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Journal of Veterinary Medicine and Research

Research Article

Gene Expression Profiling of Chicken Hypothalamus in the Response to *Campylobacter jejuni* Inoculation

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Submitted: 24 August 2022

Accepted: 10 September 2022

Published: 12 September 2022

ISSN: 2379-948X

Keywords

- Transcriptome; Hypothalamus; SPF chicken and C. *jejuni*

Abstract

Campylobacter jejuni (C. *jejuni*) is the most frequently reported bacterial food-borne pathogen, which can cause great threat to human health through the contaminated poultry products. Chicken is the main reservoir of the C. *jejuni*. It has been reported that hypothalamus-pituitary-thyroid interacts with immune system. It is worth to know the transcriptome of hypothalamus in the response to C. *jejuni* inoculation. To study the transcriptome in hypothalamus following C. *jejuni* inoculation, C. *jejuni* free 3-day old SPF white leghorn chickens were orally inoculated with C.*jejuni*. The hypothalamus was collected from inoculated and non-inoculated groups at 8 hours post inoculation for RNA sequencing. The results showed that 19,061 genes were expressed in either group with 17,446 known genes and 1,615 novel genes. There were 135 genes significantly differentially expressed between inoculated and non-inoculated groups. Functional annotation results showed that the biological process of biological adhesion, hormone secretion and cell aggregation were enriched. The Hedgehog signaling pathway, the Jak-STAT signaling pathway, the Wnt signaling pathway, and the Cytokine receptor interaction were significantly enriched following C. *jejuni* inoculation. The results herein will provide a crucial theoretical foundation to understand the molecular mechanism of response to C. *jejuni* inoculation in chicken.

INTRODUCTION

Campylobacter jejuni (C. jejuni), a Gram-negative, spiral-shaped bacterium, is recognized as a leading cause of acute bacterial gastroenteritis in human worldwide [1,2], and the most common source for human infections. Chickens can carry a high load of Campylobacter without clinical signs [3]. The chicken is the natural reservoir of *C. jejuni* [4]. Moreover, several previous studies have shown that the host genetic background plays an important role in the response to *C jejuni* infection [3,5]. Several studies have shown that stress can activate the hypothalamic-pituitary-thyroid axis, and further affect the synthesis and secretion of thyrotropin-releasing hormone (TRH), thyroid stimulating hormone (TSH), and thyroid hormone [6,7]. Hormones of the hypothalamus-pituitary-gonad axis regulate the immune system in vertebrates [8,9].

High-throughput sequencing has become a powerful tool for transcriptome analysis and gene identification. The aim of current study is to investigate the transcriptome variation involved in the response to *C. jejuni* infection in chicken hypothalamus through next generation sequencing and uncover the signaling pathways and important biological processes related to *C. jejuni* infection. We expect these differentially expressed genes may increase our understanding of disease resistance mechanism and may allow the development of innovative strategies for breeding of anti-disease breeds in chicken.

MATERIALS AND METHODS

Animals and *C. jejuni* challenges

Specific pathogen free (SPF) white leghorn was used in the current study. The *C. jejuni* inoculation was described in our previous study [10]. Briefly, fifty-six three-day old *C. jejuni* negative SPF White Leghorn chickens were randomly divided into non-inoculated group and inoculated group and raised in two separate isolators. Chickens in the inoculated group were orally inoculated 500μl inoculant with 1×10^8 CFU *C. jejuni*, chickens in the non-inoculated group were mock inoculated 500μl sterile PBS. Four chickens in each group was randomly sacrificed at 4, 8, 12, 16, 20, 24, and 48 h post inoculation (hpi). Hypothalamus samples collected at 8 hpi was used in the current study. All animal procedures were approved by Shandong Agricultural University Animal Care and Use Committee (SDAUA-2014-010).

Total RNA isolation, RNA library construction and transcriptome sequencing

Individual hypothalamus tissue of each chicken from inoculated group and the non-inoculated groups (3 chickens in each group) were randomly selected to extract total RNA using Trizol Reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity, concentration and integrity of RNA samples were detected using Nanodrop and Agilent 2100 respectively, then cDNA libraries

were constructed. These six cDNA libraries were sequenced using the Illumina HiSeq2500 (Biomarker Technologies Corporation, Beijing, China).

Bioinformatics analysis

The adaptors and low quality sequences were trimmed to get clean data. The clean reads were aligned with chicken genome sequence using TopHat2. The mapped reads were assembled using Cufflinks and aligned with chicken reference genome (Gallus_gallus-5.0) sequence to find the novel genes and transcripts. The novel genes and transcripts were annotated through aligning with NR, Swiss-Prot, GO, COG, KEGG database. The expression levels of transcripts and genes were estimated using FPKM. The expression differences between inoculated and non-inoculated groups were analyzed using DESeq2 package. The fold change >1.5 and P <0.05 were considered as significance. Gene Ontology (GO) for those differentially expressed genes was analyzed using WEGO [11]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathway analysis for those differentially expressed genes was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.8. Health QTL data were retrieved from Animal QTL Database [12], and joined with differentially expressed genes through AnimalGene2QTL (https://github.com/liuyufong/AnimalGene2QTL) to get genes associated with health QTL. All the data have been deposited into the National Genomics Data Center with the accession number of PRJCA007854.

RESULTS

Sequence assembly and gene identification

In total, 28.74 Gb clean data were obtained. Average number of read counts in inoculated and non-inoculated groups was 20,365,328 and 17,672,488, respectively. More than 77% clean reads were mapped to the reference genome (galGal5). The results showed that 19,061 genes were expressed in either group including 1,615 novel genes.

Identification of differentially expressed genes

Differentially expressed genes were identified through DESeq2. There were totally 135 genes differentially expressed between the inoculated and non-inoculated groups which included 102 up-regulated genes and 33 down-regulated genes (P < 0.05, Additional file 1). The heat map and hierarchical clustering results showed that expression of detected genes was distinct between inoculated group and non-inoculated group (Figure 1). The differentially expressed genes were clustered into 4 subgroups based on expression levels between inoculated group and non-inoculated group. Subgroup A consisted of genes with higher expression in non-inoculated group and lower expression in inoculated group. Subgroup B consisted of genes with higher expression in inoculated group and lower expression in non-inoculated group. Subgroup C consisted of genes with higher expression in both inoculated and non-inoculated groups. Subgroup D consisted of genes with lower expression in both inoculated and non-inoculated groups. All DEGs were mapped to Animal QTL database (https://www.animalgenome.org/QTLdb). There were 1,437 QTLs associated with the DEGs. Of those QTLs, 96 health QTLs from 11 traits were identified and associated with

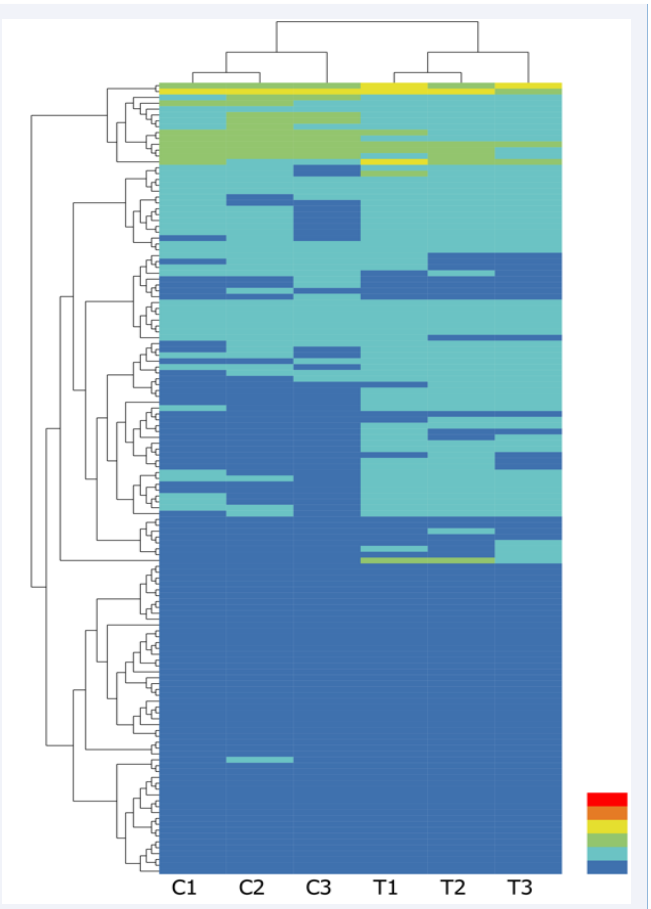

Figure 1 Cluster of differentially expressed genes.

41 DEGs (Additional file 2). Antibody titer to LPS antigen consisted of 3 genes which were IQGAP2, FSIP1 and SCNN1B. Cloacal bacterial burden after challenge with Salmonella E consisted of BG8, C5orf22, DSP, DPEP2 and NPVF. Antibody response to SRBC antigen consisted of PERP2, BG8, HMOX1, ELFN2, LOC423321, LOC423321, SYT14, FSIP1, CPED1, ANKDD1A, ACAN, PAX2 and KNTC1. There were 23 DEGs associated with Marek's disease related traits (Table 1).

Functional annotation of DEGs

GO and pathway annotation of those differentially expressed genes were performed. In term of cellular component, functional terms of membrane part, extracellular region, extracellular region part, cell junction, synapse and extracellular matrix were enriched, however, collagen trimer, nucleoid, and virion were absent. In terms of molecular function, functional terms of receptor activity, enzyme regulator activity, receptor regulator activity and morphogen activity were enriched, however, channel regulator activity, antioxidant activity, translation regulator activity, chemoattractant activity, chemorepellent activity were absent. In term of biological process, functional terms of reproductive process, locomotion, biological adhension, growth, hormone secretion and cell aggregation were enriched, however, cell killing was absent (Figure 2).

KEGG analysis results showed that immune-related pathways

Table 1: Differentially expressed genes associated with healthy traits.

Trait name associated with QTL	Differentially expressed genes
Antibody response to KLH antigen	PERP2、LOC101748058、NPVF、AGMO、SYT14、THNSL1、LOC420491、VIPR2、SCNN1B
Antibody response to MB antigen	BG8、SCNN1B
Antibody response to SRBC antigen	PERP2、BG8、HMOX1、ELFN2、LOC423321、SYT14、FSIP1、CPED1、ANKDD1A、ACAN、PAX2、KNTC1
Antibody titer to LPS antigen	IQGAP2、FSIP1、SCNN1B
Antibody titer to LTA antigen	FOXB1、IQGAP2、CYP19A1、LOC423321、SYT14、FSIP1、SCNN1B
Body temperature	SYT2、CENPE、SLC39A8、PAX2
Cloacal bacterial burden after challenge with Salmonella E	BG8、C5orf22、DSP、DPEP2、NPVF
Marek's disease-related traits	FOXB2、SLCO4C1、TAL1、CNTNAP4、LOC101748058、PDE6B、IQGAP2、ELFN2、WNT6、FAP、AGMO、LOC423321、DDX60、FSIP1、LOC424998、SFRP2、THNSL1、LOC420491、VIPR2、VIPR2、CCDC18、KNTC1、FRZB
Plasma coloration	OSR1、PAX2
Time to achieve maximum antibody response to SRBC	LOC423321
Total mortality	ACAN

like Hedgehog signaling pathway, Jak-STAT signaling pathway, Wnt signaling pathway, Cytokine-cytokine receptor interaction and Neuroactive ligand-receptor interaction were enriched. Metabolism-related pathways like Butaoate metabolism, other glycan degradation, Ether lipid metabolism, Steroid hormone biosynthesis, Porphyrin and chlorophyll metabolism and Purine metabolism were enriched (Figure 3).

The functional annotation of DEGs showed that the up-regulated genes were significantly enriched in biological processes of digestive tract morphogenesis, chondrocyte development ($P<0.05$). The down-regulated genes were significantly enriched in development related biological process terms such as inferior colliculus development, urogenital system development, cell fate determination, anatomical structure formation involved in morphogenesis, cell maturation, negative regulation of fat cell differentiation, digestive tract development, inner ear morphogenesis, organ morphogenesis, cell development and cell fate commitment (Table 2). GATA2, GATA3 and FOXB1 were top three genes involved in significantly enriched GO terms.

Network construction of DEGs

The network of DEGs was constructed using Ingenuity Pathways Analysis (IPA) (Figure 4). Twenty-one DEGs was built into one network including 15 up-regulated genes and 6 down-regulated genes. Of those up-regulated genes, NPVF was associated with Cloacal bacterial burden after challenge with Salmonella E, FAP and PDE6B which were associated with

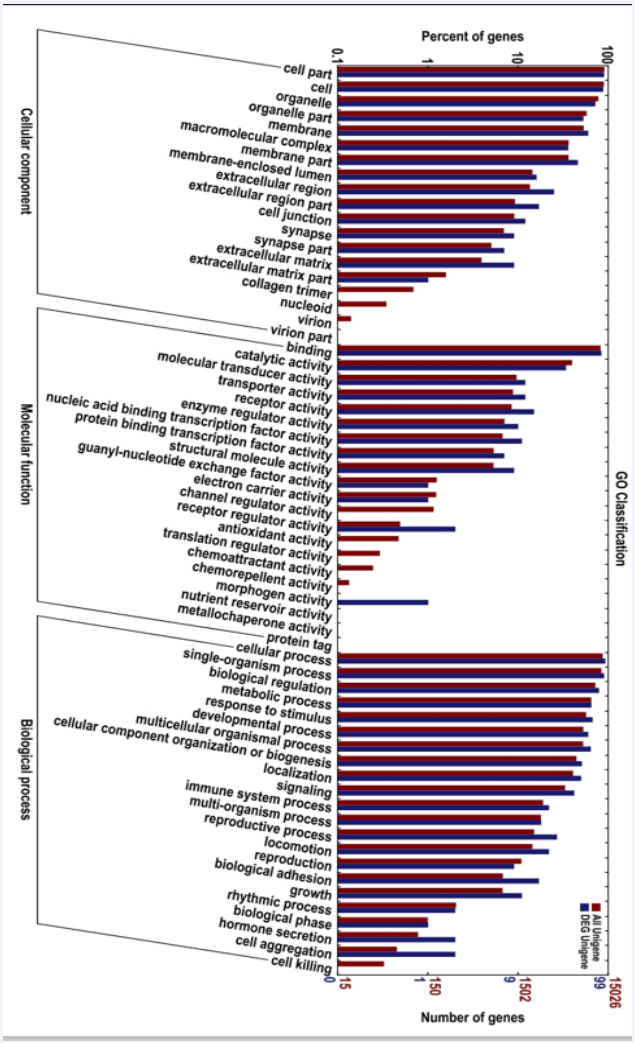

Figure 2 GO annotation of differentially expressed genes.

Table 2: Enriched GO-BP terms associated with down -regulated genes.

GO-BP Term	Fold Enrichment
Inferior colliculus development	499.67
urogenital system development	142.76
cell fate determination	90.85
anatomical structure formation involved in morphogenesis	90.85
cell maturation	52.60
negative regulation of fat cell differentiation	45.42
digestive tract development	43.45
inner ear morphogenesis	33.31
organ morphogenesis	33.31
cell development	29.39
cell fate commitment	29.39

Marek's disease-related traits, SCNN1B was associated with antibody titer to LPS antigen and LTA antigen, CYP19A1 was associated with Antibody titer to LTA antigen. Of those down-regulated genes, SYT2 was associated with Body temperature, HMOX1 was associated with Antibody response to SRBC antigen.

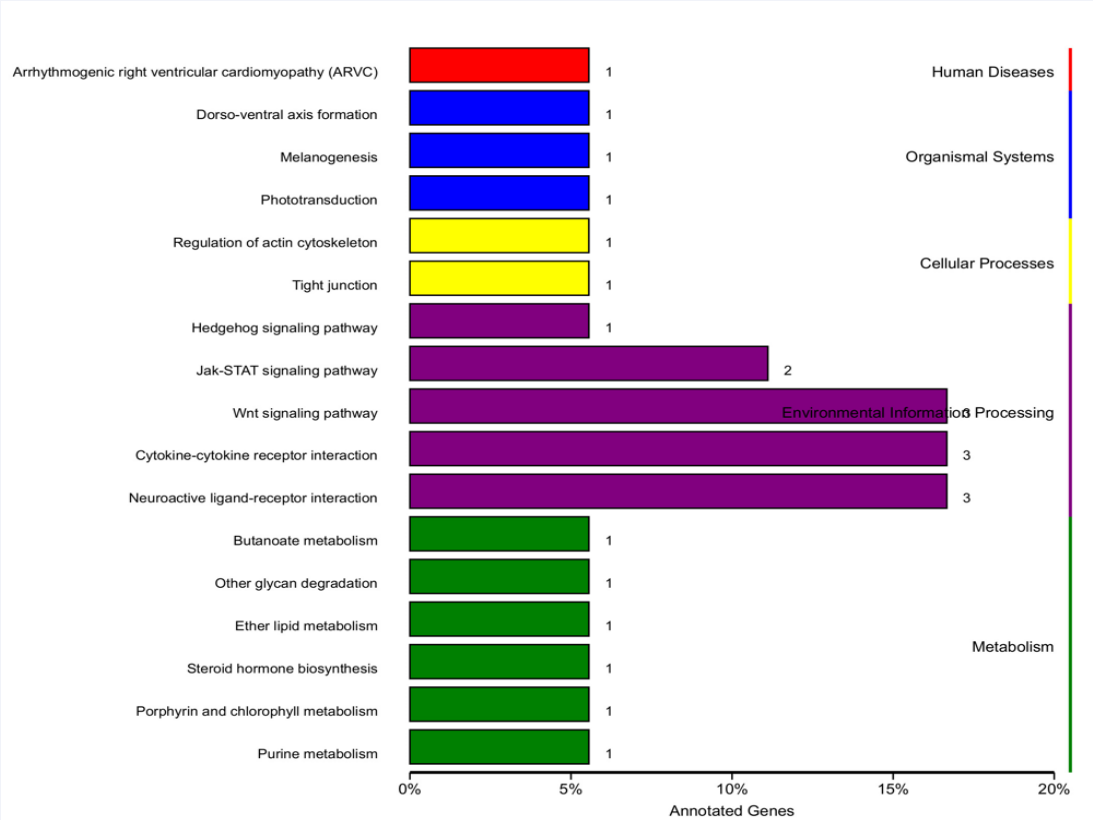

Figure 3 KEGG pathway annotation.

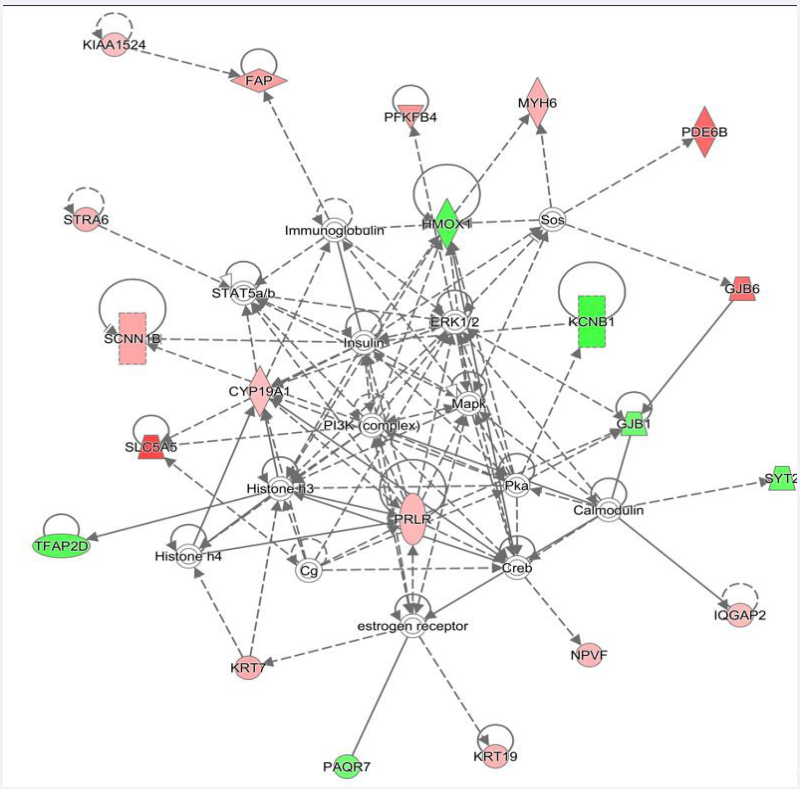

Figure 4 Network constructed through IPA.

DISCUSSION

The expression of mRNA and miRNAs related to *C. jejuni* inoculation in immunogical tissue have been reported [13-15]. Both bacteria and viruses have been implicated in a variety of neuropsychiatric disorders [16]. It has been reported that Guillain-Barré syndrome is caused by Campylobacter infection through contaminated food [17]. The gene expression profile responding to *C. jejuni* inoculation in hypothalamus has been investigated, several pathways and genes involved in the response to *C. jejuni* inoculation identified.

Wnt signaling pathway is one of important pathways in cell development, stem cell and cancer [18], and essential for B-cell development in chicken [19]. It was reported that Wnt receptor signaling pathway is associated with up-regulated genes following *C. jejuni* inoculation in chicken cecum [20]. The Wnt gene family is composed of structurally related genes that encode 19 secretory signaling proteins rich in cysteine. Wnt protein is a secretory signal transduction factor, which has many functions in the development of tumor. As a member of the Wnt signaling family, Wnt6 is involved in promoting the proliferation and differentiation of embryonic palatal mesenchymal cells, macrophages, and stromal cells. Wnt6 is also involved in tumorigenesis [21]. Wnt6 mRNA and protein are significantly up-regulated in patients with osteosarcoma [22], which is consistent with the current results. SFRP2 and WIF1 are inhibitors of Wnt signaling pathway [23], and frequently hyper methylated in tumor tissues [24,25]. SFRP2 inhibits Wnt6 in adult cardiac progenitor cell proliferation [26] and involved in Marek's disease-related traits. Wnt6 and SFRP2 co-express in mesoderm of ceca and cloaca [27]. WIF1 can prevent proliferation and keratinization of BCC-related keratinocytes [28]. The SFRP2 and WIF1 may regulate Wnt signaling pathway through methylation during *C. jejuni* inoculation.

The transcription factor FOXB1, as a new participant in the development of oligocytes, promotes neuronal formation and cell differentiation [29]. FOXB1 is involved in the formation of postmortem nerve tissue and inhibits pre-mortem development through FGF and Wnt pathways. FOXB1 inhibits BMP-dependent epidermal formation and promotes neural induction, thereby regulating ectoderm formation. The downstream and synergistic action of FOXB1 by the BMP inhibitor OCT-25 forms the regulatory network necessary to induce and maintain neural tissue [30]. FOXB1 is expressed in the neuroepithelium of the tail of the hypothalamus, which enables neurons to generate the later hypothalamic nuclei [31]. FOXB1 associated with antibody titer to LTA antigen was down-regulated following *C. jejuni* inoculation. The FOXB1 could regulate the Wnt signaling pathway through BMP in the response to *C. jejuni* inoculation in hypothalamus.

CYP19A1 gene, a member of the cytochrome P450 gene family, encodes the aromatase enzyme, which is involved in estrogen biosynthesis, as it promotes androgen aromatization in estrogens [32,33]. CYP19A1 plays a significant role in the sex-specific differentiation of brain and peripheral conversion of androgen to estrogen. Dysregulation of CYP19A1 can lead to disordered E2 secretion, abnormal GC status, ovarian dysfunction, and even infertility [34]. Inhibition of estrogens by anti-aromatase in embryos may induce a permanent female-male sex reversal [35]. The immune function and association with disease of CYP19A1 has been reported. CYP19A1 is a prognostic marker of breast cancer in human [33]. Locus of rs749292 of CYP19A1 is associated with breast cancer [36]. CYP19A1 associated with Antibody titer to LTA antigen was significantly up-expressed following *C. jejuni* inoculation. Female chickens were used in the current study, the gender could influence the response to *C. jejuni* inoculation. The CYP19A1 may play special role in the response to *C. jejuni* inoculation in female.

CONCLUSION

In conclusion, *C. jejuni* inoculation affects the transcriptome profile in hypothalamus. Wnt signaling pathway and CYP19A1 play important roles in *C. jejuni* inoculation in chicken. The findings herein will pave the foundation of the response of hypothalamus to *C. jejuni* inoculation in chicken. The effect of chicken gender on the response to *C. jejuni* inoculation will be interested.

ACKNOWLEDGEMENTS

This project was supported by the National Natural Science Foundation of China (31872343, 31172204), Shandong Modern Agricultural Industry & Technology System (SDAIT-11-02), National Key Research and Development Program of China (2021YFD1300102), and Shandong Province Agricultural Seed Project (2017LZN007).

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