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Nanopore direct RNA sequencing reveals the m^{5}C methylomes of tomato fruits in response to *Botrytis cinerea*

Xinyao Cai[1,2], Baowen Huang[1,2], Yulin Cheng[1,2], Jianghua Cai[1,2], Hua Cassan-Wang[3], Guozheng Qin[4], Ying Gao[1,2*] and Leilei Zhou[1,2*]

[1] *Center of Plant Functional Genomics and Synthetic Biology, Institute of Advanced Interdisciplinary Studies, Chongqing University, Chongqing 401331, China*
[2] *Key Laboratory of Plant Hormones Regulation and Molecular Breeding of Chongqing, School of Life Sciences, Chongqing University, Chongqing 401331, China*
[3] *University of Toulouse, CNRS, Toulouse INP, LRSV, Toulouse 31320, France*
[4] *College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China*
* Correspondence: yinggao@cqu.edu.cn (Gao Y); zhouleilei@cqu.edu.cn (Zhou L)

Abstract

RNA methylation, particularly 5-methylcytosine (m^{5}C) modification, has emerged as a pivotal regulatory layer governing plant development and stress responses. However, the role of m^{5}C in mediating the resistance of tomato (*Solanum lycopersicum*) fruit to the fungal pathogen *Botrytis cinerea* remains unclear. In this study, we utilized Nanopore direct RNA sequencing to generate the transcriptome-wide m^{5}C methylomes in *B. cinerea*-infected tomato fruits. Our results revealed that m^{5}C sites are preferentially enriched in coding sequences (CDSs) and 3' untranslated regions (3' UTRs). Notably, *B. cinerea* infection induced site-specific m^{5}C changes without altering the global modification level. Integrated omics analysis identified a transcriptome-wide negative correlation between m^{5}C modification and gene expression. Importantly, a large number of ethylene signaling pathway and defense-related genes exhibited reduced m^{5}C levels, accompanied by significantly increased transcript abundance, implying that m^{5}C may act as a novel regulator of fruit's resistance to *B. cinerea* by coordinating hormone signaling and defense response pathways. Furthermore, the putative m^{5}C demethylase gene *SlALKBH1* and the reader protein gene *SlALY4* were transcriptionally downregulated and upregulated by *B. cinerea* infection, respectively. Collectively, this study establishes the first comprehensive m^{5}C methylomes of tomato fruits upon *B. cinerea* infection and provides new insights into RNA-based epigenetic regulation of hormone signaling during plant–pathogen interactions.

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Introduction

RNA modifications, including *N*^{6}-methyladenosine (m^{6}A), *N*^{7}-methylguanosine(m^{7}G),5-methylcytosine(m^{5}C),*N*^{1}-methyladenosine (m^{1}A), pseudouridine (Ψ), and *N*^{4}-acetylcytidine (ac4C), play vital roles in regulating fundamental RNA metabolism, such as mRNA's stability and translation efficiency, thus playing essential roles in modulating various plant development and stress responses[1,2]. Among these, m^{5}C is a conserved RNA methylation modification formed by the addition of a methyl (-CH_3) group to the fifth carbon position of cytosine residues. It is widespread in transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and messenger RNAs (mRNAs) across eukaryotes, archaea, and some bacteria and is generally regulated by three key classes of proteins: Writers (methyltransferases), erasers (demethylases), and reader proteins[3,4]. In contrast to m^{6}A modification, whose writers, erasers, and reader proteins have been well characterized and proven to play pivotal regulatory roles in various physiological processes[2,5–7], the molecular components underlying m^{5}C methylation remain largely elusive in plants. To date, only a limited number of m^{5}C methyltransferases have been identified in plants, including transfer RNA aspartic acid methyltransferase 1 (AtTRDMT1) and tRNA-specific methyltransferase 4A/B (AtTRM4A/B) in *Arabidopsis thaliana* (orthologs of human DNMT2 and NSUN2, respectively), as well as the m^{5}C methyltransferase OsNSUN2 in rice (*Oryza sativa*)[8,9]. The *A. thaliana* Alkylation B (ALKB) domain-containing protein AtALKBH6 has been shown to directly bind to m^{5}C-modified RNAs *in vitro*, thus qualifying as a putative m^{5}C demethylase[10]. Additionally, two Aly/REF export factor (ALYREF) family proteins in *A. thaliana*, ALY2 and ALY4, have been identified as m^{5}C reader proteins[11,12]. It is distinctly different from m^{6}A modification, which is recognized by YT521-B homology (YTH)-domain proteins, such as YTHDF1-3[13].

Emerging evidence indicates that m^{5}C methylation participates in plant development and plants' abiotic stress responses. For instance, AtTRM4B in *A. thaliana* regulates root development by modulating the stability and abundance of m^{5}C-modified transcripts of key root-development genes, including *Short hypocotyl 2* (*SHY2*) and *Indoleacetic acid-induced protein 16* (*IAA16*)[14]. The OsNSUN2 mutation led to shortened root length in rice, further supporting a role for m^{5}C in developmental regulation, although the underlying molecular mechanism remains elusive[9]. Moreover, *attrm4b* mutants of *A. thaliana* exhibit enhanced sensitivity to oxidative stress[8], whereas *osnsun2* mutants of rice display a pronounced heat-sensitive phenotype, associated with reactive oxygen species (ROS) accumulation caused by photosystem defects[9]. Collectively, these studies demonstrate that m^{5}C methylation possesses broad regulatory functions comparable with m^{6}A modifications, particularly in modulating plants' development and abiotic stress responses. However, the role of m^{5}C in plants' responses to biotic stress remains largely unclear.

Tomato (*Solanum lycopersicum*) is one of the most economically important horticultural crops worldwide, valued for its essential nutritional components and strong adaptability to diverse growth environments. However, postharvest decay caused by fungal pathogens remains a major constraint in tomato production and storage, leading to an annual global yield loss of 15%–25%[15]. Among these, *B. cinerea*, a ubiquitous necrotrophic fungus, causes gray mold disease, characterized by water-soaked lesions and rapid fruit decay, severely compromising fruit quality and commercial value[16]. To defend against *B. cinerea* infection, tomato fruits have

evolved complex defense mechanisms, including transcriptional activation of defense-related genes[17], protein kinase-mediated signal transduction[18], synthesis of antimicrobial compounds[19], and modulation of hormone signaling pathways, particularly those involving jasmonic acid (JA) and ethylene[20–22].

The activation of defense-related genes is tightly controlled by multiple layers of gene expression regulation. Several key transcription factors, such as the helix–loop–helix (bHLH) transcription factor SlMYC2[23], the R2R3 MYB transcription factor SlMYB75[24], and the B-box (BBX) family transcription factor SlBBX20[25], have been demonstrated to directly bind to and activate defense-related genes at the transcriptional level. Additionally, epigenetic modifications, such as DNA methylation[26], noncoding RNAs[27], and microRNAs[28], have also been proven to function in upregulating the expression of defense-related genes. In recent, m^{6}A methylation was shown to enhance resistance to *B. cinerea* in leaves of *A. thaliana* by stabilizing defense-related genes at the post-transcriptional level[29]. These findings highlight RNA modification as a critical regulatory layer in plants' defense responses and raise the possibility of other RNA modifications. However, the role of m^{5}C modification in *B. cinerea* invasion, particularly during the postharvest stage of tomato fruits, remains unknown.

Traditional methods for detecting m^{5}C modifications, such as bisulfite sequencing, suffer from limited resolution and an inability to capture full-length RNA molecules. To overcome these limitations, Nanopore direct RNA sequencing (DRS) has emerged as a powerful technology that enables single-molecule, full-length RNA sequencing and direct detection of RNA modifications based on characteristic current signals, without chemical treatment[30]. In this study, we used Nanopore DRS technology to systematically profile the m^{5}C methylomes in *B. cinerea*-infected and mock-inoculated tomato fruits. We aimed to characterize the global landscape of m^{5}C modification during *B. cinerea* infection, identify dynamic changes in the m^{5}C sites associated with hormone- and defense-related genes, and uncover key m^{5}C-modified transcripts in tomato's resistance to *B. cinerea*. This study provides new insights into the RNA epigenetic regulatory networks underlying tomato fruit's defense against *B. cinerea* and establish a foundation for improving postharvest resistance in tomato.

Materials and methods

Plant materials and culture conditions

Tomato seedlings (*Solanum lycopersicum* cv. 'Micro-Tom') were planted in a greenhouse under standard cultivation conditions (23 °C, 16 h light/8 h dark photoperiod, and 60% relative humidity). Mature green tomato fruits at approximately 40 days postanthesis (DPA) were harvested. The fruits were surface-sterilized with a NaClO solution (2%, v/v) for 2 min, rinsed with sterile water three times, and surface-dried for subsequent use.

B. cinerea culture and inoculation

B. cinerea (B05.10) was cultured on solid potato dextrose agar (PDA) medium for 7–14 days. The culture condition was set at 25 °C under a 16 h light/8 h dark photoperiod. Conidia were collected in a half-strength liquid potato dextrose broth (PDB) medium, and the conidial concentration was adjusted to 5×10^5 spores per mL. For inoculation, the conidial suspension (5 μL) was dropped onto the surface of tomato fruits. Mock-inoculated fruits (control group) were treated with 5 μL of half-strength PDB medium without conidia. All inoculated fruits were placed in a growth chamber with a conditions of 25 °C and 80% relative humidity. At 48 h postinoculation, pericarp tissues with an area approximately 2–3 mm thick around the lesions were collected for RNA extraction.

Total RNA extraction

Total RNAs were extracted from the pericarp tissues of *B. cinerea*-infected and control fruits using the hot phenol method as described by Moore et al[31]. Briefly, 0.5 g of the powdered sample was mixed with 1 mL of an RNA extraction buffer (100 mM Tris-HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid [EDTA], 100 mM LiCl, 1% (w/v) sodium dodecyl sulfate [SDS], and 50% (v/v) water-saturated phenol) in a 2-mL centrifugation tube, followed by vortexing at room temperature for 10 min. After the addition of 500 μL of chloroform, the mixture was vortexed at room temperature for another 10 min, followed by centrifugation at 15,000×*g* for 20 min. The supernatant was transferred to a new 1.5-mL centrifugation tube and extracted once again with 500 μL of chloroform. Subsequently, total RNAs were precipitated with 2 M LiCl, washed twice with 75% (v/v) ethanol, and dissolved in Diethyl pyrocarbonate (DEPC)-treated water. The purity and integrity of total RNAs were evaluated using a NanoDrop 2000 spectrophotometer and a 1.5% agarose gel electrophoresis, respectively.

Nanopore direct RNA sequencing

Nanopore direct RNA sequencing (DRS) was performed by Benagen Technology Co., Ltd. (Wuhan, China), following the method described by Song et al.[32]. In brief, total RNA was used to isolate mRNAs using the mRNA Dynabeads Kit (Thermo Fisher, 61006). The enriched mRNAs were then ligated to Nanopore RT adapters using T4 DNA ligase (NEB, M0202M). The resulting ligation products were purified using Agencourt RNAClean™ XP beads (Beckman Coulter, California, USA). Subsequently, after the addition of the RNA adapter, the obtained products were purified again with Agencourt RNAClean™ XP beads. The prepared library was loaded into an R9.4 sequencing flow cell and then sequenced on a PromethION sequencer (Nanopore Technologies, Oxford, UK). Three independent biological replicates were subjected to generate real-time single-molecule sequencing data.

m^{5}C data analysis

Raw sequencing data in FAST5 format were converted to FASTQ format following base calling by GUPPY software (version 3.2.6). Raw reads were filtered using NanoFilt (version 2.8.0) to remove adapter sequences and low-quality reads (quality score < 10). The filtered clean reads were then mapped to the ITAG3.2_release reference annotation (https://ftp.solgenomics.net/tomato_genome/) using Minimap2 software (version 2.2.17)[33], with the tomato genome build SL3.0 as the reference genome. Statistical comparisons of the mapping results were conducted using Samtools (version 1.11)[34]. Subsequently, m^{5}C modification sites were identified using Modkit software (version 0.4.1) with the 'detect_modifications' module, which detects RNA modifications on the basis of current signal deviations from the reference sequence. m^{5}C sites with a fraction score > 0.7 were defined as high-confidence sites. Differential m^{5}C sites were identified using the R package 'DESeq2' with the criterion of $p < 0.05$. Motif analysis was performed using MEME software (version 5.5.1)[35]. Gene Ontology (GO) enrichment analysis was carried out on the Gene Ontology Consortium platform (https://www.geneontology.org/). GO terms that with a Fisher's exact false discovery rate (FDR) < 0.05 were statistically significant.

Transcriptome-wide gene expression analysis

The raw DRS sequencing data were concurrently used to perform gene expression analysis. The transcript abundance of gene transcripts was calculated as transcripts per million (TPM) using Salmon software (version 1.9.0)[36]. Differential expression analysis was conducted using the R package 'DESeq2' with the criteria of $\log_2$(fold change) ≥ 1 and an adjusted *p*-value < 0.05.

Phylogenetic analysis

The protein sequence from *A. thaliana* AtALKBH6 were downloaded from the Arabidopsis Information Resource (TAIR; www.arabidopsis.org). Using this sequence as a query, putative homologous proteins across multiple plant species were identified in the Phytozome database (https://phytozome-next.jgi.doe.gov). Multiple protein sequence alignments were performed using Clustal X (version 2.1) with the default settings. These resulting alignment data were imported into MEGA (version 5.2) for phylogenetic tree construction via the neighbor-joining algorithm, with 1,000 bootstrap replicates for assessing the reliability.

Statistical analysis

All experiments in this study were carried out with three independently biological replicates. Microsoft Excel and GraphPad Prism 8.0 software were used to process and visualize the experimental data. GraphPad Prism 8.0 was used to analyze statistical significance (two-tailed Student's *t*-test).

Results

m^{5}C methylome of tomato fruits as revealed by Nanopore DRS

To characterize the m^{5}C methylome landscapes in tomato fruits in response to *B. cinerea* infection, Micro-Tom fruits at the mature green stage were harvested and inoculated with *B. cinerea*. Pericarp tissues approximately 2–3 mm thick surrounding the disease lesions were collected at 48 h postinoculation (Fig. 1a). Total RNA was extracted from three biological replicates of both mock-inoculated (control) and *B. cinerea*-infected fruit pericarps, followed by mRNA enrichment and library preparation. The constructed RNA libraries were subjected to Nanopore DRS, and the raw data were filtered and aligned to the tomato reference genome SL3.0. The mapped reads were of high quality, with an average length of approximately 1,000 nt and a mapping rate above 90% (Supplementary Table S1), supporting the reliability of the sequencing data.

The m^{5}C modification sites were identified on the basis of their fraction scores, which represent the modification ratio and reflect the relative m^{5}C methylation level. Sites with a fraction score > 0.7

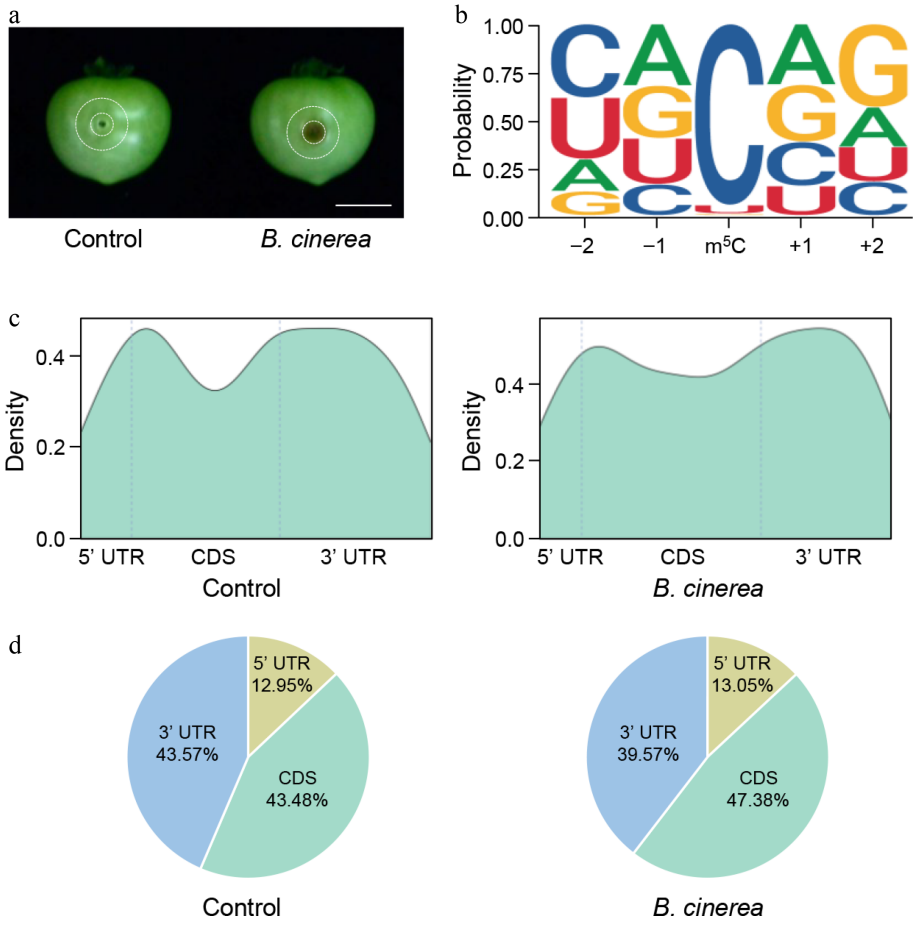

Fig. 1 m^{5}C methylome profiles of tomato fruits under *Botrytis cinerea* infection. (a) Representative photographs of mock-inoculated and *B. cinerea*-infected tomato fruits at 48 h postinoculation. Micro-Tom fruits at the mature green stage were inoculated with *B. cinerea*, and pericarp tissues between the two indicated circles were collected as experimental materials. Scale bar = 1 cm. (b) Sequence motif identified around m^{5}C sites using MEME software. All m^{5}C sites with a fraction score > 0.7 were used for motif prediction analysis. (c) Metagenomic profiles of m^{5}C site distributions in the 5' untranslated region (5' UTR), coding sequence (CDS), and 3' UTR region. (d) Pie charts showing the proportion of m^{5}C sites in the 5' UTR, CDS, and 3' UTR.

were regarded as high-confidence m^{5}C sites, and a total of 221, 233, and 242 high-confidence m^{5}C sites were identified in the three control replicates, whereas 209, 177, and 201 sites were identified in the *B. cinerea*-infected tomato fruits. To clarify the nucleotide preference of m^{5}C modifications, motif analysis was performed on these high-confidence sites. No obvious nucleotide preference was found at the −1 and +1 positions flanking the m^{5}C site (Fig. 1b). By contrast, the −2 position was predominantly enriched in cytosine (C) and uracil (U), and the +2 position showed a significant enrichment of guanine (G) (Fig. 1b). This conserved motif pattern was comparable between the control and *B. cinerea*-infected groups, suggesting that the sequence preference of m^{5}C modification in tomato fruits is an intrinsic feature and is not substantially altered by *B. cinerea* infection.

To further assess the positional preference of m^{5}C sites across gene transcripts, each transcript was divided into three functional regions: the 5' untranscribed region (UTR), the coding sequence (CDS), and the 3' UTR. The distribution pattern of m^{5}C sites was highly similar between control and *B. cinerea*-infected samples (Fig. 1c, d). In both groups, m^{5}C sites were mainly distributed in the CDS and 3' UTR regions, each accounting for approximately 40% of the total m^{5}C sites. In comparison, only almost 13% of m^{5}C sites were distributed in the 5' UTR (Fig. 1c, d). This distribution pattern may be attributed to the shorter length of the 5' UTR and thus fewer potential modification sites. Collectively, these results indicate that *B. cinerea* infection does not alter the regional distribution pattern of m^{5}C sites in tomato fruit mRNAs, and m^{5}C modification has no obvious regional preference along gene transcripts.

Analysis of differential m^{5}C sites upon *B. cinerea* infection

To investigate the effect of *B. cinerea* infection on m^{5}C modification levels, differential m^{5}C sites were identified by comparing the control and infected groups. m^{5}C sites with a fraction score ≥ 0.1 in at least one group were defined as reliable m^{5}C sites. In total, 39,610 reliable m^{5}C sites were identified, which were distributed across 8,580 gene transcripts. Among these, 354 m^{5}C sites were significantly upregulated and 341 were significantly downregulated in *B. cinerea*-infected fruits relative to the control group ($p < 0.05$; Fig. 2a), corresponding to 340 and 324 distinct gene transcripts, respectively (Supplementary Tables S2 and S3). These results suggest that *B. cinerea* infection triggers specific changes in m^{5}C methylation levels across the tomato fruit transcriptome.

Further statistical analysis of transcripts harboring differential m^{5}C sites revealed that the vast majority (approximately 95%) contained only one differential m^{5}C site, whereas only a small proportion (around 5%) harbored two or more differential m^{5}C sites (Fig. 2b).

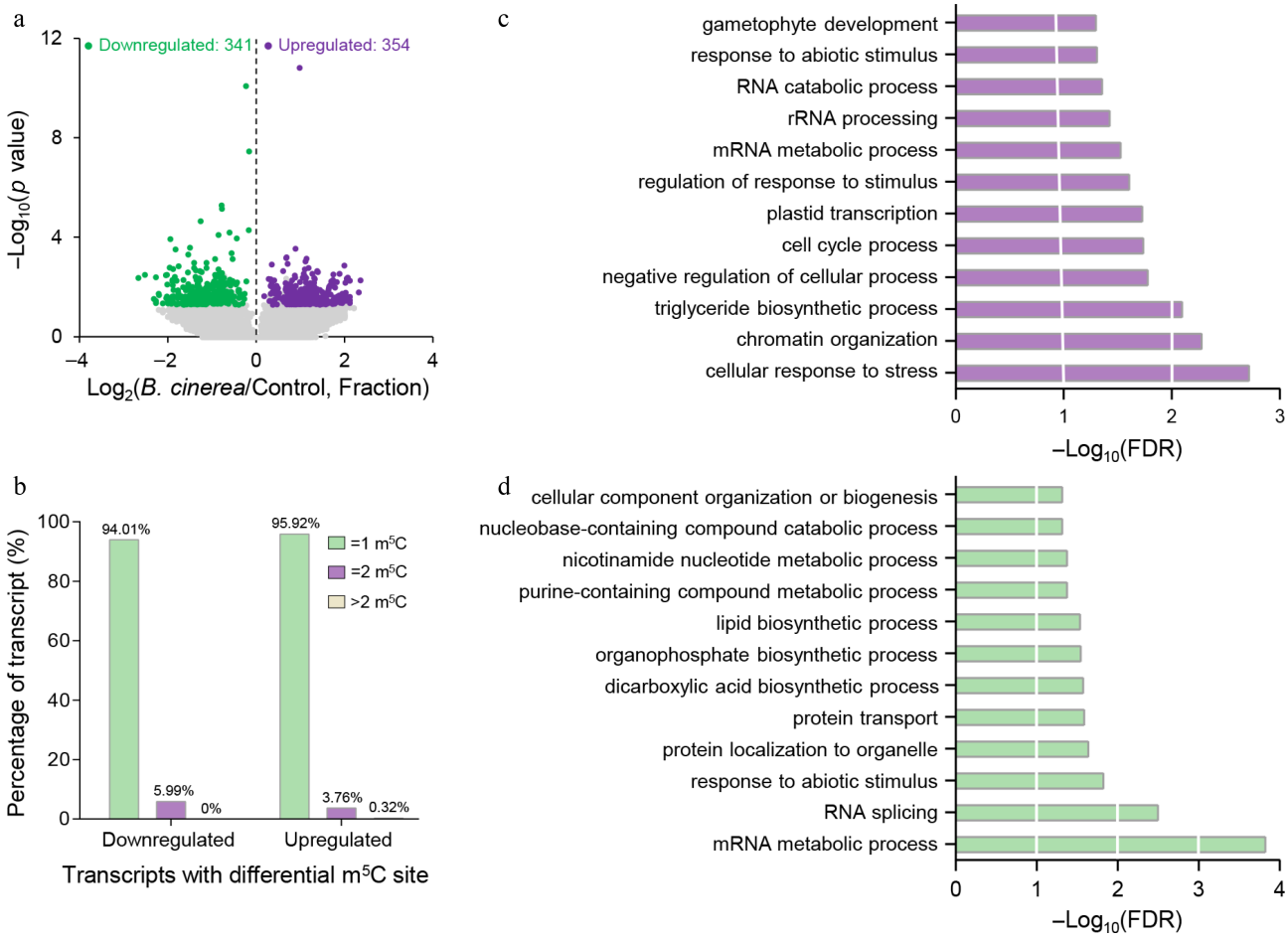

Fig. 2 Identification of differential m^{5}C sites in *B. cinerea*-infected tomato fruits. (a) Volcano plot showing significantly unregulated (purple) and downregulated (green) m^{5}C sites in *B. cinerea*-infected tomato fruits compared with the control group. (b) Proportions of the differential m^{5}C-modified transcripts containing different m^{5}C site numbers. (c) Gene Ontology (GO) enrichment analysis of biological processes for gene transcripts harboring significantly upregulated m^{5}C sites. (d) GO enrichment analysis of biological processes for gene transcripts harboring significantly downregulated m^{5}C sites. FDR, false discovery rate.

This observation indicates that the regulatory effect of *B. cinerea* infection on m^{5}C modification in tomato fruits occurs primarily through site-specific local regulation, rather than coordinated modification changes across multiple sites within the same transcript.

To uncover the biological processes regulated by m^{5}C modification in response to *B. cinerea* infection, GO enrichment analysis was performed on genes exhibiting differential m^{5}C methylation. The results showed that genes harboring upregulated m^{5}C sites were significantly enriched in several biological processes, including RNA metabolism (e.g., RNA catabolic process, rRNA processing, and mRNA metabolic process), responses to external stimuli (e.g., response to abiotic stimulus, regulation of responses to stimuli, and cellular response to stress), chromatin organization, and plastid transcription (Fig. 2c). By contrast, genes with downregulated m^{5}C sites were not only enriched in RNA metabolism (e.g., RNA splicing and mRNA metabolic process) and responses to external stimuli (e.g., responses to abiotic stimuli) but also significantly enriched in protein localization and transport (e.g., protein transport and protein localization to organelles), as well as the biosynthesis and metabolism of diverse organic compounds including lipids, organophosphates, and dicarboxylic acids (Fig. 2d). These results suggest that m^{5}C modification is broadly implicated in the regulation of multiple biological processes in tomato fruits during *B. cinerea* infection, implying multifaceted roles for m^{5}C modification in mediating tomato fruit's resistance to *B. cinerea*.

m^{5}C methylation is generally negatively correlated with gene expression upon *B. cinerea* infection

To explore the putative regulatory role of m^{5}C modification in gene expression, we next analyzed the correlation between m^{5}C modification levels and transcript expression abundance. A parallel transcriptome-wide gene expression analysis (RNA-seq) was performed in conjunction with Nanopore DRS analysis, and we identified 7,509 upregulated and 7,255 downregulated transcripts in the *B. cinerea*-infected group compared with the control group, respectively (Supplementary Table S4). Subsequently, an integrated analysis was conducted to associate transcripts harboring differential m^{5}C sites with those exhibiting significantly altered expression levels. The results showed that among transcripts with upregulated m^{5}C sites, 113 transcripts exhibited significantly decreased expression levels, whereas only 38 transcripts showed significantly increased expression levels ($\log_2$(fold change) ≥ 1; adjusted p-value < 0.05) in *B. cinerea*-infected fruits (Fig. 3a; Supplementary Table S5). By contrast, among transcripts with downregulated m^{5}C modification, 48 transcripts had significantly decreased expression levels and 97 transcripts displayed significantly increased expression levels (Fig. 3b; Supplementary Table S6). Furthermore, when this analysis was extended to the whole transcriptome, cumulative fraction analysis revealed that gene transcripts harboring downregulated m^{5}C sites had an obvious higher proportion of increased gene expression (relative to the control group) compared with those containing upregulated m^{5}C sites (Fig. 3c). Collectively, these results indicate an overall negative correlation between m^{5}C modification and gene expression, suggesting that m^{5}C modification may predominantly act as a negative regulator of gene expression in tomato fruits during *B. cinerea* infection.

Ethylene signaling pathway genes exhibit differential m^{5}C modification and transcript level during *B. cinerea* infection

The phytohormone ethylene plays a pivotal role in regulating plant growth and development, as well as in mediating responses to biotic and abiotic stresses[37,38]. Previous studies have demonstrated that the ethylene signaling pathway is involved in defending tomato leaves and fruits against *B. cinerea* infection by regulating the expression of downstream defense-related genes[21,37,38]. In the present study, we analyzed changes in m^{5}C modification and expression levels of ethylene signaling pathway genes, and identified three such genes that exhibited coordinated changes in m^{5}C modification and transcript abundance. Compared with the control group, one ethylene response factor (ERF) domain-containing protein gene (*Solyc03g093610*) and one ethylene insensitive 3 (EIN3)-like protein gene (*Solyc01g014480*) harbored downregulated m^{5}C sites and significantly increased transcript levels in the *B. cinerea*-infected tomato fruits (Fig. 4). In addition, another ERF domain-containing protein gene (*Solyc03g093560*) exhibited upregulated m^{5}C modification accompanied by decreased gene expression (Fig. 4). These findings indicate that m^{5}C modification may participate in modulating the ethylene signaling pathway to regulate tomato fruit's resistance against *B. cinerea* infection.

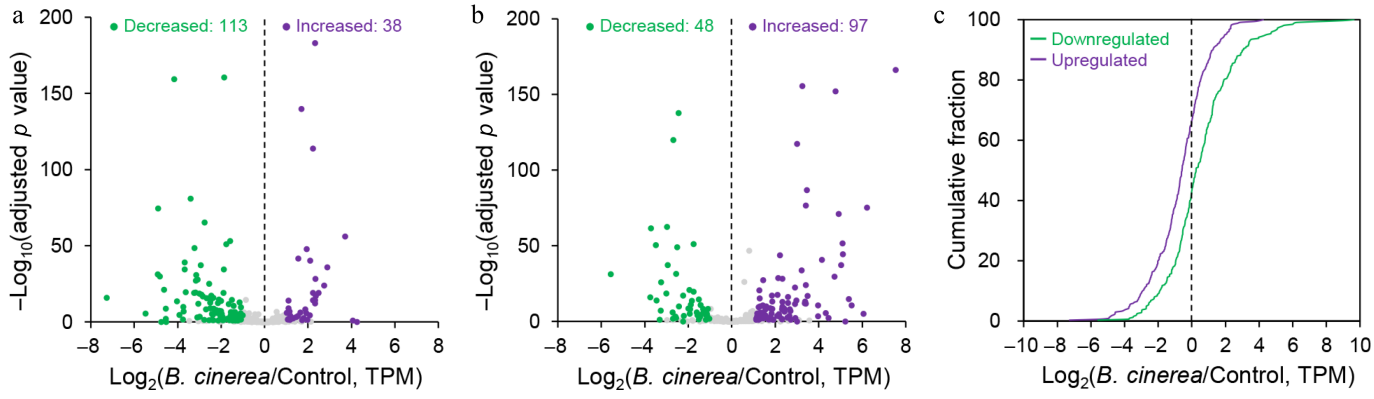

Fig. 3 m^{5}C methylation is globally negatively correlated with transcript abundance. (a) and (b) Volcano plots displaying the expression ratios of transcripts containing upregulated and downregulated m^{5}C sites in *B. cinerea*-infected tomato fruits compared with the control group, respectively. Transcripts with significantly increased and decreased mRNA levels ($\log_2$(fold change) ≥ 1; adjusted p-value < 0.05) in *B. cinerea*-infected tomato fruits are highlighted in purple and green, respectively. (c) Cumulative distribution of gene expression changes in transcripts harboring upregulated and downregulated m^{5}C sites in *B. cinerea*-infected tomato fruits.

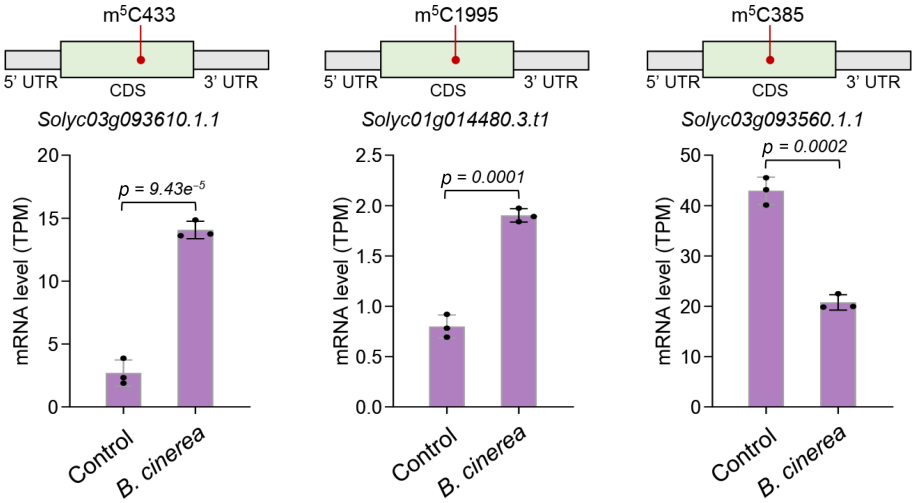

Fig. 4 Ethylene signaling pathway genes exhibited differential m^{5}C methylation and expression abundance under *B. cinerea* infection. The transcript levels of three ethylene signaling pathway genes, including two ethylene response factor genes (*Solyc03g093610* and *Solyc01g014480*) and one ethylene insensitive 3-like DNA-binding domain-containing protein gene (*Solyc03g093560*) were determined by RNA-seq analysis. Error bars represent the standard deviation of three independent experiments. Significance analysis was performed using Student's *t*-test, with *p*-values indicated. For each gene transcript, the position of differential m^{5}C site is shown.

Defense-related genes exhibit differential m^{5}C modification and transcript levels during *B. cinerea* infection

By integrating differential m^{5}C modification and gene expression analyses, we found that the transcripts of numerous defense-related genes harbor downregulated m^{5}C sites and significantly increased expression abundance in *B. cinerea*-infected fruits compared with the control group, which further verified the negative regulatory relationship between m^{5}C modification and gene expression. Among these genes, protein kinase genes were the most prominent category. In total, 12 protein kinase genes showed decreased m^{5}C modification and elevated transcript levels (Supplementary Table S6). Three of these protein kinase genes displayed a dramatic increase in expression abundance (at least a 10-fold increase; Fig. 5a), including two protein kinase domain-containing protein genes (*Solyc03g115610* and *Solyc04g075000*) and one nonspecific serine/threonine protein kinase gene (*Solyc03g078520*). Previous studies have demonstrated that protein kinase genes mediate signal transduction activated by fungal pathogen infection and regulate fruit's resistance to pathogenic fungi[18,39,40]. Thus, these genes may be direct targets of m^{5}C methylation and play a vital role in defending against *B. cinerea* infection as key defense-related genes. In addition, four redox-related genes, including two peroxidase genes (*Solyc02g092580* and *Solyc04g071890*) involved in ROS metabolism, one procollagen-proline 4-dioxygenase gene (*Solyc02g067530*), and one MsrB domain-containing protein gene (*Solyc02g083360*), as well as one toxin-resistant protein gene (*Solyc03g118970*), exhibited decreased m^{5}C modification and elevated transcript levels (Fig. 5b, c). It is possible that under *B. cinerea* infection, tomato fruits enhance their resistance by reducing the m^{5}C modification levels of these potentially targeted defense-related genes, thereby promoting their expression.

Expression of m^{5}C methyltransferase, demethylase, and reader protein genes in response to *B. cinerea* infection

The dynamic changes in and molecular functions of m^{5}C methylation *in vivo* depend on the coordinated regulation of m^{5}C methyltransferases, demethylases, and reader proteins[41]. However, in plants, only a limited number of m^{5}C methyltransferase genes have been identified, and the coding genes of m^{5}C demethylases and reader proteins remain largely uncharacterized. In *A. thaliana*, the reported m^{5}C methyltransferase genes include *AtTRDMT*, *AtTRM4A*, and *AtTRM4B*[14,41]. Their homologous genes in tomato were identified as *SlTRDMT*, *SlTRM4A*, and *SlTRM4B* through BLASTP analysis[42,43]. Gene expression analysis revealed that the mRNA levels of *SlTRDMT*, *SlTRM4A*, and *SlTRM4B* in *B. cinerea*-infected fruits were not significantly different from those in the control group (Fig. 6a), indicating that these three putative m^{5}C methyltransferase genes do not respond significantly to *B. cinerea* infection at the transcriptional level.

In *A. thaliana*, an ALKB domain-containing protein encoded by *AtALKBH6* has been shown to directly bind to m^{5}C-modified RNAs, thereby qualifying as a putative m^{5}C demethylase[10]. Given the amino acid sequence of AtALKBH6, we searched for its homologous proteins across multiple plant species, and identified a ALKB domain-containing protein with the ID Solyc01g057570 in tomato, which we previously named SlALKBH1 and characterized as a putative m^{6}A demethylase[44]. Phylogenetic analysis demonstrated that SlALKBH1exhibits high similarity to those from other Solanaceous crops including potato (*Solanum tuberosum*) and tobacco (*Nicotiana benthamiana*) (Fig. 6b). Furthermore, the expression level of *SlALKBH1* was significantly decreased under *B. cinerea* infection (Fig. 6c), implying that it may play a critical regulatory role in mediating tomato's resistance to *B. cinerea* through m^{5}C demethylation. Additionally, on the basis of the amino acid sequences of the reported m^{5}C reader proteins ALY2 and ALY4 in *A. thaliana*[11,12], we identified two putative tomato m^{5}C reader proteins via sequence homology analysis, designated SlALY2 and SlALY4. Gene expression analysis showed that *SlALY4* was significantly upregulated upon *B. cinerea* infection, whereas *SlALY2* was expressed at low and stable levels (Fig. 6d), suggesting that SlALY4 may be functionally important for the defense response of tomato fruits to *B. cinerea*.

Discussion

m^{5}C methylation is a conserved epigenetic modification present in various RNA species, playing crucial roles in regulating gene

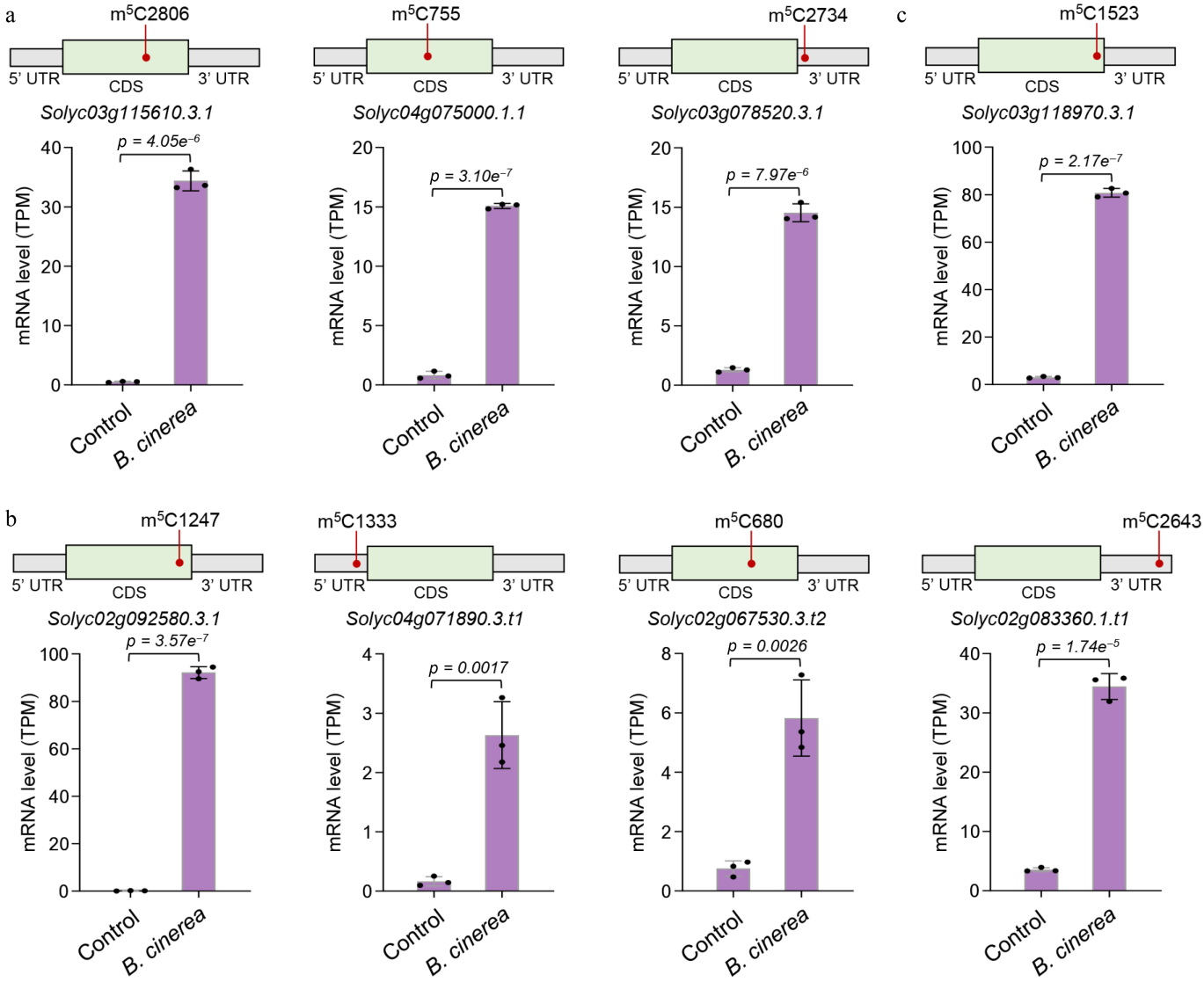

Fig. 5 Defense-related genes exhibited downregulated m^{5}C modification and increased expression abundance under *B. cinerea* infection. (a) Changes in the transcript levels of three protein kinase genes. (b) Changes in the transcript levels of four redox-related genes. (c) Changes in the transcript levels of one toxin-resistant protein gene. Gene expression levels were determined by RNA-seq analysis. Error bars represent the standard deviation of three independent experiments. Significance analysis was performed using Student's *t*-test, with *p*-values indicated. For each gene transcript, the position of downregulated m^{5}C site is shown.

expression and RNA metabolism[45,46]. In this study, we used Nanopore DRS to profile the m^{5}C methylomes of tomato fruits in response to *B. cinerea* infection for the first time, thereby revealing the dynamic changes in m^{5}C modification and its regulatory roles in tomato fruit's reaction to *B. cinerea*. Our findings provide new insights into the RNA epigenetic mechanisms underlying plant–pathogen interactions, particularly highlighting the involvement of m^{5}C methylation in mediating the defense responses of postharvest tomato fruits.

The distribution of m^{5}C sites across mRNA regions is closely associated with their regulatory functions. For instance, the m^{5}C modification localized in the 3' UTR and stop codon regions could coordinate with m^{6}A methylation to regulate translation efficiency and mRNA stability[47]. In the present study, m^{5}C methylome profiling revealed that the m^{5}C sites in tomato fruits are preferentially distributed within the CDS and 3' UTR, with only a small proportion located in the 5' UTR (Fig. 1c, d). This distribution pattern is consistent with that reported in *A. thaliana* seedlings and Ailsa Craig tomato fruits[8,47], as revealed by bisulfite RNA sequencing (bsRNA-seq) and m^{5}C-RNA immunoprecipitation sequencing (RIP-seq) analysis, respectively, but differs from that in tomato leaves as revealed by m^{5}C-RIP-seq analysis, where m^{5}C is mainly distributed in the CDS region[42]. The low proportion of m^{5}C sites in the 5' UTR may be attributed to the short length of the 5' UTR and the limited number of modifiable cytosine residues. Notably, *B. cinerea* infection did not alter the distribution pattern of m^{5}C sites, which is distinct from the effect of tomato spotted wilt virus in tomato leaves, whose infection induces significant alterations in the distribution preference of m^{5}C peaks[42]. This observation indicates that the regional distribution of m^{5}C modification in tomato fruits is stable and not affected by *B. cinerea* infection, which may be associated with the fundamental regulatory roles of m^{5}C modification in RNA metabolism.

Differential m^{5}C modification analysis revealed that *B. cinerea* infection induces specific changes in the m^{5}C modification levels of tomato fruit mRNAs, with 354 upregulated and 341 downregulated m^{5}C sites (Fig. 2a). The similar number of increased and decreased m^{5}C sites suggests that *B. cinerea* infection does not cause an

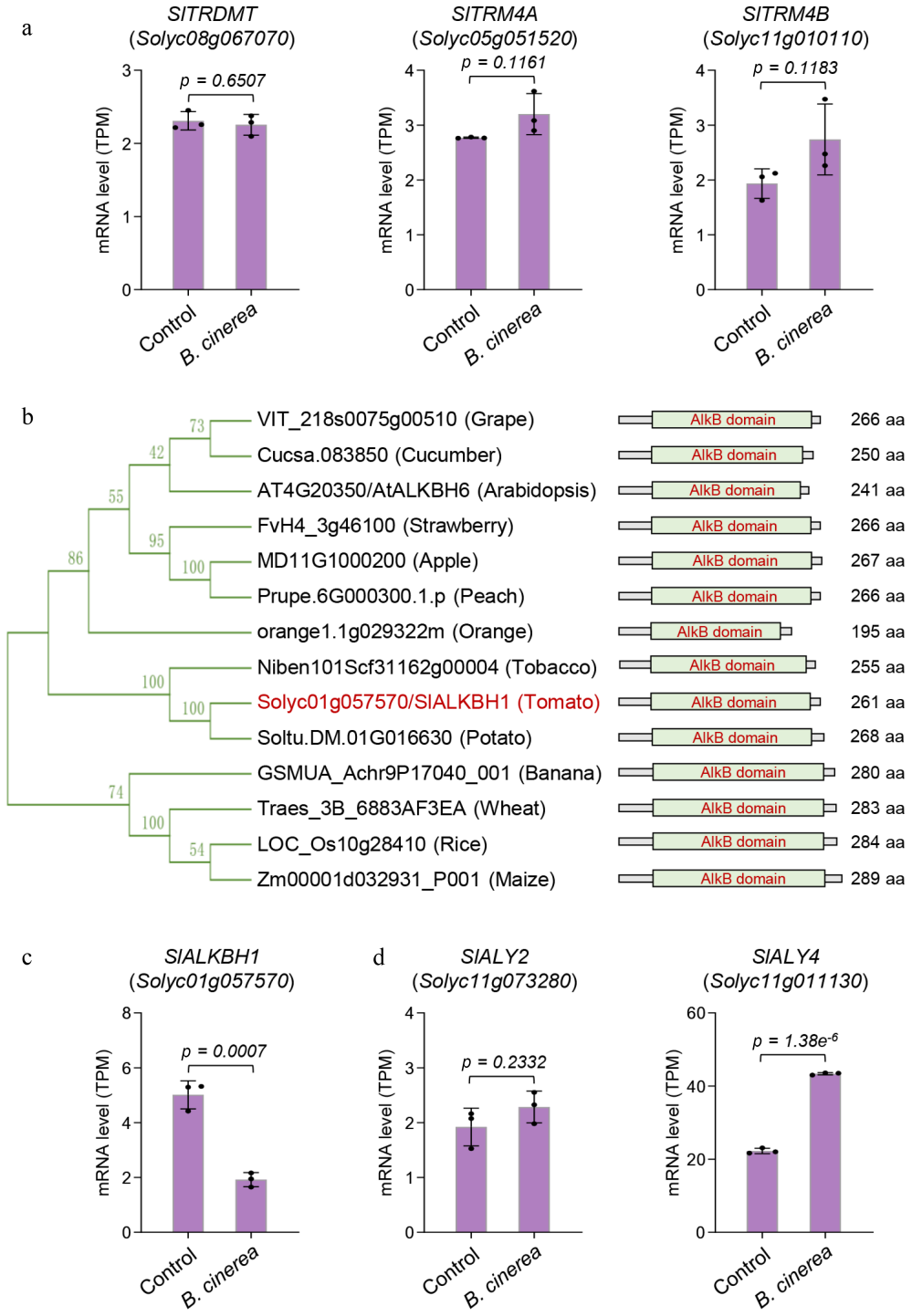

Fig. 6 Changes in the transcript levels of putative tomato m^{5}C methyltransferase, demethylase, and reader protein genes. (a) The transcript levels of three putative m^{5}C methyltransferase genes (*SlTRDMT*, *SlTRM4A*, and *SlTRM4B*) did not significantly alter upon *B. cinerea* infection. (b) Phylogenetic analysis of the putative m^{5}C demethylase gene among various plant species. The phylogenetic tree was generated using MEGA 5.2 software. Bootstrap values from 1,000 replications are shown for each branch. aa, amino acid. (c) The transcript level of the putative m^{5}C demethylase gene *SlALKBH1* significantly decreased upon *B. cinerea* infection. (d) The transcript level of the putative m^{5}C reader protein gene *SlALY4*, but not that of the *SlALY2*, significantly increased upon *B. cinerea* infection. Error bars represent the standard deviation of three independent experiments. Significance analysis was performed using Student's *t*-test, with *p*-values indicated.

overall change in m^{5}C modification levels, but rather elicits site-specific local changes. The correlation between m^{5}C modification and gene expression is a key issue for understanding the regulatory function of m^{5}C methylation. In this study, we identified a negative correlation between m^{5}C modification levels and gene expression levels (Fig. 3a–c). This finding is inconsistent with previous studies in *A. thaliana* roots and tomato leaves, where m^{5}C modification was positively correlated with transcript levels, as mutation of the m^{5}C methyltransferase gene *AtTRM4B and SlTRM4B* led to reduced m^{5}C modification and decreased expression of m^{5}C-targeted genes[8,14,42]. This discrepancy implies that the regulatory role of m^{5}C modification in gene expression may be organ-specific in plants. In fact, the causal mechanism by which m^{5}C modification negatively regulates gene expression remains largely uncharacterized.

Previous studies have demonstrated that m^{6}A modification can recruit the CCR4-NOT RNA degradation complex to specifically mediate the decay of target mRNAs within cytoplasmic P-bodies[48]. In addition, m^{6}A triggers ribosome stalling and collision, induces premature termination of translation, and promotes mRNA degradation, thereby reducing overall mRNA stability and translational efficiency[49]. Whether m^{5}C represses gene expression through a similar mechanism or distinct regulatory pathways warrants further investigation.

GO enrichment analysis demonstrated that genes harboring differential m^{5}C sites were significantly enriched in multiple biological processes, including RNA metabolism and responses to external stimuli (Fig. 2c, d). The enrichment of genes associated with the response to external stimuli highlights the involvement of m^{5}C modification in the defense response of tomato fruits against *B. cinerea* infection. Specifically, we found that several defense-related genes, encompassing redox-related genes, toxin-resistant protein genes, and protein kinase genes, exhibited reduced m^{5}C modification and elevated expression levels following *B. cinerea* infection (Fig. 5a–c). Previous studies have demonstrated that ROS and reactive nitrogen species act as both cytotoxic agents and key signaling molecules in plants' responses to environmental cues[50]. Redox-related genes targeted by m^{5}C modification include multiple peroxidase genes responsible for ROS scavenging. This process is critical for plants' defense against pathogen invasion by alleviating oxidative damage[51–53]. Notably, *A. thaliana* peroxidases PRX33 and PRX34 are essential for H_2O_2 accumulation during defense responses against pathogens[50,54]. Accordingly, we propose that m^{5}C may modulate tomato fruit's resistance to *B. cinerea* by regulating H_2O_2 content. Toxin-resistant protein genes are responsible for the degradation and clearance of mycotoxins produced by *B. cinerea*[55], whereas protein kinase genes can sense pathogen-associated molecular patterns (PAMPs) and trigger downstream defense signaling cascades[56]. The increased expression of these genes is likely to enhance the ability of tomato fruits to resist *B. cinerea* infection.

In addition, our results revealed that m^{5}C modification potentially targets and modulates the expression of ethylene signaling pathway genes, including those encoding ethylene response factors and EIN3-like protein genes (Fig. 4). Previous studies have demonstrated that the ethylene signaling pathway contributes to the defense of tomato leaves and fruits against *B. cinerea* infection by regulating the transcription of downstream defense-related genes[24,38,41]. To our knowledge, this study is the first to document that m^{5}C modification is involved in the regulation of the ethylene signaling pathway in *B. cinerea*-infected tomato fruits, thereby expanding our understanding of the regulatory mechanisms underlying the ethylene-mediated defense response.

Notably, no significant differentially methylated m^{5}C sites were detected in transcripts of the jasmonic acid (JA) signaling pathway, an indispensable hormonal cascade regulating tomato's resistance to *B. cinerea*. Additionally, auxins have been implicated in activating defense mechanisms against pathogens by reinforcing the cell walls' integrity and are widely recognized as crucial hormones for plants' defense responses[57]. However, no expressed auxin-related gene transcripts (TPM ≥ 1) exhibited significant differential m ^{5}C modification during *B. cinerea* infection (Supplementary Tables S5 and S6). These results indicate that transcripts associated with the JA and auxin signaling pathways may not be direct targets of m^{5}C modification in tomato fruits upon *B. cinerea* challenge. It would be intriguing to further explore whether these hormone-related transcripts are regulated by other RNA modifications, such as m^{6}A and m^{1}A, during tomato fruit's defense against fungal infection, which warrants further investigation.

The dynamics of m^{5}C modification is tightly orchestrated by three classes of key regulators: Methyltransferases, demethylases, and reader proteins. In *A. thaliana*, AtTRDMT, AtTRM4A, and AtTRM4B have been characterized as functional m^{5}C methyltransferases[8,17,44]. In the present study, we identified their homologous genes in tomato, designated as *SlTRDMT*, *SlTRM4A*, and *SlTRM4B*. However, *B. cinerea* infection did not significantly alter the transcript levels of these three genes, suggesting that these methyltransferases may not participate in the regulation of m^{5}C modification during *B. cinerea* infection. Alternatively, their regulatory effects might be mediated at the post-transcriptional or post-translational level, which requires further investigation.

It should be noted that this study has several inherent limitations. First, our analysis of the m^{5}C methylome was restricted to a single time point (48 h postinoculation), leaving the dynamic changes in the m^{5}C methylome across different stages of *B. cinerea* infection largely unexplored. Second, the regulatory mechanisms through which m^{5}C modification modulates gene expression, such as its potential effects on mRNA stability or translation efficiency, have not been validated using *in vitro* experimental approaches Third, how ethylene-mediated hormone signaling is regulated by m^{5}C methylation and participates in the defense response remains to be further investigated. Fourth, the specific roles of m^{5}C methyltransferases, demethylases, and reader proteins in mediating tomato fruit's resistance to *B. cinerea* infection remain elusive. In future research, we will focus on addressing these issues to further delineate the regulatory mechanisms by which m^{5}C modification governs tomato fruit's defense responses against *B. cinerea*, which may provide novel targets for improving tomato fruit's postharvest resistance to gray mold disease.

Conclusions

In this study, we profiled the m^{5}C methylomes of *B. cinerea*-infected and mock-inoculated tomato fruits via Nanopore DRS. Our results demonstrated that m^{5}C modification in tomato fruit's mRNAs exhibits a specific nucleotide preference and is predominantly distributed in the CDS region and 3' UTR. Notably, *B. cinerea* infection induces site-specific changes in m^{5}C modification but does not alter the global m^{5}C modification level. Globally, *B. cinerea*-triggered m^{5}C modification negatively regulates gene expression, and genes with differential m^{5}C modifications are involved in multiple biological processes, including RNA metabolism and responses to external stimuli. Importantly, a large number of ethylene signaling pathway and defense-related genes are directly targeted by m^{5}C modification, implying its critical involvement in mediating tomato fruit's resistance to *B. cinerea*. Additionally, the putative tomato m^{5}C demethylase gene *SlALKBH1* and the reader protein gene *SlALY4*, but not the three m^{5}C methyltransferase genes, respond significantly to *B. cinerea* infection at the transcriptional level. Collectively, this study provides the first comprehensive m^{5}C methylome profile of tomato fruits during *B. cinerea* infection, advancing our understanding of RNA-based epigenetic regulation in plant–pathogen interactions and offering novel targets for improving postharvest resistance in tomato fruit.

Author contributions

The authors confirm their contributions to this study as follows: conceived and designed the experiments: Zhou L, Gao Y; performed

the experiment and analyzed the data: Cai X, Zhou L; provided critical discussions: Qin G, Cassan-Wang H, Cai J, Huang B, Cheng Y; wrote the manuscript: Zhou L; contributed to the writing: Gao Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Conflict of interest

The authors declare that they have no conflict of interest.

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