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The transcriptional mechanism responding to air particulate matter in Laurus nobilis (L.)

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ABSTRACT

Air levels of particulate matter (PM) are dangerously increasing in urban environment due to high levels of industrialization. The use of plants to reduce pollutants is well-known although molecular mechanisms underlying this phenomenon have been scarcely investigated. The aim of this work was to shed lights into the molecular physiological responses of leaves of a typical plant ornamental species (Laurus nobilis L.), to identify key PM-modulated genes of early leaf responses and to optimize phylloremediation strategies in polluted areas. The transcriptome and the PM accumulation in leaf tissues were analysed in plants grown in pots for three months under two different areas: rural and traffic. Differentially expressed genes in common between different pairwise comparisons were highly effective in discriminating leaves at high PM levels after three months of exposure. Thirty of these genes showed contrasting trend of expression between rural and traffic conditions. Higher PM levels highlighted significant repression of genes and gene set categories involved in cell wall and membrane modification. In addition, a repression of genes involved in photosynthesis reactions, glycolysis, gluconeogenesis, TCA, fermentation was observed. Some key members of C2H2, WRKYs and CCAAT-HAP2 were induced as well as downstream defense response genes. The results allowed to develop a general model of gene regulatory networks in response to particulate matter in plants and innovative biotechnological approaches for phylloremediation strategies.

1. Introduction

Particulate matter (PM) is a complex mixture of solid and liquid particles composed of organic and inorganic substances. Currently, it represents one of the most dangerous pollutant for humans due to its structure, especially in highly urbanized areas (Sgrigna et al., 2015). It is estimated that only in Europe, PM contamination causes about 0.4 million cases of cancerogenic diseases per year (EEA 2019). Indeed, the International Agency for Research on Cancer (IARC) classified indoor and outdoor air pollution as carcinogenic to humans (Loomis et al., 2013). The PM is commonly divided into three main groups according to the diameter of the particles: i) coarse particles (PM_{10}), the largest one, with a diameter of 10 μm or less, ii) fine particles ($PM_{2.5}$) characterized by a width of less than 2.5 μm, and iii) ultrafine particles ($PM_{0.1}$), with an aerodynamic of 0.1 μm or tiniest (Mühlfeld et al., 2008). The smallest ones are the most dangerous in terms of dispersion and penetration into living organisms, including plants (De Kok et al., 2006).

It is undebated that plants growing nearby highly polluted areas greatly contribute to reduce PMs concentrations in the air, protecting the health of the inhabitants. In particular, road vegetation is reported to reduce the concentration of various pollutants by up to 60% (Abhijith et al., 2017). Indeed, phyllo-remediation is a widespread, environmentally-friendly, and low-cost approach to counteract air pollution and protect the inhabitant's health based both on the metabolic processes activated by the plants and on the ability to act as physical barriers able to retain air particles on the leaf surfaces (Kim et al., 2020). Pollutants are then capable of passing through the pores into the lymph, where they can widespread and interact with the

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metabolic processes of the plants (Papazian and Blande, 2020), causing responses typical of abiotic and biotic stresses (Castagna and Ranieri, 2009; Papazian et al., 2016). Several studies have highlighted how the physiological and structural modifications of plants subjected to different pollutants are strongly correlated with their functional characteristics (Gostin, 2009). Macroscopically, plants are directly involved, through the leaves, in trapping particles and in the consequent reduction of their environmental concentration (Han et al., 2022). Different studies demonstrated that leaf morphology directly impacts the adsorption power (Bandehali et al., 2021; Sgrigna et al., 2020; Weerakkody et al., 2018; Yang et al., 2015). Generally, the most effective plant morphological characteristics involved in the sequestration of airborne debris are the surface roughness, the density of stomata, the presence of surface hairs, and the leaf size (Chen et al., 2017).

To date, to our knowledge, the molecular mechanisms underlying the plant's capability to contain, adapt and process these environmental pollutants are mostly unknown. A recent study investigated the relationship between transcript and phenotypical modifications in *Carpinus putoensis putoensis* under high levels of NO_2 (Sheng et al., 2021). Other authors highlighted similar findings concerning several air pollutants such as O_3 and SO_2 (Zhao et al., 2017). In particular, Wu et al. (2020) highlighted the involvement of ethylene in response to O_3 accumulation.

Evergreen broad-leaved shrubs are commonly planted in urban green areas of the Mediterranean because they are powerful remediation systems during the most polluted winter season, when buildings heating is added to road traffic as a source of pollution (Freer-Smith et al., 2005). Moreover, their compact dimensions allow for greater management in confined spaces and provide better particulate motility in highly polluted environments. However, although some works were performed to highlight the most suitable species involved in outdoor air remediation (Lee et al., 2021), evidences are lacking, due to the difficulties of finding univocally a compromise between benefits and disadvantages.

Laurus nobilis L. is a large evergreen shrub (ranging from 2 to 20 m in height) endemic to the Mediterranean region and belonging to the Lauraceae family (Patrakar et al., 2012). Phenotypically, leaves are oval, dark green, sharp, and present a thick cuticle on a uni-layer epidermis. Flowers present multiple inflorescences and are categorized into unisexual and bisexual types (Endress and Lorence, 2020). Therefore, many studies were carried out focusing on the chemical characteristics of *L. nobilis*, dealing with its usage as an essential oil in medicine, food industries, and postharvest agricultural processes (Ercin et al., 2022; Ordoudi et al., 2022; Papanikolaou et al., 2022). Due to the large shape of the leaves, this plant may be more useful than conifers in air remediation although the complex molecular mechanisms to particulate matter in all kind of plants (including Laurus nobilis L.) is completely unknown. The association between PMs adsorption and transcriptomic changes in plants is still lacking. RNA-seq represents the premier "omic" approach to shed light on under-investigated complex plant physiological mechanisms in a deep manner, thanks to the availability of closely-related genomic resources. Transcriptomic approaches have been widely used to gain insight into the complex molecular mechanisms modulating plant responses to environmental stresses (Martinelli et al., 2015; Rizzini et al., 2010). Investigating the gene regulatory networks through functional "omics" approaches is necessary to fully understand the relationship between genetic expression and outdoor air remediation. In particular, the study of how autochthonous species can improve air quality for each specific macro- and micro-climatic environment will be essential and could bring to the identification of which local plant species can contribute to reducing the air particulate matter. These studies would allow sustainably designing for future urban green management.

The present work aimed to shed light on the gene regulatory networks underlying plant leaf responses to air particulate matter. Our investigation focused on autochthonous shrubs of *L. nobilis* L. that were grown in pots located in two close-by contrasting areas (within the same municipality), a highly polluted traffic road and rural countryside within the same town (Altopascio, Lucca, Italy). RNA-seq data were interpreted in relation to the leaf accumulation of air particulate matter allowed to identify key players in the model of plant transcriptional responses to air particulate matter in plants.

2. Material and methods

2.1. Samples collection

Ten plants of *L. nobilis* were planted in pots using commercial soils and placed in 2 different areas of the city of Altopascio (Lucca, Tuscany, Italy). Plants had a height of around 1.5 m and were bought in the same plant nursery to reduce plants' physiological and agronomic variability at the beginning of the trial. Plants were divided into two groups: 5 plants were grown in a rural area (named "R") sparsely inhabited, with significant lower levels of PM (control) compared to 5 plants exposed to higher levels of PM (named "T"; "traffic", close to a traffic light with a high number of moving cars). Plants were irrigated weekly and routinely inspected to determine any eventual symptoms of biotic and abiotic stresses. Plants remain healthy during the three months of the experimental trial. Healthy, fully-expanded, green leaves were sampled from the 5 plants from each zone at the time of planting (R1 and T1; 15th March 2021) and three months after planting (R1 and T1; 15th June 2021). Leaves were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. PM analysis and morphological analysis

Leaves samples from the different areas (R and T) were collected at the two time points, before the exposure (T0) and at the end of the exposure period (T1). We assumed that the PM accumulated on the leaves refers to the period before the last precipitation above 10 mm (24/05/2021).

Before starting the filtration, the leaves were disposed in a white sheet and photographed for assessing the total leaf area using the program ImageJ Pro (https://imagej.nih.gov/ij/). An average of 260 cm^2 of each sample were used to perform the filtration methodology following the procedures described in Mori et al. (2018). Three filters with different retention capacities (retention 10 μm, retention 2.5 μm, and PTFE membrane - retention 0.2 μm) were used. Each filter was dried at 60 °C for 30 min and then left for 60 min at a constant relative air humidity (50%) for weight stabilization and then pre-weighed. Each leaf sample was washed for 60 s with 150 ml of deionized water under agitation. The washing solution was then sequentially filtered using the three filters. Filtration was carried out using an apparatus equipped with a 47 mm glass filter funnel connected to a vacuum pump. After filtration, filters were dried, left to stabilize, and weighed again. At the end of the filtration procedure, particle of different sizes was divided as large: > 10 μm (PM_{10}), coarse: 2.5–10 μm ($PM_{2.5}$), fine: 0.2–2.5 μm ($PM_{0.2}$), and PM_s = Total sum of PM_{10} +$PM_{2.5}$ +$PM_{0.2}$. A high-resolution picture of 5 leaves per sample using white background was processed using ImageJ Pro (https://imagej.nih.gov/ij/) to measure the following leaf traits: leaf surface area (LA); leaf roundness (Roundness = (4 * leaf area)/[π * (mayor axis)2]; leaf perimeter, used to calculate the leaf dissection index [LDI = leaf perimeter / (leaf area)− 2] and leaf mass per area (LMA) performed on five discs (8 mm) per leaf of each sample dry until constant weight used to calculate the specific leaf area (gram dry weight per cm^{-2} of leaf area, SLA) following the methodology described by Muhammad et al. (2019). Two-Way ANOVA was applied considering the effect of Area and Time in the accumulation of PM, divided by the particle size and the leaf traits.

2.3. Transcriptomic analysis

Four pools of 5–7 mature leaves collected from 4 single plants for both the locations were analyzed using the RNA-seq approach for each

time point for a total of 16 samples. Frozen leaves were grounded to a fine powder with a previously sterilized mortar and pestle using liquid nitrogen to prevent RNA degradation. A CTAB-modified protocol was used (Doyle and Doyle, 1987). The pellet obtained from the extraction procedure was resuspended in 130 μL of nuclease-free water, and the RNA was treated with DNaseI (NEB). Subsequently, the RNA was purified using a silica-based method (Monarch RNA Cleanup kit – NEB) and eluted twice in 15 μL of nuclease-free water. Qualitative and quantitative assessments were respectively performed with Agilent 2100 Bioanalyzer (RNA 6000 nano kit - Agilent Technologies) and Qubit™ 4 Fluorometer (RNA BR Assay Kit - Invitrogen).

Sixteen transcriptomic libraries were prepared using Illumina mRNA Prep kit, following the manufacturer instructions and a unique dual index combinations were used for each sample for barcoding. The concentration of each of the libraries was determined using Qubit™ 4 Fluorometer (dsDNA High Sensitivity Kit - Invitrogen). Libraries were sequenced on the Illumina Novaseq6000 platform. Sequencing was performed using Novaseq 6000 SP Reagent Kit ($2 \times 100 + 10 + 10$ bp parameters). Four replicates were used for each group (rural and traffic areas) and sampling time (T0 and T1). Samples were run in a single flow cell.

2.4. Transcriptome assembling and differential expression analyses

RNA-Seq data in bcl format were converted to fastq files using Illumina bcl2fastq v2.20. After assessing the raw reads quality with FastQC v0.11.5 (Andrews, 2010), adaptors sequences and low quality bases were removed using Trimmomatic v0.39 with these parameters: HEADCROP:1 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:18 MINLEN:40 (Bolger et al., 2014). The filtered reads of the sixteen RNA libraries were used for a *de novo* transcriptome assembling with Trinity v2.1.1 (Grabherr et al., 2011), and CD-hit v4.7 (Fu et al., 2012) with default parameters to cluster the assembled transcripts to produce a set of 'non-redundant' transcriptome representative sequences. Statistics of *de novo* assembled and clustered transcriptome were obtained with 'n50' Perl script (https://metacpan.org/pod/ distribution/Proch-N50/bin/n50).

The filtered RNA reads of the 16 libraries were mapped to the assembled and clustered laurel transcriptome, and the expression quantification of each transcript was carried out using the Trinity v2.2.1 utility "align_and_estimate_abundance.pl" with Bowtie aligning method (Grabherr et al., 2011). The assembled and clustered laurel transcripts were aligned to *Arabidopsis thaliana* L. reference transcriptome using BlastX against Araport11 dataset with an expectation value cutoff of 0.001 through the Galaxy portal (Afgan et al., 2018; Cheng et al., 2017). For each laurel transcript, the best hit was considered as *Arabidopsis* putative orthologue.

Differential expression (DE) analyses for three transcriptomes pairwise comparisons (Rural at time 1 *Vs.* Rural at time 0 (R1vs R0); Traffic at time 1 vs. Traffic at time 0 (T1 vs T0); Traffic at time 1 *Vs.* Rural at time 1 (T1 vs R1) were carried out using Bioconductor EdgeR v3.38.1 (Robinson et al., 2010) with EdgeR likelihood test. Before the DE analyses, raw counts were normalized basing on each library number of reads, and not expressed or poorly expressed transcripts (a transcript was considered 'active' if CPM (counts per million mapped reads) was $>$ 1 in at least three libraries) were filtered out. False discovery rate (FDR) lower than 0.05 and $\log_2$FC (fold change) lower than -1 or higher than 1 were considered as the conditions to state the transcripts as differentially expressed.

2.5. Blasting, mapping, and annotation

The Blast2GO software suite v5.2 (https://www.blast2go.com/) was used to perform homology searches for analysed sequences (BLASTX and BLASTN) on nonredundant (nr) database, in addition corresponding Gene Ontology terms for unique sequence and functional annotation (GO; http://www.geneontology.org/) were retrieved. Protein sequence analysis and classification were carried out by using InterPro (InterPro, EBI, https://www.ebi.ac.uk/interpro/).

Sequences were blasted against a nonredundant (nr) protein database corresponding to the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) via BLASTx-fast. InterPro was performed in parallel with the blasting phase, followed by the Gene Ontology (GO) mapping and gene annotation. Subsequently, the Gene Ontology terms that were derived from the BLAST and InterPro steps were merged. Go-slim reduction was applied. BLAST2GO was also used for assigning the genes to cellular processes, biological functions, and cellular components, as well as other salutary statistics.

2.6. Functional data mining and enrichment analyses

According to the DE analyses results, functional data mining was performed by analyzing single lists of DEGs (Differentially Expressed Genes) in the pairwise comparisons between T1 and T0 for each location (Traffic, T1 vs. T0; Rural, R1 vs. R0). The pairwise comparison at T1 between Traffic and Rural was also conducted (T1 vs. R1) and compared with the previous two pairwise comparisons. Hierarchical clustering and QT-clust analysis of data, based on the three values attributed, was carried out using MeV v4.6.2 software (http://www.tm4.org/). Our matrix was presented graphically by coloring different genes expression result on the basis of measured color range: lower limit "− 5" was colored by green, upper limit "5" was colored by red and midpoint value "0" was colored by black. The generated heat map displays single gene expressions under the three conditions. Common and exclusive DEGs between the two different locations comparing different time points were determined to dissect the complex gene regulatory networks underlying plant responses to different air particulate matter. MapMan 3.6.0RC1 software was used with the available *Arabidopsis thaliana* mapping file (http://mapman.gabipd.org/) to identify and visualize genes in functional overviews of cell pathways and gene categories (Thimm et al., 2004). The gene set enrichment analysis using Pageman was carried out with the same list of DE genes (http://mapman.gabipd.org/). The analysis was performed through the Wilcoxon test without correction and with a cutoff value of 1. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) v.6.8 (https://david.ncifcrf.gov/) (Dennis et al., 2003) was used to get the gene ontology information related to the biological processes modulated in response to environmental stresses such as abiotic stresses. The analysis of the gene-gene interaction network among the commonly regulated genes between T1 vs T0 and T1 vs R1 was carried out using STRING v.11 web-tool (https://string-db.org/), setting a confident score cutoff = 0.7 (Szklarczyk et al., 2018). Principal components analysis carried out on the different groups of common genes are calculated using one of the methods in pcaMethods R package and according with Nipals PCA will iteratively find components by leaving out missing values when calculating inner products. Vector scaling methods were used to data pre-processing which divides the values by the Euclidean norm of the values (square root of sum of squared values).

2.7. WGCNA co-expression network analysis and phenotypical correlation

Expression values (CPM) of DEGs obtained by RNA-Seq analysis for the two pairwise comparison between traffic and rural area and the two time points (R0 vs R1; T0 vs. T1) for *L. nobilis* were further investigated by using WGCNA algorithm (V. 1.70–3; (Langfelder and Horvath, 2008)) in order to detect possible gene network based on expression values. Analyses were conducted as specified by manual, in particular soft threshold values were implemented by the gradient method, testing different power values (as suggested by WGNA manual) and the minimum number of transcripts per module was set to 15. In addition, correlation analysis between eigengenes of each co-expression module (here labelled with the name of different colours) and the particulate

level detected for the PM, in the two different areas and time point, was performed using the WGCNA pipeline, in order to assess statistical linear regression with corresponding P-Value for each module.

3. Results

3.1. PMs accumulation and leaf traits

The levels of PM_{10} and $PM_{2.5}$ registered in the air during the experimental period were, on average 12.11 and 10.63 μg m^{-3} (PM_{10}) and 8.43 and 6.05 μg m^{-3} ($PM_{2.5}$) in the R and T areas respectively (Fig. S1). However, the last precipitation above 10 mm was recorded on 24th May, and the accumulation of PM_{10} and $PM_{2.5}$ after that period until the end of the experimental was higher in T (PM_{10} = 266.32 μg m^{-3} and $PM_{2.5}$ =185.48 μg m^{-3}) compared to the R area (PM_{10} = 233.93 μg m^{-3} and $PM_{2.5}$ =133.06 μg m^{-3}). An interaction between time and area factors was verified for all the PMs fractions analyzed (Table 1A). Generally, T1 presented a higher amount of all fractions of PMs than T0. However, PM_{10} and PMs did not differ between T0 and T1 in the rural area as they did for the traffic area (Fig. 1A-B). For $PM_{2.5}$ and $PM_{0.2}$, the values registered at T1 were higher than for T0 for both areas, with the higher values registered in the traffic area (T1, Fig. 1C-D).

3.2. RNA sequencing and de novo transcriptome assembling

Sixteen RNA libraries (4 experimental conditions with 4 biological replicates each) were sequenced, and the resulting fastq reads were deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-12332. Overall, the number of resulting paired reads ranged from 13.4 to 23.9 million across the libraries, for a total of 288.8 million paired reads (Table 2). After assessing the high quality of the RNA libraries, adapters and low-quality reads were filtered out, resulting in a percentage of 'survived' reads ranging from 89% to 94% (Table 2). Filtered reads of the sixteen libraries were *de novo* assembled, resulting in 307,522 transcripts with an N50 di 1032 for a total of 197,957,903 nucleotides. Assembled transcripts were then clustered to reduce redundancy, resulting in 242,263 transcripts for a total of 144,613,304 nucleotides: this is the transcriptome that was used for the downstream analyses. A run with BUSCO v5.3.2 (Simao et al., 2015) using the lineage dataset 'viridiplantae_odb10' indicated that the completeness of the transcriptome was 96.2%, which is considered a high parameter.

3.3. Transcript expression estimation and DE analyses

The filtered reads of the sixteen libraries were mapped to the laurel *de novo* assembled and clustered transcriptome, and the reads mapping to each transcript were counted. The most probable Arabidopsis orthologues of the laurel transcripts were searched, and 19,986 Arabidopsis proteins were found to have at least one orthologue in the laurel assembled transcriptome (Supplementary Table 1). The downstream analyses were carried out considering the summed reads counts of the laurel transcripts according to Arabidopsis orthology. After removing unexpressed and poorly expressed transcripts, 14,841 active transcripts were normalized according to libraries dimensions, and a multi-dimensional scaling (MDS) analysis was performed to visualize the samples clustering. MDS chart (Fig. S2) shows that samples are clearly separated by their time points. Moreover, as expected, R0 and T0 samples were not clustering separately, while, after three months of exposure to different levels of PM, R1 and T1 samples were clearly separated.

Differential expression analyses were carried out for three pairwise comparisons: R1vsR0, T1vsT0 and T1vsR1. Differentially expressed transcripts (DETs) detected in the three comparisons were 1332 (562 down-regulated; 770 up-regulated), 1424 (820 down-regulated; 604 up-regulated) and 225 (217 down-regulated; 8 up-regulated), respectively. Detailed results of the three DE analyses were reported on Supplementary Table 2. The PCA analysis carried out with the common DEGs (Fig. 2 between R1vsR0, T1vsT0 and R1vsT1 (panel A) were clearly able to separate well T1 from the rest of the samples and so they might be candidate biomarkers of response to PM. At the same time, in panel D data of T0 DEGs showed a specific cluster remark that these DEGs can be considered specific biomarkers of leaf development.

Table 1
A) Results of the Two-Way ANOVA considering the effect of Time (T0 and T1) and Area (Traffic and Rural) in the accumulation of particulate matters (PMs), divided by the particle size, in *L. nobilis* leaves collected. B) Results of the Two-Way ANOVA considering the effect of Time (T0 and T1) and Area (Traffic and Rural) in the leaf traits analyzed: Leaf surface area (LA); leaf roundness (LR); specific leaf area (SLA) and leaf dissection index (LDI).

	Effect	PM_{10}	$PM_{2.5}$	$PM_{0.2}$	PMs Total
A)	Time	* **	* **	* **	* **
	Area	* **	* **	*	* **
	Time*Area	* *	* **	*	* **
		LA	**LR**	**SLA**	**LDI**
B)	Time	*	*	n.s	n.s
	Area	n.s	n.s	* *	n.s
	Time*Area	n.s	n.s	n.s	n.s

* = $p < 0.05$, * * = $p < 0.001$, * ** = $p < 0.001$, n.s = non significant

3.4. Co-expression analysis

Venn diagrams were constructed to determine the number of DEGs unique and in common between each of the three pairwise comparisons (Fig. 3). Common genes between T1 vs T0 and T1 vs R1 were investigated as strongly associated to PMs and less affected by other external environmental factors. Indeed, the co-expression analysis of these commonly regulated genes allows to define which genes are signatures of the responses to the different environmental states.

A total of 2026 DEGs were analyzed. Among them, 43 genes were common to the three experimental conditions (R1 vs R0, T1 vs T0 and R1 vs T1), 66 genes were shared between R1 vs R0 and T1 vs T0 conditions, 501 genes were unique to R1 vs R0 and finally 578 genes were only found in T1 vs T0 condition (Fig. 3). In addition, 722 DEGs were in common between T1 vs R1 and R1 vs R0 while 81 were commonly expressed between T1 vs T0 and T1 vs R1.

Heat map was conducted for the differentially expressed transcripts in common between the three pairwise comparisons (Fig. S3). A first main group was composed by genes upregulated R1 vs R0 and repressed in T1 vs T0. Another group of genes was composed by genes upregulated in T1 vs R1 comparison.

The co-expression analysis showed that 30 genes were upregulated in R1 vs R0 and repressed in the other two comparisons (cluster 1) highlighting that these genes should be the most affected by air particulate matter (Fig. S4). They might represent a signature of plant responses to air pollution. Indeed, they distinguished the rural and traffic conditions since all these genes were repressed by higher level of pollution. Among them, it is worth mentioning some genes involved in oxidative stress responses such as glutathione-dependent formaldehyde activating enzymes, *formate dehydrogenase* and two oxidases. Cluster 2 and cluster 3 were characterized by set of genes that were mainly downregulated in all the experimental condition. A very different set of genes were grouped in cluster 3, like *GDT1-like protein 1* involved in calcium and manganese ions transport or the protein FAR1-RELATED SEQUENCE 5-like that encodes a nuclear localized protein involved in far red-light response signaling. The co-expression analysis of the 81 DEGs common between T1 vs R1 and R1 vs R0 showed that these genes were upregulated in R1 vs R0 and repressed in T1 vs R1 (Fig. S4, cluster 4). Several genes of this cluster are involved in small molecule metabolic processes and response to inorganic substances (Supplementary Table 3). Data derived from the 81 DEGs common to T1 vs T0 and T1vs R1 (cluster 5 and cluster 6) showed that all genes were mainly repressed

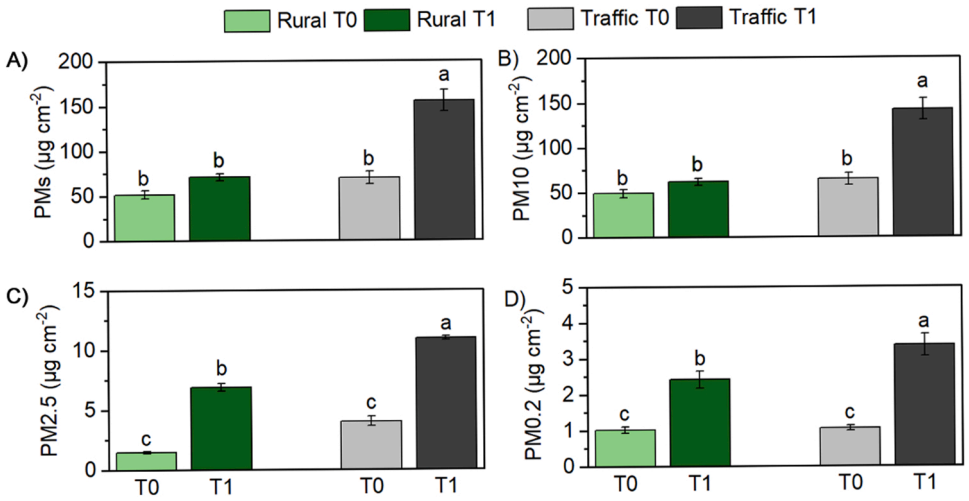

Fig. 1. A-D) PM divided by the particle size accumulated in *L. nobilis* leaves collected from two different areas of study (Traffic and Rural) at two different times (T0 and T1) during the summer season of 2021. A) Total PM (PMs). B) PM10. C) PM2.5. D) PM0.2. Letters represent differences between areas and time (Tukey test, $p < 0.05$). The total amounts of each PM fractions were expressed in µg/cm^2).

Table 2
RNA sequencing and filtering statistics. For each RNA library, the environmental condition, time point, number of raw reads pairs and number and percentage of survived reads after filtering were reported.

RNA library ID	Condition	Time-point	Read pairs	Read pairs survived to filtering (#)	Read pairs survived to filtering (%)
R0_1	rural area	0	20,778,316	19,339,126	93.07%
R0_2	rural area	0	16,314,214	15,097,189	92.54%
R0_3	rural area	0	21,030,050	19,556,575	92.99%
R0_4	rural area	0	18,528,362	17,316,311	93.46%
R1_1	rural area	1	23,934,419	22,333,260	93.31%
R1_2	rural area	1	18,386,769	16,923,781	92.04%
R1_3	rural area	1	19,772,276	18,491,702	93.52%
R1_4	rural area	1	18,110,382	17,103,553	94.44%
T0_1	traffic area	0	15,350,147	14,316,736	93.27%
T0_2	traffic area	0	18,213,864	16,840,358	92.46%
T0_3	traffic area	0	13,458,181	12,672,308	94.16%
T0_4	traffic area	0	15,462,836	13,830,863	89.45%
T1_1	traffic area	1	16,416,972	15,319,283	93.31%
T1_2	traffic area	1	13,712,556	12,842,902	93.66%
T1_3	traffic area	1	17,417,482	16,418,401	94.26%
T1_4	traffic area	1	21,888,827	19,879,770	90.82%

and were involved in different metabolic and transport processes (Supplementary Table 3). The analysis on the 723 DEGs common to T1 vs T0 and R1 vs R0 showed that these genes were split in two big clusters (Fig. S4, cluster 7 and cluster 8) in which 428 DEGs were repressed and 295 were upregulated.

3.5. Gene set enrichment analysis and metabolic pathways

GO-term enrichment analysis using DAVID functional annotation web-tool highlighted several differences between the transcriptomic responses to traffic and rural area implying a link with particulate matter air levels (Tables 2–4). In rural conditions a higher number of significant GO-term related to protein unfolding and binding processes through the use of chaperone activities were observed in comparison to traffic conditions. In addition, also photosynthesis-related, light harvesting photosystem I and II, chlorophyll binding, and protein chromophore binding genes were upregulated in rural conditions and not in traffic ones. Oxidoreductase processes were enhanced in rural conditions. As concerning the repressed GO-term, traffic conditions affected ribosome-related processes and vacuolar membrane, while rural conditions showed less repressed GO-terms. Lignin related processes were upregulated in traffic conditions while they were repressed in rural ones.

The gene set enrichment analysis highlighted differences between traffic and rural areas. In traffic conditions gluconeogenesis and cell wall modification were repressed while in rural area an induction of mitochondrial electron transport, lipid metabolism and protein-related pathways were upregulated (Fig. 4). Differentially expressed genes in the two pairwise comparisons (T1 vs T0 and R1 vs R0) involved in metabolism overview were shown in Fig. 5. Most of the genes showed a similar trend of expression between the two pairwise comparisons. However, some genes belonging to different categories were modulated in a contrasting way. Cell wall categories were one of the most functional groups that were mainly affected by a different trend of expression comparing the two conditions (Supplementary Table 4). Some key genes were downregulated in T and unregulated in R such as *Plant invertase/pectin methylesterase inhibitor superfamily*, *expansin A1 xyloglucan endotransglucosylase/hydrolase 25*. In addition, several genes were repressed in T and not affected in R such as *Fasciclin-like arabinogalactan 6*, *NAD (P)-linked oxidoreductase superfamily protein*, *Mannose-6-phosphate isomerase*. On the other hand, some genes were upregulated in T and not in R: the most interesting of these are *xyloglucan endotransglucosylase/hydrolase 24* and *Pectin lyase-like superfamily protein*. Some genes were shown to be downregulated in T and upregulated in R; examples of this trend were reported for *delta9 desaturase 1* and *2*, a fatty acid desaturase family protein. In addition, some key lipid metabolism genes like *ADS1*, *FAD5* and *FAD7* were strongly induced in rural conditions while they were downregulated in traffic ones. These genes were encoding FA

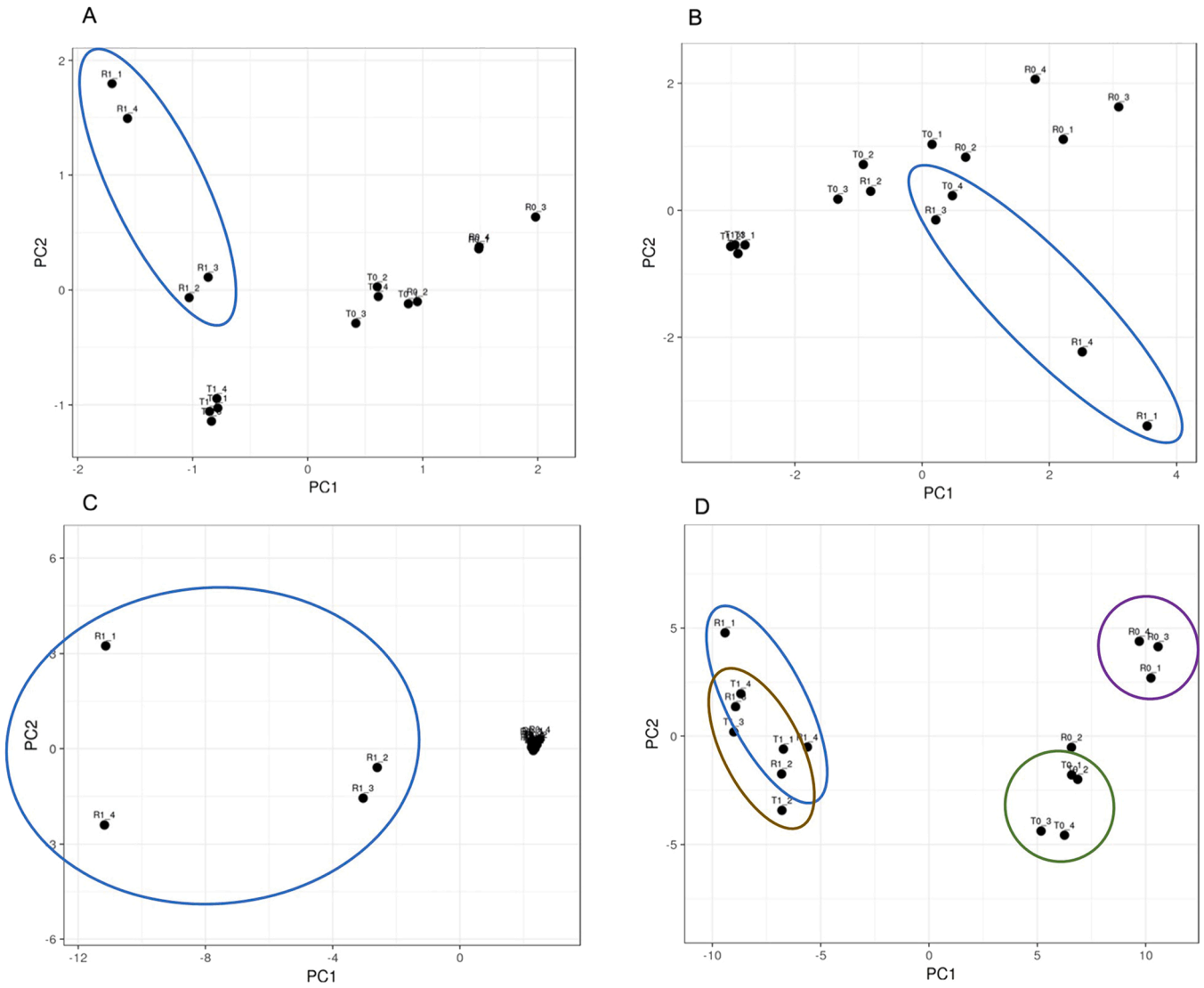

Fig. 2. The principal component analysis (PCA) of different groups of common genes: A) 41 genes common to the three experimental conditions (R1vsR0, T1vsT0 and R1vsT1), B) 157 genes common between T1 vs T0 and R1 vs R0, C) 393 genes common between R1 vs R0 and T1 vs R1 and D) 1625 genes common to R1vsR0 and T1vsT0.

desaturation desaturases. The number of DEGs involved in biosynthesis and metabolism of amino acids were lower than cell wall although some genes showed to have dissimilar expression among the two comparisons such as methionine synthase 2 (repressed in T, upregulated in R), S-adenosyl-L-homocysteine hydrolase (repressed in T1, not modulated in R), glutamate decarboxylase 5 (upregulated in T, not modulated in R).

Among genes involved in starch and sucrose, glycolysis, OPP and fermentation pathways, *UDP-glucose pyrophosphorylase* (glycolysis) and *6-phosphogluconate dehydrogenase* family protein (*OPPs*) were upregulated in R, while *glucose-6-phosphate dehydrogenase 5* were repressed in T. A fermentation aldehyde dehydrogenase was upregulated in rural environment and repressed in traffic one. Many contrasting differences were observed for TCA cycle. TCA cycle was mostly upregulated or unregulated in R (DEGs modulated into this pathway are *malate synthase*, *phosphoenolpyruvate carboxykinase 2*, *citrate synthase 5*, *succinate dehydrogenase 2–1* and *1–2*). Genes involved in photosynthesis were mostly induced in the conditions with less PM (rural) while they were not affected in the traffic area implying the air particulate has a negative effect on genes involved in light reactions. Another clear effect was observed for genes involved in mitochondrial electron transport: many genes were induced in R and not in T. Some key differences were also observed for genes involved in secondary metabolism. Generally lower amounts of PM activated genes in terpenes (*3-ketoacyl-coa synthase 10*, *terpene synthase 14*) and other genes involved in phenylpropanoid-related pathways such as *flavin-monooxygenase glucosinolate s-oxygenase 5*, *nicotinamidase 3*, *cinnamyl-alcohol dehydrogenase*, *elicitor-activated oxidoreductase*. On the same way, high levels of PM repressed the expression of some key genes such as *cinnamyl alcohol dehydrogenase homolog 2,3* and *6* (phenols and phenypropanoids) and *methylthioalkylmalate synthase 1* (S-misc). In general air particulate negatively modulated genes involved in transport of NO_3 (*glutamate synthase 1*) and C-1 (*Dimethylmenaquinone methyltransferase, glycine decarboxylase P-protein 2*).

Although there were no great differences in the number of DEGs in the two conditions (T and R), it is worth noticing that some genes involved in hormone biosynthesis, metabolism and signaling behaved in different way (Fig. 6). For example, several genes were inhibited by traffic conditions such as *iron ion binding / monooxygenase/ oxygen binding* (auxins), *cytokinin oxidase 5* (cytokinins, details), *12-oxophytodienoate reductase* (Jasmonates), *S-adenosylmethionine-dependent methyltransferase* (Salicylic acid) (Supplementary Table 5). Concerning redox categories, there were slight differences. In general, some genes were

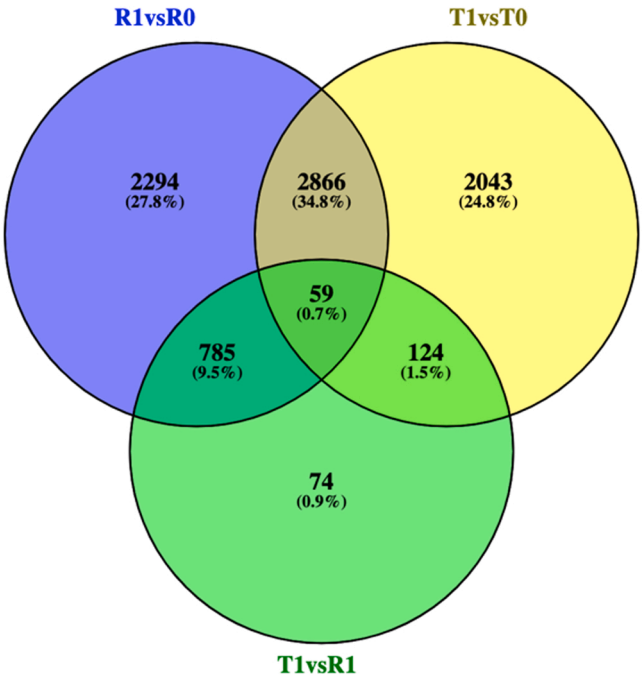

Fig. 3. Number of differentially expressed genes for each pairwise comparisons: R1 vs R0, T1 vs T0 and T1 vs R1 conditions. The number of uniquely PM-modulated genes or in common between the three pairwise comparisons were also shown with the percentage of genes for each unique or overlapping sub-groups.

repressed by T, encoding thioredoxin-related genes (15, 17) and G-proteins. Lower PM induced genes encoding catalase and ascorbate/glutathione. Generally redox pathways were slightly more activated in Rural conditions as shown by the induction of several genes (*cytochrome b5 isoform c*, *copper chaperone for sod1*, *Catalase 3*) (Fig. 7, Supplementary Table 6).

With regards to genes involved in environmental stress responses, high levels of air PM showed to repress a slighter number of genes involved in drought responses as well as thioredoxin (*CXXS1, WCRKC1, ATTRX4*) (Fig. 7). Most of the genes upregulated in both traffic and rural conditions were related to heat stress and encoding heat shock proteins (20-like, 70, 18.2). When air quality was better (rural, less polluted), plants showed to be more actively responding to environmental stresses as shown by the activation of genes encoding dismutase (*Catalase 3*), miscellaneous (*extensin family protein*, *germin-like protein 7*), and ascorbate/glutathione (Supplementary Table 6). These results may be explained by considering that this study investigates a long-time response (3 months) and not a rapid response. These results are therefore a picture of the leaf metabolism during a chronic and continuous exposure that induces senescence and general repression of cell pathways. Although leaves in T were not symptomatic, a light repression of pathways involved in photosynthesis, energy, defense responses are expected to occour.

In general, air particulate matter showed a higher number of upregulated genes belonging to the transcription factor families *CCAT-HAP2* and *C2H2* (Fig. 8). On the other hand, T inhibited more genes encoding bHLH factors. In addition, there were some specific genes that showed contrasting pattern of expression among the two pairwise comparisons. The following genes were repressed at higher levels of PMs while they were not regulated in R: *Constans-like 9* (*C2C2-CO-like*), *indole-3-acetic acid inducible 33* (*Aux/IAA*), *Lateral organ boundaries* (*LOB*) domain family protein (*AS2*), and Eukaryotic aspartyl protease family protein (unspecific). Similarly, some genes were higher in abundance in R than in T implying a similar effect of air particulate such as Homeodomain-like superfamily protein (*G2-like*), *GATA transcription factor 12* (*C2C2-Gata*), *constans-like 9* (*C2C2-CO-like*), C2H2-like zinc finger protein (*C2H2*), and zinc finger (*C2H2* type) family protein (unspecific). Transcription factors (TFs) involved in ethylene signaling were differentially regulated in both conditions with some slight differences: two members were upregulated in R, one gene downregulated only in T and another one repressed only in R (*ethylene response factor 110*) (Supplementary Table 7).

3.6. Protein-protein interaction network

Protein-protein interaction analysis conducted using string showed 45 proteins encoded by DEGs in common between T1 vs T0 and T1 vs R1 (Fig. S5). These two comparisons were shown since they indicate genes affected by air particulate matter after 3 months. The presence of the genes in both comparisons strengthen the link between the expression of these genes and PM air concentration. While some of them were barely interactive, others showed a high level of interactions and were very connected each other. Among these highly interactive proteins (hubs), it is worth mentioning *BTF3, PGY1, EMB2296* and *RPS5B*. Among other genes showed in the networks, there were some other genes well-known to be involved in abiotic stress responses such as *CAD2, CAD3, CAT1, GSH2* and *SDH1*-1.

3.7. WGNA analysis

WGNA analysis was performed on the list of DEGs in both comparisons at traffic (T1 vs T0) and rural conditions (R1 vs R0) (Fig. S6 and Fig. S7) and correlated with corresponding PM concentration detected on leaves. The most correlated clusters with PM leaf accumulation at traffic conditions were those named “darkred”, “mediumpurple3”,

Table 3
Up-regulated and down-regulated GO-term categories in T1 vs T0 comparison (traffic area). Enrichment score, number of genes (Count), p-value and Benjamini correction were indicated and obtained using DAVID software.

UP-Regulated categories				
Annotation Cluster 1	Enrichment Score: 8.86	Count	P_Value	Benjamini
GOTERM_BP_DIRECT	response to heat	35	1.9E-19	1.7E-16
GOTERM_BP_DIRECT	protein folding	23	1.6E-11	7.2E-9
GOTERM_BP_DIRECT	protein oligomerization	8	4.7E-8	1.4E-5
GOTERM_MF_DIRECT	unfolded protein binding	16	4.9E-8	2.1E-5
GOTERM_MF_DIRECT	protein self-association	14	7.0E-6	1.5E-3
Down-regulated categories				
Annotation Cluster 1	**Enrichment Score: 5.22**	**Count**	**P_Value**	**Benjamini**
GOTERM_CC_DIRECT	polysomal ribosome	18	3.0E-9	2.4E-7
GOTERM_CC_DIRECT	cytosolic large ribosomal subunit	21	2.2E-8	1.1E-6
GOTERM_CC_DIRECT	cytosolic ribosome	24	2.8E-8	1.1E-6
GOTERM_MF_DIRECT	structural constituent of ribosome	34	2.1E-6	1.0E-3
GOTERM_CC_DIRECT	ribosome	27	2.8E-5	7.6E-4
GOTERM_CC_DIRECT	cytosolic small ribosomal subunit	13	6.4E-5	1.1E-3
GOTERM_MF_DIRECT	mRNA binding	55	3.0E-4	2.9E-2
Annotation Cluster 2	**Enrichment Score: 3.16**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	lignin biosynthetic process	9	1.6E-4	4.0E-2
GOTERM_MF_DIRECT	sinapyl alcohol dehydrogenase activity	5	2.9E-4	2.9E-2
Annotation Cluster 3	**Enrichment Score: 2.99**	**Count**	**P_Value**	**Benjamini**
GOTERM_CC_DIRECT	vacuolar membrane	34	4.4E-5	1.0E-3

Table 4
Up-regulated and down-regulated GO-term categories in R1 vs R0 comparison (rural area). Enrichment score, number of genes (count), p-value and Benjamini correction were indicated and obtained using DAVID software.

UP-Regulated categories				
Annotation Cluster 1	Enrichment Score: 8.86	Count	P_Value	Benjamini
GOTERM_BP_DIRECT	response to heat	40	3.8E-21	3.7E-18
GOTERM_BP_DIRECT	protein folding	33	1.5E-18	7.2E-16
GOTERM_MF_DIRECT	unfolded protein binding	22	1.3E-11	6.4E-9
Annotation Cluster 2	**Enrichment Score: 6.62**	**Count**	**P_Value**	**Benjamini**
GOTERM_MF_DIRECT	unfolded protein binding	22	1.3E-11	6.4E-9
GOTERM_BP_DIRECT	cellular response to unfolded protein	9	6.8E-9	2.2E-6
GOTERM_BP_DIRECT	protein refolding	11	9.8E-9	2.4E-6
GOTERM_MF_DIRECT	protein binding involved in protein folding	10	3.3E-8	8.0E-6
GOTERM_MF_DIRECT	misfolded protein binding	9	8.3E-8	1.3E-5
GOTERM_BP_DIRECT	chaperone mediated protein folding requiring cofactor	10	6.1E-6	6.7E-4
GOTERM_MF_DIRECT	heat shock protein binding	8	7.9E-6	6.3E-4
Annotation Cluster 3	**Enrichment Score: 5.09**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	protein oligomerization	8	1.9E-7	3.2E-5
GOTERM_BP_DIRECT	response to hydrogen peroxide	12	2.3E-6	2.8E-4
Annotation Cluster 4	**Enrichment Score: 4.48**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	unsaturated fatty acid biosynthetic process	7	1.7E-5	1.3E-3
GOTERM_MF_DIRECT	oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	6	2.4E-5	1.6E-3
Annotation Cluster 5	**Enrichment Score: 4.4**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	photosynthesis, light harvesting in photosystem I	9	1.2E-7	2.3E-5
GOTERM_MF_DIRECT	protein domain specific binding	13	5.6E-7	6.7E-5
GOTERM_BP_DIRECT	photosynthesis, light harvesting	8	1.5E-6	2.1E-4
GOTERM_CC_DIRECT	photosystem I	9	1.8E-6	1.2E-4
GOTERM_CC_DIRECT	plastoglobule	12	8.8E-6	4.2E-2
GOTERM_BP_DIRECT	protein-chromophore linkage	10	1.4E-5	1.3E-3
GOTERM_MF_DIRECT	chlorophyll binding	8	5.2E-5	2.8E-3
GOTERM_CC_DIRECT	photosystem II	8	2.4E-4	4.8E-3
Annotation Cluster 6	**Enrichment Score: 3.18**	**Count**	**P_Value**	**Benjamini**
GOTERM_MF_DIRECT	Hsp90 protein binding	7	1.6E-6	1.6E-4
Annotation Cluster 7	**Enrichment Score: 2.62**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	lignin biosynthetic process	9	6.7E-5	4.4E-3
Down-regulated categories				
Annotation Cluster 1	**Enrichment Score: 5.22**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_DIRECT	regulation of transcription, DNA-templated	59	1.2E-4	1.3E-2
Annotation Cluster 2	**Enrichment Score: 3.16**	**Count**	**P_Value**	**Benjamini**
GOTERM_CC_DIRECT	nucleosome	8	2.4E-4	1.5E-2

"turquoise". DEGs of the two pairwise comparisons belonged to different clusters and involved in primary metabolism, hormone regulation, abiotic stress, redox and transcription factors were indicated in Supplementary Table 8 and 9. Members of the first cluster were phosphoenolpyruvate carboxylase kinase 1, alpha-crystallin domain 32.1, HSP20-like chaperones superfamily protein, GRAS family transcription factor, B-box type zinc finger family protein. The following primary metabolism genes belonged to the "mediumpurple3" cluster: glyoxylate reductase 2, Glyceraldehyde-3-phosphate dehydrogenase-like family protein and glutamate receptor 3.4. The third highly correlated cluster "turquoise" was the most represented. Genes involved in primary metabolism and belonged to this cluster were the following: Leucine-rich repeat (LRR) family protein, aldehyde dehydrogenase 11A3, UDP-Glycosyltransferase superfamily protein, beta-amylase 1, NAD(P)H dehydrogenase B2, cellulose synthase 1. In addition, this latter cluster contained genes involved in hormone pathways such as cysteine-rich RLK (RECEPTOR-like protein kinase) 3, phytochrome kinase substrate 1, phosphoinositide 4-kinase gamma 7, sacI homology domain-containing protein, Histone H3 K4-specific methyltransferase SET7, Calcium-binding endonuclease/exonuclease/phosphatase family. Finally several transcription factors belonged to "turquoise" cluster such as two homeodomain-like superfamily proteins, CONSTANS-like 9, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein, B-box type zinc finger family protein, and auxin response factor 8.

4. Discussion

Typically, the choice of ornamental plant species grown in the urban environment is based only on decorative and functional effect. However, recent findings have also highlighted other possible criteria of plant selection based on the phylloremediation capability to remove air PM (Popek et al., 2018). Experimental works on plants cultivated in urban conditions under a real impact of PM are still scarce and more research on this topic is required. This work represents the first attempt to analyze the transcriptome responses of leaves from plants subjected to different PM levels trying to link transcriptional changes with the accumulation on leaves of different particulate matter sizes. The aim was to shed lights into the gene regulatory networks underlying plant molecular responses to particulate matter levels in urban environment, so as to: 1) identify key molecular markers (differentially expressed genes; DEGs) helpful to determine the health stress status of plants under high levels of air PMs, 2) find out genes involved in tolerance to air PMs and helpful for phytoremediation of air pollution in urban environment.

4.1. *Effects on primary metabolism*

Results dealing with PM showed that the two examined areas had significant differences of PM_{10} and PM_s with the traffic area having the highest levels of all PM sizes compared to rural area. Morphological leaf features such as hairs and waxes may enhance PM content (Jouraeva et al., 2002; Leonard et al., 2016). It has been hypothesized that PM present in the leaves may absorb, reducing free access of light rays to the chloroplasts (Hirano et al., 1995). Low size PM_s may clog the stomata, decreasing gas exchange (Cape et al., 2009), while chemically-active PM may influence physiological plant processes (Chauhan, 2010). Taking together all the transcriptome data, only a slight separation was observed between rural and traffic areas at T1 (three months of exposure) while the two plant groups were unseparated with high data

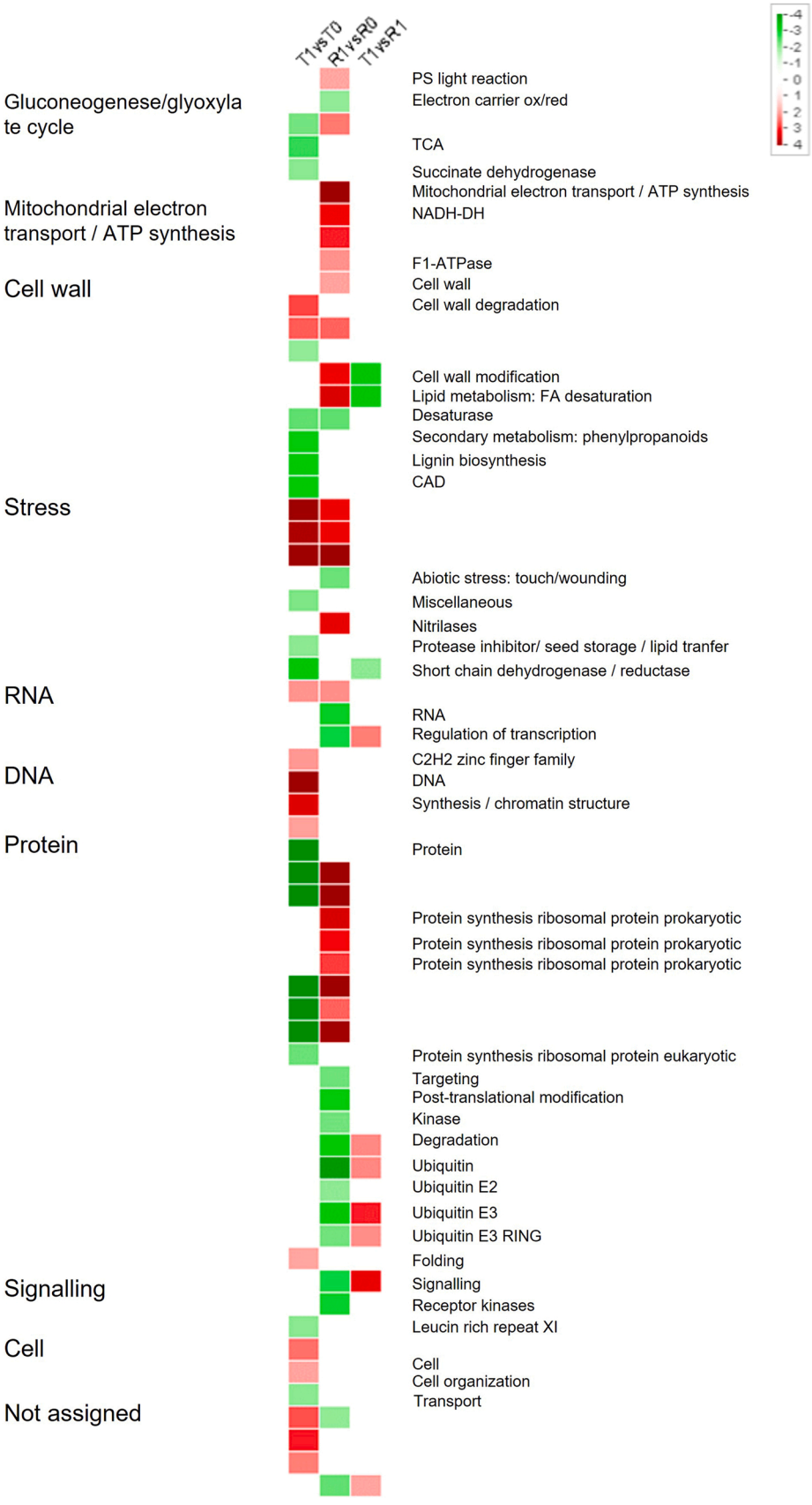

Fig. 4. Gene set enrichment categories for the three pairwise comparisons using Pageman web-tool. The gene categories upregulated (red) and down-regulated (green) in the three pairwse comparisons at two time-points in the two areas: traffic and rural, respectively T1 vs T0 and R1 vs R0. A third comparison was indicated at the same time point (1) between traffic and rural areas (T1 vs R1). Red means upregulated while green means down-regulated categories. The intensity of colour is correlated with the statistical change of significance based on Wilcoxon test default settings of Pageman.

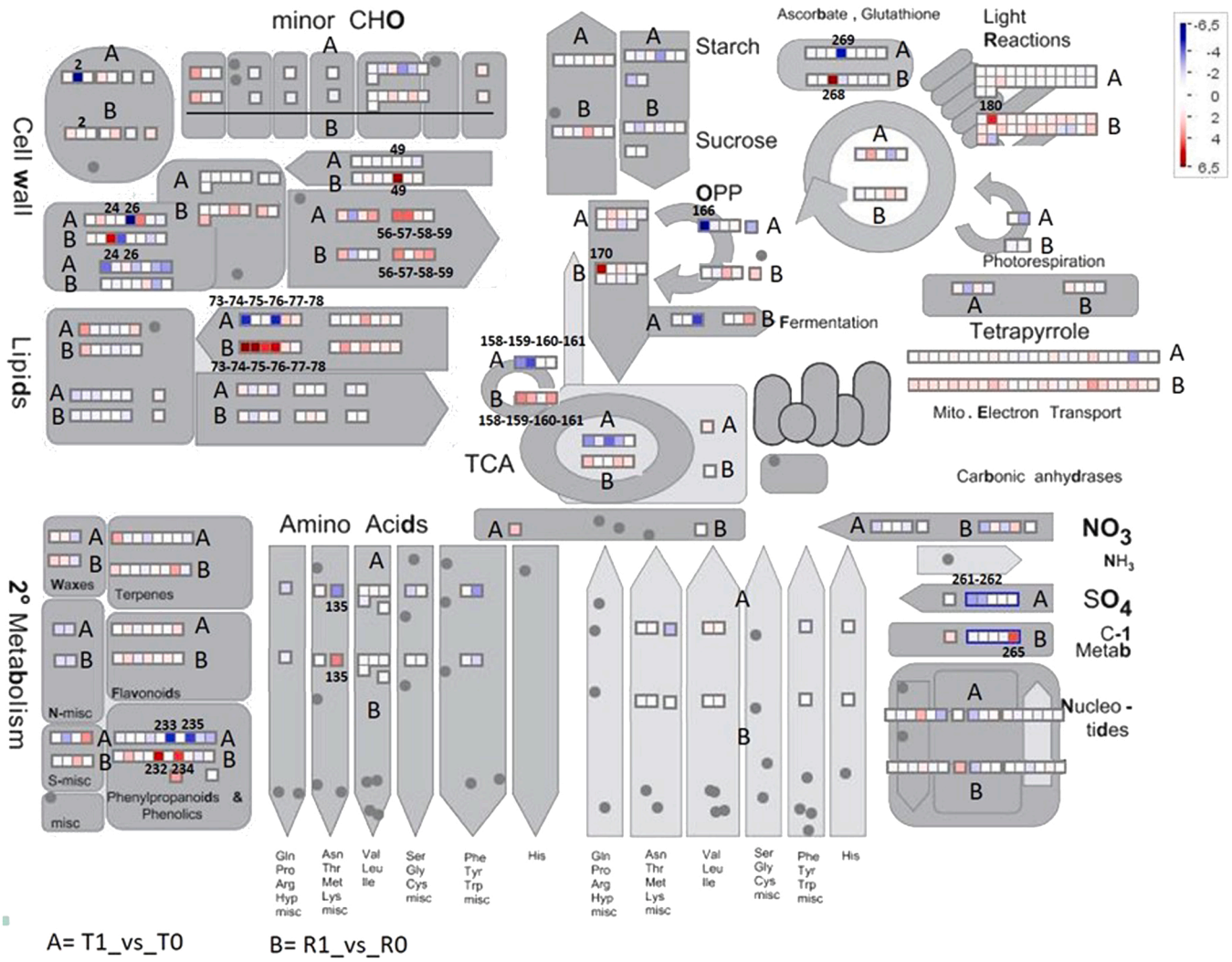

Fig. 5. Metabolism overview of differentially expressed genes in T1 vs T0 and R1 vs R0 comparisons. Red means upregulated and blue means downregulated in A (T1 vs T0) and B (R1 vs R0). Numbers in the figure refer to differentially regulated genes (red or blue) listed and annotated in Supplementary Table 4. In this supplementary file, the exact logFC is indicated for each gene highlighted with a number in this figure.

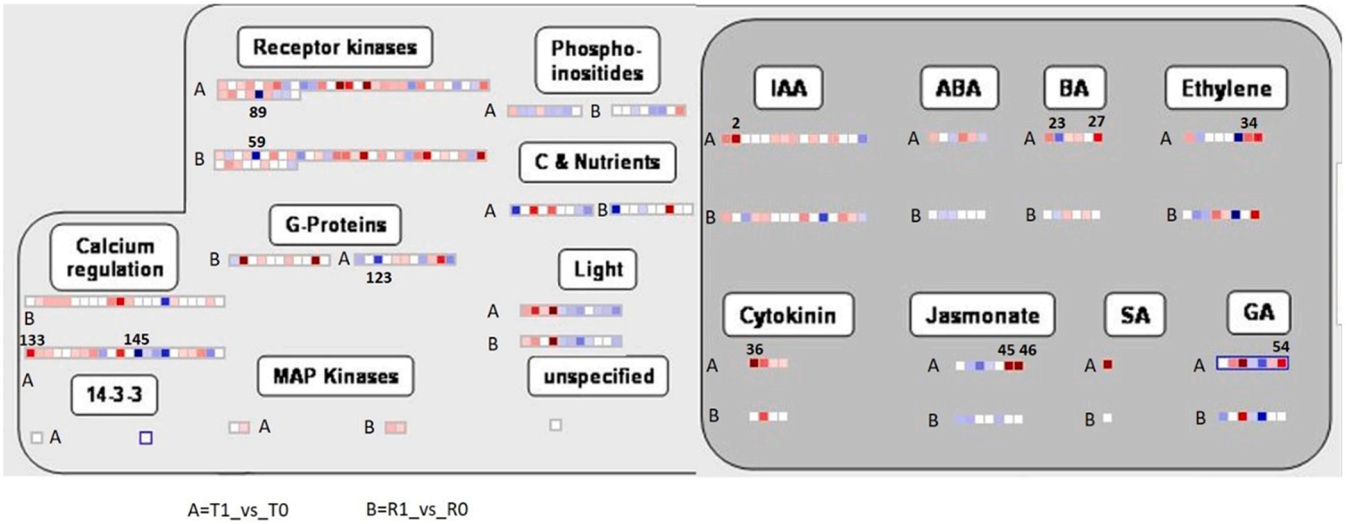

Fig. 6. Hormones and signaling. Regulation overview of the two pairwise comparisons (T1 vs T0 and R1 vs R0). Red means upregulated and blue means downregulated in A (T1 vs T0) and B (R1 vs R0). Numbers in the figure refer to differentially regulated genes (red or blue) listed and annotated in Supplementary Table 5. In this supplementary file, the exact logFC is indicated for each gene numbered and highlighted in the figure.

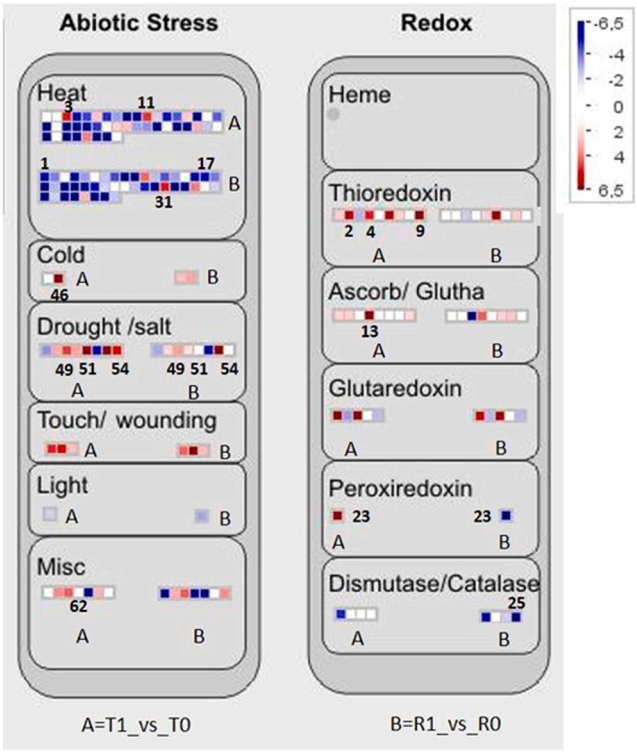

Fig. 7. Cellular responses. Regulation overview of the two pairwise comparisons (T1 vs T0 and R1 vs R0. Red means upregulated and blue means down-regulated in A (T1 vs T0) and B (R1 vs R0). Numbers in the figure refer to differentially regulated genes (red or blue) listed and annotated in Supplementary Table 6. In this supplementary file, the exact logFC is indicated for each gene numbered and highlighted in the figure.

variability at T0. This evidence has been found also for other abiotic and biotic stresses (Martinelli et al., 2012). However, the PCA performed on selected genes in common between two or three pairwise comparisons (R1 vs R0; T1 vs T0; T1 vs R1) showed a better separation of Traffic and Rural finding out genes that might be good candidate markers of leaf responses and health status to PM levels. PM seem to have a negative inhibitory effect on gene expression involved in photosynthesis. This finding was expected and might be related to the higher levels of PM deposited on leaf, tissues which consequently reduce the amounts of light received by the leaf chloroplasts and the photosynthetic machinery. This hypothesis found several corroborating data that have been published dealing with the impact of particulate matter accumulation on the photosynthetic apparatus of woody plants in urban conditions (Popek et al., 2018). This previous study showed that plants presented a lowered efficiency of their photosynthetic apparatus under higher PM_s, with a reduced photosynthesis rate and enhanced stomatal resistance. In addition, plants cultivated at higher PM showed a reduction of the F_v/F_m ratio and no changes in the total chlorophyll content. The photosynthetic rate in urban environment might be decreased by several factors such as pollution and soil structure, water availability, light and space limitations, and heat stress (Hanslin et al., 2017). A decreased rate of photosynthesis due to PM has previously been found in plants dusted with different compounds (Hirano et al., 1995; Kuki et al., 2008). A decrease of photosynthetic rate in two *Prunus* species was correlated with the different capability to accumulate PM (Popek et al., 2018). It has been hypothesized that PM may affect optical properties of leaves and reduce radiation levels reaching the chlorophyll antenna due to its absorption or reflection (Naidoo and Chirkoot, 2004; Nanos and Ilias, 2007). In addition, the inhibitory effect of PM on photosynthesis might be also linked to reduced gas exchange and disturbed light accumulation (Popek et al., 2018). We have also found some key genes repressed by PM that are involved in carbon assimilation and energy production through glycolysis, TCA and fermentation such as *glucose-6-phosphate dehydrogenase 5*, *6-phosphogluconate dehydrogenase family protein*, *aldehyde dehydrogenase 2B7*, *malate synthase*, *phosphoenolpyruvate carboxykinase 2*, *isocitrate lyase*. Carbohydrates are the direct metabolites produced by photosynthetic light and dark reactions. The repression of genes due to PMs implied that downstream pathways from photosynthesis involved in C assimilation are inhibited by PMs. Unfortunately the effects of air particulate matter produced mainly by smog of vehicles have been scarcely investigated at the transcriptomic level. Other dust particulate matter (iron) has shown a reduced pH and consequently impairs of the glycolysis process in two costal plant species (Kuki et al., 2008). Particulate matter levels were showed to affect the activity of several enzymes involved in photorespiration such as *glycolate oxidase* and *phosphoenol pyruvate carboxylase* (Mandal, 2006). This latter enzyme implies shifting from C3 to C4 pathway and subsequent decarboxylation of C4 acid to increase the CO_2 level inside the mesophyll cells, which is revealed here. Similar results were found in the pea leaves fumigated by SO_2 (Rao et al., 1983). The photorespiration increases should be due to the use of the excess amount of ATP produced in the light reactions to reduce the damage of photosynthetic apparatus due to over production of ATP (Lazar, 2003). However, our finding did not show any clear induction of photorespiration genes in response to higher PM. We observed higher expression of *UDP-glucose pyrophosphorylase* in rural environment while previous authors showed that under stress this enzyme together with *G6PD* were enhanced (Lazar, 2003). PM which is black in colour, may decrease incident light on the leaf surfaces thereby negatively affecting photosynthetic capacity and sugar content (Shabnam et al., 2021). This evidence is corroborated with our data, which showed higher transcript abundance for several key genes involved in pentose phosphate, glycolysis, starch biosynthesis and TCA in rural compared to traffic conditions. All these pathways consequently enhance levels of carbohydrates so the repression due to PM should be a consequence of the reduced photosynthetic activity of leaves due to shading, producing less reducing power and consequently inhibiting dark reactions of photosynthesis. The heavy metals present in PM_s and accumulated in the leaves, as well as shading, also reduce photosynthetic declining of the sugar content in the leaves causing smaller sized leaves. Another class of primary metabolism genes clearly repressed by higher PM encoded fatty acid desaturases (*Fatty acid desaturase family protein*, *16:0delta9 desaturase 2*, *delta 9 desaturase 1*). These genes are involved in the desaturation of cell membrane lipids allowing to enhance the fluidity and permeability of cell membranes. Fatty acids are important components of cellular membranes, suberin, and cutin waxes producing structural barriers to the environment (Beisson et al., 2007) and contributing to inducible stress resistance through the remodeling of membrane fluidity suitable for the function of important proteins involved in the photosynthetic machinery, during stresses (Iba, 2002). The modified levels of unsaturated fatty acids allow adjusting membrane lipid fluidity thanks to the modulated activity of fatty acid desaturases. In addition, free linolenic acid is a stress signal and the precursor for phytooxylipin biosynthesis (Blé-Castillo et al., 2002). Heavy metal accumulation typically upregulates enzymatic peroxidase activities and increased peroxidation of polyunsaturated fatty acids which may undergo resulting in damage and loss of membrane integrity.

4.2. *Effects on secondary metabolism*

Lower PM levels led to the expression of some key genes involved in phenylpropanoids and phenolics, flavonoids (*flavin-monooxygenase glucosinolate S-oxygenase 5*) and the induction of the *terpene synthase 14*. Phenylalanine and tryptophan are required for protein biosynthesis. In addition, phenylalanine is also a substrate for the phenylpropanoid pathway and the biosynthesis of secondary plant products, such anthocyanins, lignin, growth inhibitors and phenolics. The repression of some genes involved in amino acid biosynthesis by high levels of PM

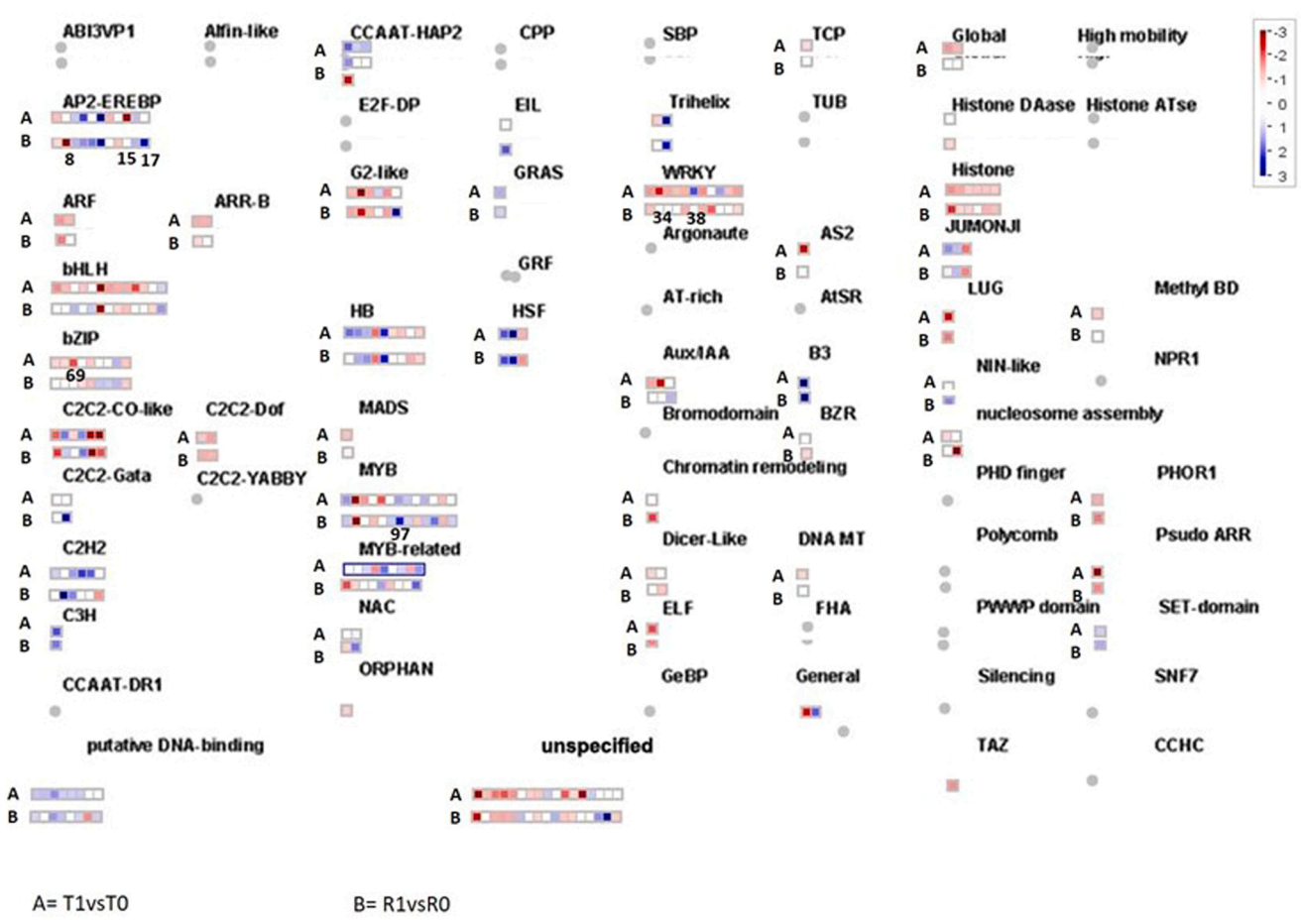

Fig. 8. Transcription factors of differentially expressed genes in T1 vs T0 and R1 vs T0 comparisons. Red means upregulated and blue means downregulated in A (T1 vs T0) and B (R1 vs R0). Numbers in the figure refer to differentially regulated genes (red or blue) listed and annotated in Supplementary Table 6. In this supplementary file, the exact logFC is indicated for each gene numbered and highlighted in the figure.

affected the secondary metabolism and the phenylpropanoid pathways. This was expected since we have examined a long-term response to chronic stress (three months). Indeed, plants that are subjected to PM stress for a long time with a consequent chronic inhibitory effect due to shading as previously described causes a reduction of photosynthesis and primary metabolism.

4.3. Hormone-related pathways

Relating to hormone pathways, we observed some key genes repressed by higher PM involved in cytokinin (*cytokinin oxidase 5*, *12-oxophytodienoate reductase*, *gibberellin carboxyl-O-methyltransferase*). In general, there were no clear differential modulation of any class of hormone-related genes. *Cytokinin oxidase 5* is a key gene involved in cytokinin metabolism. This class of hormones have not traditionally considered part of the physiological stress responses although recently, its crosstalk with ethylene and other "stress hormones" (jasmonate, salicylic acid and abscisic acid) has been documented as a possible receptor in abiotic stresses (O'Brien and Benkova, 2013). Cytokinins are known to protect the photosynthetic machinery, induce antioxidant systems, improve water balance control, modulate plant growth and cell differentiation, and contribute to the fine-tuning of stress-related phytohormones (Pavlů et al., 2018). Gibberellins and salicylic acid crosstalk play a key role in modulating plant tolerance to abiotic stress. They induce antioxidant activities in ROS scavenging, enhancing photosynthetic functions such as stomatal conductance, photosynthetic activity and efficiency of carboxylation. In addition, gibberellins modulate cell division and expansion of cell growth in plants under stress. Exogenous GA_3 can upregulate biosynthesis of SA enhancing plant defense response to environmental abiotic stresses (Emamverdian et al., 2020). Indeed, the repression of *gibberellin carboxyl-O-methyltransferase* at higher PM levels agrees with the repression of photosynthesis and primary metabolism previously described.

4.4. Environmental stress-related genes

Relating to the cellular stress responses, both conditions (rural and traffic) showed the induction of genes involved in heat stress, such as *HSP17.8*, *HSP17.6II*, *HSP15.7*, *HSPA2*, *HSP70*. This was expected since the experimental trial was started in spring and concluded at the beginning of summer, when temperature was higher. Indeed, the induction of different members of heat shock proteins were expected as protectant of unfolding processes of cellular proteins caused by increased temperature (Yang et al., 2020). A very similar trend of expression between the two areas was observed here, suggesting that different levels of PMs are not responsible for activating heat stress categories. Abiotic stress caused by PMs did not seem to be linked to the folding protection typically caused by well-known chaperones and other proteins in previous studies (Zhou et al., 2022; Xue et al., 2015). A similar trend of expression was also observed between T and R for genes well-known to be involved in cold and drought/salt stresses such as *cold shock domain protein 1* (repressed), *ERD4* and *ERD15* (repressed), *RD22* (induced), and *wound-responsive family proteins* (induced) (Zhang et al., 2020; Ma et al., 2021). Conversely, particulate matter is expected to

promote an oxidative process due to the presence of heavy metals deposited in the leaves. Heavy metals stress causes oxidative damage in plant tissues with several phyto-toxic effects such as the inactivation of enzymes,and biomolecules (AbdElgawad et al., 2020; Huihui et al., 2020). In addition, different heavy metals (including those found in PM such as lead (Pb) and cadmium (Cd)) are responsible for extensive genome-wide modulation of the plant transcriptome (Dubey et al., 2014). Oxidative stress is typically due to higher levels of free radicals and molecules containing reactive oxygen species (ROS) (Mishra et al., 2021). Their presence induces lack of electrons with a consequent damage to cell compounds and growth inhibition (Demidchik, 2015). Although we observed slight differences between rural and traffic conditions, some transcripts encoding cytochrome B5 isoform C, key catalases (*Catalase 2* and *3*) and *peroxiredoxin type 2* were higher in abundance in rural rather than of traffic conditions. In addition, some of the redox genes were repressed or less induced in the traffic area compared to the rural area such as *CXXS1* (*protein disulfide isomerase*), *ATTRX4* (*oxidoreductase*), and *peroxiredoxin type 2*. Unfortunately, there are no reports for the direct effects of PMs on the modulation of these genes. However, these genes have been shown to be linked to responses to heavy metal stresses. Cytochromes P450 (P450s) are one of the largest families of genes that played a key role in evolution in relation to the plant capability to produce plant secondary metabolism and in response to abiotic stresses (Paquette et al., 2009). Functional works in A. thaliana have demonstrated that P450-type metabolizing FA enzymes are involved in the biosynthesis of protective biopolymers (cutin, suberin and sporopollenin) and they are key player in the crosstalk between abiotic and biotic stress responses (Pandian et al., 2020a, 2020b). Particularly, the transgenic over-expression of CYP50-lke gene in Arabidopsis showed to enhance plant tolerance to heavy metals (Rai et al., 2016). In addition, genes belonging to the cytochrome B5 families, expressed in transgenic Medicago sativa, showed to enhance the plant capability to withstand a PM-related mercury contamination via enhanced metabolism (Zhang et al., 2013). Catalases showed to play important roles in mechanisms of response/tolerance against Cu (+2) and Zn(+2) heavy metal stresses in eggplant (Soydam-Aydın et al., 2015), nanoparticles in tomato (Noori et al., 2020), cadmium stress in Arundo donax L. (Sicilia et al., 2020). Peroxiredoxin genes showed to alleviate cadmium stress during BABA priming (Hossain et al., 2012) and thiol-reductase activity enhanced heavy metal stresses in Arabidopsis (Cha et al., 2022). The slighter high upregulation of these genes in R compared to T might be considered contradictory at first glance. However, it may be explained with the type of stress that we have analyzed. Plants were chronically exposed by PMs causing a depressive effect on the plant transcriptome. It is expected that plant transcriptional regulation should have been repressed by traffic conditions during the three-month period while these plants should have activated defense response at early stage (range of few days from the transplantation). These negative effects were observed at morphological level. We speculate that the expected increase of oxidative stress responsive genes might generally occur at shorter time when plants are healthy and actively respond to abiotic stresses. On the other hand, rural conditions are not pollution-free as we have previously highlighted. The levels of PM significantly increased after three months even in this area. Indeed, the presence of PM in rural conditions highlighted a response to plants against oxidative stress and this explained the induction of several key oxidative stress categories implying that these limits are probably under the inhibitory threshold.

4.5. Transcription factors

Regarding transcription factors, some minor differences were observed dealing with members of the AP2-EREBP family which resulted to be more upregulated at rural conditions in comparison to traffic ones. Members of the AP2-EREBP family have been linked to different abiotic stress through the recognition of specific *cis* elements in target promoters (Kizis et al., 2001) and involved in hormone-dependent gene expression. This family of TFs are unique to plants and are characterized by highly conserved regions of about 60–70 amino acids (AP2 domain). DRE-binding proteins contain specific residues within their AP2 domain that may determine their ability to bind the DRE/C-repeat element (Shinozaki and Yamaguchi-Shinozaki, 2000). Both DREB1 and DREB2 factors are modulated by drought or cold stress. The *DREB1* genes are induced by cold stress whereas the *DREB2* homologues are upregulated by drought and high salt stress (Gilmour et al., 1998). The upregulation of these genes in response to particulate matter highlights the different roles of these members for responses to different environmental stresses. The evidence that two of these genes were induced at rural conditions and not in traffic one suggest that they should play a role in the modulation of downstream defense genes in relation to moderate levels of PM, while higher levels may repress them as occur for most of the previously cited genes. *ERF110* is a gene responding to ethylene signaling, mediating ethylene-regulated transcription of genes involved in the biosynthesis of ethylene (Tao et al., 2018). Indeed, the repression of this gene in rural conditions and not in traffic area suggest that higher PM ethylene might activate defense responses involved in the activation of defense responses against negative effects of air pollution.

In addition, some bZIPs, C2H2, C2C2-Gata and putative DNA binding members were slightly induced at rural conditions. Except for these differences, a similar trend of expression was observed for most of the other families. A zinc finger (C2H2 type) family protein was enhanced at lower PM conditions. This gene belonged to the plant C2H2 zinc finger transcription factors which are involved in plant development and the responses to environmental stresses (Zhang et al., 2020). Many C2H2-type TFs act as transcriptional activators or repressors to regulate plant responses to different abiotic stresses such as salt stress and drought by increasing the levels of osmotic adjustment molecules. They are also involved in cold stress tolerance directly regulating downstream cold-related genes in plants (Han et al., 2014). Abiotic stress typically enhances the production of ROS causing secondary damage to plants. C2H2 zinc finger proteins may have a role in helping to maintain stable ROS levels in plants (Gadjev et al., 2006) and by modulating the expression of antioxidase genes linked to ROS scavenging, such as *Ascorbate peroxidase 1* which is an important scavenger of H_2O_2 in plants (Liu et al., 2017). It has been hypothesized that different C2H2-type zinc finger proteins are key players in the modulation of antioxidative stress responses via ABA or MAPK signaling. C2H2 TFs modulate early responses to SA and multiple abiotic stresses (Jiang and Pan, 2012). The different modulation of C2H2 members between the two locations suggest that they play a role in the response to air particulate matter.

4.6. Protein-protein interaction networks in response to PM

The protein-protein interaction network revealed that *BTF3* was clearly affected by air PM. This gene in known to be induced to salt stress and the transgenic expression of *BTF3* enhances tolerance of transgenic plants to reactive oxygen species (ROS) through the activation of scavenging activity, enhancing higher cell membrane stability and seedling survival under osmotic stress (Pruthvi et al., 2017). *BTF3* is also involved in the modulation of cold stress response. The phosphorylation of *BTF3* promoted the interaction of this gene with CBF proteins, increasing their stability and positively modulating CBF genes involved in the activation of defense responses to cold stress (Praat et al., 2021). *PGY1* has been linked to oxidative stress (Bansal et al., 2021). Indeed, a possible role in antioxidant responses against air PM in plants may be hypothesized. The PPI network showed the presence of two cinnamyl alcohol dehydrogenase (CAD) genes. These genes are involved in the final step of lignin biosynthesis, reducing hydroxycin- namaldehyde precursors into their corresponding hydroxycinnamyl alcohols (monolignols) (Martin et al., 2019). These compounds are then exported to cell walls through oxidative radical coupling mediated by laccases and peroxidases (Tobimatsu and Schuetz, 2019). Based on these previous findings we

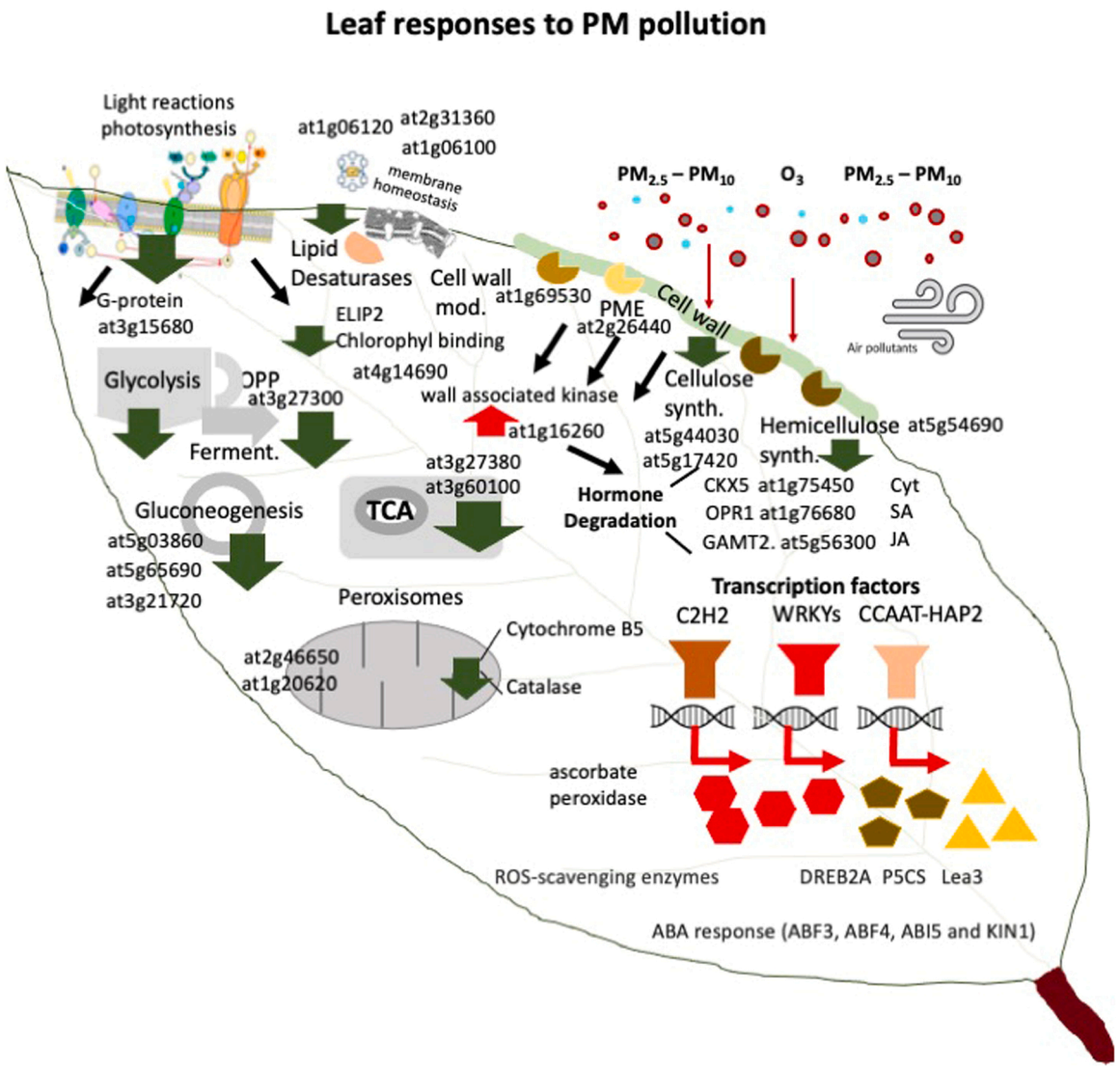

Fig. 9. Main transcriptomic changes in response to PM in leaves of *Laurus nobilis* (L.). Green down arrows mean repression while red up arrows mean general induction. Arabidopsis orthologs of key DEGs for the main classes of gene categories were indicated. PM generally decrease transcript abundance of genes belonging to key categories of the primary metabolism (photosynthesis, glycolysis, gluconeogenesis, TCA) and involved in cell wall modification (cellulose synthases, hemicellulose synthases) while some transcription factor families were upregulated.

hypothesize that air particulate matter should promote a restructuring process of the cell wall through the modulation of CAD genes which interact with other proteins (*HER2*, *ALDH10A8*, Groes-like zinc binding dehydrogenase family protein) as shown by the PPI network.

5. Conclusions

A summarized picture of the main gene regulatory networks affected by PM levels in leaf cells is provided in Fig. 9. Our results suggest that higher PMs induced an intense cell wall restructuring due to the main repression of several key genes encoding pectin-methyl esterase, cellulose and hemicellulose synthases. The other clear effect was on primary metabolism: a repression of several key genes involved in glycolysis, gluconeogenesis and TCA cycle was observed. This may lead to the repression of defense responses (i.e. catalases and other peroxisome-related genes). On the other hand, some members of the WRKYs, C2H2, CAAT-HAP2 transcription factors were induced by higher PM levels and these might affect the typical defense responses against abiotic stresses such as the proline and soluble sugars accumulation. This study sets the stage to the identification of key genes involved in PM response in leaves in order to improve biotechnological strategies for enhancing phylloremediation of air PMs in highly polluted urban environments.

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CRediT authorship contribution statement

Chiara Vergata: Validation, Investigation, Writing – original draft, Writing – review & editing. **Felice Contaldi:** Validation, Software, Investigation, Writing – review & editing, Validation, Writing – original draft. **Ivan Baccelli:** Investigation. Writing – review & editing. Funding acquisition. **Matteo Buti:** Software, Investigation, Writing – review & editing. **Alberto Vangelisti:** Software, Writing – review & editing. **Tommaso Giordani:** Software, Writing – review & editing. **Francesco Ferrini:** Investigation, Writing - review & editing. **Federico Martinelli:** Conceptualization, Investigation, Writing – original draft, Writing –

review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2023.105304.

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