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N6-methyladenine DNA modification modulates pathogen virulence in nematodes

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Understanding the global regulatory mechanisms that control pathogen virulence gene expression is essential for elucidating the molecular basis of pathogenicity. N6-methyladenine (6 mA) plays a crucial role in regulating gene expression in response to various environmental stresses; however, its role in pathogen virulence remains largely unexplored. Here, we report the widespread occurrence of 6 mA across 17 nematode isolates and map its genomic landscape in six notorious agriculturally important pathogen root-knot nematodes (RKNs). We demonstrated that 6 mA is characterized by a conserved GAG motif across nematodes, but exhibits species-specific distribution patterns and distinct effects on gene expression. In particular, its enrichment in transposable elements (TEs) differs between polyploid and diploid nematodes, suggesting lineage-specific epigenetic regulation potentially associated with polyploidy. We further identified two functional 6 mA demethylases, MinMAD-1 and MinMAD-2, and confirmed their catalytic activity and active sites. Host-induced gene silencing (HIGS) of *minmad-1* significantly increased plant resistance to three polyploid RKN species. A detailed functional analysis revealed that *minmad-1* knockdown broadly affected gene expression during the parasitic stage, including genes involved in virulence, thereby reducing nematode infectivity. Together, our findings suggest 6 mA demethylase as a key epigenetic regulator of RKNs' virulence, providing new insights into nematode biology and offering promising targets for the development of sustainable control strategies.

DNA 6 mA | pathogen virulence | RKNs

The regulation of pathogen virulence gene expression is crucial to understanding pathogenicity mechanisms. While current research has largely focused on the functional analysis of individual virulence genes, little is known about the broader mechanisms underlying global virulence regulation in pathogens. DNA methylation, one of the earliest discovered DNA modifications, influences chromatin structure, DNA conformation, stability, and protein interactions, thereby modulating gene expression (1, 2). The role of 6 mA in bacterial virulence is well established (3–5). In eukaryotes, evidence remains limited, with only a few studies reporting 6 mA-regulated virulence in fungi. For example, 6 mA methyltransferase is critical for the pathogenicity of the plant fungal pathogen *Cryphonectria parasitica* (6). However, most investigations linking 6 mA to virulence have focused on animal pathogens, leaving its role in plant pathogens largely unexplored. Furthermore, while functional studies have predominantly examined 6 mA methyltransferases, the roles of demethylases remain poorly understood.

Recently, DNA 6 mA modification has been reported in an increasing number of eukaryotes, including *Caenorhabditis elegans* (7), *Bursaphelenchus xylophilus* (8), *Drosophila* (9), *Chlamydomonas reinhardtii* (10), fungi (11, 12), plant (13–15), and mammal (16). In eukaryotes, 6 mA methylation occurs in both symmetric and asymmetric contexts, and asymmetric 6 mA predominates in nematodes. Eukaryotic 6 mA has been shown to be transmitted via a semiconservative mechanism (17) and incorporated into mammalian genomes by DNA polymerase (18, 19). Functionally, 6 mA has been linked to disease onset (20–22), embryonic development (9), and environmental stress responses (14, 23). As a key epigenetic modulator, 6 mA demethylases influence diverse biological processes, including reproduction (7), ovarian development (9), DNA replication and repair (24), vascular calcification (25), and neurodevelopment (26). The role of 6 mA demethylase in pathogen infection has also been preliminarily reported in *Mucor lusitanicus* (27). However, it is worth noting that some studies have reported the presence of 6 mA in cultured cells, where the detected 6 mA was later shown to largely arise from bacterial DNA

Significance

6 mA methyltransferase regulates the expression of virulence genes in certain pathogenic bacteria and plays a critical role in their infectivity. However, the regulation of virulence gene expression in eukaryotic pathogens, particularly plant pathogens, remains poorly understood. Most studies on pathogen virulence have focused on individual effectors, with little insight into global regulatory mechanisms. Here, we demonstrate that 6 mA broadly shapes virulence gene expression patterns in polyploid root-knot nematodes (RKNs). Moreover, transgenic tobacco, tomato, and rice plants expressing dsRNA against nematode demethylases showed significantly enhanced resistance to RKNs. These findings establish 6 mA demethylase as a promising epigenetic target for controlling plant-parasitic nematodes.

Competing interest statement: The authors have applied for Chinese patents related to the use of the *minmad-1/2* genes for the control of root-knot nematodes.

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contamination (28). Thus, stringent control of bacterial contamination is critical for accurate detection of 6 mA in eukaryotes.

Nevertheless, the mechanism by which 6 mA regulates parasitism in eukaryotic pathogens—particularly the link between demethylases and virulence—remains unclear. Plant-parasitic nematodes (PPNs) cause devastating agricultural losses estimated to exceed \$100 billion annually (29). Among them, root-knot nematodes (RKNs) are the most destructive (30), drawing increasing attention due to their broad host range and complex ploidy (31). Infective second-stage juveniles (J2s) penetrate host roots near the tip, migrate intercellularly, and establish giant cells within the vascular cylinder. A diverse suite of nematode effectors facilitate the formation and maintenance of giant cells (32). However, the epigenetic regulation of these effector genes remains largely unexplored. Previous studies of RKN epigenetics have mainly focused on histone modifications (33–35) and microRNA (36). Homology-based analysis of methyltransferases and demethylases suggested that the *Meloidogyne incognita* (*M. incognita*, Mi) lacks DNA 6 mA modification machinery (37), raising doubts about the presence of 6 mA in the RKN genomes. Alternatively, these enzymes may have diverged substantially from known homologs, warranting further investigation.

In this study, we investigated the presence of 6 mA in 17 nematode isolates using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) and confirmed DNA 6 mA modification in two diploids (*Meloidogyne graminicola* and *Meloidogyne hapla*), two allotriploids (*M. incognita* and *Meloidogyne arenaria* 3n), and two allotetraploids (*M. arenaria* 4n and *Meloidogyne javanica*)

through SMRT-seq, N6-methyladenine DNA immunoprecipitation sequencing (6 mA-DIP-seq), and DNA Dot blot assays. We found that 6 mA is enriched in actively transcribed genes and exhibits distinct distribution patterns across RKN species, particularly within transposable elements (TEs), where striking differences were observed between diploid and polyploid RKNs. In *M. incognita*, low-methylation-level sites were concentrated in the A₂ subgenome and associated with genes critical for cellular function, such as G protein-coupled receptors (GPCR). Importantly, we identified two putative 6 mA demethylases (MiNMAD-1 and MiNMAD-2) and pinpointed their catalytic sites through structural prediction and biochemical validation. Remarkably, host-induced gene silencing (HIGS) of *minmad-1* significantly enhanced resistance of transgenic host plants to three polyploid RKN species. Further analyses revealed that MiNMAD-1 plays a global role in regulating gene expression, including genes associated with virulence. Collectively, our findings establish a direct link between epigenetic regulation and virulence in a eukaryotic pathogen.

Results

6 mA Widespread in Nematode Genome. To investigate the presence of DNA 6 mA in nematodes, we analyzed 17 isolates, including seven RKNs (*M. graminicola*, Mi, *M. arenaria* 3n, *M. arenaria* 4n, *M. javanica*, *M. enterolobii*, and *M. hapla*), four isolates of *Heterodera glycines*, three migratory PPNs (*Aphelenchus avenae*, *Ditylenchus destructor*, and *Bursaphelenchus xylophilus*),

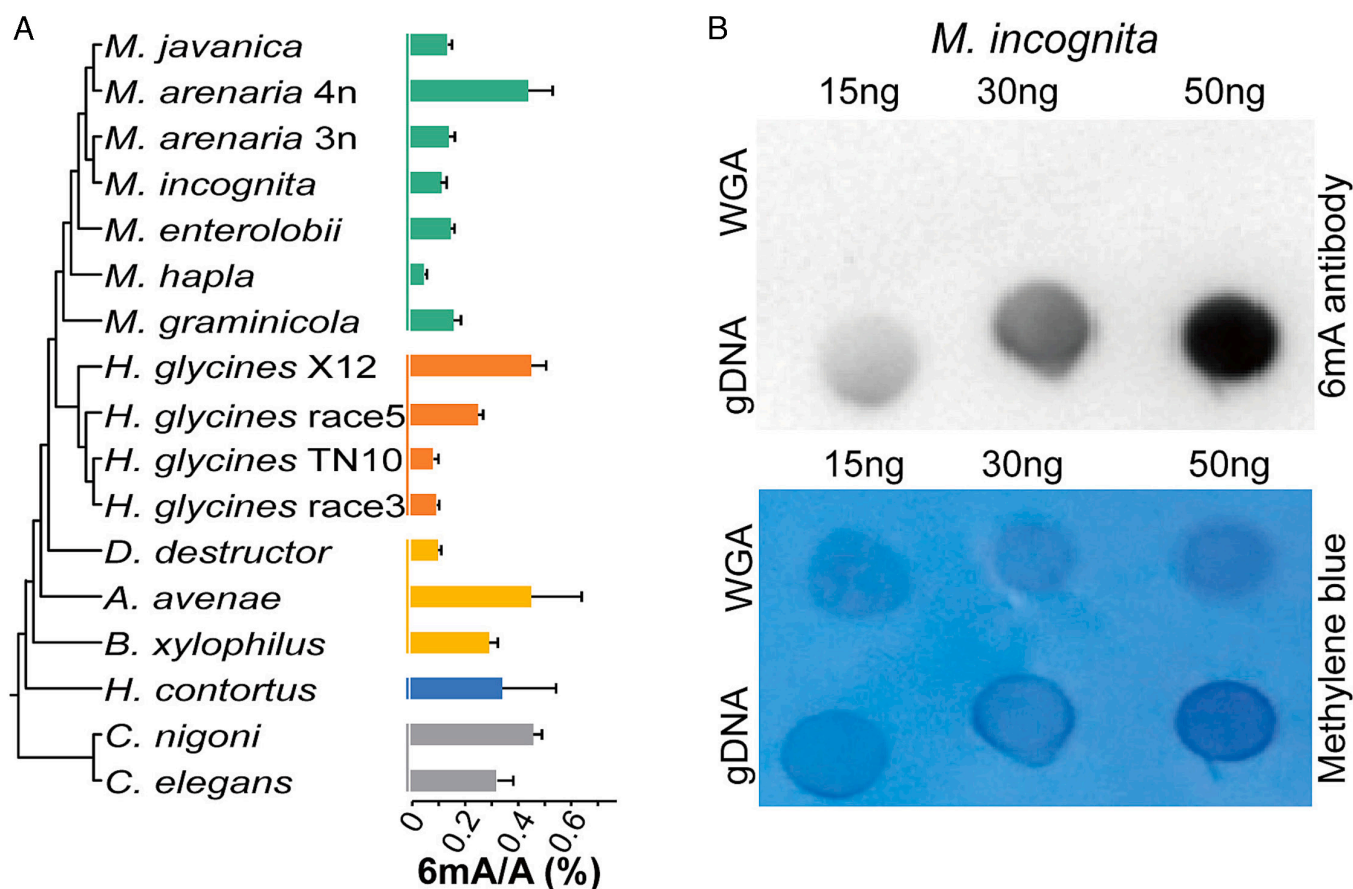


Fig. 1. 6 mA DNA methylation is widespread in nematodes. (A) 6 mA levels identified by LC–MS/MS in different nematodes. The green bar plot means 7 species of RKNs, the orange bar plot means 4 isolates of cyst nematodes, the yellow bar plot means migratory nematodes, the blue bar plot means animal-parasitic nematode, and the gray bar plot means free-living nematodes. Bars represent mean values $\pm$ SD from three independent biological replicates ($N = 3$). (B) Genomic DNA 6 mA dot blot analysis of *Meloidogyne incognita*. The upper panel shows the membrane after antibody incubation and chemiluminescent detection, followed by methylene blue staining in the lower panel. Whole genome amplified (WGA) DNA from *M. incognita* gDNA, generated using Phi29 polymerase, was used as a negative control.

one animal-parasitic nematode (*Haemonchus contortus*), and two free-living nematodes (*C. elegans* and *C. nigoni*). To minimize bacterial DNA contamination during LC-MS/MS detection, we extracted DNA from sterilized eggs and verified the absence of bacterial contamination using 16S rRNA primers amplification (27F, 1492R) (*SI Appendix, Fig. S1*). Finally, a clear 6 mA signal was detected in all tested species except for the whole-genome amplification (WGA) negative control (*Fig. 1A* and *SI Appendix, Fig. S2*).

To further understand the role of 6 mA methylation in the life history of PPNs, we focused on several species of RKNs (*SI Appendix, Tables S1 and S2*). PacBio sequencing is widely used to detect 6 mA modifications at single-base resolution; however, false positives caused by bacterial contamination remain a major concern. To address this, we applied the 6mASCOPE approach (28) to quantify potential contamination by aligning PacBio sequel I reads to the NCBI nt database. The analysis showed that only 0.0004 to 0.11% of reads were of bacterial origin (*SI Appendix, Tables S3*). To further validate these estimates, we generated HiFi sequencing data for Mi, *M. arenaria* 4n (Ma 4n), and *M. javanica* (Mj) and analyzed them with 6mASCOPE. The results revealed that the proportions of fungal and bacterial reads were 0.018%, 0.011%, and 0.011%, respectively (*SI Appendix, Tables S4–S6*). The 6 mASCOPE evaluation confirmed that the contamination rate in our data was extremely low. We then analyzed the 6mA content in our previously assembled genomes of *M. graminicola* (Mg), Mi, *M. arenaria* 3n (Ma 3n), Ma 4n, and Mj (38) using PacBio sequel I data, and the results were consistent with the LC-MS/MS measurements. In addition, we generated a chromosome-level genome assembly for the diploid *M. hapla* (Mh, *SI Appendix, Tables S7*) using PacBio, Illumina, and Hi-C data (*SI Appendix, Tables S8*). The genome size of this assembly is consistent with the previously reported version (39) (53.1 Mb vs. 53.6 Mb), but demonstrates markedly higher quality, as reflected by a substantially improved scaffold N50 (2.8 Mb vs. 0.08 Mb) and a BUSCO completeness of 66.8%. Furthermore, we identified 6 mA modifications at single-base resolution in Mh using PacBio sequel I data. To minimize false positives, 6 mA sites with low sequencing coverage were excluded using sample-specific minimum coverage thresholds (15× to 50×), as previously described (7, 11). After this filtering step, numerous 6 mA sites were identified across the six genomes (*SI Appendix, Tables S9*). Subsequently, to further minimize false positives in 6 mA analysis, we employed WGA samples sequenced by PacBio HiFi as negative controls, thereby providing an estimate of the false-positive rate (*SI Appendix, Tables S9 and S10*). We observed that samples with minimal bacterial contamination—such as Ma 4n—had very few false-positive 6 mA sites. In contrast, WGA samples with higher contamination levels yielded substantially more false-positive sites (*SI Appendix, Tables S10 and S11*). These findings highlight the importance of controlling for contamination when interpreting 6 mA methylation data.

To further validate the presence of 6 mA in RKN genomes, we first excluded potential RNA m6A contamination (*SI Appendix, Fig. S3*, see *Materials and Methods*). We then performed 6 mA dot blot assay on egg genomic DNA from Mi, which displayed clear 6 mA signal (*Fig. 1B*). In contrast, no signal was detected in the WGA negative control (*Fig. 1B*). Additionally, we performed 6 mA-DIP-seq on Mi genomic DNA and Mi-WGA DNA (*SI Appendix, Fig. S4A*). Pearson correlation analysis confirmed high reproducibility between replicates (*SI Appendix, Fig. S4B*). The results verified the presence of 6 mA in the Mi genome, with 53% of the peaks overlapping those identified by SMRT sequencing (*SI Appendix, Fig. S4 C and D*). By contrast, only a few peaks

were identified in the Mi-WGA 6 mA-DIP-seq data (*SI Appendix, Tables S12*). Collectively, these results provide strong evidence for the presence of 6 mA in RKN genomes.

6 mA Distribution Landscape in Nematodes Genome. DNA methylation site distribution has been reported to be associated with gene expression regulation (40). We categorized all 6 mA-modified genes according to whether the sites were located in promoter, gene body, or distal intergenic regions. Most 6 mA sites were found in gene bodies and promoters, with *C. elegans* (Ce) showing the highest proportion of 6 mA distribution in promoters (59.93%) (*SI Appendix, Fig. S5*). We found the motifs of 6 mA sites in promoters, gene bodies, and intergenic regions were largely similar (*SI Appendix, Fig. S6*). By contrast, cross-species comparisons revealed that motifs were highly conserved among RKNs but markedly different between RKNs and Ce (*SI Appendix, Fig. S6*). Specifically, the core motif in RKNs was GAG, whereas in Ce it was ApT, consistent with previous observations in fungi (11). Notably, the motifs identified in specific genomic contexts represent region-specific patterns and do not contradict the GAGG motif reported in previous global analyses. This finding suggests that 6 mA may regulate gene expression through distinct contexts in RKNs and Ce.

To investigate the functional implications of different 6 mA methylation levels, we classified 6 mA sites into three categories: low (at a specific locus, <30% of reads methylated across all sequencing data), moderate (30 to 70%), and high (70 to 100%). Moderate-methylation sites were the most abundant in Ce, Mi, Ma 3n, and Mj, accounting for ~76% of all 6 mA sites. In Ma 4n, Mg, and Mh, approximately 50% of the sites showed moderate methylation, whereas only a small fraction exhibited high or low methylation (*Fig. 2A*). Motif analysis of the 8-bp flanking sequences revealed three distinct motifs corresponding to the methylation categories in each nematode (*Fig. 2B*). Among them, the GAG motif was most enriched at high-methylation sites in four of the six RKNs (Mi, Mj, Mg, and Mh), consistent with previous findings in Ce (7) and rice (13, 14). By contrast, motifs associated with moderate and low methylation levels varied considerably among species (*Fig. 2B*), suggesting that different levels of 6 mA methylation may be regulated by distinct modifying enzymes and may serve diverse biological functions.

Subsequently, we analyzed the distribution density of 6 mA sites in gene regions and TE regions across these nematode genomes. We found that the gene regions generally exhibited a low density of 6 mA in all nematodes except Mh (*Fig. 2C*), a pattern reminiscent of the distribution characteristics of 5mC (13). By contrast, TE regions showed significant differences in the distribution density of 6 mA sites between polyploid and diploid species (*Fig. 2D*). These observations suggest potential lineage-specific patterns of 6 mA-TE distribution, which may warrant further investigation in the context of polyploidization.

To further investigate the distribution patterns of 6 mA in TE regions of polyploid and diploid nematodes, we examined 6 mA enrichment across different TE classes. In polyploid nematodes, 6 mA was densely distributed in DNA transposons, MITEs, and LTRs, but showed a low density in low-complexity regions (*SI Appendix, Figs. S7–S10*). We noticed that both the content and composition of TE differed significantly between polyploid and diploid RKNs (*SI Appendix, Tables S13*). In polyploid RKNs, TEs accounted for 28 to 33% of the genome, with 8 to 10% classified as Low-complexity elements (including simple repeat). While in diploid RKNs, only 8 to 10% of the genome consisted of TEs, of which 41 to 81% were low-complexity elements. However, differences in TE composition alone cannot explain the pronounced discrepancy in 6 mA abundance between polyploid

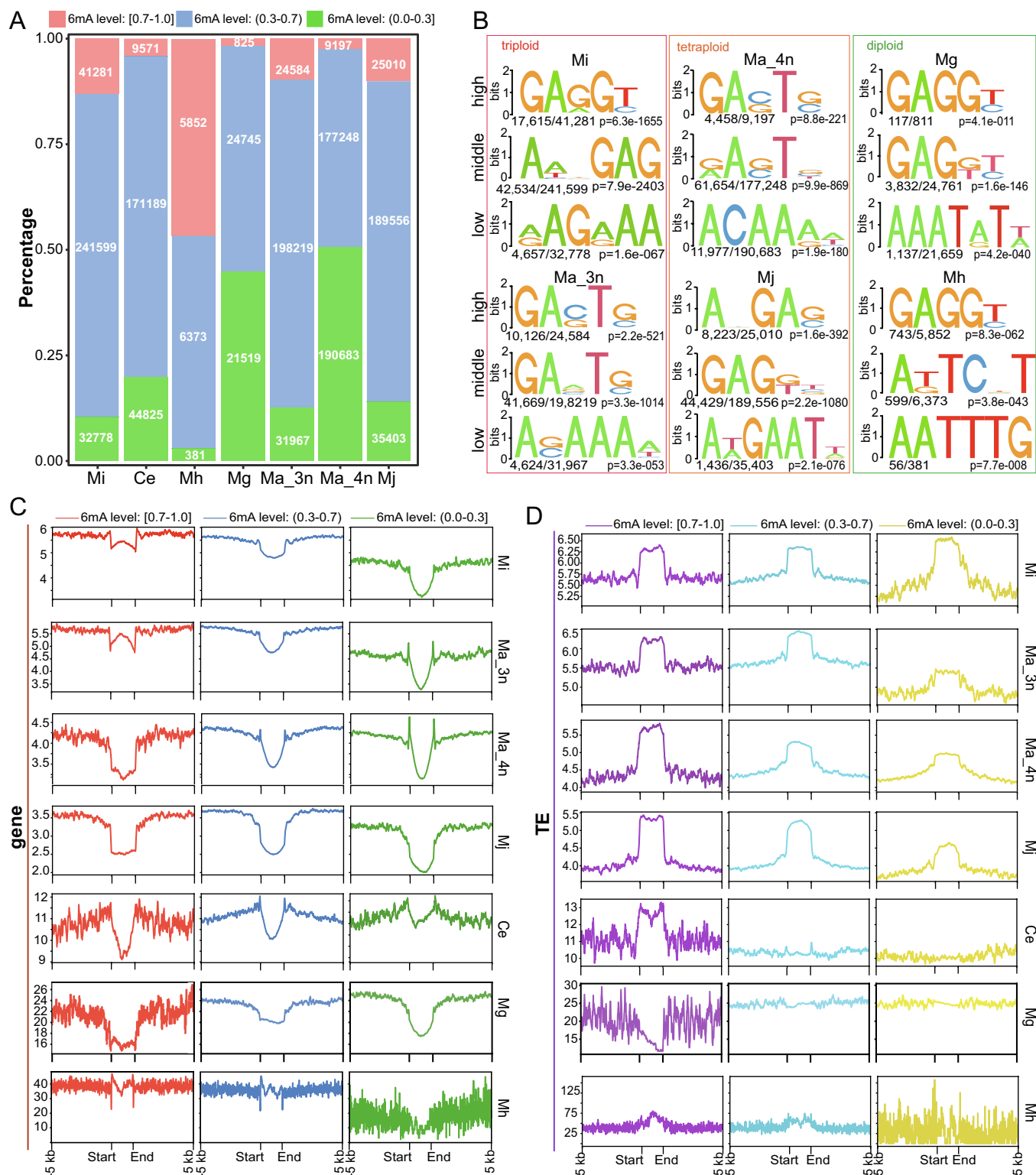


Fig. 2. The distribution landscape of 6 mA in RKNs genome. (A) Percentage of the different modification level categories of 6 mA in Mi, Ma 3n, Ma 4n, Mj, Mg, and Mh, respectively. (B) The motif of different methylation categories of Mi, Ma 3n, Ma 4n, Mj, Mg, and Mh. *P* values were calculated by two-sided Wilcoxon rank sum tests. (C) The distribution density of 6 mA on gene regions within 100 bp genome window (Mi, Ma 3n, Ma 4n, Mj, Ce, Mg, and Mh). (D) The distribution density of 6 mA on transposable element (TE) region within 100 bp genome window. The y-axis represents the average number of 6 mA per 100 bp genome window.

and diploid nematodes. For instance, although diploid nematodes harbor a substantial proportion of DNA transposons, MITE-type elements in these species still lacked the high-density 6 mA modification observed in polyploids (SI Appendix, Figs. S11–S13). For example, although *C. elegans* exhibits a TE composition similar to that of polyploid RKNs (SI Appendix, Tables S13), its 6 mA

distribution across TEs resembled that of diploid nematodes (SI Appendix, Fig. S13). To verify the authenticity of our observations, we repeated the analysis using false-positive 6 mA sites predicted from WGA sequencing data. These false-positive sites showed neither increased density in the TE regions nor a decreased density in gene regions (SI Appendix, Fig. S14), but instead

appeared randomly distributed across the genome. To further confirm the enrichment of 6 mA in TE regions, we compared 6 mA-DIP-seq peaks with the annotated TE regions in Mi. The results revealed that 74.8% of merged 6 mA-DIP-seq peaks overlapped with the TE regions, whereas TEs comprise only 28.18% of the Mi genome. Together, these results indicate that the high-density 6 mA feature of TEs in polyploid RKNs is not directly associated with TE type. Instead, it may reflect broader genomic processes, such as TE activity or mechanisms contributing to genome stability.

6 mA Regulates Gene Expression in RKNs. To assess the impact of 6 mA methylation on gene expression, we examined the association between methylation status and transcript levels from multiple perspectives. Firstly, we compared the expression levels of 6 mA-methylated and unmethylated genes in the egg stage (same stage as collecting SMRT sequencing data) across six RKN species. The results showed that in nearly all RKNs, 6 mA-methylated genes exhibited significantly higher expression levels than unmethylated genes, whereas the opposite trend was observed in Mh (*SI Appendix, Fig. S15A*). In polyploid RKNs, unmethylated genes were predominantly expressed at low levels (TPM < 1), while a larger proportion of methylated genes displayed higher expression levels (TPM > 5) (*SI Appendix, Fig. S15B*). By contrast, in diploid RKNs, most genes—regardless of methylation status—showed expression levels exceeding TPM > 5. In the free-living nematode Ce, however, the majority of genes exhibited low expression (TPM < 1) (*SI Appendix, Fig. S15B*). Collectively, these findings suggest that 6 mA methylation is predominantly associated with transcriptional activation in polyploid species, while no such trend is evident in diploid nematodes.

Subsequently, we performed motif enrichment analysis of 6 mA sites in genes with TPM < 1, 1 < TPM < 5, and TPM > 5. The results showed that within the same species, genes across different expression levels exhibited similar motif enrichment patterns, and motifs among RKNs were relatively conserved, characterized by a core GAG motif (*SI Appendix, Fig. S16*). In contrast, a marked divergence was observed between Ce and RKNs, with the core motif in Ce being ApT (*SI Appendix, Fig. S16*). Notably, the motif patterns associated with genes of different expression levels are similar to those observed for 6 mA sites located in different genomic regions (*SI Appendix, Fig. S6*).

Thirdly, we examined genes modified by 6 mA at only one of the three positions (promoter, gene body, or intergenic) as well as those simultaneously modified at all three positions (*SI Appendix, Fig. S17*) to explore expression differences among these categories. The analysis revealed that in polyploid RKNs, genes methylated in the gene body showed higher expression levels than the average of total genes (*SI Appendix, Fig. S15C*). In contrast, in diploid RKNs, promoter-methylated genes exhibited higher expression levels than the average of total genes (*SI Appendix, Fig. S15C*). Moreover, in Ce, both promoter- and gene body-methylated genes were expressed at higher levels than the average of total genes (*SI Appendix, Fig. S18*), which differs from the patterns in RKNs. These results suggest that 6 mA exerts distinct regulatory effects on gene expression in polyploid versus diploid RKNs, which may reflect differences in ploidy level and reproductive strategy.

Fourthly, we analyzed the expression levels of genes associated with different 6 mA methylation levels. The results showed that in Mi, Ma 3n, and Mg, genes with moderate methylation levels exhibited the highest expression, whereas in Ce and Mh, genes with high methylation levels displayed the highest expression (*SI Appendix, Fig. S15D*). These findings indicate that the impact on gene expression differs among nematode species.

Finally, we compared the expression levels of transposable element-related genes (TEGs) and 6 mA-marked genes, as previously described (13). Genes unmodified by either 6 mA or TE shows the lowest expression levels in all six nematodes except Mh. The triploid RKNs (Mi and Ma 3n) displayed similar patterns, whereas diploid and tetraploid RKNs did not (*SI Appendix, Fig. S15E*). In Ma 3n, Ma 4n, Mj, Mg, and Ce, 6 mA-modified genes with nearby TE showed higher expression than those without, while the opposite trend was observed in Mi and Mh (*SI Appendix, Fig. S19*). Moreover, 6 mA-marked TEGs consistently exhibited significantly higher expression than unmarked TEGs (*SI Appendix, Fig. S19*). These findings indicate that 6 mA exerts context-dependent effects on gene expression.

Low-Methylation-Level 6 mA Sites Play a Crucial Role in Mi.

We examined the genomic distribution of 6 mA sites at different methylation levels in RKNs (*SI Appendix, Fig. S20A*). Overall, sites of all three methylation levels were evenly distributed across chromosome, except for low-methylation-level sites in Mi, which showed a distinct enrichment (green circle in *SI Appendix, Fig. S20A*). Specifically, 72% of high-density-regions (HDRs, >60 sites per 50 kb genome window) of low-methylation-level 6 mA were located in the A₂ subgenome. We then extracted genes from these HDRs in the A₂ subgenome, along with orthologous regions in A₁ and B₁, to compare their expression levels at the egg stage. Notably, the genes from A₂ consistently exhibited higher expression than those from A₁ and B₁ (*SI Appendix, Fig. S21*). Moreover, these A₂ genes derived from HDRs maintained higher expression not only in the egg stage (the stage used for 6 mA profiling), but also across other developmental stages of Mi (*SI Appendix, Fig. S20B*), suggesting a subgenome-specific regulatory role of 6 mA.

To explore functional differences in genes associated with HDRs of low-methylation-level 6 mA sites between A₂ and A₁/B₁ subgenomes, we compared their enriched Pfam domain. The results showed that the genes from A₂ subgenome were enriched in GPCR (*SI Appendix, Fig. S20C*), consistent with previous GO enrichment results of the genes with high methylation density in humans (16). The GPCR superfamily represents one of the largest chemoreceptor families; in nematodes, the absence of GPCR renders them “blind” and “deaf” (41). GPCRs are also critical for fungal infection (42, 43) and may play essential roles in host recognition and effector secretion in RKNs (44). Furthermore, GO analysis revealed that A₂-associated genes were predominantly enriched in key molecule function, such as pharyngeal gland morphogenesis in Mi (*SI Appendix, Fig. S20D*). These findings suggest that low-methylation 6 mA modified genes in A₂ subgenome participate in vital biological processes and exert a stronger regulatory influence in A₂ than in the A₁ or B₁ subgenomes.

MinMAD-1/2 are Involved in 6 mA Demethylation. A previous study identified three 6 mA methyltransferases from Mi, but the corresponding demethylase remained unknown (37). To test whether Mi possesses 6 mA demethylase activity, we synthesized a biotin-labeled DNA oligos containing 6 mA and measured its methylation level after incubation with a suspension of disrupted Mi eggs. Compared with the heat-inactivated egg suspension control and the BSA control, the 6 mA content in the treatment group was significantly reduced (*SI Appendix, Fig. S22A*), indicating the presence of one or more putative 6 mA demethylases in Mi.

To identify putative demethylase genes in Mi, we collected six genes containing 2OG-FeII_Oxy_2 domain based on Pfam annotation, together with three genes showing the highest sequence similarity to *C. elegans* demethylase gene *nmad-1*. Phylogenetic

analysis revealed that these nine putative demethylase genes (including three alleles) clustered into the *alkbh1*, *alkbh6*, and *alkbh7* families (Fig. 3A and SI Appendix, Fig. S23). To assess whether these genes function as 6 mA demethylases, we measured genomic DNA 6 mA levels after RNAi-mediated knockdown of each gene in Mi eggs using LC–MS/MS. The results showed that knockdown of *Mi_14752.1* and *Mi_00010.1* significantly increased 6 mA levels, whereas knockdown of *Mi_23849.1* had no effect (Fig. 3B and SI Appendix, Fig. S22B).

We purified these three candidate proteins and tested their demethylation activity *in vitro*. Both *Mi_14752.1* and *Mi_00010.1* were able to demethylate Mi genomic DNA (Fig. 3C and SI Appendix, Fig. S24). To further validate their activity, synthetic DNA oligos carrying 6 mA modifications were treated with the purified proteins, and significant demethylation activity was again observed for *Mi_14752.1* and *Mi_00010.1* (Fig. 3D). In addition, comparative sequence analysis revealed that although demethylases from different species show substantial divergence at the overall sequence level, their catalytic active sites are highly conserved (SI Appendix, Fig. S25A). We predicted the 3D structure of *Mi_14752.1*, *Mi_00010.1*, *Mi_23849.1*, and CeNMAD-1 using AlphaFold2 (45). Structural comparison revealed that *Mi_14752.1* closely resembles human ALKBH7, with a Rms deviations (RMSD) of 0.067 Å (SI Appendix, Fig. S25B). Moreover, the catalytic sites of CeNMAD-1 overlap with those of *Mi_14752.1* and *Mi_00010.1* but not with *Mi_23849.1* (SI Appendix, Fig. S25 C–E). The RMSD values between CeNMAD-1 and *Mi_14752.1*, *Mi_00010.1*, and *Mi_23849.1* were 2.392, 1.066, and 12.625 Å, respectively (SI Appendix, Fig. S25 C–E). To determine whether the demethylase activity is intrinsic to *Mi_14752.1* and *Mi_00010.1*, we introduced point mutations (H153A and D155A in *Mi_14752.1*; H104A and D106A in *Mi_00010.1*). These mutations completely abolished their demethylation activity (Fig. 3E). Accordingly, we designated *Mi_14752.1* as Mi N6-methyladenine demethylase 1 (MiNMAD-1) and *Mi_00010.1* as MiNMAD-2, reflecting their identified demethylation functions.

HIGS of *minmad-1/2* reduces infectivity of MIG. Considering that most stages of the RKN life cycle occur within host plants, we generated transgenic tobacco lines expressing dsRNA targeting *minmad-1/2*, *Mi_23849.1* (to assess its demethylase activity during infection), and *gfp* (control) to investigate the regulatory role of 6 mA during the parasitic stage of the *M. incognita* group (MIG, including Mi, Mj, and Ma). In three-week pot experiments, *minmad-1/2*-HIGS tobacco plants exhibited significantly enhanced resistance to Mi compared with *gfp*-HIGS and CK (wild-type) controls, whereas *Mi_23849.1*-HIGS plants showed no significant difference (Fig. 4A). The eight-week pot experiment showed that the gall numbers in the high-expression *minmad-1/2*-HIGS tobacco line were reduced approximately tenfold compared with those in *gfp*-HIGS and CK plants (Fig. 4B and SI Appendix, Fig. S26 A and B). As anticipated, Mi genomic DNA exhibited a significant increase in 6 mA levels upon infestation of *minmad-1/2*-HIGS tobacco relative to the control (Fig. 4C), confirming the demethylase activity of MiNMAD-1/2 in Mi. Subsequently, according to galls number, we divided the nematode-infested *minmad-1/2*-, and *Mi_23849.1*-HIGS tobacco plants into three groups including high-, medium-, low-gall groups (SI Appendix, Fig. S27 A–C). After confirming the absence of bacterial contamination, DNA from females and eggs of the three groups was extracted for 6 mA quantification. The results showed a negative correlation between 6 mA content and gall number (Fig. 4D), indicating that demethylase silencing reduces the virulence of Mi. Additionally, the 6 mA level of females was much higher than that of eggs (Fig. 4D), indicating a stage-specific difference in

6 mA levels during development. To further explore the resistance conferred by demethylase silencing in different hosts, we generated *minmad-1*-HIGS tomato lines. Pot experiments demonstrated that RNAi targeting the demethylase enhanced tomato resistance to Mi (SI Appendix, Fig. S28 A–C), and the 6 mA level in Mi feeding on transgenic plants increased significantly (SI Appendix, Fig. S28D). These results highlight the feasibility of using transgenic plants expressing Mi demethylase dsRNA to confer resistance against Mi.

Additionally, *minmad-1* sequences are completely conserved among polyploid RKNs (SI Appendix, Fig. S29), suggesting that the transgenic plants expressing Mi demethylase dsRNA could potentially confer resistance to multiple polyploid RKNs. To test this hypothesis, we evaluated the infection ability of other polyploid RKNs on transgenic tobacco. The results showed that *minmad-1*-HIGS tobacco plants displayed significant resistance to the tetraploid RKNs Mj and Ma 4n compared with CK and *gfp*-HIGS controls (Fig. 4 E and F and SI Appendix, Fig. S30 A and B). Consistently, the 6 mA levels of genomic DNA from Mj and Ma 4n were significantly increased in *minmad-1*-HIGS group (Fig. 4 G and H). Finally, to assess the role of demethylases in diploid RKNs, we generated transgenic rice expressing Mg putative demethylase dsRNA. However, these plants did not exhibit resistance to Mg, despite the significant elevation of 6 mA levels in nematodes feeding on them (SI Appendix, Fig. S30 C–F). Together, these results suggest that demethylases regulate the virulence of polyploid RKNs, whereas their functional impact on diploid RKNs remains to be further explored.

6 mA Regulates the Expression Pattern of the Mi Virulence Gene.

To further investigate the regulatory effect of the 6 mA demethylase on Mi, we grew 80 *minmad-1*-HIGS tobacco plants inoculated with Mi. Based on gall formation, we divided the 80 tobacco plants into three categories: high-gall plants (groups 1 to 2, with numerous galls but fewer than those in the control), medium-gall plants (groups 3 to 6), and low-gall plants (groups 7 to 8). Infective third/fourth-stage juveniles (J3/J4) were then collected from these three categories for RNA-seq analysis (SI Appendix, Tables S14). We identified 7,198, 8,978, and 8,660 DEGs in high-, medium-, and low-gall plants, respectively (SI Appendix, Fig. S31). Among them, 4,667 DEGs were shared across all three groups (SI Appendix, Fig. S32A) and exhibited two distinct expression patterns (SI Appendix, Fig. S32B). The numbers of DEGs unique to the high-, medium-, and low-gall plants were 832, 1685, and 1491, respectively (SI Appendix, Fig. S32A). Functional enrichment analysis revealed that a large proportion of downregulated DEGs unique to the medium-gall plants were ribosome genes (SI Appendix, Fig. S32C), suggesting that demethylase activity may influence ribosomal function, thereby modulating mRNA translation efficiency and ultimately reducing Mi infectivity.

To explore the primary cause of the reduced infectivity of Mi in *minmad-1*-HIGS tobacco plants, we focused on the nematode's virulence gene (effectors), which constitute its key molecular weapons for host parasitism. From the 4,667 DEGs shared by the three groups, we identified 520 putative effector genes (SI Appendix, Fig. S32A). The putative effector genes exhibited consistently lower expression levels across all three gall groups compared with the control (SI Appendix, Fig. S32D), with the low-gall group showing the most pronounced downregulation (SI Appendix, Fig. S32E). These results indicate a negative correlation between transgenic plant resistance and the expression levels of Mi effectors.

Nematode invasion requires diverse effectors, and their proper expression is essential for host infestation (46). Expression profiling revealed that all putative secreted protein genes clustered into 11 groups, with RNAi plants displaying an expression pattern

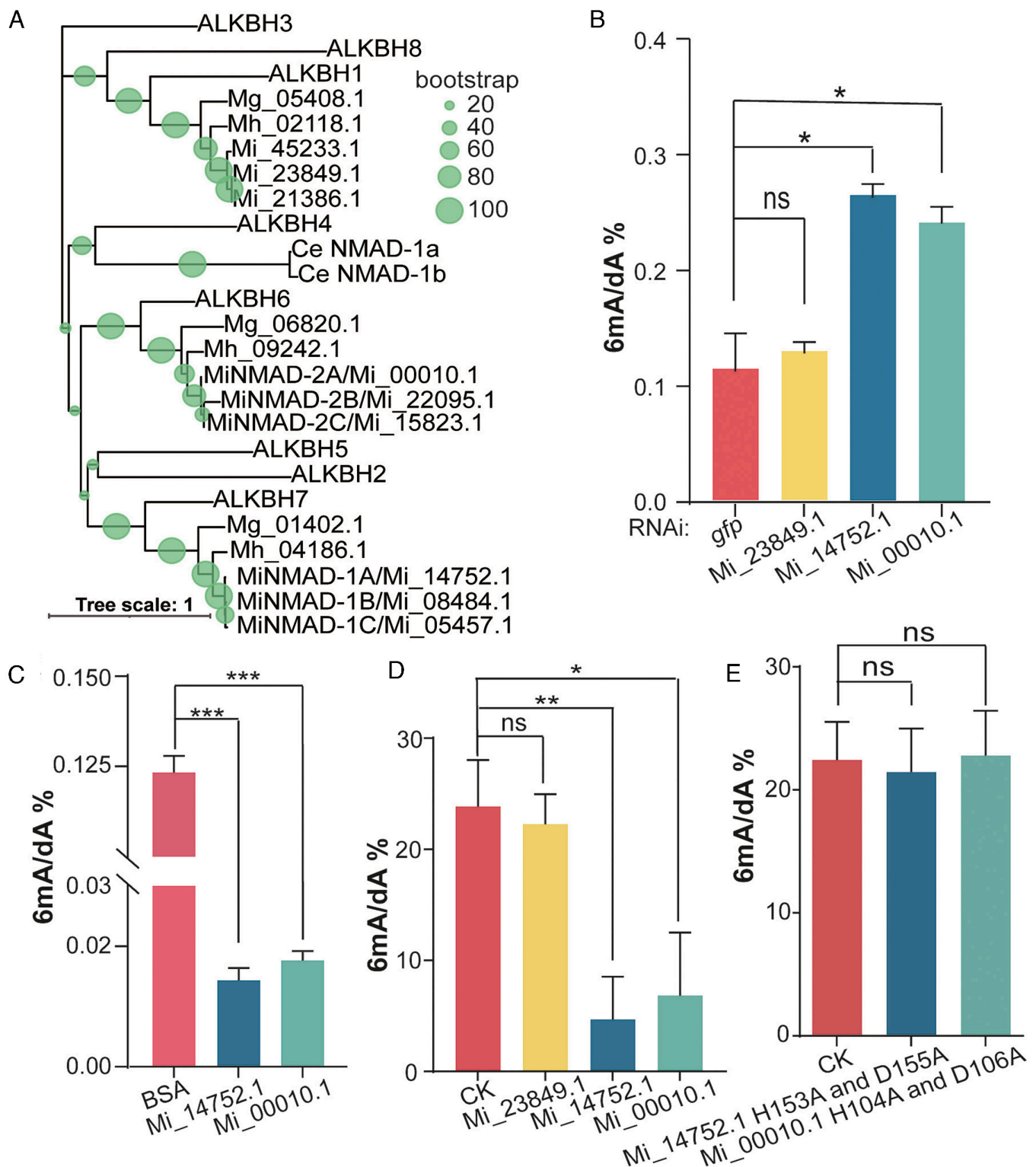


Fig. 3. MiNMAD-1/2 are involved in 6 mA demethylation in *M. incognita*. (A) The maximum-likelihood tree is constructed from the protein sequence of RKNs, *C. elegans* demethylase, and human ALKBH family members; bootstraps are shown in green dot. (B) Knockdown of 3 ALKBH family members present a significant increase of 6 mA level in two of them (Mi_14752.1, Mi_00010.1). The 6 mA level was determined by LC-MS/MS. (C) In vitro demethylation assays demonstrating that purified Mi_14752.1 and Mi_00010.1 remove DNA methylation from Mi WHF4-1 egg genomic DNA, with BSA as a negative control; 6 mA levels were quantified by LC-MS/MS. (D) LC-MS/MS detected the DNA oligos with 6 mA modified was demethylated by purified Mi_14752.1 and Mi_00010.1 proteins in vitro. (E) Mutation of the catalytic domain of Mi_14752.1 and Mi_00010.1 abrogates their ability to demethylate 6 mA-premethylated DNA oligos. Bars represent mean values $\pm$ SD from three independent biological replicates (N = 3). All P values were calculated by two-tailed unpaired Student's t test. * $P < 0.05$, *** $P < 0.01$, **** $P < 0.001$.

nearly opposite to the control (Fig. 5A). These results indicate that demethylases globally alter effector expression, thereby reducing Mi infectivity. To further examine the regulatory effect of demethylase on effectors, we measured the expression of several reported

nematode effectors in *minmad-1*-HIGS and *gfp*-HIGS control tobacco plants by RT-qPCR. The results showed that, compared with the *gfp*-HIGS control, the expression levels of Mi-XY11, Mi-CRT, Mi8D05, MiPFN3, 16D10, MiSGCR1, and MiIDL1

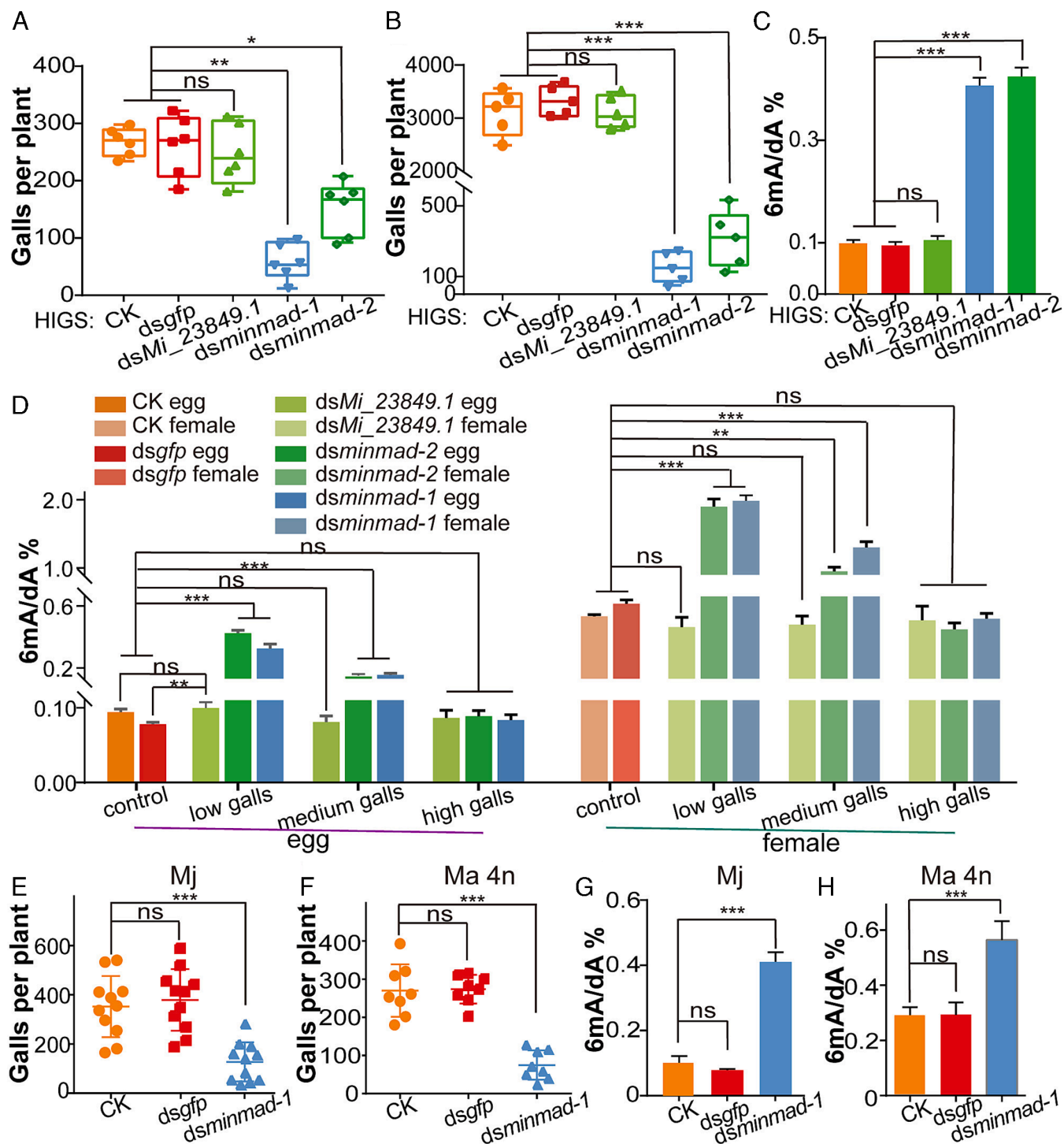


Fig. 4. Host-induced gene silencing (HIGS) of *minmad-1* reduces the infectivity of polyploid RKNs. (A) Three weeks pot experiment of transgenic plants show a resistance of Mi for *minmad-1* RNAi, but no difference for *Mi_23849.1* RNAi. Data were collected from two independent transgenic lines, with three independent pot experiments performed for each line (total N = 6). (B) Eight weeks' pot experiment show an extremely significant resistance of expression *minmad-1/2* dsRNA transgenic tobacco to Mi. Data were collected from a single transgenic line with the highest expression level, with five independent pot experiments performed (N = 5). (C) The 6 mA level of Mi is significantly increased after infection of transgenic tobacco expression *minmad-1/2* dsRNA. Bars represent mean values $\pm$ SD. For each treatment, nematodes were independently collected from five individual pot-grown plants, followed by independent DNA extraction and LC-MS/MS analysis (N = 5). (D) Plants were grouped according to gall numbers (low, medium, and high galls) following nematode infection. 6 mA levels in the corresponding *M. incognita* decreased with increasing gall numbers, and were consistently higher in females than in eggs. Bars represent mean values $\pm$ SD. For each bar, DNA was independently extracted from nematodes collected from 3 to 6 individual plants and analyzed separately by LC-MS/MS (N = 3 to 6). (E) The number of galls after 1,000 *M. javanica* (Mj) J2 infection two months of transgenic tobacco expressing *minmad-1* dsRNA. Data were collected from two independent transgenic lines, with five or six independent pot experiments performed for each line (total N = 11). (F) The number of galls after 1,000 *M. arenaria* 4n (Ma 4n) J2 infection of two months of transgenic tobacco expressing *minmad-1* dsRNA. Data were collected from two independent transgenic lines, with four independent pot experiments performed for each line (total N = 8). (G) The genomic DNA 6 mA content of the Mj after infecting the *minmad-1* transgenic tobaccos was significantly higher than that of the control. (H) The genomic DNA 6 mA content of the Ma 4n after infecting the *minmad-1* transgenic tobaccos was significantly higher than that of the control. For (G and H), bars represent mean values $\pm$ SD. For each treatment, nematodes were independently collected from five individual pot-grown plants, followed by independent DNA extraction and LC-MS/MS analysis (N = 5). All P values were calculated by two-tailed unpaired Student's *t* test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

6 mA-DIP-seq on Mi J2s to further assess whether 6 mA directly marks the candidate effector genes. Approximately 40% of the candidate effector genes carried 6 mA modifications, and notably, 47.4% of these overlapped with demethylase ChIP-seq targets (*SI Appendix, Fig. S35*). Together, these findings demonstrate that the enzyme directly acts on effector genes and may influence their transcriptional regulation.

Collectively, these results support a model in which 6 mA methyltransferases and demethylases dynamically regulate effector methylation in a spatiotemporal manner, thereby shaping their expression across developmental stages (*Fig. 5F*). Such regulation likely represents a major epigenetic mechanism underlying the parasitic ability of RKNs. Importantly, whether 6 mA also acts through transcription factors to exert broader control over effector networks remains to be determined, and will be an important direction for future research.

Discussion

In recent years, the existence of 6 mA in eukaryotes has been debated, largely due to concerns about bacterial DNA contamination. To address this, we implemented multiple strategies to minimize false positives: DNA for 6 mA analysis was extracted from axenic nematode eggs and other sterilized tissues verified free of microbial contamination (culture tests, 16S PCR, and 6 mASCOPE analysis), sequenced with PacBio-based methods under stringent coverage thresholds and false-positive filtering (including WGA and 6 mA-DIP-seq), and validated by LC-MS/MS. These results confirm that contamination in our datasets is negligible, strengthening the reliability of our findings. Importantly, WGA HiFi data also showed that samples with higher bacterial contamination yielded more false-positive 6 mA calls, which may explain earlier misinterpretations of 6mA in eukaryotes. Our analyses verified that the contamination ratio was as low as 0.01%, supporting the high reliability of our results.

Methylation is an epigenetic modification that influences gene expression and genome stability. The enrichment of 6 mA sites in TE regions may affect their stability, expression, and chromatin structure, thereby shaping the three-dimensional organization of the genome and modulating chromatin accessibility. Moreover, TE regions in polyploid nematodes harbor high-density 6 mA methylation, whereas diploid nematodes lack such enrichment. Given that TEs are often associated with phenotypic diversity and stress responses (47), a comparative analysis of 6 mA distribution between polyploid and diploid RKNs may help uncover the mechanistic driving these differences.

The AlkB family of dealkylating enzymes is conserved from bacteria to humans (48, 49). Humans encode nine AlkB proteins (AlkB1-8 and FTO), among which ALKBH1 and ALKBH4 are most strongly associated with demethylase activity. ALKBH1 has been confirmed as a demethylase in humans (16), mice (50), and rice (13), while NMAD-1 in *C. elegans*, a homolog of ALKBH4, performs a similar role (7). In this study, we identified three proteins containing the 2OG-FeII_Oxy_2 domain from Mi. Our in vivo and in vitro assays demonstrated that two proteins, homologous to ALKBH6 and ALKBH7, act as putative 6 mA demethylases in triploid Mi. Moreover, we found that genomic regions with different methylation levels corresponded to distinct sequence motifs, suggesting that these motifs may be recognized by different modifying enzyme systems.

Finally, our data suggest that 6 mA can directly affect the expression of nematode virulence genes, but it is also likely to influence virulence indirectly through modulating of key transcription factors. Importantly, 6 mA is a genome-wide epigenetic mark, and

its role in nematode biology extends beyond virulence regulation to broader essential processes such as development, reproduction, and stress responses, consistent with its known effects on shaping chromatin accessibility and transcriptional dynamics. Further studies of 6 mA analysis in additional parthenogenetic and sexual RKNs will be essential to further clarify its role across reproductive modes and ploidy levels. In summary, this study establishes a link between epigenetic regulation and pathogen virulence, and highlights 6 mA as a promising target for nematode control, with broader implications for the management of other agricultural pathogens.

Materials and Methods

Nematode Materials. Nematodes were propagated on Rutgers tomato plants under greenhouse conditions at 25 °C. Eggs, J3/J4, and females were collected after six weeks for downstream experiments. DNA used for SMRT-seq, LC-MS/MS, and dot blot assays was extracted from surface-sterilized J2s that had been inoculated into aseptically cultured tobacco plants grown on MS medium.

6 mA Identification by SMRT Sequencing. All 6 mA data from SMRT-seq analyses in this study were obtained from DNA extracted from eggs. The eggs used for DNA extraction were rigorously sterilized to minimize bacterial DNA contamination. SMRTLink versions 7.0.1 and 13.1.0 were used to identify 6 mA modifications.

LC-MS/MS Analysis of 6 mA. Mass spectrometric detection was performed using a triple-quadrupole tandem mass spectrometer (Shimadzu, LC-MS 8050) operated in multiple reaction monitoring (MRM) mode with positive electrospray ionization. Standards and samples were analyzed simultaneously using established methods based on ion mass transitions of m/z 266.1-150.0 for 6 mA and m/z 252.1-136.0 for adenine (A). The ratio of 6 mA to adenine (6 mA/A) was subsequently calculated.

Verification of Endogenous Nematode 6 mA Demethylase. Verification of endogenous nematode 6 mA demethylase activity was performed using protein extracts from WHF4-1 eggs. Protein extracts were incubated with biotin-labeled oligonucleotide DNA, followed by pull-down using Dynabeads MyOne Streptavidin C1. The bound DNA was then purified and analyzed to assess demethylase activity.

Generate of Transgenic Plants. Tobacco transformation was performed using *Nicotiana benthamiana*. Tomato transformation was carried out using the "MoneyMaker" cultivar following the same procedure as for tobacco, except that cotyledons and hypocotyls were used as explants. Cotyledons and hypocotyls from 5-d-old tomato seedlings were used for transformation. Transgenic rice plants were generated by Wuhan Towin Biotechnology Co., Ltd.

More detailed experimental procedures are provided in *SI Appendix*.

Data, Materials, and Software Availability. All sequencing data have been deposited in NCBI BioProject under accession numbers PRJNA897862 (51), PRJNA896710 (52), PRJNA1208558 (53), and PRJNA936407 (54). The genome assembly of *Meloidogyne hapla* MhF1-2 has been submitted to the China National Center for Bioinformation Genome Warehouse (<https://ngdc.cnca.ac.cn/gwh/>) (55) under accession number GWHJF100000000.1.

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