RESEARCH ARTICLE

Changes of Host DNA Methylation in Domestic Chickens Infected with *Salmonella enterica*

FEI WANG*,JIANCHAO LI*,QINGHE LI,RANRAN LIU, MAIQINGZHENG, QIAO WANG, JIE WEN AND GUIPING ZHAO[1]

Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, P.R. China

*These authors contributed equally to this work.

[1]To whom correspondence should be addressed.Institute of Animal Sciences, Chinese Academy ofAgricultural Sciences, No2 Yuanmingyuan West Road, Beijing 100193, China.

E-mail: zhaoguiping@caas.cn

Abstract

Cytosine methylation is an effective way to modulate gene transcription. Little is known aboutthe epigenetic changes in the host infectedwith*Salmonellaenterica*. In this study,we used methylated DNA immunoprecipitation sequencingto analyze the genome-wide DNA methylation changes in domestic chickens after infection with *Salmonella*.The level of DNA methylation was slightly higher in the genomic regions around the transcription start termination sites in a *Salmonella*-infectedgroup compared to the controls. In all, 879 peaks were differentially methylated between *Salmonella*-infected and control groups, among which 135 were located in the gene promoter regions.Genes including MHC class IV antigen, GABARAPL1, MR1 and KDM1B were shown to be methylated more heavily after infection with *Salmonella*, whereasDYNLRB2, SEC14L3 and ANKIB1 tended to have

fewermethylated cytosine residues in the promoter regions. Gene interaction network analysis of differentially methylated genes in the promoter regions revealed extensive connections with immune-related genes, indicating the possible impact of infection with *Salmonella* on the epigenetic statusof the host.

Introduction

DNA methylation is involved in many biological activities, including regulation of gene expression, genomic imprinting, cell differentiation and silencing of transposons. Bedsides the methylation ofcytosine in CpGdinucleotides, CHG or CHH (where H is A, T or C) was found to have methylated cytosine residues. A variety ofDNA modifications have beenreported in recent years, including5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine(Ito et al., 2011),however, methylation ofthe fifth cytosine residue remains an efficient and dominant DNA modification that regulates gene expression. Promoter DNAis the main target of methylation for suppressing gene transcription activity. Methylation of promoter DNA usually leads to correspondingsuppressive modifications of histones inthe same region and, thus, forms a condensed chromatin conformationthatsuppresses gene expression. Genome-wide studies have provided sufficient evidence to show the negative correlation between promoter DNA methylation and the level of gene expression. By contrast, methylation in a gene body is always correlated positively with thelevel of gene expression.

Salmonella enterica, a major cause of infectious diseases,infects humans and other animals and is a great threat to public health and animal production worldwide. The domestic chicken (*Gallus gallusdomesticus*) is an important host of *S. enterica*, which causes a great loss for avian production every year, so it is important to have a comprehensive

understanding of how *S. enterica* infects chickens and how the host antagonizes this exogenous pathogen. The pathogenicity of *S. enterica*relies heavily on its virulence genes, which are termed *Salmonella*pathogenicity islands(Srikanth et al., 2011). *Salmonellaenterica*secretes effectors that promote the entry of bacteria and control host inflammatory responses. For example, SipA of the bacteria is activated by caspase-3 and has a crucial role in bacterial entry(Giacomodonato et al., 2007),SopE promotes the activation of Rho GTPases and initiates the process of invasion(Hardt et al., 1998). Host innate immune responses are triggered by the *S. enterica*infection;NF-KB, ERK and p38 are induced strongly upon infection. Toll-like receptors recognize the infection of and activate transcription responses to *S. enterica*, leadingfinally to the release of inflammatory factors, including interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) from the macrophages(Srikanth et al., 2011).Using high-throughput approaches, many studies have provided comprehensive transcriptome-wide gene expression analyses in chickens challenged with *Salmonella*. Luan et al have identified hundreds of differentially expressed genes in *Salmonella*-challenged chickens, which wererepresented by key genes, including IL-8, chemokine (C-C motif) ligand 5 (CCL5) and B-cell CLL/lymphoma 2(Luan et al., 2012). Avidinimmune responsive gene 1and chemokine ah221were highly induced in both spleen and cecum in *Salmonella*-infected chickens(Matulova et al., 2012). Genes such as IgG,interferon γ,induciblenitric oxide synthase 2, IL-1β and IL-17 would have inductions even >100-fold inchicken cecum after infection with *Salmonella*(Matulova et al., 2013).Although there have been many investigations of the transcriptional changes in chickens infected with *Salmonella*, there are few reports of the molecular mechanisms

underlying the transcription variations.

With the development of high-throughput sequencing or DNA array technologies, further analyses have been carried out on the epigenetic changes upon infectionbypathogens. Through a genome-wide DNA methylation analysis, Xu et al showed DNA methylation changes in chickens challenged with *Escherichiacoli*,the transcription of IL-8, IL 2 receptorβand IL 1 receptor accessory protein-like 1were found to be epigenetically regulated in response to bacterial infection(Xu et al., 2015). Uropathogenic infectionby *E. coli* in humansresulted in hypermethylation of CDKN2A and downregulation of its transcription(Tolg et al., 2011) and*Legionella pneumophila* induced the expression of IL-8 by promoting acetylation of IL-8 promoter(Schmeck et al., 2008). Besides the epigenetic changes induced by bacterial infection, the components of bacterial pathogens canmodulate the epigenetic status of host genes. The nuclear targeted protein A (LntA) of *Listeria monocytogenes* prevented binding ofthe bromo adjacent homology domain containing 1chromatin silencing complex to upregulate the expression of interferon-stimulated genes(Lebreton et al., 2011).*Listeriamonocytogenes*increased the acetylation levels of H3K14 and H4K8 of the promoter of IL-8(Schmeck et al., 2008).The investigations mentioned above have demonstrated the influence of bacterial infection on host epigenetic modifications;however, details of the host epigenetic changes after infection by *Salmonella*areunknown.

Materials and Methods

Ethics statement

This research complied with the Animal welfare guidelines for experimental

animalsestablished by the Ministry of Science and Technology of China.

Animals

Eighty one-day-old White Leghorn chickens were raised in an isolation tank.At day 12, the chickenswere divided randomly into two groups.One group was used as the control and the other was challenged with *Salmonella*.Chickens were sacrificed 24 h postinfection. Three individuals in articulo mortis from the infected group and three from the control groups were used for the isolation of DNA from blood samples.The DNA of three individuals in each group was pooled for methylated DNA immunoprecipitation (meDIP) analysis.

Methylated DNA immunoprecipitation sequencing (MeDIP-seq)

Genomic DNA was extracted using a DNeasy Blood & Tissue Kit(QIAGEN). The DNA was sheared into 400–1000 bp fragments by sonication(Bioruptor®, Diagenode]) and subjected to MeDIP-seq assay as described(Li et al., 2015, Li et al., 2011, Zhang et al., 2006).Briefly, 2 mg of sonicated DNA was denatured and incubated with 5 mg of mousemonoclonal anti-5-methylcytosineantibody (Diagenode) in 500 ml of IP buffer (0.5%(v/v) NP40, 1.1% (v/v) Triton X-100, 1.5 mM EDTA, 50 mMTris–HCl and 150 mMNaCl) at 4ºCfor 4 h on a rotating wheel. Themixture was incubated with 50 ml of magneticbeads coupled to anti-mouse IgG (Bangs Laboratories Inc.,) at 4ºC for 2 h by end-over-endrotation and washed three times with 700 ml of IP buffer. Methylated DNA was recovered using proteinase K digestion at 50ºC for 3 h andpurified by extraction withphenol/chloroformfollowed by precipitation in ethanol.The methylated DNA was used for the construction of a sequencing library and sequenced by HiSeq 2000Illumina. The chicken GalGal4 genome release was used for reads alignment.Model-based analysis of ChIP-Seq (MACS) was used to scan the

methylated peaks in the genome.

Pathway enrichment analysis

Genes with differentially methylated promoters afterinfection with Salmonella were subjectedto pathway analysis using the online tool KEGG Orthology Based Annotation System (KOBAS)(Xie et al., 2011),whichprovided pathway enrichment analysis based uponthe Kyoto Encyclopedia of Genes and Genomes (KEGG) database.Only pathways with $P<0.05$ were reserved.

Results

Genome-wide DNA methylation analysis by MeDIP-seq

To decipher the variations in DNA methylation after infection with *Salmonella*, DNA from threesamples of both the *Salmonella*-infected (SE) and the control (NC) groups werepooled for MeDIP-seq analysis. A total of 45×10^6 raw reads were generated for both groups, of which >74% could be mapped onto the chicken genome and 66% were mapped uniquely(Table 1).

MeDIP-seqis a methylated DNA enrichment-based technique that cannot give single-base resolution; however, it is reliable and is widely used for DNA methylation analysis.Further, methylation peak calling is a well-recognized approach for methylation difference analysis.We used MACS software to scan methylation peaks across the chicken genome. In all,65,697 and 58,943 methylation peaks were found in the SE and NC groups covering 11.5% and 10.5% of the chicken genome, respectively (Table 2).

We further investigated the DNA methylation distribution around the transcription start site (TSS) andterminationsite (TTS) and part of the gene body.The level of DNA methylation

decreased towards TSS (Fig. 1) andthe overall level of DNA methylation of the SE group was slightly higher around both TSS and TTScompared to the NC group (Fig. 1).

Identification of differentially methylated genes after infection with Salmonella

Withinclusioncriteriaof fold change>1.5 and $P<0.05$, we identified 879 peaks methylated differentially between the SE and the NCgroup. These peaks were further divided according to thelocation in thepromoter, in the gene body or in other genomic positions.We defined the promoter as the genomic regions from TSS to 2 kb upstream. In all, 120 peaks were located in promoters with 71 upregulated and 49 downregulated in the SE group (Fig 2 and Table 3). There were 544 differentially methylated peaks in the gene body regions and more than half were upregulated in the SE group (Table 3).

Promoter DNA methylation is the most effective way to downregulate the level of gene expression.We assumed genes with significant DNA methylation changes in promoter regions as crucial genes affected by *Salmonella* infection and termed these differentially methylated genes (DMGs).In thisstudy,135 genes were differentially methylated after infection with *Salmonella*. This number was less than the number of differentially methylated peaks because some peaks occurredin the same gene. Alarge portion of the DMGs werenot well annotated in the chicken genome but many genes with important biological functions wereincluded in the DMGs;for example,methylation levels of the promoter of major histocompatibility complex (MHC) class IV antigen,GABA(A) receptor-associated protein like 1(GABARAPL1), major histocompatibility complex, class I-related (MR1) and lysine (K)-specific demethylase 1B (KDM1B) were increased in the SE group. Dyneinlight chain, roadblock-type 2 (DYNLRB2), SEC14-like 3(SEC14L3), and ankyrinrepeat and IBR domain containing 1 (ANKIB1)

showed decreased levels ofmethylation in promoter regions.

Expression of differentially methylated genes in Salmonella-infected chicken spleen

We undertook transcriptome analysis of *Salmonella*-infected chicken spleen by high-throughput sequencing (data not shown). We knew DNA methylation and gene expression werevaried amongdifferent tissues even in the same individual; however,we thought it mightprovidesome clues for understanding the mechanism underlying*Salmonella* infection if we combined the methylation data fromthe blood sample with the gene expression data for thespleen.Of the 135 differentially methylated genes, 25 had reverse trends of gene expression change in the spleen (Pearson's correlation coefficient r=–0.51) (Fig.3 and Table S1).

Gene interaction networks of differentially methylated genes

We investigated the gene interaction networks of DMGs in blood samples. OnlyDNA-directed polymeraseβ (POLB), GABARAPL1, catalase (CAT) and proteasome subunit, βtype, 3 (PSMB3)were found to have interaction partners (Fig 4).We used data for the genes of these four interaction proteins to constructgene interaction networks. GABARAPL1, CAT and POLB interacted with ten,nine and fourgenes, respectively. Proliferating cell nuclear antigen (PCNA)was involved in synthesis of the leading strand during DNA replication(Chilkova et al., 2007).In thisstudy, it was associated with POLB, which is a DNA polymerase responsible for base excision and repair. GABARAPL1 was associated with phosphoinositide-3-kinaseclass 3 (PIK3C3), whichis the catalytic subunit of the PI3K complex that hasa crucial role in the initiation and maturation of autophagosomes(Roberts et al., 2013). Fas ligand(FASLG) induced cell apoptosis when

triggered by the FAS/FASLG signaling pathway;we found FASLG interacted with both CAT and GABARAPL1, which were differentially methylated genes in the promoter upon Salmonella infection.

Discussion

We present the results of genome-wide DNA methylation analysis of *Salmonella*-infected chicken.We found DNA methylation levels of a substantial number of genes were altered by the infection with *Salmonella*.Methylation of promoter DNA has a crucial impact on gene expression.In thisstudy, we found 135 genes were differentially methylated in promoter regions, among which many were host immune response-related genes.For example, promoters of MHC and MHC class IV antigen were modified with more methylated cytosine residues compared to the controls. Our results revealed the impact of *Salmonella* infection on the methylation of immune-related genes.

The promoter region of a gene is crucial for regulation of gene expression. After *Salmonella* infection, promoters of MHC class IV antigen, GABARAPL1, MR1, KDM1B, DYNLRB2, SEC14L3 and ANKIB1 showed altered levels of DNA methylation. Transcription of GABARAPL1, which might be involved in maturation of the autophagosome, was upregulated inhuman enteroendocrine cells followinginfection with *Chlamydia trachomatis*(Dlugosz et al., 2014).The dynein complex is involved in the intracellular transport of various membranous organelles, including lysosomes, endosomes, phagosomesand the Golgi complex(Hirokawa, 1998).The dynein complex might also be involved in the delivery of viral or bacterial components after infection(Merino-Gracia et al., 2011).

We appliedgene interaction network analysis for DMGsin the promoter after infection with *Salmonella*. The gene interaction network reflects the reciprocal relations between genes, such as inhibition, activation, binding or phosphorylation.Owingto the lack of full annotation of the chicken genes, only a small subset of differentially methylated genes was included in the gene interaction network. CAT, POLB, PSMB3 and GABARAPL1 were the core of the network. Host factors, including PCNA, PIK3C3 and FASLG, were found to interact with the DMGs after infection with *Salmonella*. POLB interacted with PCNA and both were involved in the replication of DNA.PCNA was shown to have a crucial impact on the replication of herpes simplex virus(Sanders et al., 2015). Our results indicated infection with*Salmonella* might affect the activity of genes involved in DNA replication.Autophagosomesare involved in the intracellular degradation of cytoplasmic contentsand invading microorganisms. We found GABARAPL1, which was associated with formation of the autophagosomal vacuole, was methylated differentially after infection with *Salmonella*.Through the gene interaction network, we showed GABARAPL1 was associated with several genes,including FASLG and PIK3C3, with function related to an immune response, indicating apossible impact on the related immune processes of *Salmonella* infection.

References:

Chilkova, O., Stenlund, P., Isoz, I., Stith, C. M., Grabowski, P. & Lundstroem, E., *et al.* 2007. The eukaryotic leading and lagging strand DNA polymerases are loaded onto primer-ends via separate mechanisms but have comparable processivity in the presence of PCNA. *Nucleic Acids Research,***35,** 6588-6597.

Dlugosz, A., Muschiol, S., Zakikhany, K., Assadi, G., D'Amato, M. & Lindberg, G. 2014. Human enteroendocrine cell responses to infection with Chlamydia trachomatis: a microarray

study. *Gut Pathogens,***6**.

Giacomodonato, M. N., Uzzau, S., Bacciu, D., Caccuri, R., Sarnacki, S. H. & Rubino, S., *et al.* 2007. SipA, SopA, SopB, SopD and SopE2 effector proteins of Salmonella enterica serovar Typhimurium are synthesized at late stages of infection in mice. *Microbiology-Sgm,***153,** 1221-1228.

Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R. & Galan, J. E. 1998. S-typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell,***93,** 815-826.

Hirokawa, N. 1998. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science,***279,** 519-526.

Ito, S., Shen, L., Dai, Q., Wu, S. C., Collins, L. B. & Swenberg, J. A., *et al.* 2011. Tet Proteins Can Convert 5-Methylcytosine to 5-Formylcytosine and 5-Carboxylcytosine. *Science,***333,** 1300-1303.

Lebreton, A., Lakisic, G., Job, V., Fritsch, L., To Nam, T. & Camejo, A., *et al.* 2011. A Bacterial Protein Targets the BAHD1 Chromatin Complex to Stimulate Type III Interferon Response. *Science,***331,** 1319-1321.

Li, Q., Li, N., Hu, X., Li, J., Du, Z. & Chen, L., *et al.* 2011. Genome-Wide Mapping of DNA Methylation in Chicken. *Plos One,***6**.

Li, Q., Wang, Y., Hu, X., Zhao, Y. & Li, N. 2015. Genome-wide Mapping Reveals Conservation of Promoter DNA Methylation Following Chicken Domestication. *Scientific Reports,***5**.

Luan, D. Q., Chang, G. B., Sheng, Z. W., Zhang, Y., Zhou, W. & Li, Z. Z., *et al.* 2012. Analysis of Gene Expression Responses to a Salmonella Infection in Rugao Chicken Intestine Using GeneChips. *Asian-Australasian Journal of Animal Sciences,***25,** 278-285.

Matulova, M., Rajova, J., Vlasatikova, L., Volf, J., Stepanova, H. & Havlickova, H., *et al.* 2012. Characterization of Chicken Spleen Transcriptome after Infection with Salmonella enterica Serovar Enteritidis. *Plos One,***7**.

Matulova, M., Varmuzova, K., Sisak, F., Havlickova, H., Babak, V. & Stejskal, K., *et al.* 2013. Chicken innate immune response to oral infection with Salmonella enterica serovar Enteritidis. *Veterinary Research,***44**.

Merino-Gracia, J.Garcia-Mayoral, M. F. & Rodriguez-Crespo, I. 2011. The association of viral proteins with host cell dynein components during virus infection. *Febs Journal*,**278,** 2997-3011.

Roberts, R., Al-Jamal, W. T., Whelband, M., Thomas, P., Jefferson, M. & van den Bossche, J.*, et al.* 2013. Autophagy and formation of tubulovesicular autophagosomes provide a barrier against nonviral gene delivery. *Autophagy*,**9,** 667-682.

Sanders, I.Boyer, M. & Fraser, N. W. 2015. Early nucleosome deposition on, and replication of, HSV DNA requires cell factor PCNA. *Journal of Neurovirology*,**21,** 358-369.

Schmeck, B., Lorenz, J., N'Guessan, P. D., Opitz, B., van Laak, V. & Zahlten, J.*, et al.* 2008. Histone acetylation and flagellin are essential for Legionella pneumophila-induced cytokine expression. *Journal of Immunology*,**181,** 940-947.

Srikanth, C. V., Mercado-Lubo, R., Hallstrom, K. & McCormick, B. A. 2011. Salmonella effector proteins and host-cell responses. *Cellular and Molecular Life Sciences*,**68,** 3687-3697.

Tolg, C., Sabha, N., Cortese, R., Panchal, T., Ahsan, A. & Soliman, A.*, et al.* 2011. Uropathogenic E. coli infection provokes epigenetic downregulation of CDKN2A (p16INK4A) in uroepithelial cells. *Laboratory Investigation*,**91,** 825-836.

Xie, C., Mao, X., Huang, J., Ding, Y., Wu, J. & Dong, S.*, et al.* 2011. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Research*,**39,** W316-W322.

Xu, H., Zhu, X., Hu, Y., Li, Z., Zhang, X. & Nie, Q.*, et al.* 2015. DNA methylome in spleen of avian pathogenic escherichia coli-challenged broilers and integration with mRNA expression (vol 4, 4299, 2014). *Scientific Reports*,**5**.

Zhang, X., Yazaki, J., Sundaresan, A., Cokus, S., Chan, S. W. L. & Chen, H.*, et al.* 2006. Genome-wide high-resolution mapping and functional analysis of DNA methylation in Arabidopsis. *Cell*,**126,** 1189-1201.

Received 3 February 2016, in final revised form 6 November 2016; accepted 14 November 2016
Unedited version published online: 17 November 2016

Figure legends

Fig 1.DNA methylation around gene flanking regions.The level of DNA methylation was calculated by the reads that were aligned to the corresponding genomic regions. The 2kb regions upstream and downstream from the transcription start site and termination site were included in the analysis. The regions were split into 20 regions not overlapped and the average depth of coverage was calculated for each window.

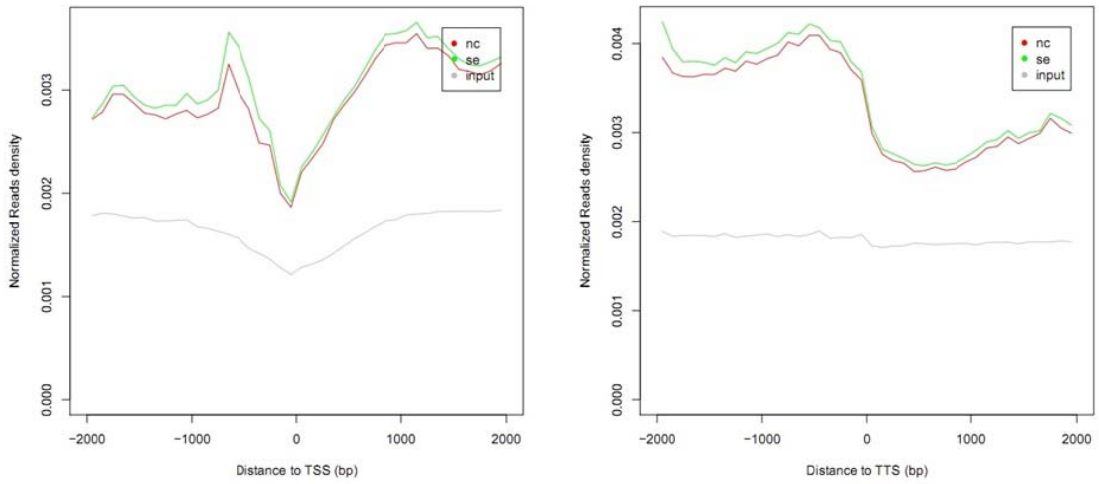


Fig 2.A scatter plot showing the correlation between the control group and the *Salmonella*-infected group.The red dotsindicate differentially methylated peaks.

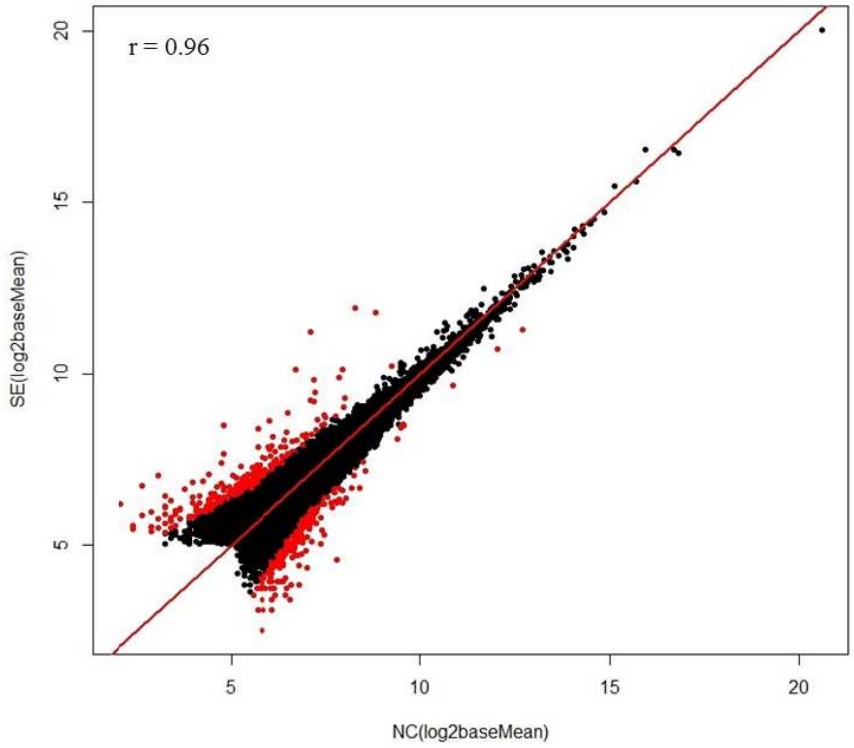


Fig 3.Gene interaction networks of the differentially methylated genes after infection with Salmonella. Act, activate; exp, transcription regulation; ind, indirect interaction; +P, phosphorylate; inh, inhibit.

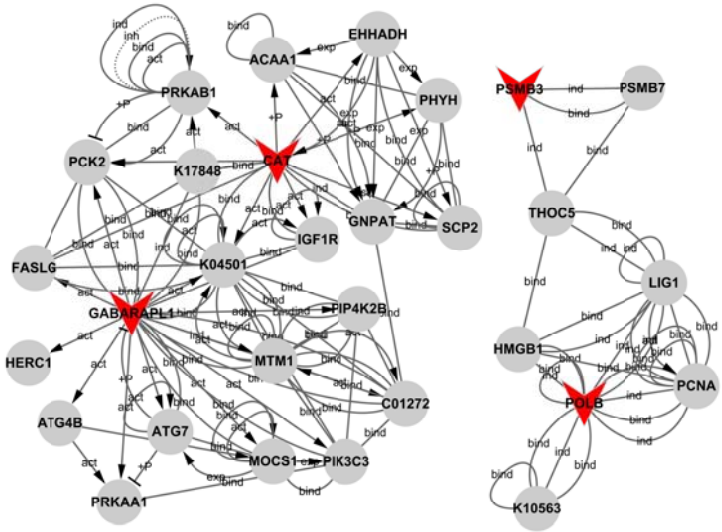
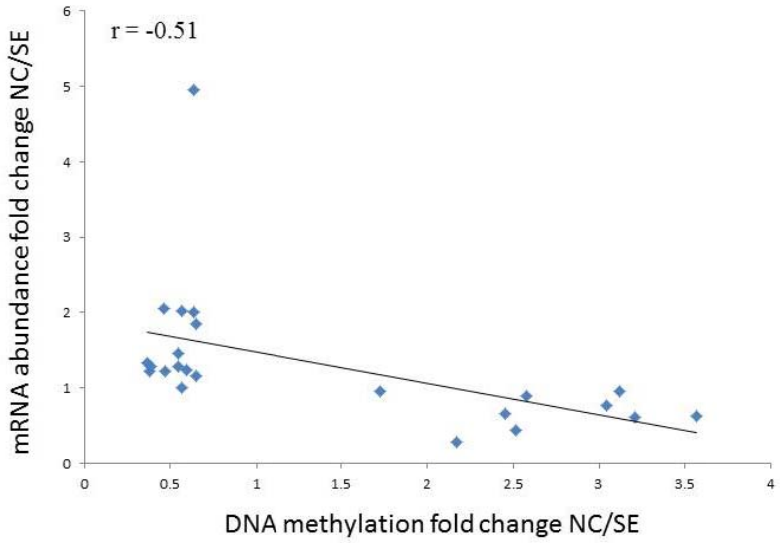


Fig 4.Correlation between the gene expression changes and DNA methylation changes of the differentially methylated genes.



Authors' contributions

JL, QL and GZ performed the experiments, analyzed the data, prepared the figures anddrafted the manuscript. MZ and QW analyzed the data. JL, QL, RL, JW and GZ conceived and designed the experiments. All authors read and approvedthe final manuscript.

Acknowledgments

This research was supported by the National High-tech R&D Program (2015BAD03B03),China Agriculture Research System (CARS-42) and the Agricultural Science and Technology Innovation Program (ASTIPIAS04) of Chinese Academy of Agricultural Sciences.

Table 1.Summary of meDIP-Seq data.

Sample	Total reads number	Number of mapped reads	Percentage of mapped reads	Number of uniquely mapped reads	Percentage of uniquely mapped reads
SE	45088386	34,476,442	74.3%	30,350,306	67.3%
NC	45177104	34,369,760	76.1%	30,127,376	66.7%

Table 2. Distribution of methylation peaks.

Sample	Peak number	Mean length of peak	Peak coverage of genome
SE	65697	1783.2	11.5%
NC	58943	1818.6	10.5%

Table 3. Summary of differentially methylated peaks between *Salmonella* infected and control groups.

Genomic regions	Upregulated peaks	Downregulated peaks	Total number of peaks
Promoter	71	49	120
Gene Body	286	258	544