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TITLE:

Genome-wide Analysis of Histone Modifications Distribution using the Chromatin Immunoprecipitation Sequencing Method in *Magnaporthe oryzae*

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ChIP-seq, *Magnaporthe oryzae*, Histone modification, H3K4me3, Genome-wide analysis, Target gene

SUMMARY:

Here, we present a protocol to analyze the genome-wide distribution of histone modifications, which can identify new target genes in the pathogenesis of M. oryzae and other filamentous fungi.

ABSTRACT:

Chromatin immunoprecipitation sequencing (ChIP-seq) is a powerful and widely used molecular technique for mapping whole genome locations of transcription factors (TFs), chromatin regulators, and histone modifications, as well as detecting entire genomes for uncovering TF binding patterns and histone posttranslational modifications. Chromatin-modifying activities, such as histone methylation, are often recruited to specific gene regulatory sequences, causing localized changes in chromatin structures and resulting in specific transcriptional effects. The rice blast is a devastating fungal disease on rice throughout the world and is a model system for studying fungus-plant interaction. However, the molecular mechanisms in how the histone modifications regulate their virulence genes in *Magnaporthe oryzae* remain elusive. More researchers need to use ChIP-seq to study how histone epigenetic modification regulates their target genes. ChIP-seq is also widely used to

study the interaction between protein and DNA in animals and plants, but it is less used in the field of plant pathology and has not been well developed. In this paper, we describe the experimental process and operation method of ChIP-seq to identify the genome-wide distribution of histone methylation (such as H3K4me3) that binds to the functional target genes in *M. oryzae.* Here, we present a protocol to analyze the genome-wide distribution of histone modifications, which can identify new target genes in the pathogenesis of M. oryzae and other filamentous fungi.

INTRODUCTION:

Epigenetics is a branch of genetic research that refers to the heritable change of gene expression without changing the nucleotide sequence of genes. An increasing number of studies have shown that epigenetic regulation plays an important role in the growth and development of eukaryotic cells, including chromatin that regulates and affects gene expression through the dynamic process of folding and assembly into higher-order structures[1,2]. Chromatin remodeling and covalent histone modification affect and regulate the function and structure of chromatin through the variation of chromatin polymers, thereby achieving the function of regulating gene expression[3–6]. Posttranslational modifications of histone include acetylation, phosphorylation, methylation, monoubiquitination, sumoylation, and ADP ribosylation[7–9]. Histone H3K4 methylation, particularly trimethylation, has been mapped to transcription start sites where it is associated with transcription replication, recombination, repair, and RNA processing in eukaryotes[10,11].

ChIP-seq technology was introduced in 2007 and has become the experimental standard for the genome-wide analysis of transcriptional regulation and epigenetic mechanisms[12,13]. This method is suitable at the genome-wide scale and for obtaining histone or transcription factor interaction information, including DNA segments of DNA binding proteins. Any DNA sequences crosslinked to proteins of interest will coprecipitate as a part of the chromatin complex. New-generation sequencing techniques are also used to sequence 36–100 bp of DNA, which are then matched to the corresponding target genome.

In phytopathogenic fungi, research has recently begun to study how histone methylation modifications regulate their target genes in the process of pathogenicity. Some previous studies proved that the regulation of histone methylase-related genes is mainly reflected in gene silencing and catalyzing the production of Secondary Metabolites (SM). MoSet1 is the H3K4 methylase in *M. oryzae.* Knockout of this gene results in the complete deletion of H3K4me3 modification[14]. Compared with the wild-type strain, the expression of the gene *MoCEL7C* in the mutant is inhibited in the CMC-induced state and in the non-induced state (glucose or cellobiose), the expression of *MoCEL7C* increased[15]. In *Fusarium graminearum*, KMT6 can catalyze the methylation modification of H3K27me3, regulate the normal development of fungi, and help regulate the "cryptic genome" containing the SM gene cluster[16–19]. In 2013, Connolly reported that H3K9 and H3K27 methylation regulates the pathogenic process of fungi through secondary metabolites and effector factors that regulate the inhibition of target genes[20]. In *Aspergillus*, the modification of histones

H3K4me2 and H3K4me3 is related to gene activation and plays an important role in controlling the chromatin level regulation of SM gene clusters[21]. In *M. oryzae*, Tig1 (homologous to Tig1 in yeast and mammals) is an HADC (histone deacetylase)[22]. Knockout of the *Tig1* gene leads to the complete loss of pathogenicity and spore production ability in the null mutant. It is more sensitive to a peroxygen environment, which cannot produce infective hyphae[22].

The rice blast caused by *M. oryzae.* is one of the most serious rice diseases in most rice-growing areas in the world[19]. Due to its representative infection process, *M. oryzae* is similar to the infection process of many important pathogenic fungi. As it can easily carry out molecular genetic operations, the fungus has become a model organism for studying fungal-plant interactions[23]. Blocking every step of the infection process of *M. oryzae* may result in unsuccessful infection. The morphological changes during the infection process are strictly regulated by the entire genome function and gene transcription. Among them, epigenetic modifications such as histone methylation play an essential role in the transcriptional regulation of functional genes[24,25]. However, so far, few studies have been done on the molecular mechanism of epigenetic modifications such as histone methylation and histone acetylation in the transcription of pathogenesis genes in *M. oryzae.* Therefore, further developing the epigenetic regulation mechanism of the rice blast fungus while researchinh the upstream and downstream regulatory network of these pathogenic related genes will help develop rice blast prevention and control strategies.

With the development of functional genomics such as ChIP-seq, especially in epigenetics, this high-throughput data acquisition method has accelerated research on chromosomes. Using the ChIP-seq experimental technology, the genome-wide distribution of histone methylation (such as H3K4me3, H3K27me3, H3K9me3) in *M. oryzae* and other filamentous fungi can be identified. Therefore, this method can help elucidate the molecular mechanisms underlying how epigenetic modifications regulate their candidate target genes during fungal pathogenesis in plant pathology.

PROTOCOL

1. Preparation of protoplasts from *M. oryzae*

1.1. Prepare the oatmeal-tomato agar (OTA).

1.1.1. Weigh 30-50 g of oatmeal and boil it in 800 mL of water (ddH_2O) for 20 min. Filter through two layers of gauze and take the filtrate.

1.1.2. Pick ripe tomatoes and peel them. Squeeze the juice, and filter through two layers of gauze to collect 150 mL of the filtered juice.

1.1.3. Mix all the tomato juice and the prepared oat filtrate thoroughly and add ddH_2O up to 1000 mL.

1.1.4. Add 250 mL of the OTA and 2.5 g of agar powder to a 500 mL conical flask, and autoclave for 20 min. Store at 25 °C.

1.2. Pour 25 mL of the autoclaved OTA into a glass 5 cm x 5 cm Petri dish. Prepare 10 of these Petri dishes total. After the OTA has solidified on the Petri dishes, store the dishes upside down at 25 °C.

1.3. Use a sterilized toothpick to dig out a small piece of mycelium from *M. oryzae* (the wild-type strain P131, knockout strains *Δmobre2, Δmospp1, and Δmoswd2*) and place them on the prepared OTA dishes. Culture them for 4-6 days at 28 °C under light conditions.

NOTE: Turn the Petri dish upside down to prevent pollution.

1.4. Add 1000 µL of liquid Complete Medium (CM) (0.6% yeast extract, 0.3% enzymatic casein hydrolysate, 0.3% acidic casein hydrolysate, 1% glucose) to the OTA dishes using a 1000 µL pipette.

NOTE: The hyphae grew on the OTA dishes for 4-6 days.

1.5. Scrape the mycelia of the wild-type strain and knockout strain with an inoculation loop.

1.6. Collect the mycelia debris and transfer them to 250 mL of liquid Complete Medium (CM).

1.7. Grow the fungal debris in a triangular flask at 28 °C for 36 h with shaking at 150 rpm.

1.8. Use a funnel to filter and collect the fungal hyphae.

1.9. Wash the fungal hyphae with 500 mL of 0.7 M NaCl solution.

1.10. Collect the fungal mycelium and weigh it.

NOTE: The mycelium does not need to be dried before weighing.

1.11. Add ~1 mL of lysis enzyme permeation solution per 1 g of the fungal mycelium.

1.11.1. Prepare the 20 mg/mL lysis enzyme permeation solution by dissolving the lysis enzyme from *Trichoderma harzianum* in 0.7 M NaCl.

1.12. Place the hyphae for lysing at 28 °C for 3–4 h with shaking at 150 rpm.

1.13. Wash the lysed hyphae with 50 mL of 0.7 M NaCl solution.

1.14. Collect the protoplasts and centrifuge for 15 min at 2,000 x *g* and 4 °C.

1.15. After centrifugation, discard the supernatant carefully. Resuspend the protoplasts in 20 mL of 0.7 M NaCl buffer at 4 °C.

2. In vivo crosslinking and sonication

2.1. Add 55 µL of 37% formaldehyde (add formaldehyde drop by drop until the final concentration is 1%) to 2 mL of NaCl buffer containing protoplast for crosslinking.

2.2. Incubate the protoplasts at 25 °C for 10 min.

2.3. Add 20 µL of 10x glycine to each tube to quench the unreacted formaldehyde.

2.4. Swirl to mix and incubate at 25 °C for 5 min.

2.5. Centrifuge for 15 min at 2,000 x *g* and 4 °C.

2.6. After centrifugation, discard the supernatant carefully. Resuspend the pellet in 1 mL of 0.7 M NaCl solution.

2.7. Centrifuge for 10 min at 2,000 x *g* and 4 °C.

2.8. After centrifugation, discard the supernatant carefully. Resuspend the pellet in 750 µL of RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA pH 8, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors).

2.9. Add 37.5 µL of 20x protease inhibitor.

2.10. Transfer the protoplasts from the previous step to a 1.5 mL centrifuge tube.

2.11. Perform sonication (25% W, output 3 s, stop 5 s, 4 °C) immediately with a sonicator for about 10 min. The purpose of this step is to sonicate the cross-linked lysate for a period of time to determine the best conditions.

NOTE: The lysate can be frozen at -80 °C at this step.

2.12. If optimal conditions for sonication have already been determined, proceed to the next step.

2.13. If desired, remove 5 µL of protoplasts for agarose gel analysis (unsheared DNA).

2.14. Shear the chromatin by sonication with an ultrasonic homogenizer for 8 min (25% W,

output 3 s, stop 5 s, 4 °C).

2.15. After the sample is ultrasonically broken, take out a part of the sample as “input”.. The input does not perform the ChIP experiment and contains all DNA and protein released after the sample is sonicated.

2.16. After sonication, run a 1% agarose gel electrophoresis to analyze the length of the DNA fragments.

NOTE: The agarose gel electrophoresis results show that the length of the DNA fragment is 200–500 bp (**Figure 4**).

2.17. Place the sonicated tube on ice to prevent protein degradation.

2.18. Centrifuge for 10 min at 10,000 x *g* and 4 °C.

2.19. After centrifugation, transfer the centrifuged supernatant to a new 1.5 mL centrifuge tube and store it at -80 °C for later use. The chromatin solution obtained in this step can be used for subsequent IP.

2.20. Before performing the IP experiment, dilute each chromatin sample to a ratio of 1:10 with 1x RIPA buffer (e.g., add 10 µL of chromatin sample to 1 µL of 1×RIPA buffer).

3. IP of crosslinked protein/DNA

3.1. Pipette 50 µL of superparamagnetic protein beads into a 2 mL centrifuge tube. Place the tubes on a magnetic stand. Let the magnetic beads precipitate. Remove the supernatant.

3.2. Add 1 mL of 1x RIPA buffer (pre-cooled on ice) to the tube and wash superparamagnetic protein beads thrice. After the wash, place the tubes on a magnetic stand and remove the supernatant. Add 100 µL of 1x RIPA buffer to each tube.

3.3. Add 300 µL of chormatin sample (2 x 10^7 cells were used), 100 µL of superparamagnetic protein beads and 4 µL of H3K4me3 antibody to the tube.

3.4. Use samples with Mouse IgG as the negative control.

NOTE: Mouse IgG used in this protocol contains 0.01 M phosphate buffer and 0.15 M NaCl, and will remain frozen below 20 °C (see **Table of Materials**).

3.5. After mixing well, place the samples on a rotary shaker and incubate overnight at 4 °C, 30 x *g*.

NOTE: It may be possible to reduce the incubation time of the immunoprecipitation (IP). The

incubation time depends on different factors (e.g., the antibody, gene target, cell type, etc.) and will need to be empirically tested.

4. Collecting and rinsing the IP products

4.1. Pellet the superparamagnetic protein beads by placing them on a magnetic stand. Aspirate and discard the supernatant.

4.2. Wash the superparamagnetic protein bead-antibody/chromatin complex by resuspending the beads in 1 mL of 1x RIPA buffer.

4.3. Rinse the tube on a rotary shaker for 5 min and remove the supernatant at 30 x *g*.

4.4. Add 1 mL of low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, and 150 mM NaCl).

4.5. Rinse the tube on a rotary shaker for 5 min and remove the supernatant.

4.6. Add 1 mL of high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, and 500 mM NaCl) to the centrifuge tube.

4.7. Place the tube on a rotary shaker for 5 min and remove the supernatant.

4.8. Rinse with 1 mL of LiCl (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid, 1 mM EDTA, and 10 mM Tris-HCl pH 8.1) in the centrifuge tube.

4.9. Place the tube on a rotary shaker for 5 min and remove the supernatant.

4.10. Rinse the test tube again with 1 mL of 0.25 M LiCl buffer, remove the supernatant with a pipette, and then discard the supernatant.

4.11. Add 1 mL of TE buffer (10 mM Tris-HCl pH 8.1, 1 mM EDTA).

4.12. Place the tube on a rotary shaker for 5 min at 30 x *g*.

4.13. Wash the tube again with 1 mL TE buffer and remove the supernatant with a pipette.

4.14. Collect the beads.

5. Elution of protein/DNA complexes

5.1. Prepare the elution buffer (10 µL of 20% SDS, 20 µL of 1 M $NaHCO_3$, 170 µL of sterile distilled H_2O) for all the IP and input tubes.

5.2. Add 100 µL of elution buffer to each centrifuge tube.

5.3. Elution at 65 °C for 15 min.

5.4. Centrifuge for 1 min at 10,000 x *g* and 4 °C and collect the supernatant into new centrifuge tubes.

5.5. Repeat steps 5.2 – 5.4 and combine the eluates. Add 190 µL of elution buffer to the 10 µL of the input DNA. (total volume = 200 µL).

6. Reverse crosslinking of protein/DNA complexes

6.1. Add 8 µL of 5 M NaCl to all the tubes and incubate at 65 °C for 4–5 h or overnight to reverse the DNA-protein crosslinks.

NOTE: After this step, store the samples at -20 °C, and continue the protocol the following day, if necessary.

6.2. Add 1 µL of RNase A and incubate for 30 min at 37 °C.

6.3. Add 4 µL of Proteinase K (dissolved in H_2O at 20 mg/mL and stored at -20 °C) to each tube and incubate at 45 °C for 1–2 h.

7. Purification and recovery of DNA

7.1. Add 550 µL of phenol/chloroform/isoamyl alcohol mixture (ratio of 25:24:1) to the centrifuge tube.

7.2. Thoroughly vortex the mixture for 1 min.

7.3. Centrifuge for 15 min at 10,000 x *g* and aspirate the supernatant.

7.4. Transfer the extracted supernatant from the previous step to a new 1.5 mL centrifuge tube.

7.5. Add 1/10 volume of 3 M sodium acetate solution, 2.5 volumes of absolute ethanol, and 3 µL of glycogen (20 mg/mL) to the tube.

7.6. Place the sample in a refrigerator at -20 °C overnight for precipitation.

7.7. Centrifuge for 15 min at 10,000 x *g* and 4 °C.

7.8. Discard the supernatant after centrifugation. Wash the pellet three times with 1 mL of

75% ethanol (needs to be prepared fresh) at 10,000 x g.

7.9. Place the washed precipitate on a clean bench to let the alcohol dry.

7.10. Add 50 µL of sterile deionized H_2O to sufficiently dissolve the precipitate.

7.11. Ligate the sequencing adaptor to the DNA fragment and use a high-throughput sequencing platform to sequence the DNA.

8. DNA repair and Solexa library construction

8.1. Repair the DNA ends to generate blunt-ended DNA using a DNA end-repair kit (1–34 µL DNA, 5 µL of 10x end-repair buffer, 5 µL of 2.5 mM each dNTP, 5 µL of 10 mM ATP, 1 µL of end-repair enzyme mix, and H_2O to adjust the reaction volume to 49 µL).

8.2. Use a PCR purification kit or phenol: chloroform extraction to purify the DNA. Elute or resuspend the DNA in 30 µL of 1x TE pH 7.4.

8.3. Add "A" to 3' ends (30 µL of DNA from step 2, 2 µL of H_2O, 5 µL of 10x Taq buffer, 10 µL of 1 mM dATP, and 3 µL of Taq DNA polymerase). Add the reagents to a 0.2 mL PCR centrifuge tube, mix well, and react in a PCR machine at 72 °C for 10 min.

8.4. Perform linker ligation by mixing 10 µL of DNA, 9.9 µL of H_2O, 2.5 µL of T4 DNA ligase buffer, 0.1 µL of adapter oligo mix, and 2.5 µL of T4 DNA ligase. Add the reagents to a 0.2 mL PCR centrifuge tube and mix well. Incubate them at 16 °C for 4 h.

8.5. Purify the DNA using a PCR purification kit according to manufacturer's protocol. Elute with 20–25 µL of elution buffer.

8.6. Before the DNA library is established, identify the concentration of the purified DNA to confirm its usage for the subsequent sequencing experiments.

8.7. Detect the DNA concentration using a fluorometer. After melting the sample on ice, mix it thoroughly and centrifuge for 30 s at 1000 x *g* and 4 °C. Then take an appropriate amount of sample and measure it in a fluorometer with a wavelength of 260 nm.

8.8. Place DNA samples with qualified quality and concentration on the Illumina sequencing platform for sequencing.

8.9. Before sequencing, amplify the DNA using PCR primers, PE1.0 and PE2.0, and 2x high fidelity master mix (10.5 µL of DNA, 12.5 µL of 2x high fidelity master mix, 1 µL of PCR primer PE1.0, and 1 µL of PCR primer PE2.0). Add the reagents to a 0.2 mL PCR centrifuge tube and mix well.

8.10. Run the PCR reaction in the PCR machine: 95 °C predenaturation for 2 min; then 35 cycles of 95 °C denaturation for 10 s, annealing at 60 °C for 15 s, extension at 72 °C for 5 s; a final extension at 72 °C for 5 min. Finally, incubate the reaction at 4 °C.

8.11. Use the DNA for cluster generation and perform sequencing-by-synthesis on an Illumina Hiseq 2000.

NOTE: In this protocol, Illumina flow cells were used for cluster generation. The sequencing-by-synthesis was performed on an Illumina Genome Analyzer following the manufacturer's instructions.

REPRESENTATIVE RESULTS:

The whole flow chart of the ChIP-seq method is shown in **Figure 1**. ChIP-seq experiments were performed using antibodies against H3K4me3 in the wild-type strain P131 and three null mutant strains that were devoid of *mobre2*, *mospp1*, and *moswd2* gene to verify the whole genome-wide profile of histone H3K4me3 distribution in *M. oryzae*. The protoplasts of the wild-type strain, Δ*mobre2*, Δ*mospp1*, and Δ*moswd2,* were prepared and sonicated at 25% W, output 3 s, stop 5 s, at 4 °C. Further, the chromatin was immunopurified with H3K4me3 antibody and Dynabeads protein A/G. Subsequently, DNA fragments were extracted using the phenol-chloroform method for constructing a sequencing library and sequenced with single ends on a high-throughput sequencing platform.

The representative results of the wild-type, Δ*mobre2*, Δ*mospp1*, and Δ*moswd2* strains with ChIP-seq method using the H3K4me3 antibody are shown in **Figure 2**. The H3K4me3 signals of the Δ*mobre2*, Δ*mospp1*, and Δ*moswd2* deletion mutants were significantly decreased at its functional target regions. As shown in **Figure 2**, some selected candidate target genes, including MGG_14897, MGG_04237, MGG_04236, and MGG_04235, were mapped for H3K4me3 distribution. Compared to the wild-type strain P131, the signals of enriched H3K4me3-ChIP-seq reads in the Δ*mobre2,* Δ*mospp1*, and Δ*moswd2* deletion mutants were largely decreased (**Figure 2**)[26]. These results suggest that the H3K4me3 modification plays important roles in regulating target gene expression in *M. oryzae.*

FIGURE AND TABLE LEGENDS:

Figure 1. The flow chart of the ChIP-seq method in *M. oryzae.* (A) The genomic DNA of *M. oryzae* was crosslinked with 1% formaldehyde. **(B)** Lysed blast fungus cells, broken DNA, free DNA, and histone binding DNA were subsequently obtained. **(C)** DNA fragments bound to histones and were extracted by specific binding to the H3K4me3 antibody. **(D)** Through reverse crosslinking, purified DNA subsequently obtained DNA fragments modified by H3K4me3 histones. **(E-F)** DNA fragments were sequenced, the sequencing results were compared, and sequences were identified in the *M. oryzae* DNA group. **(G)** Specific genes and loci of H3K4me3 histones in *M. oryzae* were retrieved.

Figure 2. The Δ*mobre2*, Δ*mospp1*, and Δ*moswd2* deletion mutants significantly decreased H3K4me3 profiles in their target regions. The H3K4me3-ChIP-seq distribution of enriched peaks around the coding regions of overlapped genes in Δ*mobre2*, Δ*mospp1*, and Δ*moswd2* deletion mutants are decresased compared to the wild-type strain in the MGG_14897,

MGG_04237, MGG_04236 and MGG_04235 genes[26]. The number in WT (input) labelled as [0-2074] signify means the results of ChIP in the range of genomic DNA [0-2074]. [0-2074] refers to 0-2074bp of Chromosome 6. The figure shows a random selection of the sequencing results, which only represents the DNA distribution on Chromosome 6. The complete sequencing results have been submitted to Genbank. (https//www.ncbi.nlm.nih.gov/bioproject/ accession 649321)[26].

Table 1. The total amount of DNA in this experiment. The total amount of input P131(2) is 2.7948 μg, the total amount of Δ*mobre2*(3) (input) is 2.4748 μg, the total amount of Δ*mospp1*(4) (input) is 3.22 μg, the total amount of Δ*moswd2* (5) (input) is 3.97 μg, and the total amount of P131(2) is 0.0735 μg, the total amount of Δ*mobre2*(3) is 0.0491μg, the total amount of Δ*mospp1*(4) is 0.0288 μg, the total amount of Δ*moswd2*(5) is 0.0527 μg.

Figure 3. Electrophoresis detection of DNA after ultrasound. After ultrasound sonication, the DNA is subjected to a 1% agarose gel experiment to analyze the length of DNA fragments. The sonicated DNA fragment length is from 200–500 bp, and these DNA fragments can be used for the following steps of ChIP-seq.

Figure 4. **Bioanalyzer trace of Input and ChIP samples.** The figure shows the fragment distribution of each sample, where the abscissa represents the fragment size, and the ordinate represents the peak size. The samples running on the bioanalyzer are input P131(2), Δ*mobre2*(3) (input), Δ*mospp1*(4) (input), Δ*moswd2*(5) (input), P131(2), Δ*mobre2*(3), Δ*mospp1*(4), Δ*moswd*(5). Among them, the distribution of input P131(2), Δ*mobre2*(3) (input), Δ*mospp1*(4) (input), Δ*moswd2*(5) (input) shows the main peak is below 100 bp, but there is DNA distribution at 100–500 bp. The true distribution of P131(2), Δ*mobre2*(3), Δ*mospp1*(4), and Δ*moswd2*(5) is between 100–500 bp as the main peak[26].

DISCUSSION:

Recently, ChIP-seq has become a widely used genomic analysis method for determining the binding sites of TFs or enrichment sites modified by specific histones. Compared to previous ChIP-seq technology, new ChIP-seq technology is highly sensitive and flexible. Results are provided in high resolution without negative effects, such as the noise signal caused by the non-specific hybridization of nucleic acids. Although this is a common gene expression analysis, many computational methods have been validated, and the complexity of ChIP-seq data in terms of noise and variability makes this problem particularly difficult for ChIP-seq to overcome. In terms of data analysis, managing and analyzing the large amount of data generated by ChIP-seq experiments is also a challenge that has yet to be adequately addressed.

There are several key steps in the ChIP-seq experiment. First of all, the preparation of the protoplasts is very important. It is necessary to control the collapse time so that high-quality protoplasts can be collected. Ultrasound is also very important, the ultrasound time should be controlled, too long or too short will not work. Secondly, the amount of antibody added should be sufficient to facilitate the enrichment of more DNA fragments that bind to the protein. When verifying the quality and quantity of DNA precipitated in the ChIP-seq experiment Qubit Fluorometer was used. Agilent 2100 was used to detect the mass

concentration and fragment distribution of DNA, which provides a basis for whether the sample can be used for subsequent library establishment and sequencing experiments.

Overall, this protocol enhances the understanding of the whole genome-wide distribution of epigenetic modifications that regulate pathogenic genes during pathogen infection. This method will contribute to identifying molecular mechanisms of epigenetic modifications and identify new target genes during fungi development and pathogen-induced pathogenesis in *M. oryzae* and other filamentous fungi.

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DISCLOSURES:

The authors have declared that no competing interests exist.

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22. Ding, S. L. et al. The Tig1 histone deacetylase complex regulates infectious growth in the rice blast fungus *Magnaporthe oryzae*. *The Plant Cell.* **22** (7), 2495–2508 (2010).
23. Dean, R. A. et al. The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature*. **434** (7036), 980–986 (2005).
24. Allis, C. D. et al. New nomenclature for chromatin-modifying enzymes. *Cell.* **131** (4), 633–636 (2007).
25. Shilatifard, A. The COMPASS family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis. *Annual Review of Biochemistry.* **81**, 65–95 (2012).
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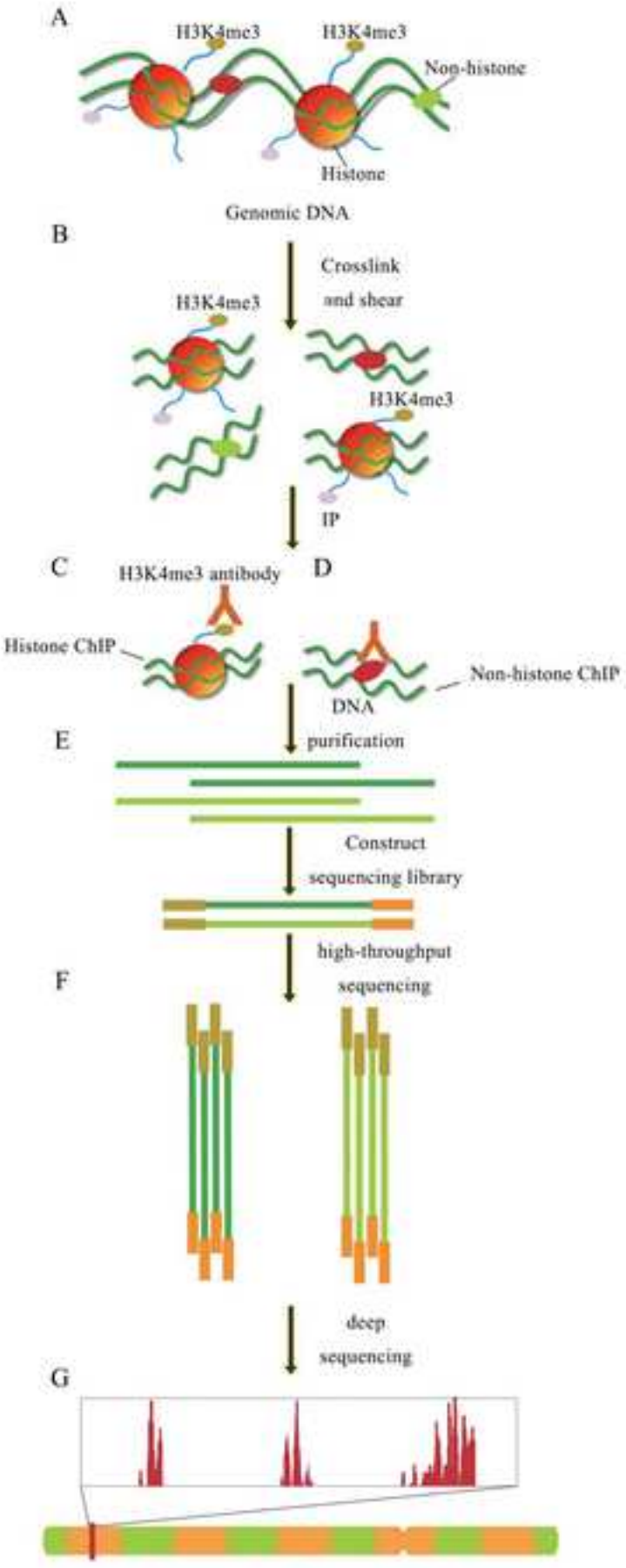
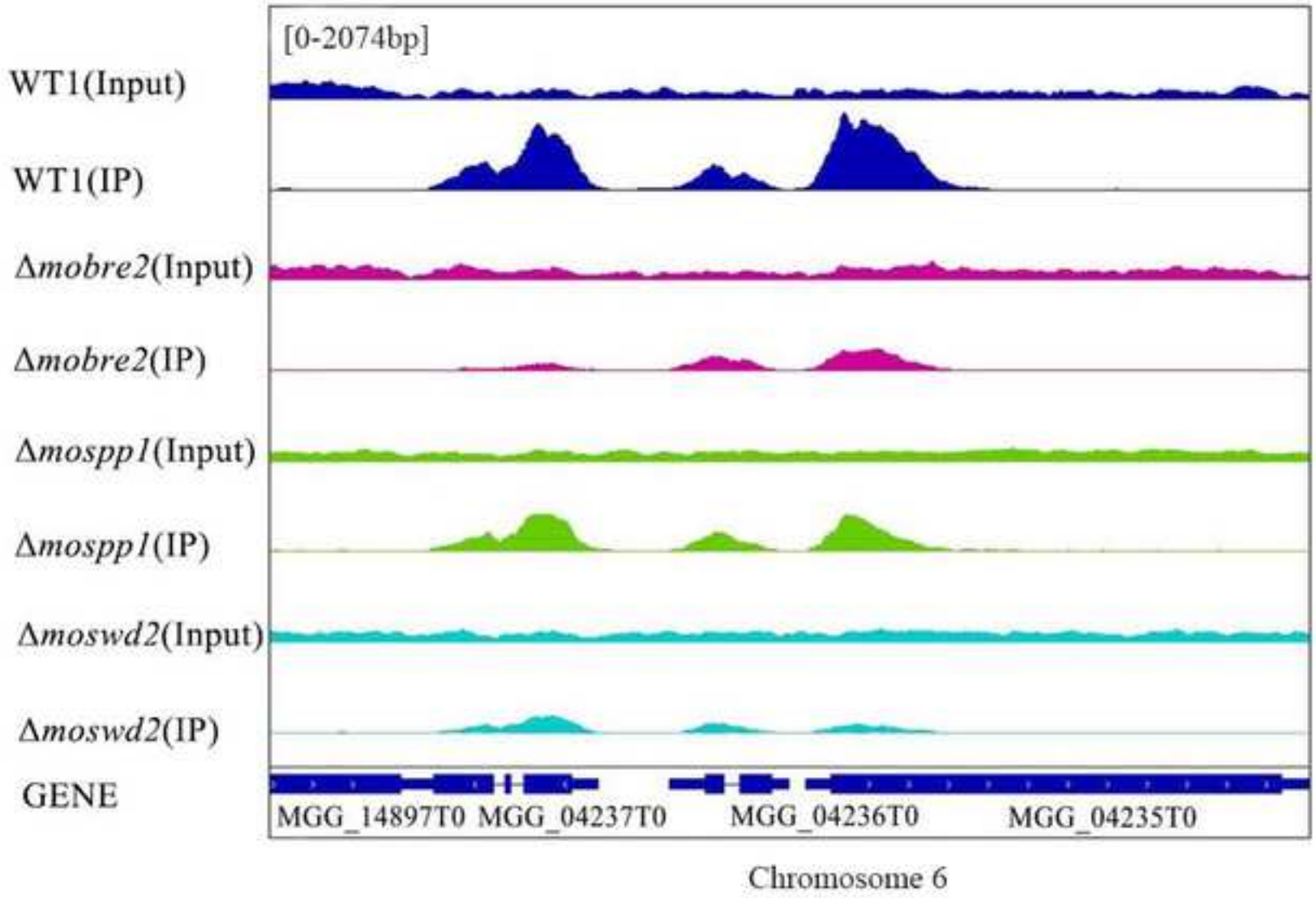
Figure

Click here to access/download;Figure;Fig 1.psd



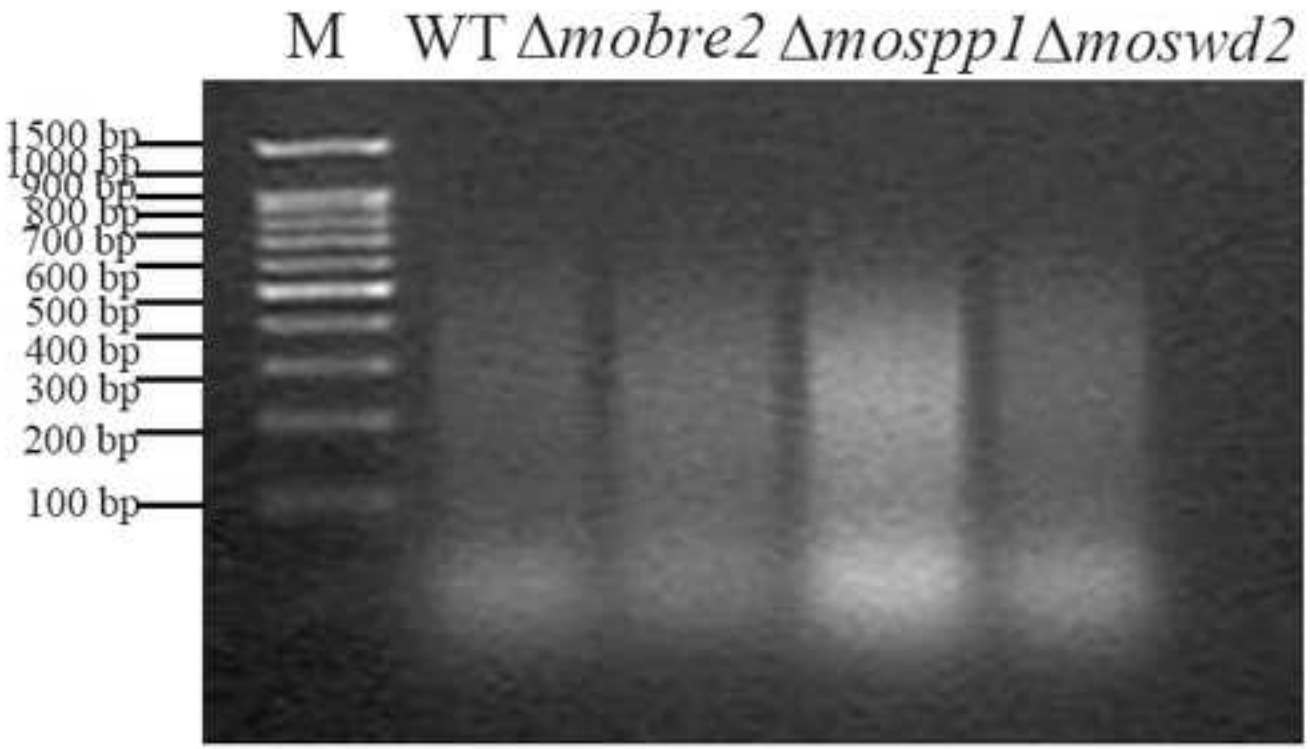
Figure

Click here to access/download;Figure;Fig 2.psd



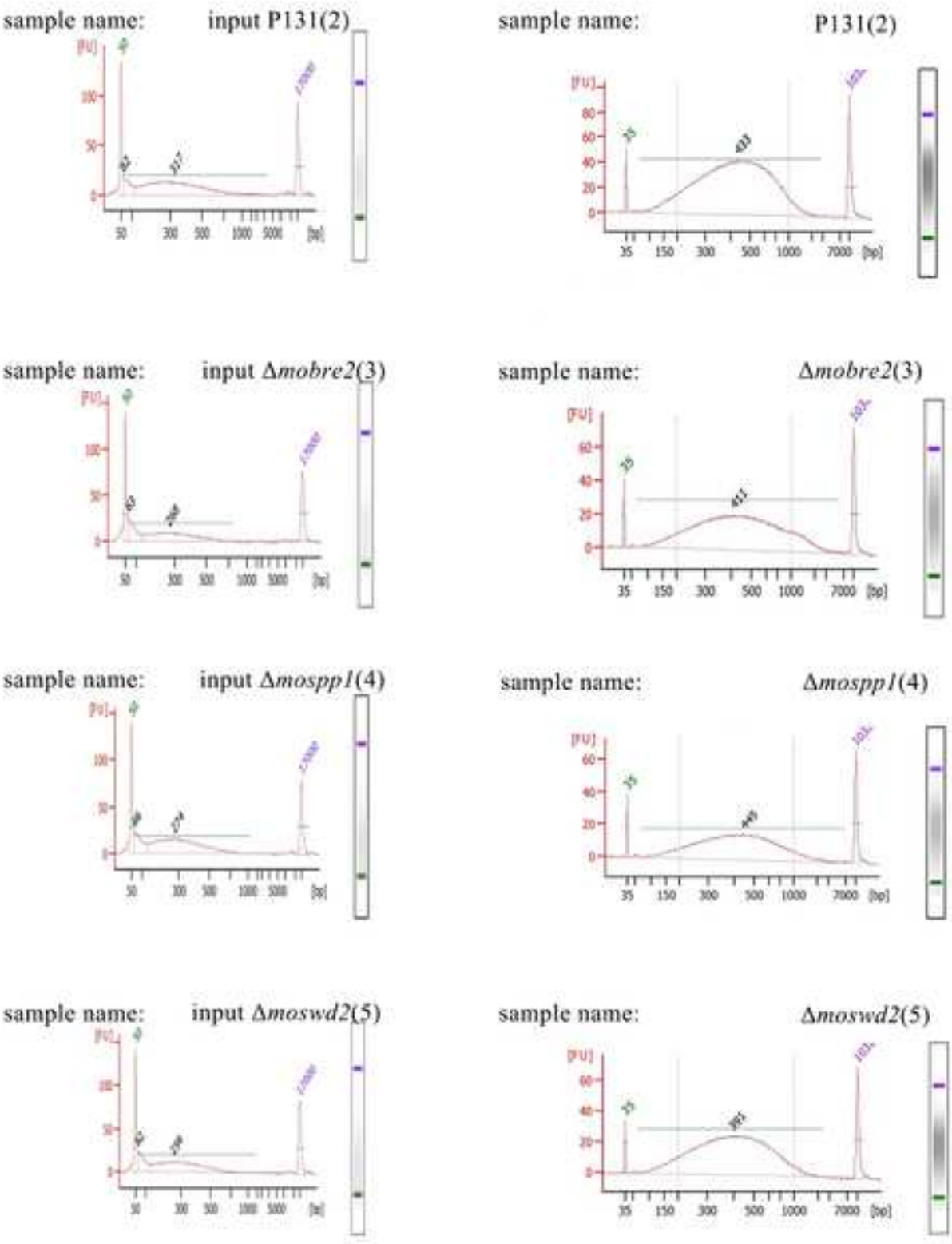
Figure

Click here to access/download;Figure;Fig 3.psd



Figure

Click here to access/download;Figure;Fig 4.psd



Serial number	Sample name	Sample serial number	Number of tubes	Total (μg)	Fragment distribution	Database type	Remarks
1	input P131(2)	WH1703004169	1	2.7948	The main peak is below 100bp, but there is DNA distribution between 100bp-500bp	ChIP-seq	Fragment is too small
2	Input Δ*mobre2* (3)	WH1703004170	1	2.4748	The main peak is below 100bp, but there is DNA distribution between 100bp-500bp	ChIP-seq	Fragment is too small
3	input Δ*mospp1* (4)	WH1703004171	1	3.22	The main peak is below 100bp, but there is DNA distribution between 100bp-500bp	ChIP-seq	Fragment is too small
4	input Δ*moswd* (5)	WH1703004172	1	3.97	The main peak is below 100bp, but there is DNA distribution between 100bp-500bp	ChIP-seq	Fragment is too small
5	P131(2)	WH1703004174	1	0.0735	The main peak is between 100bp-	ChIP-seq	
					The main peak is		

6	Δ*mobre2* (3)	WH1703004175	1	0.0491	between 100bp-	ChIP-seq	
7	Δ*mospp1* (4)	WH1703004176	1	0.0288	The main peak is between 100bp-	ChIP-seq	
8	Δ*moswd* (5)	WH1703004177	1	0.0527	The main peak is between 100bp-	ChIP-seq	

Name of Material/Equipment	Company	Catalog Number	Comments/Description
acidic casein hydrolysate	WAKO	65072-00-6	Medium configuration
agar powder	scientan	9002-18-0	Medium configuration
deoxycholic acid	MedChemExpress	83-44-3	protein and dissolution
DNA End-Repair kit	NovoBiotec	ER81050	Repair DNA or cDNA damaged by enzymatic or mechanical shearing
Dynabeads	Invitrogen	no.100.02D	Binding target
EB buffer	JIMEI	JC2174	Membrane and liquid
EDTA	ThermoFisher	AM9912	protease inhibitor
enzymatic casein hydrolysate	Sigma	91079-40-2	Medium configuration
glucose	Sigma	50-99-7	Medium configuration
glycogen	ThermoFisher	AM9510	Precipitant action
H3K4me3 antibodies	Abcam	ab8580	Immune response to H3K4me3 protein
illumina Genome Analyzer	illumina	illumina Hiseq 2000	Large configuration
Illumina PCR primers	illumina	CleanPlex	Random universal primer
isoamyl alcohol	chemical book	30899-19-5	Purified DNA
LiCl	ThermoFisher	AM9480	specific removal RNA
lysing enzymes	Sigma	L1412-10G	cell lysis buffer
Mouse IgG	Yeasen	36111ES10	Animal normal immunoglobulin
NaCl solution	ThermoFisher	7647-14-5	Medium configuration
$NaHCO_3$	Seebio	SH30173.08*	preparation of protein complex eluent
NP-40	ThermoFisher	85124	cell lysate to promote cell lysis
PCR Purification kit	Qiagen	28004	The purification procedure removes primers from DNA samples
protease inhibitors	ThermoFisher	A32965	A protein inhibitor that decreases protein activity
Proteinase K	ThermoFisher	AM2546	DNA Extraction Reagent
Qubit 4.0	ThermoFisher	Q33226	Medium configuration
RIPA buffer	ThermoFisher	9806S	cell lysis buffer
RNase A	ThermoFisher	AM2271	Purified DNA
SDS	ThermoFisher	AM9820	cover up the charge differences
sodium acetate solution	ThermoFisher	R1181	Acetic acid buffer
sodium deoxycholate	ThermoFisher	89904	inhibition of protease degradation

T4 DNA ligase	ThermoFisher	EL0011	Under the condition of ATP as coenzyme, DNA ligase
T4 DNA ligase buffer	ThermoFisher	B69	DNA ligase buffer
Tris-HCl	ThermoFisher	1185-53-1	buffer action
Triton X-100	ThermoFisher	HFH10	keep the membrane protein stable
yeast extract	OXOID	LP0021	Medium configuration

Point-by-point response to reviewers' comments: all the changes are highlighted in the new revised version by blue text.

1. Please employ professional copyediting services as there are many grammatical errors in the manuscript that significantly affects the comprehension of the

Response: Thanks for your suggestion, we have revised as suggested.

2. Please revise the title to "Genome-wide Analysis of Histone Modifications Distribution using Chromatin Immunoprecipitation Sequencing in Magnaporthe oryzae"

Response: Thanks for your suggestion, we have revised as suggested.

3. Please revise the following lines to avoid previously published works: 227-229, 366-368.

Response: Thanks for your suggestion, we have revised as suggested.

4. Line 28: Please add a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 47-50）:

Here, we present a protocol to analysis the genome-wide distribution of histone modifications, which is also suitable for identifying its new target genes in the process of pathogenesis in *M. oryzae* and other filamentous fungi.

5. Line 119-122: Please provide more details to the steps. Prepare how? Boil 30 g of oatmeal? Filter with what? How many grams of tomatoes? Is all the tomato juice mixed with the oat filtrate? How is the sterilization performed?

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 122-127）:

Weigh 30-50 g of oatmeal and boil it in 800 mL of water(ddH2O) for 20 min, filter through two layers of gauze and take the filtrate, pick ripe tomatoes, peel them

and squeeze the juice, filter through two layers of gauze to collect 150 mL of the filtered juice, mix the tomato juice and the prepared oat filtrate thoroughly, and add ddH2O up to 1000 mL. Finally, dispens into 500 mL conical flasks, add 250 mL and 2.5 g agar powder to each bottle, and autoclave for 20 min. Store at 25 °C.

6. Line 124-127: Please mention how the OTA plates are prepared. How much OTA per plate? What type of plate is used? At what temperature is the plate maintained? How is the wild-type strain P131 inoculated? What are the growth conditions for the P131? Grow for how long? 4 days?

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 129-137）:

After heating and melting 250 mL of OTA, pour it into 10 the glass petri dishe with a diameter of 5 cm×5 cm, each of which contains about 25 mL of OTA. After it has solidified, store it upside down at 25 °C.

Use a sterilized toothpick to dig out a small piece of mycelium from the old M. oryzae （wild-type strain P131，knockout strain *Δmobre2, Δmospp1, and Δmoswd2*）and place them on the new OTA plates, and culture them for 4-6 days at 28°C under light conditions.

NOTE: We need to turn the petri dish upside down to prevent pollution.

7. Line 143: Is the mycelium dried before weighing it?

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 158）:

NOTE: Mycelium does not need to be dried before weighing.

8. Line 151/168/172: What happens after centrifugation? Aspiration?

Response: Thanks for your suggestion,We have added relevant information in the steps.

(Lines: 170）:

After centrifugation, discard the supernatant carefully.

(Lines: 186）:

After centrifugation, discard the supernatant carefully.

(Lines: 191）:

After centrifugation, discard the supernatant carefully.

9. Line 170: Resuspend the pellet in what? What volume of 0.7 M NaCl is used?

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 186-187）:

After centrifugation, discard the supernatant carefully. Resuspend the pellet in 1 mL of 0.7 M NaCI solution.

10. Line 180: How long is the sonication performed?

Response: Thanks for your suggestion,We have added relevant information in the steps.

(Lines: 199-201）:

for about 10 min. (the purpose of this step is to sonicate the cross-linked lysate for a period of time to determine the best conditions.)

11. Line 206: Please specify what is meant by "adjust the chromatin sample to the same concentration. Same concentration as what?

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 231-232）:

Before performing the IP experiment, dilute each chromatin sample at a ratio of 1:10 with 1x RIPA buffer (e.g., add 10 uL of chromatin sample to 1 uL of 1×RIPA buffer).

12. Line 217-218: what does "from 107 protoplasts" mean.

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 243）:

($2x10^7$ cells were used)

13. Line 225/239/261: Please mention the rpm set for the rotary shaker.

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 251-252）:

After mixing well, place the samples on a rotary shaker and incubate overnight at

4 °C, 30 x g.

(Lines: 266）:

at 30 x g.

(Lines: 288）:

at 30 x g.

14. Line 256/263: Wash the tube again with what? LiCl?

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 283-284）:

Rinse the test tube again with 1 mL 0.25 M LiCI buffer, remove the supernatant with a pipette, and then discard the supernatant.

(Lines: 290-291）:

Wash the tube again with 1 mL TE buffer and remove the supernatant with a pipette.

15. Line 276: Please specify for how long the centrifugation is performed.

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 304）:

at 4 °C for 1 min.

16. Line 332-333/335-336/349-351: Please include the reaction conditions.

Response: Thanks for your suggestion,We have added relevant information in the steps.

(Lines: 362-363）:

Add the reagents to a 0.2 mL PCR centrifuge tube, mix well, and react in a PCR machine at 4 °C for 10 min.

(Lines: 366-367）:

Add the reagents to a 0.2 mL PCR centrifuge tube and mix well, connect them at 16 °C for 4 h.

(Lines: 383-388）:

Add the reagents to a 0.2 mL PCR centrifuge tube and mix well, put them in a PCR instrument for PCR reaction.

Reaction program in PCR machine: The first step, reactant in 95 °C Predenaturation for 2 min. The second step, reactant in 95 °C denaturation for 10 s, refolding at 60 °C for 15 s, extend at 72 °C for 5 s (this step needs to be repeated 35 cycles). The third step, reactant in 72 °C extend for 5 min.In the end,save them at 4 °C.

17. Line 343-345: Please mention the wavelength used. What is the centrifugation speed? How long is the centrifugation performed?

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 374-376）:

Detect the DNA concentration using a Fluorometer. After melting the sample on ice, mix it thoroughly and centrifuge at 4 °C for 30 s at 1000 x g. Then take an appropriate amount of sample and measure it in a fluorometer with a wavelength of 260 nm.

18. Line 347: How is the sequencing performed?

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 378-379）:

place DNA samples with qualified quality and concentration on the Illumina sequencing platform for sequencing.

19. Based on the flow of protocol, Figure 4 needs to be discussed before Figure 3. Please revise the figure numbers accordingly. Ensure all the results are discussed in the representative results.

Response: Thanks for your suggestion, we have revised as suggested.

20. Please include the details of the Illumina PCR primers, Qbit fluorometer, and Illumina Genome Analyzer in the Table of Materials.

Response: Thanks for your suggestion,We have added relevant information in the Table of Materials.

21. In the current version, the highlighted/ underlined content is 4 pages. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized

to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: Thanks for your suggestion, we have revised as suggested.

Point-by-point response to reviewers' comments: all the changes are highlighted in the new revised version by blue text.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thanks for your suggestion, we have revised as suggested.

2. Please revise the following lines to avoid previously published work: 214-217, 270-272, 338-340, 350-352, 364-366.

Response: Thanks for your suggestion, we have revised as suggested.

3. Please define all abbreviations before use (IP, etc.)

Response: Thanks for your suggestion, we have revised as suggested.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: scientan, Sigma, Scientzjy, Dynabeads, Invitrogen, Abcam, ab8580, Yeasen, NovoBiotec, Qiagen, MinElute, Illumina, Phusion, etc.

Response: Thanks for your suggestion, we have revised as suggested.

We have summarized them in a new table(Table_of_Materials).

5. Line 159/171: Please provide the details of sonication. How long is it performed? What frequency, pulse, cycle is used for sonication?

Response: Thanks for your suggestion, We have added relevant details in the article.

(Lines: 145-146）:

Shear the chromatin by sonication with a JY 92-IIDN ultrasonic homogenizer (Scientzjy, Shanghai, China) for 8 min (25% W, output 3 s, stop 5 s, 4°C).

6. Line 171: Please mention how the fragment length (200-500 bp) is confirmed after

sonication.

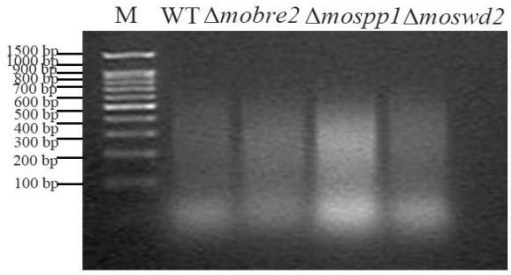
Response: Thanks for your suggestion. We have added relevant information to the article.

(Lines: 147-149）:

After sonication, 1% agarose gel electrophoresis was performed to analyze the length of the DNA fragments. The results showed that the length of the DNA fragment was 200–500 bp (Fig. 4).

(Lines: 359-364）:

Figure 4. This figure shows the electrophoresis detection of DNA after ultrasound. After ultrasound, the DNA is subjected to a 1% agarose gel experiment to analyze the length of DNA fragments. As shown in Figure 4, The sonicated DNA fragment length is from 200 bp to 500 bp, and these DNA fragments can be used for the following steps of ChIP-seq. WT: the wild-type strain.



7. Line 191: What type of magnetic force is used. How long is the tube placed on the magnetic force?

Response: Thanks for your suggestion, we have revised as suggested.

8. Line 195: Add more details to complete the protocol step. How are the Dynabeads obtained? “not rinsing” what?

Response: Thanks for your suggestion, we have revised as suggested.

9. Line 197: How is the same concentration and volume adjusted?

Response: Thanks for your suggestion, we have revised as suggested.

10. Line 227/232/237/242: Is the solution in the tube discarded after each rinsing step?

Response: Thanks for your suggestion, we have revised as suggested.

11. Line 298: Please specify the volume of 75% ethanol used during the washing step.

Response: Thanks for your suggestion, we have revised as suggested.

12. Line 322-336: Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: Thanks for your suggestion, we have revised as suggested.

13. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: Thanks for your suggestion, we have revised as suggested.

In the section of experimental steps, we underlined the important steps of visualization.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: Thanks for your suggestion, we have revised as suggested.

(Lines: 54-60）:

There are several key steps in the ChIP-seq experiment. First of all, the preparation of protoplasts is very important. It is necessary to control the collapse time so that high-quality protoplasts can be collected. Ultrasound is also very important, we need to control the ultrasound time, too long or too short will not work. Secondly, the amount of antibody added should be sufficient to facilitate the enrichment of more DNA fragments that bind to the protein. When verifying the quality and quantity of DNA precipitated in the ChIP-seq experiment, we use Qubit Fluorometer and Agilent 2100 to detect the mass concentration and fragment distribution of DNA, which

provides a basis for whether the sample can be used for subsequent library establishment and sequencing experiments.

Taken together, this protocol enhances our understanding of the whole genome-wide distribution of epigenetic modifications that regulate pathogenic genes during pathogen infection. This method will not only contribute to identify molecular mechanisms of epigenetic modifications, but can be used to identify new target genes during fungi development and pathogen-induced pathogenesis in *M. oryzae* and other filamentous fungi.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: Thanks for your suggestion, The pictures we used in the article have not been published, and the relevant citations have indicated the source.

16. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). In-text references must be numbered in the order of citation, and when repeated, they must be given in the numbered order used in the list of references.

Response: Thanks for your suggestion, we have revised as suggested.

17. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Response: Thanks for your suggestion, we have revised as suggested.

18. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).

Response: Thanks for your suggestions, we have revised as suggested.

19. Please sort the Table of Materials in alphabetical order.

Response: Thanks for your suggestion, we have revised as suggested.

Reviewers' comments:
Referee: 1

Minor Concerns:

Line 68: I am unsure of this sentence and the ones that follow. Needs to be reworded. The last two paragraphs of introduction can be written into one with better wording.

Response: Let me first thank you for carefully reading our manuscript. We sincerely thank you for your comments. Thanks for suggestion, we have revised as suggested.

Line 81-83 could be your introductory sentence
Response: Thanks for your suggestion, we have revised as suggested.

Line 78-80 I am not sure what this part of the sentence is trying to achieve

Response: Thanks for your suggestion, we have revised as suggested.

Line 89 please delete etc.

Response: Thanks for your suggestion, we have revised as suggested.

Line 96-97 I am unable to follow.

Response: Thanks for your suggestion, we have revised as suggested.

Steps 1.2 I had previously mentioned that the growth conditions are missing. They are still missing.

Response: Thanks for your suggestion, we have revised as suggested.

Step 3.1: what volume of dynabeads? (Invitrogen no.100.02D).

Response: Thanks for your suggestion, we have revised as suggested.

I am unable to follow steps 3.1-3.6.

Response: Thanks for your suggestion, we have revised as suggested.

Does step 4.1 need to be repeated in between 4.3 and 4.4, 4.5 and 4.6, 4.7 and 4.8, 4.9 and 4.10?

Response: Thanks for your suggestion, we have revised as suggested.

7.8 please add centrifugation information.

Response: Thanks for your suggestion, we have revised as suggested.

7.9: is this step necessary?

Response: Thanks for your suggestion, we have revised as suggested.

7.12 what does this step mean?

Response: Thanks for your suggestion, we have revised as suggested.

7.13 not required

Response: Thanks for your suggestion, we have revised as suggested.

Line 346: reference

Response: Thanks for your suggestion, we have revised as suggested.

Line 348: correct sonicated

Response: Thanks for your suggestion, we have revised as suggested.

Table 1: Keep a heading and delete the rest of the information as it is in the table. Also what is the reference to class A or C in the table?

Response: Thanks for your suggestion, we have revised as suggested.

Figure 3: Heading: Bioanalyzer trace of Input and ChIP samples.
The figure shows the fragment distribution of each sample, where the 394 abscissa represents the fragment size, and the ordinate represents the peak size.
Response: Thanks for your suggestion, we have revised as suggested.

Referee: 2

Comments to the Author

The article by Wang et al. on "Genome-wide analysis of histone modifications using the chromatin immunoprecipitation sequencing method in Magnaporthe oryzae" describes an operational method of ChIP-seq for genome-wide mapping of histone methylation, especially H3K4me3, in the filamentous fungus M. oryzae. The authors have described the methods in detail. The authors have addressed most of the points raised. However, at many places, the authors have not given a direct response to the reviewer's questions. These should be addressed and require further clarifications.

Response:

Let me first thank you for carefully reading our manuscript. We sincerely thank you for your comments.We have tried to address all the concerns.

Major Concerns:

1. In the introduction section, the significance of studying epigenetic histone modifications in *Magnaporthe oryzae* is missing. The importance of these modifications has been studied in few of the filamentous fungus like Fusarium oxysporum and Zymoseptoria tritici to name a few and should be discussed to put forward the relevance of studying such modifications in the pathogenic fungus.

Response:

Thanks for your suggestions. We have added the information in the section of introduction.

(Lines: 54-60）:

In phytopathogenic fungi, research has recently begun to study how histone methylation modifications regulate their target genes in the process of pathogenicity. Some previous studies proved that the regulation of histone methylase-related genes is mainly reflected in gene silencing and catalyzing the production of Secondary Metabolites (SM).

(Lines: 64-69）:

In *Fusarium graminearum*, KMT6 can catalyze the methylation modification of H3K27me3, regulate the normal development of fungi, and help regulate the "cryptic genome" containing the SM gene cluster[16-19]. In 2013, Connolly reported that H3K9 and H3K27 methylation regulates the pathogenic process of fungi through secondary metabolites and effector factors that regulate the inhibition of target genes[20].

(Lines: 77-91）:

Due to its representative infection process, *M. oryzae* is similar to the infection process of many important pathogenic fungi, and is easy to carry out molecular genetic operations, the fungus has become a model organism for studying fungal-plant interactions[23]. Blocking every step of the infection process of *M. oryzae* may result in unsuccessful infection. The morphological changes during the infection process are strictly regulated by the entire genome function and gene transcription. Among them, epigenetic modifications such as histone methylation play an important role in the transcriptional regulation of functional genes[24,25]. However, so far, there have been few study on the molecular mechanism of epigenetic modifications such as histone methylation, histone acetylation in the transcription of pathogenesis genes in *M. oryzae*. Therefore, further developing the epigenetic regulation mechanism of the rice blast fungus, searching for and discovering the upstream and downstream regulatory network of these pathogenic related genes, will provide new enlightenment for the development of rice blast prevention and control strategies.

The authors have tried to describe some aspects of these in M. oryzae itself. However, the lines correspond to line 68-83 in the revised manuscript. Still, it is difficult to understand the significance of these modifications in filamentous fungi. Why the authors don't read and see the significance of these studies in other filamentous fungi and cite these references accordingly.

Response:

Thanks for your suggestions. We have added the information in the section of introduction.

(Lines: 69-75):

In *Aspergillus*, the modification of histones H3K4me2 and H3K4me3 is related to gene activation and plays an important role in controlling the chromatin level

regulation of SM gene clusters[21]. In *M. oryzae*, Tig1 (homologous to Tig1 in yeast and mammals) is a HADC (histone deacetylase)[22]. Knockout of *Tig1* gene leads to the complete loss of pathogenicity and spore production ability in the null mutant, and it is more sensitive to peroxygen environment, which can not produce infective hyphae[22].

2. One of the main steps which the authors have neglected in this method is checking the efficacy of the ChIP by keeping both semi quantitative and qPCR with +Ab, -Ab, and Input DNA (control). This should be included and discussed.

Response:

Thanks for your suggestions. We have added relevant information to the article.
What we are doing this time is ChIP-seq. The samples we obtained through the Chip-seq experiment are tested by Qubit Fluorometer and Agilent 2100 to test the DNA mass concentration and fragment distribution, and the qualified samples are used for DNA library establishment.
We have not perform the ChIP-qPCR and semi quantative PCR in ChIP-seq. All the precipitaed DNAs are used to sonstruct the DNA library for sequencing.

One cannot proceed for library preparation without knowing whether the ChIP has worked in the first place. It is one of the most important steps in the ChIP-seq before proceeding for library preparation.

Response:

Thanks for your suggestions. We have added relevant information to the article.
(Lines: 291-295):
When verifying the quality and quantity of DNA precipitated in the ChIP-seq experiment, we use Qubit Fluorometer and Agilent 2100 to detect the mass concentration and fragment distribution of DNA, which provides a basis for whether the sample can be used for subsequent library establishment and sequencing experiments.

On lines 314-318, I don't see that the information has been added.

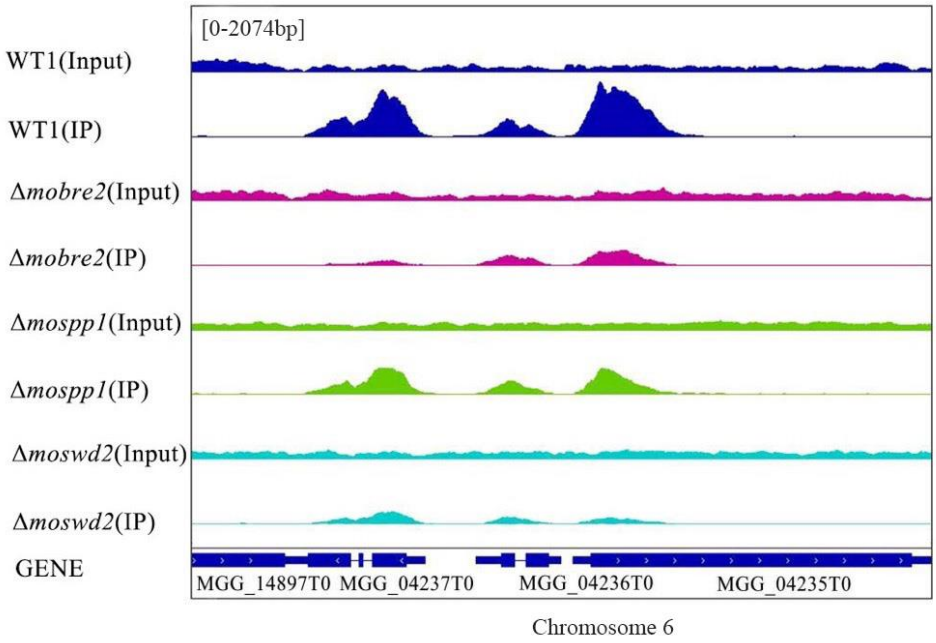
Response:

Thanks for your suggestions.We have added relevant information in this article.

(**Lines: 337-340):**

As shown in Figure 2, the signals of hisone H3K4me3 distribution on some selected target gene in Chromosome 6 are shown here in the wild-type and three null mutan strains. All the results of the whole genome are already submitted the complete sequencing results to Genbank（https//www.ncbi.nlm.nih.gov/bioproject/accession

649321）[26].



The authors in bold mention that selected genes show the expression in WT decreased compared with deletion mutants. The authors should be careful in making such statements and that too when it's highlighted in bold. However, the authors correct themselves in the subsequent line. This is so inconsistent and carelessly written response.

Response:

Thanks for your suggestions. We have made changes in the corresponding part of the article.

Figure 4, which was put in the previous manuscript, is the qPCR for detecting gene expression of some selected target genes of H34me3 modification in RNA-seq experiments. This is inappropriate to put it in this ChIP-seq method article, and the relevant ones have been deleted.

3. The work flow for ChIP protocol in M. oryzae has been shown as Figure 1. This figure lacks labelling from A-G, which has been poorly described in the figure legends.

Response:

Thanks for your suggestions. We have made changes in the corresponding part of

the article.

(**Lines: 321-329):**

We have labelled the figure from A-G (Fig 1). The flow chart of the ChIP-seq method in *M. oryzae*. (A) The genomic DNA of *M. oryzae* was crosslinked with 1% formaldehyde. (B) Lysed blast fungus cells, broken DNA, free DNA, and histone binding DNA were subsequently obtained. (C) DNA fragments bound to histones and were extracted by specific binding to the H3K4me3 antibody. (D) Through reverse crosslinking, purified DNA subsequently obtained DNA fragments modified by H3K4me3 histones. (E-F) DNA fragments were sequenced, the sequencing results were compared, and sequences were identified in the *M. oryzae* DNA group. (G) Specific genes and loci of H3K4me3 histones in *M. oryzae* were retrieved.

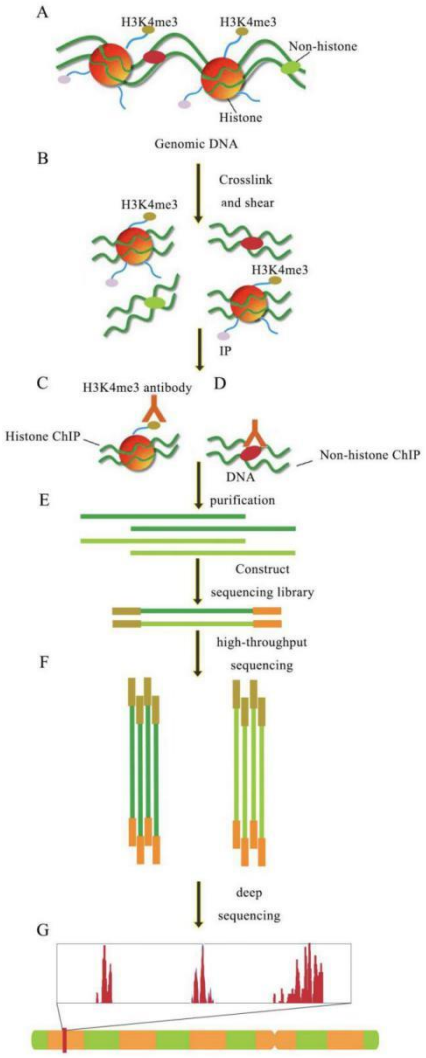


Figure 2, as mentioned in line 224, says "the whole genome-wide distribution of H3K4me3 are mapped". However, the figure does not represent genome-wide view of

any chromosome, rather it represents just a chunk of specific chromosome.

Response:

As shown in Figure 2, the signals of hisone H3K4me3 distribution on some selected target gene in Chromosome 6 are shown here in the wild-type and three null mutan strains. All the results of the whole genome are already submitted the complete sequencing results to Genbank（https//www.ncbi.nlm.nih.gov/bioproject/accession 649321）[26].

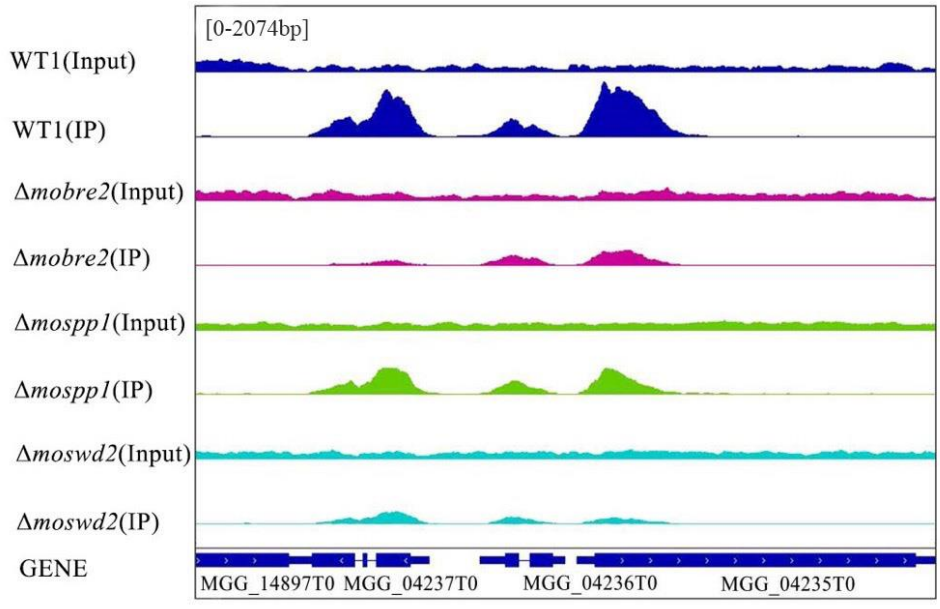
What does the number in WT (input) labelled as [0-2074] signify? The coordinates representing a chromosome should have been mentioned.

Response:

Thanks for your suggestions. We have made changes in the corresponding part of the article.

(Lines: 336-338):

The number in WT (input) labelled as [0-2074] signify means the results of ChIP in the range of genomic DNA [0-2074]. [0-2074] refers to 0-2074bp of Chromosome 6.



Chromosome 6

Emphasis should also have been given as to how to calculate enrichment in IP samples over input samples.

Response: Thanks for your suggestion, we have revised as suggested.

This time we use Qubit Fluorometer and Agilent 2100 to verify the results of our chip experiment, and calculate the enrichment degree of our IP samples through the size of the DNA mass concentration and the peak value of the sample in the fragment distribution.

The authors should clearly mention if the Figure 2 has been taken from Da et al., 2020. The authors have not included this in the reference. The authors have included labelling in the figure 1. However, why have the authors not considered the suggestions as suggested for figure 2 and included the corresponding changes for figure 2?

Response: Thank you very much for your suggestions, we have made corresponding changes, and the relevant references have been added in the new prepared manuscript.

Point-by-point response to reviewers' comments: all the changes are highlighted in the new revised version by blue text.

Reviewers' comments:
Referee: 1

Comments to the Author

The authors have described a protocol for studying histone modification in Magnoporthe Oryzae. The protocol is important and it will likely help other scientists preparing ChIP-seq for similar fungi.
However, the manuscript requires major revision.

Response: Let me first thank you for carefully reading our manuscript. We sincerely thank you for your comments.We have tried to address all the concerns.

1) Line 21-23 Magnaporthe oryzae is the best-studied phytopathogenic fungus and one of the top 10 plant fungal pathogens causing disease.

Response: Thanks for suggestion, we have revised as suggested.

2) Line 50-51: Rice blast caused by M. oryzae, is one of the most serious rice diseases in most rice-growing areas worldwide.

Response: Thanks for suggestion, we have revised as suggested.

3) Line 59: no preparation steps given to oatmeal media prep.

Response: Thanks for suggestion, we have revised as suggested.

4)Line 59-60: growth stage steps missing between step 1 and 2.

Response: Thanks for suggestion, we have revised as suggested.

5)Line 77: lysed mycelium? Consistency between the use of mycelium and hyphae.

Response: Thanks for suggestion, we have revised as suggested.

6)Line 80: temperature?

Response: Thanks for suggestion, we have revised as suggested.

7)Line 97: Steps 12-14 could be worded better. The sonication temperature?

Response: Thanks for suggestion, we have revised as suggested.

8)Line 105: are we re-sonicating? Or this step should be removed?

Response: Thanks for suggestion, we have revised as suggested.

9)Line 113: what volume of dynabeads?

Response: Thanks for suggestion, we have revised as suggested.

10)Line 116: Rinse with what?

Response: Thanks for suggestion, we have revised as suggested.

11)Line 113-121: very confusing.

Response: Thanks for suggestion, we have revised as suggested.

12)Line 129: This line needs further clarification with the brand name for mouse IgG.

Response: Thanks for suggestion, we have revised as suggested.

13)Line 164: centrifuge speed

Response: Thanks for suggestion, we have revised as suggested.

14)Lines 188-192: out of sequence and out of place.

Response: Thanks for suggestion, we have revised as suggested.

15)Line 200: cycle information missing.

Response: Thanks for suggestion, we have revised as suggested.

16)Line 4: oligo details and incubation time is missing.

Response: Thanks for suggestion, we have revised as suggested.

17)Line 206: concentration of primer mix is missing.

Response: Thanks for suggestion, we have revised as suggested.

Referee: 2

Comments to the Author

The article by Wang et al. on "Genome-wide analysis of histone modifications using the chromatin immunoprecipitation sequencing method in Magnaporthe oryzae" describes a detailed and operational method of ChIP-seq for genome-wide mapping of histone methylation, especially H3K4me3, in the filamentous fungus M. oryzae. The authors have tried to describe the methods in detail. However, at many places, the authors have skipped certain key steps, failed to even point out/highlight these steps along with the precautions. These should be addressed and require further improvement and clarification.

Response:

Let me first thank you for carefully reading our manuscript. We sincerely thank you for your comments.We have tried to address all the concerns.

Major Concerns:

1. In the introduction section, the significance of studying epigenetic histone modifications in Magnaporthe oryzae is missing. The importance of these modifications has been studied in few of the filamentous fungus like Fusarium oxysporum and Zymoseptoria tritici to name a few and should be discussed to put

forward the relevance of studying such modifications in the pathogenic fungus.

Response:

This is a good idea. We have added the information in the section of introduction (**Lines: 51-66):** The epigenetic part of Magnaporthe grisea is still less studied. Set1 is the H3K4 methylase. Knockout of this gene results in the complete deletion of H3K4me2. Compared with the wild type, the expression of the gene MoCEL7C in the mutant is inhibited in the CMC-induced state, and in the non-induced state (glucose or cellobiose), the expression of MoCEL7C increased (Vu et al., 2013). Tig1 (homologous to Tig1 in yeast and mammals) is a HADC (histone deacetylase). Knockout of this gene leads to the complete loss of pathogenicity and spore production ability of the mutant, and it is more sensitive to peroxygen environment, Can not produce infective hyphae in plant cells (Ding et al., 2010). Through DNA methylation analysis at the genomic level, it was found that DNA methylation was distributed throughout the rice blast fungus genome, and further studies were made on the involvement of single-line DNA methylation in the development of the rice blast fungus and the protection of the genome (Jeon et al., 2015). Epigenetics in yeast or other filamentous fungi plays an important role in the assembly and maintenance of heterochromatin and euchromatin, gene transcription activation and silence suppression.

2. One of the major concerns I have is the rpm used (150 rpm) at line 75-76 for generating the protoplast from lysing the hyphae using lysing enzyme. From my experience of working with both yeast and fungal systems, lysing is always done at a lower rpm, to avoid cell lysis of protoplasts, as these are devoid of cell wall and hence are very delicate. Note or Precautions are not mentioned anywhere in the whole article.

Response:

Let me first thank you for carefully reading our manuscript. We sincerely thank you for your suggestion. The rpm used (150 rpm) is what we have done in the past experiment.

3. Line 80-82: The protoplasts were resuspended in 20 mL RIPA buffer. However, in line 84-86, only 2 mL of protoplasts were used for subsequent steps. Any specific reasons for using lower volume should be highlighted. The importance of maintaining a specific number of protoplasts for this kind of analysis should be highlighted as well and is missing in the article.

Response:

This is a good idea. We have made 2 mL per tube and a total of 10 tubes. So in the first the protoplasts were resuspended in 20 mL RIPA buffer.

4. One of the main steps which the authors have neglected in this method is checking the efficacy of the ChIP by keeping both semi quantitative and qPCR with +Ab, -Ab, and Input DNA (control). This should be included and discussed. One cannot proceed for library preparation without knowing whether the ChIP has worked in the first place. It is one of the most important steps in the ChIP-seq before proceeding for library preparation.

Response:

This is a good idea. We have added the information in the section of introduction (**Lines: 314-318): Figure 4. qRT-PCR experiment for selected genes show the expression in WT decreased compared with deletion mutants.** Identified genes show decreased signals and the expression level was reduced in deletion mutants(Zhou et al., 2021). The figure is the use of qPCR technology to verify the ChIP-seq results. The results showed that the expression level of the genes in the three knockouts was significantly lower than that of the wild type, which was consistent with the results of ChIP-seq.

5. There are certain buffers which needs to be prepared fresh for preparing samples for ChIP. It has not been mentioned anywhere in the text. It is very important to highlight this information. Also, the amount of IP DNA necessary for making the library should be mentioned. The reason being, at many a times getting the appropriate amount of IP-ed DNA is the limiting factor, especially working with filamentous fungi and it should be highlighted and discussed. There is no mention of running the prepared library samples on bioanalyzer to know the true distribution of the sizes of the sheared samples.

Response:

Thanks for suggestion, we have revised as suggested. **Table 1.** The total amount of DNA in this experiment incloudes: the total amount of input P131(2) is 2.7948 μg, the total amount of Δ*mobre2*(3)(input) is 2.4748μg, the total amount of Δ*mospp1*(4)(input) is 3.22μg, the total amount of Δ*moswd2*(5)(input) is 3.97μg, and the total amount of P131(2) is 0.0735μg, the total amount of Δ*mobre2*(3) is 0.0491μg, the total amount of Δ*mospp1*(4) is 0.0288μg, the total amount of Δ*moswd*(5) is 0.0527μg. **Table 1.** The samples running on the bioanalyzer are input P131(2),

Δ*mobre2*(3)(input), Δ*mospp1*(4)(input), Δ*moswd2*(4)(input), P131(2), Δ*mobre2*(3), Δ*mospp1*(4),Δ*moswd*(5)(input), Among them, the distribution of input P131(2), Δ*mobre2*(3)(*input*), **Δ***mospp1*(4)(input), Δ*moswd*(5)(input) is that the main peak is below 100bp, but there is DNA distribution at 100bp-500bp. P131(2), Δ*mobre2*(3), Δ*mospp1*(4), Δ*moswd*(5), their true distribution is between 100bp-500bp as the main peak.

6. The work flow for ChIP protocol in M. oryzae has been shown as Figure 1. This figure lacks labelling from A-G, which has been poorly described in the figure legends. Figure 2, as mentioned in line 224, says "the whole genome-wide distribution of H3K4me3 are mapped". However, the figure does not represent genome-wide view of any chromosome, rather it represents just a chunk of specific chromosome. What does the number in WT (input) labelled as [0-2074] signify? The coordinates representing a chromosome should have been mentioned. Emphasis should also have been given as to how to calculate enrichment in IP samples over input samples. The authors should clearly mention if the Figure 2 has been taken from Da et al., 2020. The authors have not included this in the reference.

Response:

Thanks for suggestion, we have revised as suggested. The number in WT (input) labelled as [0-2074] signify means the results of ChIP in the range of genomic DNA [0-2074].

Minor Concerns:

1. Line 16: transcription factors (TFs) in the bracket should be mentioned as the authors use this acronym later in the abstract

Response: Thanks for suggestion, we have revised as suggested.

2. Line 25, 253: M. oryzae is spelled incorrectly (oryzea) in the abstract and discussion

Response: Thanks for suggestion, we have revised as suggested.

3. Line 29-30: Chromatin regulates and affects gene expression and development in eukaryotic cells. Please remove comma and include and instead

Response: Thanks for suggestion, we have revised as suggested.

4. Line 32: The references are present in different sizes/type

Response: Thanks for suggestion, we have revised as suggested.

5. Line 32-36: it should be reframed

Response: Thanks for suggestion, we have revised as suggested.

6. Line 42-44: A suitable reference should be cited

Response: Thanks for suggestion, we have revised as suggested.

7. Line 59: The catalogue number and make should be mentioned for oatmeal tomato agar

Response: Thanks for suggestion, we have revised as suggested.

8. Line 60: How can 100 µL be pipetted out using a 0.5-5 mL pipette? Please correct the pipette type used

Response: Thanks for suggestion, we have revised as suggested.

9. The authors should mention that the wild-type culture was grown for 4 days. Any reasons specifically?

Response: Thanks for suggestion, we have revised as suggested.

10. Line 66: After the mycelia debris were transferred to 250 mL liquid complete medium, reframe the sentence to "the fungal biomass was grown at 28°C for 36 h at 150 rpm". It does not make any sense to culture the plates at constant shaking.

Response: Thanks for suggestion, we have revised as suggested.

11. Line 68-69: Reframe the sentence to "Filter the fungal biomass using sterilized filter paper"

Response: Thanks for suggestion, we have revised as suggested.

12. Line 75-76: Reframe the sentence. Hyphae was kept for lysing at 28°C for 3-4 h at 150 rpm.

Response: Thanks for suggestion, we have revised as suggested.

13. Line 77: Lysis of the mycelium is done to form the protoplasts. Reframe the sentence to include the word protoplast

Response: Thanks for suggestion, we have revised as suggested.

14. Line 79, 91, 119, 135, 139, 143, 147, 152, 156, 158, 184: remove of from "Discard of the supernatant"

Response: Thanks for suggestion, we have revised as suggested.

15. Line 80: Replace with to in "Resuspend protoplast in"

Response: Thanks for suggestion, we have revised as suggested.

16. Line 84-86: Add formaldehyde to 2 mL RIPA buffer containing protoplast for crosslinking. Include the highlighted words.

Response: Thanks for suggestion, we have revised as suggested.

17. Line 92: Remove s from "Resuspend the pellets"

Response: Thanks for suggestion, we have revised as suggested.

18. Line 94: Resuspend the pellet in 750 µL; replace with

Response: Thanks for suggestion, we have revised as suggested.

19. Line 103-104: this step should come before sonication so as to distinguish between unsheared and sheared DNA

20. Line 105-107: Remove sentence after 200-500 bp in length. No need to repeat sonicator type and duration which has been mentioned previously

Response: Thanks for suggestion, we have revised as suggested.

21. Line 120: Obtain the rinsed Dynabeads and not rinsing

Response: Thanks for suggestion, we have revised as suggested.

22. Line 121: The authors should clarify what they mean by "Adjust each sample to the same concentration" concentration of what? Is it the same volume of Dynabeads?

Response: Thanks for suggestion, we have revised as suggested.

23. Line 122: It should be made clearer to the reader that of the total volume of 750 µL of sheared chromatic DNA, equal volume of 100 µL should be distributed in separate centrifuge tubes

Response: Thanks for suggestion, we have revised as suggested.

24. Line 129: Remove the word "normal"

Response: Thanks for suggestion, we have revised as suggested.

25. Line 160: 1 M NaHCO3 and not NAHCO3. Please change this from table as well

Response: Thanks for suggestion, we have revised as suggested.

26. Line 165: The authors should make it clear to the reader that 190 µL of elution buffer was added to the 10 µL of input DNA kept as a control kept on line 111.

Response: Thanks for suggestion, we have revised as suggested.

27. Line 171: Correct H2O to H2O

Response: Thanks for suggestion, we have revised as suggested.

28. Line 174: Why the authors have added 550 mL of phenol/chloroform/isoamyl alcohol? It should be 550 µL.

Response: Thanks for suggestion, we have revised as suggested.

29. Line 188: What do the authors mean by "Sequence the obtained ChIP-DNA product"

Response: Thanks for suggestion, we have revised as suggested.

30. The authors should mention the catalogue number and details of the make for DNA End-repair kit (line 194) and MinElute PCR Purification kit (line 198)

Response: Thanks for suggestion, we have revised as suggested.

31. Line 204: What's EB buffer? Elution buffer

Response: Thanks for suggestion, we have revised as suggested.

32. Line 219: remove full stop after homogenizer

Response: Thanks for suggestion, we have revised as suggested.

33. Line 219-220: reframe

Response: Thanks for suggestion, we have revised as suggested.

34. Line 226: were mapped for H3K4me3 distribution

Response: Thanks for suggestion, we have revised as suggested.

35. Line 227: wild type to wild-type

Response: Thanks for suggestion, we have revised as suggested.

36. Line 230: important roles in the regulation of gene expression. Include the highlighted words

Response: Thanks for suggestion, we have revised as suggested.

37. Line 319: remove oso from PLOS

Response: Thanks for suggestion, we have revised as suggested.

38. Line 327: spacing between Chromatin structure: are repeating

Response: Thanks for suggestion, we have revised as suggested.

39. Line 351: spacing between associated with

Response: Thanks for suggestion, we have revised as suggested.

40. There are few references which have been used in the text. However, these are not present in the reference section. Few examples include: Wilson 2009 has been included in line 52, but is not listed in the reference section. Similarly, line 53, Ryder et al., 2019 is not included in the reference. The authors should be very careful about this.

Response: Thanks for suggestion, we have revised as suggested.