GENOME-WIDE ANALYSIS OF DNA METHYLATION PATTERNING IN ALFALFA (*MEDICAGO SATIVA* L.) UNDER MUTAGENIC TREATMENT USING BISULFITE-SEQUENCING (BS-SEQ)

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Abstract. DNA methylation is an important type of epigenetic modification that plays crucial roles in many biological processes. To investigate the epigenetic effect mechanism of ethyl methanesulfonate (EMS) mutation treatment on alfalfa, the whole genome DNA methylation profile of normal growth alfalfa and EMS mutation treatment alfalfa were analyzed by whole-genome bisulfite sequencing (WGBS) technology. The results showed that there were three main types of alfalfa DNA methylation: mCG, mCHH and mCHG; after alfalfa was mutagenized, the rate of methylation at the C site increased, and the methylation rates of CHH, CG and CHG all increased. In addition, DNA methylation mainly occurs in the CG sequence; a total of 8707 DMRs were detected in the differential methylation region (DMR) identification study. Among them, there were 5221 and 3486 hypermethylated DMRs and hypomethylated DMRs after alfalfa mutagenesis. Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses revealed some DMGs related to the occurrence of physiological metabolic processes. Finally, we found that 33 DMGs, including MTR_2g015550, MTR_6g088795, MTR_4g011180 and MTR_0034s0170, are more likely to be involved in plant carbon metabolism, nitrogen metabolism pathways, various amino acid metabolisms and glucose metabolism.
Keywords: *genome-wide DNA methylation, alfalfa, ethyl methane sulfonate, differentially methylated region, methylation profile*

Introduction

Alfalfa (*Medicago sativa* L.) is known as "the King of Forage" because of its high yield, high quality and wide adaptability in the livestock industry. The "Document No. 1" of the Central Government clearly stated: "Accelerate the development of the forage industry, support the cultivation of alfalfa and silage, carry out a combination of grain-feeding and planting, and promote the coordinated development of the ternary planting structure of grain, cash crops, and forage." To develop and grow the forage industry, we must first solve the problem of supplying a large number of high-quality forage. However, the bottleneck of alfalfa industry development in China is mainly the lack of resistant germplasm and high-yield variety resources. High-quality alfalfa species still depend on foreign imports. With the development of animal husbandry in China, animal husbandry in different regions is developing and growing, which has higher requirements for alfalfa yield and quality. China's animal husbandry is mainly concentrated in the northeast and western regions. However, there is a problem of low overwintering rate of alfalfa in the northeast and other alpine regions, which seriously affects the yield and quality of alfalfa and reduces the economic benefits of growers. In

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the northwest and other saline alkali areas, the poor salt-tolerance ability of domestic alfalfa also seriously affected the yield of alfalfa, resulting in the low quality and small quantity of domestic alfalfa. In view of the above problems, this study used mutation method to treat alfalfa in order to screen out different resistant or high-yielding variant strains, and analyze the epigenetic characteristics before and after the mutation to provide a theoretical basis for enriching alfalfa germplasm resources. Therefore, it is necessary improve the quality of domestic varieties through improving and enriching forage germplasm resources by using innovative scientific and technological means. Practice has proved that the use of artificial mutation technology is one of the important means of enriching crop germplasm resources, creating new materials and breeding new species (Mashinsky et al., 2001). Mutation breeding has become a method widely used by breeding scholars. In the study of biological effects, mutagenesis mainly studies the changes of cell phenotypes or physiological and biochemical characteristics caused by mutagenesis (Singh et al., 2006; Li et al., 2013; Shi et al., 2010), but in the existing reports, there is a lack of mutagenesis studies on plant epigenetics.

DNA methylation has the function of protecting the genome from exogenous inserted sequences and regulating gene expression in plants, and at the same time, it can control gene expression, maintain genome stability, and play an important role in heterosis. In recent years, with the rapid development of second-generation sequencing technology, genome-wide DNA methylation research has also developed rapidly, and its research has played an important role in many fields such as biology, medicine, agriculture, and the environment (Xue, 2017). Studies by Mirbahai and Grafi (Mirbahai et al., 2014; Grafi. 2011) showed that plant cytosine DNA methylation changes under stress conditions, indicating that plants are involved in DNA methylation in the process of resisting stress environments. Kim et al. (2013) found that radiation can cause changes in plant DNA methylation: the level of genomic DNA methylation in wild-type *Arabidopsis* decreases with increasing dose of gamma radiation. Studies by Shi Jinming et al. (2009) and Zhao et al. (2016) showed that the epigenetic effect of dried rice seeds under heavy ion radiation was significant, and the rate of methylation change of cytosine was higher than that of demethylation.

At present, genome-wide DNA methylation research is mainly concentrated in the fields of human diseases and livestock and poultry. In terms of crops, there are many studies on the methylation changes of single genes or specific sites, but there are few studies on the analysis of the DNA methylation status and methylation patterns of mutational stress alfalfa at the whole genome level. Previous studies have shown that whole-genome bisulfite sequencing (WGBS) is the most comprehensive of the existing methods. In this study, we investigated DNA methylation profiles of alfalfa before and after EMS mutagenesis during the seedling stage using WGBS technology. Our research systematically analyzed the molecular differences of alfalfa tissue methylation levels before and after mutagenesis. In addition, our findings will advance knowledge and understanding of the alfalfa methylome.

Materials and methods

Plant material and mutation treatment

Longmu 806, a main alfalfa variety planted in Heilongjiang Province, P. R. of China. It was selected at the Heilongjiang Province Animal Husbandry Research Institute.

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Ethyl methanesulfonate (EMS) treatment (Shen et al., 2018): Select 300 full-fledged mature alfalfa seeds, treat with concentrated sulfuric acid for 5 min, and then rinse with distilled water multiple times. The seeds were soaked in phosphate buffer solution (100 mmol • L^{-1}, pH 7.0) at 4 ℃ for 12 h, and the seeds were completely submerged in water to swell. Phosphate buffer solution was used to prepare a 0.4% EMS solution (preliminary tests have screened out the most treatment concentration) (Shen et al., 2018; Shen, 2018). The seeds were treated at room temperature for 15 h under dark conditions, and then repeatedly washed with distilled water to remove the residual EMS solution on the surface of the seeds. Untreated seeds were used as controls, and three experimental replicates were set for each experimental treatment.

DNA extraction, WGBS library construction and sequencing

Collect a mixture of leaves with consistent growth and development at the three-leaf stage, 10 strains as a sample, and the test samples were control group (A34_1A) and EMS treatment group (A34_2A). The construction of whole-genome bisulfite sequencing (WGBS) library mainly includes the following four aspects: the genomic DNA was extracted by CTAB method; Genomic DNA was interrupted into 300-700 bp fragments by Bioruptor Pico ultrasound; and then bisulfite conversion was performed using the EZ DNA Methylation Gold Kit; the Accel-NGS® Methyl-Seq DNA Library Kit is used to build single-stranded DNA libraries. After library quality detection, pair-end sequencing was performed with an Illumina XTen (Illumina, San Diego, CA, USA) sequencer, and the specific method was referred to the literature (Guo et al., 2017). All operations were conducted following the Wuhan Kangtest Bioinformation Technology Co., Ltd.'s recommended instructions.

Filtering, comparison and analysis of sequencing data

The peak signal produced by the Illumina HiSeq was transformed into base sequence by base calling as Raw Data or Raw Reads. The Raw Reads were then filtered for subsequent information analysis to ensure the quality of information analysis, including the removal of reads that have adapters and filtration of reads with more than 10% N content or more than 50% low quality bases. The final filtered data are called clean reads.

The sequencing reads need to be aligned with the reference genome (the reference genome selected *Medicago truncatula*, derived from ftp://ftp.ensemblgenomes.org/pub/release-33/plants/fasta/medicago_truncatula/dna/Medicago_truncatula.MedtrA17_4.0.dna.toplevel.fa.gz) before conducting the methylation analysis. Bismark software was used to perform a comparison of the alignments of bisulfite-treated reads to a reference genome using the default parameters (Krueger, 2011).

Estimating methylation levels and the identification of DMRs

To detecting the different methylated C sites in a region, we defined C_i as the number of supporting methylation reads at a single C site, T_i as the number of supporting unmethylation reads at a single C site, i as the position of C. The methylation level of a C site was counted as follows (Schultz, 2012):

$$\text{Methylation level of C site} = C_i / (C_i + T_i) \times 100 \quad \text{(Eq.1)}$$

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Use swDMR software (version 1.0.0) to detect DMR (Yang, 2020). swDMR uses a sliding window (sliding window size: 1000 bp; step length: 100 bp) to detect DMR. Fisher's exact test was used for two samples in this study.

Enrichment analysis of DMR gene KEGG pathway

Pathway enrichment analysis takes KEGG Pathway as the unit (Young, 2010), and applies hypergeometric test to find the pathways that are significantly enriched in DMR-related genes compared with the background of the entire alfalfa genome. KOBAS (Mao, 2005) was used to perform gene pathway enrichment analysis on the gene list where DMR is located, and the pathways with pathway enrichment P-value (or corrected *P*-value) less than 0.05 and the number of genes in this pathway of the query gene were more than 2 were screened out (Xie et al., 2011).

Results

Analysis of alfalfa whole genome DNA methylation sequencing data

A total of 21.46 G and 17.91 G raw bases were generated on average for the A34-1A and A34-2A respectively by using the Illumina XTen sequencing platform. After data filtering, approximately 43 G of Clean reads were obtained (*Table 1*). Use the software SOAPnuke (version 1.6.0) to align the clean reads obtained from the A34-1A and A34-2A samples to the alfalfa genome sequence. The alignment rates are 90.40% and 88.72%, respectively. The average sequencing depth of the whole genome is 50.71× and 42.93× respectively.

Table 1. *Statistical results of sequencing data and clean reads map to reference genome results*

Samples	Raw reads (G)	Clean reads (G)	Mapped reads (G)	Mapped rate (%)	Average depth (×)
A34_1A	21.46	21.44	19.38	90.40%	50.71×
A34_2A	17.91	17.89	15.87	88.72%	42.93×

The characteristics of genome-wide methylated cytosine C in alfalfa

This study found that 32.00% and 35.79% of C sites in the control sample (A34_1A) and EMS sample (A34_2A) were methylated, respectively. There are three main types of DNA methylation at the C site: mCG, mCHG and mCHH. The number and composition ratio of these three types reflect the characteristics of the whole genome methylation of a specific species. Among them, the methylation ratio of C site of A34_2A is higher than that of A34_1A (*Table 2*). The results of the distribution ratio of CG, CHG and CHH in methylated C bases show that the proportion of mCHH sites is the highest (all above 63%) after alfalfa mutagenesis, the frequency of mCG locus and mCHG locus decreased in turn (*Fig. 1*).

Analysis of sequence characteristics near methylated C in CG, CHG and CHH

We extracted the 9 bp sequence near the methylation to study the sequence features near the methylation site. In the alfalfa genome, for CG, we define sites with a methylation level greater than 75% as hypermethylated sites, and sites with

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less than 75% as hypomethylated sites; for CHG and CHH, methyl sites with a level of greater than 25% are defined as hypermethylated sites, and sites with a level of less than 25% are defined as hypomethylated sites (Ryan et al., 2009). As shown in *Figure 2*, the frequency of the bases in the upstream and downstream of pyrimidine was similar, and the sequences of the three motifs of CG, CHG and CHH have no obvious preference.

Table 2. *The number and proportion methylated C in alfalfa*

Samples	Context	Covered	Methylation	C site methylation ratio
A34_1A	C	35259145	11282721	32.00
	CG	3145374	2306626	73.33
	CHG	4641999	1821643	39.24
	CHH	27471772	7154452	26.04
A34_2A	C	25214733	9025061	35.79
	CG	2307826	1747850	75.74
	CHG	3354013	1467649	43.76
	CHH	19552894	5809562	29.71

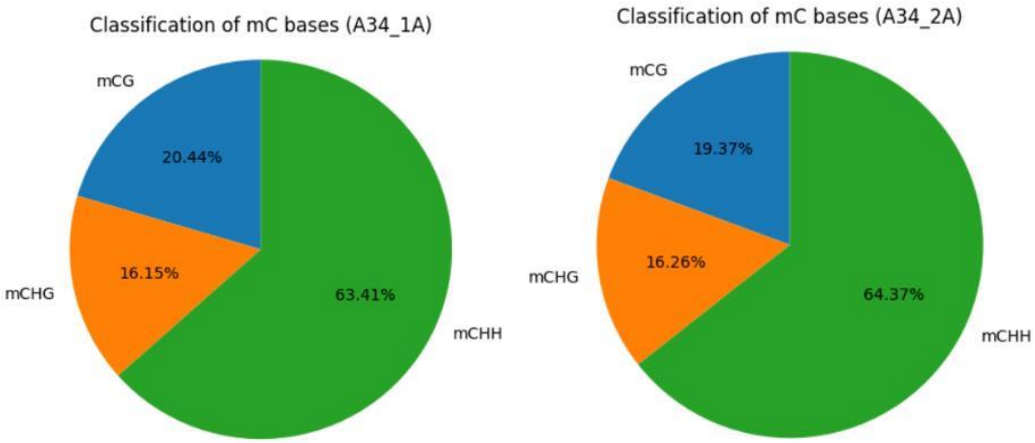

Figure 1. *The distribution of mCG, mCHG and mCHH in all methylcytosine*

Analysis of alfalfa whole genome DNA methylation level

To study the changes in the whole genome methylation level of alfalfa mutagenesis treatment and control treatment. The experiment performed a statistical analysis of the methylation levels of CG, CHG and CHH sequences in the whole genome (*Fig. 3*). The most CG sites in the alfalfa genome were in a state of 80-100% high methylation level, or are not methylated, regardless of the mutagenesis treatment or the control treatment. This is because CG methylation is the most abundant type of DNA methylation (Kanehisa, 2016). It was found that among all methylated cytosine sites, the degree of methylation of CHG and CHH was relatively low, and the methylation level was mainly distributed below 40%. The change trend of CHG sites was in the range of 20-100%, and the change trend was gentle. CHH sites were generally un-methylated or distributed in the 20-40% range, and the methylation level was relatively low; while the methylation level of CG at 80%-100% was higher than that of CHG and CHH. It

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showed that methylation of alfalfa genome mainly occurs at CG sites, and this trend was also a characteristic of alfalfa genome DNA.

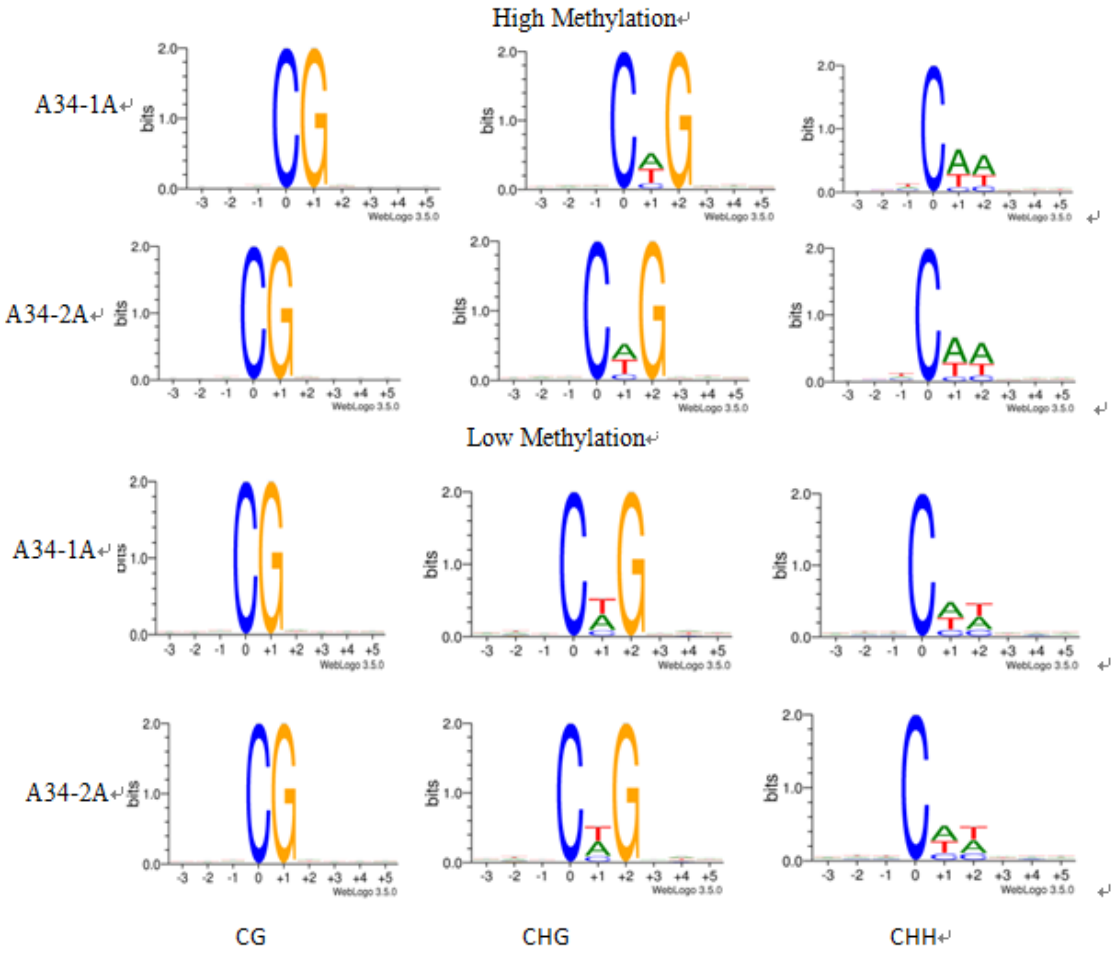

Figure 2. *Methylation preferences in 9 bp spanning CG, CHG, and CHH methylcytosine sites. H = A, C or T. The abscissa is the base number of the methylation site, the total height of each position is the sequence conservation of the base, which represents the relative frequency of the base at that position*

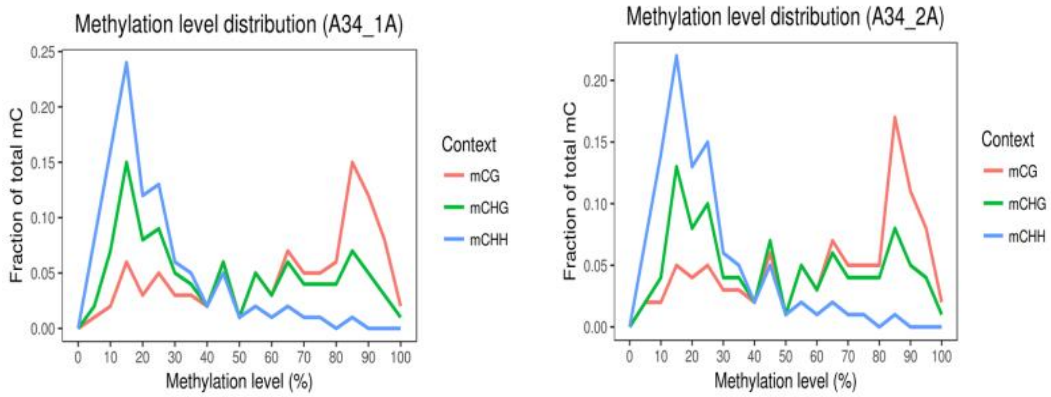

Figure 3. *The methylation levels of CG, CHG and CHH sequences*

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DNA methylation levels of different functional regions

We divided all mC into specific gene features: promoter, 5'UTR (Untranslated Regions), exons, introns and 3'UTR. The methylation levels were evaluated in these functional regions. The methylation levels obtained according to *Equation 1* is shown in *Figure 4*, the trend of methylation levels in the specified regions of the two treatments were similar, and the methylation levels for the CG type were higher than those for the CHG and CHH types. The CG methylation level of many gene regions increased after mutagenesis. It shows that mutagenesis may promote the methylation process of certain sites. Moreover, the results of this study showed that the methylation level of intron was the highest during the mutagenesis of alfalfa, followed by the exon and the promoter regions.

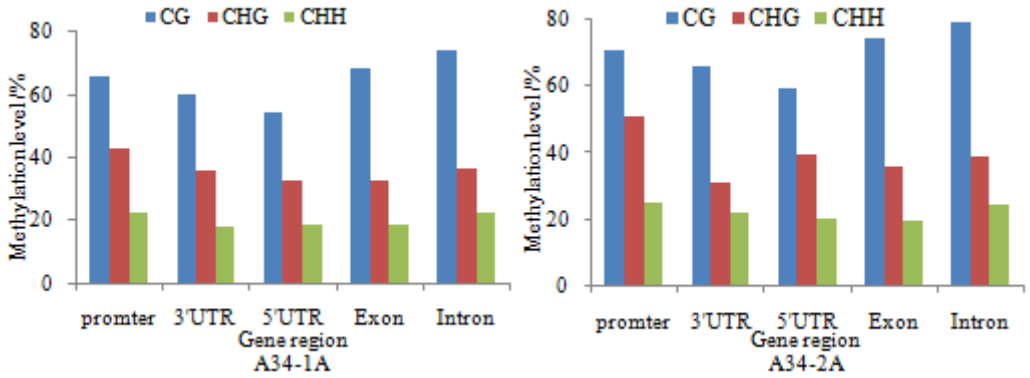

Figure 4. *Methylation level of various types of C in different gene regions*

DMRs analysis for the control and EMS mutagenesis treatments

A total of 8707 DMRs were detected in A34_1A and A34_2A on the difference of methylation regions (*Table 3*). There were 5221 hyper-DMR and 3486 hypo-DMR during the mutagenesis of alfalfa. The CG, CHG and CHH sequences all have higher rates of hypermethylation than that of hypomethylation. The highest rate of hypermethylation was the CHH sequence, which is 64.38%, and the lowest proportion of hypomethylation was CG sequence, which was 41.42%. The comparison of DMR in A34_1A and A34_2A showed that the methylation level generally increased after EMS mutagenesis.

The results of the study on the distribution of DMR in different regions of genes were shown in *Figure 5*. For all methylation types, the ratio of DMRs located in introns and promoters were the highest except for those in distal intergenic regions. This indicates that the promoter and intron regions have a high level of methylation and may participate in the process of methylation regulation.

Table 3. *Details of DMR during A34-1A VS.A34-2A*

Type	Hyper–DMR	Hypo–DMR	Hyper ratio	Hypo ratio
CG–DMR	1635	1156	58.58	41.42
CHG–DMR	1556	1207	56.32	43.68
CHH–DMR	2030	1123	64.38	35.62
Total	5221	3486	59.96	40.04

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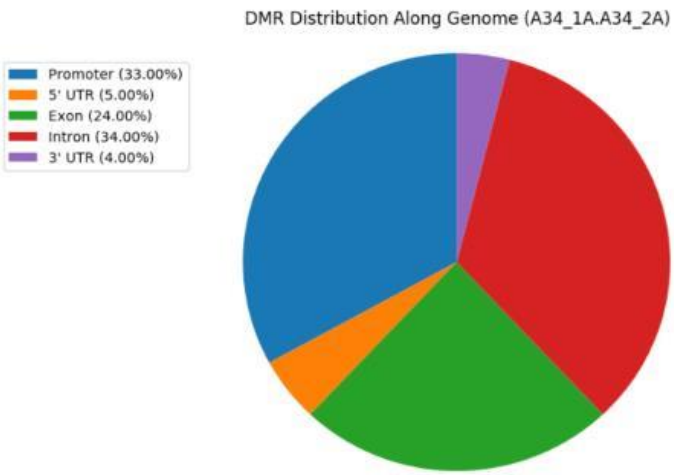

Figure 5. *Proportion of DMR of different sequence types in the genome region*

KEGG pathway enrichment analysis

To probe changes in the methylation status of gene functions after mutagenesis in alfalfa. KOBAS was used to carry on the analysis of gene pathway enrichment on the gene list where DMR is located. Screen the pathways whose pathway enrichment P-value is less than 0.05 and the number of query genes in this pathway is greater than 2 (Xie et al., 2011). The KEGG analysis revealed that there are 20 pathways ($P < 0.1$) were enriched, of which the main enrichment was in starch and sucrose metabolism, sugar metabolism pathways include galactose metabolism, fructose and mannose metabolism, amino acid sugar and nucleotide sugar metabolism, nitrogen metabolism, Pyrimidine metabolism, Oxidative phosphorylation, Arginine and proline metabolism, etc. (*Fig. 6*). There are 33 genes involved in these metabolisms (*Table 4*). The above research results indicate that the differentially methylated genes of alfalfa after mutation may be closely related to the physiological and biochemical metabolic processes such as carbon metabolism and amino acid metabolism.

Discussion

DNA methylation is the main feature of the epigenetic regulatory mechanism that plays an important role in the regulation of gene expression (Yan et al., 2017). As an important epigenetic modification method, DNA methylation can cause heritable changes in gene expression and play an important regulatory role in the growth and development of plants. The level of DNA methylation varies significantly in different plant species, as well as between different species of the same species. Previously, some studies have been conducted to describe DNA methylation for alfalfa (Russo et al., 2013; Barboni et al., 2011; Colosimo et al., 2009; Russo, 2007), but few reports analyzed from the alfalfa mutagenesis genome-wide methylation pattern (Cao et al., 2016). Mutagenesis can cause changes in the organism's genome (Xu et al., 2006; Li et al., 2007), expression group (Hwang et al., 2014), proteome (Wang et al., 2008), and metabolome (Zhang et al., 2011; Kim et al., 2012). Analyzing the changes of alfalfa genomic methylation level before and after mutagenesis can help to study the expression regulation of functional genes and the molecular mechanism of alfalfa

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adaptation to adversity under mutagenic stress. In this study, we used WGBS to investigate the DNA methylation profiles of the genome in leaf tissues between A34_1A and A34_2A to discover the methylation changes before and after mutagenesis. Further correlation analysis indicated that several DMR-related genes were most likely involved in alfalfa physiological resistance.

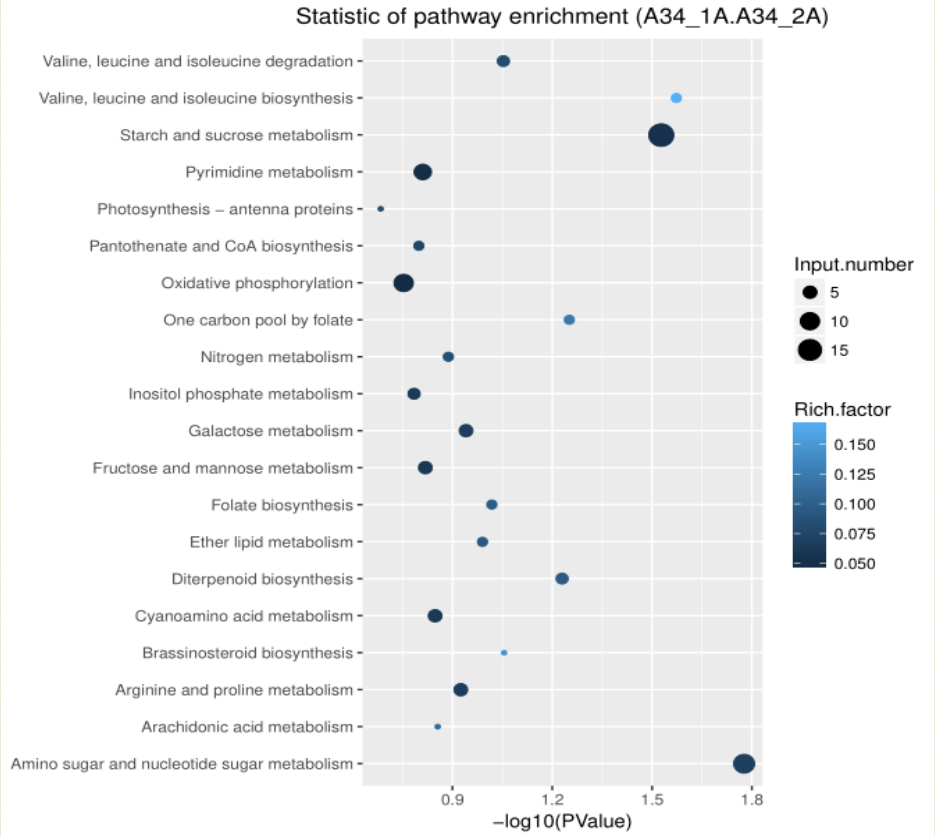

Figure 6. *The enrichment analysis of DGEs in the comparison of A34-1A VS.A34-2A*

Table 4. *The pathways and genes involved in physiological metabolism*

Sample	Pathways	P value	Number	genes
A34-1A VS. A34-2A	Amino sugar and nucleotide sugar metabolism	0.0167	12	MTR_2g015550|MTR_6g088795|MTR_0046s0180|MTR_6g029470|MTR_2g006790|MTR_4g131760|MTR_0354s0040|MTR_8g044160|MTR_3g064490|MTR_0034s0060|MTR_5g021760|MTR_4g070430
	Valine, leucine and isoleucine biosynthesis	0.0267	3	MTR_4g011180|MTR_3g103580|MTR_7g033125
	Starch and sucrose metabolism	0.0297	18	MTR_0034s0170|MTR_7g012840|MTR_4g120280|MTR_4g036685|MTR_4g081530|MTR_4g066590|MTR_2g006790|MTR_4g131760|MTR_0354s0040|MTR_7g113910|MTR_6g088795|MTR_3g064490|MTR_1g015970|MTR_8g032250|MTR_0046s0180|MTR_2g015550|MTR_6g088810|MTR_4g070430

In the *Arabidopsis* methylation group, both the upstream and downstream methylated cytosines have sequence specificity. The preference for the second base upstream of CG for cytosine C is 13 times higher than the preference for adenine A in the case of high methylation levels. At low methylation levels, the upstream of the CHG motif is not

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biased and the downstream always follows a methylated cytosine C. In which, the most obvious phenomenon is that the CHH motif always follows the TA combination, but the second base upstream of the CHH motif is rarely adenine A. (Cokus et al., 2008). Previous studies have shown that there is no preference analysis for plant methylation except for the specificity of upstream and downstream sequences of methylated cytosine in *Arabidopsis*. In this study, the methylation levels of methylated cytosine mC sites were divided into two groups in the alfalfa genome: High Methylation and Low Methylation. The frequency of the bases is similar, and there is no obvious preference for the sequences of the three motifs of CG, CHG and CHH.

DNA methylation level plays an important role in plant development (Zhong et al., 2013). In this study, the proportion of CG-type methylated cytosines was 73.33%-75.74%, and about 30% of methylated cytosines were non-CG types (mCHG, mCHH), indicating that methylation of the alfalfa genome mainly occurred at the CG position point. This is consistent with the findings of Shi et al. (2014) that the DNA methylation of CG site is more than that of CNG site for mature stage rice under heavy ion irradiation treatment. The results of the study showed that during the mutagenesis of alfalfa, the methylation level of the intron region was higher, which may be caused by the involvement of introns in the regulation of gene expression and the prevention of abnormal transcription of intron sequences. These results were consistent with previous reports (Zemach et al., 2013). In addition, related research shown that CNG site methylation plays an important role in plant growth and resistance to external stress (Zhuang. 2008; Liang et al., 2014; Xiao et al., 2006). Kim et al. (2013) found that after gamma irradiation, the DNA methylation of CNG sites in *Arabidopsis* genome was higher than that of CG sites. These differences may be caused by different stages of crop development.

The comparison of the DNA methylation of A34_1A and A34_2A showed that the overall methylation level of A34_2A showed an upward trend. In the 8707 DMRs obtained, CG, CHG and CHH all had hypermethylation, and the methylation was mainly in the CG sequence. We found that the DNA methylation level in the functional element regions of the genome, promoters, introns and other elements did not change much before the alfalfa mutagenesis, but after the EMS mutagenesis, the CG methylation levels of many gene structures has increased, which indicates that there are multiple sites of methylation during the mutagenesis process, which will affect the expression of genes.

DNA methylation can change the physiology and metabolism of plants, promote or inhibit the metabolism and conversion of certain energy substances in the process of plant growth, thereby affecting the vitality of plants and helping plants resist adversity (Lauria et al., 2011). In our previous research, EMS mutagenesis treatment was used to explore the physiological and biochemical characteristics of alfalfa and its effect on alfalfa stress resistance. The results showed that the physiological indexes of alfalfa stress resistance were significantly improved after the mutation. The effect of mutagenesis on improving plant stress resistance was revealed at the physiological and biochemical level (Shen et al., 2018). In this study, the effect of mutagenesis treatment on alfalfa methylation was conducted. Through path enrichment analysis, it was found that there were 33 differentially methylated genes in the three physiological pathways related to stress after alfalfa mutagenesis. We will validate those DMR-related genes from this study in different stages of growth development in the future. The results of this study will further revealed the mechanism that mutagenesis is contribute to increase

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alfalfa resistance at the molecular level. It provides a valuable reference for the study of alfalfa resistance breeding by using mutagenesis technology.

Conclusion

In this study, WGBS technology was used to study the whole genome DNA methylation map of alfalfa mutated by EMS. The levels and patterns of DNA methylation before and after mutation were analyzed, and DMRs/DMGs that may be related to stress physiology were found. The results will help to better understand the epigenetic regulation of alfalfa before and after mutation stress. In the future, DNA methylation and transcriptome association analysis will be used to identify specific up-regulated or down regulated genes, and explore the relationship between methylation and gene expression, so as to provide theoretical basis for epigenetic research of alfalfa.

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