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Article:

Matthys, J., Naessens, I., Van Nieuwerburgh, F. et al. (2026) Distinct targeting of 5mC and 6mA DNA methylation during the onset of Flg22-induced immunity between Arabidopsis and rice. New Phytologist. ISSN: 0028-646X

<https://doi.org/10.1111/nph.71600>

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MANUSCRIPT TITLE

Distinct targeting of 5mC and 6mA DNA methylation during the onset of Flg22-induced immunity between Arabidopsis and rice

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SOCIAL MEDIA PROMOTION:

Genome-wide profiling reveals 5mC and 6mA dynamics during Flg22-induced plant immunity

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WORD COUNTS

Introduction: 803

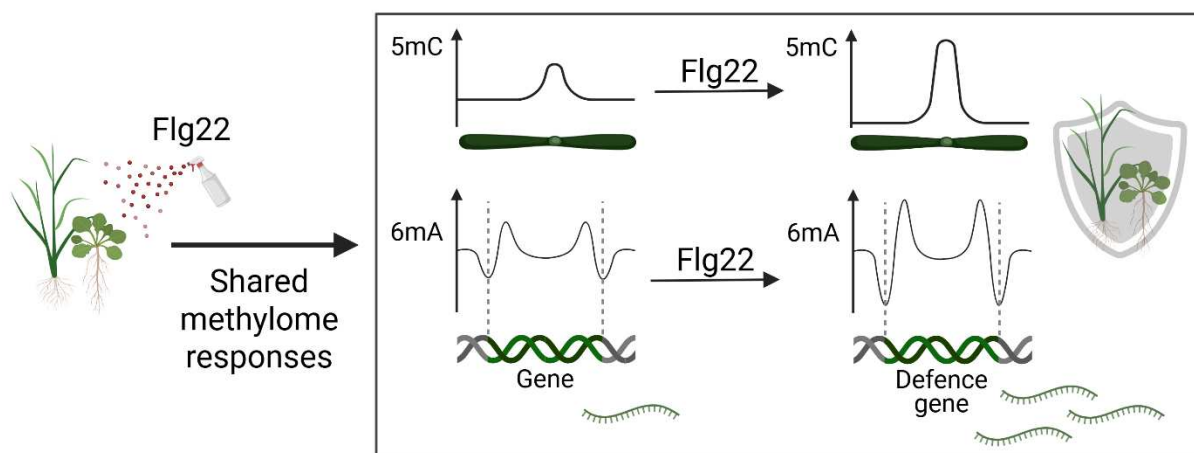
Materials & Methods: 2042

Results: 2918

Discussion: 1699

Total: 7462

GRAPHICAL ABSTRACT



Using Oxford Nanopore sequencing, genome-wide DNA methylation responses to Flg22 were compared between *Arabidopsis thaliana* and *Oryza sativa*. In both species, immune activation was marked by increased relative 5mC accumulation at (peri)centromeric regions compared with chromosome arms. In parallel, defence-responsive genes displayed an enhanced bimodal 6mA distribution flanking transcription start and termination sites following Flg22 elicitation. These findings identify shared methylation features associated with immune activation across two evolutionarily distinct plant species and associate 6mA remodelling with activation of the immune-responsive transcriptome. Created in BioRender. Kyndt, T. (2026). <https://BioRender.com/smipv3o>

SUMMARY

- Cytosine methylation drives transcriptional reprogramming during plant innate and acquired immunity. Beyond RNA adenosine methylation, DNA adenine methylation (6mA) has recently emerged as a candidate regulator of gene expression, though its role in plant immunity is unknown.
- Using the bacterial elicitor Flg22, we examined genome-wide DNA methylation responses of *Arabidopsis thaliana* and *Oryza sativa* to identify shared epigenetic responses across divergent genomes. High-resolution Oxford Nanopore sequencing enabled simultaneous detection of 5-methylcytosine (5mC) and 6mA DNA modifications.
- In both species, early immune activation was marked by non-CG 5mC hypomethylation at short euchromatic transposable elements. By 72 hpt, both species showed elevated 5mC levels at (peri)centromeric regions, targeted to pericentromeric non-CG sites in *Arabidopsis* and accompanied by lasting chromosomal arm hypomethylation in rice. Despite species-specific global 6mA responses, both species exhibited a conserved bimodal 6mA distribution flanking transcription start and termination sites of genes, intensified at defence-related genes following Flg22 elicitation. In *Arabidopsis*, these 6mA signatures correlated with Flg22-primed gene activation, while mutation of 6mA demethylase ALKBH1D increased susceptibility to *Pseudomonas syringae* and *Meloidogyne incognita*.
- Together, our findings reveal conserved 5mC dynamics during immune activation and highlight a role for 6mA in modulating defence-related transcriptional reprogramming.

Keywords: plant immunity, flagellin, epigenetics, DNA methylation, Transposable elements, adenine methylation, *Arabidopsis thaliana*, *Oryza sativa*

INTRODUCTION

Susceptible plants can acquire an enhanced defensive capacity after an early-life exposure to an immunity-activating stimulus, enabling them to mount faster and stronger responses to subsequent attacks (1). This acquired resistance can protect plants against a broad spectrum of biotic stresses over both short and extended timescales, including inter- and transgenerational resistance (2–6). Research into the establishment and persistence of this acquired immunity has underscored the importance of epigenetic modifications, particularly DNA cytosine methylation (5mC) (7–12). One proposed mechanism underlying this immune memory is defence gene priming, where selected genes are maintained in a potentiated state via epigenetic mechanisms, such as DNA methylation and the action of non-coding RNAs (3, 13, 14).

The importance of 5mC in plant immunity has been established in various studies. Specifically DNA hypomethylation has been proposed as a key regulator of plant immunity (7, 15–19). These 5mC methylation changes are mostly directed towards transposable elements (TEs) and repetitive regions, underscoring the importance of TE-mediated *trans*-regulatory mechanisms (5, 16, 20). For instance, a study identified four hypomethylated epigenetic quantitative trait loci, explaining more than 60% of the variance in *Arabidopsis* susceptibility to *Hyaloperonospora arabidopsidis*, which were located at TE-rich pericentromeric regions (21). Altered methylation patterns at TEs can regulate immune genes in *trans* by generating small RNAs (16). These small RNAs may silence immune genes or their repressors by guiding DNA methylation by the RNA-directed DNA methylation (RdDM) pathway (20, 22), but may also activate defence genes via interaction with AGO1 (7, 23). Various studies have shown that both active DNA demethylation and remethylation by RdDM are essential to establish short- and long-term immune memories that are at least partially transmittable to subsequent generation(s) (7–9, 12, 24, 25). However, the exact methylation changes that occur during the onset of plant immunity remain poorly understood.

Recent interest has emerged in – the less abundant – adenine methylation of genomic DNA, specifically *N*⁶-methyl-2'-deoxyadenosine (6mA). Unlike the extensively studied m⁶A RNA modification (26, 27), the regulatory relevance of 6mA in plant genomes is only beginning to be resolved. In various plant species, 6mA gene body methylation has been correlated to active gene expression (28–33). However, its role in environmental response remains unclear. In rice, the 6mA methylomes of the two cultivars Nipponbare (subsp. *japonica*) and 93-11 (subsp. *indica*) responded distinctly to heat, cold and salt stress (31). While salt stress increased the global 6mA levels in both cultivars, heat stress led to 2.6 times more increase in the heat-tolerant cultivar 93-11. Similarly, upon exposure to cold, global 6mA levels decreased in both cultivars, but more strongly in the cold-tolerant Nipponbare (31), showing that 6mA levels in rice respond to environmental stress in a dose-dependent manner, with response magnitude correlating with stress tolerance. By contrast, an independent study reported that both *Arabidopsis* and rice subjected to cold showed increased 6mA levels both globally and at key cold-responsive genes (34). Finally, natural salt tolerance of the halophyte *Paspalum vaginatum* was linked to moderate 6mA levels at salt-tolerance genes (35). These observations show that 6mA may regulate the abiotic stress response, warranting research into its role in plant immunity. 6mA has been linked to immunity in *Caenorhabditis elegans*, where the specific 6mA methyltransferase METL-9 contributed to innate immunity by increasing global 6mA levels in response to pathogen infection (36). These 6mA increases were enriched at both

upregulated and defence genes, while mutation of *METL-9* abolished immune transcriptional activation, showing their importance in transcriptional reprogramming in response to infection. To the best of our knowledge, no elaborate research has been conducted into the role of 6mA DNA methylation in plant immunity.

Bacteria are important plant pathogens, and as a result, plants have evolved immune receptors to detect conserved bacterial molecules (pathogen-associated molecular patterns, PAMPs) such as flagellin. The 22-amino-acid peptide Flg22, which represents the immunogenic epitope of bacterial flagellin, is well known for triggering rapid and transient immune responses in plants, including acquired immunity and longer-term priming of defence mechanisms (37, 38).

To unravel the epigenetic changes set upon PAMP recognition in plants, we investigated the dynamic DNA methylome changes at the onset of Flg22-induced immunity in two model plants, Arabidopsis and rice. We leveraged long-read Oxford Nanopore Technologies (ONT) sequencing to improve mapping at TEs and repetitive regions, given their importance to plant immunity (16). The base modification features provided during ONT-seq allowed to characterise both 5mC and 6mA changes during the plant immune response. Although Arabidopsis and rice exhibited divergent methylome landscapes under basal conditions, they shared conserved DNA methylation responses to Flg22. In both plant species, Flg22 initially triggered euchromatic TE non-CG hypomethylation, followed by a relative increase in non-CG methylation at the (peri)centromeres compared to the chromosome arms. Furthermore, both species displayed a bimodal gene-bordering 6mA profile, which was sharpened upon Flg22 treatment and associated with immune-responsive transcriptomes.

MATERIAL AND METHODS

Plant materials and growth conditions

***Arabidopsis thaliana* (L.) Heynh. (Arabidopsis)** accession Col-0 (NASC) was used for all experiments, unless specified otherwise. Prior to sowing, seeds were sterilised using 70% ethanol and bleach, and were thoroughly air-dried. Each seed was sown on a 50 μ L $\frac{1}{2}$ MS medium plug (0.6% microagar) suspended in $\frac{1}{2}$ Hoagland solution to establish a sterile hydroponic growth system. Sown seeds were stratified for four days at 4°C in the dark. Plants were grown for three weeks in a growth chamber under 16h/8h light/dark at 21°C in a sterile, closed hydroponic system. After two weeks of growth, the $\frac{1}{2}$ Hoagland solution was refreshed every two days.

***Oryza sativa* L. cv. 'Nipponbare'** (GSOR-100, USDA) seeds were dehusked and sterilised with 70% ethanol and bleach. Seeds were germinated in the dark at 30°C on $\frac{1}{2}$ MS plates. Germinated seeds were grown hydroponically in $\frac{1}{2}$ Hoagland solution, refreshed every two days, for one week in a growth chamber under 12h/12h light/dark at 28°C.

Treatments

Arabidopsis (3-weeks-old) and rice (1-week-old) plants were treated with 1 μ M Flg22 (Proteogenix) or distilled water (mock), both supplemented with 0.02 % (v/v) Tween-20 (P1379, Sigma-Aldrich). Treatments were applied 1.5 (*Arabidopsis*) or 3.5 (rice) hours after the start of the photoperiod (in both cases at 8:30 AM) by immersing roots in treatment solution for one hour and simultaneously spraying shoots (0.5 mL per *Arabidopsis* plant; 1.25 mL per rice plant). After treatment, plants were returned to growth conditions.

The experimental set-up with treatments, timing and samples is illustrated in detail in Supplementary Figure 1A. *Arabidopsis* plants received either mock or Flg22 treatments on two occasions separated by 72 hours, generating four conditions: mock (mock + mock), 12 hours post-treatment (hpt) Flg22 (mock + Flg22), 84 hpt Flg22 (Flg22 + mock) and defence priming (Flg22 + Flg22). In addition, a separate set of plants received a single Flg22 treatment and was sampled at 72 hpt. Each sample consisted of twenty pooled whole plants, snap-frozen in liquid nitrogen, and was used for both methylome and transcriptome analyses.

For rice, methylome and transcriptome analyses were performed in separate experiments. For methylome analyses, plants were treated once with mock or Flg22 and sampled at 12 and 72 hpt. For transcriptome analyses, the same two-treatment design as in *Arabidopsis* was applied, with treatments separated by 72 hours, generating mock (mock + mock), 12 hpt Flg22 (mock + Flg22), 84 hpt Flg22 (Flg22 + mock) and defence priming (Flg22 + Flg22) conditions. Each rice sample consisted of ten pooled whole plants, snap-frozen in liquid nitrogen.

Oxford Nanopore Technology (ONT) library preparation and sequencing

Plant material was ground using mortar and pestle, cooled by liquid nitrogen. For *Arabidopsis*, three biological replicates per condition (mock, 12 and 72 hpt Flg22) were processed for sequencing, while for rice only one biological replicate was used per time point (mock, 12 and 72 hpt Flg22). High molecular weight (HMW) DNA was extracted using the NucleoBond® HMW DNA kit (MN740160.20, Macherey Nagel) according to the manufacturer's instructions. DNA quality and size distribution were assessed using the HS Genomic DNA 50 kb kit on the

Fragment Analyzer 5200 (DNF-468-0500, Agilent Technologies). Concentration of the genomic DNA was measured with the Qubit dsDNA HS Assay Kit (Q32851, Thermo Fisher Scientific). For Arabidopsis and rice samples, respectively, 400 ng and 600 ng extracted HMW genomic DNA was used as input for the library preparation with the Native Barcoding Kit v14 (SQK_NBD114.24, Oxford Nanopore Technologies), following manufacturer's instructions with some minor adaptations. Specifically, after the Native Barcoding Ligation step, samples were each purified separately with Ampure XP beads (ratio 0.4x; A63881, Beckman Coulter) and eluted in 8 µl molecular biology grade water before pooling them for the Native Adapter ligation step. In addition, pooling was performed using an equimolar mass of each purified sample, calculated from Qubit concentration measurements and Fragment Analyzer size profiles obtained after clean-up. All nine Arabidopsis samples were pooled together into a single library. Rice samples were pooled into two libraries, each comprising one mock- and one Flg22-treated sample collected at the same time point (12 or 72 hpt). The prepared library pools were entirely, with a maximum of 30 fmol, loaded into a PromethION flow cell (FC) (R10.4.1) on a PromethION sequencer (ONT). After 1-2 days, the FC was washed using the Flow Cell Wash Kit (EXP-WSH004, ONT) to recover pore activity, and a fresh library was loaded. The FC was then run to exhaustion. Sequencing yielded reads with an N50 of approximately 17 kb.

Cytosine and adenine methylation calling from ONT-data

Raw .pod5 files were basecalled and demultiplexed using Dorado and the modified basecalling models 'dna_r10.4.1_e8.2_400bps_sup@v4.3.0_5mC_5hmC@v1' and 'dna_r10.4.1_e8.2_400bps_sup@v4.3.0_6mA@v2' (<https://github.com/nanoporetech/dorado>; accessed: 10/03/2025), for 5mC and 6mA, respectively. Reads were then aligned to the *Arabidopsis thaliana* (Col-CEN v1.2, <https://github.com/schatzlab/Col-CEN/tree/main/v1.2>) (39) and *Oryza sativa* ssp. *Japonica* reference genome (build MSU7.0/IRGSP1.0) using minimap2 (version 2.26-r1175).

Summary counts of modified and canonical bases in bedMethyl format were generated using modkit (version 0.2.2). For cytosine methylation, calls were also summarised in CG, CHG and CHH contexts (function: modkit pileup --ref reference_genome.fasta --motif CG 0 --motif CHG 0 --motif CHH 0). For cytosine methylation, the unmethylated plastid genome was used for estimating false-positive methylation calls. For Arabidopsis, samples showed an average plastic methylation percentage of 0.85%, when excluding positions with less than 5x coverage. For rice, the average plastic methylation percentage was higher at 1.90%, when excluding positions with less than 20x coverage. For reference, these values are below the accepted non-conversion rate of WGBS data (< 2%) (40). Details about the analysis of global DNA methylation patterns, DNA methylation levels at genes and TEs, and differentially methylated regions (DMRs) are described in the Supplementary Methods.

RNA extraction, sequencing and data analysis

For each condition (mock, 12 hpt Flg22, 84 hpt Flg22 and primed), three biological replicates consisting of the pooled material of twenty Arabidopsis or ten rice plants were analysed. For rice, three sequencing runs were performed, each including three samples per condition, to improve transcriptome robustness. Plant material was ground using mortar and pestle cooled by liquid nitrogen. Total RNA was extracted using the RNeasy Plant Mini Kit (74904, Qiagen) according to the manufacturer's protocol. RNA was treated with DNase I (30 min at 37°C;

EN0521, Thermo Fisher Scientific) and subsequently purified by sodium acetate/ethanol precipitation. Quality and concentration of the input total RNA was checked with 'Quant-it ribogreen RNA assay' (R11490, Life Technologies) and with the DNF-472 HS RNA (15 nt) kit on the Fragment Analyzer (Agilent Technologies), respectively. The RIN values were 7.20 ± 0.40 (average $\pm$ standard deviation). Subsequently, 170 ng of RNA was used to perform an Illumina sequencing library preparation using the QuantSeq 3' mRNA-Seq Library Prep FWD Kit (Lexogen) including Unique Molecular Identifiers (UMI) according to the manufacturer's protocol using 15 enrichment PCR cycles. The QuantSeq 3' mRNA-Seq protocol is optimised for gene-level expression analysis, with limited resolution for comprehensive transcript isoform characterisation. Libraries were quantified by qPCR, according to Illumina's protocol 'Sequencing Library qPCR Quantification protocol guide'. With a DNF-474 HS NGS Fragment (1-6000 bp) kit on Fragment Analyzer (Agilent Technologies) the library's size distribution and quality were evaluated. Libraries were equimolarly pooled and sequenced on an Element Biosciences Aviti sequencer together with 5% PhiX Sequencing Control library, generating 100 bp single-end reads.

Reads were trimmed with Trimmomatic using following settings: ILLUMINA-CLIP: TruSeq3-SE.fa: 3:30:10, SLIDINGWINDOW:5:20, MINLEN:20 (version 0.36) (41) and polyA-tails were removed using TrimGalore (version 0.6.0). The resulting reads were mapped to an index generated from the TAIR10 genome using Araport11 annotations for Arabidopsis, or from the *Oryza sativa* ssp. *Japonica* reference genome (build MSU7.0/IRGSP1.0) using STAR (version 2.5.2a) (42). UMI-adapters were used to remove duplicate reads using umi_tools dedup (version 1.1.4) (43). Count tables were generated by the SummariseOverlaps function in the Genomic Alignments R package (version 1.42.0) (44) on the Araport11 and IRGSP-1.0.42 annotations. Differential expression analysis was performed using the edgeR package (version 4.4.2) (45) according to the limma-voom pipeline. Lowly expressed genes were filtered using filterByExpr, followed by library size normalisation using the TMM method (calcNormFactors). Normalised counts were transformed with the voom method, and linear modelling was performed using lmFit, followed by empirical Bayes moderation (eBayes). For rice, sequencing run was included as a covariate in the linear model. P-value distributions were checked to ensure proper distribution of the observations. Genes with an FDR < 0.05 (Benjamini-Hochberg adjusted) were considered differentially expressed compared to the control group. Details about the cross-referencing of the ONT and RNA-seq datasets, and about determining defence primed genes are described in the Supplementary Methods and Supplementary Figure 1.

Arabidopsis Pst-LUX infection assay

Arabidopsis plants were grown in a modified hydroponics set-up. Col-0 (wild type) and *alkbh1d* (SALK_021477C, obtained from NASC) sterile seeds were sown into sterile 12-well plates (5-7 seeds per well) containing 2 mL liquid $\frac{1}{2}$ MS medium with 0.5% sucrose. Plates were stratified at 4°C in darkness for four days. Seedlings were then transferred to a long-day growth chamber (16h/8h light/dark at 21°C). On day five, each well was thinned to contain a single seedling per well and the medium was refreshed with the same formulation to maintain nutrient balance. Twelve biological replicates were used for each genotype. On day nine, the medium was removed and replaced with 2 mL of a *Pseudomonas syringae* pv. *tomato* DC3000::LUX (*Pst*-LUX) suspension ($\sim 4 \times 10^6$ CFU/mL in $\frac{1}{2}$ MS without sucrose + 0.025% Silwet L-77 (Lehle Seeds)). After 3 minutes of incubation at room temperature, the inoculum was

removed, wells were rinsed twice with sterile distilled water, and 500 μ L of $\frac{1}{2}$ MS (no sucrose) was added per well. *Pst*-LUX cultures were grown overnight at 28°C with shaking (190 rpm) in King's B medium supplemented with rifampicin (10 μ g/mL) and kanamycin (50 μ g/mL). Bacteria were pelleted, washed twice with sterile $\frac{1}{2}$ MS medium, and adjusted to OD₆₀₀ = 0.02 ($\sim 4 \times 10^6$ CFU/mL) before inoculation.

Bioluminescence was captured daily from day 10 to day 13 (1-4 dpi, days post infection) using a GelDoc imaging system (G:BOX, Syngene; Dataset S1). Plates were placed centrally on the imaging platform with a scale reference. After 8 minutes in darkness, images were captured with a 4-minute exposure. Quantification was performed using ImageJ software. Brightfield images were used to define regions of interest (ROIs) corresponding to individual plants, and the scale was set using a coin of known diameter. ROIs were applied to corresponding bioluminescent image stacks. Background signal was estimated from twenty non-leaf areas and used to define a threshold (mean + 3 x SD) for background subtraction. Bioluminescence within ROIs was quantified post-thresholding per mm². Statistical analysis was performed using a linear mixed-effects model (R-package lme4, function: lmer, including a random intercept for SampleID to account for repeated measurements per individual plant) and Type III ANOVA.

Arabidopsis *Meloidogyne incognita* infection assay

Col-0 (wild type) and *alkbh1d* (SALK_021477C, obtained from NASC) sterile seeds were sown on square plates containing MS20 medium. After two-day stratification at 4°C, seedlings were transferred to a long-day growth chamber (16h/8h light/dark at 21°C). Thirty biological replicates were used for each genotype.

Meloidogyne incognita race 3 cultures were maintained on tomato (*Solanum lycopersicum* L. 'MoneyMaker') plants. Eggs were extracted by cutting infected roots into 5 cm pieces and vigorously shaking them in 0.5% bleach for 3 min, followed by filtration through a stack of 1000, 250, 100, 50 and 25 μ m sieves and rinsing with water. Eggs were surface-sterilised in 10% bleach for 3 min, washed and incubated in 0.2% sodium azide solution for 20 min. After removing residual sodium azide, eggs were transferred to a 25 μ m hatching sieve in a glass petri dish containing 1.5 mg/mL gentamycin and 0.05 mg/mL nystatin, and kept in the dark at room temperature for five days. Second-stage juveniles (J2s) were sterilised using 0.002% Triton X-100, 0.004% NaN₃ and 0.004% HgCl₂ for 15 min, rinsed, and resuspended in sterile 0.7% gelrite to obtain a density of 15 J2s/ μ L.

Eight-day-old seedlings were inoculated with 150 J2s each. Plates were partially covered with foil to reduce light exposure to inoculated roots. Galls were counted at 6 and 14 dpi using a stereomicroscope (SMZ1270, Nikon; Supplementary Figure 2). Statistical analysis was performed using a linear mixed-effects model (R-package lme4, function: lmer, including random intercepts for SampleID and PlateID to account for repeated measurements per individual plant and plate effects, respectively) and Type III ANOVA.

RESULTS

To examine the methylome and transcriptome dynamics at the onset of plant immunity responses, Arabidopsis and rice were treated with the well-characterised immune elicitor Flg22 (37) (Supplementary Figure 1A). In both species, Flg22 induced transcriptional reprogramming at 12 hours post treatment (hpt), comprising 1587 up- and 982 downregulated genes in Arabidopsis and 414 up- and 656 downregulated genes in rice. These effects persisted at 84 hpt, with 2672 up- and 2326 downregulated genes in Arabidopsis and 585 up- and 790 downregulated genes in rice. However, defence pathways showed partial attenuation, consistent with negative feedback regulation (Supplementary Figure 3 & 4). In Arabidopsis, double Flg22 treatment (Supplementary Figure 1A) resulted in defence gene priming (as defined in Supplementary Figure 1B), with 685 up- and 1251 downregulated defence primed genes (Supplementary Figure 3C), suggesting the formation of an acquired immune memory. In rice, a similar double Flg22 treatment did not reveal a clear priming-associated transcriptional response under our experimental conditions.

Flg22 induces early non-CG hypomethylation of euchromatic TEs on the chromosome arms followed by late pericentromeric hypermethylation in Arabidopsis

To investigate how the methylation landscape in Arabidopsis is altered at the onset of (acquired) immunity, we performed whole-genome sequencing using ONT on DNA from plants treated with Flg22 and sampled at 12 and 72 hpt. ONT improves mapping in repetitive regions, including pericentromeres, providing high-resolution methylation profiles across the genome. At each time point, three biological replicates, each consisting of 20 pooled plants, were taken. The Arabidopsis genome was well covered with an average per-sample coverage of 74x, with only 0.08% of the genome covered by less than 20 reads. As expected, 5mC levels within the centromere and adjacent pericentromeric regions (hereafter collectively referred to as the pericentromere throughout the Arabidopsis analyses) were significantly higher than those at the chromosome arms ($p = 8.1e-5$, Figure 1) (39).

5mC differentially methylated regions (DMRs) in response to Flg22 treatment were most abundant in the CHH context (Figure 2A), which is the least methylated under basal conditions (2.73% for CHH vs. 32.19% for CG and 10.32% for CHG). As a measure of methylome variability, we compared DMRs between treatment groups (consistent DMRs, 3 vs. 3 comparison) and across all nine pairwise mock-Flg22 comparisons (stochastic DMRs, 1 vs. 1 comparison) (7). Methylome reprogramming appeared highly variable between replicates, with up to 27.5-fold more stochastic than consistent DMRs, particularly in the CHG context (Figure 2A). This variability was also observed between mock-treated controls, indicating that it reflects general methylome dynamics. As environmentally induced epigenetic responses are frequently characterised by high replicate-to-replicate variability (7, 46), subsequent analyses focussed on stochastic DMRs. Because replicate-level DMRs may reflect both biological heterogeneity and technical variation, all downstream analyses were additionally performed using consistent DMRs, yielding highly similar qualitative trends (Supplementary Figures 5-6).

To assess the chromosomal distribution of 5mC changes upon Flg22 treatment, we generated chromosome metaplots (Figure 2B,C). At 12 hpt, no clear chromosome-wide patterns were detected. By 72 hpt, CG methylation showed significant chromosome-wide hypermethylation

($p = 0.0002$), while CHG and CHH hypermethylation was concentrated at pericentromeric regions (CHG: $p = 0.0001$; CHH: $p = 0.0007$) and largely absent from gene-rich arms. Consequently, the arm/pericentromere methylation ratio significantly decreased in CHG and CHH contexts (CHG: $p = 0.005$; CHH: $p = 0.008$), indicating a relative non-CG methylation shift towards the TE-rich pericentromeres. At both time points, DMRs were enriched in pericentromeric regions and depleted in chromosome arms, with CG DMRs more frequent on arms and CHG DMRs concentrated in pericentromeres (Supplementary Figure 5A&B). Moreover, DMRs were strongly enriched at TEs and depleted from genic regions, regardless of sequence context or time point (Figure 2D and Supplementary Figure 6), suggesting that 5mC changes primarily act through TE-mediated *trans*-regulatory mechanisms. However, slight enrichment at TE-containing genic regions indicates that *cis*-regulatory effects may also contribute.

Given the strong enrichment of DMRs at TEs, we plotted the average relative changes in 5mC methylation at TEs, depending on their localisation at the pericentromere or on the chromosome arms, and their size (short TEs < 1000 bp, long TEs > 4000 bp; Figure 3A&B and Supplementary Figure 7) (47). At 12 hpt, short TEs on chromosome arms were hypomethylated in the CHG and CHH contexts but slightly hypermethylated in the CG context, while pericentromeric short TEs were hypermethylated in the CG context. By 72 hpt, short TEs on arms retained CG hypermethylation, whereas pericentromeric short TEs were hypermethylated in all contexts. Long TEs on arms showed no early changes but became hypermethylated in the CG and CHG contexts at 72 hpt, while pericentromeric long TEs were hypermethylated in all contexts at this time point. These results indicate transient demethylation of euchromatic TEs (short TEs on the arms) early after Flg22 treatment, followed by widespread hypermethylation of pericentromeric TEs, suggesting that TE methylation dynamics underlie the chromosome-wide methylation patterns (Figure 2B&C).

Only 16-17% of 5mC DMRs were located within gene bodies or promoters, and no functional enrichment was detected among the associated genes. In addition, sparse overlap of DMRs with Flg22-responsive genes (2-3%) suggests that 5mC-mediated regulation primarily acts through indirect, TE-driven mechanisms rather than direct *cis*-regulation of gene expression.

Flg22 induces early CHH hypomethylation of euchromatic TEs on the chromosome arms followed by late centromeric enrichment of non-CG methylation in rice

To test conservation of the patterns observed in Arabidopsis, we performed a similar experiment in rice using Flg22 treatment and sampling at 12 and 72 hpt. A single biological replicate of 10 pooled plants per treatment was used, achieving high coverage (average 56x) with only 0.17% of the genome below 20x coverage. This enabled precise estimation of population-level and stochastic methylation patterns, particularly for low-abundance modifications such as 6mA. As analyses are restricted to descriptive and comparative profiles, this high coverage design supports cautious interpretation despite the absence of independent biological replicates. As expected, centromeric regions displayed significantly

higher 5mC methylation than chromosome arms ($p = 1.2e-8$, Figure 1). Compared to Arabidopsis, rice exhibited a more uniform methylation landscape across chromosomes, lacking the strong pericentromeric clustering observed in Arabidopsis. This difference likely reflects contrasting TE distributions, as rice TEs are more evenly dispersed rather than concentrated at pericentromeres (Figure 1). These findings highlight species-specific methylome organisation and support the use of high-coverage single replicates to capture stochastic methylation changes, which are lost when averaging biological replicates.

In rice, Flg22-induced DMRs were predominantly in the CHH context, with a directional shift towards hypomethylation at 72 hpt (Figure 2E), contrasting with the hypermethylation trend in Arabidopsis (Figure 2A). Unlike Arabidopsis, DMRs in rice were distributed uniformly across chromosomes (Supplementary Figure 5C) and showed slightly lower overlap with TEs (Figure 2E), reflecting its more even methylome organisation. These findings suggest that the genomic architecture of both species strongly influences the spatial distribution of these immune-induced changes. Hence, changes shared between these divergent genomes are more likely to be key to the plant epigenetic immune response.

Chromosome-wide analysis in rice revealed no methylation shifts at 12 hpt, but by 72 hpt significant hypomethylation occurred in CHG and CHH contexts (CHG: $p = 2.8e-7$; CHH: $p = 9.9e-13$; Figure 2F&G), particularly at gene-rich chromosome arms. This reduced the arm-to-centromere methylation ratio (CHG: $p = 3.2e-5$; CHH: $p = 0.0003$), indicating a relative enrichment of methylation at centromeres. Despite opposite global trends - hypermethylation in Arabidopsis versus hypomethylation in rice (Fig 2C&G) - both species exhibited a similar chromosomal 5mC distribution, where (peri)centromeric regions (i.e. the centromeric/pericentromeric domain in Arabidopsis and the centromeric region in rice) became relatively more methylated than arms. This suggests that a relative shift in methylation between arms and (peri)centromeres may represent a conserved feature of the immune-induced methylation response.

In rice, DMRs were strongly enriched at TEs and depleted from genic regions, across all investigated contexts and time points (Figure 2H and Supplementary Figure 6). When genic regions overlapped with TEs, the DMR depletion was less pronounced, suggesting TE-associated methylation changes may influence gene regulation. At 12 hpt, short TEs on chromosome arms were significantly hypomethylated in the CHH context, while long or centromeric TEs remained unaffected (Figure 3C&D and Supplementary Figure 7). By 72 hpt, hypomethylation extended to both short and long TEs in CHG and CHH contexts, with the strongest effect at euchromatic short TEs on arms and the weakest at heterochromatic centromeric long TEs. This progressive spread from euchromatic to heterochromatic TEs mirrors chromosome-level patterns and parallels Arabidopsis, where early non-CG hypomethylation of arm-localised short TEs (Figure 3) and relative (peri)centromeric methylation increases were also observed (Figure 2C). These findings suggest a conserved early hypomethylation response targeting euchromatic TEs, followed by broader methylation reprogramming.

Similar to Arabidopsis, only 18-19% of Flg22-induced DMRs in rice were located within gene bodies or promoters, while these showed minimal overlap with stress-related genes. Cross-referencing DMR-containing genes with the Flg22-responsive transcriptome revealed sparse overlap (0.5-2%), supporting the notion that 5mC-mediated gene regulation occurs indirectly via TE reprogramming (5, 16).

Flg22 induces global 6mA hypomethylation in Arabidopsis, sharpening the bimodal gene-bordering profile at upregulated and defence primed genes

In addition to providing high resolution at TEs and (peri)centromeres, ONT-sequencing enabled detection of 6mA across the Arabidopsis genome (Figure 1), with significantly higher levels at pericentromeres (0.76%) compared to chromosome arms (0.49%, $p = 3.2e-5$), averaging 0.59% genome-wide. This distribution closely mirrored the 5mC landscape and aligned with previous reports (28). Following Flg22 treatment, 6mA levels across the chromosomes decreased slightly but uniformly at 12 hpt ($p = 0.03$; Figure 4A) and more strongly by 72 hpt ($p = 0.0004$).

Although TEs were the main targets for Flg22-induced 5mC changes, 6mA changes at TEs were uniform across TE-types and consistent with the chromosomal methylation changes (Supplementary Figure 8), indicating no TE-specific deviations. Figure 4B shows the absolute 6mA levels at TEs and flanking regions, revealing that Arabidopsis TE bodies are enriched in 6mA - mirroring their well-known 5mC methylation profile (47).

Basal 6mA profiles at Arabidopsis protein-coding genes showed sharp depressions at the transcription start site (TSS) and transcription termination site (TTS), flanked by peaks within gene bodies, suggesting a structured distribution of this mark (Figure 5A). Flg22 treatment did not alter this pattern at 12 hpt, but 6mA uniformly decreased across genes by 72 hpt (Supplementary Figure 9). To capture changes to this bimodal methylation profile, we focussed on relative methylation shifts at the TSS region (500 bp downstream of TSS), TTS region (1000 bp upstream of TTS) and gene bodies (Supplementary Figure 10). At 12 hpt, genes with $\geq 30\%$ higher 6mA increase at the TSS region than the gene body were enriched for defence-related GO-terms, particularly when gene bodies were simultaneously hypomethylated. Similar patterns were observed at 72 hpt with a $\geq 10\%$ TSS bias, suggesting that both 6mA addition at the TSS and removal from gene bodies contribute to immune reprogramming. However, genes with the same TSS bias were not enriched for defence-related GO-terms when gene bodies were simultaneously hypermethylated, highlighting the importance of coordinated 6mA dynamics in regulating defence genes.

To relate 6mA redistribution to transcriptional outcomes, we analysed gene expression at matched time points (12 hpt) to capture immediate associations. We additionally compared gene expression at 84 hpt with earlier 6mA methylation patterns (72 hpt), allowing assessment of delayed associations between 6mA dynamics and gene expression. Cross-referencing 6mA changes with transcriptome data revealed that at 12 hpt, upregulated genes significantly overlapped with those showing relative 6mA increases at TSS (and to a lesser extent TTS) compared to gene bodies, particularly when gene bodies were demethylated (Figure 6). This indicates that a sharper bimodal 6mA profile may contribute to immune gene activation. Downregulated genes showed minimal overlap with tested 6mA patterns at this time point. At 84 hpt, upregulated genes maintained this association, while downregulated genes overlapped with relative TSS/TTS hypermethylation combined with gene body hypomethylation, and in some cases with relative TSS demethylation. These contrasting

patterns likely reflect transient expression dynamics, where TSS/TTS hypermethylation in downregulated genes may indicate prior activation and subsequent homeostasis, whereas TSS demethylation likely contributes to active silencing. Indeed, genes related to defence responses were downregulated at 84 hpt (Supplementary Figure 3B), indicating a negative feedback response.

To explore 6mA's association with immune memory, we cross-referenced the transcriptome of primed plants, which received a second Flg22 treatment, with their 6mA methylome just before second treatment (72 hpt), to investigate if defence primed genes are marked by 6mA prior to second induction. Defence primed genes were defined as genes showing significantly stronger expression 12 hours after a second Flg22 treatment compared to 12 hours after single treatment, excluding genes whose expression was already elevated at this time point (84 hpt) compared to single Flg22 treated plants (12 hpt) (Supplementary Figure 1B). Primed upregulated genes consistently overlapped with those showing strong relative 6mA increases at the TSS combined with gene body demethylation at 72 hpt (Figure 6). This association, absent in primed downregulated genes, indicates that coordinated 6mA redistribution may act as an epigenetic marker for defence priming. Moreover, at 72 hpt, primed upregulated genes on average showed strong 6mA demethylation at the TSS (-1000 to +100 bp) and further downstream in the gene body (>1600 bp), while the region immediately downstream of the TSS (+300 to +1300 bp) was only slightly hypomethylated, accentuating the TSS-proximal 6mA peak (Figure 5D and Supplementary Figure 11). In contrast, primed downregulated genes displayed the opposite pattern, whereas non-primed genes exhibited uniform demethylation. TTS regions were largely unaffected.

To validate the role of 6mA in Arabidopsis immunity, we infected mutants in the 6mA demethylase *ALKB HOMOLOG 1D* (*ALKBH1D*), which exhibit elevated genomic 6mA levels (48). These mutants showed drastically increased susceptibility to *Pst* and *Meloidogyne incognita* infection (respectively $p = 1.4e-7$ and $p = 9.6e-5$, Figure 7), supporting a role for 6mA in immunity.

Flg22 induces global 6mA hypermethylation in rice, sharpening the bimodal gene-bordering profile at upregulated genes

In rice, 6mA was detected genome-wide with significantly higher levels at centromeres compared to chromosome arms ($p = 2.6e-8$; Figure 1). The average genome-wide 6mA level was 2.09% - approximately 3.5 times higher than in Arabidopsis (0.59%). Similar as for 5mC, 6mA was more uniformly spread across the rice genome, lacking the strong pericentromeric clustering observed in Arabidopsis. Following Flg22 treatment, 6mA levels increased at 12 hpt ($p = 5.1e-12$), particularly at chromosome arms, resulting in a higher arm/centromere methylation ratio ($p = 0.0002$, Figure 4C). At 72 hpt, hypermethylation persisted ($p = 0.0006$) but was less pronounced and evenly distributed, suggesting a transient, genome-wide 6mA response to immune stimulation.

Unlike 5mC DMRs, which were enriched at TEs and depleted from genic regions (Figure 2H), 6mA DMRs in rice were strongly enriched at genic regions - particularly at the TSS - and

depleted from introns and TEs (Figure 5C and Supplementary Figure 12). Similar to Arabidopsis, most TE-types in rice exhibited 6mA changes consistent with chromosome-wide patterns (Supplementary Figure 8), suggesting no major TE-specific deviations. However, at 12 hpt, 6mA changes at short TEs on chromosome arms were exclusively detected in their flanking regions, but not within the TE body. This suggests that 6mA modifications may be restricted from these sites to avoid interference with early CHH hypomethylation patterns (Figure 3C). Rice TEs displayed a distinct basal 6mA profile compared to Arabidopsis, with short TEs marked by pronounced 6mA depressions at their boundaries and centres (Figure 4D), unlike the elevated methylation typically seen for 5mC (49). 6mA levels at long rice TEs were similar to those in flanking regions, whereas long Arabidopsis TEs exhibited strong enrichment relative to their surroundings (Figure 4B&D). These distinct patterns suggest that 6mA may have evolved different roles in TE regulation across these two divergent species, potentially linked to their varying genome size and TE density.

Rice protein-coding genes displayed a basal 6mA profile similar to Arabidopsis, with clear gene-bordering patterns marked by a TTS depression and a peak just upstream of it (Figure 5B). However, unlike Arabidopsis, rice lacked strong TSS depletion and instead showed a pronounced 6mA peak within 500 bp downstream of the TSS, suggesting subtle species-specific differences in 6mA distribution. In rice, Flg22 treatment strengthened the gene-bordering profile on average by increasing the genic 6mA levels at 12 hpt, particularly at TSS and TTS regions, an effect largely lost by 72 hpt (Figure 5E). This contrasts with Arabidopsis, where 6mA was globally unaffected by Flg22 at genes at 12 hpt, but was decreased uniformly by 72 hpt (Supplementary Figure 9), indicating species-specific timing and mechanisms of 6mA reprogramming. Similar to Arabidopsis, genes with pronounced Flg22-induced 6mA increases were linked to plant defence, particularly at 12 hpt where genes with stronger increases at TSS and/or TTS regions than the gene body were enriched for phenylpropanoid, and more specifically lignin, biosynthetic pathways (Supplementary Figure 13). At 72 hpt, defence-related enrichment persisted only for genes with $\geq 50\%$ higher 6mA at the TTS compared to the gene body, suggesting a dynamic, region-specific role of 6mA in stress regulation.

Similar to Arabidopsis, at both 12 and 84 hpt, upregulated genes significantly overlapped with those showing relative 6mA increases at the TSS compared to gene bodies (Figure 8). For instance, around 11% of the upregulated genes showed $\geq 50\%$ increase at the TSS compared to the gene body. At 84 hpt, downregulated genes also significantly overlapped with those showing relative 6mA decreases at the TSS compared to gene bodies. Together with the Arabidopsis observations, our data indicate that 6mA changes sharpening the bimodal gene-bordering profile, particularly at the TSS, are associated with immune gene activation.

DISCUSSION

Our research compared Arabidopsis and rice methylomes under Flg22 immune stimulation, revealing both shared and species-specific patterns. While Arabidopsis has been pivotal for epigenetic research, its atypical genome architecture, characterised by low TE content and distinct pericentromeric regions (Figure 1), limits generalisation to other plants (50–52). In contrast, rice exhibits a more uniform TE and methylation distribution (Figure 1), reflecting broad (epi)genomic diversity among angiosperms. These differences underscore the value of cross-species analyses: species-specific patterns highlight genome-architecture effects, whereas conserved features likely represent core components of the plant epigenetic immune response.

Flg22-triggered immune stimulation in both Arabidopsis and rice predominantly induced DMRs in the CHH context (Figure 2A&E), the least methylated genomic context (52), highlighting its role in environmental response regulation (7, 17, 53, 54). In line with existing literature (10, 55, 56), 5mC DMRs were enriched at TEs and depleted from genic regions (Figure 2D&H). Notably, this depletion was not observed for TE-containing genic regions, suggesting a role for *cis*-regulation of genes by nearby TEs. However, gene-distal TEs harboured the majority of DMRs, supporting the hypothesis that immune-induced 5mC changes mainly regulate the immune response through *trans*-regulatory mechanisms (5, 7, 16, 21). Although the high-depth ONT sequencing provided robust methylation estimates for the pooled rice sample, the lack of independent biological replicates limits inference regarding biological variability, and the rice DMRs should therefore be regarded as observations requiring future validation. Future studies using multiple individually sequenced plants may distinguish consistent Flg22-responsive DMRs from stochastic individual-specific methylation changes and better resolve technical versus biological sources of variation. Such heterogeneity may reflect differences in response intensity, individual-specific epigenetic plasticity, or cell-type-specific immune activation. While stress-associated epigenetic states may enhance resistance to recurrent challenges, they can also increase susceptibility to alternative stresses (2, 7). Consequently, heterogeneous methylation responses may generate a spectrum of defence-associated states within a population, potentially contributing to bet-hedging under unpredictable environmental conditions (46, 57).

Flg22-induced TE methylation dynamics differed between Arabidopsis and rice. In Arabidopsis, short euchromatic TEs were initially non-CG hypomethylated at 12 hpt, then remethylated by 72 hpt, while long and pericentromeric TEs became hypermethylated (Figure 3). In rice, early CHH hypomethylation of euchromatic TEs was followed by broader non-CG hypomethylation across all TE-types, but strongest at euchromatic TEs. Despite these differences, both species shared a common early CHH (and CHG) hypomethylation at euchromatic TEs, the core targets of RdDM (47) which has been proposed to contribute to immune memory formation (7–9, 12, 24, 25). Our findings extend this concept by demonstrating a shared euchromatic TE hypomethylation between Arabidopsis and rice which may reflect an early regulatory event supporting plant immunity.

At 72 hpt, Arabidopsis TEs showed remethylation or hypermethylation (Figure 3A&B), contrasting previous reports of hypomethylation associated with plant immunity (10, 16, 17). This may reflect late feedback regulation or result from improved pericentromeric resolution via long-read sequencing, allowing to detect these novel methylation patterns. While highly

repetitive regions can still be affected by residual mapping uncertainty and k-mer-dependent modification-calling biases (58, 59), the long reads generated here and recent improvements in R10 chemistry are expected to limit such effects (60), making them unlikely to substantially influence the aggregate repeat-level patterns reported here. We hypothesise that RdDM activity shifts from chromosome arms to pericentromeres, explaining both the observed euchromatic TE non-CG hypomethylation and pericentromeric hypermethylation (Figure 2). In rice, global hypomethylation was strongest at chromosome arms, creating a similar relative increase in centromeric methylation (Figure 2). These parallels suggest that balancing methylation between chromosome arms and (peri)centromeres is a core feature of the Flg22-induced immune response.

Although 5mC is essential for long-term and intergenerational immune memory (7–9, 12, 24, 25), its changes rarely correlate directly with defence gene expression (9, 21, 61), consistent with our observations. Instead, methylation reprogramming primarily targets TEs, suggesting regulation occurs via *trans* mechanisms (5, 16). For instance, the hypothesised RdDM shift from euchromatic TEs towards the (peri)centromeres may generate new siRNA regulatory pathways. In Arabidopsis, pericentromeric TEs have greater potential to become transcriptionally active in the absence of DNA methylation, threatening genome stability (62–64). Upon immune-induced demethylation, this may recruit RdDM to the (peri)centromeres to ensure genome stability. Active TEs are targeted by non-canonical RdDM, generating 21–22 nt siRNAs associated with AGO1 and AGO6 (65, 66). Interestingly, in Flg22-treated Arabidopsis, TE-derived 21–22 nt siRNAs loaded into AGO1 were found to *trans*-activate defence gene expression (23), indicating that the observed (peri)centromeric relative hypermethylation may drive defence gene activation. In support, in *Pst*-infected Arabidopsis, pericentromeric TEs were transcriptionally active after 3–7 hours and resiled after 24 hours (20).

We sought to explore the role of 6mA in plants by investigating its behaviour under immune stress induced by Flg22. In line with the literature, we observed that 6mA is enriched at (peri)centromeric regions in both Arabidopsis and rice (28, 31). As for 5mC, Arabidopsis exhibits a distinct pericentromeric 6mA peak, whereas rice displays a more uniform chromosomal 6mA distribution (Figure 1). Notably, overall 6mA levels in rice were approximately 3.5-fold higher than in Arabidopsis, consistent with previous reports (28, 31, 67, 68). Upon Flg22 stimulation, Arabidopsis showed global 6mA hypomethylation at 72 hpt, while rice exhibited transient hypermethylation at gene-rich chromosome arms at 12 hpt, which diminished by 72 hpt (Figure 4). These contrasting dynamics highlight species-specific regulatory mechanisms of 6mA in plant immunity.

Accurate quantification of 6mA in eukaryotic genomes remains challenging. Its low abundance, the absence of a gold-standard detection method, and limitations of available approaches have contributed to substantial variation in reported 6mA levels between studies (67, 69, 70). Differences between detection methods, including susceptibility to contamination, antibody specificity issues and challenges in accurately detecting rare modifications, complicate the distinction between true biological variation and methodological effects (67, 70–72). Therefore, absolute estimates of genomic 6mA abundance require careful interpretation, particularly in genomes where the modification occurs at low frequency. Recent benchmarking showed that ONT R10/Dorado provides robust 6mA profiling, with reliable discrimination between modified and unmodified sites and stable performance at sufficient coverage, although SMRT-sequencing remains more accurate for absolute site-level quantification,

particularly at low methylation frequencies (73). Our analyses focussed on aggregated methylation signals across genomic features, reducing the impact of stochastic classification errors. Nevertheless, ONT-based approaches remain limited in their ability to accurately quantify absolute 6mA levels, highlighting the need for orthogonal validation.

In literature, 6mA has been hypothesised as a gene expression activating mark in plants (28–33). Here we observed 6mA enrichment at gene boundaries in both *Arabidopsis* and rice (Figure 5A&B). In *Arabidopsis*, 6mA levels exhibited a depression at the TSS and TTS, flanked by peaks within the gene body. Rice displayed a similar pattern at the TTS but showed a pronounced peak downstream of the TSS without a preceding depression. This aligns with earlier reports in *Arabidopsis* and rice linking 6mA to the TSS and TTS, particularly in highly expressed genes (34).

Despite contrasting global 6mA responses to Flg22, both species exhibited similar gene-level patterns. Genes displaying elevated 6mA at the TSS (and TTS) relative to the gene body, especially when coupled with gene body demethylation, were associated with defence functions and upregulation (Figure 6 and 8, Supplementary Figure 10 and 12). Flg22 re-exposure in *Arabidopsis* revealed that primed upregulated genes retained 6mA immediately downstream of the TSS, flanked by demethylated regions, reinforcing this gene-bordering profile, whereas downregulated genes showed the inverse pattern (Figure 5D). As this gene-bordering profile prior to second induction marks genes for defence priming in *Arabidopsis*, it may indicate a novel role for 6mA in immune memory generation. Our findings align with previous reports linking strong 6mA boundary patterns to highly expressed genes in *Arabidopsis*, rice and *P. vaginatum* (34, 35), but expand this observation to environmentally responsive genes and defence gene priming. Whether these 6mA patterns actively contribute to transcriptional regulation or instead arise as a consequence of transcriptional activity remains unresolved. Our data cannot distinguish between these possibilities. However, the priming experiment provides tentative support for a regulatory role, as gene-bordering 6mA profiles detected prior to the second immune challenge were associated with genes that subsequently displayed enhanced transcriptional responses. This temporal relationship is consistent with a role for 6mA in establishing or maintaining a primed chromatin state, although additional evidence will be required to firmly establish causality.

To explore the functional relevance of 6mA in immunity, we examined the *Arabidopsis alkbl1d* mutant, which lacks a validated 6mA demethylase (48). These mutants exhibited increased susceptibility to *Pst* and the root-parasitic nematode *M. incognita* (Figure 7), reinforcing the relevance of 6mA (de)methylation in immunity. Our data suggest that precise 6mA profiling at gene boundaries contributes to defence gene regulation, a process disrupted in *alkbl1d*. While potential RNA m⁶A alterations may confound interpretation, as AlkB-family enzymes often show activity towards both DNA and RNA (74), our results clearly implicate 6mA demethylation in plant immunity. However, further studies combining genome-wide 6mA profiling with transcriptome analysis in *alkbl1d*, together with the identification and functional characterisation of additional 6mA writers and erasers, will be crucial to establish a causal link and directionality between 6mA dynamics and gene expression.

Our findings suggest that 6mA may regulate gene expression via the sharpness of its gene-bordering profile, which closely resembles nucleosome occupancy patterns around TSS and TTS in plants (75–78). Both features are associated with highly expressed genes, and evidence from other species indicates that 6mA may affect nucleosome positioning (33, 79–82). We

hypothesise that sharper 6mA profiles at the TSS could contribute to nucleosome phasing, thereby promoting gene expression.

Together, Flg22 triggers extensive reprogramming of both 5mC and 6mA methylomes during immune activation in Arabidopsis and rice. For 5mC, changes predominantly target TEs in the CHG and CHH contexts, with early hypomethylation of euchromatic TEs possibly redirecting RdDM towards heterochromatic TEs at (peri)centromeres. These shifts may influence defence gene expression through *trans*-regulatory mechanisms. In parallel, 6mA displays a bimodal distribution at gene boundaries, sharpened by Flg22 at defence-activated genes and at primed genes in Arabidopsis. Both downstream TSS 6mA methylation and gene body demethylation appear integral to this process. The increased susceptibility of the *alkbh1d* mutant underscores the functional importance of 6mA demethylation in immunity, highlighting the 6mA DNA methylome as a key component of pathogen resistance.

ACKNOWLEDGEMENTS

The authors thank Helen Hipperson for support in optimising ONT-sequencing, Louis Tirot for training and assistance with *Pst* infection assays, and Lien De Smet for technical assistance with laboratory experiments. The graphical abstract and Supplementary Figure 1 were created using BioRender.com. Arabidopsis and rice icons were sourced from BioRender.com.

This work was supported by Fonds Wetenschappelijk Onderzoek [grant 1134122N to J.M.]; and Bijzonder Onderzoeks Fonds of Ghent University.

Generative AI tools were used exclusively to assist with language editing and textual revision of the manuscript. All scientific content, data interpretation, and conclusions were reviewed and approved by the authors.

AUTHOR CONTRIBUTIONS

Jasper Matthys: Conceptualisation, Formal analysis, Methodology, Writing. Ibe Naessens: *Meloidogyne* infection. Filip Van Nieuwerburgh: Methodological assistance with ONT-sequencing. Jurriaan Ton: Conceptualisation, Review, Editing. Tina Kyndt: Conceptualisation, Review, Editing.

SUPPLEMENTARY DATA

Supplementary Methods and Data are available at *New Phytologist* Online.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY

The raw mRNA-seq and modified-base-called ONT-seq data underlying this article are available in SRA at <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1395842>, and can be accessed with PRJNA1395842.

Figure legends

Figure 1: Global distribution of 5mC (all contexts) and 6mA basal methylation in the rice (*Os*) and Arabidopsis (*At*) genomes. Plotted data for all tracks was binned into 50 kb bins.

The outer two tracks represent the 6mA (green, between 0-6%) and 5mC (red, between 0-26%) methylation percentage across the rice genome ($n = 2$). Centromeres are represented by grey boxes. The centromeres showed significantly higher levels of both 6mA and 5mC methylation ($p = 2.6e-8$ and $1.2e-8$, respectively; paired Student's t-test). The third track shows the TE coverage within each bin, using a colour scale between white (minimal coverage) and dark green (maximal coverage). The fourth and fifth tracks represent the 6mA (green, between 0-1.8%) and 5mC (red, between 0-33%) methylation percentage across the Arabidopsis genome, respectively ($n = 3$). Pericentromeres are represented by grey boxes. The pericentromeres showed significantly higher levels of both 6mA and 5mC methylation ($p = 3.2e-5$ and $8.1e-5$, respectively; paired Student's t-test). The sixth track shows the TE coverage within each bin, using a colour scale between white (minimal coverage) and dark green (maximal coverage).

Figure 2: Comparison of sequence context-dependent global changes in 5mC methylation in Arabidopsis and rice treated with Flg22. A,E) Differentially methylated region (DMR) distribution across sequence contexts in Flg22-stimulated Arabidopsis (A) and rice (E) at 12 and 72 hpt. "3 vs. 3 comparison" denotes DMRs obtained by comparing the mock to the treated group ($n = 3$), whereas "1 vs. 1 comparison" denotes pairwise comparison of individual mock and treated samples, thereby including stochastically variable DMRs. For rice, the 1 vs. 1 comparison ($n = 1$) is visualised. Overlap of DMRs with transposable elements (TEs) is depicted by dark red and dark blue for hyper- and hypomethylated DMRs, respectively. B,C,F,G) Chromosome metaplots showing relative 5mC changes across sequence contexts at 12 (B,F) and 72 hpt (C,G) relative to controls. Dashed lines represent the pericentromeric (Arabidopsis) or centromeric (rice) borders. PERI = pericentromere (grey), CEN = centromere (grey). Asterisks indicate significance of the GLOBAL change, or of changes in the chromosome ARMS/PERIcentromere (Arabidopsis) or chromosome ARMS/CENtromere (rice) ratio (** $p < 0.01$; paired Student's t-test). (Arabidopsis: $n = 3$; rice: $n = 1$). D,H) Significance and $\log_2$ -fold enrichment (LFE) of stochastic Arabidopsis (D; $n = 3$) and rice (H; $n = 1$) DMRs at genetic elements at 12 hpt (two-sided Fisher's exact test). DMRs simultaneously overlapping a genetic element and a TE are coloured red. DMRs were merged in the CG, CHG, CHH and 5mC sequence contexts. Promoters and downstream regions correspond to the 1000 bp upstream and downstream of annotated genes, respectively. For visualisation, $-\log_{10}(q\text{-value})$ was capped at 300.

Figure 3: TE metaplots showing the relative methylation changes in the CHG and CHH contexts in Arabidopsis and rice treated with Flg22. Relative changes in methylation upon Flg22 treatment (vs. control) were binned at TEs and their 1 kb surrounding regions. A) Arabidopsis TE metaplots for short TEs (< 1000 bp) either on the chromosome arms or at the pericentromere ($n = 3$). B) Arabidopsis TE metaplots for long TEs (> 4000 bp) either on the chromosome arms or at the pericentromere ($n = 3$). C) Rice TE metaplots for short TEs (< 1000 bp) either on the chromosome arms or at the centromere ($n = 1$). D) Rice TE metaplots for long TEs (> 4000 bp) either on the chromosome arms or at the centromere ($n = 1$). (** $p < 1e-4$; asterisk colour indicates the time point at which the comparison was significant, if both time points were significant, the asterisks are shown in black; paired Student's t-test)

Figure 4: Flg22-immune stimulation in Arabidopsis and rice induces distinct changes to the 6mA methylome. A,C) Chromosome metaplots showing relative 6mA changes upon Flg22 treatment in Arabidopsis (A; $n = 3$) and rice (C; $n = 1$) (vs. control). Dashed lines represent the pericentromeric (Arabidopsis) or centromeric (rice) borders. PERI = pericentromere (grey),

CEN = centromere (grey). Asterisks indicate significance of the GLOBAL change, or of changes in the chromosome ARMS/PERIcentromere (Arabidopsis; ** $p < 0.05$) or chromosome ARMS/CENtromere (rice; ** $p < 1e-3$) ratio (paired Student's t-test). B) TE metaplots showing the 6mA methylation levels around Arabidopsis TEs at 72 hpt with Flg22 ($n = 3$). The top row shows short TEs (< 1000 bp), the bottom row long TEs (> 4000 bp), either on the chromosome arms or at the pericentromere. (** $p < 1e-4$) D) TE metaplots showing the 6mA methylation levels around rice TEs at 12 hpt with Flg22 ($n = 1$). (** in the middle of the plot: $p < 1e-4$; ** on the edges: $p < 1e-3$ when comparing the ratio of the relative change at the TE surroundings to the relative change at the TE body; paired Student's t-test)

Figure 5: 6mA methylation shows distinct patterns at Arabidopsis and rice genes. A,B) Average basal 6mA levels at all Arabidopsis (A; $n = 3$) or rice (B; $n = 2$) protein-coding genes, based on their relative position compared to the transcription start site (TSS) and transcription termination site (TTS). bp = base pair. -1000 bp as relative position denotes the 1000 bp surrounding region outside the gene body. C) Enrichment of rice 6mA DMRs at genetic elements at 12 hpt with Flg22, showing both the significance and $\log_2$ -fold change (LFE) of the enrichment ($n = 1$) (two-sided Fisher's exact test). Genic elements are shown as triangles (green shades), other elements as dots. TSS denotes the region 500 bp downstream of the TSS. TTS denotes the region 1000 bp upstream of the TTS. Promoters and downstream regions correspond to the 1000 bp upstream of the TSS and downstream of the TTS, respectively. D) The relative 6mA changes at Arabidopsis genes at 72 hpt with Flg22 (vs. control) ($n = 3$). Genes were grouped based on their expression profile following second treatment with Flg22 (12 hpt) after 72 hpt with the initial stimulation: primed upregulated, primed downregulated and unprimed genes. Plots show a LOESS smoothed curve (span = 0.6) of the relative 6mA levels binned per 100 bp relative to the TSS or TTS. Confidence intervals and TTS-region are shown in Supplementary Figure 11. E) Average relative 6mA changes at all rice genes upon Flg22 treatment (vs. control), relative for their position to the TSS and TTS (binned per 50 bp) ($n = 1$).

Figure 6: Flg22-induced genic 6mA methylation changes are associated with transcriptomic responses in Arabidopsis. Overlap analysis between genes with relative changes in 6mA methylation and DEGs ($n = 3$). 6mA levels were assessed 12 and 72 hpt with Flg22, and compared to the transcriptomes collected at 12 and 84 hpt, respectively. Primed genes were defined as those showing significantly stronger expression 12 hours after second Flg22 treatment (72 hours after the first) compared to 12 hours after single treatment, excluding genes whose expression was already elevated at 84 hours compared to 12 hours in singly treated plants. Graphs on the left represent a simplified view of the effect of each assessed profile. The colour gradient represents the $-\log_{10}(p)$ values of the overlap (one-sided Fisher's exact test). GB = gene body. TSS = region between TSS and 500 bp downstream. TTS = region between TTS and 1000 bp upstream. Δ in ' Δ TSS' refers to the relative difference in 6mA methylation compared to the control. ' Δ TSS- Δ GB' signifies the difference in methylation change between the TSS and GB regions.

Figure 7: The Arabidopsis *alkbh1d* mutant shows increased basal susceptibility to *Pst* and *M. incognita* infection. A) Progress of *Pst* infection in Col-0 and *alkbh1d* mutants. Infection was quantified as bioluminescence (*Pst-LUX*) corrected per mm^2 plant. Statistics were obtained by type III ANOVA of the fitted linear mixed-effects model. Time:Genotype ($p = 1.4e-7$): significance of the time-genotype interaction in the model. Error bars represent the standard deviation. ($n = 12$) B) Example of bioluminescence by a hydroponic Arabidopsis

culture infected by *Pst-LUX* at 2 days post infection (dpi). C) Progress of *M. incognita* infection in Col-0 and *alkbh1d* mutants. Infection was quantified as number of galls per root system. Statistics were obtained by type III ANOVA of the fitted linear mixed-effects model. Time:Genotype ($p = 9.6e-5$): significance of the time-genotype interaction in the model. Error bars represent the standard deviation. ($n = 30$)

Figure 8: Flg22-induced genic 6mA methylation changes associate with transcriptomic responses in rice. Overlap analysis between genes with relative changes in 6mA methylation ($n = 1$) and DEGs ($n = 9$). 6mA levels were assessed 12 and 72 hpt with Flg22, and compared to the transcriptomes collected at 12 and 84 hpt, respectively. Graphs on the left represent a simplified view of the effect of each assessed profile. The colour gradient represents the $-\log_{10}(p)$ values of the overlap (one-sided Fisher's exact test). GB = gene body. TSS = region between TSS and 500 bp downstream. TTS = region between TTS and 1000 bp upstream. Δ in 'ΔTSS' refers to the relative difference in 6mA methylation compared to the control. 'ΔTSS-ΔGB' signifies the difference in methylation change between the TSS and GB regions.

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Supporting Information

Supplementary Methods:

Detailed description of the data analysis and plots presented in this publication.

Supplementary Figures:

Supplementary Figure 1: Experimental overview of Arabidopsis immune elicitation in hydroponics and the definition of defence primed genes detected by RNA-seq. Created in BioRender. Kyndt, T. (2026) <https://BioRender.com/wexbmip>

Supplementary Figure 2: Representative gall phenotype induced by *Meloidogyne incognita* infection on wild-type (*Arabidopsis thaliana* Col-0) roots.

Supplementary Figure 3: GO-enrichment analysis of the Arabidopsis Flg22-responsive transcriptome.

Supplementary Figure 4: GO-enrichment analysis of the rice Flg22-responsive transcriptome.

Supplementary Figure 5: Proportion of Arabidopsis and rice DMRs within the centromeric and pericentromeric regions, and chromosome arms.

Supplementary Figure 6: 5mC DMR enrichment at Arabidopsis and rice genetic elements in different sequence contexts at different time points.

Supplementary Figure 7: TE metaplots showing the relative methylation changes in all cytosine contexts in Arabidopsis and rice treated with Flg22.

Supplementary Figure 8: TE metaplots showing the relative 6mA methylation changes in Arabidopsis and rice treated with Flg22.

Supplementary Figure 9: Average relative change of 6mA methylation at all Arabidopsis genes upon Flg22 treatment.

Supplementary Figure 10: Genic 6mA methylation changes upon Flg22 treatment are linked to the Arabidopsis immune response.

Supplementary Figure 11: Relative average 6mA changes at Flg22-primed genes in Arabidopsis (detailed).

Supplementary Figure 12: 6mA DMR enrichment at rice genetic elements at different time points upon Flg22 treatment.

Supplementary Figure 13: Genic 6mA methylation changes upon Flg22 treatment are linked to the rice immune response.

Supplementary Datasets:

Dataset S1: Regular and bioluminescence images of Arabidopsis plants (Col-0 and *alkbh1d*) infected with *Pst*-LUX.