Tree Physiology 42, 1463–1480
https://doi.org/10.1093/treephys/tpac012

Research paper

Systemic effects of *Tuber melanosporum* inoculation in two *Corylus avellana* genotypes

Fabiano Sillo[1], Cecilia Brunetti[2], Fabio Marroni[3], Federico Vita[4], Luana Beatriz dos Santos Nascimento[5], Alfredo Vizzini[6,7], Antonietta Mello[7,†] and Raffaella Balestrini[1,8,†]

[1]National Research Council - Institute for Sustainable Plant Protection (CNR-IPSP), Strada della Cacce 73, 10135 Torino, Italy; [2]National Research Council - Institute for Sustainable Plant Protection (CNR-IPSP), Via Madonna del Piano 10, 50019 Firenze, Italy; [3]Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Via delle Scienze 206, 33100 Udine, Italy; [4]Department of Biology, University of Bari Aldo Moro, Via E. Orabona 4, 70125 Bari, Italy; [5]Department of Agriculture, Food, Environment and Forestry, University of Florence, 50019 Florence, Italy; [6]Department of Life Sciences and Systems Biology, University of Torino, Viale P.A. Mattioli 25, 10125 Torino, Italy; [7]National Research Council - Institute for Sustainable Plant Protection (CNR-IPSP), Viale Mattioli 25, 10125 Torino, Italy; [8]Corresponding author (raffaella.balestrini@ipsp.cnr.it)

Received September 26, 2021; accepted January 28, 2022; handling Editor Andrea Polle

Roots of the European hazelnut (*Corylus avellana* L.), i.e., one of the most economically important nut species, form symbiosis with ectomycorrhizal (ECM) fungi, including truffles. Although physical interactions only occur in roots, the presence of mycorrhizal fungi can lead to metabolic changes at a systemic level, i.e., in leaves. However, how root colonization by ECM fungi modifies these processes in the host plant has so far not been widely studied. This work aimed to investigate the response in two *C. avellana* genotypes, focusing on leaves from plants inoculated with the black truffle *Tuber melanosporum Vittad*. Transcriptomic profiles of leaves of colonized plants were compared with those of non-colonized plants, as well as sugar and polyphenolic content. Results suggested that *T. melanosporum* has the potential to support plants in stressed conditions, leading to the systemic regulation of several genes involved in signaling and defense responses. Although further confirmation is needed, our results open new perspectives for future research aimed to highlight novel aspects in ECM symbiosis.

Keywords: hazelnut, RNA-seq, symbiosis, transcriptomics, truffle.

Introduction

European hazelnut (*Corylus avellana* L.) is one of the most economically important nut species, belonging to the Betulaceae family, a group of six plant genera including birches and alders. Members of this family produce fruits in the form of kernels. *Corylus avellana* is the most widely cultivated species, which was bred mainly for high fruit yield for a long time, probably predating the Roman era (Li et al. 2021). Its kernels, the hazelnuts, both raw and roasted, are in demand by consumers worldwide as they are widely used in a variety of butters, candies, chocolate spreads and confectionary pastes (Alasalvar and Shahidi 2008), making this species an economically relevant woody crop. Hazelnuts have a high overall content of fatty acids (~60% of the hazelnut kernel), mainly oleic acid (~80% of the fatty acids), which have an importance as dietary components for humans (Lamberti et al. 2021), particularly considering that the last is usually regarded as a healthy fatty acid, reported to reduce the risk of cardiovascular disease (Li et al. 2021). Hazelnuts are also high in fiber, contain several essential vitamins and the derived oils have potential for use as biofuels (Moser 2012). The shells can be used for both landscaping and ground cover (Rowley et al. 2018). In addition, the European hazelnut is a host plant for ectomycorrhizal (ECM) fungi belonging to the genus *Tuber* P. Micheli ex F.H. Wigg. (e.g., *Tuber melanosporum* Vittad., *T. borchii* Vittad., *T. macrosporum* Vittad.) which, as symbiotic fungi, can offer

[†]Co-senior authors.

nutritional advantages to the host plant, but also represent themselves a priced food product, the 'truffles' (Lefevre and Hall 2001). In ECM symbiosis, in fact, the plant and the fungal partners significantly improve their nutrition because the fungus has access to photosynthetic products of the host plant and completes its life cycle, while the plant takes advantage through an improved mineral nutrition (mainly nitrogen (N)) and water supply (Willmann et al. 2014, Mello and Balestrini 2018). Additionally, *C. avellana's* reputation as 'an excellent carrier of mycorrhizae' (Chevalier 1998) makes it one of the species whose seedlings are commonly inoculated for truffle cultivation (Lefevre and Hall 2001). At least two other *Corylus* species can be also successfully inoculated with *T. melanosporum*: *C. colurna* L. (Chevalier 1998) and *C. heterophylla* Fisch. ex Trautv. (Craddock 1992, Chevalier 1998). Although the impact on the composition of the plant cell wall in hazelnut roots colonized by *T. melanosporum* has been deeply investigated (Sillo et al. 2016), the absence of a high-quality genome assembly until 2021 (Lucas et al. 2021) limited the information on the *C. avellana* genes involved in the interaction with ECM fungi such as *T. melanosporum* (Kohler et al. 2016). Conversely, thanks to the release of the *T. melanosporum* genome sequence (Martin et al. 2010), several papers have focused on the characterization of the fungal genes involved in the symbiosis (Balestrini et al. 2012, Balestrini and Mello 2015 and references therein, Mello and Balestrini 2018 and references therein).

Although physical interactions only occur in roots, mycorrhizal fungi are known to change processes at a systemic level, i.e., in leaf metabolism (Smith and Read 2008). Leaves produce carbohydrates, and their transfer from plants to fungal partners is one of the most important factors in mycorrhizal symbiosis. Notably, several works have been focused on verifying the impact of arbuscular mycorrhizal (AM) symbiosis in leaves of diverse crops (maize, rice, tomato) and model plants (*Medicago truncatula* Gaertn.) (Liu et al. 2007, Aloui et al. 2011, Campos-Soriano et al. 2012, Lingua et al. 2012, Song et al. 2015, Cervantes-Gámez et al. 2016, Adolfsson et al. 2017), allowing demonstration of the effects of the AM fungus in the aerial parts of the plants, including the remodulation of plant hormones and a potential bioprotective effect. Interestingly, in mycorrhizal maize plants, leaves experienced an increase in carbon vs N metabolism, suggesting a redistribution of metabolic pools, including carbohydrates and amino acids, as well as an induction of systemic defense gene expression and the accumulation of secondary metabolites, suggesting a mycorrhizal-specific systemic response (Gerlach et al. 2015).

In contrast, how root colonization by ECM fungi modifies the metabolic processes in the distal part of the host plant is less understood and knowledge is still patchy. It was already reported that ECM symbiosis can lead to altered leaf anatomies, for example by increasing leaf sizes, thus enhancing photosynthetic ability (Martins et al. 1997). Ectomycorrhizal fungi are known also to influence the N status of leaves, resulting in an increase (Hobbie and Colpaert 2003) or decrease (Hobbie and Colpaert 2003, Koele et al. 2012, Näsholm et al. 2013, Szuba et al. 2019) in foliar N concentration. The exchange between carbon (C) and N is probably one of the most important aspects in the ECM symbiosis, considering that the outcome of the interaction is determined by C/N balance (Corrêa and Martins-Loução 2011). Ectomycorrhizal root colonization was found to affect carbohydrate-nutrient exchange, via modulation of the plant N uptake and via the fungal-induced 'carbon sink' due to the C demand of the fungus (Corrêa and Martins-Loução 2011, Dosskey et al. 1990). However, resource exchanges are very complex in mutualistic relationships and might depend on many factors (Veneault-Fourrey and Martin 2011). In poplar, a higher quantum yield of photochemistry and an increased concentration of phosphorus and potassium have been documented in leaves of plants colonized by the ECM fungus *Paxillus involutus* (Batsch) Fr., compared with non-ECM plants (Luo et al. 2011). Moreover, these leaves showed altered amino acid, sugar and fatty acid contents, highlighting the systemic plant response during ECM fungal colonization (Luo et al. 2011).

Furthermore, according to recent studies, mycorrhizal symbioses not only improve plant nutrition but also enhance plant resistance against abiotic and biotic stresses (Dreischhoff et al. 2020). This effect has been called 'mycorrhiza-induced resistance' (MIR) (Cameron et al. 2013, Mauch-Mani et al. 2017). MIR has similarities with both systemic-acquired resistance (SAR), induced after pathogen attack and the induced systemic resistance, conferred by other beneficial soil microbes (Dreischhoff et al. 2020). MIR in fact was reported as a mechanism that induces systemic effects in distal tissues, i.e., leaves, which are not in direct contact with the fungus but trigger local responses in roots (Dreischhoff et al. 2020). MIR response to pathogen attacks involves the production of defense enzymes such as lipoxygenases (LOX) (Baysal and Demirdöven 2007) and protease inhibitors that are an important class of proteins in defense, acting as a biocidal compound against insect herbivores (Zhu-Salzman and Zeng 2015). Other compounds mainly involved in enhancing plant tolerance or resistance to pathogens can be chemically categorized as terpenes, phenolic compounds, nitrogenous and sulfurous compounds (Wink 2018). Terpenes and terpenoids are ecologically important compounds, including volatile organic compounds (VOCs), that can be induced in plants by diverse abiotic and biotic stresses (Rosenkranz et al. 2021). Phenolic compounds are part of the plant defense arsenal and often higher in mycorrhizal than in non-mycorrhizal plants (Schweiger et al. 2014). The increase in tannins, flavonoids, phenolic glycosides and proanthocyanins in ECM-colonized poplar suggests that MIR triggers a metabolic shift from C-based to N-based defense (Kaling et al. 2018).

The main objective of this work is to decipher the response to colonization with the ECM fungus *T. melanosporum* in two genotypes of *C. avellana* leaves. Transcriptomic profiles of leaves of colonized plants were compared with those of non-colonized plants, as well as sugar and polyphenolic content. Currently, there is growing evidence that plant benefits deriving from symbiosis with mycorrhizal fungi are not only linked to plant and fungal species, but also to the involved plant/fungal genotypes, although results have been mainly obtained on AM symbiosis (Bazghaleh et al. 2018). Specifically, two cultivars have been considered, i.e., plantlets germinated from seeds (called wild) and plantlets belonging to the 'Tonda Gentile delle Langhe' (TGL) cv, propagated by cuttings to avoid genetic variation. The first one, commonly propagated by seeds, is the most utilized to obtain mycorrhizal plants with several truffle species in plant nurseries, while TGL is recognized as one of the best hazelnut cultivars worldwide, particularly appreciated by the processing industry for the organoleptic and nutritional characteristics of its nuts (Valentini et al. 2014, Silvestri et al. 2021). Results could be relevant also for applicative purposes, i.e., for the production of plants inoculated with truffles, providing information that is useful for applying the best mycorrhization strategy in diverse hazelnut genotypes.

Materials and methods

Biological materials

Hazelnut plants of about 1-year-old, non-mycorrhized and mycorrhized with *T. melanosporum*, were purchased from the nursery 'Tartufaie-Produzione Piante Tartufigene' (now Agrotecnologie srls, San Bonifacio, Verona, Italy) and grown in the IPSP-CNR greenhouse (Torino, Italy) from April 2018 (25 plants in total for each condition). Inoculum for the preparation of mycorrhizal plants was prepared and applied to produce mycorrhizal plants by the abovementioned nursery. In detail, starting from selected *T. melanosporum* fruiting bodies plants were inoculated (i) after about 6 months from the sowing in sterilized substrate in greenhouse (to allow the germination) for wild and (ii) on micropropagated cuttings kept in a growth chamber for 30 days for TGL. At sampling (May 2018), fully expanded leaves were harvested, frozen in liquid N and stored at −80 °C. Upon plants' arrival and at the moment of the collections of leaves, plants were checked for the presence of *T. melanosporum* ECMs; colonization was evaluated in terms of homogeneity and cut-off percentage of truffle ECMs. In detail, the root system of six hazelnut seedlings inoculated with *T. melanosporum* (three for cultivar) was rinsed in water and observed under a stereo dissecting microscope to look for the presence of the *T. melanosporum* ECMs (Agerer et al. 1996-2001). The same operation was also done at the moment of the leaf sampling to confirm the presence of a good level of *T. melanosporum* ECMs (i.e., at least a colonization degree of 40–60%) in the root apparatus of considered plants (in total eight plants for each hazelnut type, three for the RNAseq and five for the biochemical analyses). When necessary, hand-made cross sections were made and examined under a light microscope to confirm the features of this truffle species. The mycorrhization level of *T. melanosporum* on each plant was assessed according to Donnini et al. (2014). The whole root system was inspected for the presence of other unwanted ECM fungi. Contaminant ascomycetous ECM fungi, such as *Sphaerosporella brunnea* (Alb. & Schwein.) Svrček & Kubička and *Pulvinula constellatio* (Berk. & Broome) Boud. usually occurring during the first phase of growth in climatically controlled greenhouses (Amicucci et al. 2001), were not present. The root system of the control plants was randomly checked with the same procedure to exclude the presence of mycorrhization. No ECM tips were observed in control plants. Ectomycorrhizal tips were also sampled for the subsequent RT-qPCR considering fungal genes involved in symbiosis (see RT-qPCR analyses on fungal genes expressed in ECM paragraph).

RNA extraction

For transcriptomic analysis, three biological replicates were selected for each condition: *T. melanosporum* inoculated and non-inoculated wild (propagated by seeds) and TGL (propagated by cuttings) cultivars. Frozen leaves (12 samples) were lyophilized (starting from 500 mg of fresh material) and then ground in 2-ml tubes by using steel beads (diameter 0.4 mm) and homogenized through a Tissue Lyzer (Qiagen, Valencia, CA, USA). RNA extraction was carried out using Spectrum Plant Total RNA Kit (Sigma-Aldrich, S Louis, MO, USA), with some modifications. Particularly, the lysis buffer for sample was increased to 1000 μl, the washing buffers were increased to 500 μl and all the washing steps were performed twice. Final elution was carried out in 30 μl. After extraction, the RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Hudson, NH, USA), and approximately 1.2 μg per sample was sent to sequencing service for the Next Generation Sequecing.

Libraries preparation, sequencing and RNA-seq analysis

Transcriptomic profiles of the two genotypes in different conditions have been evaluated through an RNA-seq experiment. Library preparation and sequencing were performed by IGA technology services s.r.l. laboratories located in Udine (Italy). In detail, Universal Plus mRNA-Seq kit (Tecan Genomics, Redwood City, CA, USA) has been used for library preparation following the manufacturer's instructions (library type: fr-second strand). RNA samples were quantified, and quality was tested by Agilent 2100 Bioanalyzer RNA assay (Agilent technologies, Santa Clara, CA, USA) or Caliper (PerkinElmer, Waltham, MA, USA). Final libraries were checked with both Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and Agilent Bioanalyzer DNA

assay or Caliper (PerkinElmer). Libraries were then prepared for sequencing and sequenced on single-end 75 bp mode on NextSeq 500 (Illumina, San Diego, CA, USA). Base-calling and demultiplexing were performed by using Bcl2Fastq 2.20 version of the Illumina pipeline. Adapter sequences were masked with Cutadapt v1.11 from raw fastq data using the following parameters: anywhere (on both adapter sequences)—overlap 5—times 2—minimum-length 35—mask-adapter (Martin 2011).

For the purposes of our work, the *C. avellana* cultivar 'Jefferson' genome (Rowley et al. 2018) has been used as a reference. This genome derived from two PE and one MP Illumina library collectively representing the 93× genome coverage and a *de novo* genome assembly, capturing 91% of the genome (345 Mbp) with a contig N50 of 21,540 bp, has been completed (Rowley et al. 2018).

Raw reads have been aligned to the reference genome using STAR 2.7v (Spliced Transcripts Alignment to a Reference; Dobin et al. 2013). SAM files were converted into BAM files and indexed by Samtools 1.11v (Li et al. 2009). The aligned BAM file was processed using HTseq-count 0.11.1v to count reads mapping on the reference genome (Anders et al. 2015). The intersection mode (strict) was used to map exon junctions and gene overlaps. Differential expression analysis was performed using DEseq2 (Love et al. 2014). The variance on reads count was calculated based on the three biological replicates per condition.

Principal components analysis (PCA) was performed on normalized gene counts using DESeq2 (Love et al. 2014) (regularized log transformation of normalized data). Two sets of differentially expressed genes (DEGs) were identified: DEGs obtained from the comparison between inoculated wild (myc_wild) vs not inoculated wild (wild), and DEGs deriving from the comparison between inoculated TGL (myc_TGL) vs not inoculated TGL (TGL). Identified DEGs were considered significant if adjusted *P*-value was ≤0.05 and were visualized as heatmap generated by using R package 'ggplot2'. In order to identify shared and common DEGs, Venn diagrams showing intersections of the comparisons were generated through the R package 'ggvenn', and a shared/common set of DEGs was listed. Among the DEGs with adjusted $P \leq 0.05$, the predicted products of the 25 showing the highest $\log_2$ fold change were further characterized through *blastp* against the Refseq protein database and PFAM (http://pfam.xfam.org/), and conserved and functional motifs were analyzed.

Differentially expressed genes were associated by their IDs in the reference genome and the sequences were annotated with Blast2GO v.5 (Conesa et al. 2005) to determine the related Gene Ontology (GO) terms. All gene models of the C. *avellana* reference genome were annotated through Blast2GO to obtain an updated functional annotation of all gene models. Fisher enrichment tests for significant DEGs obtained from the four comparisons were performed with Blast2GO to search for significant differences (False Discovery Rate ≤ 0.05) in frequencies of GO terms compared with all *C. avellana* gene models (overrepresentation). The R package 'GOplot' v. 1.0.2 was used to visualize the enriched GO terms in the four comparisons as bubble plots. For Fisher enrichment results, the z-score for each enriched category was calculated with the following formula: $z\text{-score} = (n^\circ\ up - n^\circ\ down)/\sqrt{count}$, where '$n^\circ$ *up*' and 'n° *down*' are the number of assigned genes upregulated (logFC > 0) or downregulated (logFC < 0), respectively, and 'count' is the number of genes assigned to a term (Walter et al. 2015).

The negative logarithm of the adjusted *P*-value (corresponding to the significance of the term) is displayed on the *y*-axis. The area of the plotted circles is proportional to the number of genes assigned to the term.

Analysis of polyphenols

Lyophilized leaves of *C. avellana* (from five plants for each condition) were ground in liquid N and 0.1 g of dry weight were extracted with 3 × 5.0 ml ethanol 75% (pH 2.5, adjusted with formic acid—SigmaAldrich®—Merck® KGaA, Darmstadt, Germany) in an ultrasonic bath (BioClass® CP104), at 5 °C, using a constant frequency of 39 kHz and an input power of 100 W, for 30 min. After the extraction, the different samples were centrifuged (5 min, 9000 r.p.m., 5 °C-ALC® 4239R, Milan, Italy) and the supernatants partitioned with 3 × 5 ml *n*-hexane to remove chlorophylls and other compounds that could interfere in the analysis of the polyphenols. Then, the hydroethanolic phase was reduced to dryness using a rotavapor (BUCHI® P12, Cornaredo, Italy; coupled to a vacuum controller V-855) and the residue was resuspended with 1.0 ml of MeOH: Milli-Q water solution (1:1 v/v, pH 2.5 adjusted with formic acid).

Aliquots of the resuspended samples (10 μl) were injected into a Perkin® Elmer Flexar liquid chromatography equipped with a quaternary 200Q/410 pump and an LC 200 diode array detector (DAD) (from Perkin Elmer®, Bradford®, CT, USA). The separation and quantification of polyphenols were carried out using an Agilent® Zorbax® C18 analytical column (250 mm × 4.6 mm, 5 μm) kept at 30 °C. The mobile phase consisted of (A) Milli-Q water (acidified with 0.1% formic acid) and (B) acetonitrile (acidified with 0.1% formic acid). The following gradient mode was applied at a flow rate of 0.6 ml min^{-1}: 0–1 min: 3% B, 1–56 min: 3–40% B, 56–61 min: 40% B, 61–62 min: 3% B. A 10-min step of conditioning was used to return to the initial conditions of the method. All the samples were analyzed in triplicate, and chromatograms were recorded at 280, 330 and 350 nm, with the spectral data from all peaks accumulated in the 210–590 nm range.

The identification of the polyphenols was carried out on the basis of their retention time, UV–vis spectral characteristics, literature data (Amaral et al. 2005, Oliveira et al. 2007, Riethmüller et al. 2013) and comparison with the following

authentic standards: gallic, chlorogenic, caffeic, caffeoyltartaric and *p*-coumaroyltartaric acids, myricetin-3-*O*-rhamnoside and quercetin-3-*O*-rhamnoside (all from SigmaAldrich®—Merck® KGaA, Darmstadt, Germany). The quantification of the polyphenols was performed using five-point calibration curves of these external standards, and if a commercial standard was not available, the quantification was performed using the calibration curve of the most similar one. All the results were expressed as mean ± standard deviation ($n = 5$).

Analysis of soluble carbohydrates

Soluble carbohydrates were quantified by HPLC-RI analysis following the protocol of (Kelly et al. 2017). Briefly, 30 mg of lyophilized samples were grounded and extracted with 1 ml of ethanol 80% in a 60–65 °C water bath for 60 min. The extracts were then centrifuged (10 min, 9000 r.p.m., ALC® 4239R, Milan, Italy) and the supernatant was reduced to dryness by a vacuum centrifugation in a Concentrator plus (Eppendorf, Italy) set to the low drying rate. The dry extracts were rinsed in 200 μl of ultrapure water and injected in a Series 250 LC binary pump equipped with an LC 30-RI detector (Perkin-Elmer, Bradfrod, CT, USA). Soluble carbohydrates were separated on an 8 × 300 mm SC1011 column (Showa Denko, Tokyo, Japan) maintained at 85 ± 1 °C and equipped with a 6 × 50 mm SC1011 precolumn. Eluent was ultrapure water at a flow rate of 0.5 ml min^{-1} during a 25-min run. Individual carbohydrates were identified by comparison of retention times with those of authentic carbohydrate standards (Sigma-Aldrich, Milano, Italy). The quantification of the individual carbohydrates was performed using five-point calibration curves of glucose, fructose and sucrose and the results expressed as mean ± standard deviation ($n = 5$).

RT-qPCR analyses on fungal genes expressed in ECM

Tuber melanosporum/*C. avellana* ECM tips from both hazelnut types (wild and TGL) were used as starting material for the total RNA extraction. In detail, about 100–150 mg of fresh ECM tips were collected from four plants for each hazelnut type, including the three considered for the RNAseq on leaves. With the aim to reach a sufficient amount of starting material, samples from two plants were pooled with the aim to obtain two independent biological replicates. RNA was extracted using Sigma Spectrum Plant Total RNA kit (Sigma-Aldrich, Munich, Germany) according to the manufacturer's instructions. Final elution was carried out in 30 μl of RNAse-free water. Genomic DNA was removed using a TURBO™ DNase kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Total RNA was used for each sample to synthesize the cDNA, according to the SuperScriptII—Reverse Transcriptase (Invitrogen) procedure. Expression of two *T. melanosporum* genes (GSTUMT00005025001 and GSTUMT00005678001), strongly upregulated in ECMcompartments in fully established ECMs (Hacquard et al. 2013), was assessed on cDNA by RT-qPCR. Primers were designed using Primer 3 plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and are listed in Table S1 (available as Supplementary data at *Tree Physiology* Online). The two reference genes *EF1B* and *RPS3* were used as previously reported by Zarivi et al. (2015). RT-qPCR was carried out with the ConnectTM Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each PCR was conducted on a total volume of 10 μl, containing 1-μl cDNA, 5-μl Sensifast sybr norox (Bioline) and 2 μl of each primer (3 μM), using a 96-well plate. Primer sequences with their optimal annealing temperature are listed in Tables S1A and B (available as Supplementary data at *Tree Physiology* Online). The following PCR program, which includes the calculation of a melting curve, was used: 95 °C for 30 s, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, ramp from 65 to 95 °C with a temperature increment of 0.5 °C and a read plate every 2 s. All the reactions were performed for two independent biological and two technical replicates. The baseline range and C_t values were automatically calculated using the Bio-Rad CFX Manager software. In order to compare the data from different PCR runs or cDNA samples, the C_t values of all the genes were normalized to the C_t value of the reference gene. The gene expression was normalized to that of the reference gene by subtracting the C_t value of the reference gene from the C_t value of the candidate gene efficiency correction, from the equation $2 - \Delta\Delta Ct$ (Livak and Schmittgen 2001), where $\Delta\Delta Ct$ represents the ΔCt sample $- \Delta Ct$ control (TGL and wild, respectively).

Statistical analysis

After verification of normal distribution of data by a Shapiro–Wilk test, a two-way ANOVA procedure was used to compare biochemical data among the four conditions (wild, TGL, myc_wild, myc_TGL). Mean separation was performed using the Tukey HSD test, adopting a probability level of $P \leq 0.05$. All tests were conducted by using R (basic and WRS2 packages). Data from sugar and polyphenol quantification were then analyzed using a PCA with the aim to better define the contribution of each variable in the differentiation of samples. Multivariate analysis was done using the software XLSTAT version 2016.02 (Addinsoft, Paris, France). Significant differences in gene expression for RT-qPCR experiments were assessed through the Pair Wise Fixed Reallocation Randomisation Test ©35 performed by using the Relative Expression Software Tool REST © 2009 v.2.0.13.

Results

Analyses of RNA-seq data: reads number, transcriptome coverage and total expressed genes

A total of 384,682,612 raw reads were generated after Illumina NextSeq sequencing from 12 sequencing libraries. Statistics of read mapping to reference genome, Htseq-count and DESeq2 normalized count results are reported in Tables S2–S4 (available as Supplementary data at *Tree Physiology* Online).

Read mapping of wild and TGL samples was similar. Proportions of reads mapping to reference ranged from 82.97 to 94.64%. Higher differences in read mapping proportion were found within wild samples compared with TGL samples. The PCA showed that RNA samples of inoculated wild and inoculated TGL clustered independently from each other and showed limited variability (see Figure S1 available as Supplementary data at *Tree Physiology* Online). Samples of not inoculated wild were grouped distinctly from inoculated ones, like inoculated TGL with not inoculated TGL samples, with one exception. Sample 1 (replicate #1 TGL) was in fact positioned close to samples from myc_TGL, and for this reason, it has been excluded from subsequent analyses. Although two replicates are not ideal, the outlier TGL sample has been removed from the analyses to avoid confounding the results. The BAM files resulting from mapping were deposited at the EMBL database ENA—European Nucleotide Archive as a project under the accession PRJEB46391 (secondary accession ERP130574).

A look at the whole-leaf transcriptome and at the DEGs: inoculated vs uninoculated plants

RNAseq analysis allowed identification of DEGs putatively related to the systemic response to symbiosis in the two studied genotypes. The comparison between inoculated wild vs not inoculated one allowed to find 1333 DEGs, 613 upregulated and 720 downregulated; on the other hand, a total of 5616 DEGs were identified in inoculated TGL vs not inoculated TGL, 3027 upregulated and 2589 downregulated (Figure 1; Tables S5–S8 available as Supplementary data at *Tree Physiology* Online). To have an overview of the regulation of the main metabolic processes and signaling pathways involved in the different comparisons, we conducted GO enrichment analysis. A significant enrichment in GO terms was observed in DEGs of myc_wild and myc_TGL. The comparison between GO terms assigned to DEGs of myc_wild and all *C. avellana* gene models showed a significant overrepresentation of 29 terms mostly related to photosystem I, photosystem II, response to stimulus, cell communication, sulfur compound metabolic process and integral component of membranes (Figure 2). These enriched GO terms showed a z-score >0, indicating they were mostly associated with upregulated genes. Fifteen out of 29 enriched GO terms showing a z-score <0 (associated with downregulation) was mainly related to oxidation–reduction processes.

On the other hand, enriched GO categories in myc_TGL DEGs were 59, 45 with a positive z-score. These terms were mainly related to phenylpropanoid biosynthetic process, response to salt stress and response to fungus, sulfur compound metabolic process, photosystem I and signal transduction (Figure 2). Enriched GO terms associated with oxidation–reduction process and lipid and carbohydrate processes showed a negative z-score in myc_TGL (Figure 2).

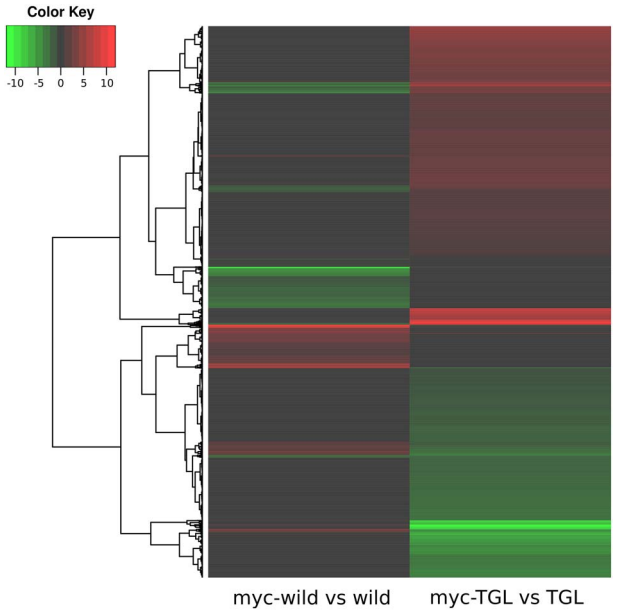

Figure 1. Heat map based on the Euclidean distances among samples. Data were hierarchically clustered based on sample distances. Columns indicate different comparisons (myc_wild vs wild and TGL vs myc_TGL), whereas rows indicate genes and their expression profiles.

Among the upregulated genes in the two sets (myc_wild vs wild and myc_TGL vs TGL) of DEGs, 37 genes were found to be shared between the comparisons (Figure 3). This shared set includes genes coding for several proteins involved in diverse functions (Table 1, Table S9 available as Supplementary data at *Tree Physiology* Online), mainly related to signaling pathways and transcriptional regulation. Among them, one transcription factor, two calcium-binding proteins, several receptor-like protein kinases, a calcium-transporting ATPase, a syntaxin, a cytochrome P450 in addition to genes involved in sugar and cell wall metabolism (i.e., a galactinol synthase and a cellulose synthase-like), a phototropin-1 and an asparaginase (Table 1). The extent of upregulation in the shared DEG was generally comparable in the two genotypes, but in some cases, a very different fold change was found, such as for the gene (g17613.t1) coding for a putative syntaxin (t-SNARE) protein, which showed a $\log_2$FC more than nine in wild and less than one in TGL leaves.

In contrast, 52 downregulated genes were shared between the two sets (myc_wild vs wild and myc_TGL vs TGL; Figure 3; Table S10 available as Supplementary data at *Tree Physiology* Online). Among them, a gene coding for a protein that showed homology with transcription factor SQUAMOSA promoter-binding protein-like (SPL), which is a class of plant-specific regulatory genes involved in plant growth and development (Xu et al. 2020). It was mainly downregulated in myc_TGL ($\text{Log}_2\text{FC} = -4.85$ vs 0.26 in myc_wild). Conversely, a gene coding for a protein involved in geraniol metabolism (g31865.t1) was particularly downregulated in myc-wild (Log2FC = − 5.85). Blastp analysis showed a homology with

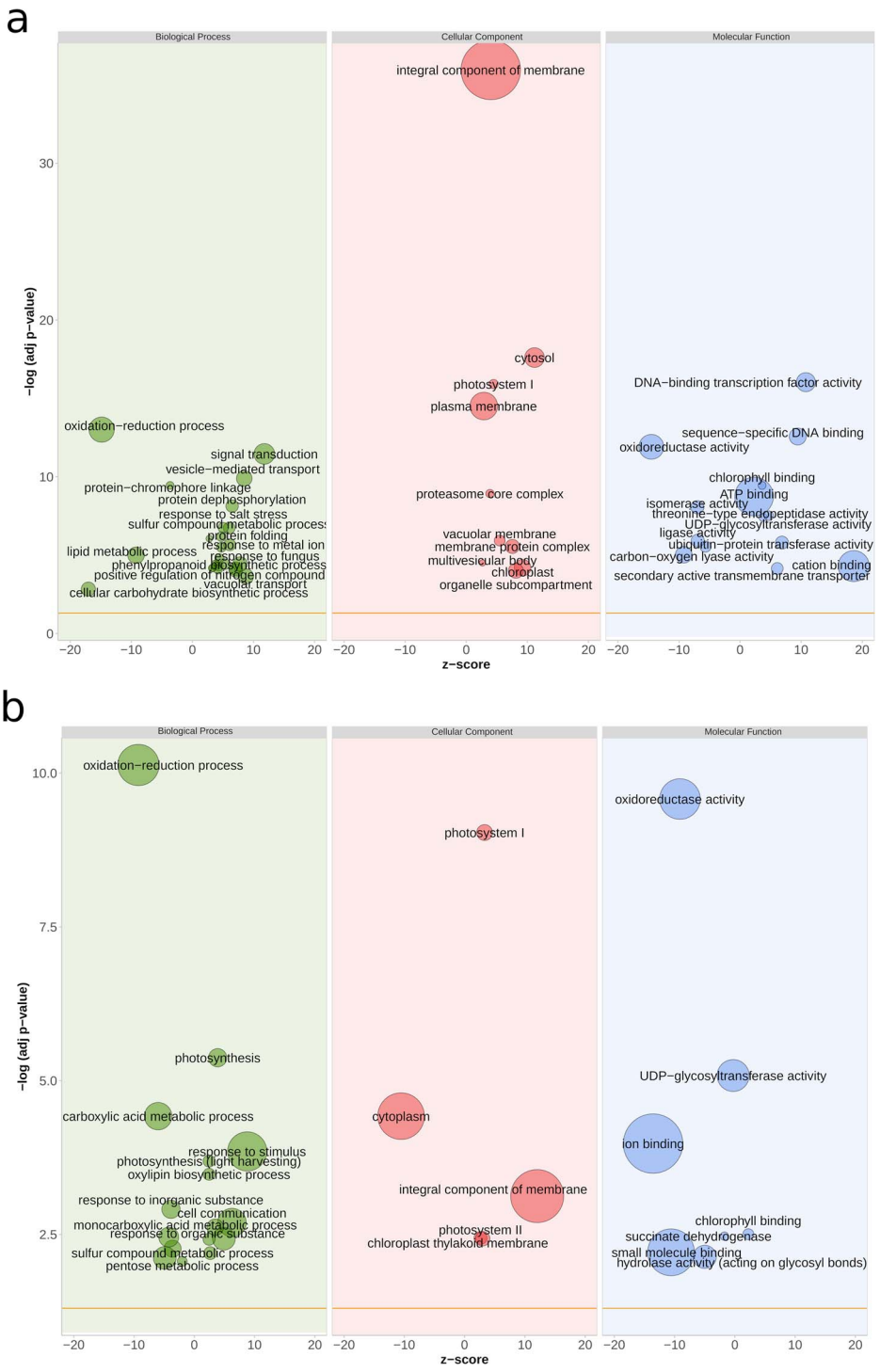

Figure 2. The GO-enriched terms in all detected DEGs. The reported GO annotations were classified based on molecular function, biological process and cellular component type. In (a), enriched terms in myc_wild vs wild and in (b) enriched terms in myc_TGL vs TGL. The *x*-axis shows the *z*-score, while the *y*-axis indicates the significance of the enriched term (−log10 adjusted *P*-value). Areas of plotted bubbles are proportional to the number of genes assigned to the related GO term.

a geraniol 8-hydroxylase (G8H)-like of *Juglans regia* (query coverage 93%, e-value 0.0, identity 71.27%). Similarly, a gene reported as uncharacterized in UNIREF50 (g19925.t1) showed homology with G8H-like of *Morella rubra* (query coverage 98%, e-value $1e^{-126}$, identity 62.12%) and resulted as downregulated mainly in myc_wild (Log2FC = −8.66).

Specific responses to T. melanosporum colonization in TGL vs wild plants

Looking at the 25 DEGs showing the highest fold change ($log_2FC > 8,0$), in myc_TGL, the most upregulated gene (g30703) codes for an uncharacterized protein with similarity with a flavonol synthase ($log_2FC = 15.97$). Blastp analysis

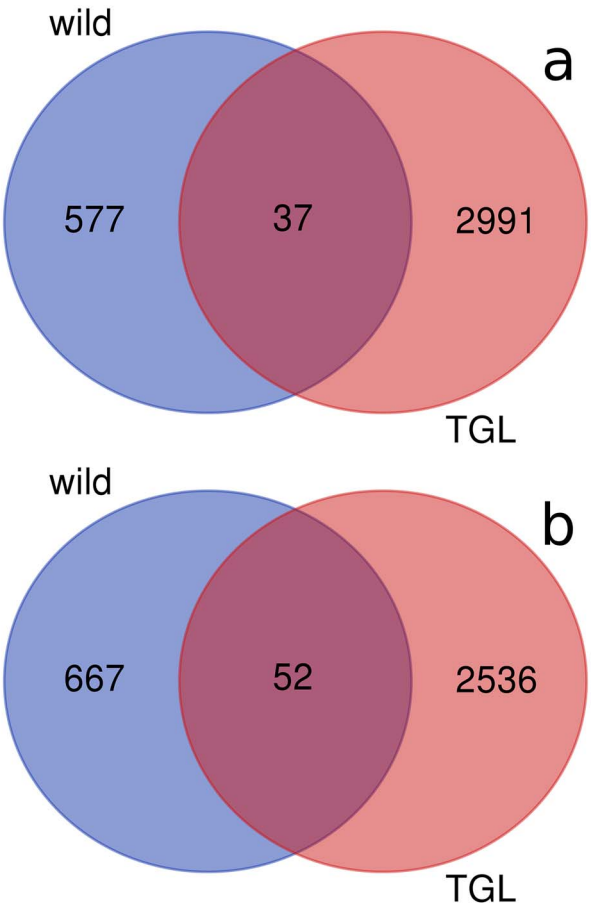

Figure 3. Venn diagrams related to differentially expressed genes (Padj ≤ 0.05). (a) Upregulated genes in myc_wild vs wild and TGL vs myc_TGL. (b) Downregulated genes in myc_wild vs wild and TGL vs myc_TGL.

showed a homology with a flavonol synthase/flavanone 3-hydroxylase-like of *Quercus lobata* Née (query coverage 93%, e-value $3e^{-159}$, identity 80.75%).

The list included genes coding for a putative protein belonging to the heavy metal transport/detoxification superfamily (g12880.t1), a putative hexosyl transferase (g15667.t1), a disease resistance protein (g840.t1), an anthocyanidin 3-*O*-glucosyltransferase (g30434.t1), an early light-induced protein (g30443.t1), two glutathione S-transferases (g35123.t1 and g19412.t1) and a putative *O*-methyltransferase (g27253.t1) belonging to a family of plant methyltransferases that contains enzymes that act on various substrates, including salicylic and jasmonic acid (see Table S5 available as Supplementary data at *Tree Physiology* Online).

Looking at the biosynthetic pathway of flavonoid (Shoeva et al. 2016), in addition to the putative flavonol synthase, it is worth note the upregulation of three genes coding for chalcone synthases (g35222.t1, g33581, 21,086; $\log_2$FC = 3.1, 2.8 and 2.3, respectively), a gene coding for a putative flavanone 3-dioxygenase (g23858.t1; $\log_2$FC = 2.5) and a gene coding for a putative chalcone isomerase (g13598.t1; $\log_2$FC = 2.3). An additional anthocyanidin 3-O-glucosyltransferase (g32605.t1) was also upregulated in myc_TGL. Additionally, three phenylalanine ammonia-lyases (i.e., PAL), involved in the general phenylpropanoid pathway, were also upregulated, as well as two caffeoyl-CoA O-methyltransferases (g8545.t1 and g8546.t1; $\log_2$FC = 2.5 and 1.2, respectively), both with a potential role in plant defense.

Although GO analysis showed carbohydrate process as more associated with downregulation, several genes involved in sugar metabolism were found to be significantly upregulated in myc_TGL, including a gene coding for a putative alkaline/neutral invertase (g23834.t1; $\log_2$FC = 1,1). Blastp analysis showed homology with a neutral/alkaline invertase 3 (chloroplastic-like) of *Quercus suber* (query coverage 60%, e-value 0.0, identity 88.82%).

Additionally, it is worth noting that among the genes with a $\text{Log}_2\text{FC} > 7.5$ there are also a gene coding for a putative disease resistance protein (g840.t1) and a gene (g12221.t1) putatively involved in abscisic acid metabolism, whose product shows homology with an EID1-like F-box protein 3 of *Vitis riparia* (query coverage 100%, e-value $4e^{-113}$, identity 74.32%).

In myc_wild, the 25 most upregulated DEGs ($\log_2\text{FC} > 7.5$) were represented by genes linked to terpene synthesis (g10000.t1), several genes putatively involved in nucleic acid binding, a methyltransferase gene (g16295.t1) and a gene (g6898.t1) coding for a major latex protein-like protein 43 (MLP43). These proteins have been reported to have a role as a positive regulator during abscisic acid responses mediating drought tolerance in *Arabidopsis thaliana* (L.) Heynh (Wang et al. 2016; see Table S7 available as Supplementary data at *Tree Physiology* Online). Particularly, the gene with the highest expression change ($\log_2$FC = 14.03) was an α-farnesene synthase-like, putatively involved in VOCs production. Conversely, the same gene resulted to be slightly downregulated in myc_TGL, confirming a specific genotype-related response (see Table S6 available as Supplementary data at *Tree Physiology* Online).

Interestingly, a gene coding for a putative phosphate transporter PHO1 (g2325.t1) was also slightly upregulated in myc_wild, as well as a gene coding for a putative caffeoyl-CoA O-methyltransferase (CCOMT, g21014.t1; $\log_2$FC = 3.2).

It is worth noting that genes coding for LOX, which are enzymes involved in the synthesis of jasmonic acid reported being involved in response to biotic and abiotic stresses, and genes involved in trehalose biosynthesis/metabolism were also found to be upregulated in both genotypes, although with a species-specific regulation (see Tables S5 and S7 available as Supplementary data at *Tree Physiology* Online).

Table 1. List of the DEGs shared by the two hazelnut genotypes and putatively involved in a systemic response to *T. melanosporum* root colonization.

ID	Log2 FC myc_wild	Log2 FC myc_TGL	Blastp (best identified match) [*species*]	Query coverage (%)	Identity (%)	*e*-value	Conserved domains (PFAM)
g14478.t1	9.43	0.54	Syntaxin-121 isoform X2 [*Q. lobata*]	79.00%	50.94%	$1e^{-71}$	PF00804: Syntaxin superfamily
g17613.t1	7.62	2.46	G-type lectin S-receptor-like serine/threonine-protein kinase At1g11330 [*J. regia*]	93.00%	69.19%	$3e^{-81}$	PF00954: S-locus glycoprotein domain
g27826.t1	3.18	9.24	Cysteine-rich receptor-like protein kinase 10 [*Q. lobata*]	98.00%	65.27%	0.0	PF01657: Domain involved in salt stress response and antifungal activity
g34419.t1	3.12	1.55	Uncharacterized protein LOC112030708 [*Q. suber*]	91.00%	29.81%	$5e^{-25}$	PF03108: uDR family transposase
g33469.t1	3.01	1.50	G-type lectin S-receptor-like serine/threonine-protein kinase At1g11330 [*J. regia*]	95.00%	50.29%	$2e^{-98}$	PF00954: S-locus glycoprotein domain
g29789.t1	2.74	3.06	Cytochrome P450 CYP82D47-like [*Q. lobata*]	98.00%	69.40%	0.0	PF00067: Cytochrome P450
g21171.t1	2.61	2.33	No significant similarity found	–	–	–	–
g30501.t1	2.56	1.44	G-type lectin S-receptor-like serine/threonine-protein kinase At1g11330 [*Q. suber*]	91.00%	50.22%	0.0	PF01453: D-mannose binding lectin PF00954: S-locus glycoprotein domain. PF11883: domain of unknown function (DUF3403)
g17472.t1	2.50	2.90	No significant similarity found	–	–	–	–
g30483.t1	2.28	2.04	G-type lectin S-receptor-like serine/threonine-protein kinase At1g11330 [*J. regia*]	69.00%	70.66%	$1e^{-74}$	PF01453: D-mannose binding lectin domain
g8531.t1	2.02	1.76	Galactinol synthase 2-like [*J. regia*]	100.00%	87.30%	$7e^{-174}$	PF01501: Glycosyltransferase family 8
g23256.t1	1.72	1.67	UDP-glycosyltransferase 90A1-like [*Q. lobata*]	98.00%	55.98%	$2e^{-168}$	PF00201: UDP-glucoronosyl and UDP-glucosyl transferase
g14650.t1	1.66	3.36	Multiprotein-bridging factor 1c [*J. regia*]	100.00%	86.81%	$2e^{-86}$	PF08523: Multiprotein bridging factor 1
g24081.t1	1.46	4.90	No significant similarity found	–	–	–	–
g30513.t1	1.39	1.30	Probable isoaspartyl peptidase/L-asparaginase 2 [*J. regia*]	100.00%	92.64%	0.0	PF01112: Asparaginase_2
g12226.t1	1.23	0.75	Phototropin-1 isoform X1 [*J. regia*]	100.00%	75.58%	0.0	PF00069: Protein kinase domain PF13426: PAS domain
g26274.t1	1.19	0.75	Cellulose synthase-like protein H1 [*Q. suber*]	93.00%	66.30%	$2e^{-65}$	PF03552: Cellulose synthase
g26614.t1	1.14	2.21	G-type lectin S-receptor-like serine/threonine-protein kinase At4g27290 isoform X2 [*J. regia*]	64.00%	68.46%	$4e^{-58}$	PF01453: D-mannose binding lectin domain PF00954: S-locus glycoprotein domain
g21819.t1	1.13	3.50	F-box protein SKIP27-like [*J. regia*]	99.00%	58.26%	$6e^{-72}$	PF17253: Family of unknown function (DUF5320)
g15220.t1	1.11	0.94	Uncharacterized protein LOC109018321 [*J. regia*]	98.00%	66.67%	$2e^{-96}$	–
g20220.t1	1.09	1.66	Uncharacterized protein LOC110763764 [*Prunus avium*]	95.00%	46.81%	$4e^{-166}$	PF10358: N-terminal C2 in EEIG1 and EHBP1 proteins
g16566.t1	1.09	1.33	Protein ALUMINUM SENSITIVE 3 [*J. regia*]	85.00%	88.81%	$4e^{-176}$	PF03649: Uncharacterized protein family (UPF0014)
g30941.t1	1.09	1.41	L-type lectin-domain containing receptor kinase VII.1-like [*Q. suber*]	100.00%	83.20%	$9e^{-137}$	PF00139: Legume lectin domain PF00069: Protein kinase domain
g26422.t1	0.97	2.32	Myosin-11-like [*J. regia*]	100.00%	78.86%	$6e^{-133}$	PF07889: Protein of unknown function (DUF1664)
g9582.t1	0.94	1.80	Calcium-binding protein PBP1-like [*Q. suber*]	98%	93.04%	$2e^{-70}$	PF13833: EF-hand domain pair with calcium-binding motif
g1792.t1	0.94	1.13	Calcium-transporting ATPase 9, plasma membrane-type-like isoform X2 [*J. regia*]	91.00%	85.71%	$3e^{-104}$	PF12515: Ca2 + -ATPase N terminal autoinhibitory domain
g9329.t1	0.94	0.63	Uncharacterized protein LOC111302697 [*Durio zibethinus*]	81.00%	50.09%	$3E^{-40}$	PF15377: Domain of unknown function (DUF4604)
g9450.t1	0.92	0.72	Uncharacterized protein LOC115975329 [*Q. lobata*]	100.00%	71.75%	0.0	PF11331: Probable zinc-ribbon domain
g15600.t1	0.87	0.79	DEAD-box ATP-dependent RNA helicase 36 [*J. regia*]	98.00%	81.35%	0.0	PF00270: DEAD/DEAH box helicase
g26116.t1	0.87	1.05	Peroxidase 17-like [*J. regia*]	100.00%	77.06%	0.0	PF00141: Peroxidase
g12784.t1	0.85	1.47	Protein DETOXIFICATION 54 [*J. regia*]	100.00%	80.80%	0.0	PF01554: Multi antimicrobial extrusion protein
g11179.t1	0.79	0.74	Two-component response regulator-like APRR9 isoform X1 [*Q. suber*]	95.00%	74.65%	0.0	PF00072: Response regulator receiver domain PF06203: CCT motif
g27676.t1	0.77	1.74	Myosin-binding protein 1-like [*J. regia*]	98.00%	63.53%	0.0	PF04576: Zein-binding
g26006.t1	0.70	1.95	Uncharacterized protein LOC108995872 isoform X2 [*J. regia*]	99.00%	88.35%	$5e^{-175}$	PF03097: BRO1-like domain
g12244.t1	0.70	2.23	dnaJ homolog subfamily B member 4-like [*J. regia*]	100.00%	89.16%	$8e^{-108}$	PF00226: Chaperone DnaJ (Hsp40)
g13906.t1	0.69	0.60	bZIP transcription factor 12-like [*J. regia*]	98.00%	57.59%	$7e^{-70}$	PF00170: bZIP transcription factor
g21601.t1	0.66	1.02	Scarecrow-like protein 21 isoform X1 [*Q. lobata*]	100.00%	82.59%	0.0	PF03514: GRAS domain family

Content of sugars and polyphenols

Considering the regulation of genes involved in sugar metabolism mainly in TGL genotype, levels of soluble sugars were measured by HPLC-RID. The most abundant soluble sugar in leaves was sucrose (ranging from 31 to 53 mg g^{-1}), which was not significantly affected by the ECM fungus in both genotypes ($P \geq 0.05$). Conversely, glucose and fructose were significantly higher in leaves of wild compared with myc_wild

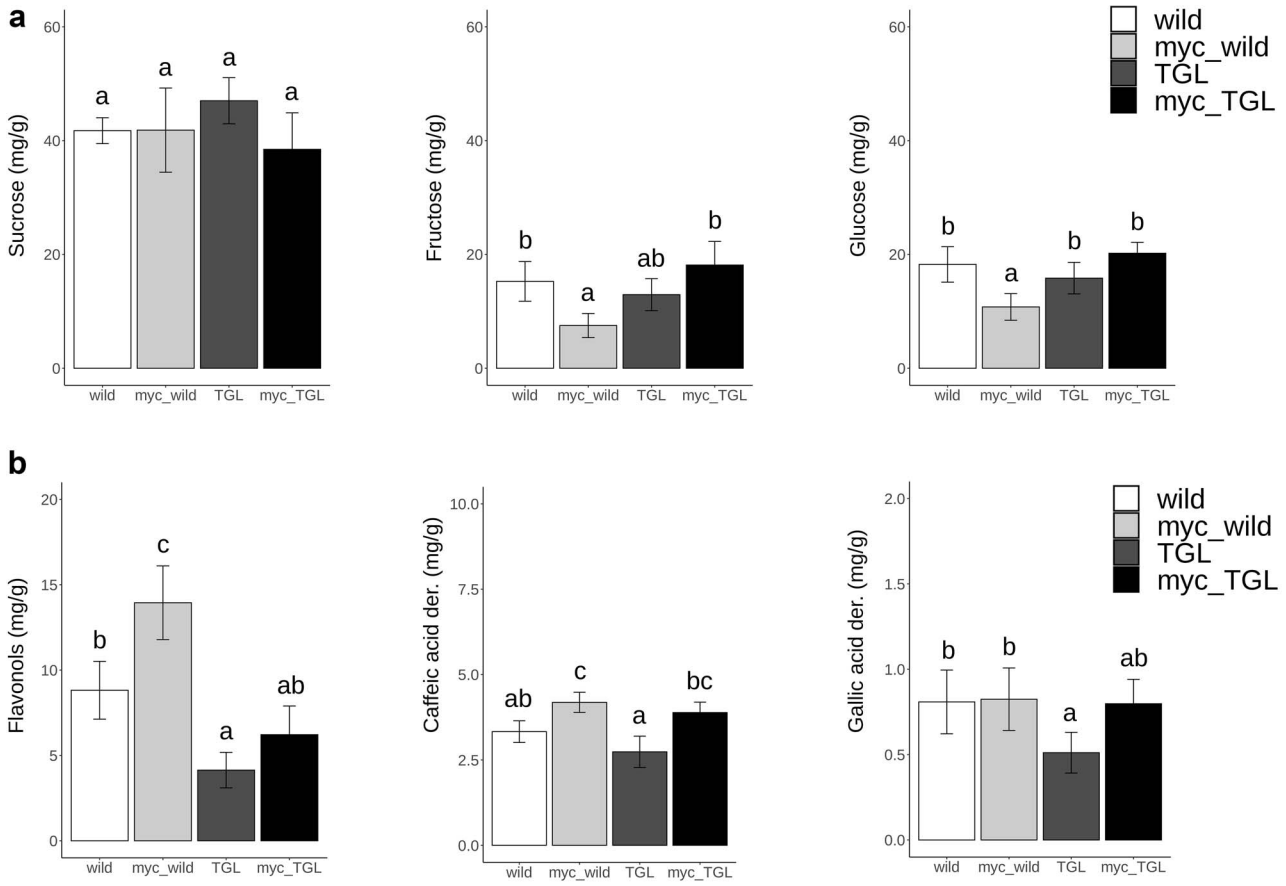

Figure 4. Quantification results related to HPLC-DAD analysis on sugars and polyphenols. (a) Fructose and sucrose levels are reported according to chromatographic data. (b) Determination of flavonols (as sum of myricetin-3-*O*-hexoside, myricetin-3-*O*-rhamnoside, quercetin-3-*O*-hexoside and quercetin-3-*O*-rhamnoside), caffeic acid derivates and gallic acid derivates is reported. Letters are plotted according to outcomes to Tukey HSD test.

($P \leq 0.05$). No significant differences were observed between TGL and myc_TGL leaves (Figure 4; see Table S11 available as Supplementary data at *Tree Physiology* Online).

Looking at the polyphenols, leaves of wild contained higher quantity of flavonols, i.e., myricetin-3-*O*-hexoside, myricetin-3-*O*-rhamnoside, quercetin-3-*O*-hexoside and quercetin-3-*O*-rhamnoside, compared with TGL (Figure 4; see Table S12 available as Supplementary data at *Tree Physiology* Online). Moreover, leaves of myc_wild showed a significant increase in the amount of flavonols compared with the wild, while myc_TGL showed a slight increase with respect to not-inoculated plants, although not significant ($P \geq 0.05$). The content of caffeic acid derivatives was similar for both wild and TGL, and a significant increase was found in myc_wild and myc_TGL, compared with leaves from not-inoculated plants (Figure 4; see Table S13 available as Supplementary data at *Tree Physiology* Online). Gallic acid derivatives showed a higher content in the wild with respect to TGL, but no significant changes were found in myc_wild and myc_TGL (Figure 4; see Table S14 available as Supplementary data at *Tree Physiology* Online). According to two-way ANOVA, both mycorrhization and genotypes were factors of variance ($P \leq 0.05$), for glucose, fructose and flavonols (see Table S15 available as Supplementary data at *Tree Physiology* Online).

The PCA analysis (Figure 5a) showed that the first principal component (PC1) clearly separated TGL and myc_wild, which formed two distinctive groups. Furthermore, the second axis (PC2) allowed separating the last two groups of samples, myc_TGL and wild, with the only exception represented by the sample myc_TGL_4. The correlation circle (Figure 5b) represents the vectors of all quantitative variables employed for PCA computation, showing a projection of the variables in the space. Results showed that the variables that mostly contribute to PC1 are glucose, fructose, flavonols and caffeic acid derivates, whereas gallic acid derivates contribute to PC2. Furthermore, the polyphenol group of variables is positively correlated to PC1, whereas the sugars variables are negatively correlated. Thus, polyphenols are inversely correlated with sucrose, whereas no relationship was detected between sucrose and glucose variables.

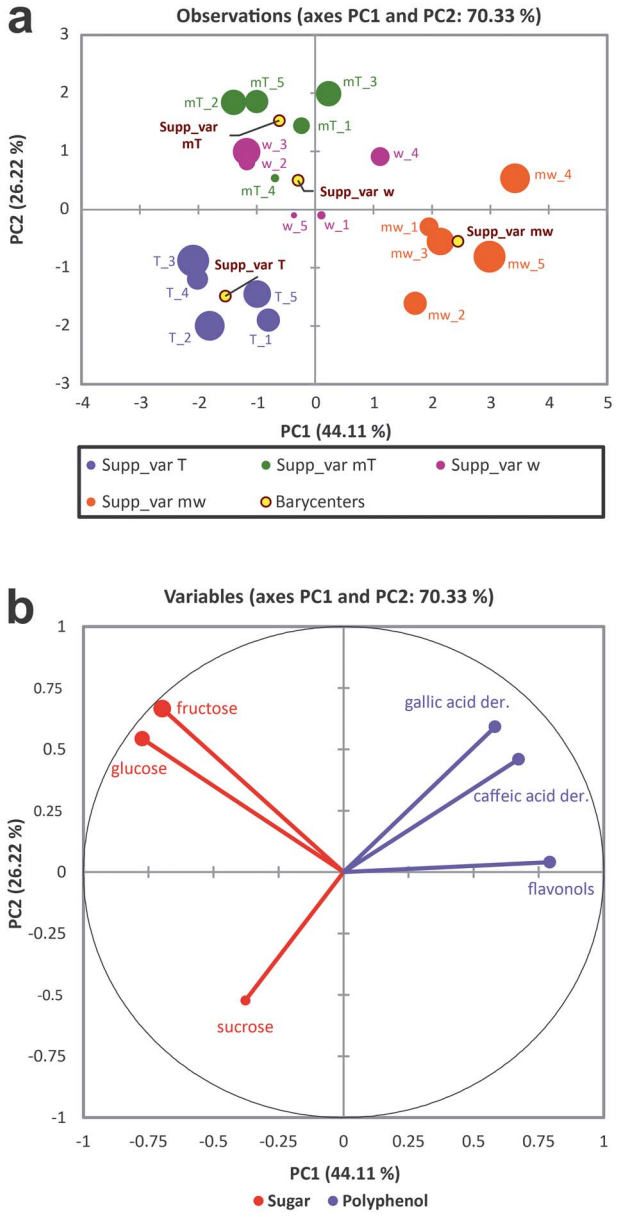

Figure 5. (a) Principal components analysis results of HPLC-DAD data (sugars and polyphenols) on samples of *C. avellana*, inoculated with the ECM fungus *T. melanosporum* and not inoculated. Dim1 = first dimension (PC1), Dim2 = second dimension (PC2). Dot size of samples is referring to squared cosines values, which are a measure of the quality representation of the observation in the map. (b) Vector representation of the variables in the factors space to the distinction of samples. The angle between two vectors or a vector and an axis represents the degree of correlation between them; adjacent, angle less than 90° = highly correlated, orthogonal (90°) = uncorrelated, and opposite (180°) = negatively correlated) (Buehler et al. 2011).

RT-qPCR on T. melanosporum genes in ECM from TGL and wild plants

To verify the functionality in the ECMs from the two genotypes, two genes previously showed to be differentially regulated in the two fungal compartments forming an ECM (the mantle and the Hartig net) were selected to be tested in RT-qPCR reactions. In particular, a gene coding for a putative ammonium transporter (AMT) channel (GSTUMT00005025001) and a gene coding for a putative lactose permease (candidate cellodextrin transporter; GSTUMT00005678001) were selected on the base of their upregulation in the mantle and in the Hartig net, respectively, in *C. avellana* ECMs (Hacquard et al. 2013). Our results, although limited to the expression of two genes, did not show any significant difference between the ECMs collected from the two genotypes (see Table S16 and Figure S2 available as Supplementary data at *Tree Physiology* Online). Although limited to two biological replicates, a consistent expression trend has been observed, thus suggesting that ECM fungus was functionally active in both genotypes.

Discussion

The main objective of this work was to evaluate the impact of colonization with *T. melanosporum* on the systemic response in hazelnut, considering two different genotypes, i.e., one commonly used for the truffle cultivation (wild) and the other one considered as among the most prized genotypes for nuts production (TGL). As reported by Campo and San Segundo (2020), reprogramming of host gene expression in leaves is potentially important to maintain a stable symbiotic relationship. However, to date, transcriptomics studies on mycorrhizal plants have been focused on changes in transcript levels in roots, more than on the modifications at a systemic level. In this work, comparison between leaf transcriptomes of the two genotypes by RNAseq allowed to identify DEGs associated with the differences in the response to mycorrhizal colonization at the systemic level. In colonized plants, leaf large-scale transcriptomics revealed a significant expression repatterning of both hazelnut genotypes after fungal colonization, with a higher number of DEGs in TGL compared with wild (5616 vs 1333 out of 25,588 *C. avellana* predicted gene models). The two genotypes shared a small set of DEGs (37) putatively involved in the systemic response to *T. melanosporum* root colonization. Genes related to development, signaling and response to stress and environmental stimuli were found to be activated in leaves of both genotypes by ECM fungi, as highlighted by Fisher enrichment tests.

Tuber melanosporum influences the expression of genes involved in plant immunity, response to abiotic stresses and light perception

Among the 37 genes activated in colonized plants, independently from the genotypes, eight genes putatively coding for receptor-like kinases (RLKs) were found. These proteins, characterized by diverse extracellular domains involved in signal perception, recognize extracellular (and internal) stimuli and activate the downstream signaling pathways, having an important role in signal transduction and leading to the expression of

specific genes (Burdiak et al. 2015). Interestingly, several plant RLKs have a role as pattern recognition receptors and play a role at the cell surface during pathogen-associated molecular patterns (PAMP) triggered immunity (He and Wu 2016, Sun et al. 2020).

Here, the list of upregulated RLK proteins included seven putative G-type lectin receptor-like kinases (LecLRKs) and a cysteine-rich receptor-like protein kinase (CRK). In plants, LecRLKs typically have a lectin domain in the N-terminal extracellular domain, a transmembrane domain and an intracellular kinase domain, and play crucial roles in sensing outside signals and abiotic/biotic stress responses (Sun et al. 2020). A role for LecRLKs in the plant–microbe symbiotic relationship has documented, and a G-type LecRLK has been reported to mediate the symbiotic interaction between *Populus* species and the ECM fungus *Laccaria bicolor* (Maire) P.D. Orton (Labbé et al. 2019). Our results suggested a regulation of these proteins at a systemic level, in particular supporting the hypothesis that the ECM fungus *T. melanosporum* might play a role in inducing PAMP-triggered immunity. In addition, the upregulation of a CRK that showed homology with a CRK10 of *Q. lobata* (% identity 65.27, accession number XP_030930902.1), previously reported to be induced by salicylic acid (SA) which is a key factor in the activation of SAR, suggested a role in SA-mediated transduction pathways. Altogether, our results suggest that *T. melanosporum* led to a constitutive activation of genes involved in plant immunity, as already suggested for the AM symbiosis (Cervantes-Gámez et al. 2016) where the upregulation of several RLKs in leaves from mycorrhizal plants was found. Among the genes commonly upregulated, also a syntaxin-121 (SYP121) was detected. SNARE syntaxin proteins are involved in vesicle trafficking pathway, driving vesicle fusion (Huisman et al. 2020). These proteins have been demonstrated to be implicated in mediating defense-related secretion in plants, as well as during arbuscule formation in *M. truncatula* mycorrhizal roots (Huisman et al. 2016). Particularly, SYP121 was reported as involved in resistance to the fungal pathogen *Blumeria graminis* (DC.) Speer f. sp. *hordei* in *A. thaliana* (Zhang et al. 2007).

A role of AM symbiosis in influencing hormonal balance in leaves of mycorrhizal plants has been reported, including a regulation of abscisic acid (ABA; Ouledali et al. 2019). Looking at our list of DEGs, some genes correlated to ABA were found to be significantly regulated. This is the case of two contigs (g12221.t1 and g14650.t1), showing homology with an EID1-like F-box protein and a multiprotein-bridging factor 1c (*J. regia* L., query coverage 100%, e-value $1e^{-84}$, identity 86.81%), respectively, upregulated only in myc_TGL, and a gene coding for a putative multiprotein-bridging factor 1c (MBF1), upregulated in both myc_TGL and myc_wild. Notably, these proteins have been reported to be involved in the response to biotic and abiotic stresses and a *Vitis vinifera* L. MBF1 (VvMBF1) increased its expression in leaf tissues in response to ABA and dehydration stress (Yan et al. 2014).

Several transcription factors (TFs) have been reported to be regulated during symbiosis both at local (roots) and systemic (leaves) levels (Fiorilli et al. 2009, Liu et al. 2007). Thirty-four transcription factor (TF)-encoding genes were modulated in tomato shoots from AM-colonized plants. Particularly, two zinc finger TFs were upregulated in the shoots, while six were downregulated (Fiorilli et al. 2009). In our system, several TFs have been found to be regulated. Particularly, a gene coding for a basic region/leucine zipper motif (bZIP) TF was found to be significantly upregulated in both genotypes. This large TF family has been reported to play an important role in plant growth, development and abiotic and biotic stress responses (Amorim et al. 2017, Gai et al. 2020, Hossain et al. 2010, Jakoby et al. 2002, Singh et al. 2002, Sornaraj et al. 2016). These results agree with those reported by Cervantes-Gámez et al. (2016) in tomato leaf transcriptomics during AM colonization, such as the modification in the expression of genes involved in transcription regulation and defense responses. Although further analyses should be done to verify the impact of the symbiotic fungus on hazelnut pest and disease resistance and abiotic stress tolerance, our results suggest that the inoculation with *T. melanosporum* might lead to a potential increase in defense, supporting the idea that *Tuber* species could provide an additional value to the hazelnut plants not only for the production of the precious ascomata but also as symbiotic fungi able to stimulate plant immunity.

Plant GRAS proteins also have important functions in different processes such as signal transduction and development (Bolle 2004). Among this group of plant proteins, a hazelnut gene coding for a protein that showed homology with a scarecrow-like 21 has been found to be significantly upregulated (log_2FC = 0.66 in myc_wild and 1.02 in myc_TGL). SCARECROW-LIKE21 (SCL21), together with the PHYTOCHROME A SIGNAL TRANSDUCTION1 (PAT1), have been reported to be positive regulators of phytochrome A signal transduction for diverse high-irradiance responses in *Arabidopsis* (Torres-Galea et al. 2013). In agreement with the effect of the fungal symbiont on light perception, a gene coding for a putative phototropin resulted also to be upregulated in both the genotypes. Phototropins, which are light-activated serine/threonine protein kinases, respond to blue-light playing a role in controlling several responses that can lead to an optimization of plant photosynthetic efficiency, including phototropism, light-induced stomatal opening, chloroplast movements and leaf expansion due to changes in light intensity (Christie 2007). Interestingly, phototropins can interact with circadian clock genes and proteins (Matsoukas 2017) and, upon low blue light, they can increase light-use efficiency and promote photosynthesis (Takemiya et al. 2005, Christie 2007, Boccalandro et al. 2012, from Kong and Zheng 2020). It is also

worth noting that a gene coding a putative early light-induced protein was present among the 25 genes more activated in TGL_myc in addition to two genes coding for glutathione S-transferase, reported having a role in response to biotic stresses (Gullner et al. 2018). Interestingly, early light-inducible proteins (ELIPs), which are in thylakoid membranes, are reported to play a role in protecting photosynthetic machinery from various environmental stresses in higher plants.

Impact of T. melanosporum on genes involved in carbohydrate and nitrogen metabolism

Increase in photosynthetic production might be related to an increase in the photosynthetic activity per leaf area as well as to an increase in the surface for sunlight capture, as already demonstrated in *M. truncatula* AM-colonized plants (Adolfsson et al. 2015). Although morphometric and ecophysiological data have not been collected, the upregulation of genes that might be involved in this process (e.g., a cellulose synthase and a phototropin) opens this hypothesis also for the hazelnut plants inoculated with *T. melanosporum*. Sugar levels in leaves have been verified in our system, showing that total sugar content did not change among the several theses. Although not always statistically supported, it is worth noting that glucose and fructose showed an increasing trend in TGL_myc with respect to the not inoculated plants, while in the wild_myc a significant decreasing was found. An important role of diverse sugars such as glucose, sucrose and trehalose in regulating plant defense-related metabolic pathways is always more evident. Interestingly, a role for glucose to induce genes coding enzymes involved in the production of defense-related secondary metabolites, such as chalcone synthase and phenylalanine ammonia-lyase (PAL), has been already shown (Xiao et al. 2000). As described above, genes coding for chalcone synthases and PAL were differentially regulated in the presence of the symbiotic fungus at least in TGL plants. Furthermore, a role for sucrose to promote host defense response by enhancing the expression of anthocyanin biosynthesis genes and stimulating the accumulation of isoflavonoids has been also reported (Morkunas et al. 2005, Solfanelli et al. 2006).

Sucrose and its hydrolytic hexoses, such as glucose and fructose, were reported to promote osmotic adjustments and to act as signaling molecules in response to stressed conditions, thus playing a positive role in abiotic stress adjustments and influencing plant growth and development (Dahro et al. 2016). It is worth noting the upregulation of a gene coding for a putative A/N invertase in myc_TGL. Invertases, which are present in sink tissue, act in hydrolyzing sucrose into glucose and fructose (reviewed in Tauzin and Giardina 2014). Additionally, a sucrose synthase gene was found to be upregulated in myc_TGL and a putative galactinol synthase gene was regulated in both genotypes. These enzymes have been reported to play important roles in desiccation tolerance acquisition during *Arabidopsis* seed development, and galactinol is a crucial protective compound in the response to desiccation stress and can improve the ability of the fruits to cope with the oxidative processes (Taji et al. 2002). Interestingly, genes related to trehalose metabolism were also upregulated in both genotypes. The synthesis of trehalose, a non-reducing disaccharide composed of two glucose moieties, is a two-step process in plants involving the production of trehalose-6-phosphate catalyzed by trehalose-6-phosphate synthase (TPS) and its consecutive dephosphorylation to trehalose, catalyzed by trehalose-6-phosphate phosphatase (TPP). T6P has been reported as an important signaling metabolite, regulating carbon assimilation and sugar status in plants, and it has been also demonstrated to have an essential role in plant development (Ponnu et al. 2011). In detail, the inoculation with the ECM fungus led to the upregulation of a TPP gene and TPS in myc_TGL, while in myc_wild genes coding for two TPSs resulted to be activated. Despite the upregulation of some genes involved in sugar metabolism, it is worth noting that carbohydrate process was more associated with downregulation in GO analysis. Overall, results are in agreement with the weak reprogramming of sugar metabolism observed in leaves in the presence of the symbiotic fungus.

A gene coding for a putative asparaginase was also found among the upregulated genes. Asparagine is a crucial N transport and storage compound in plants (García-Calderón et al. 2017). These enzymes are mainly found in developing leaves and seeds where the enzyme catalyzes the conversion of asparagine into aspartate and ammonia, which is then reassimilated by glutamine synthetase (Cánovas et al. 2007, Lea et al. 2007). Plant phloem transports N compounds from source to sink organs (Raven and Smith 1976), and these enzymes have been mainly reported to modulate asparagine metabolism in both the sink and source organs (Yabuki et al. 2017). However, the influence of *T. melanosporum* inoculation on the C:N ratio, which is an indicator of the nitrogen-use efficiency, is a point that still needs to be investigated.

Tuber melanosporum impacts secondary metabolites production and expression of cell wall genes

Ectomycorrhizal symbiosis led to a significant increase in leaf content of flavonols, although only in the wild, whereas the ECM fungus does not affect the content of other polyphenols such as gallic acid derivatives in any of the studied hazelnut types. The observed profiles in not-inoculated plants are like those already reported by HPLC/DAD and HPLC/DAD/MS/MS—ESI (Amaral et al. 2005). These authors analyzed 10 hazelnut cultivars, including the TGL, showing that all the analyzed samples showed a similar phenolic profile, with myricetin 3-*O*-rhamnoside and quercetin 3-*O*-rhamnoside as the major compounds. In poplar, the ECM *L. bicolor*

induced a systemic transcriptional repatterning that involves the regulation of phytohormones and flavonoid metabolism (Goossens et al. 2016). Changes in plant secondary metabolites' production at a systemic level has been already observed in AM symbiosis (Avio et al. 2018), e.g., mycorrhizal colonization triggered the increase of total phenolic content in artichoke leaves (Ceccarelli et al. 2010), suggesting a role of symbiosis in increasing plant tolerance to environmental stresses and improving product quality. Four compounds with significant antioxidant properties, such as ferulic acid, asiatic acid, carnosol and vanillin, were related to mycorrhizal rosemary plants, while caffeic and chlorogenic acids were more influenced in non-mycorrhizal plants (Seró et al. 2019). Our results, showing a significant increase in caffeic acid derivates in inoculated plants, independently from the type (wild and TGL), confirm the impact of the fungal inoculation on defense processes, considering that these compounds have been reported to have a role in plant biotic and abiotic stress response (Martin 2011, Riaz et al. 2019). It is worth noting that a contig corresponding to a Caffeoyl-CoA O-methyltransferase (CCOMT, g21014.t1) was upregulated in myc_wild and two contigs coding for the same enzymes (g8545.t1 and g8546.t1), which have a role in phenylpropanoid pathway and lignin production, were upregulated in myc_TGL. These enzymes have been reported to have a role in lignin biosynthesis and a homolog maize gene has been reported to be involved in resistance to pathogens (Yang et al. 2017). Considering genes involved in cell wall modification, pectinesterase genes resulted to be downregulated in both the hazelnut types, suggesting an effect on the cell wall firmness. The upregulation of pectinesterases, which have a role in pectin demethylation, has been associated with a lower pericarp firmness in tomato fruit (Cruz-Mendívil et al. 2015). Interestingly, genes encoding for these enzymes resulted to be downregulated in tomato fruit from mycorrhizal plants, suggesting that the mechanisms that lead to softening are delayed (Zouari et al. 2014).

Additionally, in our study, the most upregulated gene in myc_TGL was a putative flavonol synthase, which is the key enzyme of the biosynthesis of flavonols that can be regulated by developmental and environmental conditions (Sun et al. 2019). Particularly, flavonols are the most abundant and widely distributed flavonoids in nature and have a range of biological activity, including an antioxidative effect. Looking at the biosynthetic pathway, a gene coding for a putative anthocyanidin 3-*O*-glucosyltransferase, involved in the qualitative modification of phenylpropanoids (Jaakola 2013), also appeared to be upregulated, suggesting a change in this compound in the presence of the ECM fungus. Conversely, the most upregulated gene in myc_wild was a putative α-farnesene synthase-like. Previous studies allowed inference of a link between synthesis of α-farnesene and ethylene production, because of regulation of the expression of α-farnesene synthase 1 mediated by this hormone (Karagiannis et al. 2018). Interestingly, α-farnesene belongs to volatile sesquiterpenes, with a possible role in plant defense (Köllner et al. 2017). Interestingly, root colonization of *in vitro*-grown grapevines by the bacteria *Bacillus licheniformis* (Weigmann) Chester Rt4M10 and *Pseudomonas fluorescens* (Flügge) Migula Rt6M10 increases the content of volatile diterpenes, monoterpenes and sesquiterpenes, including α-farnesene in leaves (*cv*. Malbec) (Salomon et al. 2014, 2017). Additionally, α-farnesene was suggested to be a putative biomarker of the grapevine response to the sulfated laminarin (PS3)-induced resistance (Chalal et al. 2015). Conversely, two putative G8H genes resulted to be downregulated in both the genotypes, although with relevant expression values only in myc_wild, supporting the differences observed in the two genotypes. Geraniol is the precursor for the secoiridoid pathway, and it is hydroxylated to the 8-hydroxygeraniol by the G8H, which is a membrane-bound cytochrome-P450 monooxygenase (CYP) belonging to the CYP76 family (Sintupachee et al. 2015). Considering that several cytochrome-P450 genes were instead upregulated in our dataset, mainly in wild inoculated plants, the role of these enzymes is worth further investigation.

Overall, the data obtained will provide novel information about the impact of an ECM fungus on hazelnut metabolism at a systemic level that could also be useful for developing hazelnut cultivation associated with truffle production. Although further analyses are needed to verify defense/tolerance traits in the presence of the symbiotic fungus, results highlight for the first time that truffle might be considered as adding value not only as a precious fruiting body, but also for its potential role to support host plant in stressed conditions, leading to the systemic regulation of several genes involved in signaling and defense responses. Moreover, both transcriptomic and biochemical analyses allowed the differentiation of the samples on mycorrhizal treatment and genotype, emphasizing the differences between two hazelnut genotypes. Particularly, wild genotype responds less to inoculation with the ECM fungus, suggesting a probable intrinsic higher genetic tolerance to environmental stresses, i.e., biotic and abiotic ones, with respect to the TGL. Although further research is needed to find a correlation, it is also worth noting that wild genotype is the most widespread in truffle orchards, while TGL cultivar is characterized by a very specific distribution area.

Authors' contributions

A.M. and R.B. designed the research; F.S., C.B. and L.B.S.N. performed the wet experiments; F.S. and F.M. performed the RNA-seq data analysis; F.S. and F.V. performed the statistical analyses; A.V. and A.M. performed the root colonization evaluation; F.S. and R.B. organized and drafted the paper, with contributions from F.M., F.V. and A.M.; all authors contributed to the discussion of the data and approved the final text.

Supplementary data

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

Acknowledgments

The authors thank Severino Bozzano for his help during RNA-seq analysis, Walter Chitarra for the critical reading of the manuscript.

Data and materials availability

The BAM files from RNA-seq were deposited at the EMBL database ENA-European Nucleotide Archive as a project under the accession PRJEB46391 (secondary accession ERP130574; http://www.ebi.ac.uk/ena/data/view/PRJEB46391). All other data generated and analyzed during this study are included in this published article and its supplementary information files.

Conflict of interest

The authors declare no conflict of interest.

References

Adolfsson L, Solymosi K, Andersson MX, Keresztes Á, Uddling J, Schoefs B, Spetea C (2015) Mycorrhiza symbiosis increases the surface for sunlight capture in *Medicago truncatula* for better photosynthetic production. PLoS One 10:e0115314.

Adolfsson L, Nziengui H, Abreu IN et al. (2017) Enhanced secondary- and hormone metabolism in leaves of arbuscular mycorrhizal *Medicago truncatula*. Plant Physiol 175:392–411.

Agerer R, Danielson RM, Egli S, Ingleby K, Luoma D, Tre R (1996-2001) Descriptions of ectomycorrhizae. Einhorn-Verlag, Germany, pp 1–7.

Alasalvar C, Shahidi F (eds) (2008) Tree nuts composition. CRC Press, Phytochemical and Health Effects, Boca Raton, FL.

Aloui A, Recorbet G, Robert F, Schoefs B, Bertrand M, Henry C, Gianinazzi-Pearson V, Dumas-Gaudot E, Aschi-Smiti S (2011) Arbuscular mycorrhizal symbiosis elicits shoot proteome changes that are modified during cadmium stress alleviation in *Medicago truncatula*. BMC Plant Biol 11:1–17.

Amaral JS, Ferreres F, Andrade PB, Valentão P, Pinheiro C, Santos A, Seabra R (2005) Phenolic profile of hazelnut (*Corylus avellana* L) leaves cultivars grown in Portugal. Nat Prod Res 19:157–163.

Amicucci A, Zambonelli A, Guidi C, Stocchi V (2001) Morphological and molecular characterisation of *Pulvinula constellatio* ectomycorrhizae. FEMS Microbiol Lett 194:121–125.

Amorim LLB, da Fonseca Dos Santos R, JPB N, Guida-Santos M, Crovella S, Benko-Iseppon AM (2017) Transcription factors involved in plant resistance to pathogens. Curr Protein Pept Sci 18:335–351.

Anders S, Pyl PT, Huber W (2015) HTSeq—a python framework to work with high-throughput sequencing data. Bioinformatics 31:166–169.

Avio L, Turrini A, Giovannetti M, Sbrana C (2018) Designing the ideotype mycorrhizal symbionts for the production of healthy food. Front Plant Sci 9:1089.

Balestrini R, Mello A (2015) Truffle research in the post-genomics era. Food Anal Methods 8:1815–1823.

Balestrini R, Sillo F, Kohler A, Schneider G, Faccio A, Tisserant E, Martin F, Bonfante P (2012) Genome-wide analysis of cell wall-related genes in *Tuber melanosporum*. Curr Genet 58:165–177.

Baysal T, Demirdöven A (2007) Lipoxygenase in fruits and vegetables: a review. Enzyme Microb Technol 40:491–496.

Bazghaleh N, Hamel C, Gan Y, Taran B, Knight JD (2018) Genotypic variation in the response of chickpea to arbuscular mycorrhizal fungi and non-mycorrhizal fungal endophytes. Can J Microbiol 64:265–275.

Boccalandro HE, Giordano CV, Ploschuk EL, Piccoli PN, Bottini R, Casal JJ (2012) Phototropins but not cryptochromes mediate the blue light-specific promotion of stomatal conductance, while both enhance photosynthesis and transpiration under full sunlight. Plant Physiol 158:1475–1484.

Bolle C (2004) The role of GRAS proteins in plant signal transduction and development. Planta 218:683–692.

Buehler DM, Versteegh MA, Matson KD, Tieleman BI (2011) One problem, many solutions: simple statistical approaches help unravel the complexity of the immune system in an ecological context. PLoS One 6:e18592.

Burdiak P, Rusaczonek A, Witoń D, Głów D, Karpiński S (2015) Cysteine-rich receptor-like kinase CRK5 as a regulator of growth, development, and ultraviolet radiation responses in *Arabidopsis thaliana*. J Exp Bot 66:3325–3337.

Cameron DD, Neal AL, van Wees SC, Ton J (2013) Mycorrhiza-induced resistance: more than the sum of its parts? Trends Plant Sci 18:539–545.

Campo S, San Segundo B (2020) Systemic induction of phosphatidylinositol-based signaling in leaves of arbuscular mycorrhizal rice plants. Sci Rep 10:1–17.

Campos-Soriano L, García Martínez J, Segundo BS (2012) The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defence-related genes in rice leaves and confers resistance to pathogen infection. Mol Plant Pathol 13:579–592.

Cánovas FM, Avila C, Canton FR, Canas RA, de la Torre F (2007) Ammonium assimilation and amino acid metabolism in conifers. J Exp Bot 58:2307–2318.

Ceccarelli N, Curadi M, Picciarelli P et al. (2010) Globe artichoke as a functional food. Mediterr J Nutr Metab 3:197–201.

Cervantes-Gámez RG, Bueno-Ibarra MA, Cruz-Mendívil A, Calderón-Vázquez CL, Ramírez-Douriet CM, Maldonado-Mendoza IE, Villalobos-López MA, Valdez-Ortíz Á, López-Meyer M (2016) Arbuscular mycorrhizal symbiosis-induced expression changes in *Solanum lycopersicum* leaves revealed by RNA-seq analysis. Plant Mol Biol Rep 34:89–102.

Chalal M, Winkler JB, Gourrat K, Trouvelot S, Adrian M, Schnitzler JP, Jamois F, Daire X (2015) Sesquiterpene volatile organic compounds (VOCs) are markers of elicitation by sulfated laminarine in grapevine. Front Plant Sci 6:350.

Chevalier G (1998) The truffle cultivation in France: assessment of the situation after 25 years of intensive use of mycorrhizal seedlings. In: Proceedings of the First International Meeting on Ecology, Physiology and Cultivation of Edible Mycorrhizal Mushrooms. Uppsala, Sweden, July 1998.

Christie JM (2007) Phototropin blue-light receptors. Annu Rev Plant Biol 58:21–45.

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674–3676.

Corrêa A, Martins-Loução MA (2011) C:N interactions and the cost: benefit balance in ectomycorrhizae. In: Rai M, Varma A (eds) Diversity and biotechnology of ectomycorrhizae. Soil biology, Vol. 25. Springer, Berlin, Heidelberg, pp 387–403.

Craddock J (1992) Mycorrhizal association between *Corylus heterophylla* and *Tuber melanosporum*. In: III International Congress on Hazelnut, Vol. 351, pp 291–298.

Cruz-Mendívil A, López-Valenzuela JA, Calderón-Vázquez CL, Vega-García MO, Reyes-Moreno C, Valdez-Ortiz A (2015) Early transcriptional responses to chilling stress in tomato fruit with hot water pre-treatment. Postharvest Biol Technol 109:137–144.

Dahro B, Wang F, Peng T, Liu JH (2016) PtrA/NINV, an alkaline/neutral invertase gene of *Poncirus trifoliata*, confers enhanced tolerance to multiple abiotic stresses by modulating ROS levels and maintaining photosynthetic efficiency. BMC Plant Biol 16:1–18.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR (2013) STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21.

Donnini D, Benucci GMN, Bencivenga M, Baciarelli-Falini L (2014) Quality assessment of truffle-inoculated seedlings in Italy: proposing revised parameters for certification. For Syst 23: 385–393.

Dosskey MG, Linderman RG, Boersma L (1990) Carbon-sink stimulation of photosynthesis in Douglas fir seedlings by some ectomycorrhizas. New Phytol 115:269–274.

Dreischhoff S, Das IS, Jakobi M, Kasper K, Polle A (2020) Local responses and systemic induced resistance mediated by ectomycorrhizal fungi. Front Plant Sci 11:1908.

Fiorilli V, Catoni M, Miozzi L, Novero M, Accotto GP, Lanfranco L (2009) Global and cell-type gene expression profiles in tomato plants colonized by an arbuscular mycorrhizal fungus. New Phytol 184:975–987.

Gai WX, Ma X, Qiao YM, Shi BH, Ul Haq S, Li QH, Wei AM, Liu KK, Gong ZH (2020) Characterization of the bZIP transcription factor family in pepper (*Capsicum annuum* L.): CABZIP25 positively modulates the salt tolerance. Front Plant Sci 11:139.

García-Calderón M, Pérez-Delgado CM, Credali A, Vega JM, Betti M, Márquez AJ (2017) Genes for asparagine metabolism in *Lotus japonicus*: differential expression and interconnection with photorespiration. BMC Genomics 18:781.

Gerlach N, Schmitz J, Polatajko A et al. (2015) An integrated functional approach to dissect systemic responses in maize to arbuscular mycorrhizal symbiosis. Plant Cell Environ 38:1591–1612.

Goossens J, Fernández-Calvo P, Schweizer F, Goossens A (2016) Jasmonates: signal transduction components and their roles in environmental stress responses. Plant Mol Biol 91:673–689.

Gullner G, Komives T, Király L, Schröder P (2018) Glutathione S-transferase enzymes in plant–pathogen interactions. Front Plant Sci 9:1836.

Hacquard S, Tisserant E, Brun A, Legué V, Martin F, Kohler A (2013) Laser microdissection and microarray analysis of *tuber melanosporum* ectomycorrhizas reveal functional heterogeneity between mantle and Hartig net compartments. Environ Microbiol 15:1853–1869.

He K, Wu Y (2016) Receptor-like kinases and regulation of plant innate immunity. Enzyme 40:105–142.

Hobbie EA, Colpaert JV (2003) Nitrogen availability and colonization by mycorrhizal fungi correlate with nitrogen isotope patterns in plants. New Phytol 157:115–126.

Hossain MA, Lee Y, Cho JI, Ahn CH, Lee SK, Jeon JS, Kang H, Lee CH, An G, Park PB (2010) The bZIP transcription factor OsABF1 is an ABA responsive element binding factor that enhances abiotic stress signaling in rice. Plant Mol Biol 72:557–566.

Huisman R, Hontelez J, Mysore KS, Wen J, Bisseling T, Limpens E (2016) A symbiosis dedicated SYNTAXIN OF PLANTS 13II isoform controls the formation of a stable host-microbe interface in symbiosis. New Phytol 211:1338–1351.

Huisman R, Hontelez J, Bisseling T, Limpens E (2020) SNARE complexity in arbuscular mycorrhizal symbiosis. Front Plant Sci 11:354.

Jaakola L (2013) New insights into the regulation of anthocyanin biosynthesis in fruits. Trends Plant Sci 18:477–483.

Jakoby M, Weisshaar B, Dröge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F (2002) bZIP transcription factors in *Arabidopsis*. Trends Plant Sci 7:106–111.

Kaling M, Schmidt A, Moritz F, et al. (2018) Mycorrhiza-triggered transcriptomic and metabolomic networks impinge on herbivore fitness. Plant Physiol 176:2639–2656.

Karagiannis E, Michailidis M, Tanou G, Samiotaki M, Karamanoli K, Avramidou E, Ganopoulos I, Madesis P, Molassiotis A (2018) Ethylene-dependent and -independent superficial scald resistance mechanisms in 'Granny Smith' apple fruit. Sci Rep 8:1–16.

Kelly G, Sade N, Doron-Faigenboim A et al. (2017) Sugar and hexokinase suppress expression of PIP aquaporins and reduce leaf hydraulics that preserves leaf water potential. Plant J 91: 325–339.

Koele N, Dickie IA, Oleksyn J, Richardson SJ, Reich PB (2012) No globally consistent effect of ectomycorrhizal status on foliar traits. New Phytol 196:845–852.

Kohler A, Kremer A, Le Provost G, Lesur I, Lin G, Martin F, Plomion C, Wu A, Zhao F, Genomic Resources Development Consortium (2016) Genomic resources notes accepted 1 August 2015-31 September 2015. Mol Ecol Resour 16:377.

Köllner TG, Mazarei M, Guo H, Chen F (2017) An (E,-E)-α-farnesene synthase gene of soybean has a role in defence against nematodes and is involved in synthesizing insect-induced volatiles. Plant Biotechnol J 15:510–519.

Kong Y, Zheng Y (2020) Phototropin is partly involved in blue-light-mediated stem elongation, flower initiation, and leaf expansion: a comparison of phenotypic responses between wild *Arabidopsis* and its phototropin mutants. Environ Exp Bot 171:103967.

Labbé J, Muchero W, Czarnecki O et al. (2019) Mediation of plant-mycorrhizal interaction by a lectin receptor-like kinase. Nat Plants 5:676–680.

Lamberti C, Nebbia S, Antoniazzi S et al. (2021) Effect of hot air and infrared roasting on hazelnut allergenicity. Food Chem 342:128174.

Lea PJ, Sodek L, Parry MA, Shewry PR, Halford NG (2007) Asparagine in plants. Ann Appl Biol 150:1–26.

Lefevre C, Hall IR (2001) The status of truffle cultivation: a global perspective. Acta Hortic 556:513–520.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R (2009) The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079.

Li Y, Sun P, Lu Z et al. (2021) The *Corylus mandshurica* genome provides insights into the evolution of Betulaceae genomes and hazelnut breeding. Hortic Res 8:54.

Lingua G, Bona E, Todeschini V, Cattaneo C, Marsano F, Berta G, Cavaletto M (2012) Effects of heavy metals and arbuscular mycorrhiza on the leaf proteome of a selected poplar clone: a time course analysis. PLoS One 7:e38662.

Liu J, Maldonado-Mendoza I, Lopez-Meyer M, Cheung F, Town CD, Harrison MJ (2007) Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. Plant J 50:529–544.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25:402–408.

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:1–21.

Lucas SJ, Kahraman K, Avşar B, Buggs RJ, Bilge I (2021) A chromosome-scale genome assembly of European hazel (*Corylus avellana* L.) reveals targets for crop improvement. Plant J 105:1413–1430.

Luo ZB, Li K, Gai Y, Göbel C, Wildhagen H, Jiang X, Feußner I, Rennenberg H, Polle A (2011) The ectomycorrhizal fungus (*Paxillus involutus*) modulates leaf physiology of poplar towards improved salt tolerance. Environ Exp Bot 72:304–311.

Martin F, Kohler A, Murat C et al. (2010) Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. Nature 464:1033–1038.

Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnetjournal 17:10–12.

Martins A, Casimiro A, Pais MS (1997) Influence of mycorrhization on physiological parameters of micropropagated *Castanea sativa* Mill. plants. Mycorrhiza 7:161–165.

Matsoukas IG (2017) Crosstalk between photoreceptor and sugar signaling modulates floral signal transduction. Front Physiol 8:382. https://doi.org/10.3389/fphys.2017.00382.

Mauch-Mani B, Baccelli I, Luna E, Flors V (2017) Defense priming: an adaptive part of induced resistance. Annu Rev Plant Biol 68:485–512.

Mello A, Balestrini R (2018) Recent insights on biological and ecological aspects of ectomycorrhizal fungi and their interactions. Front Microbiol 9:216.

Morkunas I, Marczak Ł, Stachowiak J, Stobiecki M (2005) Sucrose-induced lupine defense against *Fusarium oxysporum*: sucrose-stimulated accumulation of isoflavonoids as a defense response of lupine to *Fusarium oxysporum*. Plant Physiol Biochem 43: 363–373.

Moser BR (2012) Preparation of fatty acid methyl esters from hazelnut, high-oleic peanut and walnut oils and evaluation as biodiesel. Fuel 92:231–238.

Näsholm T, Högberg P, Franklin O, Metcalfe D, Keel SG, Campbell C, Hurry V, Linder S, Högberg MN (2013) Are ectomycorrhizal fungi alleviating or aggravating nitrogen limitation of tree growth in boreal forests? New Phytol 198:214–221.

Oliveira I, Sousa A, Valentão P, et al. (2007) Hazel (*Corylus avellana* L.) leaves as source of antimicrobial and antioxidative compounds. Food Chem 105:1018–1025.

Ouledali S, Ennajeh M, Ferrandino A, Khemira H, Schubert A, Secchi F (2019) Influence of arbuscular mycorrhizal fungi inoculation on the control of stomata functioning by abscisic acid (ABA) in drought-stressed olive plants. S Afr J Bot 121:152–158.

Ponnu J, Wahl V, Schmid M (2011) Trehalose-6-phosphate: connecting plant metabolism and development. Front Plant Sci 2:70.

Raven JA, Smith FA (1976) Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. New Phytol 76:415–431.

Riaz U, Kharal MA, Murtaza G, Zaman Q, Javaid S, Malik HA, Aziz H, Abbas Z (2019) Prospective roles and mechanisms of caffeic acid in counter plant stress: a mini review. Pakistan J Agric Res 32:8–19.

Riethmüller E, Alberti Á, Tóth G, Béni S, Ortolano F, Kéry Á (2013) Characterisation of diarylheptanoid and flavonoid type phenolics in *Corylus avellana* l. leaves and bark by HPLC/DAD-ESI/MS. Phytochem Anal 24:493–503.

Rosenkranz M, Chen Y, Zhu P, Vlot AC (2021) Volatile terpenes - mediators of plant-to-plant communication. Plant J 108:617–631.

Rowley ER, VanBuren R, Bryant DW, Priest HD, Mehlenbacher SA, Mockler TC (2018) A draft genome and high-density genetic map of European hazelnut (*Corylus avellana* L.). In: bioRXiv, p 469015; preprint: not peer reviewed. https://doi.org/10.1101/469015.

Salomon MV, Bottini R, de Souza Filho GA, Cohen AC, Moreno D, Gil M, Piccoli P (2014) Bacteria isolated from roots and rhizosphere of *Vitis vinifera* retard water losses, induce abscisic acid accumulation and synthesis of defense-related terpenes in *in vitro* cultured grapevine. Physiol Plant 151:359–374.

Salomon MV, Piccoli P, Pinter IF, Stirk WA, Kulkarni M, van Staden J, Bottini R (2017) Bacteria and smoke-water extract improve growth and induce the synthesis of volatile defense mechanisms in *Vitis vinifera* L. Plant Physiol Biochem 120:1–9.

Schweiger R, Baier MC, Persicke M, Müller C (2014) High specificity in plant leaf metabolic responses to arbuscular mycorrhiza. Nat Commun 5:1–11.

Seró R, Núñez N, Núñez O, Camprubí A, Grases JM, Saurina J, Moyano E, Calvet C (2019) Modified distribution in the polyphenolic profile of rosemary leaves induced by plant inoculation with an arbuscular mycorrhizal fungus. J Sci Food Agr 99: 2966–2973.

Shoeva OY, Mock HP, Kukoeva TV, Börner A, Khlestkina EK (2016) Regulation of the flavonoid biosynthesis pathway genes in purple and black grains of *Hordeum vulgare*. PLoS One 11:e0163782.

Sillo F, Fangel JU, Henrissat B, Faccio A, Bonfante P, Martin F, Willats WGT, Balestrini R (2016) Understanding plant cell-wall remodelling during the symbiotic interaction between *Tuber melanosporum* and *Corylus avellana* using a carbohydrate microarray. Planta 244:347–359.

Silvestri C, Bacchetta L, Bellincontro A, Cristofori V (2021) Advances in cultivar choice, hazelnut orchard management, and nut storage to enhance product quality and safety: an overview. J Sci Food Agr 101:27–43.

Singh KB, Foley RC, Oñate-Sánchez L (2002) Transcription factors in plant defense and stress responses. Curr Opin Plant Biol 5:430–436.

Sintupachee S, Promden W, Ngamrojanavanich N, Sitthithaworn W, De-Eknamkul W (2015) Functional expression of a putative geraniol 8-hydroxylase by reconstitution of bacterially expressed plant CYP76F45 and NADPH-cytochrome P450 reductase CPR I from Croton stellatopilosus Ohba. Phytochemistry 118:204–215.

Smith SE, Read DJ (2008) Mycorrhizal Symbiosis, 3rd edn. Academic Press, London.

Solfanelli C, Poggi A, Loreti E, Alpi A, Perata P (2006) Sucrose-specific induction of the anthocyanin biosynthetic pathway in *Arabidopsis*. Plant Physiol 140:637–646.

Song Y, Chen D, Lu K, Sun Z, Zeng R (2015) Enhanced tomato disease resistance primed by arbuscular mycorrhizal fungus. Front Plant Sci 6:786.

Sornaraj P, Luang S, Lopato S, Hrmova M (2016) Basic leucine zipper (bZIP) transcription factors involved in abiotic stresses: a molecular model of a wheat bZIP factor and implications of its structure in function. Biochim Biophys Acta Gen Subj 1860:46–56.

Sun Y, Qiao Z, Muchero W, Chen J-G (2020) Lectin receptor-like kinases: the sensor and mediator at the plant cell surface. Front Plant Sci 11:596301.

Sun YJ, He JM, Kong JQ (2019) Characterization of two flavonol synthases with iron-independent flavanone 3-hydroxylase activity from *Ornithogalum caudatum* Jacq. BMC Plant Biol 19:195.

Szuba A, Marczak Ł, Karliński L, Mucha J, Tomaszewski D (2019) Regulation of the leaf proteome by inoculation of *Populus* × *canescens* with two *Paxillus involutus* isolates differing in root colonization rates. Mycorrhiza 29:503–517.

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought and cold inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J 29:417–426.

Takemiya A, Inoue SI, Doi M, Kinoshita T, Shimazaki KI (2005) Phototropins promote plant growth in response to blue light in low light environments. Plant Cell 17:1120–1127.

Tauzin AS, Giardina T (2014) Sucrose and invertases, a part of the plant defense response to the biotic stresses. Front Plant Sci 5:293.

Torres-Galea P, Hirtreiter B, Bolle C (2013) Two GRAS proteins, SCARECROW-LIKE21 and PHYTOCHROME a SIGNAL TRANSDUCTION1, function cooperatively in phytochrome a signal transduction. Plant Physiol 161:291–304.

Valentini N, Calizzano F, Boccacci P, Botta R (2014) Investigation on clonal variants within the hazelnut (*Corylus avellana* L.) cultivar 'Tonda gentile delle Langhe'. Sci Hortic 165:303–310.

Veneault-Fourrey C, Martin F (2011) Mutualistic interactions on a knife-edge between saprotrophy and pathogenesis. Curr Opin Plant Biol 14:444–450.

Walter W, Sánchez-Cabo F, Ricote M (2015) GOplot: an R package for visually combining expression data with functional analysis. Bioinformatics 31:2912–2914.

Wang Y, Yang L, Chen X, Ye T, Zhong B, Liu R, Wu Y, Chan Z (2016) Major latex protein-like protein 43 (MLP43) functions as a positive regulator during abscisic acid responses and confers drought tolerance in *Arabidopsis thaliana*. J Exp Bot 67:421–434.

Willmann A, Thomfohrde S, Haensch R, Nehls U (2014) The poplar NRT2 gene family of high affinity nitrate importers: impact of nitrogen nutrition and ectomycorrhiza formation. Environ Exp Bot 108: 79–88.

Wink M (2018) Plant secondary metabolites modulate insect behavior-steps toward addiction? Front Physiol 9:364.

Xiao W, Sheen J, Jang JC (2000) The role of hexokinase in plant sugar signal transduction and growth and development. Plant Mol Biol 44:451–461.

Xu Y, Xu H, Wall MM, Yang J (2020) Roles of transcription factor SQUAMOSA promoter binding protein-like gene family in papaya (*Carica papaya*) development and ripening. Genomics 112: 2734–2747.

Yabuki Y, Ohashi M, Imagawa F, Ishiyama K, Beier MP, Konishi N, Kojima S (2017) A temporal and spatial contribution of asparaginase to asparagine catabolism during development of rice grains. Rice 10:1–10.

Yan Q, Hou HM, Singer SD, Yan XX, Guo RR, Wang XP (2014) The grape *VvMBF1* gene improves drought stress tolerance in transgenic *Arabidopsis thaliana*. Plant Cell Tiss Org Cult 118:571–582.

Yang F, Liao D, Wu X et al. (2017) Effect of aboveground and belowground interactions on the intercrop yields in maize-soybean relay intercropping systems. Field Crop Res 203:16–23.

Zarivi O, Cesare P, Ragnelli AM, et al. (2015) Validation of reference genes for quantitative real-time PCR in Périgord black truffle (*Tuber melanosporum*) developmental stages. Phytochemistry 116:78–86.

Zhang Z, Feechan A, Pedersen C, Newman MA, Qiu JL, Olesen KL, Thordal-Christensen H (2007) A SNARE protein has opposing functions in penetration resistance and defence signalling pathways. Plant J 49:302–312.

Zhu-Salzman K, Zeng R (2015) Insect response to plant defensive protease inhibitors. Annu Rev Entomol 60:233–252.

Zouari I, Salvioli A, Chialva M et al. (2014) From root to fruit: RNA-Seq analysis shows that arbuscular mycorrhizal symbiosis may affect tomato fruit metabolism. BMC Genomics 15:221.