Genome-Wide Analysis of DNA Methylation During
Ovule Development of Female-Sterile Rice fsv1

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ABSTRACT The regulation of female fertility is an important field of rice sexual reproduction research. DNA methylation is an essential epigenetic modification that dynamically regulates gene expression during development processes. However, few reports have described the methylation profiles of female-sterile rice during ovule development. In this study, ovules were continuously acquired from the beginning of megaspore mother cell meiosis until the mature female gametophyte formation period, and global DNA methylation patterns were compared in the ovules of a high-frequency female-sterile line (fsv1) and a wild-type rice line (Gui99) using whole-genome bisulfite sequencing (WGBS). Profiling of the global DNA methylation revealed hypo-methylation, and 3471 significantly differentially methylated regions (DMRs) were observed in fsv1 ovules compared with Gui99. Based on functional annotation and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis of differentially methylated genes (DMGs), we observed more DMGs enriched in cellular component, reproduction regulation, metabolic pathway, and other pathways. In particular, many ovule development genes and plant hormone-related genes showed significantly different methylation patterns in the two rice lines, and these differences may provide important clues for revealing the mechanism of female gametophyte abortion.

KEYWORDS rice female gametophyte abortion DNA methylation whole-genome bisulfite sequencing gene expression and regulation

Rice (Oryza sativa L.) not only feeds half of the global population, but it is also useful for basic molecular and genetic studies because of its relatively small genome size and complete genome information (Goff 1999; Delseny et al. 2001). As an important participant in the sexual reproduction of rice, the fertility of male and female gametes is directly related to rice yield. In recent years, attention has been focused on the mechanism of male fertility regulation, while few studies have been conducted to elucidate the mechanism of female fertility in rice. The ovule is an important sexual reproductive organ, and previous studies have demonstrated the inseparability of ovule development and female gametophyte formation (Reiser and Fischer 1993). Generally, the process of female gametophyte formation is mainly divided into three stages: meiotic division of the megaspore mother cell (MMC), mitotic stage of functional megaspore cells, and mature stage of embryo sac. In detail, the MMC originating from a sporogenous cell derives from below the epidermis of the nucellus and divides into the linear tetrad type after meiosis. Subsequently, the megaspore near the chalaza becomes the functional megaspore, and the remaining three megaspores die during ovule development. The functional megaspore undergoes three rounds of mitotic division and finally forms the female gametophyte with eight nuclei (Yadegari and Drews 2004; Pagnussat et al. 2005). The ovule sporophyte tissue provides the necessary nutritional and mechanical support for the formation of the female gametophyte, and communication between the ovule sporophyte and the female gametophyte has been proposed (Bencivenga et al. 2011). Formation of the female gametophyte in rice is a complex process that involves multiple genes and thus is influenced by changes in ovule gene expression patterns. Moreover, changes in gene expression in female gametophytes also affect ovule development (Johnston et al. 2007; Armenta-Medina et al. 2013; Wu et al. 2015).

In our experiment, a high-frequency female-sterile mutant rice line (fsv1) was derived from a rice cultivar, Gui99, by transferring the genomic DNA of Panicum maximum via the ear-stem injecting method.
Yang et al. (2016) have found that the high-frequency female gametophyte abortion in fsv1 is caused by the abnormal functional megaspore. Furthermore, many differentially expressed genes and miRNAs involved in ovule development and female gametophyte abortion between fsv1 and Gui99 have been identified by high-throughput sequencing (Yang et al., 2016, 2017). These findings provided important clues for revealing the molecular mechanism of female gametophyte abortion and guided the direction of our research.

DNA methylation plays an important role in regulating plant growth and development, and it is widely involved in the regulation of gene expression, transposition silencing and activation, chromatin-remodeling process, and maintaining the stability of the genome (Kim and Zilberman 2014; Niederhuth and Schmitz 2017). An abnormal DNA methylation status in plants will produce obvious abnormal phenotypes (Ong-Abdullah et al., 2015), while some studies have shown that DNA methylation is involved in plant sexual reproduction (Ingouff et al., 2017). Generally, plant DNA methylation is mainly distributed in three different sequence contexts: symmetric CG and CHG (hereafter mCG and mCHG) sites and asymmetric CHH (H = A, T, or C) sites (hereafter mCHH). Recently, some techniques have been applied for whole-genome methylation profile analysis, such as methylation-sensitive amplified polymorphisms, methylated DNA immunoprecipitation sequencing (MeDIP-seq), and whole-genome bisulfite sequencing (WGBS), among which WGBS is considered the gold standard for methylation sequencing. The principle of this technique is that nonmethylated cytosine (C) nucleotides are converted to uracil (U) by bisulfite treatment and are read as thymine (T) during sequencing, while methylated cytosines are protected from conversion and are still read as cytosine, thus distinguishing the original methylated from the modified cytosine. Combined with high-throughput sequencing technology and reference sequence alignment, the methylation status of the CG, CHG, and CHH sites can be determined to achieve single-base resolution mapping of the whole-genome DNA methylation map. In addition, methylation in the miRNA gene promoter region is a cause of abnormal miRNA expression regulation. The miRNA is expressed and the target gene is inactivated or inhibited when the promoter region of the miRNA is in the demethylated state. Studies have shown that miRNAs are involved in regulating multiple growth periods of plants, including reproductive development (Xie et al., 2015; Zhang et al., 2016). Therefore, methylation of the miRNA gene promoter region associated with ovule and female gametophyte development has become the focus of our attention.

In this study, we investigated global DNA methylation alterations in ovules of the rice fsv1 and Gui99 lines using WGBS. The aim of this work was to explore the DNA methylation patterns and their influence on gene expression during female gametophyte abortion in fsv1 ovule development. The results will provide important information to characterize the role of DNA methylation in the molecular mechanisms underlying female gametophyte sterility in rice.

**MATERIALS AND METHODS**

**Plant materials**

Two lines of rice (O. sativa L.), a high-frequency female-sterile rice line (fsv1) and a rice wild-type line (Gui99), were used in this experiment. The fsv1 mutant was obtained by transfusing the genomic DNA of Panicum maximum into Gui99 by ear-stem injection. After 36 generations of self-pollination, fsv1 exhibited stable genetic traits and almost the same genetic background as Gui99. The fsv1 and Gui99 rice lines had similar morphological characteristics, such as plant height, stem diameter, tiller number, and panicle length. The pollen of fsv1 was fertile, but ~80.5% of the female gametophytes were aborted. A detailed description of the materials is provided by Zhao et al. (1998) and Yang et al. (2016). The rice plants were grown and maintained in the greenhouse of Wuhan University, Wuhan, China.

According to the correspondence between the rice floret morphology and the development of the female gametophyte (Kubo et al., 2013), we collected the ovules from meiosis of the MMC stage until the mature embryo sac stage. We removed the ovules from the ovaries with an anatomical needle under a microscope and quickly placed them in liquid nitrogen for DNA extraction. Each sample was subjected to three biological replicates, from each of which was randomly harvested at least 600 florets on 50 plants during the normal growing season.

**DNA library construction and whole-genome bisulfite sequencing**

Total genomic DNA was extracted using a Plant Genomic DNA kit (TIANGEN, China) according to the manufacturer’s instructions. Agarose gel electrophoresis and an ultraviolet spectrophotometer were used to evaluate the quality of the genomic DNA. The three biological replicates of genomic DNA were pooled together for each DNA library. The two DNA samples were sonicated to produce DNA fragments using the AIR DNA Fragmentation Kit (Bioo Scientific Corporation). Different fragments of the overhanging structure were treated with T4 DNA polymerase and Klenow enzyme to form blunt ends, followed by the addition of A-tailing to the 3’ blunt ends to produce sticky ends. After end repair and adenylation, the DNA fragments were ligated to an Illumina sequencing primer adaptor. Bisulfite treatment of the genomic DNA was performed using the Zymo EZ DNA Methylation Lightning Kit. With this method, nonmethylated cytosine nucleotides are converted to uracil (U) and read as thymine (T) when sequenced. Methylated cytosines that were protected from conversion were still read as cytosine (C). The bisulfite-treated DNA was purified on a spin column and used to prepare the sequencing library. In this procedure, bisulfite-treated single-stranded DNA was random primed using a polymerase able to read uracil nucleotides to synthesize DNA containing a specific sequence tag. These tags were then used to add Illumina adapters by PCR at the 5’ and 3’ ends of the original DNA strand. The Epigene libraries were diluted and loaded onto the cBot DNA Cluster Generation System. After cluster generation was complete, the sample was transferred to the HiSeqation 4000 System for sequencing.

**Definition of genome elements**

The raw data obtained from Illumina sequencing were first processed to filter out reads containing adapters, unknown or low-quality bases, and then the unique clean reads were mapped to the rice reference genome (https://phytozome.jgi.doe.gov/pz/portal.html#info=alias=Org_Osativa) using the Alignment software Bowtie 2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml). Identification of genome elements was performed according to the method introduced by Li et al. (2012).

**Identification of differentially methylated regions (DMRs)**

WGBS detected the methylation status of single bases, the number of methylated cytosine loci, and the methylation ratio in each genome element. Significant differences in the DNA methylation ratio in the same genome regions between fsv1 and Gui99 were evaluated using the $\chi^2$ test. The false discovery rate (FDR) method was used to determine the threshold of the $P$-value in multiple tests (Benjamini and Yekutieli 2001). DMRs between fsv1 and Gui99 were considered with an FDR ≤0.001 and at least a 2.0-fold methylation ratio change in genome...
element sequence counts. The base data manipulations and statistical analysis were performed using the R package.

**Gene ontology (GO), KEGG pathway, MapMan, and clustering analysis**

A gene was considered to be a differentially methylated gene (DMG) when at least one DMR was located on this gene. DMGs are the focus of our research, and thus their function was a focus of our attention. Using the Blast2GO software (Version 2.3.5) to obtain GO information for each DMG, we used the WEGO website (http://wego.genomics.org.cn/) to upload the annotation information for each DMG, and the DMGs were classified according to their function. The GO enrichment analysis of the functional significance applies a hyper-geometric test to map all of the DMGs to terms in the GO database, searching for significantly enriched GO terms in the differentially methylated genes compared with the complete genome. The formula used in this analysis has been described by Yang et al. (2017).

The KEGG pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways in DMGs compared with the whole genome background. The calculation formula and the applied program were the same as that used in the GO analysis. Next, an FDR (Q-value) \( \leq 0.05 \) was used as the threshold to determine the most significantly enriched pathways in the DMGs.

MapMan software http://mapman.gabipb.org was used to describe the biological pathways associated with the DMGs, as described by Gao et al. (2013).

To provide an overview of hyper- or hypo-DMGs, Cluster software and JAVA TreeView software were used to perform the clustering analysis. A document containing the DMGs and their methylation ratios in both samples was uploaded to Gene Cluster 3.0. After data filtering and log transformation, hierarchical clustering of genes and arrays were performed by the average linkage method. Using the cdt. format file generated by the Cluster software with JAVA TreeView software, we can intuitively observe the clustering results of the graphical display.

**Bisulfite sequencing PCR**

Genomic DNA was modified using an EZ DNA Methylation Gold kit (Epigenetics) according to the manufacturer’s instructions. In short, 130 µl of the C-T conversion reagent was added to 2 µg of DNA sample and then incubated at 98°C for 10 min, 64°C for 2.5 hr, and held at 4°C. The modified DNA was purified using a Zymo-Spin IC Column (Epigenetics) and stored at −80°C until use. Primers for the selected genic sequences were designed using Methyl Primer Express v1.0 and were listed in Supplemental Material, Table S1. For each PCR reaction, 4 µl of bisulfite-treated DNA was used in a 20-µl reaction system. The PCR products were gel-purified using an AsyPrep DNA Gel Extraction Kit (Axygen, Hangzhou, China) and then cloned into the PCRII pGEM-T vector and sequenced. At least 15 clones were sequenced for each sample. The methylation levels were expressed as the percentage (%) per site for each of the three types of cytosines, CG, CHG and CHH, and were calculated by dividing the number of nonconverted cytosines by the total number of cytosines within the assay. Analyses of the bisulfite sequencing results were conducted using the Kismeth website (http://katahdin.mssm.edu/kismeth).

**Data availability**

The raw data files obtained in this study by Illumina sequencing have been released in the Sequence Read Archive Database of the National Center for Biotechnology Information. The accession numbers of two sequence read archive runs were SRR5684993 and SRR5684994 (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=run_browser).

**RESULTS**

**Overview of the DNA library sequencing data**

To clarify the whole-genome DNA methylation profiles, we sequenced the bisulfite-converted adaptor-ligated genome DNA fragments from developing ovules of fsv1 and Gui99 rice lines, respectively. The bisulfite conversion efficiency was >99%, providing a reliable guarantee of the accuracy of WGBS. A total of 122.8 million raw reads were generated via Illumina sequencing from the fsv1 and Gui99 libraries, and then FastQC was applied to assess the quality of the raw data. The results revealed high-quality sequencing (Figure S1). In total, 104.9 million clean reads were obtained after removing the low-quality reads, duplicate reads, and the 5’ and 3’ adapter nulls. Each library contained ~50 million clean reads, indicating that the depth and density of sequencing were sufficient to provide a high-quality genome-wide methylation analysis. We used Bowtie2 software to map the clean reads to the rice genome, and ~57% of the reads were uniquely mapped to the rice genome in each sample (Table 1). Only the uniquely aligned reads were used in the following analysis.

**Genome-wide DNA methylation profiles of fsv1 and Gui99 ovules**

By mapping the unique clean reads to the rice genome, we can accurately obtain the methylation status of each cytosine site. Since DNA methylation mainly occurs at three different sequence sites, the CG and CHG sites as well as the CHH sites (H = C, T or A), we calculated the methylation counts and methylation ratio of the three sequence contexts in each genome element by statistical analysis. A total of 1.59 billion cytosine sites were detected throughout the whole genome, of which 21.67% of the cytosine sites were methylated in fsv1 and 22.56% of the cytosine sites were methylated in Gui99 ovules. For all the sequences, the methylation density of the CG sequence was the highest, followed by the CHG sequence and the CHH sequence (Table 2). The visualization software Circos was used to show the methylation level of the 12 chromosomes in the two rice lines. According to the Circos results, genome elements with high methylation levels were mainly distributed on Chr1, Chr5, and Chr9, while those with low methylation levels were primarily on Chr11 and Chr12 (Figure S2).

**Characterization of DMRs**

We used statistics to measure the methylation ratio changes and define DMRs with at least a 2.0-fold change and a FDR \( \leq 0.001 \). All 3471 DMRs were classified into four types according to genome elements as
Table 2 The whole-genome methylation level of *fsv1* and Gui99 ovules

| Rice Line | Chr | CpG | mCpG | mCpG% | CHG | mCHG | mCHG% | CHH | mCHH | mCHH% | Total C | Total mC | C% |
|-----------|-----|-----|------|-------|-----|------|-------|-----|------|-------|--------|----------|-----|
| Gui99 | Chr1 | 3657714 | 1496039 | 40.9 | 3228108 | 1040813 | 32.24 | 11616288 | 1936072 | 16.67 | 18502110 | 4472924 | 24.18 |
| | Chr2 | 2905484 | 1071684 | 36.88 | 2636260 | 749393 | 28.43 | 9679734 | 1384263 | 14.30 | 15221478 | 3205340 | 21.06 |
| | Chr3 | 3021244 | 1140894 | 37.76 | 2724050 | 777474 | 28.54 | 9801969 | 1492353 | 15.23 | 15547263 | 3410721 | 21.94 |
| | Chr4 | 3053058 | 1174319 | 38.46 | 2679200 | 844076 | 31.50 | 9616649 | 1274372 | 13.25 | 15348907 | 3292767 | 21.45 |
| | Chr5 | 2539476 | 1177823 | 46.38 | 2218968 | 835699 | 37.66 | 8104757 | 1304133 | 16.09 | 12863201 | 3317655 | 25.79 |
| | Chr6 | 2594974 | 1072883 | 41.34 | 2295932 | 764712 | 33.31 | 8425977 | 1269531 | 15.07 | 13316883 | 3107126 | 23.33 |
| | Chr7 | 244714 | 936023 | 38.29 | 2158950 | 661921 | 30.66 | 8021141 | 1075987 | 13.41 | 12624805 | 2673931 | 21.18 |
| | Chr8 | 2314860 | 1003047 | 43.33 | 2061584 | 722042 | 35.02 | 7690127 | 1132860 | 14.73 | 12066571 | 2857949 | 23.68 |
| | Chr9 | 1910938 | 870190 | 45.56 | 1666110 | 625696 | 37.55 | 6205602 | 984000 | 15.86 | 9782650 | 2480715 | 25.36 |
| | Chr10 | 1928028 | 799691 | 41.47 | 1693122 | 578771 | 34.18 | 6263446 | 863446 | 13.79 | 9884776 | 2241908 | 22.68 |
| | Chr11 | 2280308 | 888872 | 38.98 | 2060684 | 645369 | 31.32 | 7836803 | 946497 | 12.08 | 12177805 | 2480738 | 20.37 |
| | Chr12 | 2166398 | 812002 | 38.30 | 1954890 | 587153 | 29.97 | 7452589 | 881038 | 11.82 | 1157927 | 2280193 | 19.69 |
| Total | 30817376 | 12443896 | 40.57 | 27381918 | 8833119 | 32.53 | 100715082 | 14544952 | 14.36 | 158914376 | 35821967 | 22.56 |
| *fsv1* | Chr1 | 3657714 | 1496039 | 40.9 | 3228108 | 1040813 | 32.24 | 11616288 | 1936072 | 16.67 | 18502110 | 4472924 | 24.18 |
| | Chr2 | 2905484 | 1071684 | 36.88 | 2636260 | 749393 | 28.43 | 9679734 | 1384263 | 14.30 | 15221478 | 3205340 | 21.06 |
| | Chr3 | 3021244 | 1140894 | 37.76 | 2724050 | 777474 | 28.54 | 9801969 | 1492353 | 15.23 | 15547263 | 3410721 | 21.94 |
| | Chr4 | 3053058 | 1174319 | 38.46 | 2679200 | 844076 | 31.50 | 9616649 | 1274372 | 13.25 | 15348907 | 3292767 | 21.45 |
| | Chr5 | 2539476 | 1177823 | 46.38 | 2218968 | 835699 | 37.66 | 8104757 | 1304133 | 16.09 | 12863201 | 3317655 | 25.79 |
| | Chr6 | 2594974 | 1072883 | 41.34 | 2295932 | 764712 | 33.31 | 8425977 | 1269531 | 15.07 | 13316883 | 3107126 | 23.33 |
| | Chr7 | 244714 | 936023 | 38.29 | 2158950 | 661921 | 30.66 | 8021141 | 1075987 | 13.41 | 12624805 | 2673931 | 21.18 |
| | Chr8 | 2314860 | 1003047 | 43.33 | 2061584 | 722042 | 35.02 | 7690127 | 1132860 | 14.73 | 12066571 | 2857949 | 23.68 |
| | Chr9 | 1910938 | 870190 | 45.56 | 1666110 | 625696 | 37.55 | 6205602 | 984000 | 15.86 | 9782650 | 2480715 | 25.36 |
| | Chr10 | 1928028 | 799691 | 41.47 | 1693122 | 578771 | 34.18 | 6263446 | 863446 | 13.79 | 9884776 | 2241908 | 22.68 |
| | Chr11 | 2280308 | 888872 | 38.98 | 2060684 | 645369 | 31.32 | 7836803 | 946497 | 12.08 | 12177805 | 2480738 | 20.37 |
| | Chr12 | 2166398 | 812002 | 38.30 | 1954890 | 587153 | 29.97 | 7452589 | 881038 | 11.82 | 1157927 | 2280193 | 19.69 |
| Total | 30817376 | 12210583 | 39.81 | 27381918 | 8557617 | 31.52 | 100715082 | 13645053 | 13.46 | 158914376 | 34413253 | 21.67 |
Hyper- or hypo-methylation of DMRs influences neighboring gene expression

We obtained annotated genome elements for each sample by mapping reads to the rice reference genome, and the promoters, exons, and introns were mapped to the genes. A total of 37,792 genes were found in each sample, of which 36,745 genes were methylated in *fsv1* and 36,802 genes were methylated in Gui99. The standard methylation status of the gene elements in *fsv1* and Gui99 were shown in Figure S3. In this study, genes that overlapped with the sequencing results in the promoter, exon, and intron for the two samples were termed as methylated genes. We screened out 3471 DMRs in gene regions, and they were distributed in 2348 genes, the function of which was the focus of our attention. Genes with similar methylation patterns usually indicate functional correlations. Cluster analysis of gene expression patterns was performed using Cluster and JAVA TreeView software, and gene methylation patterns were clustered using a distance metric based on the Pearson correlation (Figure 2). Hierarchical clustering can provide an overall understanding of DMGs, and the results showed that more DMGs in *fsv1* ovules had a hypo-methylated status.

Compared with the gene methylation status in Gui99 ovules, 1039 genes were hyper-methylated and 1309 genes were hypo-methylated in *fsv1* ovules (Table S2). Most of these DMRs were observed in gene promoters, and many of them were hypo-methylated in *fsv1*. Considering the transcriptome data for *fsv1* and Gui99 ovules (Yang et al. 2016), the analysis of the relationship between DNA methylation and gene expression revealed that hyper-methylation or hypo-methylation in some regions was related to the expression of neighboring genes (Table S3). For example, the promoter regions of several genes (e.g., LOC_Os02g10220, LOC_Os04g38950, LOC_Os04g01690, and LOC_Os09g30150) were hyper-methylated in *fsv1*, and the expression levels showed a down-regulation compared with Gui99. As shown in Table S3, 56 DMGs were hyper-methylated in exon regions, of which 33 genes were up-regulated and 23 genes were down-regulated in *fsv1*. The effect of gene body methylation on gene expression was obvious, but its mechanism and correlation remained unclear (Zhang et al. 2006; Zilberman et al. 2007). Thus, we discovered genes were highly methylated in exon or intron regions, while the expression levels of the genes could be up-regulated or down-regulated.

**GO analysis of DMGs in fsv1 and Gui99 ovules**

As described above, 2348 genes were found to be DMGs between *fsv1* and Gui99 ovules. We used the WEGO website to functionally categorize the methylated genes and analyze their significant differences. As shown in Table S4, a DMG could be annotated to different GO function terms, and a GO function term could also be annotated for many DMGs. All 757 DMGs out of the 2348 DMGs between *fsv1* and Gui99 ovules were annotated to the GO terms according to the Gene Ontology database (http://www.geneontology.org/), and they were distributed in 236 terms of three main GO categories (Figure 3 and Table S4). Among

### Table 3 Numbers of DMRs distributed in CGI and CGI shore

| Genome Elements | CGI | CGI Shore |
|-----------------|-----|-----------|
| Promoter        | 100 | 132       |
| Exon            | 43  | 47        |
| Intron          | 12  | 22        |
| Intergenic      | 0   | 0         |
| Total           | 155 | 201       |
them, 336 hyper-DMGs and 421 hypo-DMGs were assigned to 217 and 196 GO terms, respectively. The most abundant GO terms in the cellular component, molecular function, and biological process category were cell (GO:0005623), binding (GO:0005488), and metabolic process (GO:0008152), respectively.

The terms organelle (GO:0043226), membrane-bound organelle (GO:0043227), intracellular organelle (GO:0043229), intracellular (GO:0005622), and intracellular part (GO:0044424) were dominant in the cellular component category, and they showed statistically significant differences between the fsv1 and Gui99 lines, suggesting that organelles and intracellular activities may play important roles in ovule and female gametophyte development. In the molecular function category, it is worth mentioning that the term catalytic (GO: 0003824) was enriched with more cytochrome P450 DMGs, such as CYP709C9 (LOC_Os07g23570), LOC_Os06g43510, LOC_Os01g52800, LOC_Os02g36150, and LOC_Os03g37080, which will be discussed later. The largest number of functional groups was distributed in the biological process category, and some of the GO terms might be related to female reproductive processes. For example, DMGs assigned to the GO terms reproductive (GO:0022414), cellular process (GO:0009987), cell division (GO:0051301), cell cycle (GO:0007049), chromosome segregation (GO:0007059), megalametogenesis (GO:0009561), and gamete generation (GO:0007276) might participate in the formation of female gametophytes. However, the DMGs in the GO terms cell death (GO:0008219), hormone metabolic (GO:0042445), and gene silencing (GO:0016458) might lead to female gametophyte abortion.

The abortion of female gametophytes is a complex process involving multiple genes, and these GO entries could provide important clues for further studies of mechanisms underlying female gametophyte abortion.

**KEGG and MapMan pathway enrichment analysis of DMGs in fsv1 and Gui99 ovules**

To generate further insight about the pathways, we performed KEGG pathway analysis of the DMGs between fsv1 and Gui99. The major biochemical metabolic pathways and signal transduction pathways of DMGs were identified by KEGG significant enrichment. As a result, 75 pathways were enriched with DMGs, including metabolic pathway, plant hormone signal transduction, biosynthesis of secondary metabolites, and phenylpropanoid biosynthesis (Table 4 and Table S5). In contrast, MapMan software [http://mapman.gabipd.org/](http://mapman.gabipd.org/) was used to describe the DMG-associated biological processes to make these pathways more intuitive and detailed (Figure 4 and Table S6). The DMGs were enriched in metabolic, protein synthesis and degradation, transcriptional regulation, and hormone metabolism pathways (Figure S4). In plant hormone-related pathways, DMGs that were mainly enriched in auxin, ethylene, and jasmonate-related genes were hypo-methylated, while those related to brassinosteroid were hyper-methylated in fsv1.
DNA methylation of transcription factor (TF) gene analysis in fsv1 and Gui99 ovules

TF is a kind of DNA-binding protein that can directly or indirectly recognize or bind to the core sequence of various cis-acting elements to regulate the transcriptional efficiency of target genes. TF genes are widely involved in the regulation of plant growth and development. In total, 97 putative TF genes were identified among the 2348 DMGs detected in fsv1 and Gui99 ovules by blasting against the rice TF database http://planttfdb.cbi.pku.edu.cn/, and they were classified into 28 gene families (Table S7). Compared with Gui99 ovules, the

Table 4 The summary of KEGG pathways with the significantly change of methylated cytosine counts in fsv1 and Gui99

| Top 10 of Hyper-methylated Pathways | Top 10 of Hypo-methylated Pathways |
|------------------------------------|-----------------------------------|
| Biosynthesis of secondary metabolites (248) | SNARE interactions in vesicular transport (−223) |
| Pyrimidine metabolism (121) | Metabolic pathways (−203) |
| Butanoate metabolism (96) | Plant–pathogen interaction (−197) |
| Ribosome (85) | Protein processing in endoplasmic reticulum (−179) |
| Ubiquinone and other terpenoid-quinone biosynthesis (82) | Glycerophospholipid metabolism (−145) |
| Carbon fixation in photosynthetic organisms (80) | Glycerolipid metabolism (−118) |
| Pantothenate and CoA biosynthesis (77) | RNA transport (−118) |
| C5-Branched dibasic acid metabolism (77) | Homologous recombination (−117) |
| Starch and sucrose metabolism (68) | Nitrogen metabolism (−114) |
| Glycolysis/gluconeogenesis (68) | Phenylalanine metabolism (−112) |

Numbers in parentheses represented the total methylated cytosine counts of the DMGs identified in the pathway in fsv1 minus that in Gui99.
methylation levels of 37 putative TF genes were significantly hyper-methylated in fsv1, and the remaining 60 putative TF genes were hypo-methylated. It is worth noting that three putative AP2 family genes, OsHAP2A (LOC_Os08g09690), OsHAP2D (LOC_Os03g48970), and OsDREB1G (LOC_Os08g43210), were hyper-methylated in fsv1, while two ARF family genes (LOC_Os01g49120, LOC_Os01g59850) were hypo-methylated. In addition, some TF gene families were assigned to more DMGs, such as the bHLH family, bZIP family, C2H2 family, FAR1 family, MYB family, NAC family, and WRKY family. These TF genes may play important roles in ovule and female gametophyte development, meriting further study.

**Methylation patterns of phytohormone-related genes in the ovules of two rice lines**

Phytohormones are well known for their important regulatory roles in plant development. In abortive hazelnut ovules, significant changes occur in indole-3-acetic acid, gibberellins, cytokinin, salicylic acid, abscisic acid, ethylene, and jasmonic acid (Cheng et al. 2016). Therefore, the genes involved in the synthesis, transport, and response of these hormones have become the focus of our attention and may play important roles in ovule and female gametophyte development. Based on the KEGG and MapMan analysis results, we found that many phytohormone-related genes were enriched in the plant hormone signal transduction and hormone metabolism pathways. Auxin plays a vital role in plant growth and development and is involved in cell division, elongation, and differentiation (Benková et al. 2016). Based on the WGBS data, auxin-related DMGs were detected, among which an auxin efflux carrier family gene LOC_Os01g51780 was hyper-methylated in fsv1 and another six auxin efflux or auxin responsive family genes (LOC_Os01g48060, LOC_Os03g25289, LOC_Os12g34450, LOC_Os12g33060, LOC_Os03g25289, LOC_Os01g59850) were hypo-methylated. Furthermore, the available evidence indicates that Jasmonic acid plays an important role in ovule development (Cheng et al. 2016; Hu et al. 2016). Based on our data, several jasmonate ZIM domain protein genes were significantly differentially methylated, of which LOC_Os03g27900 and LOC_Os08g33160 were hyper-methylated and LOC_Os07g42370 and LOC_Os07g42370 were hypo-methylated in fsv1 compared with Gui99. In addition, some important plant hormone-related genes were differentially methylated in fsv1 ovules. For example, the ethylene sensitivity gene LOC_Os05g46240 and gibberelin oxidase gene LOC_Os05g43880 were hypo-methylated while gibberelin oxidase gene LOC_Os01g55240 was hyper-methylated in fsv1.

**MiRNA gene promoter methylation profiles in ovules of fsv1 and Gui99 rice lines**

MiRNAs are a class of noncoding small RNAs with a length of 20–24 nucleotides that are important negative regulatory factors and extensively involved in the regulation of plant growth and development (He and Hannon 2004). Eukaryotic gene promoters are important regulatory elements that are indispensable for gene expression, including transcription initiation sites, TATA boxes, and upstream cis-acting elements. The upstream promoter of the miRNA gene is closely related to the tissue and the developmental period expression specificity of the plant miRNA. The abnormal methylation status of the miRNA gene promoter may affect the synthesis of miRNAs and affect the expression of its target genes, leading to abnormal growth and development of the plant. Based on our results, 84 differentially methylated miRNA gene promoters were detected in fsv1 and Gui99 ovules (Table 5), of which 37 were hyper-methylated (for example, miR156i, miR166b-3p, miR166b-5p, miR166e-3p, miR166e-5p, miR169o, miR169g, miR169r-3p, miR169r-5p, miR172a, miR1432-3p, miR1432-5p, miR5535, among others), and 47 were hypo-methylated (miR160c-5p, miR166c-3p, miR166d-3p, miR166d-5p, miR167b, miR167f, miR171d-5p, miR171d-5p, miR1425-3p, miR1425-5p, miR5339, miR5491, miR5810, among others) in fsv1 ovules. Combined with miRNA and RNA-Seq analyses (Yang et al. 2016, 2017), we found that abnormal methylation of these miRNA gene promoters could lead to transcriptional abnormalities in miRNAs, and the abnormal expression of miRNA affected the expression of their coherent target genes (Figure 5). For example, the miR5504 gene promoter was hypo-methylated in fsv1, then we found that the expression level of

**Figure 4** MapMan analysis of 2348 DMGs. Red boxes in these overviews indicate hypo-methylated genes and blue boxes indicate hyper-methylated genes. Detailed information about the overview is shown in Table S5.
**Table 5** MiRNA promoters with significantly differentially methylated status between ovules of two rice lines

| MiRNA Name          | Log2 Fold-Change | Methylation Level | P-value          | Significance |
|---------------------|------------------|-------------------|------------------|-------------|
| osa-miR1425-3p      | -3.044394119     | Hypo-methylation  | 3.3523E−06       | **          |
| osa-miR1425-5p      | -3.087462841     | Hypo-methylation  | 2.0804E−06       | **          |
| osa-miR1432-3p      | 1.874649118      | Hyper-methylation | 0.026893         | *           |
| osa-miR1432-3p      | 1.044394119      | Hyper-methylation | 0.00027986       | **          |
| osa-miR1432-5p      | 1.736965594      | Hyper-methylation | 0.044044         | *           |
| osa-miR1435         | 1.169925001      | Hyper-methylation | 0.039095         | *           |
| osa-miR156i         | 3                | Hyper-methylation | 0.016686         | *           |
| osa-miR159d         | -2.807354922     | Hypo-methylation  | 0.0034877        | **          |
| osa-miR160c-3p      | -1.144389909     | Hypo-methylation  | 0.0052627        | **          |
| osa-miR160c-5p      | -1.10433666      | Hyper-methylation | 0.0061281        | **          |
| osa-miR166b-3p      | 1.502500341      | Hyper-methylation | 0.016861         | *           |
| osa-miR166b-5p      | 1.736965594      | Hyper-methylation | 0.0044014        | **          |
| osa-miR166c-3p      | -3.169925001     | Hypo-methylation  | 0.013607         | *           |
| osa-miR166d-3p      | -3                | Hypo-methylation  | 0.022957         | *           |
| osa-miR166d-5p      | -3                | Hypo-methylation  | 0.022957         | *           |
| osa-miR166e-3p      | 1.584962501      | Hyper-methylation | 0.0059267        | **          |
| osa-miR166e-5p      | 1.584962501      | Hyper-methylation | 0.0059267        | **          |
| osa-miR167b         | 1.125308822      | Hypo-methylation  | 0.037497         | *           |
| osa-miR167f         | -1.263034406     | Hypo-methylation  | 0.022275         | *           |
| osa-miR169g         | 2                | Hyper-methylation | 0.0055546        | **          |
| osa-miR1806i        | 2.321928095      | Hyper-methylation | 0.017365         | *           |
| osa-miR1809         | 1.807354922      | Hyper-methylation | 0.014604         | *           |
| osa-miR1848         | -1.754887502     | Hypo-methylation  | 0.0019694        | **          |
| osa-miR1850.2       | 1.321928095      | Hyper-methylation | 0.039814         | *           |
| osa-miR1850.3       | 1.321928095      | Hyper-methylation | 0.039814         | *           |
| osa-miR1859         | 1.584962501      | Hyper-methylation | 0.037516         | *           |
| osa-miR2096-3p      | -2.402098444     | Hypo-methylation  | 0.000011225      | **          |
| osa-miR2100-3p      | 1.192645078      | Hyper-methylation | 0.048544         | *           |
| osa-miR2100-5p      | 3.169925001      | Hypo-methylation  | 0.013607         | *           |
| osa-miR2275a        | 2.129283017      | Hyper-methylation | 0.000021442      | **          |
| osa-miR2863b        | 1.736965594      | Hyper-methylation | 0.044044         | *           |
| osa-miR2873a        | 2.906890596      | Hyper-methylation | 0.001213         | **          |
| osa-miR2907c        | 1                 | Hypo-methylation  | 0.0018074        | **          |
| osa-miR2919         | 1.286881148      | Hyper-methylation | 0.00004735        | **          |
| osa-miR2932         | 1.584962501      | Hyper-methylation | 0.011048         | *           |
| osa-miR319a-3p.2.3p | -1.169925001     | Hypo-methylation  | 0.022704         | *           |
| osa-miR319a-3p.2.2p | -1.169925001     | Hypo-methylation  | 0.022704         | *           |
| osa-miR396b-3p      | -1.584962501     | Hypo-methylation  | 0.0065944        | **          |
| osa-miR396b-5p      | -1.222392421     | Hyper-methylation | 0.016184         | *           |
| osa-miR396c-3p      | -2.584962501     | Hypo-methylation  | 0.0093555        | **          |
| osa-miR396d         | -1.201633861     | Hypo-methylation  | 0.031607         | *           |
| osa-miR3981-3p      | 1.137503524      | Hyper-methylation | 0.025448         | *           |
| osa-miR3981-5p      | 1.070389328      | Hyper-methylation | 0.036884         | *           |
| osa-miR399c         | -1.485426827     | Hypo-methylation  | 0.047871         | *           |
| osa-miR399g         | -1.247927513     | Hypo-methylation  | 0.043997         | *           |
| osa-miR399j         | -2.169925001     | Hypo-methylation  | 0.040849         | *           |
| osa-miR415          | -1.700439718     | Hyper-methylation | 0.035614         | *           |
| osa-miR439c         | -1.502500341     | Hyper-methylation | 0.0018721        | **          |
| osa-miR439d         | -1.874649118     | Hyper-methylation | 0.00003647        | **          |
| osa-miR5071         | -1.321928095     | Hypo-methylation  | 0.030533         | *           |
| osa-miR5071         | -2.321928095     | Hypo-methylation  | 0.025026         | *           |
| osa-miR5145         | 1.298982676      | Hyper-methylation | 0.0248668        | **          |
| osa-miR5162         | -1.440572591     | Hypo-methylation  | 2.731E−10        | **          |
| osa-miR5339         | -4.058893689     | Hypo-methylation  |                   |              |
miR504 was up-regulated in miRNA data and the expression level of its coherent target gene LOC_Os03g48970 was down-regulated in fsv1 of RNA-Seq.

Validation of the WGBS data by bisulfite sequencing

In this study, the DNA methylation patterns of 10 DMR-associated genes were randomly selected to carry out bisulfite sequencing to validate the WGBS data (Figure S5), including LOC_Os08g30660 (NB-ARC domain-containing disease resistance protein), LOC_Os12g1890 (amino acid transporter 1), LOC_Os06g47350 (RNA polymerase I-specific transcription initiation factor RRN3 protein), LOC_Os10g34490 (Nucleotide-sugar transporter family protein), and LOC_Os12g08300 (glycosyltransferase family protein 2), among others (Figure 6, A–D). We found that more genes were hypo-methylated in fsv1 compared with Gu99, and the results were almost consistent with the WGBS data. For example, LOC_Os01g17310 (LRR and NB-ARC domains-containing disease resistance protein), LOC_Os12g31180 (retrotransposon protein), and LOC_Os10g34490 (nucleotide-sugar transporter family protein) showed lower methylation levels in fsv1. However, LOC_Os05g16680 (retrotransposon protein) and LOC_Os12g1890 (amino acid transporter 1) were hyper-methylated in fsv1 ovules.

DISCUSSION

DNA methylation plays an important regulatory role in plant development and fertility, and whole-genome DNA methylation surveys have been performed in thermo-sensitive male sterile rice and other plants (Vining et al. 2012; Hu et al. 2015; Ausin et al. 2016), while few studies have been reported about the methylation profiles of female-sterile rice lines (for example fsv1) during female gametophyte development. The objective of the present study was to perform a genome-wide identification of methylated genes that affect ovule development and female gametophyte formation in fsv1. To confirm the results obtained from WGBS, the methylation patterns of 10 gene regions in each sample were analyzed by bisulfite sequencing, and the methylation levels of the samples obtained by the two methods were basically consistent, indicating that the high-throughput bisulfite sequencing results were reliable.

DNA methylation plays an important role in gene expression and regulates plant growth and development. The ovules of the female-sterile rice line fsv1 displayed a slightly lower whole-genome methylation level than Gu99, while localized regions of the genome showed a significantly varied hyper-methylation or hypo-methylation status in fsv1. The abnormal methylation status in gene elements may lead to changes in gene expression levels. Generally, promoter methylation is often linked to the suppression of gene expression, whereas the role of gene body methylation is far more uncertain, demonstrating both positive and negative relationships to gene expression (Zhang et al. 2006; Zilberman et al. 2007). The development of the female gametophyte involves precise and complex gene activities, and changes in the expression levels of individual genes may lead to female gametophyte abortion (Kubo et al. 2013). Previous studies have shown that genes that regulate the female gametophyte may be derived from genes expressed by the female gametophyte itself, and may also be derived from genes expressed in the ovule sporophyte tissue (Tucker et al. 2012; Song and Chen 2015).

In the rice mutant OsAPC6, female gametophyte development is abnormal. The abnormal mitotic divisions during megagametogenesis can be attributed to the inactivation of APC6/CDC16 of the anaphase-promoting complex in rice, which is responsible for cell cycle progression (Kumar et al. 2010; Awasthi et al. 2012). In the current research, OsAPC6, as a key gene involved in the development of female gametophytes, was significantly hypo-methylated in the promoter region of fsv1 compared with Gu99. Moreover, RNA-Seq revealed that the OsAPC6 gene was up-regulated in the meiosis stage and mature embryo sac stage and down-regulated in the mitosis stage in fsv1 (Yang et al. 2016). Thus, we speculate that changes in the expression levels of the OsAPC6 gene may affect the formation of female gametophytes. In Lactuca sativa, the concentration and distribution of calmodulin are critical for the occurrence of megaspore (Qiu et al. 2008). In our study,
several key genes in the calmodulin signaling pathway, such as OsCam3, LOC_Os10g27170 (calmodulin-binding family protein), LOC_Os08g4670 (calmodulin-binding family protein), and LOC_Os09g13890 (plant calmodulin-binding protein-related), exhibited significant differences in methylation in fsv1 ovules compared with Gui99. These differential methylation regions may affect female gametophyte fertility by regulating the expression of key genes in the calmodulin signaling pathway. Moreover, previous studies have shown that cytochrome P450 is a metabolic enzyme that is widely found in plants and plays a key role in regulating the growth and development of ovules (Adamski et al. 2009; Ma et al. 2015). In Arabidopsis, a CYP450 enzyme CYP85A1 is required for the initiation of female gametogenesis (Pérez-Españo et al. 2011), and the cyp78a8 cyp78a9 loss-of-function mutant shows abnormal ovule development, resulting in abortion of the majority of female gametophytes in the mutant (Sotelo-Silveira et al. 2013). In the present study, CYP97A4 (LOC_Os02g57290), CYP709C9 (LOC_Os07g23570), and several putative cytochrome P450 family genes (such as LOC_Os01g52800, LOC_Os02g09330, LOC_Os03g25150, LOC_Os06g43510, LOC_Os11g04310, among others) were differentially methylated in fsv1 and Gui99 ovules, of which more DMGs were hypo-methylated in fsv1. Accordingly, the expression levels of CYP97A4 and LOC_Os06g46680 were down-regulated, while LOC_Os06g43510, LOC_Os03g25150, LOC_Os07g29960, LOC_Os02g09400, and LOC_Os02g36280 were up-regulated in fsv1 ovules (Yang et al. 2016), and these differentially expressed CYP genes may be associated with female gametophyte fertility.

SNF2 family proteins are ATP-dependent chromatin-remodeling factors that control many aspects of DNA events such as transcription, replication, homologous recombination, and repair. In Arabidopsis, many genes in the SNF2 family have been shown to be involved in ovule and embryo development (Bleyard et al. 2004; Huanca-Mamani et al. 2005). Embryo sac development of the atrad50 mutant is severely damaged, and this mutant does not produce any functional megaspores or viable gametes (Bleyard et al. 2004). An SNF2 family gene, CHRL1, encodes a chromatin-remodeling protein that is abundantly expressed during female gametogenesis in Arabidopsis, and specific degradation of the endogenous CHRL1 mRNA by RNA interference (RNAi) results in an increased frequency of ovule abortion and defective female gametophytes prior to completion of the mitotic haploid nuclear divisions (Huanca-Mamani et al. 2005; Yang et al. 2010). In rice, the SNF2 family of proteins exhibits a similar structure and function to Arabidopsis thaliana (Hu et al. 2013). In our study, CHR721 and CHR722 belonged to the SNF2 family gene, were hyper-methylated in intron of fsv1 ovules compared with Gui99, and the expression levels of CHR721 and CHR722 were generally down-regulated (Yang et al. 2016). Thus, we speculate that these methylation differences in genes probably cause changes in expression levels and are likely to be closely related to female gametophyte abortion in fsv1.

In addition, TFs are significantly involved in plant female sexual reproduction as an important regulatory factor in gene expression (Bencivenga et al. 2012; Makkena et al. 2012). Studies have shown that DNA methylation can regulate the expression of TF genes in floral organ differentiation and development in plants (Sheldon et al. 1999; Zhao et al. 2005). Changes in the methylation status of some TF genes may affect female gametophyte fertility-related gene expression. In Arabidopsis, the TF SPL is required for cytokinin and auxin signaling during ovule development (Bencivenga et al. 2012). In rice, OsSPL1 encodes long-chain base sphingosine-1-phosphate lyase, which acts as a signaling mediator in regulating programmed cell death (Zhang et al. 2014). In our study, two genes in the SPL TF family, OsSPL1 and SPL28, showed significant differences in methylation between fsv1 and Gui99 ovules. We hypothesize that changes in the methylation pattern of these two genes may cause programmed cell death during ovule development, leading to female gametophyte abortion. The AP2 (APETALA2) multigene family can be divided into AP2 and ANT groups, including developmentally and physiologically important TFs (Shigyo et al. 2006). In Arabidopsis, the AP2 gene is expressed in egg and synergistic cells, and strong ant mutants have ovules that fail to form integuments or a female gametophyte (Klucher et al. 1996; Wang et al. 2009). In our study, three AP2 family genes, OsHAP2A, OsHAP2D, and OsDREB1G, were significantly hyper-methylated in promoter regions in fsv1 compared with Gui99, and this high level of methylation in the promoter region may lead to a decrease in the gene expression level, which probably affects ovule development and female gametophyte fertility.
It is well known that plant hormones play essential parts in plant growth and development (Durbak et al. 2012). The available studies have shown that plant hormones are involved in ovule development (Bencivenga et al. 2012; Cheng et al. 2016). Auxin is one of the most important plant hormones and is involved in a number of biological processes of plant growth, development, and reproduction. Previous studies have shown that the expression of auxin biosynthesis and transport-related genes plays an important role in the formation of fertile female gametophytes (Panoli et al. 2015). ARF is a very important auxin response gene family, and the gene expression of this family can respond to changes in auxin (Ellis et al. 2005; Wang et al. 2007). In fsv1 and Gui99 ovules, OsARF2 was significantly hypo-methylated in fsv1 compared with Gui99, and its expression level was up-regulated during meiosis (Yang et al. 2016). In contrast, PIN genes encode efflux carrier proteins of the phytohormone auxin in rice (Wang et al. 2009; Miyashita et al. 2010), while functional megaspore development is discontinued during mitosis to mutate the vast majority of female gametophytes in the PIN1 mutant (pDEFH9;amiPIN1) of A. thaliana (Ceccato et al. 2013). We found that several PIN genes showed differential methylation in our analysis, among which OsPIN8 was significantly hyper-methylated in fsv1, and their expression levels were up-regulated during meiosis and mitosis (Yang et al. 2016). The differential methylation of these two gene families, resulting in gene expression, may result in changes in the female gametophyte fertility of fsv1. The gibberellin class of plant hormones plays a role in many processes during plant development, including seed germination, cell elongation, flowering, and fruit setting (De Jong et al. 2009). Recent studies have shown that the gibberellin level is significantly reduced in abortive hazelnut compared with wild-type ovules (Cheng et al. 2016). In our study, two gibberellin oxidase genes, OsGA2ox3 and OsGA2ox4, were significantly differentially methylated in exon regions in fsv1 compared with Gui99 ovules, leading to further changes in expression levels and abnormal ovule development. In plants, many physiological and developmental processes, such as seed germination, flower senescence, and fruit ripening, are controlled by ethylene (Rieu et al. 2003; Linkies and Leubner-Metzger 2012). Ethylene also plays a significant role in ovule development and female gametophyte fertility (Tsai et al. 2008; Clark et al. 2010). We found that an ethylene-sensitive gene, OsRTH2, and an ethylene-forming enzyme gene, OsACO3, were significantly hypo-methylated in fsv1. The RNA-Seq data showed that the two genes were up-regulated and then down-regulated during ovule development (Yang et al. 2016), suggesting that these changes might be associated with female gametophyte abortion. Furthermore, jasmonic acid (JA) signaling has been well studied in Arabidopsis, and most reports have focused on the role of JA in biological pathways such as stress resistance, trichome initiation, and anthocyanin accumulation. However, there is some evidence that JA signaling pathways are involved in female sexual reproduction. For example, changes in the expression of key genes involved in jasmonate signaling pathways affect the formation of linit and fluff fibers in cotton ovules (Kim et al. 2015; Hu et al. 2016). In hazelnut, the JA signaling pathway is involved in ovule abortion (Cheng et al. 2016), and the key enzyme in the synthesis of JA precursors in the JA signaling pathway, AOC protein, is specifically expressed in ovules of tomato (Hause et al. 2000, 2003). In our study, several key genes in the rice jasmonate pathway, such as OsJAZ3, OsJAZ14, OsJAZ15, and OsJAZ27, were significantly differentially methylated and expressed in fsv1, and we speculated that these changes likely affected the development of the female gametophyte. The miRNAs play an essential role in the growth and development of plants, including sexual reproduction (Yang et al. 2013; Ding et al. 2016). Many studies have demonstrated that changes in the expression of key genes involved in important signaling pathways during ovule development can affect the fertility of female gametophytes (Olmedo-Monfils et al. 2010; Tucker et al. 2012). miRNAs play an important regulatory role in gene expression, and their targeted genes are involved in multiple metabolic pathways during ovule development (Xie et al. 2020).
In plants, the SBP-box genes are key genes regulating sexual reproductive growth (Shikata et al. 2009). In tomato, miR156 can regulate pistil development by targeting the SBP-box gene (Silva et al. 2014). In this study, the promoter region of the miR156i gene was hyper-methylated in fsv1 ovules, which may inhibit the expression of miR156i and thus affect the expression of the SBP-box genes related to the formation of fertile female gametophytes. As mentioned above, auxin plays an important role in the regulation of plant ovule development. When some auxin-related genes are abnormally expressed in ovules, the female gametophyte becomes stagnant, and the cells differentiate abnormally, seriously affecting the female gametophytes (Ceccato et al. 2013; Panoli et al. 2015). In Arabidopsis, miRNAs can regulate the fertility of female gametophytes by regulating changes in auxin-related gene expression, in which miR160 targets multiple ARF family genes. Abnormal expression of miR160 alters the expression of auxin-related genes, leading to smaller petals and early maturation of A. thaliana, among other features (Mallory et al. 2005). In addition, miR167 controls the development of ovules and anthers by targeting the TF genes ARF6 and ARF8, and its abnormal expression in petals and stamens causes abortion (Wu et al. 2006). In the present study, the promoter regions of the miR160c-3p, miR160c-5p, miR167b, and miR167f genes were significantly hypo-methylated, potentially leading to the abnormal expression of miR160 and miR167 and thus affect the synthesis of auxin, which further influences female gametophyte formation. PHB, PHA, and CNA, which are members of the HD-ZIP TF family, are key genes in the regulation of ovule development. In the A. thaliana mutant phb-13phv-11cna-2, ovule development exhibits severe defects that lead to the abortion of the majority of female gametophytes (Prigge et al. 2005). Experiments have shown that miR166 targets the expression of HD-ZIP family genes in plants (Zhou et al. 2015). In our study, the promoter methylation statuses of the miR166b-3p, miR166b-5p, miR166c-3p, miR166d-3p, miR166d-5p, miR166e-3p, and miR166e-5p genes were abnormal in fsv1 ovules, potentially leading to changes in the expression of target genes and ultimately affecting the development of ovules and causing female gametophyte abortion. In rice, miR172 targets and regulates AP2 family member genes, and the floral organs display serious developmental defects that lead to a significant reduction in fertility in miR172-overexpressing mutant plants (Zhu et al. 2009). In our study, the miR172a gene promoter region was hyper-methylated in fsv1 compared with Gui99, potentially affecting the expression levels of AP2 family genes related to female gametophyte fertility. Rice miRNAs are closely related to fertility, and DNA methylation is very important for the regulation of miRNAs, supporting the importance of further studies.

In summary, we have systematically investigated changes in DNA methylation levels in the highly female-sterile and wild-type rice ovules by high-throughput sequencing and have obtained a large number of DMRs and genes. Based on the whole-genome DNA methylation map, hypo-methylation was observed in fsv1, and the DMGs were involved in many pathways, such as plant hormone metabolism, DNA replication and transcriptional regulation, and glycophospholipid metabolism. We propose that female gametophyte fertility is a complex process involving multiple pathways, and that changes in methylation may affect this process. We also found that some auxin-related genes, TF genes, and miRNA gene promoter regions displayed significant differences in methylation in fsv1 and Gui99 ovules, and these DMGs are being further examined in our laboratory. In conclusion, the above studies reveal the genome methylation pattern of ovules of the female-sterile rice line fsv1 and fertile rice line Gui99, providing important clues for further analyses of the molecular mechanism of female gametophyte fertility.

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