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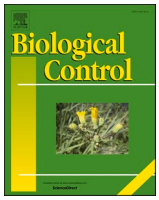

Impact of *P. Mediterranea* Pre-Treatment on Genome-Wide DNA methylation in *Plenodomus tracheiphilus*-Inoculated lemon (*Citrus limon* L.) plants

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HIGHLIGHTS

- P. mediterranea alters the methylation status of the CHH context of promoter regions.
- Genes were grouped based on the opposite trends between expression and methylation.
- Both hypo/hyper methylated genes are implicated in coinciding biological processes.
- A negative regulator of plant immunity is a promising candidate for genome editing.

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ABSTRACT

Lemon (*Citrus limon* L. Burm.) is widely cultivated in the Mediterranean and Black Sea regions where it is challenged by mal secco (MSD), a disruptive disease caused by the *Plenodomus tracheiphilus* fungus. The application of biocontrol agents (BCAs) has garnered growing interest due to the necessity of mitigating pathogen-induced yield losses while reducing the reliance on harmful substances for pathogen control. *Pseudomonas mediterranea* strain PVCT 3C pre-treatment has revealed a promising strategy to cope with mal secco disease with positive impacts mainly achieved by impeding the fungus-induced reprogramming of lemon leaf transcriptome. In this study, the effect of *P. mediterranea* PVCT 3C pre-treatment upon the DNA methylation status of fungal inoculated lemon plants was investigated via whole genome bisulfite sequencing (WGBS). The study reveals that PVCT 3C application mainly alters the methylation status of the CHH context of promoter regions. In the plants pre-treated with the biocontrol agent and *P. tracheiphilus* inoculated vs plants *P. tracheiphilus* inoculated comparison (3CPt vs Pt), a total of 1,873 differentially methylated regions (DMRs) were identified, while DESeq2 analysis of transcriptomic data detected 1,072 differentially expressed genes (DEGs). The integration of transcriptomic and methylation data allowed the retrieved genes to be grouped based on the opposite trends between expression and methylation. Most of them are implicated in coinciding biological processes, suggesting their key role in plant resilience induced by the bacterial BCA treatment. Among the hypermethylated/downregulated genes, a calcium-binding protein negatively regulating plant immunity was identified, representing a favorite candidate for genome editing application in lemon biotechnological breeding.

1. Introduction

Lemon (*Citrus limon*) is the third most important citrus species worldwide, with a global production, together with limes, amounting to 23.6 million tonnes (FAOSTAT, 2023). In the Mediterranean and Black Sea basins, Turkey is the major producing country (2.3 million tonnes in 2023), followed by Spain (1.1 million tonnes) and Italy (473 thousand tonnes). In these areas, lemon cultivation is threatened and severely

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limited by a devastating vascular disease known in the Italian language as "Mal secco" disease – literally "dry disease" (Migheli et al., 2009; Nigro et al., 2011; Catara and Catara, 2019). Mal secco disease (MSD), caused by the mitosporic fungus of the Leptospheriaceae family, *Plenodomus tracheiphilus*, is a vascular disease that can seriously affect the citrus industry worldwide due to both its dramatic economic impact and its ability to potential disseminate towards disease-free countries (Migheli et al., 2009; Nigro et al., 2011; Health (PLH), 2014; Picard et al., 2018; PM 7/048 (3) *Plenodomus tracheiphilus* (formerly Phoma tracheiphila, 2015). The pathogen is considered of quarantine concern by plant protection organizations worldwide, although it is currently present in the majority of countries in the Mediterranean basin and in some of the Black Sea (Picard et al., 2018; Zhao et al., 2021; "EPPO Global Database," 2025). Infected plants exhibit symptoms such as leaf chlorosis, wilting, defoliation, and dieback of twigs and branches. The disease causes a progressive drying of shoots, starting from the tips and moving backward and, in severe cases, the infection can lead to a significant reduction in fruit yield and even to plant death (Migheli et al., 2009; Nigro et al., 2011; Catara and Catara, 2019.). The primary host for *Plenodomus tracheiphilus* is *Citrus limon* (lemon), although the pathogen also affects other citrus species, including *C. medica* (citron), *C. bergamia* (bergamot), *C. aurantifolia* (lime), *C. aurantium* (sour orange), and *C. jambhiri* (rough lemon) (Catara and Catara, 2019; Nigro et al., 2011; Russo et al., 2021). Notably susceptible rootstocks include *C. aurantium* (sour orange), prevalent in Italy, Greece, and Turkey, *C. jambhiri* (rough lemon), *C. volkameriana* (Volkamer lemon), and *C. macrophylla* (alemow). Conversely, rootstocks such as *C. reshni* (Cleopatra mandarin), *Poncirus trifoliata* (trifoliate orange), and citranges (*C. sinensis* × *P. trifoliata*) demonstrate reduced susceptibility (Catara and Catara, 2019; Puglisi et al., 2019; Russo et al., 2020; Rovetto et al., 2024). The exploitation of genetic tolerance through less susceptible plant varieties and rootstocks is an effective strategy for managing the disease. However, despite efforts in traditional breeding and biotechnological techniques, no varieties have yet been developed that combine resistance to mal secco with high fruit quality (Gentile and La Malfa, 2019; Poles et al., 2020; Catalano et al., 2021). As a result, the management of MSD relies primarily on preventive measures, including using healthy plants from certified nurseries and applying copper-based compounds after pruning or injuries caused by natural events (Deng et al., 2009; Tamm et al., 2022). Beneficial microorganisms, such as biocontrol agents (BCAs) or plant biostimulants, are among the most promising solutions to ensure plant health and product quality. BCAs, which are microorganisms that target plant pathogens, have been shown to interfere with pathogen life cycles through direct antagonism or by inducing plant resistance, thereby exerting indirect control (Conrath et al., 2015; Kalai-Grami et al., 2016; Palmieri et al., 2022; Kelbessa et al., 2023). Recent research has demonstrated the effectiveness of various biocontrol agents (BCAs) against harmful fungal pathogens (Panebianco et al., 2025), leading to the availability of BCA-based bioformulations on the market, with some awaiting regulatory approval (Ghadamgahi et al., 2022; Lahlali et al., 2022). Lately, several studies have demonstrated the effectiveness of biocontrol agents also in combating MSD. It has been shown that commercial biological products based on *Bacillus amyloliquefaciens* are effective against *P. tracheiphilus* both in vitro and in vivo experiments (Aiello et al., 2022). Specifically, application of these biological products significantly reduces disease incidence and symptom severity in *C. volkameriana* seedlings (Aiello et al., 2022). Leaf-spray application of *Pseudomonas mediterranea* strain PVCT 3C suspensions significantly reduces disease incidence and severity in highly susceptible citrus hosts, with the strongest effects observed during the early infection stages (Dimaria et al., 2024). In addition, genome sequencing of *P. mediterranea* strain PVCT 3C identified biosynthetic clusters for known antimicrobial metabolites and potential novel compounds, highlighting its potential disease-suppressive capacity (Dimaria et al., 2024). RNA-seq analysis of lemon leaf revealed that *P. mediterranea* leaf-spray application preceding fungal inoculation is able to restrict pathogen replication during the asymptomatic phase of the disease, significantly diminishes the expected transcriptome reprogramming in response to fungus and prevents the deregulation of important metabolic pathways such as photosynthesis (Sicilia et al., 2022; 2023; 2024a; 2024b). In recent years, with the continuous advancements in genetics and molecular biology technology research, studies have revealed that epigenetic mechanisms, such as histone modification, chromatin rearrangement, DNA methylation, acetylation, and noncoding RNAs act as key players in plant defense responses against pathogens through the modification of host gene expression (Mehdi et al., 2025; Gupta and Salgotra, 2022; Hannan Parker et al., 2022). Epigenetic mechanisms also play a role in the development of stress-induced environmental epigenetic memory and priming in plants, allowing them to retain past molecular experiences and leverage this information to adapt to new conditions (Mierziak and Wojtasik, 2024). Among the epigenetic mechanisms, DNA methylation plays a crucial role in regulating gene expression involved in defense against biotic stress to enhance their resistance and boost productivity. Hence, it has been shown that treatment of rice plants with DNA methylase inhibitors such as 5-azacytidine or 5-aza-deoxycytidine causes activation of disease-resistance genes and the development of disease resistance in rice plants (Akimoto et al., 2007; Atighi et al., 2020), confirming that low levels of DNA methylation can contribute to the activation of gene expression. Similarly, studies carried out on *Arabidopsis thaliana* reveal that mutant strains bearing defective DNA demethylation process exhibit compromised MAMP-triggered immunity, resulting in susceptibility to bacteria pathogens (Yu et al., 2013; Huang et al., 2022). In contrast, mutants with impaired both RNA-directed DNA methylation (RdDM) and DNA methylation maintenance exhibit high resistance to the *Pseudomonas syringae* pv. tomato DC300 (pst) compared to the wild type (Dowen et al., 2012; Yu et al., 2013). More recently, Zou et al. (2025) reported that sDNA (extracellular self DNA) can trigger PTI responses and might contribute to DNA demethylation and enhance disease resistance in peach. In citrus, DNA methylation was shown to be a plant defense mechanism to cope with *P. citrophthora* gummosis infection (Rodrigues da Silva et al., 2021). In addition, Chen et al. (2024) demonstrated that treatment with 5-azacytidine activates defense-related enzymes and directs carbon allocation towards lignin biosynthesis, which is crucial for citrus defense against *Penicillium italicum*. In recent years, there has been a growing interest in exploring the complex interactions between BCAs and modification of plant gene expression, this being probably driven by BCA's potential applications in sustainable agriculture and crop improvement. However, direct manuscripts connecting BCA activity to specific epigenetic modifications remain still absent and this matter remains almost unexplored. In this scenario, our study aimed to identify the changes in the DNA methylation (5 mC) levels of the lemon whole-genome DNA induced by *P. mediterranea* PVCT 3C pre-treatment in *P. tracheiphilus* infected plants would fill this knowledge gap. Moreover, considering the aforementioned effect of PVCT 3C in strongly limiting the transcriptome reprogramming of fungus inoculated plants, the whole genome bisulfite sequencing (WGBS) data were integrated with lemon leaf transcriptomic data in order to detect those genes that resulted both differentially methylated and expressed in response to bacterial-treatment. As far as we know, this is the first study correlating the *P. mediterranea* biocontrol activity with epigenetic regulation of plant gene expression in order to unveil its role in avoiding or tolerating mal secco disease.

2. Materials and methods

2.1. Experimental scheme, preparation of Pseudomonas mediterranea PVCT 3C cell-based bioformulation and fungal pathogen inoculum

The experimental scheme is described in Sicilia et al. (2023). Briefly, potted lemon 'Femminello Siracusano 2Kr' plants grafted onto *Citrus aurantium* (sour orange) kept in greenhouses at Agrobiotech srl

(Catania) were used for the inoculation. To evaluate the effect of the treatment with the biocontrol agent, the following theses were defined as follows: Pt thesis, plants aerial sprayed with water (three days and one day before fungal inoculation) and *P. tracheiphilus* inoculated; 3CPt thesis, plants pre-treated with the biocontrol agent by aerial spraying (three days and one day before fungal inoculation) and *P. tracheiphilus* inoculated. The bacterial inoculum was obtained from cultures of *P. mediterranea* strain PVCT 3C grown as reported in Sicilia et al. (2023). The inoculum of *P. tracheiphilus* (Petri) Gruyter, Aveskamp & Verkley consisted of vial phialoconidia of the PVCT Pt57 strain obtained as reported in Dimaria et al. (2023). The inoculation with the fungal pathogen was carried out as reported by Oliveri et al. (2022). Each leaf was inoculated in four spots. The leaves intended either for DNA or RNA extractions (three independent biological replicates) were collected at 7 DPI (days post fungal inoculation), which coincided with the early phase of the disease characterized by the absence of symptoms (Russo et al., 2021; Sicilia et al., 2023). DNA extraction and real-time PCR assay to confirm fungal infection were performed as described in Sicilia et al. (2023).

2.2. DNA methylation library sequencing

Genomic DNA was extracted by using the CTAB extraction protocol. After the genomic DNA samples were quantified, their integrity and purity were assayed by agarose gel electrophoresis. Library preparation and sequencing were performed by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). Libraries were constructed as follows: firstly, the genomic DNA spiked with lambda DNA were fragmented to 200–400 bp. Methylation sequencing adapters were ligated, followed by double strand DNA synthesis. The library was ready after size selection and PCR amplification and checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries have been pooled and sequenced on Illumina platforms. The quality of raw reads in fastq format was checked by FastQC (v0.11.5); raw reads were then trimmed using Trimmomatic-0.36 to filter out the contaminated adapter sequence and low quality reads. Bismark software v0.16.3 (Krueger and Andrews, 2011) was used to perform alignments of bisulfite-treated reads of the *Citrus limon* L. Burm f. genome v1.0 (https://www.citrusgenomedb.org/Analysis/1470606) (Di Guardo et al., 2021) reference genome (accessed on 17 June 2022). The process of Bismark performing alignment was as follows: perform C-to-T and G-to-A conversions for both sequencing reads and the reference genome, align converted reads to the converted genome and choose the best alignment deriving from the parallel four alignment processes. Raw reads were submitted to SRA under BioProject ID PRJNA1422645.

2.3. DNA methylation data analysis and DMRs identification

After alignment, Bismark (Krueger and Andrews, 2011) was used to identify methylated sites. Firstly, PCR duplicates have been removed and then the methylation state of cytosines was identified by comparing the read base and the reference genome base at the same position. If the read base is C, it suggests that this cytosine is methylated; otherwise, it is unmethylated. The following thresholds were set to find accurate methylated sites (Habibi et al., 2013, Gifford et al., 2013): (1) the sequencing depth is greater than or equal to five; (2) q-value less than or equal to 0.01. The methylation level was calculated as: ML = mC/(mC + umC), where ML is the methylation level, mC is methylated cytosine and umC is unmethylated cytosine. The average methylated level in different cytosine contexts was calculated in the following functional genomic regions: promoter or upstream 2 kb (the 2 kb region above Transcription Start Site, TSS), exon, intron and downstream 2 kb (the 2 kb region following the Transcription End Site, TES); exons and introns are obtained from the Ensembl structure annotation files. DSS-single (DSS_2.12.0) was used to analyze differentially methylated regions (DMRs) between samples. The factors considered in DSS analysis are: the spatial correlation of the methylation site, read depth of the sites and the variance among biological replicates. Evaluation of DMR distribution and DMR calling was then performed. DMR calling used Wald test on each site to obtain the significant value (p-value). The filtering conditions for DMR determination were: smoothing.span = 20; delta = 0; p.threshold = 1e-05; minlen = 50; minCG = 3; dis.merge = 100; pct.sig = 0.5. Annotation to DMR regions such as promoter, exon, intron, CGI, CGI shore, repeat, Transcription Start Sites (TSS) and Transcription End Sites (TES) was retrieved, and the information of gene name was provided in the case that the DMR was within a gene region. Furthermore, to deduce the potential functions of the differentially methylated coding sequence (DMG), the sequences were aligned against the NCBI core nucleotide database (core_nt) and the *Citrus* genus subset of the NCBI nt database using BLASTn, with a significance cut-off E value of 10^{-5}. Gene enrichment analysis was performed on DMRs distributed in the genome in gene body regions (from TSS to TES) and in different sequence contexts (CG/CHG/CHH). ShinyGO V0.82 online tool (Ge et al., 2020) was used for GO enrichment analysis.

2.4. Transcriptomic data

Unpublished transcriptomic data from our previous experiment (Sicilia et al., 2023), publicly available in the NCBI GEO database (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE227934, were incorporated into this study to establish associations between differentially expressed genes (DEGs) and differentially methylated genomic regions in plants pre-treated with the *P. mediterranea* strain PVCT 3C as BCA. Differential expression analysis was performed using the R DESeq package (version 1.12.0, padj $\leq$ 0.05, https://bioconductor.org/packages/release/bioc/html/DESeq.html) considering the comparison 3CPt vs Pt (samples pre-treated with PVCT 3C and fungus inoculated versus samples fungus inoculated). The resulting padj values were adjusted using the Benjamini and Hochberg approach to control for the false discovery rate (FDR) (Benjamini and Hochberg, 1995). Genes with a padj value $\leq$ 0.05 ($-\log_{10}$padj $>$ 1.3) were assigned as differentially expressed. GO enrichment analysis of differentially expressed genes (either up- or down-regulated) was implemented by ShinyGO V0.82 online tool (Ge et al., 2020). The validation of the RNA-seq experiment by RT real-time PCR is reported in Sicilia et al. (2023).

2.5. Combined DNA methylation and expression analyses

The coding sequences of the DEGs obtained by the *de novo* transcriptomic analysis were aligned to the *Citrus limon* L. Burm f. genome v1.0 (https://www.citrusgenomedb.org/Analysis/1470606), the same database used for WGBS analysis. DEGs and DMGs lists were then merged using *Citrus limon* Region ID as a common column between the two datasets, allowing to obtain a unique list of genes that are both differentially expressed and differentially methylated Then, the DEGs and DMGs were combined based on their opposite trends in gene expression and methylation. Specifically, genes were classified into two groups: upregulated/hypomethylated genes and downregulated/hypermethylated genes. In this last case no thresholds for the differential expression and methylation measurement have been applied to avoid limiting the analysis only to a few genes. Statistical significance for DEGs and DMGs is maintained using specific thresholds and p-value cutoffs as detailed above.

3. Results

3.1. WGBS analysis overview of Citrus limon leaves

To explore the effect of BCA upon DNA methylation of *Plenodomus tracheiphilus* infected lemon plants, both 3CPt and Pt samples were analyzed through whole-genome bisulfite sequencing (WGBS). The clean data were collected after filtration and an average clean ratio of

89.01% was obtained (Table S1). An average of 12 million mapped reads was calculated for each sample, and the Q20 and Q30 percentages, indices of sequencing accuracy, were 96.71% and 90.24%, respectively. Furthermore, the bisulfite conversion rate was 99.74% (Table S1).

3.2. Analysis of dynamic changes in DNA methylation levels

Fig. 1 illustrates the circular genomic plots of lemon, highlighting cytosine methylation across the CG, CHH, and CHG contexts. It is also reported the gene density through the chromosomes, that corresponds to the number of transcripts in each bin. The plots compare methylation profiles of inoculated samples (Pt) and those pre-treated with the biocontrol agent *P. mediterranea* PVCT 3C prior to inoculation (3CPt). PVCT 3C exerts a clear impact on cytosine methylation in the CHH and CHG contexts. Specifically, pre-treatment (3CPt samples) results in chromosome widespread increase in cytosine methylation levels in the CHH context, especially on chromosome 2, while in the CHG context it leads to a reduction in methylation, predominantly localized on chromosomes 2 and 5. Conversely, the analysis indicates that the bacterial treatment pre-treatment slightly alters cytosine methylation patterns of the CG context, although this context was widely methylated across the genome in both conditions. The detected methylation alterations are localized in both high and low gene density regions, as shown in the bottom right circle plot (Fig. 1).

According to the location of the methylation sites on the reference genome and the gene location information on the reference genome, the genomic regions identified as upstream region (−2 kb) (also referred to as promoter), gene body (that include both exons and introns), and downstream region (+2 kb) were divided into 50 bins to count the methylation levels of different regions of the whole genome (Fig. 2). In Fig. 2A, referring to the CG context, methylation levels decrease progressively as they approach the gene body. Within the gene body, however, the methylation levels increase and remain very high, even in proximity to the 2 kb downstream region, where methylation levels fluctuate but never exceed those observed within the gene body. It is worth noting that there are no differences in the methylation profile between the Pt and 3CPt samples. In the CHG context (Fig. 2B), methylation levels in the 2 kb upstream region decrease as they approach the gene body, where they undergo a slight reduction, continuing to decline until reaching the 2 kb downstream region. In this area, a gradual increase in methylation is observed, resembling the trend seen in the CG context. As in the previous case, the methylation profile remains unchanged between the two theses. In the CHH context (Fig. 2C), the profile is similar to that observed for the CHG context,

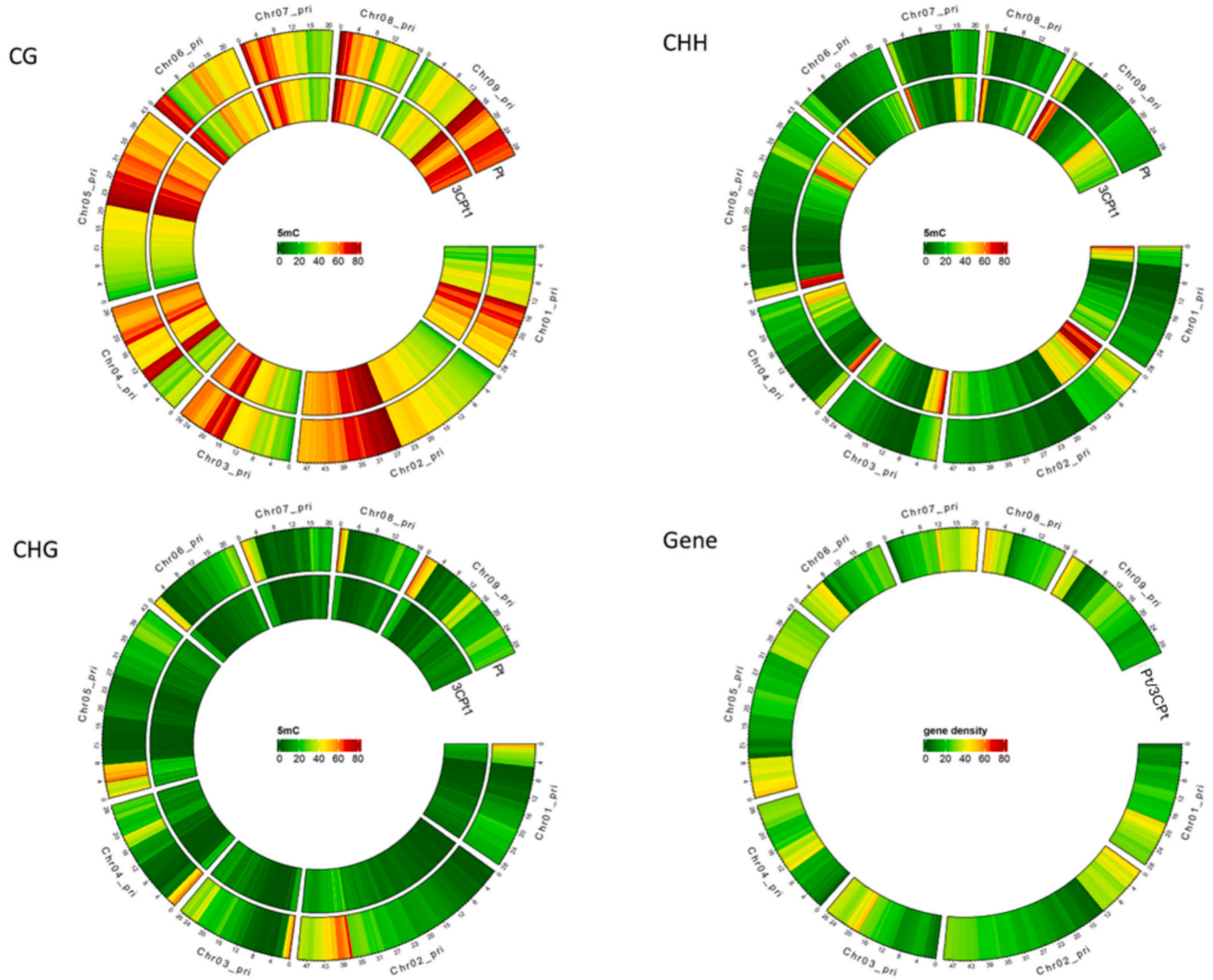

Fig. 1. Circos plots showing the methylation level on chromosomes of CG, CHH and CHG contexts and gene density of both fungus inoculated plants (Pt) and plants pre-treated with the biocontrol agent (3CPt). The green-to-red color represents the direction in which the level increased.

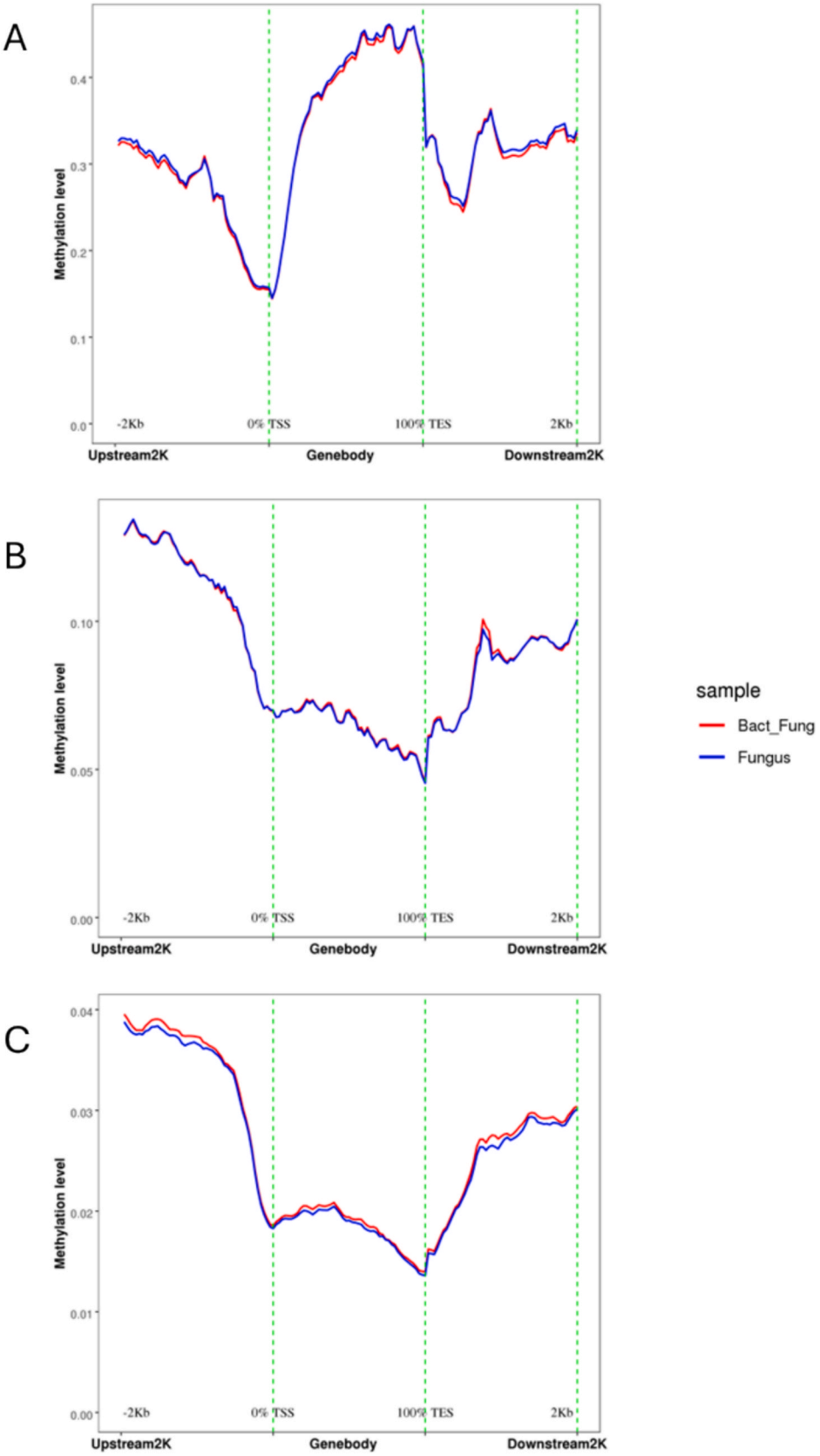

Fig. 2. Methylation levels in different regions of the genome (upstream, gene body, and downstream). A) CG methylation level, B) CHG methylation level, C) CHH methylation level. The red line corresponds to the 3CPt samples, the blue line corresponds to the Pt samples. The x-axis is the gene body and up/downstream region, which is calculated by bin, and the y-axis is the methylation level.

showing a reduction in methylation levels within the 2 kb upstream region, a slight decrease within the gene body, and an increase in methylation levels within the 2 kb downstream region as the distance from the gene body increases. However, the profile corresponding to the *P. mediterranea* PVCT 3C-treated samples (3CPt) displays higher methylation levels in this context, particularly in the 2 kb upstream and downstream regions. This result is consistent with the trends observed in the circular plots.

3.3. Differentially methylated region (DMR) analysis

A total of 1873 DMRs were identified in the 3CPt vs Pt comparison, including 992 hypomethylated and 881 hypermethylated regions (data not shown). Fig. 3 illustrates the distribution of DMRs across different CG, CHH and CHG contexts. Fig. 3A illustrates the number of hypermethylated regions in the 3CPt vs Pt comparison across the three contexts. Notably, the CHH context exhibits the highest number of hypermethylated regions (624), compared with the CG (195) and CHG (173) contexts. Most hypermethylated regions are context-specific, as only a small fraction of DMRs are shared among all three contexts. Similarly, hypomethylated regions (Fig. 3B) are predominantly found in the CHH context (397), followed by the CHG (179) and CG (143) contexts. Context specificity is also pronounced for hypomethylated regions, with few DMRs overlapping across contexts.

Differential methylation levels in the CG, CHG, and CHH contexts across promoter, intron, exon, TES, and TSS regions are shown in Fig. 4. The majority of differentially methylated sites were located within promoter regions (Fig. 4A), particularly in the CHH context (blue dots). However, the largest methylation differences, both hypo- and hypermethylation, were observed in the CG (red dots) and CHG (green dots) contexts. A similar trend was detected in intronic regions (Fig. 4B), although the number of differentially methylated regions was markedly lower than in promoters. In exonic regions (Fig. 4C), differential methylation followed an increasing trend from CHH to CHG and CG contexts, with the latter showing the highest absolute methylation changes. Only a small number of differentially methylated regions were identified in TES and TSS regions, with methylation differences evenly distributed among the three contexts (Fig. 4D).

Fig. 5 presents the results of the Gene Ontology (GO) enrichment analysis within the Biological Process category performed on differentially methylated genes (DMGs), obtained after DMRs coding sequences alignment against the NCBI nt database, within the 3CPt versus Pt comparison. The terms encompassing the largest number of genes include "Response to stress", "Macromolecule modification", "Defense response", and "Response to biotic stimulus", clearly suggesting that the BCA treatment might modulate the methylation status of genes within these categories related to BCA-lemon plant interaction.

Furthermore, DMGs whose NCBI annotation contains "Disease resistant proteins" were then filtered and are listed in Table 1. By applying a threshold of ± 0.30 of methylation difference, twenty DMGs have been retrieved, most of them (15) showing hypomethylation in the 3CPt samples. The encoded proteins (receptor-like kinases, RLKs; disease resistance proteins, NBS-LRRs and related; proteins involved in immunity signaling; receptor-like proteins, RLPs; tyrosine-protein kinases) are central to plant immunity, acting as sensors or intracellular immune receptors.

3.4. RNA-seq and WGBS integration

Taking advantage of previously performed transcriptomic analysis (Sicilia et al., 2023), DESeq2 analysis was applied, leading to the identification of 1072 differentially expressed protein-coding genes (610 upregulated and 462 downregulated DEGs) in the 3CPt vs. Pt comparison ($-\mathrm{Log}_{10}\mathrm{padj} > 1.3$) (Fig. 6A). A Gene Ontology (GO) enrichment analysis was performed on the up-regulated genes, revealing significant enrichment in Biological Processes primarily related to photosynthesis and light stimuli. Notably, categories like "Regulation of photosynthesis," "Chloroplast organization," "Photosynthesis," "Response to radiation," and "Response to light stimulus" demonstrated the highest average fold enrichment values, alongside the "Response to reactive oxygen species" category (Fig. 6B). Conversely, Fig. 6C illustrates the GO enrichment analysis for the down-regulated genes, which showed significant enrichment in Biological Processes such as "Response to jasmonic acid," "Response to fatty acid," and "Cellular response to oxygen-containing compound." Additionally, it is noteworthy that categories such as "Response to endogenous stimulus," "Response to hormones," "Cellular response to chemical stimulus", "Signal transduction," and "Signaling" were also enriched. Overall, the analysis highlights distinct functional enrichments for up-regulated genes, emphasizing processes related to photosynthesis and light stimuli, while the down-regulated genes are predominantly associated with responses to various chemical stimuli and signaling pathways.

In order to analyze the putative relationship between different levels of DNA methylation and gene expression, transcriptomic data were integrated to identify DMGs which were also DEGs in response to *P. mediterranea* strain PVCT 3C pre-treatment in *P. tracheiphilus* infected plants. Therefore, the DEG list (1072 DEGs) was intersected with the DMG list (1823 DMGs) by means of the unique *Citrus limon* L. Burm f. genome v1.0 Region ID (https://www.citrusgenomedb.org/Analysis/1470606). As a result, a total of 50 genes both differentially expressed and differentially methylated were obtained (Fig. 7A). Most of these genes are differentially methylated in the promoter region (30 genes) and in the CHH context (blue portion of the bars in the figure 7B).

Among these 50 genes, we recruited those showing an opposite trend

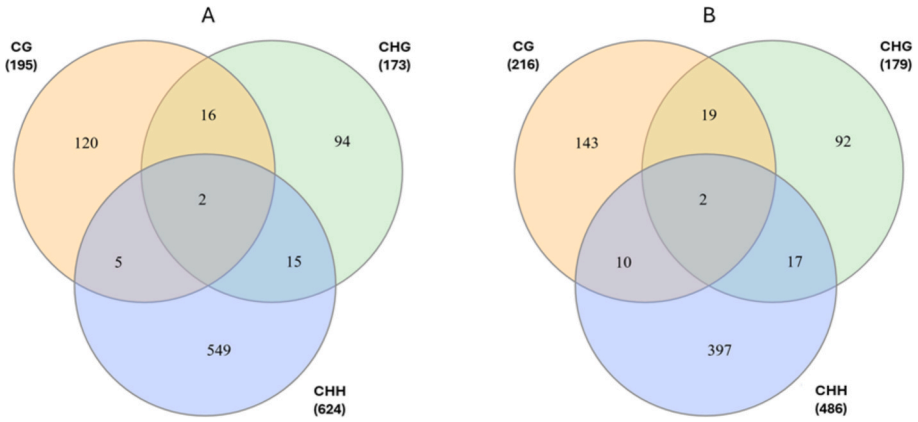

Fig. 3. Venn diagram of the differentially methylated regions (DMRs) between 3CPt and Pt samples. (A) Hypermethylated regions in 3CPt vs Pt comparison; (B) Hypomethylated regions in 3CPt vs Pt comparison. The different colors represent different methylation contexts and the numbers represent the number of DMRs.

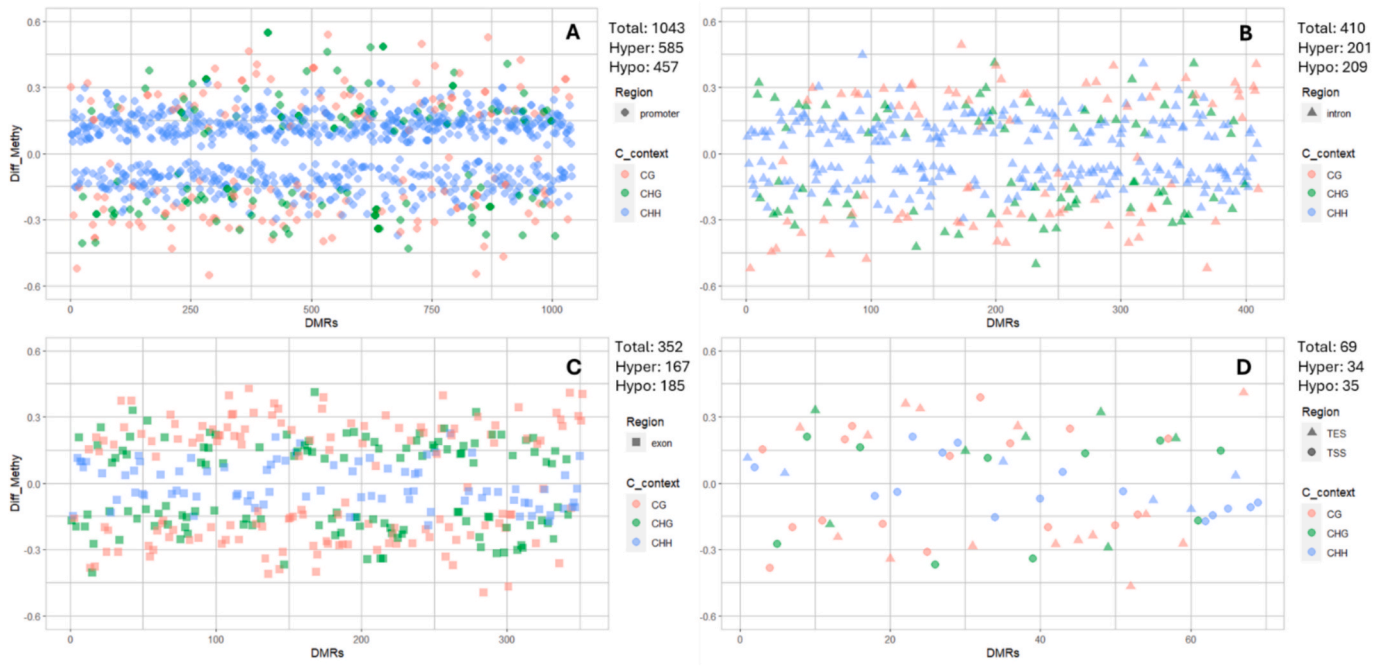

Fig. 4. Overview and distribution of the Differentially Methylated regions (DMRs). The X-axis reports the number of DMRs, the Y-axis the difference in methylation level between 3CPt and Pt. A) DMRs in promoter region; B) DMRs in intronic regions; C) DMRs in exonic regions; D) DMRs in Transcription Start Sites (TSS) and Transcription End Sites (TES).

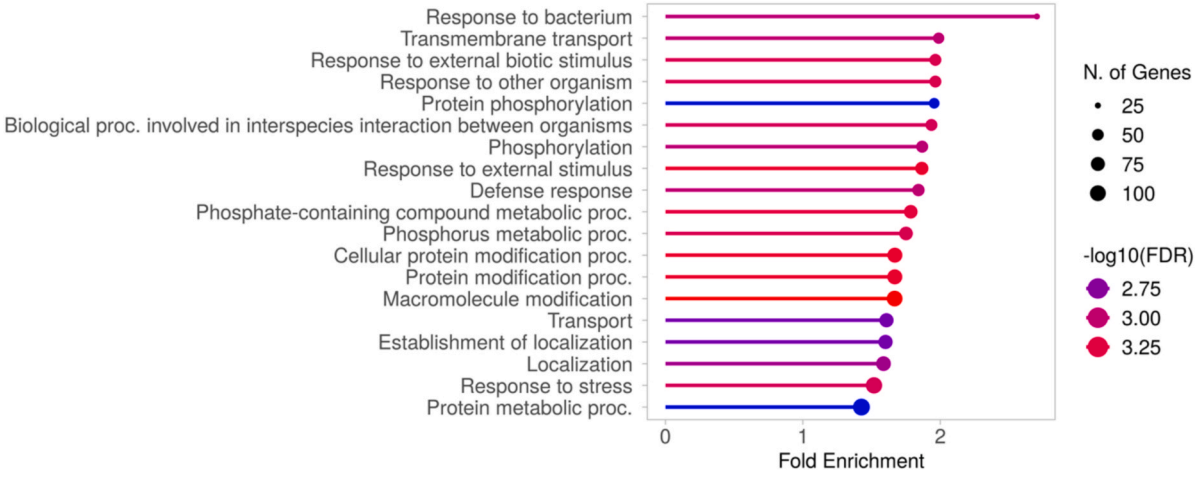

Fig. 5. GO (Biological Process) enrichment analysis scatter plot of the differentially methylated genes.

between gene expression and methylation. Consequently, they were classified into two groups based on their DNA methylation status and expression levels (Fig. 8). The first group included hypomethylated DMGs and upregulated DEGs (Fig. 8A). Similarly, the second group consisted of hypermethylated DMGs and downregulated DEGs (Fig. 8B). Based on these criteria, we identified 20 candidates in the first group (Fig. 8 A) and 11 candidates in the second group (Fig. 8 B); in both cases most of these genes were differentially methylated in CHH context and in the promoter region.

In Table 2 are listed the twenty genes resulting both hypomethylated and upregulated in the BCA-treated samples. The $\log_2$FC ranged from + 0.42 to + 2.74, while the differential methylation ranged from −0.04 to −0.5. Given the small number of genes, a detailed and precise analysis was conducted for the identification of their function. The gene encoding *C. clementine* multiple RNA-binding domain-containing protein 1 is involved in RNA binding thus influencing gene expression at the post-transcriptional level. It may be involved in the regulation of RNA processing events such as splicing, RNA editing, and polyadenylation. Under stress conditions, RNA-binding proteins can help modulate the expression of stress-responsive genes by altering RNA metabolism and translation (Ambrosone et al., 2012; Yan et al., 2022). Pentatricopeptide repeat (PPR)-containing proteins are a large family of proteins primarily known for their role in the regulation of organelle gene expression, particularly in mitochondria and chloroplasts in plants. PPR proteins are essential for the expression of genes involved in photosynthesis and respiration. Through their impact on organelle function, PPR proteins indirectly influence plant development and stress responses as proper organelle function is critical for energy metabolism and adaptation to environmental stress (Barkan and Small, 2014). A gene encoding clathrin assembly protein was also among the hypomethylated/upregulated genes. These proteins assist cells in responding to environmental stress through several mechanisms related to their roles in membrane

Table 1
List of DMGs annotated as disease resistant protein. A threshold of ± 0.30 was used for diff.Methy.

regionID	diff.Methy	C_context	region	Predicted Annotation*	e value	Identity
CL0G034387011	−0.41	CHG	promoter	*Cs* G-type lectin S-receptor-like serine/threonine-protein kinase LECRK2 (LOC102625840)	0	84.13%
CL0G035159011	−0.38	CG	promoter	*Cs* probable disease resistance protein At5g63020 (LOC107174607)	0	97.61%
CL0G035159011	−0,40	CHG	promoter	*Cs* probable disease resistance protein At5g63020 (LOC107174607)	0	97.61%
CL0G035160011	−0.38	CG	exon	*Cs* probable disease resistance protein At5g63020 (LOC107174607)	0	98.41%
CL0G035160011	−0.38	CG	TSS	*Cs* probable disease resistance protein At5g63020 (LOC107174607)	0	98.41%
CL0G035160011	−0.38	CG	promoter	*Cs* probable disease resistance protein At5g63020 (LOC107174607)	0	98.41%
CL0G035160011	−0.40	CHG	exon	*Cs* probable disease resistance protein At5g63020 (LOC107174607)	0	98.41%
CL0G035559011	−0.43	CG	intron	*Cs* disease resistance protein RPV1-like (LOC102624560)	0	93.63%
CL2G008557011	−0.31	CHG	promoter	*Cs* cysteine-rich receptor-like protein kinase 10 (LOC127900229)	5.00E-164	97.92%
CL2G008860011	+0.31	CG	intron	*Cc* probable LRR receptor-like serine/threonine-protein kinase At1g56140 (LOC18051905)	2.00E-94	99.48%
CL2G008860011	+0.31	CG	exon	*Cc* probable LRR receptor-like serine/threonine-protein kinase At1g56140 (LOC18051905)	2.00E-94	99.48%
CL3G009903011	−0.35	CHG	promoter	*Cc* disease resistance protein RGA2-like (LOC112097907)	0	96.18%
CL3G010655011	+0.31	CG	exon	*Cs* disease resistance protein At4g27190-like (LOC127902125)	0	87.83%
CL4G013782011	−0.36	CHG	promoter	*Cc* protein EDS1 (LOC18040572)	0	99.23%
CL5G019487011	−0.30	CG	exon	*Cs* disease resistance-like protein DSC1 (LOC107174807)	0	99.91%
CL7G025654011	+0.34	CHG	intron	*Cs* PTI1-like tyrosine-protein kinase 3 (LOC102619989)	4.00E-120	94.12%
CL8G028762011	+0.53	CG	promoter	*Cs* probable L-type lectin-domain containing receptor kinase S.5 (LOC102627877)	0	99.35%
CL8G028840011	−0.32	CHG	exon	*Cc* disease resistance RPP13-like protein 4 (LOC18046941)	0	97.11%
CL8G029105011	−0.31	CHG	exon	*Cs* receptor-like protein 15 (LOC102613465)	0	95.90%
CL8G029105011	−0.31	CHG	intron	*Cs* receptor-like protein 15 (LOC102613465)	0	95.90%

* *Cs* = *Citrus sinensis*; *Cc* = *Citrus clementina*.

trafficking and signaling such as regulation of receptor internalization, membrane protein turnover, nutrient uptake and redistribution, transport of stress-response molecules, signal transduction efficiency, and adaptation to osmotic stress (Chatukuta et al., 2018). Flavanone 3-dioxygenase 2-like plays a key role in the production of flavonoids, a diverse group of secondary metabolites in plants (Treutter, 2006). SDE2 (Suppressor of Defective Silencing 2) is involved in recognizing and responding to stalled replication forks, which can occur due to DNA damage or other hindrances that impede replication machinery. Its general function in replication stress response and DNA repair is crucial for protecting plants from genomic instability, especially under stress conditions that cause DNA damage (Xie et al., 2017; Lo et al., 2020). Disease resistance RPS2 proteins guard the plant against pathogens that contain an appropriate avirulence protein via an indirect interaction with this avirulence protein. That triggers a defense system including the hypersensitive response, which restricts the pathogen growth by interacting with RIN4 (Mackey et al., 2003). UDP-glycosyltransferase 79B3-like are part of the broader family of UDP-glycosyltransferases (UGTs), which play essential roles in the glycosylation processes in plants and other organisms. The key function associated with glycosylation of small molecules typically makes them more water-soluble and less reactive. UGTs can modify plant hormones thereby playing crucial roles in plant growth and development, fine-tuning the plant's defense responses, enhancing the ability to respond to specific threats (Bowles and Lim, 2010). KH domain-containing proteins are a family of RNA-binding proteins characterized by the presence of the KH (K-homology) domain playing significant roles in plant stress responses through their RNA-binding capabilities and regulatory functions contributing to stress resilience in plants (Lorković, 2009; Ambrosone et al., 2012). Cold and drought-regulated protein CORA is a type of protein typically found in plants that plays an important role in response to abiotic stresses, particularly cold and drought conditions. CORA proteins may contribute to reinforcing the general defenses, thus providing a supportive role in biotic stress contexts (Jha et al., 2021). Strictosidine synthase-like 2 (SSL2) proteins are known for their role in the biosynthesis of monoterpene indole alkaloids (MIAs). By contributing to the production of defensive alkaloids, SSL2 proteins play a role in plant defense mechanisms against pests and environmental stressors by inhibiting their growth (Wang et al., 2023). Thioredoxin reductase NTRC (NADPH-dependent thioredoxin reductase C) localizes primarily to chloroplasts, where it is involved in regulating the redox state of chloroplast enzymes. It is essential for balancing photosynthetic activity and protecting against oxidative stress induced by light, in response to oxidative stress, safeguarding plant cells against oxidative (Kim et al., 2017b). Nudix hydrolases comprise a large gene family of proteins, each containing a conserved motif capable of hydrolyzing specific substrates like ADP-glucose and NADH. These enzymes can help modulate cellular responses to stress by controlling the concentrations of important signaling molecules and metabolic byproducts that arise during oxidative stress or other stress conditions (Huang et al., 2012). Finally, plant endonuclease MutS2 proteins are crucial for responding to translational stress, such as ribosome collisions and stalling within chloroplasts. In its role in ribosome quality control (RQC), MutS2 uses its endonuclease activity to resolve collisions between ribosomes that stall during translation in the plastid (Broz et al., 2025).

In Table 3 are listed the eleven genes resulting both hypermethylated and downregulated in the BCA-treated samples. The log_2FC ranged from −0.28 to −5.14, while the differential methylation ranged from + 0.05 to + 0.2. Also in this case, given the restricted number of genes, a detailed and precise analysis was conducted for their identification. Among these hypermethylated and downregulated genes, a cytosolic sulfotransferase 15-like was discovered. Sulfotransferases assist plants in their defense against pathogens and stress by modifying small molecules, hormones, and secondary metabolites through the process of sulfation (Han et al., 2025). This action can enhance the plant's ability to respond to biotic (pathogen-related) (Baek et al., 2010) and environmental stresses (Chaudhary et al., 2024) in several ways such as modification of secondary metabolites, hormone regulation (jasmonic acid and salicylic acid), detoxification and removal of pathogen-derived toxins, strengthening of cell walls and biosynthesis and modification of molecules that act in signaling pathways, enabling rapid activation of defense mechanisms when the plant is under attack. Retinoblastoma-related protein-like (RBR) encoding genes play a role in plant stress responses (Chen et al., 2014), although their primary function is in cell cycle regulation and developmental processes. RBR proteins can interact with plant hormonal signaling pathways that are crucial for stress responses (Hamid et al., 2025), and may influence the expression of genes related to stress responses by interacting with E2F transcription factors (Zaragoza et al., 2024). In some stress scenarios, RBR proteins can participate in regulating programmed cell death (PCD) by influencing the related pathways and gene networks, and contribute to maintaining genome stability by regulating DNA repair mechanisms and ensuring accurate DNA replication once stress is alleviated (Zaragoza et al., 2024). MDIS1-interacting receptor like kinase 2-like encoding genes

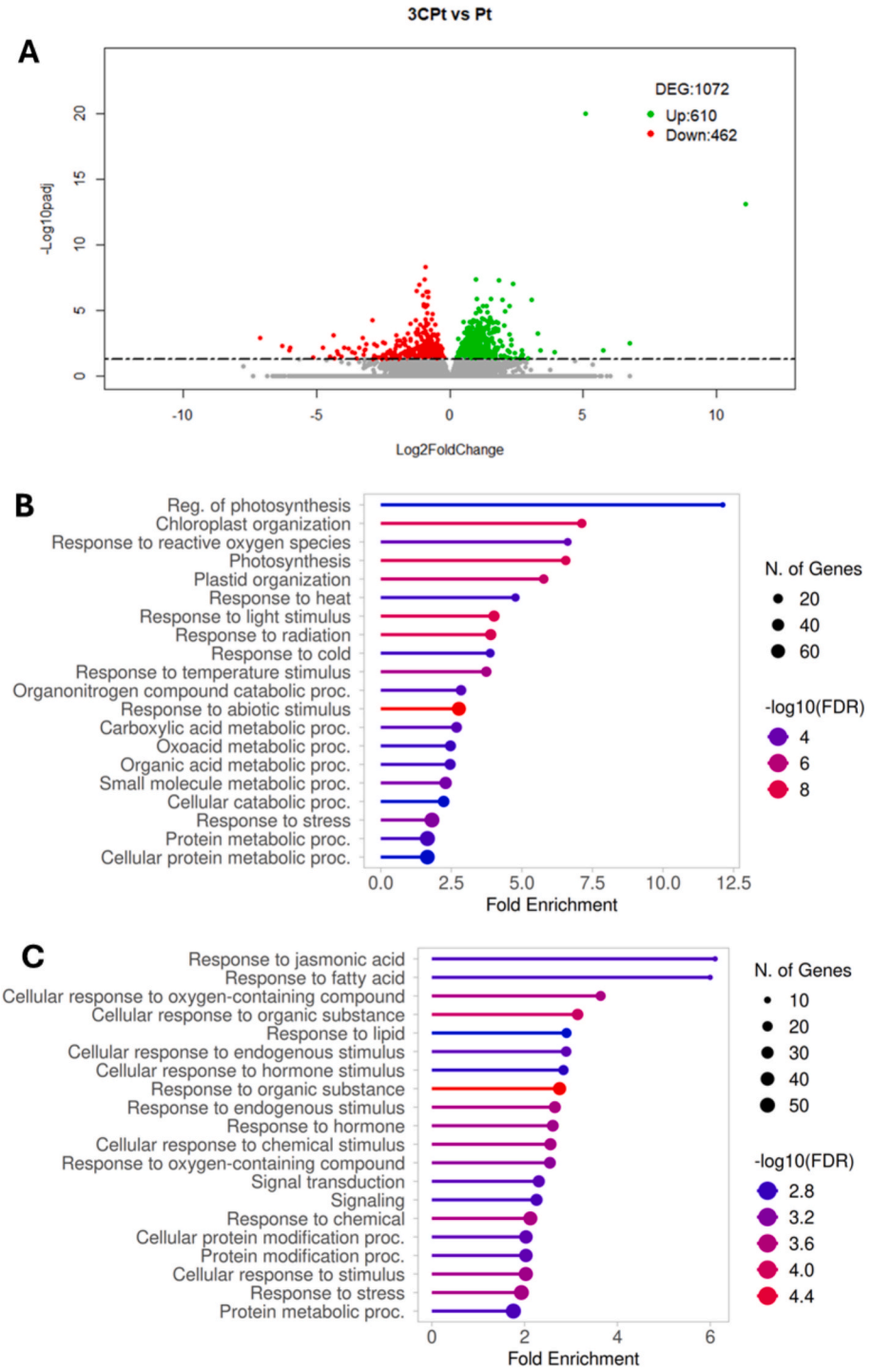

Fig. 6. A) Volcano plot of the differentially expressed genes (DEGs) in the comparison 3CPt vs Pt; Gene Ontology Biological Process functional enrichment of B) up-regulated genes and C) down-regulated genes.

(also referred to as MIRK2-like) play crucial roles in plant cellular communication and signal transduction, and are often implicated in plant immune responses (Coleman et al., 2021). They help recognize pathogen-associated molecular patterns (PAMPs) and initiate PAMP-triggered immunity (PTI), a crucial part of the plant's defense strategy (Sun and Zhang, 2020). Calcium-binding protein CML47 encodes a calcium-binding protein that likely plays a role in negative regulation of plant immunity by suppressing the accumulation of salicylic acid and immune responses (Lu et al., 2018). DNA replication licensing factor MCM7 gene is a crucial part of the machinery that ensures DNA is replicated precisely once per cell cycle. Although primarily known for its role in DNA replication, MCM7 are identified as hub genes associated with responses to both abiotic (drought, salinity) and biotic (viral, fungal) stresses, suggesting a broad role in stress adaptation (Tuteja et al., 2011; Razalli et al., 2025). A gene encoding xanthohumol 4′-O-methyltransferase was also both hypermethylated and downregulated. It plays a crucial role in the modification of flavonoids by transferring a methyl group to hydroxyl groups on flavonoid structures (Nagel et al., 2008). This modification can alter the solubility, stability, and biological activity of the compounds. PELOTA 1 is involved in a critical cellular

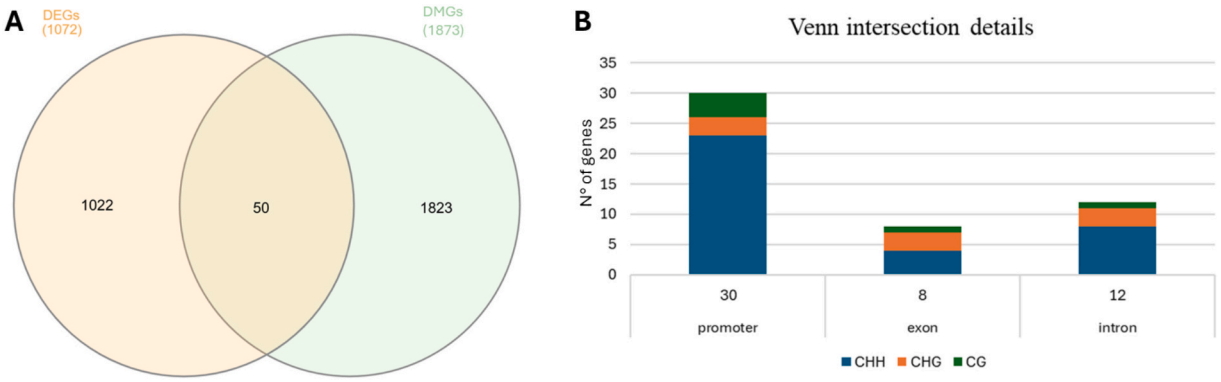

Fig. 7. A) Venn diagram of DEGs and DMGs. The intersection contains genes that are both differentially expressed and methylated. B) Context and region details of the genes in the intersection of the VENN diagram.

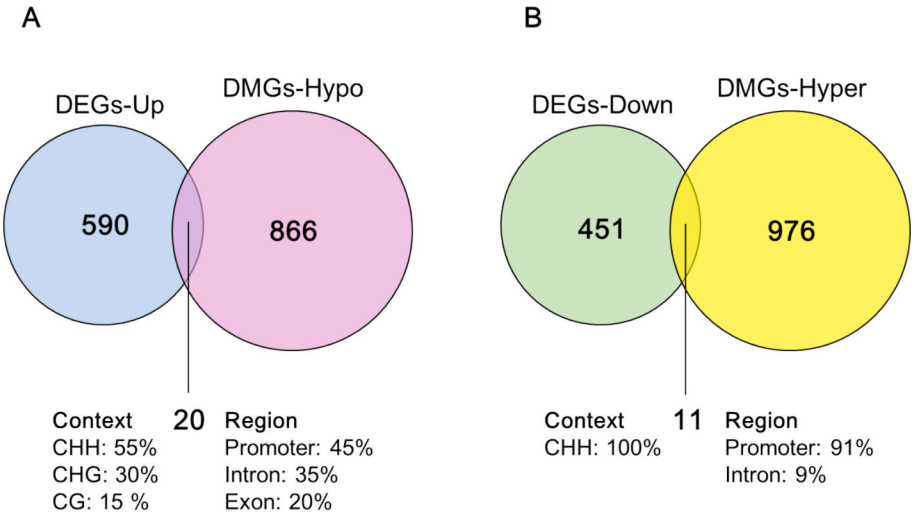

Fig. 8. Venn diagram illustrating the relationship between RNA-seq and WGBS data. (A) Overlapping sets of upregulated protein-coding genes (610) and protein-coding genes associated with hypomethylated DNA (886). (B) Overlapping set of downregulated protein-coding genes (462) and protein-coding genes associated with hypermethylated DNA (987).

Table 2
List of up-regulate protein coding genes presenting hypo-methylated regions.

regionID	region	C_context	diff.Methy	log_2FC	Predicted Annotation*
CL1G001212011	exon	CHH	−0.04	1.21	*Cc* multiple RNA-binding domain-containing protein 1 (LOC18050766)
CL9G031870011	promoter	CHH	−0.05	0.79	*Cs* 30S ribosomal protein S1 (LOC102616265), transcript variant X4
CL4G014566011	promoter	CHH	−0.06	0.60	*Cc* pentatricopeptide repeat-containing protein At2g15690, mitochondrial (LOC18055623)
CL9G031870011	intron	CHH	−0.07	0.79	*Cs* 30S ribosomal protein S1 (LOC102616265), transcript variant X4
CL3G011058011	promoter	CHH	−0.08	0.48	*Cc* putative clathrin assembly protein At4g40080 (LOC18037655)
CL3G011059011	intron	CHH	−0.08	0.70	*Cs* flavanone 3-dioxygenase 2-like (LOC102613536)
CL5G021123011	intron	CHH	−0.10	0.48	*Cs* replication stress response regulator SDE2 (LOC102619656)
CL2G007609011	promoter	CHH	−0.11	0.88	*Cs* disease resistance RPS2 protein At4g27220 (LOC112496770)
CL4G014079011	promoter	CHH	−0.1	1.32	*Cs* UDP-glycosyltransferase 79B3-like (LOC102620938)
CL5G018446011	exon	CHG	−0.15	0.84	*Cs* endonuclease MutS2 (LOC102631445), transcript variant X5
CL5G018446011	intron	CHG	−0.15	0.84	*Cs* endonuclease MutS2 (LOC102631445), transcript variant X5
CL3G009350011	promoter	CHG	−0.15	0.42	*Cs* KH domain-containing protein At1g09660/At1g09670 (LOC102624747)
CL5G016490011	exon	CHG	−0.16	0.57	*Cc* uncharacterized LOC18047795 (LOC18047795), transcript variant X2
CL7G026188011	promoter	CHH	−0.16	1.40	*Cc* cold and drought-regulated protein CORA (LOC18054952)
CL3G009380011	intron	CHH	−0.17	2.74	*Cc* uncharacterized LOC18034732 (LOC18034732)
CL9G032050011	promoter	CHG	−0.23	0.76	*Cs* protein STRICTOSIDINE SYNTHASE-LIKE 2 (LOC102621854)
CL9G032377011	promoter	CG	−0.23	0.54	*Cs* thioredoxin reductase NTRC (LOC102626278), transcript variant X1
CL4G013340011	intron	CG	−0.33	1.12	*Cs* nudix hydrolase 20, chloroplastic-like (LOC102625343)
CL0G035212011	exon	CG	−0.37	0.85	*Cs* glutelin type-D 1-like (LOC102616079), transcript variant X2
CL5G018446011	intron	CHG	−0.50	0.84	*Cs* endonuclease MutS2 (LOC102631445), transcript variant X5

* *Cs* = *Citrus sinensis*; *Cc* = *Citrus clementina*.

quality control pathway known as the stalled ribosome rescue mechanism during translation. Through this role, PELOTA 1 is crucial

Table 3
List of down-regulate protein coding genes presenting hyper-methylated regions.

regionID	region	C_context	diff.Methy	log_2FC	Predicted Annotation*
CL3G010098011	promoter	CHH	0.20	−3.98	*Cs* cytosolic sulfotransferase 15-like (LOC102616051
CL2G004604011	promoter	CHH	0.19	−0.28	*Cs* retinoblastoma-related protein-like (LOC102611926
CL9G031976011	promoter	CHH	0.16	−3.56	*Cs* MDIS1-interacting receptor like kinase 2-like (LOC107174686)
CL5G017292011	promoter	CHH	0.16	−5.14	*Cs* probable calcium-binding protein CML47 (LOC107176795)
CL5G020768011	promoter	CHH	0.16	−1.05	*Cs* DNA replication licensing factor MCM7 (LOC102629070
CL0G034964011	promoter	CHH	0.14	−0.70	*Cs* hypothetical protein (LOC102617021)
CL1G001155011	promoter	CHH	0.13	−0.70	*Cs* uncharacterized LOC102619583 (LOC102619583
CL9G031483011	promoter	CHH	0.12	−2.12	*Cc* xanthohumol 4′-O-methyltransferase (LOC18032212)
CL6G021913011	promoter	CHH	0.11	−0.79	*Cs* protein PELOTA 1 (LOC102617256)
CL2G003710011	intron	CHH	0.07	−3.81	*Cs* linoleate 13S-lipoxygenase 2–1, chloroplastic (LOC102625429
CL1G001113011	promoter	CHH	0.05	−0.86	*Cc* ADP-ribosylation factor 1 (LOC18035757)

* *Cs* = *Citrus sinensis*; *Cc* = *Citrus clementina*.

for maintaining cellular homeostasis (Li et al., 2025). Linoleate 13S-lipoxygenase 2–1, chloroplastic (LOX) plays pivotal role in contributing to plant stress responses by participating in the metabolism of polyunsaturated fatty acids (PUFAs), ensuring the efficiency and integrity of protein synthesis machinery. This process leads to selective chloroplast destruction, facilitating nutrient remobilization from aging leaves to seeds and roots. Moreover, 13-LOX initiates the biosynthesis of jasmonic acid (JA) and green leaf volatiles (GLVs), both of which are vital for plant defense and signaling. ADP-ribosylation factor 1 (ARF1) is primarily linked to vesicular trafficking and membrane dynamics, which are essential for the proper localization of stress-related proteins and hormones during stress adaptation (Ganotra et al., 2023). Small GTPases, including ARF1, also act as molecular switches in signaling pathways that help plants manage environmental stress, including pathogen attack, by interacting with downstream effectors to regulate stress-responsive genes and physiological processes, contributing to stress tolerance (Ganotra et al., 2023; Kim et al., 2017a). Considering their log_2FC, ranging from −3,56 to −5.14 (Table 3), cytosolic sulfotransferase 15-like, MDIS1-interacting receptor like kinase 2-like, linoleate 13S-lipoxygenase 2–1, and especially calcium-binding protein CML47 could play a pivotal role during plant infection.

4. Discussion

A biological control agent can suppresses or interfere with the growth, survival, or infection processes of one or more plant pathogens, thereby reducing disease incidence or severity. The application of BCAs (o BCAs-based treatments) may improve plant's ability to fend off pathogens through various mechanisms, including competition with pathogens for space and nutrients, which benefits the plant (Baker, 1974; Raymaekers et al., 2020; Lahlali et al., 2022). Previously, we assessed how pre-treatment with *P. mediterranea* strain PVCT 3C impacted the transcriptome of lemon leaves infected with *P. tracheiphilus* (Sicilia et al., 2023). Mapman analysis revealed that PVCT 3C prevented the deregulation of several genes belonging to various metabolic pathways and processes such as “Photosynthesis”, “Secondary metabolism”, “Coenzyme metabolism”, “Multiprocess regulation”, “Cellular respiration”, “Phytohormone action – biosynthesis, perception and degradation” and “External stimulus response – pathogen – effector triggered immunity (ETI) machinery” (Sicilia et al., 2023). Moreover, both the measurements of fungal DNA and disease severity showed a significant reduction following *P. mediterranea* pre-treatment, thus serving as effective indicators for both mal secco disease and the efficacy of BCA intervention (Sicilia et al., 2024b). DNA methylation, a common epigenetic regulation, mainly occurs at stable and easily accessible cytosine residues. Changes in DNA methylation in response to stimuli can be long-lasting, and even potentially be inherited onto subsequent generations (Zhang et al., 2018; Zhou et al., 2025). In plants, cytosine methylation occurs in three distinct sequence contexts: cytosine-guanine (CG), cytosine-H-guanine (CHG), and cytosine-H-H (CHH), where H indicates adenine (A), cytosine (C), or thymine (T). These adjustments are managed by DNA methyltransferases and demethylases classified into *de novo* methyltransferases and maintenance methyltransferases, each playing complementary roles in the DNA methylation dynamics (Lodhi and Srivastava, 2025). Within plant-pathogen interactions, reducing methylation levels in genes linked to defense responses is particularly crucial as it promotes their transcriptional activation, thereby boosting the plant's capacity to resist pathogens (Yu et al., 2013). In contrast, increased methylation of these genes often results in partial or complete transcriptional silencing, compromising the plant's defensive capabilities (Wang et al., 2018), although it's crucial to recognize that, regardless the significance of differential DNA methylation as an epigenetic regulator in plants, it is not the only factor influencing gene expression (Neto et al., 2017). However, a very restricted number of manuscripts reports the effect of biocontrol agents upon the DNA methylation status of the host genome, despite it is widely reported that the beneficial effect of BCAs upon plant resilience passes through a transcriptomic reprogramming (Sicilia et al., 2023, 2024b). In this study, the impact of a bacterial BCA pre-treatment on the genome-wide DNA methylation in *P. tracheiphilus* inoculated lemon (*Citrus limon* L.) plants was evaluated by whole-genome bisulfite sequencing (WGBS). Specifically, *P. mediterranea* pre-treatment (3CPt samples) results in chromosome widespread increase in cytosine methylation levels in the CHH context, especially at chromosome 2, while in the CHG context it leads to a reduction in methylation, predominantly localized on chromosomes 2 and 5. Specifically, a total of 1873 DMRs were identified in the 3CPt vs Pt comparison, with the CHH context exhibiting the highest number of either hypermethylated or hypomethylated regions. In both cases, context specificity was strongly pronounced, with few DMRs overlapping across contexts. Our data indicated that the majority of differentially methylated sites were located within the promoter regions, particularly in the CHH context, although the widest methylation differences, both hypo- and hypermethylation, were observed in the CG and CHG contexts. GO enrichment analysis of DMGs allowed the identification of genes involved in “Response to stress”, “Macromolecule modification”,”Defense response”, and “Response to biotic stimulus”, clearly suggesting that the BCA treatment might modulate the methylation status of genes within these categories establishing a primed state in the plants that increases its potential to resist fungal infection as reported in Sicilia et al. (2023). Among them, twenty gene NCBI annotated as disease resistant proteins were retrieved, most of them being hypomethylated in the *P. mediterranea* pre-treated samples (3CPt). The encoded proteins (receptor-like kinases, RLKs; disease resistance proteins, NBS-LRRs and related; proteins involved in immunity signaling; receptor-like proteins, RLPs; tyrosine-protein kinases) are implicated in detecting pathogens or stress signals and activating defense pathways, including pattern-triggered and effector-triggered immunity (Padmanabhan et al., 2009; Ercolano et al., 2022; Sutherland et al., 2025; Gao et al., 2025). The global transcriptomic analysis led to the identification of 1072 differentially expressed protein-coding genes.

However, none of the aforementioned genes have been found deregulated in the 3CPt vs Pt comparison, indicating that the hypothesized regulatory role of DNA hypomethylation was not supported by the gene expression data. In this respect, several cases on plant epigenetics document that DNA hypomethylation might occur without corresponding increases in gene expression, often due to context-specific regulation or, more likely, due to compensatory epigenetic mechanisms (Kumar and Mohapatra, 2021). Some evidence also indicates hypomethylated loci are responsible for the meiotic transmission of quantitative disease resistance (Furci et al., 2019). The putative relationship between different levels of DNA methylation and gene expression was obtained by intersecting transcriptomic data (DEGs) with the WGBS data (DMGs) in response to *P. mediterranea* pre-treatment in *P. tracheiphilus* infected plants. Consequently, from the 3CPt vs Pt comparison, a list of 50 genes was identified that exhibited both differential expression and differential methylation. Among these, genes displaying opposite trends between gene expression and methylation were selected for further analysis. Interestingly, all the genes that resulted both differentially methylated and differentially expressed play a direct or indirect role in lemon plants' response to the undergoing biotic stress. In particular, based on their function, the upregulation/hypomethylation of the cited genes suggest that *P. mediterranea* pre-treatment can result in a comprehensive enhancement of plant resilience and adaptability through: a) post transcriptional regulation of gene expression, b) regulation of mitochondria and chloroplasts gene expression, particularly of those genes involved in photosynthesis and respiration, c) balance of the photosynthetic activity and protection against oxidative stress induced by light, these latter being in strong accordance with the observed largely positive effect of *P. mediterranea* pre-treatment upon the gene related to the photosynthetic processes. Moreover, the overall beneficial effects could also arise from the induction of genes involved in the d) regulation of receptor internalization and transport of stress-response molecules and signal transduction efficiency, e) production of secondary metabolites and monoterpene indole alkaloids, f) recognizing and responding to stalled replication forks to prevent genomic instability, g) activation of a defense system including the hypersensitive response, and reinforcing the general defenses, thus providing a supportive role in biotic stress contexts. It is worthy to note that none of these genes is markedly upregulated, suggesting that likely their collective induction and not the single gene upregulation might contribute to the overall effect of bacterial BCA in enhancing the reported plant resilience, at least in the asymptomatic phase of the disease (Sicilia et al., 2023; Dimaria et al., 2024). Based on their annotation, the downregulated/hypermethylated genes might exert a critical role in several plant processes including: a) modification of small molecules, hormones and secondary metabolites, b) genome stabilization via DNA replication and repairing mechanisms, c) signal transduction, often implicated in plant immune responses d) recognizing and responding to stalled replication forks, e) modification of flavonoids by methylation altering their solubility; f) regulate stress-responsive genes and physiological processes. Interestingly, most of these functions coincide with those above mentioned as positively influenced by BCA treatment. This could indicate that the BCA pre-treatment intervenes in the host dilemma between "succumbing or resilient" during plant-pathogen interaction by playing on the concerted and balanced expression of this core group of genes. In this respect, DNA methylation fulfills a significant role in plant adaptation to environmental stresses by fine-tuning gene expression without changing the DNA sequence influencing cellular, physiological, and metabolic functions vital for stress resilience (Sicilia et al., 2020; Sicilia et al., 2021; Lodhi and Srivastava, 2025). Moreover, considering their $\log_2$FC, ranging from −3,56 to −5.14 (Table 3), cytosolic sulfotransferase 15-like, MDIS1-interacting receptor like kinase 2-like, linoleate 13S-lipoxygenase 2–1, and especially calcium-binding protein CML47 could play a pivotal role during plant infection. The strong repression of cytosolic sulfotransferase 15-like, MDIS1-interacting receptor like kinase 2-like, linoleate 13S-lipoxygenase 2–1 indicates that the defense against pathogens through the process of sulfation, the efficiency and integrity of protein synthesis machinery, and initiate PAMP-triggered immunity (PTI) are strongly impaired and lemon plants likely cannot escape from those detrimental consequences of fungal infection, despite *P. mediterranea* pre-treatment. However, Calcium-binding protein CML47 has been shown to negatively regulate plant immunity. Specifically, CML47 (along with CML46) suppresses the accumulation of salicylic acid and immune responses in *Arabidopsis* (Lu et al., 2018). Plants lacking both CML46 and CML47 display heightened resistance to the bacterial pathogen *Pseudomonas syringae*, indicating that these proteins normally act to dampen immune signaling (Lu et al., 2018). The sharp downregulation of CML47 by *P. mediterranea* pre-treatment indicates that this protein might hide the presence of the pathogen by lowering the immune signaling, at least during the asymptomatic phase of the disease (Sicilia et al., 2023, 2024b). In conclusion, *P. mediterranea* PVCT 3C pre-treatment modifies the DNA methylation status in lemon plant infected with *P. tracheiphilus*. The integration of WGBS and transcriptomic data leads to the identification of main genes involved in biological processes whose fine regulation is likely crucial to cope with the disease, at least in the early phase of the disease. Among the hypermethylated/downregulated genes, CML47 negatively regulating plant immunity, might represent a favorite candidate for genome editing application in lemon biotechnological breeding.

CRediT authorship contribution statement

A. Sicilia: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **E. Scialò:** Methodology, Investigation, Formal analysis. **F.G. Privitera:** Software, Formal analysis. **V. Catara:** Visualization, Conceptualization. **G. Dimaria:** Validation. **A. Gentile:** Visualization, Funding acquisition. **A. Pulvirenti:** Software. **A.R.Lo Piero:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation, Conceptualization.

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Data availability Statement

The *Citrus limon* WGBS data was submitted to NCBI (https://www.ncbi.nlm.nih.gov/geo/) under the accession number PRJNA1422645.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2026.106027.

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