Coordination of m6A mRNA methylation and gene transcriptome in rice response to cadmium stress

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Research Article

Keywords: N6-methyladenosine, post-transcriptional regulation, rice, cadmium stress, seedling

Posted Date: March 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-328701/v1

Version of Record: A version of this preprint was published at Rice on July 5th, 2021. See the published version at https://doi.org/10.1186/s12284-021-00502-y.

Abstract

N^6-methyladenosine (m^6A) is the most prevalent internal modification present in mRNAs of all higher eukaryotes. However, the role of the m^6A methylomes in rice is still poorly understood. With the development of MeRIP-seq technique, in-depth identification of mRNAs with m^6A modification becomes feasible. We investigated the m^6A methylomes in roots of cadmium (Cd) group and compared that with the roots in the control (CK) group by m^6A sequencing, in 9311 and Nipponbare (NIP), respectively. The results indicated that Cd leads to altered modification profile in 3,406 differential m^6A peaks in 9311, and 2,065 differential m^6A peaks in NIP. KEGG pathway analysis of genes with differentially modified m^6A peaks indicates that the “phenylalanine”, “tyrosine and tryptophan biosynthesis”, “glycine”, “adherens junctions”, “glycerophospholipid metabolism” and “threonine metabolism” signaling pathways may be associated with abnormal roots development of rice due to exposure to cadmium in 9311. “Arginine”, “proline metabolism”, “glycerolipid”, “protein processing in endoplasmic reticulum”, metabolism pathways were significantly enriched in genes with differentially modified m^6A peaks in NIP. Different from that in *Arabidopsis*, the m^6A peak (m^6A-modified nucleotide position on mRNAs) distribution exhibits preference toward both the stop codon and 3'UTRs region. These findings provide a resource for plant RNA epi-transcriptomics studies and further enlarge our knowledge on the function of RNA m^6A modification in plants.

Introduction

m^6A is one of the most important internal modifications in the mRNA of many eukaryotic species, including yeast, plants (Wei et al. 2018), flies (Lence et al. 2016), and mammals (Yang et al. 2018; De et al. 2019). In mammals, this modification is dynamic and plays important roles in the regulation of mRNA metabolism and processing (Duan et al. 2017), including alternative splicing, exportation, stability, translation, and microRNA maturation (Yang et al. 2018; Shen et al. 2016). The functions of m^6A on RNA are determined by the dynamic interplay between a conserved set of proteins which are called writers, erasers and readers (Meyer and Jaffrey 2017). METTL3 was the first m^6A methyltransferase identified in mammals that is highly conserved in plants and mammals (Yao et al. 2019). METTL14 protein was the second most-active m^6A methyltransferase enzyme in human to catalyze m^6A RNA methylation, being highly homologous to METTL3. While other components like ZFP217, RBM15, RBM158, HAKAI, ZC3H13 have been shown to directly regulate RNA modifications.

In plants, most of the progress in elucidating the methylation mechanism and function of m^6A has been made in *Arabidopsis* (Zhang et al. 2019). In *Arabidopsis*, MTA is homologous to METTL3, MTB to METTL14, and FIP37 to WTAP. Recent studies showed that ALKBH10B-mediated mRNA demethylation influences floral transition by affecting the stability of target transcripts (Duan et al. 2017). It is reported that the cytoplasmic *Arabidopsis* YTH domain proteins EVOLUTIONARILY CONSERVED C-TERMINAL REGION2/3 (ECT2/3) are required for the correct timing of leaf formation and normal leaf morphology (Arribas-Hernández et al. 2018). In addition, study showed that AtFIP37 plays an indispensable role in determining the fate of stem cells in *Arabidopsis* (Shen et al. 2016). All together, these studies indicate that the m^6A has unique functions during their life cycle in *Arabidopsis*. There is increasing evidence that m^6A is also involved in regulating responses to various abiotic and biological stresses (Yue et al. 2019). Recent studies showed that m^6A modifications were involved in the regulation of responses to salt stress in *Arabidopsis* and sweet sorghum (Anderson et al. 2018; Zheng et al. 2021). Different cellular pressures can lead to a redistribution of m^6A within the transcriptome, resulting in an increase in the number of mRNAs with 5'UTR m^6A (Meyer et al. 2015). The m^6A pattern is dynamic, and 5–30% of m^6A peaks are altered by ultraviolet light, heat shock, or interferon-gamma, thereby affecting gene expression and splicing (Meyer et al. 2012). Studies have shown that m^6A can dynamically regulate the response of cells to abiotic stress, including heat shock, ultraviolet light, hypoxia stress and oxidative stress (Parker et al. 2020).

Despite its importance, much of original work of m^6A has focused on humans and model animals such as mice, while few studies have explored rice. There was a study, which revealed for the first time that OsFIP plays an indispensable role in rice early sporogenesis (Yao et al. 2019). Rice is one of the most important food crops in China and also an important monocotyledonous model organism. Cadmium accumulation in rice grain poses a serious threat to human health and is a widespread detrimental heavy metal pollutant that poses potential chronic toxicity to living organisms (Tan et al. 2017; Cao et al. 2019). Cadmium is absorbed from the soil by the roots and transported to the shoots and accumulated into grains (Oono et al. 2017). In plants, the most obvious effect of cadmium toxicity is a reduction of plant growth related to an inhibition of photosynthesis, respiration and nitrogen metabolism, as well as to a reduction in water and nutrient uptake (Santos et al. 2012). Although epigenetic mechanisms have also been found to be related to the response of plants to Cd (Greco et al. 2012), it is not clear whether m^6A modifications may also play an important role in the response to cadmium stress. The complexity of rice cadmium transport and accumulation indicates the necessity of explaining what is responsible for cadmium accumulation divergence between *indica* and *japonica* rice subspecies.

Thus, in this work, we aimed at obtaining further understanding of the effects of cadmium on rice roots in terms of m^6A methylation in mRNA. We report here m^6A-sequencing profiling in two accessions of rice, *indica* rice 9311 and *japonica* rice Nipponbare. To investigate the different cadmium response mechanisms in different cultivars, we study enriched metabolic pathways of the differential m^6A modified peaks. Collectively, our data will constitute a comprehensive picture of m^6A methylation in mRNA in rice roots and provide the basis for future studies of its function and biological significance in rice.

Results

Rice roots growth was affected by Cd stress

The Cd stress induces phenotypic variations in rice seedlings. The roots lengths of 9311 and NIP were shortened under Cd groups compared with the CK groups (CK *vs*. Cd; *student's t-test,* p-value < 0.01 or 0.01<p-value < 0.05) (Figure 1A and 1B). Interestingly, we observed that 9311 was more sensitive to Cadmium than NIP, the seedling length of 9311 was significantly longer than NIP in control condition. In 9311, the average length of rice roots in CK groups is 3.2 cm, while in Cd groups is 1.2 cm. In NIP, the average length of rice roots in CK groups is 1.1 cm, while in Cd groups is 0.9 cm. The seedling length of 9311 was significantly longer than NIP in CK groups (9311_CK *vs*. NIP_CK; *student's t-test*, p-value < 0.01), while there were no significant differences between the two genotypes with Cd groups at same stages. (9311_Cd *vs*. NIP_ Cd; *student's t-test*, p-value > 0.05) (Figure 1C). These two materials inspired us to investigate whether, and to what extent the Cd stress changes m^6A methylation of genes in rice genotypes with two rice cultivars.

Generation of m^6A methylation profiles for rice roots

To obtain the transcriptome-wide m^6A map in rice seedlings, a series of m^6A-immunoprecipitation (IP) and the matched input (non-IP control) libraries were constructed and sequenced (Supplementary Table S1). The clean reads were obtained, resulting in 59 –77 million clean reads for each library. 8,972, 8,239, 8,706 7,813 peaks are present in at least 2 out of 3 biological replicates in 9311 or NIP under CK and Cd groups, respectively (Supplementary Table S2). These m^6A peaks from different experimental conditions were further merged into a unique set of 10,735 m^6A peaks, 92.26% (9,904) of which are present in genic regions of 9,802 genes (minimum overlap as 100 bp), accounting for an average of 1.01 m^6A peaks within transcription units from each gene. We randomly selected seven m^6A-methylated genes and validated their m^6A modification using m^6A reverse transcription quantitative PCR (RT-qPCR) (Supplementary Fig. S1). These 10,735 m^6A peaks in rice are enriched in the stop codon region (46.6% of m^6A peaks), followed by 3'UTR (19.2%) and coding region (11.2%) (Figure 2A). Similar distribution patterns of m^6A peaks were also observed in the separate analysis of m^6A-seq data from each 9311 or NIP group (Supplementary Fig. S2). The distribution pattern of m^6A peaks in rice is similar to that observed in maize (Miao et al. 2020) and *Arabidopsis* (Shen et al. 2016).

As expected, we also observed that 99.95% of 10,730 m^6A peaks contain the canonical motif RRACH (where R represents A/G, A is m^6A, and H represents A/C/U) in rice and 96.57% of 10,367 m^6A peaks contain the canonical motif URUAY (where Y represents C/U; Figure 2B)，which can also be detected from m^6A peaks from each replicate sample.

m^6A methylations are affected by Cd stress in NIP and 9311

With these two cultivars at hand, we investigated whether, and to what extent the cadmium stress changes m^6A methylations of genes in rice genotypes with different tolerance to cadmium stress. Firstly, we examined the genomic distribution of m^6A peaks in rice seedling under different experimental conditions (Figure 3A). At the genome level, 10,735 peaks were unevenly distributed across each chromosome. The majority of high confident peaks (hcpeaks) are present in all four different experimental conditions (Figure 3B). Saturation curve shows that RNA methylation levels of Cd group were lower than CK group under both 9311 and NIP (Figure 3C). We further compared all peaks under CK and the Cd groups with different rice cultivar (9311 and NIP). In 9311, 7,591 hcpeaks within mRNAs (~79% of all peaks in the CK and Cd groups) were overlapped between the CK and Cd groups, 3,406 hcpeaks were identified as statistically differentially enriched hcpeaks in Cd group compared to CK group (FDR < 0.05) (Figure 4A). Gene ontology (GO) enrichment analysis of genes in these differentially enriched m^6A hcpeaks showed that ATP binding, protein kinase activity, oxidoreductase activity and oxidation–reduction process were enriched (Figure 4B). KEGG pathway analysis of genes in these differentially enriched m^6A hcpeaks showed that, Phenylalanine, tyrosine and tryptophan biosynthesis, Glycine, serine and threonine metabolism and Cysteine and methionine metabolism pathways were enriched (Figure 4C).

While in Nipponbare, 7,383 hcpeaks within mRNAs (~80.8% of all peaks in the CK and Cd groups) were overlapped between the CK and Cd groups，2,065 hcpeaks were identified as differential methylated peaks (DMPs) (FDR < 0.05) (Figure 5A). GO terms including transferase activity, transferring glycosyl groups, defense response to bacterium and cell surface receptor signaling were particularly enriched in genes

overlapping with these differentially enriched m^{6}A hcpeaks (Figure 5B). With respect to KEGG pathways, “arginine and proline metabolism”, “protein processing in endoplasmic reticulum” and “glycerolipid metabolism” pathways were significantly enriched in genes overlapping with these differentially enriched m^{6}A hcpeaks (Figure 5C).

In order to investigate whether genes with m^{6}A methylation at different genic regions play different functions in rice, we explored the over-represented functions of genes with m^{6}A methylation at 5’-UTR or 3’-UTR. We observed that in 9311, the GO terms “mitochondrial inner membrane” and “organelle inner membrane” were specifically enriched in genes with DMPs within 5’-UTR (Figure 6A), whereas the GO terms “cellular nitrogen compound metabolic process” and “establishment of protein localization” were specifically enriched in genes within DMPs near the 3’-UTR, (Figure 6B). In NIP, the GO terms “ribosome” and “structural constituent of ribosome” were specifically enriched in genes with DMPs within 5’-UTR (Figure 6C), whereas the GO terms “cellular nitrogen compound metabolic process” and “cellular macromolecule localization” were specifically enriched in genes within statistically differentially enriched hcpeaks near the 3’-UTR (Figure 6D). These results revealed that genes containing statistically differentially enriched hcpeaks in specific genic locations play roles in distinct biological processes in 9311 and NIP.

Our study suggests that the number and extent of m^{6}A modifications on the transcripts of Cd-resistance genes may be important factors for determining and assessing the Cd tolerance of crops.

Conjoint analysis of the genes with differential m^{6}A peaks and differential expression

Differentially expressed genes (DEGs)were identified by comparing samples of the same rice cultivar in different conditions and different rice cultivar (9311 and NIP) in the same condition, two comparison groups in total (9311_cd *vs* 9311_ck, NIP_ cd *vs* NIP_ ck) were obtained. 8510 differentially expressed genes (DEGs) were identified as differential genes (FDR < 0.05) in 9311_cd *vs* 9311_ck, and among of them, 4664 are up-regulated and 3846 are down-regulated. According to peak differential analysis, 3406 significantly differential (FDR < 0.05) peaks were identified in 9311_cd compared to 9311_ck, and among of them, 1810 overlapping with 1733 genes, are up and 1596, overlapping with 1515 genes are down. The comparison between genes overlapping with differential peaks and DE genes in the comparison of "9311_cd vs 9311_ck" is shown in Figure 8A.

As the same time,7742 significantly DE (FDR < 0.05) genes were identified in NIP_ cd compared to NIP_ ck, and among of them, 4768 are up-regulated and 2974 are down-regulated. According to peak differential analysis, 2065 significantly differential (FDR < 0.05) peaks were identified in NIP_ cd compared to NIP_ ck, and among of them, 1,191 overlapping with 1,084 genes are up and 874 overlapping with 825 genes are down. The comparison between genes overlapping with differential peaks and DE genes in the comparison of "NIP_ cd *vs* NIP_ ck" is shown in Figure 8B.

It indicated that not only the cultivar but also the treatments affect the gene expression level and m^{6}A mRNA methylation level. Moreover, the number of differential peaks and DE genes in NIP_ cd *vs* NIP_ ck less than 9311_cd *vs* 9311_ck. The results further suggested that 9311 is more sensitive to Cd than NIP.

Combined analysis of differential m^{6}A methylation in NIP and 9311

To further study the different effects of m^{6}A methylome in *indica* and *japonica* rice. We sought to examine the key pathways that may be involved in rice roots shorten by Cd exposure. To eliminate the influence of rice varieties, the common genes with m^{6}A enriched in *indica* and *japonica* under cadmium stress were detected, which were enriched in various pathways. KEGG pathway analysis found that these genes were involved in multiple biological pathways, including “beta−Alanine metabolism”, “arginine and proline metabolism”, “pyruvate metabolism” and “histidine metabolism” and so on (Figure 7A). These results indicated that cadmium treatment would affect the metabolism of various amino acids and further affect their growth and development in rice.

To further investigate the effects of m^{6}A on rice growth under Cd stress, all genes with differential m^{6}A peaks in *indica* and *japonica* were detected under Cd stress, except above common genes, unique genes with differential m^{6}A peaks in *indica* or *japonica* were enriched in various pathways. In terms of KEGG pathway analysis, “gluconeogenesis”, “plant−pathogen interaction” and autophagy-other were enriched in 9311(Figure 7B). While “plant hormone signal transduction”, “serine” and “threonine” metabolism were enriched in NIP (Figure 7C). Differences in unique pathways about 9311 and NIP may explain the phenotypic differences about *indica* and *japonica* rice under cadmium stress.

Changes of RNA methylation related genes in rice response to cadmium stress

In order to further explore the effect of m^6A methylation on rice growth under cadmium stress, we checked whether RNA methylation-related genes are differentially expressed genes (DEGs) or DMGs (differentially methylated genes) when comparing Cd groups with CK groups in rice.

We collected a total of 31 genes might related to RNA methylation in rice including m^6A writers, m^6A readers and m^6A erasers (Hu et al. 2019) (Table1), and 17 RNA methylation-related genes were DMGs or DEGs in rice responsive to cadmium stress. As a result, two m^6A writers, including *LOC_Os02g45110* and *LOC_Os01g16180*, were downregulated when comparing 9311_Cd with 9311_CK, but not statistically significantly differentially expressed when comparing NIP_Cd with NIP_CK. The down-regulation of these two genes may explain the phenomenon that the root length of 9311 was significantly shortened under cadmium stress while the variation in NIP was not significant. Under cadmium stress, four genes including m^6A writers *LOC_Os10g31030* and *LOC_Os03g35340*, m^6A readers *LOC_Os06g46400* and *LOC_Os07g07490* were down-regulated in both 9311 and NIP, but there was no significant change in m^6A methylation levels. This suggests that these genes respond to cadmium in both *indica* and *japonica* rice. While m^6A level of the m^6A writer *LOC_Os06g27970* is decreased when comparing 9311_ Cd with 9311_ CK, but there was no significant change in NIP_ Cd vs NIP_ CK. The m^6A level of the m^6A writer *LOC_Os10g35190* is decreased both in 9311 and NIP under cadmium stress. In contrast, m^6A writer *LOC_Os09g29630* is enriched with m^6A methylation both in 9311 and NIP under cadmium stress. The expression levels of genes including *LOC_Os03g20180*, *LOC_Os01g48790* and *LOC_Os05g01520* are increased when comparing 9311_cd with 9311_ck. As a m^6A eraser, the m^6A level of *LOC_Os10g02760* is decreased both in 9311 and NIP under Cd stress, and its expression level is decreased when comparing NIP_ cd with NIP_ ck, but no significant change in the NIP. The changes of m^6A levels and expression levels of methylation-related genes in rice under cadmium condition, may contribute to the phenotypic difference after cadmium treatment in rice.

Discussion

Several studies have shown that different cultivars showed different responses under Cd stress (Yan et al. 2019). In recent years, Cd has fascinated great attention due to its harmful effects on plant productivity. Up to now, no data has been reported about the role of m^6A methylome in *indica* and *japonica* rice under cadmium treatment. Our data showed that during the growth of rice under cadmium stress, there are a large number of m^6A methylation modification of genes in roots tissues. Therefore, the exploitation genes in these metabolic pathways might fully explain the differences in cadmium stress between *indica* and *japonica*. The m^6A distribution could be influenced by exogenous stimulation. Importantly, we discovered the patterns of the m^6A distribution in 9311 and NIP mRNA from CK and Cd groups, respectively. In *Arabidopsis*, m^6A is exclusively enriched around the stop codon and start codon of genes. However, in our results, it is observed that the m^6A peak was clearly enriched in the stop codon and 3'UTRs region of genes in rice.

After cadmium treatment, the expression level of 11 genes related to cadmium stress both in 9311 and NIP were increased, including *OsHMA4*, *PEZ1*, *OsHsfA4a*, *OsPDR8*, *OsMAPK2*, *OsABCG43*, *OsHMA9*, *OsMSRMK2* and so on (Supplementary Table S3). In 9311, the expression level and methylation level of two genes, including *OsHIR1* and *OsNramp6*, were increased. *OsHIR1*, a RING E3 ligase gene induced by heavy metals in rice, is located on the cell membrane and can control the cadmium transport (Lim et al. 2014). Several metal ions like Zn^{2+}, Mn^{2+}, Fe^{2+}, Cd^{2+} etc. have been studied to be transported *via* NRAMP transporter proteins like *OsNramp6* in rice (Mani et al. 2018). In addition, the expression level of *OsZIP1* was increased. *OsZIP1* is abundantly expressed in roots throughout the life span and sufficiently induced by excess cadmium (Liu et al. 2019). On the contrary, the methylation level of *OsHMA3* was decreased in 9311 under cadmium stress, this gene isolates Cd^{2+} by transporting it into the vacuole, reducing Cd^{2+} transport to the ground and thus reducing cadmium toxicity (Sasaki et al. 2014). In NIP, the expression levels of *OsLCD* and *OsCDT1* were increased under cadmium stress. *OsLCD* is involved in cadmium partitioning in rice, and the *lcd* mutant showed tolerance to cadmium on agar plates and in hydroponic culture during early plant development (Shimo et al. 2011). Constitutive expression of *OsCDT1* confers cadmium -tolerance to transgenic A. thaliana plants by lowering the accumulation of cadmium in the cells. The changes in these genes further explained the phenotypic changes of the two rice varieties under cadmium stress.

Based on the combined analysis of transcriptome and differentially enriched m^6A peaks in 9311_cd vs 9311_ck and NIP_ cd vs NIP_ ck, nine differentially expressed genes containing m^6A modification, which were related to root growth in rice according to previous research, were screened (Meng et al. 2019). In 9311_cd vs 9311_ck, we found five genes including *OsGatB*, *OsNAL1*, *OsFH1*, *OsGLU3* and *OsABIL2*, which control root growth in rice, are overlapping with differentially enriched m^6A peaks. For example, *OsGatB* may promote primary root growth by maintaining mitochondrial structure and function to facilitate cell division and elongation in the root tip (Qin et al. 2016). *OsNAL1* encodes a putative trypsin-like serine/cysteine protease that affects auxin transport (Fujita et al. 2013). *OsFH1* was also found to regulate rice root hair elongation (Huang et al. 2013). *OsGLU3* encodes a putative membrane-bound endo-1,4-β-glucanase, which is necessary for root elongation in rice (Zhang et al. 2012). These genes play an active role in rice root growth, and the m^6A level of them were downregulated, and their

expressions were downregulated after cadmium treatment in 9311. However, the m^6A level of *OsABIL2* was downregulated, but the gene expression was upregulated. Plants overexpressing *OsABIL2* had attenuated ABA signaling and shorter root hairs (Wang et al. 2017), which means this gene has a negative regulatory effect on rice growth. The results were consistent with the phenotype of rice treated with cadmium in 9311. In NIP_ cd vs NIP_ ck, we found three genes including *OsCHR4*, *OsSLL1* and *OsSNDP1*, are overlapping with differentially modified peaks. *OsSLL1*, encoding a stearoyl acyl carrier protein from the fatty acid desaturase family, affects overall fatty acid desaturation (Shelley et al. 2017). *OsSNDP1*, encoding a phosphatidylinositol transfer protein (PITP), promotes root hair elongation via phospholipid signaling and metabolism (Huang et al. 2013). These two genes have a negative regulatory effect on rice growth, and their m^6A levels were decreased, but the expression levels of genes were increased. *OsCHR4* plays a role in crown root development through the auxin-signaling pathway (Zhao et al. 2012). The m^6A methylation level of OsCHR4 was upregulated, but the expression level was downregulated in NIP_ cd vs NIP_ ck. *OsAIM1* is also required for root growth in rice through promoting reactive oxygen species (ROS) accumulation (Xu et al. 2017). This gene was both in 9311 and NIP, and it indicates this gene may be a common methylation modification gene that responds to cadmium stress in rice. The m^6A methylation level of *OsAIM1* was decreased, but the expression level of genes was upregulated both in NIP_ cd vs NIP_ ck and 9311_cd vs 9311_ck. Our study suggests that the number and extent of m^6A modifications on the transcripts of cadmium -resistance genes may be important factors for determining and assessing the cadmium tolerance of crops.

Methods

Rice seedlings cultivation and treatment

Seeds of *indica* rice 9311 and *japonica* rice NIP were germinated. Each type of rice seedlings was divided into two groups. Each group was repeated three times and exposed to 0 or 50 μM $CdCl_2$ in hydroponic culture for three days, respectively. The seedlings were grown in a growth chamber at 28 °C under a 16 h light/8 h dark cycle with the light period from 6:00 AM to 10:00 PM for five days and distilled water with or without $CdCl_2$ which were changed every day. After treated for three days, rice roots from CK and Cd groups were harvested and snap frozen in liquid nitrogen and then refrigerated at −80 °C for RNA isolation and sequencing.

RNA isolation and library construction

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's procedure. The total RNA quality and quantity were analysed using Bioanalyzer 2100 and RNA 6000 Nano Lab Chip Kit (Agilent, CA, USA). Only RNAs with RIN number >7.0 were used for library construction. Approximately more than 200 ug of total RNA was subjected to isolate Poly (A) mRNA with poly-T oligo attached magnetic beads (Invitrogen). Following purification, the poly(A) mRNA fractions were fragmented into ~100-nt-long oligonucleotides using divalent cations under elevated temperature. Then the cleaved RNA fragments were subjected to be incubated for 2h at 4℃ with m6A-specific antibody (No. 202003, Synaptic Systems, Germany) in IP buffer (50 mM Tris-HCl, 750 mM NaCl and 0.5% Igepal CA-630) supplemented with BSA (0.5 μg μl−1). The mixture was then incubated with protein-A beads and eluted with elution buffer (1 × IP buffer and 6.7mM m^6A) Eluted RNA was precipitated by 75% ethanol. Eluted m^6A-containing fragments (IP) and untreated input control fragments were converted to final cDNA library in accordance with a strand-specific library preparation by dUTP method. The average insert size for the paired-end libraries was ~100±50 bp. And then we performed the paired-end 2×150bp sequencing on an Illumina Novaseq™ 6000 platform at the LC-BIO Bio-tech ltd (Hangzhou, China) following the vendor's recommended protocol.

The exposure of rice seedling 9311 and NIP, extensive phenotypic variations were observed for seedling length under Cd stress, and control condition. After the experiment was carried out continuously for 7 days, rice roots were collected for the measurement of length levels.

m^6A sequencing

Rice roots from CK and Cd^{2+} stressed rice plants were collected to extract the total RNA. Three biological replicates of m^6A RIP sequencing were performed for the four rice samples. $MACS_2$ was used to call m^6A peaks with strict standards (error detection rate (FDR) <0.05, p-value <0.01, fold change (FC)>2). Homer software is used to identify the new motifs in m6A peaks and obtain their position weight matrices and precise motif region. We assigned all modification sites to gene regions including CDS, 3'UTR, 5' UTR, intron and exon region. The genic regions were separated into six regions, including 1) 5' UTR, in which 100bp close to start codon was removed; 2) start codon region, which is a 200bp long region extracted from 5' UTR and CDS regions centered at start codon; 3) CDS region, in which 100 bp region after start codon and 100 bp region before stop codon were removed; 4) stop codon region, which is a 200bp long region extracted from CDS and 3' UTR regions centered at stop codon; 5) intron region, which includes all introns of genes. The annotation of unique peaks (unipeak) was designated to one of above 5 genic regions based on genomic coordinates with a minimum overlap of 100 bp following the priorities of 5' UTR, start codon region, CDS region, stop codon region, intron region. Peaks unable to be designated to one of 5 genic regions were classified as intergenic. To analyze the distribution profiles of m^6A peaks within mRNAs, peaks were categorized into six regions, including 1) 5' UTR, in

which 100bp close to start codon was removed; 2) start codon region, which is a 200bp long region extracted from 5' UTR and CDS regions centered at start codon; 3) CDS region, in which 100 bp region after start codon and 100 bp region before stop codon were removed; 4) stop codon region, which is a 200bp long region extracted from CDS and 3' UTR regions centered at stop codon; 5) intron region, which includes all introns of genes. Then the differentially expressed genes were identified using edgeR(Nikolayeva et al. 2014). Gene ontology (GO) enrichment analysis was performed using AgriGOv2 (Tian et al. 2014).

Processing of raw data

Raw data from sequencing was performed using fastQC (v0.11.7). R package "ngsReports" was used to summarise fastQC report. Low quality and adaptor sequences were trimmed from raw reads using trim_ galore (v0.4.4) with following parameters: –stringency 6 -a-aAAGTCGGAGGCCAAGCGGTCTTAGGAAGACAA-a2AAGTCGGATCGTAGCCATGTCGTTCTGTGAGCCAAGGAGTTG –fastqc –paired.

genome mapping

Clean reads were mapped to rice reference genome IRGSP-1.0 (https://plants.ensembl.org/Oryza_sativa/Info/Index) with gene annotation Release 48 (ftp://ftp.ensemblgenomes.org/pub/plants/release-48/gff3/oryza_sativa) using STAR (v2.7.6a) with following parameters: –outFilterMismatchNmax 6 –outFilterMismatchNoverLmax 0.03 –quantMode.

Comparison of peaks in different groups

Only unipeaks which are presented in at least two out of three biological replicates (minimum overlap as 100bp with identified peaks in each biological replicate) are considered as high confident peaks (named as hcpeak) in this group.

Quantitative real-time PCR (qRT-PCR) validation

In order to validate the RNA-seq results, different expression patterns of several genes were confirmed by quantitative real-time RT-PCR (qRT-PCR). For qRT-PCR, 1 μg of total RNA was used to synthesize cDNA using PrimeScriptTM RT reagent Kit (Perfect Real Time) (TaKaRa). The qRT-PCR was carried out using SYBR® Premix Ex Taq II (Tli RNaseH Plus; TAKARA BIO Inc., Shiga, Japan) and determined in LightCycler 480 (Roche, Basel, Switzerland) according to the manufacturer's instructions. The qRT-PCR reactions were amplified for 95 OC for 30 s, followed by 40 cycles of 95◦C for 5 s, 55◦C for 30 s and 72◦C for 30 s. All reactions were performed with three independent biological replicates for each sample and three technical replicates for each biological replicate were analyzed. The relative gene expression was calculated by the software of ABI7500 Real-Time PCR System using the $2^{-\Delta\Delta Ct}$ method. The primers used for real-time qPCR are listed in Supplementary Table S4.

Conclusion

This study revealed the phenotypic differences between Indica and Japonica Rice under cadmium stress through methylation of m6A mRNA and transcriptome analysis.

Abbreviations

N^6-methyladenosine (m^6A), cadmium (Cd), control (CK), Nipponbare (NIP), m^6A peak (m^6A-modified nucleotide position on mRNAs), immunoprecipitation (IP), input (non-IP control), high confident peaks (hcpeaks), Gene ontology (GO), differential methylated peaks (DMPs), differentially expressed genes (DEGs), DMGs (differentially methylated genes), reactive oxygen species (ROS)

Declarations

Ethical Approval and Consent to participate

(Not applicable)

Consent for publication

All authors reviewed the manuscript and agreed to publish it.

Availability of supporting data

The data sets supporting the results of this article are included within the article and its additional files.

Funding

Grant (20192BCB23010) from Project of Science and Technology Department of Jiangxi Province.

Author contributions

Q.C designed and performed experiments, analyzed data and wrote the manuscript. P.W participated in designing and performing experiment. G.W and Y.W completed the manuscript with inputs in technical support, critical writing and suggestions regarding the manuscript. J.T, C.L, X.Z, S.L, S.H, T.H, M.Y participated in performing experiments. H.H and J.B. conceived and supervised the experiments. All authors reviewed the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgments

We thank the anonymous referees for their critical comments on this manuscript. This research was supported by grant (20192BCB23010) from Project of Science and Technology Department of Jiangxi Province.

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Tables

Table 1 RNA methylation related genes in rice. “UP” means this gene is overlapping with up hcpeaks or in up genes when comparing cd to ck, vice versa. “NDE” means this gene is not in differential hcpeaks or DE genes.

RAP_id	MSU_id	gene_name	type	N9311_hcpeaks	N9311_DE_genes	NIP_hcpeaks	NIP_DE_genes
Os02g0672600	LOC_Os02g45110	MTA	writers	NDE	DOWN	NDE	NDE
Os01g0267100	LOC_Os01g16180	MTB	writers	NDE	DOWN	NDE	NDE
Os03g0147700	LOC_Os03g05420	NA	writers	NDE	NDE	NDE	NDE
Os10g0447600	LOC_Os10g31030	NA	writers	NDE	DOWN	NDE	DOWN
Os06g0474200	LOC_Os06g27970	FIP37	writers	DOWN	NDE	NDE	NDE
Os03g0554900	LOC_Os03g35340	VIRLIZER	writers	NDE	DOWN	NDE	DOWN
Os10g0494500	LOC_Os10g35190	HAKAI	writers	DOWN	NDE	DOWN	NDE
Os08g0484400	LOC_Os08g37780	TRM4A	writers	NDE	NDE	NDE	NDE
Os09g0471900	LOC_Os09g29630	TRM4B	writers	UP	NDE	UP	NDE
Os01g0329800	LOC_Os01g22630	ECT11	readers	NDE	NDE	NDE	NDE
Os08g0224200	LOC_Os08g12760	ECT9	readers	NDE	NDE	NDE	NDE
Os06g0677700	LOC_Os06g46400	CPSF30	readers	NDE	DOWN	NDE	DOWN
Os03g0317000	LOC_Os03g20180	ECT7	readers	NDE	UP	NDE	NDE
Os03g0748000	LOC_Os03g53670	ECT4	readers	NDE	NDE	NDE	NDE
Os01g0679900	LOC_Os01g48790	ECT8	readers	NDE	UP	NDE	NDE
Os04g0608800	LOC_Os04g51940	ECT1	readers	NDE	UP	NDE	UP
Os08g0556000	LOC_Os08g44200	ECT5	readers	NDE	NDE	NDE	NDE
Os07g0170300	LOC_Os07g07490	ECT2	readers	NDE	DOWN	NDE	DOWN
Os04g0608900	LOC_Os04g51950	ECT6	readers	NDE	NDE	NDE	NDE
Os05g0130600	LOC_Os05g04000	ECT10	readers	NDE	UP	NDE	UP
Os05g0105600	LOC_Os05g01520	ECT3	readers	NDE	UP	NDE	NDE
Os03g0816500	LOC_Os03g60190	ALKBH1A	erasers	NDE	NDE	NDE	NDE
Os11g0488500	LOC_Os11g29690	ALKBH1B	erasers	NDE	NDE	NDE	NDE
Os06g0286310	LOC_Os06g17830	ALKBH2	erasers	NDE	NDE	NDE	NDE
Os10g0420000	LOC_Os10g28410	ALKBH6	erasers	UP	NDE	NDE	NDE
NA	LOC_Os04G51360	ALKBH8	erasers	NDE	NDE	NDE	NDE
Os11g0657200	LOC_Os11g43610	NA	erasers	NDE	NDE	NDE	NDE
Os06g0138200	LOC_Os06g04660	ALKBH9A	erasers	UP	NDE	NDE	NDE
Os05g0401500	LOC_Os05g33310	ALKBH10A	erasers	NDE	NDE	NDE	NDE
Os10g0116900	LOC_Os10g02760	ALKBH10B	erasers	DOWN	NDE	DOWN	DOWN

Figures

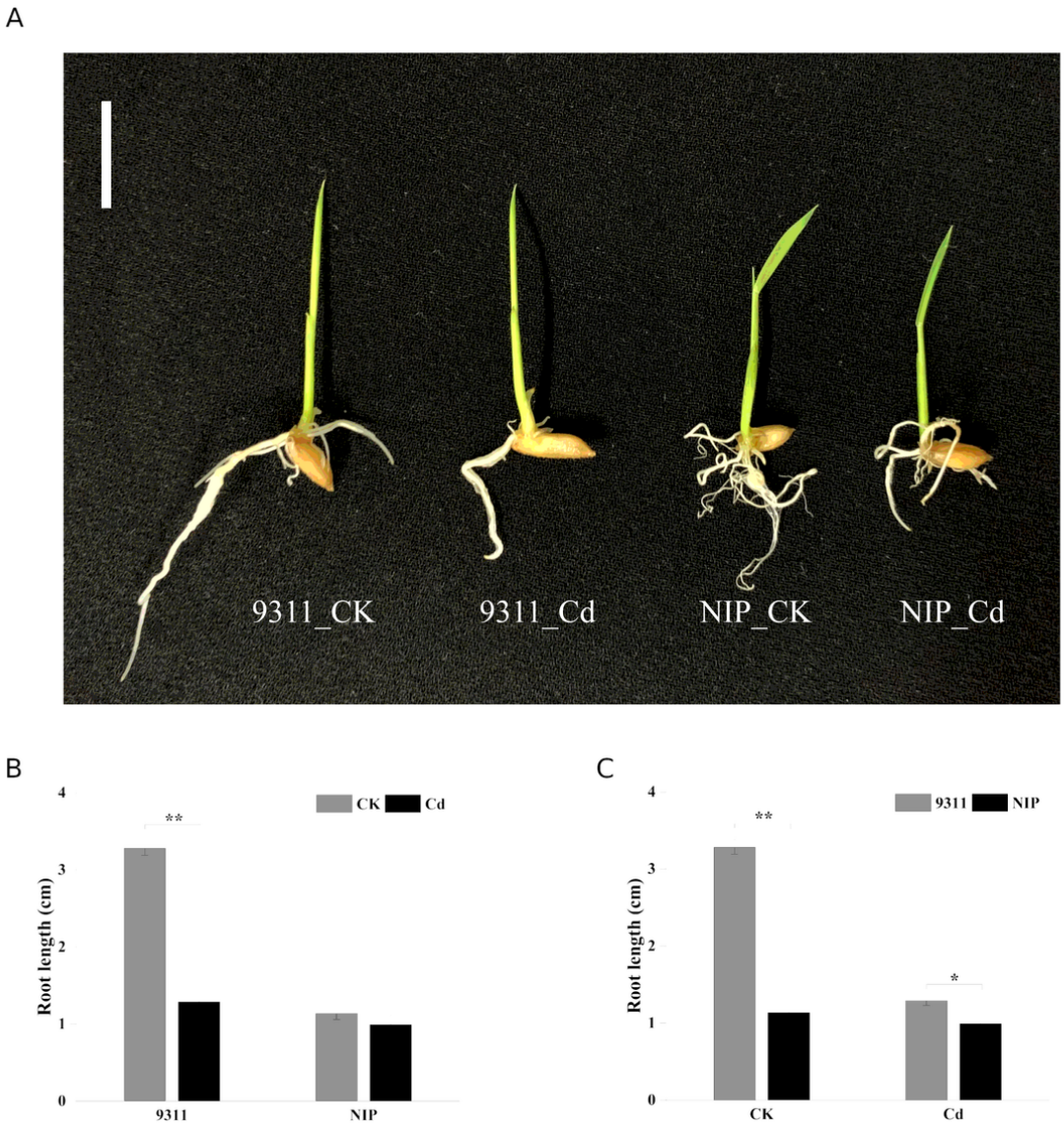

Figure 1

Phenotypes of 9311 and NIP under CK (control) and Cd (cadmium)groups. (A) The 3-day-old seedlings of 9311 and NIP under control condition and Cd stress conditions. (B) and (C) Comparison of root length, in 3-day-old seedlings of 9311 and NIP under under control condition and Cd stress conditions, respectively. Data are presented as means ± SE. n = 15. Statistical analysis was conducted using the Student's t-test. *, P-value < 0.05; **, P-value < 0.01; ***, Pvalue < 0.001. scale bar = 1 cm.

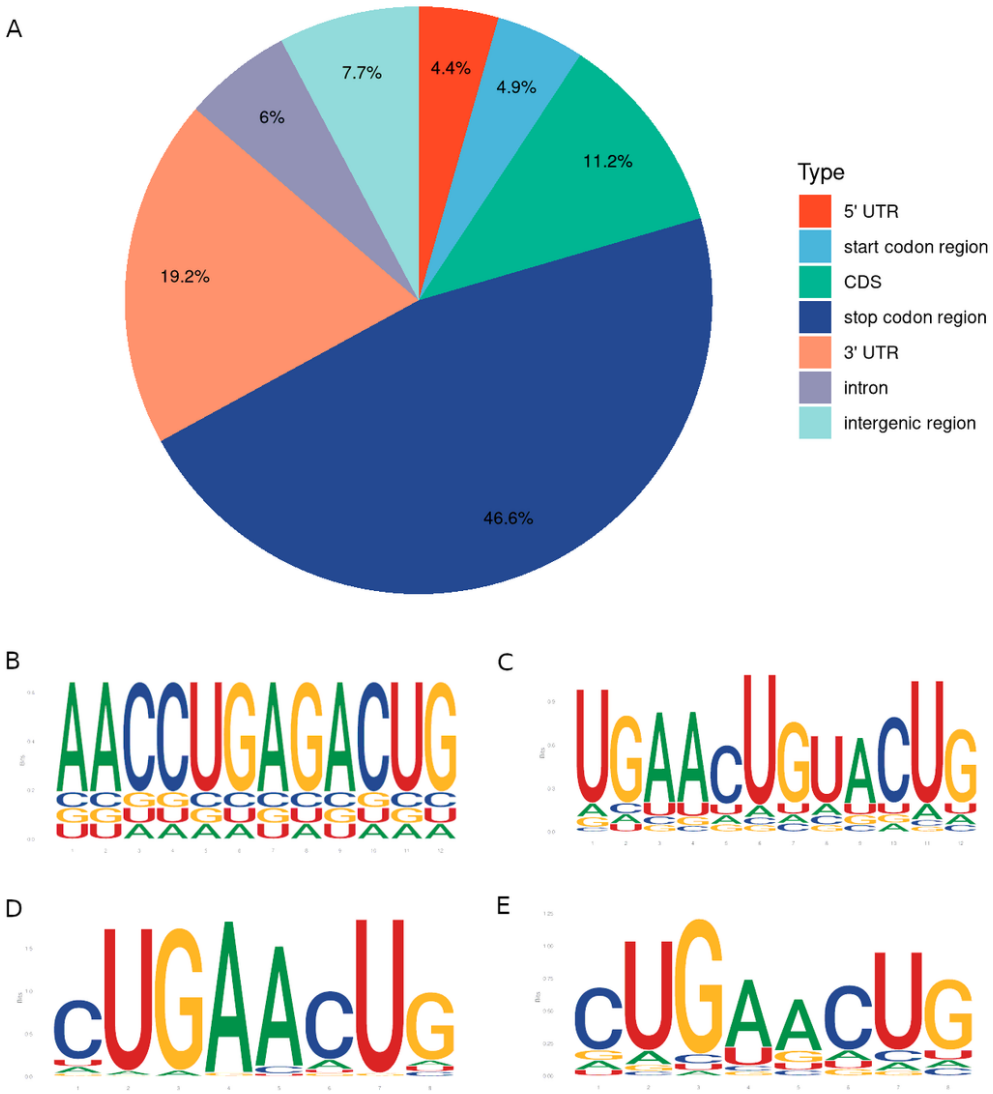

Figure 2

Overview of m6A methylome in rice. (A) Annotation of identified m6A high confident peaks (B)-(E) canonical RRACH and motif URUAY motif in four samples.

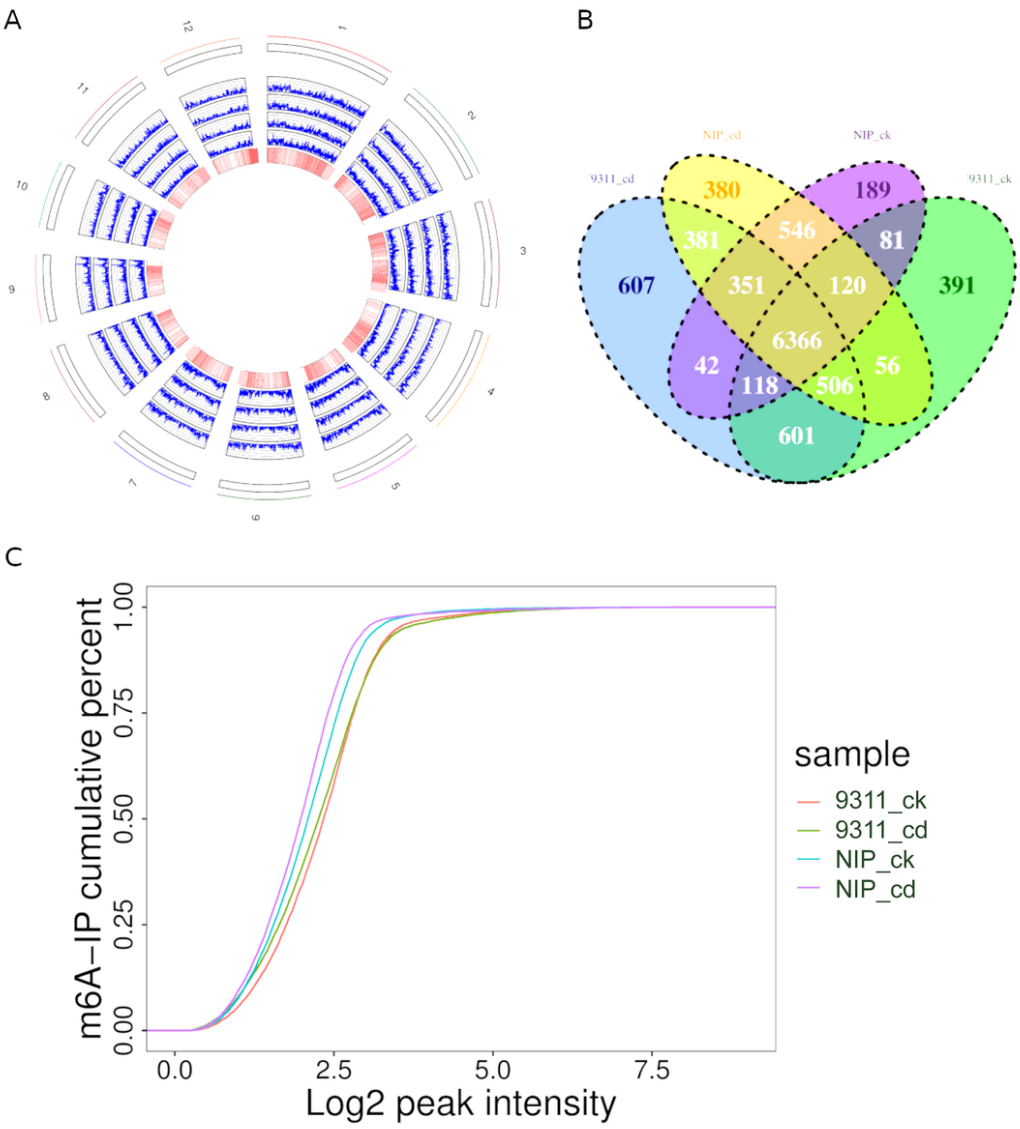

Figure 3

The m6A methylome changes under Cd stress in rice. (A) Genomic distribution of high confidience peaks (hcpeaks) in four groups. The reference genome is split into 100kb bins and the frequencies of genes or peaks located in each 100kb bin was counted and plot as line. The tracks are “gene (heatmap)”, “peaks in 9311_cd”, “peaks in 9311_ck”, “peaks in NIP_cd” and “peaks in NIP_ck” from inside outwards. (B) Comparison of high confidence peaks (hcpeaks) in 4 groups. (C) Cumulative distribution function of log2 peak intensity of m6A-modified sites under CK and Cd groups in 9311 and NIP.

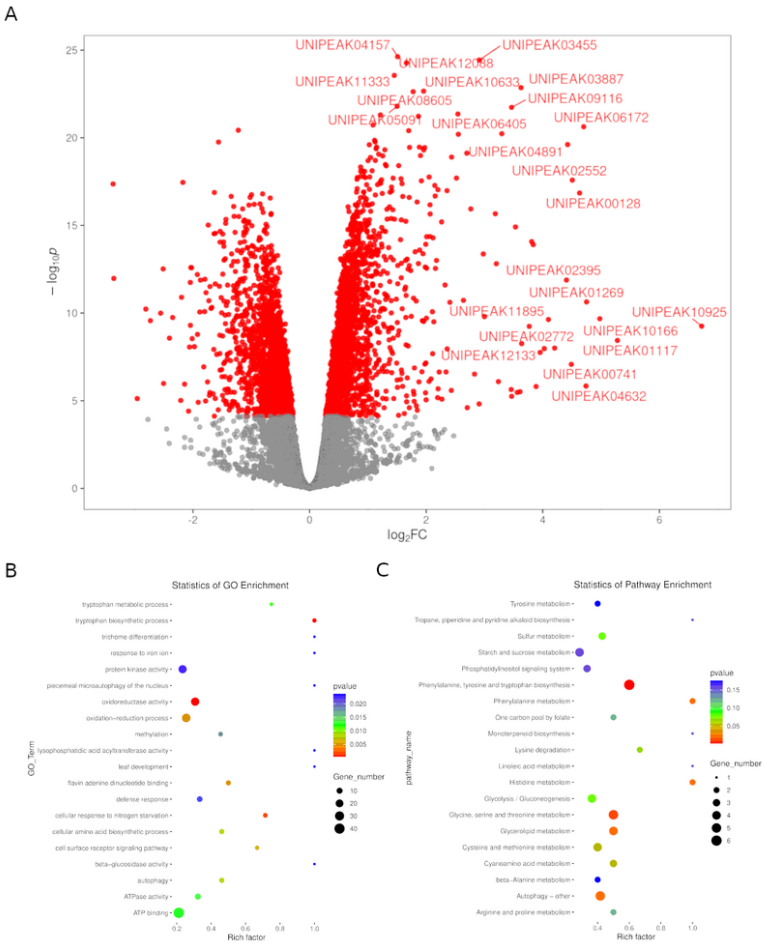

Figure 4

Gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses of coding genes containing differentially enriched m6A hcpeaks in 9311_CK vs. 9311_Cd. (A) Volcano plot showing logFC against statistical significance. Hcpeaks with FDR < 0.05 are coloured red with select points at the extremities of the plot labelled for easier visualisation (top 10 DE peaks or logFC < -4 or logFC > 4) (B) Major gene ontology terms were significantly enriched for these genes. (C) Major Kyoto Encyclopedia terms were significantly enriched for these genes.

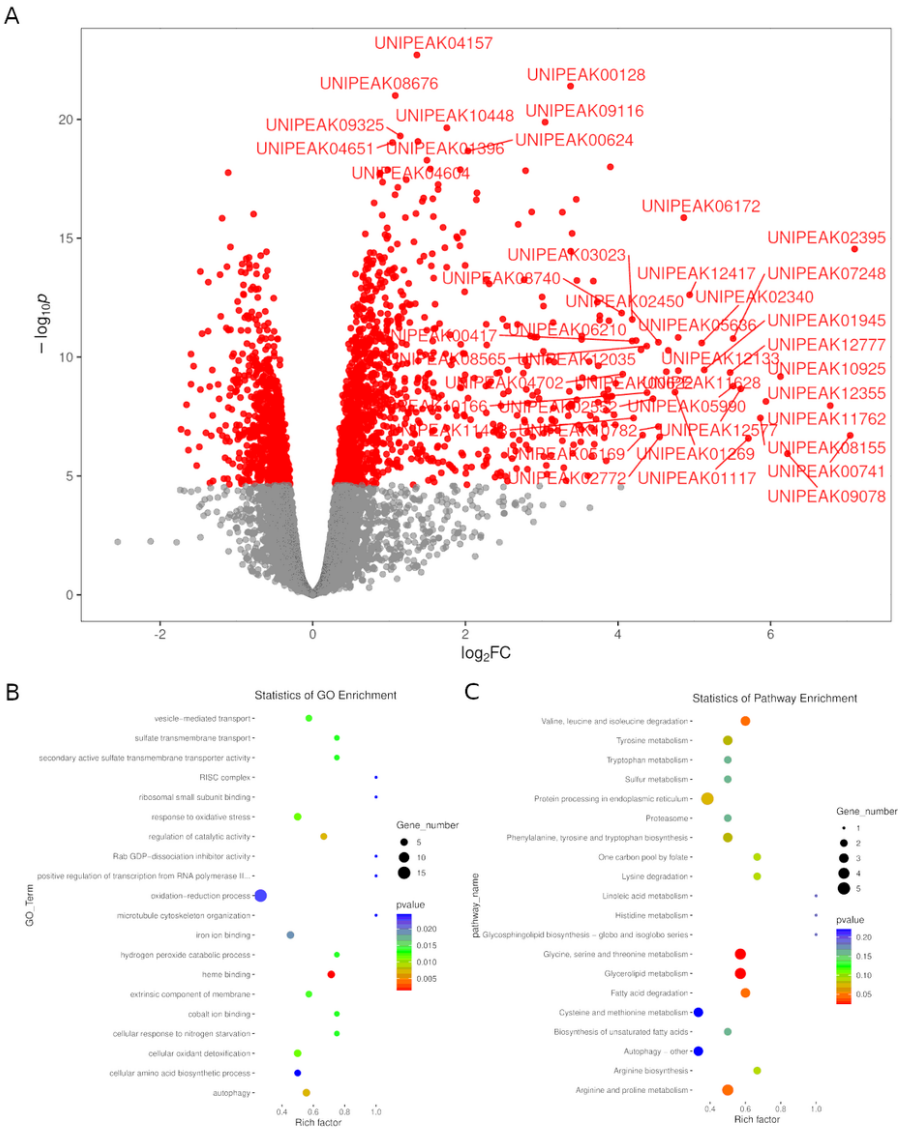

Figure 5

Gene ontology and Kyoto Encyclopedia of Genes and Genomes analyses of coding genes containing differentially enriched m6A hcpeaks in NIP_CK vs. NIP_Cd. (A) Volcano plot showing logFC against statistical significance. Hcpeaks with FDR < 0.05 are coloured red with select points at the extremities of the plot labelled for easier visualisation (top 10 DE peaks or logFC < -4 or logFC > 4) (B) Major gene ontology terms were significantly enriched for these genes. (C) Major Kyoto Encyclopedia terms were significantly enriched for these genes.

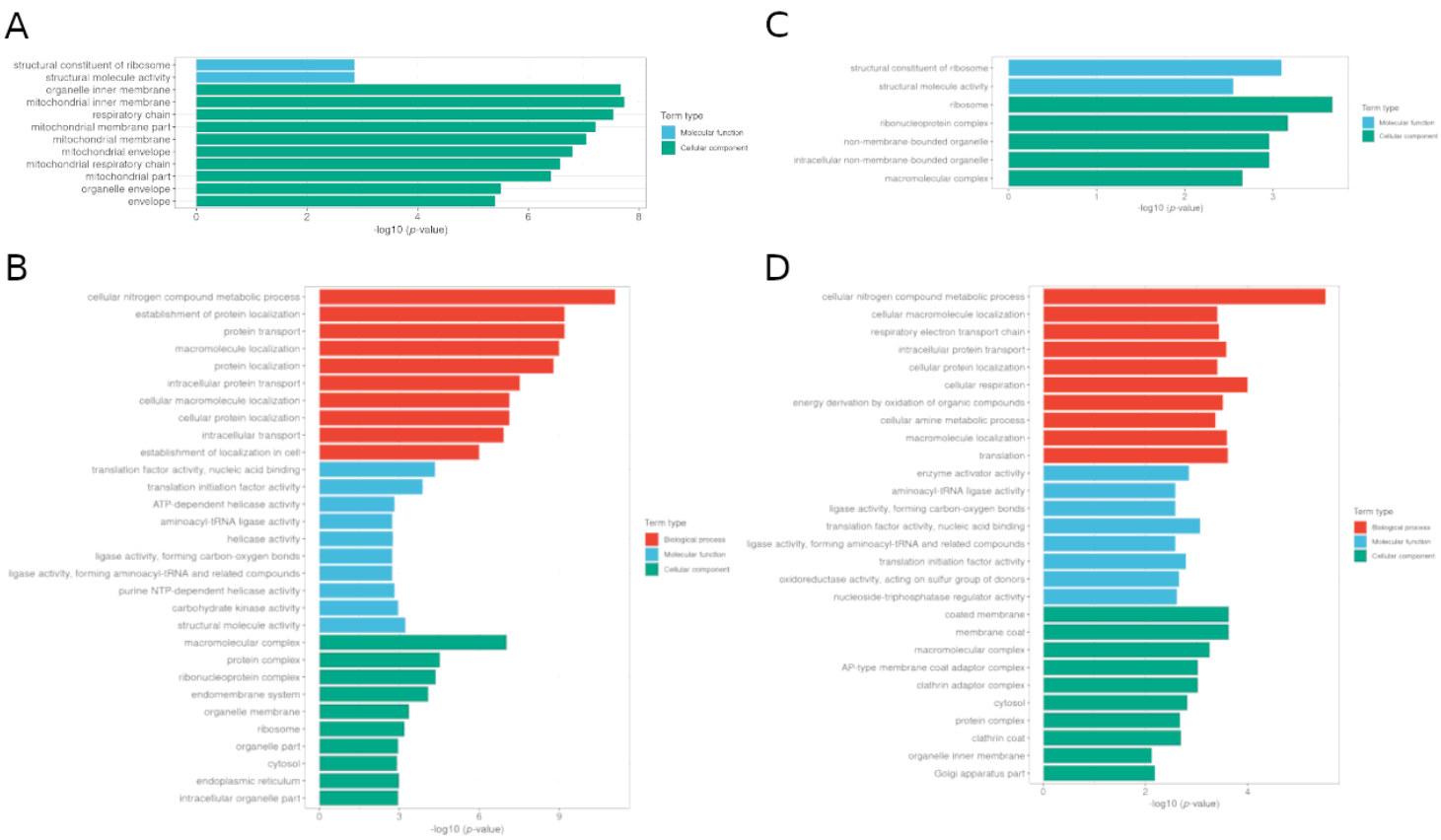

Figure 6

Functional characteristics of differentially methylated peaks (DMPs) in the context of genic location in 9311 and NIP. (A) top 10 over-represented GO term in each category at their 5'-UTR in 9311 (B) top 10 over-represented GO term in each category at their 3'-UTR in 9311(C) top 10 over-represented GO term in each category at their 5'-UTR in NIP(D) top 10 over-represented GO term in each category at their 3'-UTR in NIP.

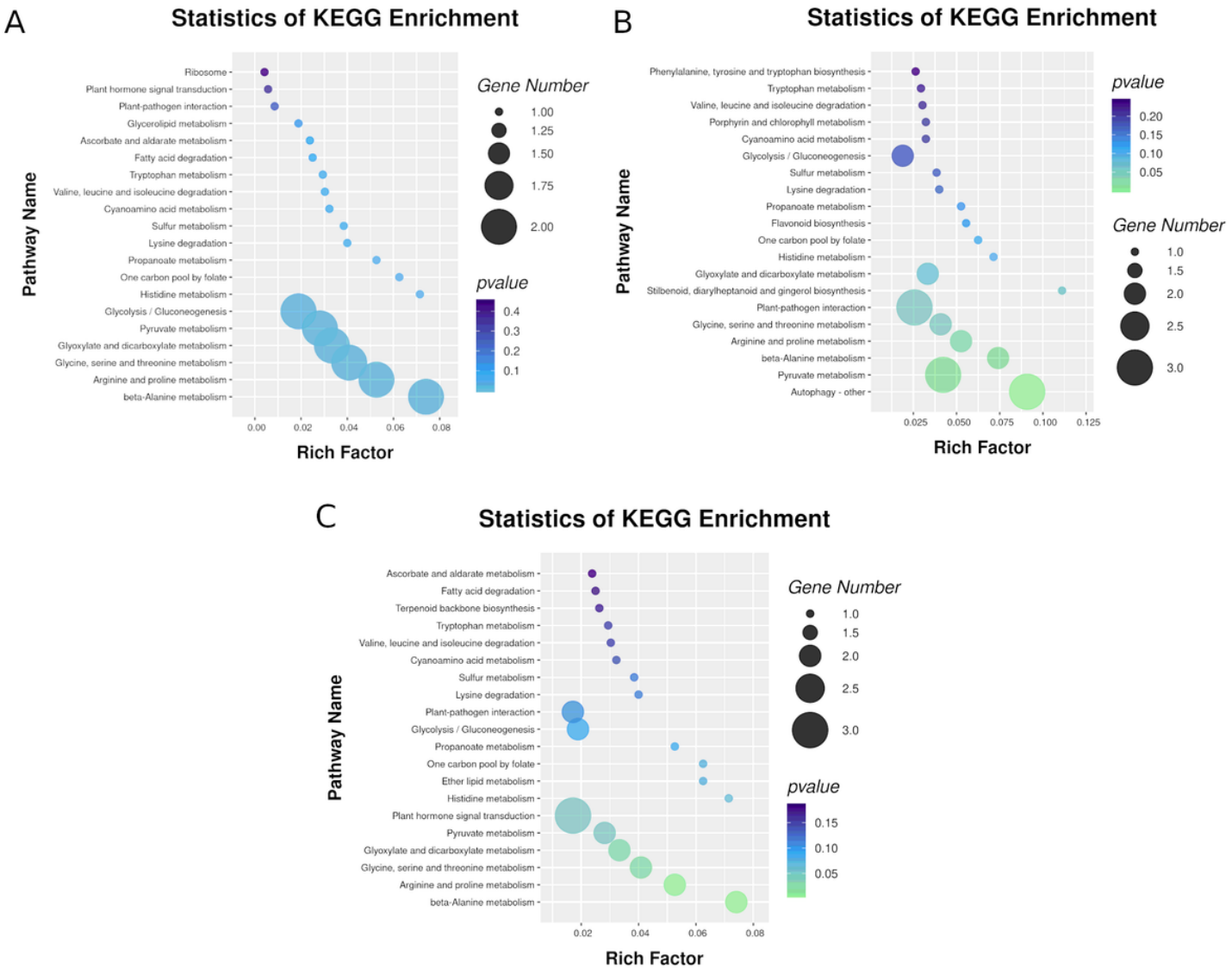

Figure 7

The KEGG terms of differential m6A methylation genes in NIP and 9311. (A) the KEGG terms of common genes with differential m6A methylation in indica and japonica under cadmium stress (B) the KEGG terms of unique genes with differential m6A peaks in indica (C) the KEGG terms of unique genes with differential m6A peaks in japonica.

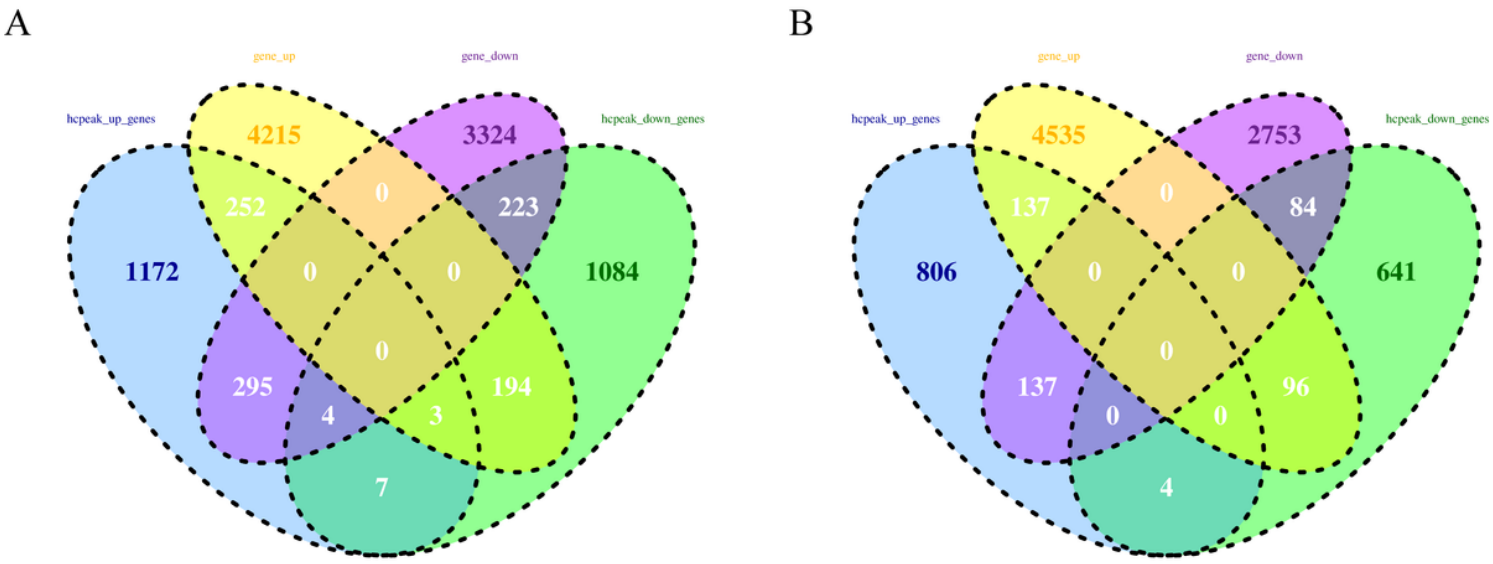

Figure 8

Compare the differential hcpeaks and DE genes. (A) comparison of genes overlapping with differential hcpeaks and DE genes in 9311_cd vs 9311_ck (B) comparison of genes overlapping with differential hcpeaks and DE genes in NIP _ cd vs NIP _ck.

Supplementary Files

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