N6-Methyladenosine Modification Changes in the Genome of *Paulownia Fortunei* Seedlings Infected With Phytoplasma

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Abstract

Background: Phytoplasmas induce diseases in more than 1,000 plant species and cause substantial ecological damage and economic losses, but the specific pathogenesis of phytoplasma has not yet been clarified. N⁶-methyladenosine sequencing (m⁶A-seq) has been applied mainly to model plants and not to woody plants.

Results: In this study, we applied m⁶A-seq to study changes in m⁶A modification in the *Paulownia fortunei* genome after phytoplasma infection. We found that the m⁶A modification level in seedlings infected with the phytoplasma that causes Paulownia witches' broom (PaWB) was slightly higher than the m⁶A modification level in PaWB-infected seedlings treated with 60 mg·L⁻¹ methyl methanesulfonate (MMS). MMS has been shown to restore PaWB-infected seedlings to their normal form and no phytoplasma can be detected in MMS-treated PaWB-infected seedlings. RNA sequencing (RNA-seq) and m⁶A-seq were used to analyze the expression of genes with m⁶A peaks and m⁶A motifs in genes, respectively. The correlation analysis between the RNA-seq and m⁶A-seq data detected that a total of 315 differentially methylated genes were predicted to be significantly differentially expressed at the transcriptome level. The functions of genes related to PaWB were predicted by functional enrichment analysis, and two genes related to maintenance of the basic mechanism of stem cells in shoot apical meristem were discovered. One of the genes encodes the receptor protein kinase CLV2 (*Paulownia_LG2G000076*), and the other gene encodes the homeobox transcription factor STM (*Paulownia_LG15G000976*). The m⁶A modification levels were higher in PaWB-infected seedlings than they were in MMS-treated seedlings. In addition, genes F-box (*Paulownia_LG17G000760*) and MSH5 (*Paulownia_LG8G001160*) had exon skipping and mutually exclusive exon types of alternative splicing in PaWB-infected seedling treated with MMS. RT-PCR verified that the alternative splicing of these two genes was related to m⁶A modification.

Conclusions: In this study, we applied m⁶A-seq to determine methylation levels in phytoplasma-infected Paulownia, and combined m⁶A-seq with transcriptome analysis to screen differentially expressed genes associated with PaWB. Also analyzed the effect of m⁶A methylation on alternative splicing. In future studies, we plan to verify genes directly related to PaWB and methylation-related enzymes in Paulownia to elucidate the pathogenicity mechanism of PaWB caused by phytoplasma invasion.

Background

N⁶-methyladenosine is the most common post-transcriptional modification of mRNA. It is regulated by methyltransferases, demethylases, and m⁶A-binding proteins (Dominissini et al. 2012; Meyer et al. 2012; Schwartz et al. 2013; Luo et al. 2014; Deng et al. 2015). The regulation of m⁶A modification involves a complex dynamic system that includes processes related to embryonic development, apoptosis, spermatogenesis, and circadian rhythm (Zhong et al. 2008; Jia et al. 2011; Zheng et al. 2013; Liu et al. 2014; Ping et al. 2014; Zhao et al. 2017; Yang et al. 2018). If components of the m⁶A modification system are defective, diseases or disorders can occur, including tumors, neurological diseases, and delayed embryonic development (Zhong et al. 2008; Liu et al. 2013; Zheng et al. 2013; Ping et al. 2014; Lin et al. 2016; Zhang et al. 2016; Li et al. 2017; Zhao et al. 2017). In addition, m⁶A is involved in a variety of RNA metabolic processes, including mRNA expression (Dominissini et al. 2012), translation efficiency (Wang et al. 2015), alternative splicing (Liu et al. 2015), and degradation (Wang et al. 2013).

Homologs of some m⁶A-related proteins in Paulownia have been identified in the model plant *Arabidopsis thaliana* (Zhong et al. 2008). For example, FIP37, a homolog of WTAP in Arabidopsis, is a core component of the methyltransferase complex that mediates methylation of the transcription factors STM and WUS, and influences the development of aboveground organs (Shen et al. 2016). ALKBH10B is an RNA m⁶A demethylase that regulates inflorescence transformation in Arabidopsis (Duan et al. 2017). The m⁶A reader ECT2 recognizes the site of m⁶A methylation and regulates the morphogenesis of Arabidopsis trichomes (Wei et al. 2018). In tobacco, the RNA demethylase ALKBH5 is associated with tobacco mosaic virus (Li et al. 2018). Until now, m⁶A modification has rarely been investigated in woody plants.

Paulownia is an important fast-growing timber and ornamental tree species indigenous to China, but now planted worldwide. PaWB, a plant disease caused by phytoplasma infection, seriously affects the growth and development of Paulownia and even causes death, resulting in serious economic loss and ecological damage. Phytoplasmas are parasitic prokaryotes that have no cell wall and are transmitted by insect vectors such as psyllids, planthoppers (Kosovac et al. 2018), and woodlice. Phytoplasmas are the causal agents of more than 1,000 plant diseases, including Jujube witches' broom, PaWB, and Aster yellow witches' broom, which are

extremely harmful to plants (Geng et al. 2015). Advances in biotechnological techniques have made it possible to study post-transcriptional mRNA modifications, especially m⁶A modifications, which have become the focus of much research. Given the indispensable function of RNA m⁶A modification in regulating gene expression and the involvement in various bioprocesses, it is reasonable to speculate that PaWB might also be associated with the regulation of m⁶A modification. Previous studies have shown that phytoplasma-infected Paulownia seedlings treated with 60 mg·L⁻¹ methyl methanesulfonate (MMS) were restored to the normal morphology, and the phytoplasma was no longer detected (Wang et al. 2018). In this study, we used terminal buds collected from untreated PaWB-infected seedlings (PFI) and PaWB-infected seedlings treated with 60 mg L⁻¹ MMS (PFIM60) for N⁶-methyladenosine sequencing (m⁶A-seq), RNA sequencing (RNA-seq), and transcriptome analysis. We constructed an m⁶A methylation map of Paulownia and screened for genes involved in the recovery process of PaWB disease by combining m⁶A-seq with transcriptome analysis. Genes that were found to be alternatively spliced because of m⁶A modification were analyzed to gain insights into gene expression changes in Paulownia in response to phytoplasma infection. Understanding the changes in m⁶A modification in the Paulownia genome after phytoplasma infection will lay a solid scientific foundation for discovering the pathogenesis and epigenetic regulation mechanisms associated with PaWB.

Results

The m⁶A modification landscape of *P. fortunei*

PaWB-infected *P. fortunei* (PFI) seedlings and PFI seedlings treated with 60 mg·L⁻¹ MMS (PFIM60) were used for m⁶A-seq, RNA-seq (total fragmented RNA as the control for m⁶A-seq), and transcriptome analysis, with two replicates for each. Heatmap correlation coefficient analysis between the biological replicates confirmed their high repeatability (Figure 1). We obtained 44–56 and 73–83 million reads for the RNA-seq and m⁶A-seq libraries, respectively (Supplementary Materials: Tables S1), and 23–28 and 47–50 million of them uniquely aligned to the *P. fortunei* reference genome (Supplementary Materials: Tables S2). Almost 94% of the reads that were aligned to the reference genome were locating in exons; the others were located in introns or intergenic regions (Supplementary Materials: Figure S1, Supplementary Materials: Tables S3).

We constructed a m⁶A methylation map of *P. fortunei* that shows the distribution of m⁶A on the chromosomes, and the distribution of the PFI and PFIM60 m⁶A peak positions on the chromosomes (Figure 2). The number of m⁶A-modified genes on the chromosomes ranged from 472 to 1,014; chromosome 9 (1,014) and chromosome 2 (1,002) had more modified genes than the other chromosomes. The highest number of m⁶A peaks (1,506) was on chromosome 2 and the lowest number (702) was on chromosome 14. The numbers of methylated genes and peaks on the chromosomes were similar in PFI and PFIM60.

We obtained 6,606 high-confidence m⁶A peaks located in 13,837 and 13,505 genes in PFI and PFIM60, respectively, using ChIPseeker; 12,554 of the genes were common to both samples (Supplementary Materials: Tables S4). Notably, there was greater enrichment of peaks at the transcription start and stop sites of genes in both samples (Figure 3a), suggesting m⁶A modification may play a key role in regulating mRNA degradation and microRNA processing in Paulownia. We compared and analyzed the differences in the observed m⁶A peaks between PFI and PFIM60. The results showed there were 306 genes with reduced m⁶A methylation in PFIM60 compared with their m⁶A methylation in PFI; 36.59% of them were located in 3' UTRs, 17.41% were in 5' UTRs, 21.58% were in first exons, and 24.15% were in other exons. This distribution is consistent with the m⁶A enrichment of all the genes in the *P. fortunei* genome (Figure 3b).

Differential modified genes between PFI and PFIM60

To determine the sequence specificity of the hypermethylated m⁶A peaks, we used HOMER for motif prediction based on the difference peak analysis, and compared the predicted motif to the microRNA sequences in miRBase ($1e^{-20} < p < 1e^{-10}$). A total of 10 highly enriched motif sequences were identified. Among them, the motif RRACH (R = A/G, H = A/C/U), which was identified in Paulownia, has not yet been confirmed in mammals and yeast. The most significantly enriched sequence was UUGUUUUGUACU (Figure 4), which is similar to the UGUAYY (Y = C/U) motif that is specific to m⁶A modifications in tomato, rice, corn, and other plants, indicating that the prediction results have a high degree of confidence. These motif sequences are recognized and bound by m⁶A-related enzymes, which in turn affect gene expression.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed on the peak-related genes with weakened m⁶A methylation modification detected in the PFIM60 samples. The results showed that the auxin biosynthesis, hormone signal transduction, and MAPK signal pathways were some of the most enriched pathways (Figure 5; Supplementary Materials: Tables S5). The auxin biosynthesis pathway involves three genes, *Paulownia_LG2G000124*, *Paulownia_LG2G000125*, and *Paulownia_LG2G000128*. The plant hormone (auxin, abscisic acid, and ethylene) signal transduction pathways involve eight genes, *Paulownia_LG11G000159*, *Paulownia_LG12G000675*, *Paulownia_LG17G000489*, *Paulownia_LG17G000559*, *Paulownia_LG2G001457*, *Paulownia_LG4G000193* and *Paulownia_LG4G000193000451*. The MAPK signaling pathway involves six genes, *Paulownia_LG10G000799*, *Paulownia_LG11G000159*, *Paulownia_LG17G000559*, *Paulownia_LG2G001457*, *Paulownia_LG4G000451*, and *Paulownia_LG9G000737*.

Extent of m⁶A methylation versus gene transcript levels in PFI and PFIM60

First, the transcription levels of the mRNAs in the PFI and PFIM60 libraries were analyzed by calculating the FPKM (FPKM=[total exon fragments/mapped reads (millions)×exon length (kb)]). Differentially expressed mRNAs (DEGs) were identified using the edgeR package in R (log₂ (fold change) >1 or <-1, and p value <0.05). A total of 5,899 DEGs were detected between the PFI and PFIM60 libraries; 3,130 were up-regulated and 2,769 were down-regulated in PFIM60. Gene ontology (GO) enrichment analysis showed that the DEGs were involved mainly in membrane, cell wall, transcriptional regulation using DNA as a template, secondary metabolite biosynthetic process, DNA binding transcription factor activity, and oxidoreductase activity (Figure 6a). The KEGG pathway enrichment analysis showed that the DEGs were involved mainly in plant circadian rhythm, phenylpropanoid biosynthesis, flavonoid biosynthesis, and glycerolipid metabolism pathways (Figure 6b).

To examine the extent of m⁶A methylation versus gene transcript levels in the PFI and PFIM60 samples, we correlated the m⁶A-seq data with the transcriptome sequences. In the transcriptome, differences in transcript abundance were categorized as up- and down-regulation, whereas in the m⁶A-seq data changes in peak abundance indicated the m⁶A methylation levels of up-regulated genes. A total of 315 differentially methylated genes were predicted to be significantly differentially expressed at the transcriptome level. Four types of relationships between the m⁶A methylation levels and gene expression were identified: 1) m⁶A modification levels and transcription levels both up-regulated (133 genes); 2) m⁶A modification levels up-regulated and transcription levels down-regulated (58 genes); 3) m⁶A modification levels down-regulated and transcription levels up-regulated (44 genes); and 4) m⁶A modification levels and transcription levels both down-regulated (80 genes) (Figure 7; Supplemental Data Table S6). The GO enrichment analysis of these differentially methylated genes showed plasma membrane and chloroplast were enriched under the cellular component category, DNA-based template for transcriptional regulation and response to light stimulus were enriched under the biological process category, and DNA-binding transcription factor activity and sequence-specific DNA binding function were enriched under the molecular function category (Figure 8a). The KEGG pathway analysis showed that these differentially methylated genes were involved mainly in signal pathways such as ABC transport, diterpenoid biosynthesis, plant hormone signal transduction, and phenylpropanoid biosynthesis (Figure 8b).

m⁶A modification affects alternative splicing

Alternative splicing (AS) is involved in many physiological processes, including responses to abiotic and biotic stresses (Barbazuk et al. 2008). m⁶A modification can affect AS by promoting the retention of exons at the transcriptional level; therefore, we jointly analyzed m⁶A modification and AS. In PFIM60, 282 genes had significant exon skipping-type AS differences and 84 genes had significant mutually exclusive exon-type AS differences compared with the corresponding genes in PFI. When the degree of m⁶A methylation increased in PFIM60 compared with that in PFI, we found the AS of three genes was correlated with m⁶A modification, namely F-box (*Paulownia_LG17G000760*) and MSH5 (*Paulownia_LG8G001160*). To verify the AS of these two genes in PFI and PFIM60, we performed an RT-PCR analysis. We found that for PFIM60, when the degree of m⁶A methylation increased, the bands of two genes were weakened compared with those for PFI, indicating that the degree of m⁶A methylation affected AS events (Figure 9; Supplemental Data Figure S2).

Identification of potential candidate genes associated with PaWB

In the process of invading a host, inherent pathogen-associated molecular patterns called PAMPs or damage-related molecular patterns of the phytoplasma are recognized by pattern recognition receptors in the host defense system, which induces PAMP-

triggered immunity and activates the downstream defense pathway. To resist PAMP-triggered immunity, phytoplasmas release effectors as signal molecules to regulate signal transduction and disrupt the host defense response. This in turn triggers a series of physiological and biochemical reactions, which results in metabolic disruption in the host plant and development of disease symptoms. Some of the identified DEGs were related to plant–pathogen interactions, plant hormone signal transduction, and plant tillering. Among them, four significantly DEGs were involved in the plant hormone signal transduction pathway: *Paulownia14027*, which encoded SHORT-ROOT (LSH4); *Paulownia26350*, which encodes LIGHT-DEPENDENT SHORT HYPOCOTYLS 4 (LSH4); *Paulownia_LG17G000277*, which encodes histidine-containing phosphotransfer protein 1-like; and *Paulownia_LG2G000140*, which encodes regulatory protein NPR5-like. One DEG was involved in the plant–pathogen interaction pathway: *Paulownia000609*, which encodes disease resistance protein RPM1-like. Two DEGs were involved in the carotenoid biosynthesis pathway: *Paulownia_LG11G000307*, which encodes phytoene synthase 2; and *Paulownia_LG18G000559*, which encodes carotenoid cleavage dioxygenase 4.

The most prominent symptom of PaWB is axillary bud clustering, so we also focused on genes that affect branching and tillering in plants, namely CLAVATA1 (*CLV1*), *CLV2*, *CLV3* (Nikolaev et al. 2007), WUSCHEL (*WUS*) (Lenhard et al. 2002), Arabidopsis TOPLESS (TPL)-like transcriptional corepressor (*ASP1*), SHOOT MERISTEMLESS (*STM*), ARABIDOPSIS RESPONSE REGULATOR 5 (*ARR5*), *ARR6*, *ARR7*, and *ARR15* (Leibfried et al. 2005). In Arabidopsis, these genes encode proteins that are involved in the WUSCHEL – CLAVATA negative feedback loop, which is the basic mechanism for maintaining stem cells in shoot apical meristem. We found *Paulownia* homologs of *CLV2* (*Paulownia_LG2G000076*) and *STM* (*Paulownia_LG15G000976*) by mapping the Arabidopsis genes to the *Paulownia* reference genome using a local BLAST program. These two genes showed significant differences in m⁶A methylation and expression levels after phytoplasma infection.

We performed a m⁶A methylated RNA immunoprecipitation quantitative reverse transcription PCR (MeRIP-qRT-PCR) verification on these two candidate genes. The results were consistent with the sequencing results, which verified the accuracy of the sequencing results (Figure 10).

STM and CLV2 maintain the dynamic balance of shoot apical meristem regulated by m⁶A methylation modification

Conserved domains were identified in the proteins encoded by the *Paulownia* homologs of *STM* and *CLV2* (Table 1). Similar to *STM*, *Paulownia_LG15G000976* encodes a homeobox transcription factor with four conserved domains, KNOX2, KNOX1, Homeobox_KN, and ELK. Similar to *CLV2*, *Paulownia_LG2G000076* encodes a protein kinase that contains the domain PLN00113 superfamily. Previous studies have shown that *STM* and *WUS* bind to the *CLV3* promoter and activate its transcription, and *CLV3*, *CLV1*, and *CLV2* together maintain the number of undifferentiated stem cells in the shoot apical meristem tissue and regulate the production of normal stem ends. The transcriptome data analysis showed that *STM* was more highly expressed in PFI than it was in PFIM60, and the m⁶A-seq data analysis showed that *CLV1*, *CLV2*, and *CLV3* had higher methylation levels in PFI than in PFIM60.

Table 1
conserved domain analysis for two protein related to PaWB

Gene	Gene ID	Significant	m6A	gene	Incomplete	Incomplete	Incomplete	Incomplete
STM	AT1G62360	-	-	-	KNOX2	KNOX1	Homeobox_KN	ELK
	Paulownia_LG15G000976	yes	down	down	KNOX2	KNOX1	Homeobox_KN	ELK
	Paulownia_LG14G000617	yes	down	down	KNOX2	KNOX1	Homeobox_KN	ELK
	Paulownia_LG7G001667	no	up	down	KNOX2	KNOX1	Homeobox_KN	ELK
CLV2	AT1G65380	-	-	-	PLN00113 superfamily	-	-	-
	Paulownia_LG2G000076	yes	down	down	PLN00113 superfamily	-	-	-

Discussion

m⁶A modification of genes in PFI and PFIM60

The m⁶A-seq of the PFI and PFIM60 samples detected 13,837 and 13,505 genes, respectively, that were methylated; overall 12,554 unique genes were m⁶A methylated. Among the shared m⁶A methylation-modified genes, 1,886 methylation sites in 1,662 genes were significantly differentially modified between PFI and PFIM60, accounting for 92% of the total differential methylation sites. Among them, 202 DEGs had significantly different m⁶A methylation. The overall methylation level was slightly higher in the PFI samples compared with the level in the PFIM60 samples. However, the regions in which methylation was different were similar in the two samples, with nearly 40% of the modified regions located in the 3' UTRs.

m⁶A methylation modification regulates the expression of genes related to PaWB

Phytoplasma infection of *P. fortunei* releases effectors that disturb the plant's immune response. The effectors can interact directly with key plant immune-related genes or can disrupt the immune response by disrupting immune signal transduction. The association analysis of the m⁶A-seq data and transcriptome sequences identified genes related to plant–pathogen interaction, plant hormone signal transduction, and plant tillering. *LSH4* (*Paulownia26350*) was predicted to be involved in the plant–pathogen interaction and signal transduction pathways. Constitutive expression of *LSH4* in the shoot apex inhibited leaf growth in the vegetative phase as well as the formation of extra shoots or shoot organs in flowers in the reproductive phase. The methylation modification and transcription levels of *LSH4* were significantly up-regulated in PFI compared with the levels in PFIM60. We speculated that phytoplasma infection of *Paulownia* increased the m⁶A modification level in the 3' UTR region of *LSH4*, which enhanced the stability of *LSH4* mRNA and increased its expression level, thereby promoting the occurrence of PaWB.

The symptom of axillary bud clustering caused by PaWB is closely related to the development of shoot apical meristem. In *Arabidopsis*, three master regulators, STM, WUS, and CLV3, are critical for stem cell specification in shoot apical meristem. STM interacts with WUS in vivo and recruits CLV3 to express shoot apical meristem. In *Paulownia*, *WUS* expression was relatively low and was similar in PFI and PFIM60, whereas *STM* expression was higher in PFI than it was in PFIM60. We speculated that the increased methylation level in the 3' UTR region of *STM* in PFI maintained its stable expression. In *Arabidopsis*, the m⁶A methylase FIP37 affected the expression of *WUS* and *STM* and regulated the development of shoot apical meristem. These results suggested that a m⁶A methylase may also be involved in regulating *STM* expression in *Paulownia*. However, this possibility needs to be confirmed in further studies.

The relationship between m⁶A modification and alternative splicing of PaWB-related genes

Alternative splicing is a key regulatory mechanism that participates in many physiological processes, controls plant development, and increases the complexity of the proteome and transcriptome. In addition, alternative splicing plays a vital role in plant defense response and photosynthesis (Wang & Brendel 2006). Compared with PFI, PFIM60 found a total of 282 SE-type alternative splicing genes and 84 MXE-type alternative splicing genes. To determine the relationship between alternative splicing and PaWB, we studied the genes associated with alternative splicing events in PaWB-infected *Paulownia*. The combined analysis of m⁶A modification and alternative splicing found that when the degree of m⁶A methylation increased, there was a correlation between alternative splicing and m⁶A modification of the two genes, namely F-box (*Paulownia_LG17G000760*) and MSH5 (*Paulownia_LG8G001160*). The gene encoding F-box is involved in alternative splicing events. The F-box protein is a subunit of the SCF complex and plays an important role in plant hormone signal transduction and regulation of plant development and growth (Gonzalez et al. 2017). JA can regulate the growth of most plants, including their ability to adapt to adversity and to resist the invasion of pathogens. The F-box protein COI1 is the receptor of JA, and the response with JA dependence as the core is mediated by the Skp1/Cullin/F-box E3 ubiquitin ligase complex containing COI1 (Gonzalez et al. 2017). The SCF complex can degrade JA, causing MYC2 to be released, thereby expressing genes that respond to JA (Liu et al. 2017). In short, F-box protein may be involved in regulating the growth of *Paulownia* to improve its ability to adapt to stress. Due to the lack of reliable data, this speculation still needs further research to verify. MSH5 is a homologue of DNA mismatch recognition protein (Muts). DNA mismatch repair (MMR) is a highly conserved biological pathway that can improve the fidelity of DNA replication and recombination. When the MutS protein recognizes small loops of mismatched and unpaired nucleotides, it initiates MMR (Jiricny 2013; Spampinato 2017). Previous studies have shown that *Arabidopsis* and other plants encode MutS protein homologues (MSH) that are conserved in other eukaryotes. Plants lacking MSH7 show significantly faster germination rates and longer germination rates during the juvenile vegetative period. In primary roots, the number of stem leaves and axillary and lateral inflorescences is higher than that of the wild type (Chirinos-Arias & Spampinato 2020; Gonzalez & Spampinato 2020). These findings indicate that the growth of mutant plants seems to be caused by damage to cell cycle

checkpoints, which allow cells to divide without proper DNA repair, and its role in the process of phytoplasma infection of Paulownia needs further development explore.

Conclusions

We applied m⁶A-seq to determine methylation levels in phytoplasma-infected Paulownia. We found that the m⁶A modifications differed from those previously reported in model plants. We speculated that the Paulownia genome had a novel m⁶A modification motif. DEGs associated with PaWB were detected by transcriptomic analysis. Also analyzed the effect of m⁶A methylation on alternative splicing. In future studies, we plan to verify genes directly related to PaWB and methylation-related enzymes in Paulownia to elucidate the pathogenicity mechanism of PaWB caused by phytoplasma invasion.

Methods

Plant materials

The experimental plant materials used in this study were obtained from the Forest Biotechnology Laboratory of the Institute of Paulownia, Henan Agricultural University, Zhengzhou, China, and the permission to collect the *P. fortunei* has been obtained. The plant materials were grown on 1/2 MS medium in 100-ml Erlenmeyer flasks for 30 days. Then, 1.5-cm terminal buds from the PFI plants were transferred into 1/2 MS medium contain 60 mg·L⁻¹ MMS (PFIM60 samples) (Figure 11a) or 0 mg·L⁻¹ MMS (PFI samples) (Figure 11b). Two seedlings were cultured in each bottle and 90 bottles were cultivated for each sample. After 30 days, the obtained samples were frozen in liquid nitrogen and stored at -80°C until used.

Antibody enrichment and sequencing library construction

Total RNA extraction and quality and quantity assessing were performed as described previously. More than 200 µg total RNA was used to isolate poly(A) mRNA with poly(T) oligo-attached magnetic beads (Invitrogen). After purification, the mRNA fractions were fragmented into approximately 100-nt long oligonucleotides using divalent cations under elevated temperature. The cleaved RNA fragments were incubated for 2 h at 4°C with m⁶A-specific antibody (No. 202003, Synaptic Systems, Germany) in IP buffer (50 mM Tris-HCl, 750 mM NaCl, 0.5% Igepal CA-630) supplemented with bovine serum albumin (0.5 µg·µL⁻¹). The mixture was incubated with protein-A beads and eluted with elution buffer (1 × IP buffer and 6.7 mM m⁶A). The eluted RNA was precipitated with 75% ethanol. The eluted m⁶A-containing fragments (IP) and untreated input control fragments were converted to form the final cDNA library according to the strand-specific library preparation dUTP method. We constructed four m⁶A-seq libraries (PFI_1_IP, PFI_2_IP, PFI-M60_1_IP, PFI-M60_2_IP) and four RNA-seq libraries (PFI_1_IP, PFI_2_IP, PFI-M60_1_IP, PFI-M60_2_IP). The average insert size for the paired-end libraries was approximately 100 ± 50 bp. Paired-end 2×150 bp sequencing was performed on an Illumina Novaseq™ 6000 (Biotech Ltd, Hangzhou, China) platform following the manufacturer's recommended protocol.

Alignment of reads to the reference genome and visualization of m⁶A peaks

Cutadapt (Martin et al. 2011) and perl in-house scripts were used to remove reads that contained adaptor contamination, low quality bases, and/or undetermined bases. Sequence quality was verified using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Bowtie (Langmead et al. 2012) (with default parameters) was used to map reads to the *P. fortunei* reference genome. Mapped reads of the IP and input libraries were input into the exomePeak package in R (Meng et al. 2014) to identify m⁶A peaks (in BED or BAM format) for visualization using the UCSC Genome Browser or Integrative Genomics Viewer (IGV) software (<http://www.igv.org/>).

Transcription level analysis

StringTie (Pertea et al. 2015) software was used to determine the mRNA expression levels in the input libraries by calculating FPKM ($\text{FPKM} = [\text{total exon fragments} / \text{mapped reads (millions)} \times \text{exon length (kb)}]$). The differentially expressed mRNAs were selected using the edgeR package in R with a threshold of $\log_2$ (fold change) >1 or <-1 and p value <0.05 (Robinson et al. 2010).

Discernment of m⁶A topological patterns

MEME (Bailey et al. 2009) and HOMER (Heinz et al. 2010) were used to find *de novo* and known motifs in the differential modified genes. Localization of the motifs with respect to peak summit was performed using in-house perl scripts. Called peaks were annotated by intersection with gene architecture using ChIPseeker (Yu et al. 2015).

m⁶A MeRIP-qRT-PCR

The m⁶A methylated RNA immunoprecipitation (MeRIP) assay was adapted from a previously reported protocol (Dominissini et al. 2013). Briefly, approximately 300 mg total RNA was extracted and isolated using an mRNA screening kit. Each biological replicate for m⁶A-seq started with 400 mg of total RNA, which yielded approximately 10 mg of double poly(A) selected mRNA. Then, the mRNA was treated with mRNA fragmentation reagent and fragmentation of the 300 mL poly(A) RNA solution was performed at 94°C for exactly 5 min using a thermocycler. The fragmentation reaction was stopped by adding 50 mL of stop buffer to a final volume of 350 mL and immediately put on ice. Approximately 100 ng mRNA was taken out as the input sample and 150 mL of pre-equilibrated m⁶A-Dynabeads was added to the remaining fragmented RNA to a final volume of 500 mL. The fragmented RNA was allowed to bind to the m⁶A-Dynabeads at 4°C while rotating (tail-over-head) at seven rotations per minute for 2 h. Then, the tubes containing the samples were placed on a magnet and the bead complexes were allowed to cluster until the solution became clear. The 500-mL liquid phase or supernatant was discarded because it contained m⁶A negative fragments that were not captured by the anti-m⁶A antibody. The m⁶A-Dynabeads–RNA complexes were resuspended in 500 mL m⁶A binding buffer, incubated for 3 min at room temperature, and the clear supernatant was removed after placing the beads on a magnet. This step was repeated with 500 mL low salt buffer, then with 500 mL high salt buffer. (The 3-min incubation time should not be exceeded to prevent release of the RNA from the beads.) This step was then repeated twice with 500 mL TET buffer. The obtained immunoprecipitated product was prepared by resuspending in 100 mL of proteinase K buffer and the obtained input sample was thawed. Then, 107 mL of RIP wash buffer, 15 mL of 10% SDS, and 18 mL of proteinase K were added to a total volume of 150 mL, and the mixture was incubated at 55°C for 30 min with shaking to digest the antibody from the magnetic beads. After incubation, the supernatant was removed and 250 mL of RIP wash buffer was added to the supernatant. Next, 400 mL of phenol/chloroform was added to each tube, which was then vortexed for 15 s, and 0.1 times the volume was added. RNA precipitation was promoted by adding 3 M NaAc and 2 mL GlycoBlue at –20°C for 1 h. The tubes were centrifuged at 14,000 rpm for 20 min at 4°C, 75% ethanol precipitation was performed twice, and the pellet was blown dry, then dissolved in 10 mL RNA-free water and tested.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by the authors. Experimental research and field studies on plants, including the collection of plant material, were carried out in accordance with relevant guidelines and regulations. We confirm that all experimental protocols were approved by the Forest Biotechnology Laboratory of the Institute of Paulownia, Henan Agricultural University, Zhengzhou, China.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the [NCBI] repository, [SRA accession: PRJNA783736 and PRJNA624264]. The *P. fortunei* used in this study came from the Forest Biotechnology Laboratory of the Institute of Paulownia, Henan Agricultural University, Zhengzhou, China., and the permission to collect the *P. fortunei* has been obtained.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

GF conceived and designed the experiments. HY and YC provided suggestions on the experimental design and analyses. PX and SH analyzed the data and wrote the paper. ZX, XL, HY, YC and GF revised the manuscript. All authors read and approved the manuscript.

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Authors' information

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Figures

Spearman Correlation of Read Counts

Figure 1

Heatmap correlation coefficient analysis between biological replicates

Figure 2

m⁶A modification map of *P. fortune*. the outermost circle is the chromosome distribution, the second circle is the distribution of PFI m⁶A peak positions on the chromosomes, and the third circle is the distribution of PFIM60 m⁶A peak positions on the chromosomes.

Figure 3

Peak enrichment area. (a), Enrichment of peak at the transcription start site in 4 samples, and the Peak distribution of the bondable region near the TSS is shown in the form of a heat map. (b), Peak enrichment on genetic elements.

Figure 4

A higher proportion of motifs: UUGUUUUGUACU.

Figure 5

KEGG Enrichment analysis of m⁶A methylation attenuated gene after 60 mg·L⁻¹ MMS treatment.

Figure 6

Functional annotation and pathways enrichment of differentially expressed genes. (a), GO function annotation of differentially expressed genes. (b), statistics of KEGG Pathway enrichment.

Figure 7

Four relationships were detected between the m6A methylation levels and gene expression.

Figure 8

Functional annotation and pathways of differential peak and differentially expressed genes

Figure 9

RT-PCR verification of F-box and MSH5 in PFI and PFIM60, respectively. 18s rRNA is an internal reference gene.

Figure 10

m⁶A MeRIP-qRT-PCR verification

Figure 11

Morphological changes of *P. fortunei* seedling infected with phytoplasma. (a), phytoplasma-infected *P. fortunei* seedling. The diseased seedlings have obvious symptoms of witches' broom. (b), 60 mg·L⁻¹ MMS treated seedling.

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