRKYs were up-regulated by *L. dispar* than by *T. viridana* feeding. *Lymantria dispar* induced the differential expression of four candidate genes annotated as bzip transcription factors (mainly downregulation), while feeding by *T. viridana* did not induce bzip expression at the time point analysed. A higher number of genes up-regulated by *L. dispar* was also obvious for genes assigned to 'carrier mediated transport' and 'channels'. There is a striking difference in 'phosphorylation' between the two feeding experiments with many down-regulated candidate genes in the *L. dispar* feeding experiment but only up-regulated candidate genes in the *T. viridana* feeding experiment (Figure 4).

The intersection of candidate genes from both feeding experiments (Fed vs Control, each) includes 603 up-regulated genes (Figure S10 available as Supplementary Data at *Tree Physiology* Online). In total, 148 or 568 genes are exclusively up-regulated in the *T. viridana* or the *L. dispar* feeding experiment, respectively. The MapMan annotation of the 568 *L. dispar*-only candidate genes revealed 176 sub-bins (Table S7 available as Supplementary Data at *Tree Physiology* Online) that were identified only assigned to this set, but not to the set of *T. viridana*-only candidate genes or to the intersection. In contrast, 32 exclusive MapMan sub-bins (Table S8 available as Supplementary Data at *Tree Physiology* Online) were identified assigned to the *T. viridana*-only candidate genes. Candidate genes assigned to exclusive sub-bins related to the MapMan bin 'RNA biosynthesis and DNA-binding transcriptional regulation' are presented in Table 2 for the *L. dispar* and in Table 3 for the *T. viridana* feeding experiment.

A total of 21 transcription factors and transcriptional regulators have been assigned to exclusive sub-bins related to the *L. dispar* experiment (Table 2), whereas only four genes have been assigned to exclusive sub-bins related to the *T. viridana* experiment (Table 3). In both experiments, two different MYB transcription factors were exclusively identified to be up-regulated by feeding. The bHLH transcription factors *bHLH118-like*, transcription factor *bHLH162-like* and *MTB1* were only identified in the *L. dispar* experiment among other transcription factors and transcriptional regulators.

Only the following three genes were commonly down-regulated in both feeding experiments (Figure S11 available as Supplementary Data at *Tree Physiology* Online): LOC126696655 (*protein vapyrin-like*), LOC126706674 (*small auxin up-regulated RNA (SAUR) 12-like*) and LOC126702565 (*protein rice salt sensitive 3-like*). An exclusive downregulation was detected for 317 genes in the *L. dispar* und 21 genes in the *T. viridana* feeding experiment, respectively.

The STRING hub networks of the most connected *A. thaliana* homologues of all up-regulated *Q. robur* candidate genes from the comparisons *L. dispar*—Fed vs Control and *T. viridana*—Fed vs Control support the trend that more candidate genes are up-regulated in the *L. dispar* than in the *T. viridana* feeding experiment (Figure 5; Tables S9 and S10 available as Supplementary Data at *Tree Physiology* Online), especially for genes assigned to the KEGG pathway 'Biosynthesis of secondary metabolites' (highlighted in green). Most of these genes form a separate cluster in the *L. dispar* hub network (Figure 5a). Again, *WRKY33* is the top 1-hub in both networks (see also Figure 2). Whereas *WRKY33*, *MYC2* and *WRKY40* formed dense hubs in the *T. viridana* network, *WRKY40* was not identified in the *L. dispar* hub network (Figure 5; top hubs in *L. dispar*: *WRKY33*, *MYC2* and *ZAT10*).

Functional characterization of *QrTPS1*—one of the terpene synthases acting downstream of *MYC2*

Activation of MYC2 (Figures 2 and 5) triggers downstream responses, including the induction of secondary metabolism genes (Figure 1b), e.g., *QrTPS1*, a putative germacrene D synthase-encoding gene (LOC126694768; Table S3 available as Supplementary Data at *Tree Physiology* Online), which was also identified in a previous study (Kersten et al. 2013). *QrTPS1* was significantly up-regulated by feeding in general (adjusted *P*-value of 2.4E-06, $\log_2$ fold change of 2.5, VIP score of 2.5), particularly after infestation by *L. dispar* (adjusted *P*-value of 2.9E-09, $\log_2$ fold change of 3.7, VIP score of 1.8; Table S3 available as Supplementary Data at *Tree Physiology* Online).

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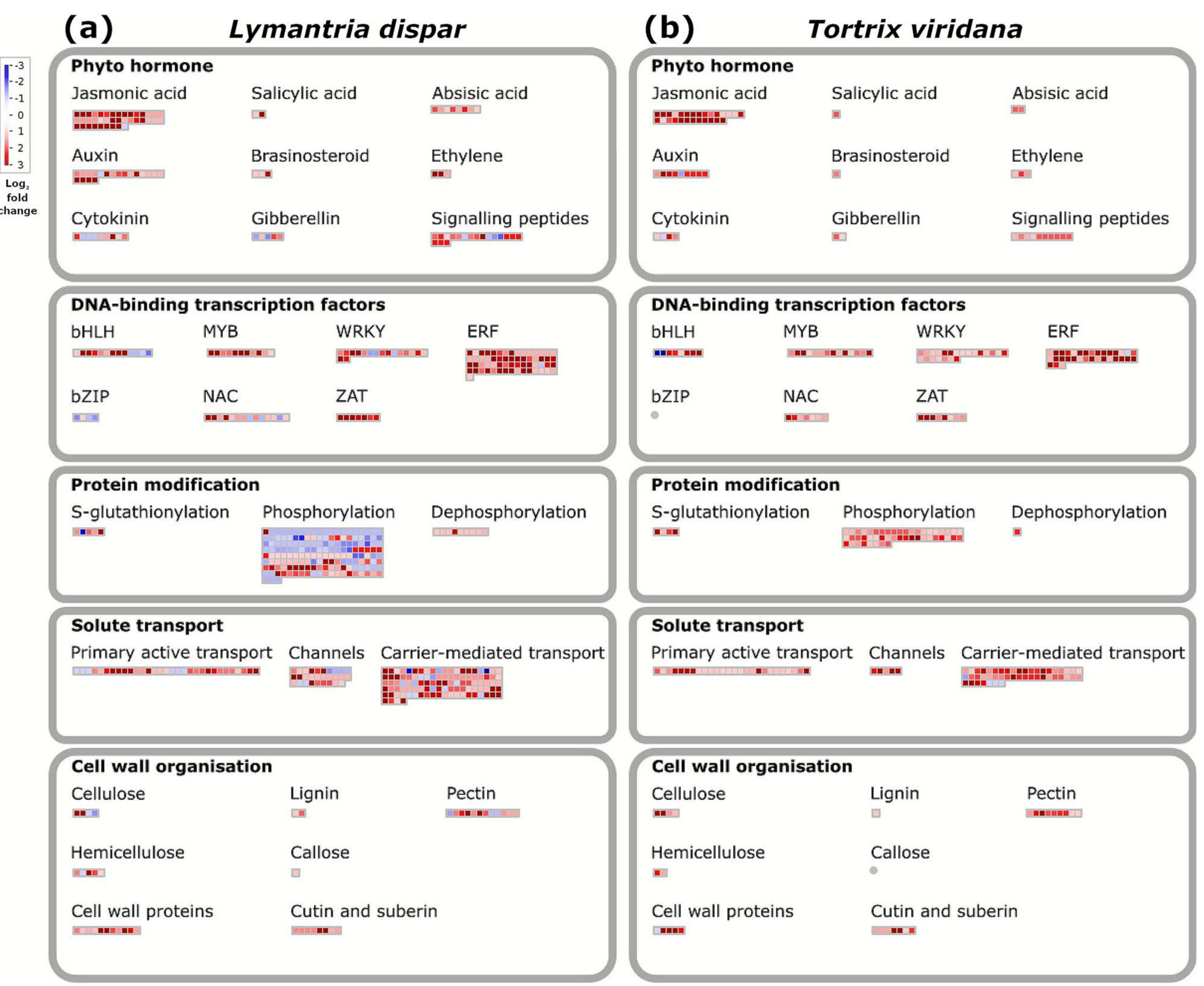

Figure 4. MapMan analyses showing the differences of candidate gene expression between (a) '*Lymantria dispar*' and (b) '*Tortrix viridana*'. Differential gene expression was estimated in the *Quercus robur* leaves fed to each herbivore relative to undamaged control leaves. In total, five distinct MapMan-bins are shown (phytohormone, DNA-binding transcription factors, protein modification, solute transport and cell wall organization) with up-regulated genes (small red boxes) and down-regulated genes (small blue boxes) depicted in sub-bins. The colour code of a box represents the $\log_2$ fold change of the respective gene in the presented comparison. Candidate genes for this comparison and their assignment to MapMan bins are provided in the list of all DEGs in Table S3 available as Supplementary Data at *Tree Physiology* Online. The Mercator annotation of all *Q. robur* genes assigning them to MapMan bins is available at the Open Science Framework (OSF). A MapMan analysis showing the differences in candidate gene expression between fed samples in comparison between *L. dispar* and *T. viridana* is presented in Figure S7 available as Supplementary Data at *Tree Physiology* Online.

We investigated the biochemical function of the *QrTPS1* gene through heterologous expression in *E. coli* and analysis of in vitro enzyme activity with the substrate farnesyl pyrophosphate (Nosenko et al. 2023). This analysis revealed that *QrTPS1* encodes a functional sesquiterpene (SQT) synthase, with germacrene D identified as the primary product (Figure S12 available as Supplementary Data at *Tree Physiology* Online).

Comparison of transcriptional responses between oak genotypes with contrasting herbivore-resistance profiles

In order to investigate the differences in the transcriptome responses between oak genotypes with contrasting resistance profiles to *T. viridana*, we compared S- and T-oaks in each feeding experiment. The differences were more pronounced under the *T. viridana* herbivory, where much more DEGs were identified (1477) than in the *L. dispar* feeding experiment (536) (Figure 6a; Tables S2 and S3; Figures S13–S16 available as Supplementary Data at *Tree Physiology* Online).

The top-3 DEGs, which were identified as higher expressed in S-oaks compared with T-oaks after feeding by *L. dispar,* were uncharacterized/unknown genes (Table 1). The top-3 DEGs in the respective *T. viridana* feeding experiment include a *putative calmodulin-like protein 3-encoding* gene (Table 1). Calmodulin-like proteins are calcium-sensing plant-specific proteins that play important roles in plant defence against insect attack (Yadav et al. 2022).

Among the DEGs with higher expression in T-oaks than in S-oaks after feeding by *L. dispar* a gene annotated as *mitochondrial-like glycine dehydrogenase (decarboxylating) 2* was among the top-3 DEGs (Table 1). A gene encoding a *putative glutamate receptor 2.1-like* protein represents the top-1

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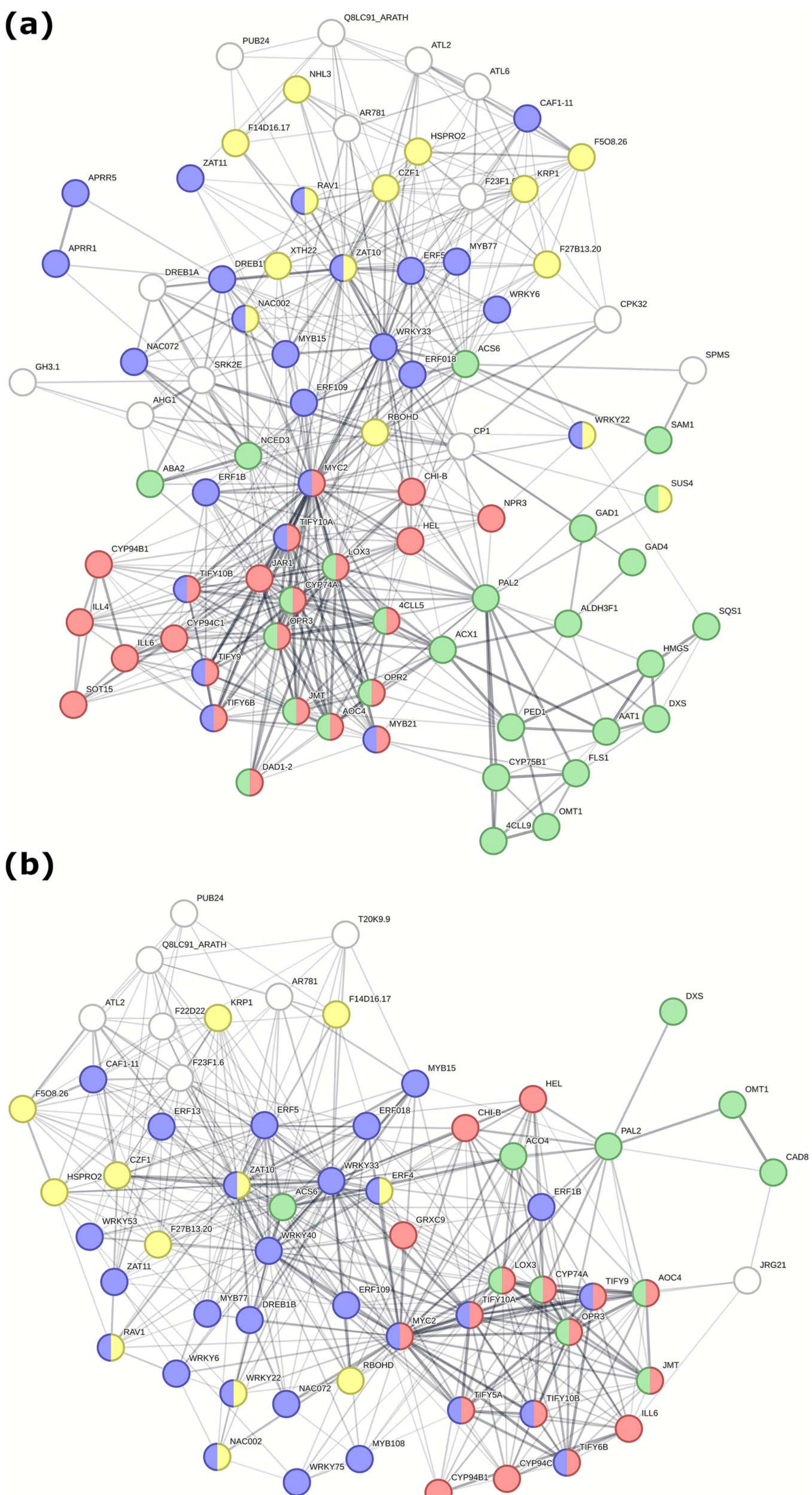

Figure 5. (a) STRING functional association network of the most connected *Arabidopsis thaliana* homologues of all up-regulated *Quercus robur* candidate genes (as defined in Material and methods) from the comparison '*Lymantria*—Fed vs Control' and (b) '*Tortrix*—Fed vs Control' (see Table S3 available as Supplementary Data at *Tree Physiology* Online for up-regulated *Q. robur* DEGs and assigned STRING node names and IDs). Genes with at least 10 connections were selected based on a STRING analysis (STRING_Consortium_2025; Szklarczyk et al. 2019) of the *Arabidopsis thaliana* homologues of the up-regulated *Q. robur* candidate genes in each comparison (Tables S10 and S11 available as Supplementary Data at *Tree Physiology* Online). Nodes assigned to different gene groups are highlighted as follows: red, local network cluster (STRING) 'Mixed, incl. Jasmonic acid signalling pathway and α-Linolenic acid pathway'; blue, Annotated Keywords (UniProt) 'Transcription regulation'; green, KEGG Pathway 'Biosynthesis of secondary metabolites'; yellow, GO Biological Process 'Cellular response to hypoxia'. All remaining nodes (white) are presented without the assignment of any functional category.

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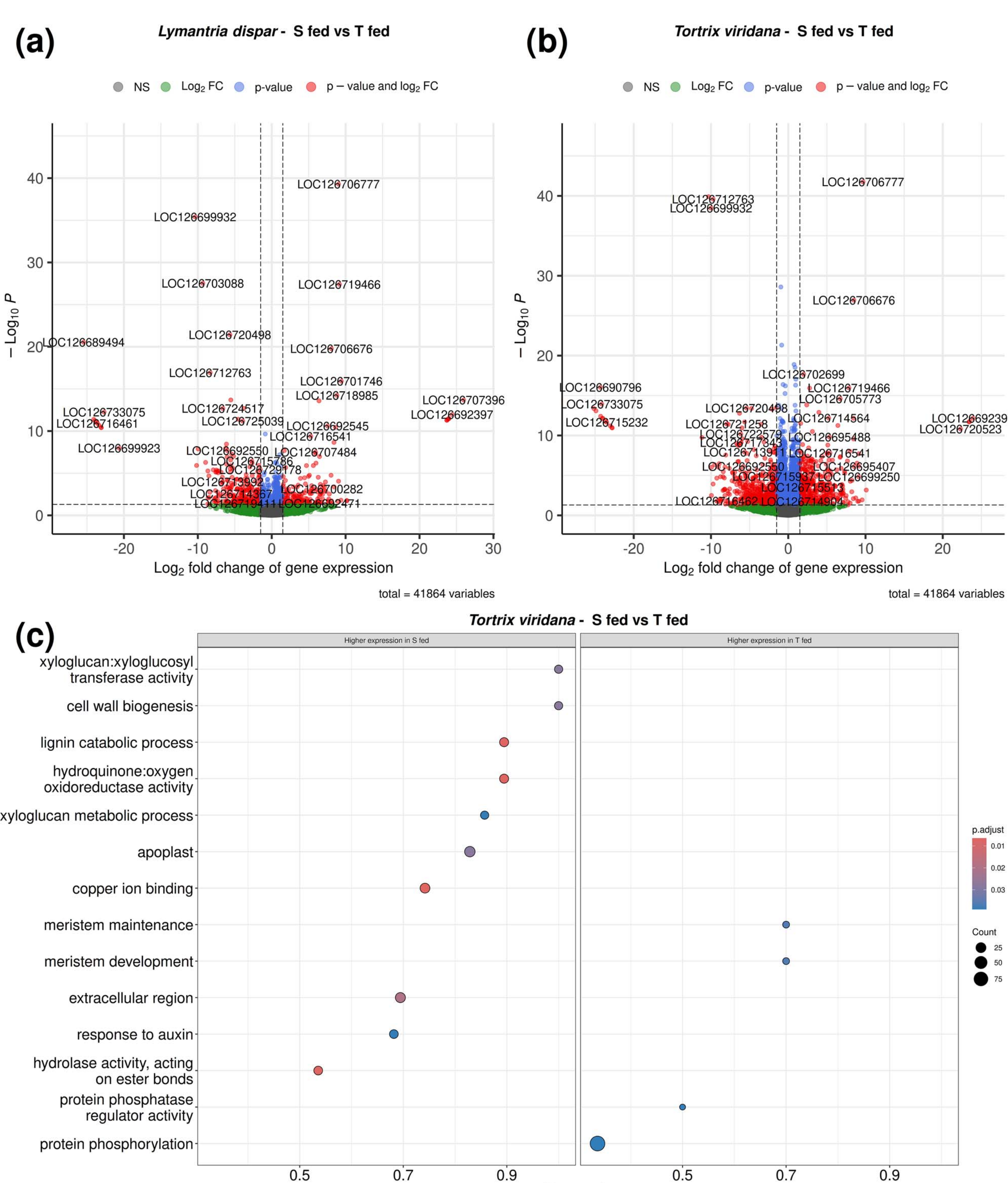

Figure 6. (a) Volcano plots of differentially expressed genes for the comparison of S- and T-oaks after feeding (Sfed vs Tfed) by *Lymantria dispar* or (b) by *Tortrix viridana*, respectively. Differentially expressed genes with a $|\log_2$ fold change$| > 1$ and respective adjusted P-value < 0.05 are presented as red dots (listed in Table S3, Figures S13–S16 available as Supplementary Data at *Tree Physiology* Online). Blue dots show differentially expressed genes with $\log_2$ fold changes between -1 and $+1$ with adjusted P-value < 0.05. Grey dots denote genes whose expression was not significantly changed. In total, 41,864 genes were analysed. (c) Dot plot visualization of gene set enrichment analysis based on the differential gene expression analysis of the groups Sfed vs Tfed in the *T. viridana* feeding experiment. The size of dots corresponds to the number of genes in the core enrichment set. Dot colour encodes the enrichment significance. Dots are ordered by gene ratio, which is defined by the number of core enrichment genes divided by all annotated genes associated with the GO term.

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Table 2. Transcription factors or transcriptional regulators identified as up-regulated DEGs in *Quercus robur* leaves only in response to *Lymantria dispar* but not to *Tortrix viridana* (genes are related to exclusive MapMan sub-bins only assigned to genes up-regulated by *L. dispar*, not by *T. viridana*).

Gene ID	Protein ID	Gene description in *Q. robur*	MapMan sub-bin within 'RNA biosynthesis.DNA-binding transcriptional regulation'
LOC126710120	xp_050266453.1	*Transcription factor bHLH118-like*	'basic DNA-binding domain.basic helix–loop–helix (bHLH) domain.bHLH class-Ib transcription factor'
LOC126703051	xp_050257913.1	*Transcription factor bHLH162-like*	
LOC126721173	xp_050280153.1	*Transcription factor MTB1*	'basic DNA-binding domain.basic helix–loop–helix (bHLH) domain.bHLH class-IIId transcription factor'
LOC126707675	xp_050263458.1	*bZIP transcription factor 44-like*	'basic DNA-binding domain.basic leucine zipper (bZIP) domain.bZIP class-S/SE transcription factor'
LOC126727435	xp_050289067.1	*B3 domain-containing protein Os07g0563300-like*	'beta-barrel DNA-binding domain.LAV-VAL transcription factor'
LOC126733058	xp_050292133.1	*AP2-like ethylene-responsive transcription factor AIL6*	'beta-hairpin exposed by alpha/beta-scaffold structure.AP2/ERF family.AP2 subfamily.transcription factor ∗(WRI/AIL)'
LOC126732985	xp_050292043.1	*Ethylene-responsive transcription factor RAP2–7*	
LOC126697341	xp_050250260.1	*Ethylene-responsive transcription factor RAP2–7-like*	
LOC126729073	xp_050290767.1	*Ethylene-responsive transcription factor ERF003-like*	'beta-hairpin exposed by alpha/beta-scaffold structure.AP2/ERF family.ERF subfamily.subgroup ERF-V transcription factor ∗(SHN)'
LOC126689871	xp_050240997.1	*VQ motif-containing protein 8%2C chloroplastic-like*	'beta-hairpin exposed by alpha/beta-scaffold structure.WRKY transcription factor activity.regulatory protein ∗(MKS1)'
LOC126729178	xp_050290827.1	*Transcription factor PCL1*	'helix-turn-helix DNA-binding domain.GARP-G2-like domain.transcription factor ∗(LUX/BOA)'
LOC126712318	xp_050267562.1	*BEL1-like homeodomain protein 7%2C transcript variant X1*	'helix-turn-helix DNA-binding domain.HOMEO domain.TALE transcription factor family.BEL transcription factor'
LOC126691922	xp_050243217.1	*Transcription factor MYB27-like*	'helix-turn-helix DNA-binding domain.MYB transcription factor family.R2R3-MYB transcription factor family.subgroup transcription factor ∗(MYB27/48/59)'
LOC126688523	xp_050239198.1	*Transcription factor MYB24*	'helix-turn-helix DNA-binding domain.MYB transcription factor family.R2R3-MYB transcription factor family.subgroup transcription factor ∗(MYB71/79/121)'
LOC126689461	xp_050240619.1	*Zinc finger protein CONSTANS-LIKE 7*	'other all-alpha-helix DNA-binding domain.BBX family.class-I transcription factor activity.transcriptional co-regulator ∗(CIA2)'
LOC126696611	xp_050249268.1	*B-box zinc finger protein 22-like*	'other all-alpha-helix DNA-binding domain.BBX family.class-IV transcription factor'
LOC126727530	xp_050289181.1	*B-box zinc finger protein 32-like*	'other all-alpha-helix DNA-binding domain.BBX family.class-V transcription factor'
LOC126695426	xp_050248132.1	*Zinc finger protein CONSTANS-LIKE 9-like*	
LOC126694846	xp_050247317.1	*Zinc finger protein CONSTANS-LIKE 2*	
LOC126715515	xp_050272103.1	*Probable protein phosphatase 2C 27*	'undefined DNA-binding domain.DBP phosphatase-domain transcription factor'

DEG with higher expression in T-oaks than in S-oaks in the *T. viridana* experiment (Table S4 available as Supplementary Data at *Tree Physiology* Online).

GSEA identified no enriched GO terms for the Sfed (susceptible oaks fed by the insects) versus Tfed (resistant oaks fed by the insects) comparison in the *L. dispar* feeding experiment. In contrast, 13 enriched GO terms with genes showing higher expression values in S-oaks compared with T-oaks (NES between 1.59 and 2.11) and four enriched GO terms with genes showing lower expression values in S-oaks compared with T-oaks were identified in the *T. viridana* experiment (NES between −1.45 and −1.79; Figure 6b; Table S11). The top five GO terms containing genes more prominently expressed in S-oaks than in T-oaks after feeding by *T. viridana* were 'copper ion binding', 'hydrolase activity, acting on ester bonds', 'lignin catabolic process', 'hydroquinone:oxygen oxidoreductase activity' and 'extracellular region'. The four GO terms containing genes more prominently expressed in T-oaks than in S-oaks were 'protein phosphorylation', 'protein phosphatase regulator activity', 'meristem maintenance' and 'meristem development'.

A comparison of the candidate genes in the two feeding experiments with the software MapMan showed similar trends in the differential expression of genes, with more genes being differentially expressed in the *T. viridana* feeding experiment than in the *L. dispar* feeding experiment, when the presented MapMan sub-bins were considered (Figure 7). Phytohormone-related candidate genes tended to

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Table 3. Transcription factors or transcriptional regulators identified as up-regulated DEGs in *Quercus robur* leaves only in response to *Tortrix viridana* but not to *Lymantria dispar* (genes are related to exclusive MapMan sub-bins only assigned to genes up-regulated by *T. viridana*, not by *L. dispar*).

Gene ID	Protein ID	Gene description in *Q. robur*	MapMan sub-bin within 'RNA biosynthesis.DNA-binding transcriptional regulation'
LOC126722979	xp_050282108.1	*transcription factor FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR*	'basic DNA-binding domain.basic helixloophelix (bHLH) domain.bHLH class-IIIa transcription factor'
LOC126717830	xp_050275706.1	*myb family transcription factor MOF1*	'helix-turn-helix DNA-binding domain.GARP-G2-like domain.transcription factor *(CLAUSA)'
LOC126720641	xp_050279325.1	*transcription factor MYB63*	'helix-turn-helix DNA-binding domain.MYB transcription factor family.R2R3-MYB transcription factor family.subgroup-3 transcription factor'
LOC126698471	xp_050251680.1	*protein LIGHT-DEPENDENT SHORT HYPOCOTYLS 3-like*	'undefined DNA-binding domain.ALOG transcription factor'

be expressed at higher levels in S-oaks than in T-oaks fed by *T. viridana*, particularly those related to auxin- and signalling peptides. In contrast, only weak differential expression was evident in the *L. dispar* experiment. In case of transcription factors, ERFs were higher expressed in S-oaks than in T-oks fed by *T. viridana*. An impressively higher differential expression was detected for phosphorylation- and cell wall organization-related candidate genes in the *T. viridana* experiment compared with the *L. dispar* experiment. After feeding by *T. viridana*, the phosphorylation-related candidate genes showed a trend to higher expression in T-oaks than in S-oaks, whereas the cell wall organization–related candidates showed the opposite trend.

Discussion

Plants have evolved complex defence systems to cope with herbivory by insects. These systems are coordinated by signalling pathways involving JA, ethylene (ET) and abscisic acid (ABA), as well as their downstream transcriptional regulators. In this study, we investigated the transcriptional responses of *Q. robur* to herbivory by larvae of two moth species: the oak specialist *T. viridana* and the generalist *L. dispar*. We observed significant transcriptional activation of the defence response to these herbivores in oak leaves, with genes differentially expressed in response to feeding (1068 DEGs) representing 2.55% of all genes annotated in the *Q. robur* reference genome (NCBI_RefSeq). The majority of the DEGs were up-regulated (992); only 76 DEGs were down-regulated (Figure 1a). A similar pattern has been observed in other studies investigating oak responses to chewing herbivores (Kersten et al. 2013, Müller et al. 2019, Li et al. 2021, Montesinos et al. 2024, among others).

Hormonal signalling and transcription factor networks involved in response to herbivory in oak leaves

The key hormonal pathways that underpin defence responses—JA, ET and ABA—were activated in both feeding scenarios (Figures 1, 2 and 8).

Jasmonic acid and MYC transcription factors

Our data indicate strong upregulation of JA-related genes, which is consistent with the previous finding that JA hormones, such as JA, methyl jasmonate (MeJA) and the wound hormone jasmonoyl-L-isoleucine (JA-Ile), act as primary regulators of defence against chewing insects (Erb et al. 2012, Wasternack and Hause 2013). Genes assigned to JA signalling and the pathway for the synthesis of α-linolenic acid, the precursor of JA biosynthesis, dominate one cluster of a functional association network of hub genes up-regulated by feeding with *L. dispar* or *T. viridana* (Figure 2). The transcription factor *MYC2* emerged as a central hub in this defence network, supporting its role as a master regulator in the JA signalling pathway (reviewed in Kazan and Manners 2013, Gimenez-Ibanez et al. 2015, Song et al. 2022; summarized in Figure 8).

Activation of *MYC2* triggers downstream responses, including the induction of secondary metabolism genes (Figure 1b). For instance, *QrTPS1*, which encodes a germacrene D synthase-encoding gene (LOC126694768; Table S3 available as Supplementary Data at *Tree Physiology* Online), was first observed in our previous study (Kersten et al. 2013). Since SQT emissions are a key component of the plant's stress response to herbivory, and since herbivory-resistant genotypes (T-oaks) emit significantly higher levels of SQTs than sensitive S-oaks (Ghirardo et al. 2012), we investigated the biochemical function of the *QrTPS1* gene through heterologous expression in *E. coli* and in vitro enzyme activity assay (see Figure S12 available as Supplementary Data at *Tree Physiology* Online). This functional characterization analysis revealed that *QrTPS1* encodes an SQT synthase, with germacrene D as the primary product.

JAZ repressors, including *JAZ1* (*TIFY10A*), *JAZ3* (*TIFY6B*) and *JAZ10* (*TIFY9*), which are known to be sensitive to JA-Ile in *A. thaliana*, were also significantly up-regulated (Figure 2), highlighting their role in the feedback control of JA responses (Gimenez-Ibanez et al. 2015). In the resting state of JA signalling (absence of JA-Ile), *MYC2* is repressed by JAZ repressors (reviewed in Gimenez-Ibanez et al. 2015, Song et al. 2022). Once JA-Ile is present as an activating signal, *coronatine-insensitive 1* (*COI1*), JA-Ile and JAZ proteins form a co-receptor complex. *COI1* regulates ubiquitin-dependent JAZ degradation via the 26S proteasome, releasing *MYC2* from repression, allowing it to recruit a subunit of the mediator complex (*MED25*) protein and then activate the transcription of JA-responsive genes via binding to the G-box in their promoters (reviewed in Gimenez-Ibanez et al. 2015, Song et al. 2022; summarized in Figure 8). Interestingly, we identified also *JAZ8* (*TIFY5A*; Figures 2 and 8), which lacks a functional Jas domain in *A. thaliana* (Shyu et al. 2012). Thus, *JAZ8* disables the association with *COI1* in the presence of JA-Ile and prevents the overactivation of JA-mediated transcription (Gimenez-Ibanez et al. 2015), indicating a finely

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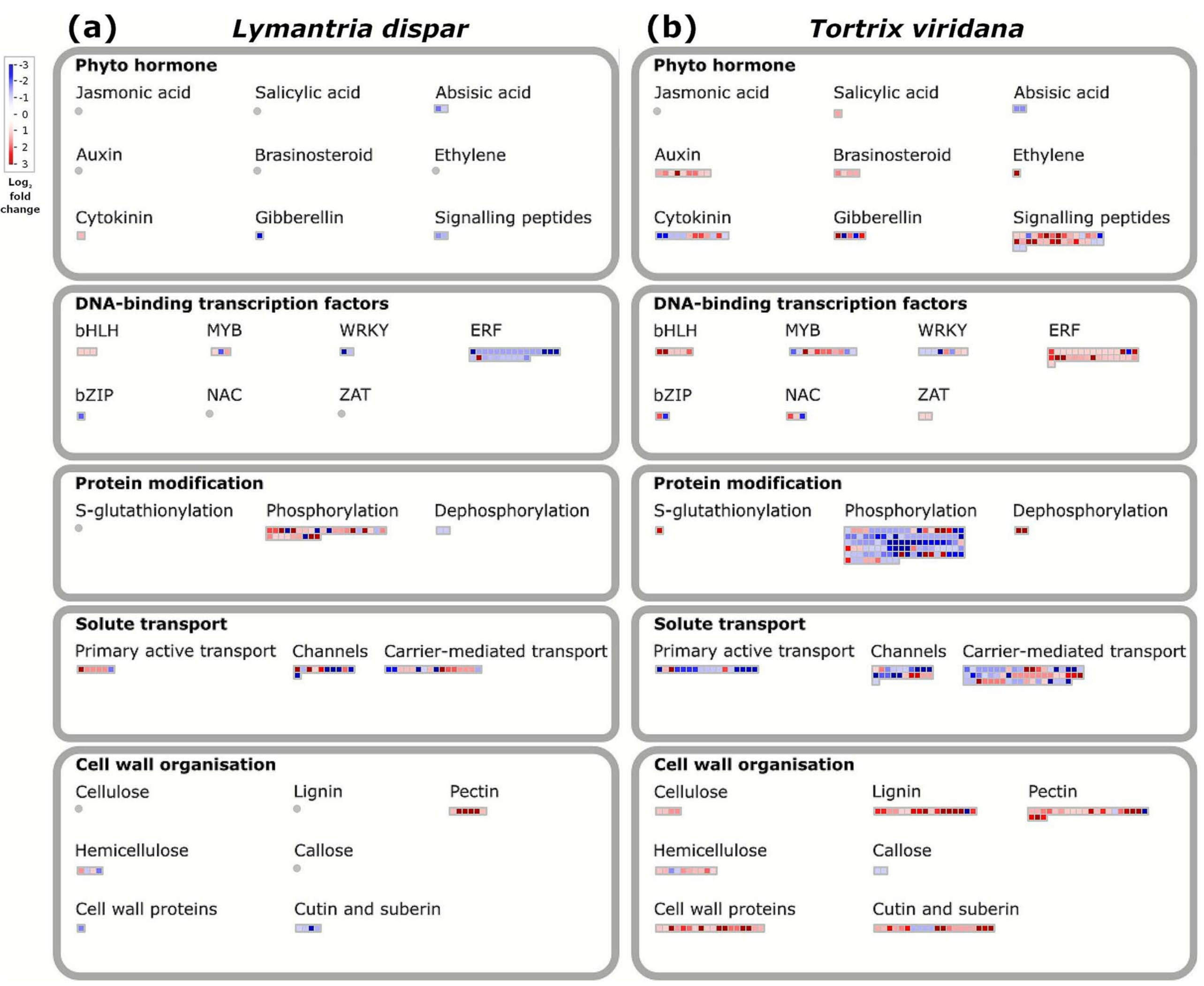

Figure 7. MapMan analyses showing the differences in candidate gene expression between the feeding experiments (a) *Lymantria* dispar and (b) *Torix viridana*. Differential gene expression was estimated in the leaves of S-oaks relative to the leaves of T-oaks fed to each herbivore. In total, five distinct MapMan-bins are shown (phyto hormone, DNA-binding transcription factors, protein modification, solute transport and cell wall organization) with up-regulated genes (small red boxes) and down-regulated genes (small blue boxes) depicted in sub-bins. The colour code of a box represents the $\log_2$ fold change of the respective gene in the presented comparison. Candidate genes for this comparison and their assignment to MapMan bins are included in the list of all DEGs in Table S3 available as Supplementary Data at *Tree Physiology* Online. The Mercator annotation of all *Quercus robur* genes assigning them to MapMan bins is available at the Open Science Framework (OSF).

regulated defence network balancing metabolic costs with defence efficacy.

Ethylene signalling

Our results also point to a strong induction of ET signalling by herbivory (Figures 1, 2 and 8). ET is a complex regulator of plant responses. In *A. thaliana*, the nuclear transcription factor *ethylene insensitive 3* (*EIN3*) plays a key role in ET signalling by initiating downstream transcriptional cascades for ET responses. It binds a primary ET response element present in the *ethylene response factor 1* (*ERF1*) promoter, which seems to be a key integrator of ET and JA signalling (Lorenzo et al. 2003). We identified *ERF1B* (other name of *ERF1* according to (TAIR)) in the hub network of genes upregulated by herbivory (Figures 2 and 4a), suggesting a previous upregulation of *EIN3* supporting cross-talk between the JA and ET pathways (Figure 8). Previous work by Song et al. (2014) demonstrated that *MYC2* and *EIN3* physically interact and antagonize each other's functions, thereby modulating the spectrum of induced defences (Song et al. 2014). Synergistic as well as antagonistic effects between JA and ET signalling were reported in herbivory (reviewed in Gimenez-Ibanez et al. 2015, Bungala et al. 2024).

ABA signalling

The upregulation of ABA–signalling related genes by herbivory in *Q. robur* (Figure 1b) is in line with literature findings showing that chewing herbivores also induce ABA. ABA induces MYC, activates herbivory defence–related genes and suppress JA (reviewed in Bungala et al. 2024). Our study supports cross-regulatory interactions between JA, ET and ABA and suggests that *Q. robur* integrates multiple hormonal signals to tailor its response according to the herbivore type (generalist vs specialist) and intensity.

WRKY transcription factors

Several WRKY transcription factors (i.e., *WRKY6*, *WRKY22*, *WRKY33*, *WRKY40*, *WRKY75*) occupy STRING network hubs in the transcriptional network of the oak response to herbivores reconstructed in this study (Figure 2). WRKYs, which

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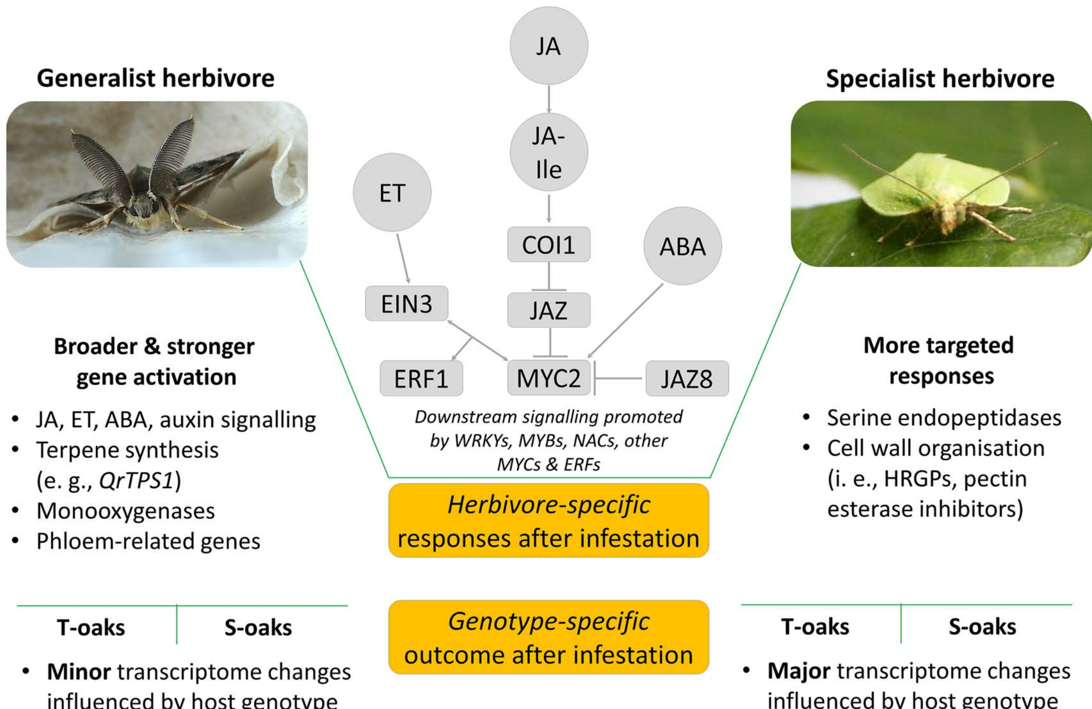

Figure 8. Herbivory-induced defence responses in *Quercus robur* and differential responses to the generalist *Lymantria dispar* and the specialist *Tortrix viridana*. In total, 1025 differentially expressed genes (DEGs) were higher expressed after infestation by the generalist compared with the specialist whereas 1117 DEGs showed higher expression after infestation by the specialist than the generalist (Table S2 available as Supplementary Data at *Tree Physiology* Online). Feeding by the generalist led to broader transcriptome changes compared with controls (1491 DEGs) than feeding by the specialist (775 DEGs; Table S2 available as Supplementary Data at *Tree Physiology* Online). The model of the hormone signalling after feeding (regardless of insect species) includes adopted elements of the regulatory network of a previously published jasmonic acid signalling pathway model (Ruan et al. 2019). The double arrow between *EIN3* and *MYC2* indicates a potential interaction between the expressed proteins as described for *Arabidopsis thaliana* (Song et al. 2014). *MYC2* was identified as a central regulatory hub in both *Q. robur* responses, to the generalist and to the specialist (Figure 5), as well as in the global herbivory interaction network (Figure 2). *JAZ8* (*TIFY5A*; Figure 2)—while lacking a functional Jas domain in *A. thaliana* (Shyu et al. 2012)—prevents the association with *COI1* in the presence of jasmonoyl-L-isoleucine, thus further repressing *MYC2* and preventing the overactivation of jasmonic acid–mediated transcription (Gimenez-Ibanez et al. 2015). JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; ET, ethylene; ABA, abscisic acid; HRGPs, hydroxyproline-rich glycoproteins; *COI1*, *coronatine-insensitive 1*; *EIN3*, *ethylene insensitive 3*; *JAZ*, *jasmonate ZIM-domain protein*; *ERF1*, *ethylene response factor 1*; *MYC2*, *basic helix–loop–helix (bHLH) transcription factor MYC2*; *JAZ8*, *jasmonate ZIM-domain protein 8* (*TIFY5A*).

specifically interact with the W box—a frequently occurring elicitor-responsive *cis*-acting element—have been reported to play an important role in the plant defence against herbivores (reviewed in Kundu and Vadassery 2021). A co-expression network analysis of genes up-regulated in poplars in response to the *Phratora vitellinae* feeding also highlighted WRKY transcription factors (Müller et al. 2019). The phosphorylation-dependent activation of *WRKY33* (our top-1 hub; Figure 2) in *A. thaliana*, by *MPK3* and *MPK6* upon wounding leads to the up-regulation of the *ACS* gene (also hub in Figure 2), which is the key enzyme of ET biosynthesis (reviewed in Kundu and Vadassery 2021). In *A. thaliana*, *WRKY40* provides resistance against a wide range of herbivores, besides *WRKY18* and *WRKY60* (reviewed in Kundu and Vadassery 2021). *MYC2* binding sites are predicted on promoters of *WRKY40* (Figure 2) and *WRKY18* in *A. thaliana* (reviewed in Kundu and Vadassery 2021). In general, the STRING network analysis (Szklarczyk et al. 2019), which was also applied in a few other plant transcriptomic studies (Davin et al. 2016, Das et al. 2022, Mader et al. 2023) points to the key players in the *Q. robur* response to herbivory, with the top hubs *WRKY33*, *MYC2*, *WRKY44* and *JAZ1* (*TIFY10A*) connected with each other (Figure 2).

Transcriptional changes in the oak leaf photosynthesis and growth caused by herbivory

As defence mechanisms against insect feeding increased (see above), the expression of the photosynthesis/photosystem II genes was downregulated, suggesting reduction of resources and energy for plant growth (Figure S3 available as Supplementary Data at *Tree Physiology* Online). This seems to indicate that oaks are allocating their carbon and energy resources to withstand herbivory including signalling danger to other plant tissues, rather than investing in growth, as the leaves are being consumed by the herbivores. Down-regulated genes potentially involved in growth in oak include homologues of the *A. thaliana* genes *SAUR12* and *SAUR38*, among others. The SAUR gene family is a family of auxin-responsive genes with ~60–140 members in most higher plant species, which are important in the regulation of dynamic and adaptive growth (Stortenbeker and Bemer 2019). Growth-defence trade-offs were obvious in several molecular studies on herbivory and were considered a result of regulatory 'decisions' by the plant, enabling it to fine-tune its phenotype in response to diverse environmental challenges (reviewed by Züst and Agrawal 2017).

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Oak leaves respond differently to the herbivory by the specialist and the generalist insect

Our comparative analysis revealed significant differences in the number and identity of the genes that were induced in response to *L. dispar* and *T. viridana*. Feeding by the generalist *L. dispar* resulted in broader transcriptional changes, including the upregulation of genes involved in secondary metabolism, e.g., genes with terpene synthase activity (Figures 3b–5 and 8). The tendency of generalist herbivores to elicit more generalized chemical defences due to their broad host range has been observed in several plant species (Voelckel and Baldwin 2004). In a comprehensive study with nearly 200 insect species from three orders feeding on willows or aspen using classical methods (chemical, morphological and phylogenetic features), it has been proven that trees show different reactions on account of the feeding insect (Volf et al. 2015). The authors interpreted their findings as suggesting that diversification of plant responses is needed for effective protection against a broad range of herbivores.

It might appear that the defence response to the generalist is broader because of the obviously larger body size of the *L. dispar* larvae and therefore its higher consumption of leaf material. On the other hand, it may simply be a more intense reaction of the oak to the generalist, regardless of the amount of material consumed. The latter hypothesis is supported by the results of a study by Zong and Wang (2007), who investigated responses of *Nicotiana* to two *Helicoverpa* species—one specialist and one generalist. Although both insects were of the same size, a less intensive defence response to the specialist has been found as well (Zong and Wang 2007). In another *Nicotiana attenuata* study, the transcriptional response of the plant to two different chewing generalists was more similar than the response to a chewing specialist (Voelckel and Baldwin 2004). In contrast, Reymond et al. (2004), who compared transcriptome changes in *A. thaliana* in response with larvae of the generalist *Spodoptera littoralis* and specialist *Pieris rapae,* observed almost identical transcript profiles in response to both chewing herbivores (Reymond et al. 2004). However, it has to be considered that this study used a DNA microarray representing only a portion of the *A. thaliana* transcripts. Rehrig et al. (2014) analysed the same experimental system including *Arabidopsis*, *S. littoralis* and *P. rapae* with the focus on the expression of transcription factors after feeding (Rehrig et al. 2014). These authors not only found differences in the identity of transcription factors induced by the generalist and the specialist but also a much higher number of transcription factors affected by feeding of the generalist. This is consistent with the results that we observed in oak in response to the *L. dispar* and *T. viridana* feeding (Figure 4).

Stronger induction of ethylene signalling after feeding of the generalist

Our results point to stronger induction in ET signalling after feeding by the generalist compared with the specialist (Figures 3b and 4; three members of the AP2 subfamily of ERF transcription factors in Table 2). Synergistic as well as antagonistic effects between JA and ET signalling were reported in herbivory (Bungala et al. 2024) (see also section ‘Hormonal signalling and transcription factor networks in herbivory’). If the stronger activation of ET signalling after generalist’s feeding compared with specialist leads to a stronger repression of JA signalling remains an open question.

Lower expression of key components of the photosystem II after feeding of the generalist

The direct comparison of the *Q. robur* transcriptomes after infestation by the generalist and the specialist revealed a lower expression of components of the core complex of photosystem II (Figure 3a) after the generalist’s feeding. This is not unexpected, because feeding by larvae of *L. dispar* leads to a stronger reduction of leaf area over time than feeding by *T. viridana* due to the considerably larger body mass of *L. dispar*. Thus, the loss of photosynthetic tissue due to the leaf area consumed by the generalist is higher. However, it has to be considered that—beside this direct effect—indirect effects may also contribute to reduced photosynthetic rates in the remaining leaf tissue (reviewed in Nabity et al. 2009). In line with our finding on a stronger reduction of photosynthesis by the generalist and the expected consequences for growth, it could also be shown that sap-feeding generalists imposed stronger effects on their hosts than sap-feeding specialists (Zvereva et al. 2010). This led the authors to the speculation that specialized sap-feeders might impose stronger selective pressure on their hosts during their coevolutionary history than did generalists, resulting in a better adaptation of plants to tolerate damage by highly specialized than by generalist pests.

Reduced and more specific responses after feeding of the specialist

In contrast to the generalist *L. dispar*, the specialist *T. viridana* elicited a comparatively reduced and more specific plant defence response. This might reflect the evolved ability of *Tortrix* to mitigate the plant’s defensive strategy (Ghirardo et al. 2012). Our results indicated that the specialist suppressed JA responses and instead triggered alternative mechanisms related to cell-wall structure and reinforcement, as seen by lower activation of JA-related genes and stronger induction of genes related to cell wall biosynthesis and organization than the generalist (Figures 3b, 7 and 8; Figure S7 available as Supplementary Data at *Tree Physiology* Online). Plant cell walls, which consist mainly of polysaccharides (i.e., cellulose, hemicelluloses and pectins), play an important role in defending plants against pathogens in general. Enzymes modifying polysaccharides may influence cell wall structure, strength and organization. Among the pectin-modifying enzymes, pectin methylesterases (PMEs) play an important role by modifying the degree of methylesterification of pectins. In *Q. robur*, five putative PMEs showed higher expression after infestation by *T. viridana* than by *L. dispar* (Table S3 available as Supplementary Data at *Tree Physiology* Online; assignment of these genes to MapMan bin ‘cell wall organisation.pectin’). The products of this reaction might be directly or indirectly involved in different defence responses (reviewed in Pelloux et al. 2007). However, in response to cell wall–degrading enzymes secreted by insects, defence responses of plants may also include inhibitors of these enzymes, such as inhibitors of pectin-degrading enzymes, e.g., pectinmethyl esterase inhibitors (‘pectinesterase inhibitor activity’ among the top-five GO terms assigned to genes more prominently expressed after infestation by *T. viridana*).

The best-known protein components of the plant cell wall have highly repetitive, proline-rich sequences. These protein groups include hydroxyproline-rich glycoproteins (HRGPs)

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or extensins, proline-rich proteins (PRPs) and glycine-rich proteins (GRPs) (Jose and Puigdomenech 1993). A total of six *Q. robur* genes encoding putative HRGPs showed higher expression after feeding by the specialist than by the generalist (Table S3 available as Supplementary Data at *Tree Physiology* Online; assignment of these genes to MapMan bin 'cell wall organisation.cell wall proteins.hydroxyproline-rich glycoprotein activities'). HRGPs are involved in cell wall self-assembly and may rapidly polymerize upon wounding, indicating a specific function in creating a tightly bound wall in stress conditions (Bradley et al. 1992).

Genotypic differences in the oak transcriptional responses to generalist and specialist herbivores

Our data also revealed genotypic differences in defence responses of oaks to generalist or specialist herbivores between T-oaks and S-oaks. Comparison of the gene expression profiles between the two different oak genotypes after feeding revealed a remarkably higher differential response when fed by the specialist than by the generalist (Figures 6 and 8). This finding is not surprising, as the phenotypic classification of S- and T-oaks is based on their tolerance to *T. viridana* feeding (Bertic et al. 2021). In an earlier study, it has been shown that T-oaks exhibited more coordinated and constitutive activation of defence pathways, including higher constitutive expression of defence genes related to the biosynthesis of terpenoids, flavonoids and tannins, than S-oaks, which more often react with induced defence mechanisms (Kersten et al. 2013). These findings align with those of Ghirardo et al. (2012) who demonstrated that T-oaks emit higher levels of sesquiterpenes, such as germacrene D and α-farnesene, in response to herbivory. Additionally, the levels of polyphenols, particularly flavonoids, varied between S- and T-oaks (Ghirardo et al. 2012, Bertic et al. 2021, Bertic et al. 2023). These compounds act as deterrents or signals to natural enemies.

For the ERF transcription factor family, a higher expression in S-oaks than in T-oaks after feeding of *T. viridana* could be observed (Figure 7), which is in accordance with earlier results (Kersten et al. 2013).

Overall, it seems that the specialized *T. viridana* tends to prefer leaves where it performs best, as it was already shown earlier for *T. viridana* on S-oaks (Ghirardo et al. 2012, Orgel et al. 2021), which can be a benefit for the host plants when having a high variability in its defence level (Thiel et al. 2020). (Bertic et al. (2023)) reported that larvae feeding on T-oaks required greater leaf consumption to reach the same pupal weight compared with larvae fed on S-oaks. Moreover, catabolism (digestion) of specific compound classes is more pronounced in S-oaks rather than T-oaks. These findings highlight the ecological relevance of the transcriptional differences presented in the study.

Conclusion

The findings of this study demonstrate that *Q. robur* exhibits complex, hormone-mediated transcriptional responses to herbivory, which are influenced by herbivore specialization and host genotype (the latter especially in the case of the specialist; Figure 8). Key regulators such as transcription factors *MYC2*, *JAZ* and *ERF* activate defence gene expression mainly via the JA and ET pathways after feeding by the generalist or the specialist. Comparative analysis revealed that feeding by the generalist *L. dispar* induced broader transcriptional gene activations, including the upregulation of genes involved in secondary metabolism, whereas the specialist *T. viridana* elicited more localized responses characterized by strong activation of genes involved in cell wall biosynthesis and reinforcement, suggesting a role for structural defences against specialist herbivores. These insights deepen our understanding of oak–insect interactions and have practical implications for forest management and the selection of insect-resistant genotypes in a changing climate. An easy-to-use molecular marker set for identification of potential T-oaks already in the seedling state is currently under development (Schroeder et al. 2021) and may enable the targeted selection of resistant genotypes for reforestation of severely infected stands. Future studies including additional specialist and generalist species, as well as time-course analyses of transcriptional changes, will further elucidate the dynamic regulation of oak defences in response to herbivory.

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Supplementary Data

Supplementary data for this article are available at *Tree Physiology* Online.

Author contributions

B.K., H.S., J.-P.S., A.G. and M.F. designed the study. M.M. performed the bioinformatic and statistical analyses with contributions of B.K. and T.N. F.O. and H.S. performed the feeding experiments and sampling. T.N., I.Z. and A.G. examined the enzymatic activity of *QrTPS1*. M.M., B.K., H.S., J.-P.S. and T.N. wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

J.-P.S. holds the position of Editor for *Tree Physiology* and has not peer reviewed or made any editorial decisions for this paper.

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Data availability

Illumina sequencing reads for all 72 samples from both feeding experiments are available for download from ENA (BioProject accession PRJEB87436). Read counts of all mapped reads against the dhQueRobu3.1 gene annotation (for all samples) as well as the assignment of dhQueRobu3.1 gene identifier and protein identifier to MapMan-Bins with Mercator are available at the Open Science Framework (OSF).

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