Bangladesh J. Bot. **54**(2): 377-387, 2025 (June) DOI: https://doi.org/10.3329/bjb.v54i2.82359

IMPACT OF PUMPKIN ROOTSTOCK ON DNA METHYLATION AND EPIGENETIC PROFILE OF GRAFTED SELF-BRED CUCUMBER PROGENY

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Keywords: Cucumber scion, Pumpkin rootstock, Grafting, Methylation

Abstract

To study the effect of pumpkin rootstock on the variation of self-bred progeny of grafted cucumber, "Zhenghuang 1409" was used as scion, and "Liangzhen No.1" pumpkin was used as rootstock. The alterations in genome DNA methylation of self-bred progeny of grafted cucumber were examined utilizing MSAP technology with the differential bands subsequently cloned and sequenced. The results showed that the methylation levels of two types of mutant materials in the first and second generations of grafted cucumber inbred lines increased compared to cucumber scion inbred lines, but the methylation levels of single-chain extracellular cytosine or double chain intracellular cytosine in the mutant materials showed almost opposite changes from the first generation (G1) to the second generation (G2) compared to cucumber scion inbred lines; The methylation status of variant types shows opposite changes from G1 to G2. In G1, the degree of methylation of variant type I is greater than the degree of demethylation, while in G2, the degree of demethylation of variant type I is greater than the degree of methylation. The degree of demethylation of variant type II in G1 is greater than that of methylation, while in G2, the degree of methylation of variant type II is greater than that of demethylation. Gene sequence homology analysis of specific fragments of MSAP found that the homologous genes mainly correspond to proteins containing specific domains, enzyme proteins, and carrier proteins, etc. These functional proteins are involved in post-transcriptional regulation, cellular amino acid synthesis, redox, and other processes, etc. The specific fragment of MSAP indicates that the methylation status of its homologous gene is mutated, which may affect the functional expression of its homologous gene, which may affect the growth and development process of the self-bred progeny of grafted cucumber and even affect the phenotypic traits.

Introduction

DNA methylation is a process where eukaryotes use S-adenosylmethionine (SAM) as the methyl donor, and under the action of DNA methyltransferase (DNMT), transfer methyl to the cytosine 5 carbon position of the genomic CpG dinucleotide to covalently combine with a methyl group to form 5-methylcytosine (5mC) (Iqbal *et al.* 2011). DNA methylation is an important epigenetic modification of genomic DNA, which is widespread in higher plants, and the methylation level of different organs in different species at different development stages is different (Martienssen *et al.* 2001). DNA methylation mainly includes full methylation (CG), semi-methylation (CHG), and hypermethylation (CHH). Methylation is related to transposon inactivation, miRNA expression, gene imprinting, and also to biotic and abiotic stress (Eccleston *et al.* 2007). The ways of DNA methylation in plants mainly include *de novo* methylation and maintenance methylation, which can help plants resist stress, effectively regulate genes, and ensure the stability of genes. At the same time, it can also affect the growth and development of plants, changing their flowering period (Duan *et al.* 2018, Ferreira *et al.* 2015).

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The main methods for detecting plant DNA methylation include whole genome high-performance liquid chromatography (HPLC) (Kuo *et al.* 1980), methylation sensitive amplification polymorphism (MSAP) (Xiong *et al.* 1999), whole genome bisulfite sequencing (WGBS) (Baubec *et al.* 2016), reduced representation bisulfite sequencing (RRBS) (Martin *et al.* 2017), and methylated DNA immunoprecipitation sequencing (MeDIP-seq) (Aniruddha *et al.* 2017, Xing *et al.* 2018). Among them, MSAP technology is an important method to detect DNA methylation in plant genomes, and is widely used in plant germplasm identification, plant improvement, population genetic structure analysis, and other fields (Li *et al.* 2011, Herrera *et al.* 2011, Xin *et al.* 2015).

Cucumber (*Cucumis sativus* L.) is an important vegetable crop, currently mainly cultivated in protected areas. However, with the expansion of planting scale, the time interval between rotations has been shortened, and the high-density planting method has made the problem of pests and diseases of cucumbers cultivated in protected areas increasingly prominent. In the production of cucumber protected areas, grafting cultivation methods using pumpkin as the rootstock are often used to overcome continuous cropping obstacles (Avramidou *et al.* 2015). A large number of studies have shown that grafting can not only improve plant resistance, yield and quality (Moncada *et al.* 2013, Liu *et al.* 2015, Kumar *et al.* 2017), but also affect plant DNA methylation, and DNA methylation can also change plant gene expression (Xie *et al.* 2020). In recent years, there have been more and more studies on epigenetic modification, especially on plant DNA methylation (Henderson *et al.* 2007). The main base is in plant stress (Mastan *et al.* 2012, Shan *et al.* 2013, Wang *et al.* 2014). There are also studies on grafting between different plants, mainly on grafted plants after grafting, changes in grafting level and mode of sexual offspring of grafted plants (Law et al. 2010, Athanasios *et al.* 2012, Wu *et al.* 2013,Tsaballa *et al.* 2013, Avramidou *et al.* 2015). Studying the epigenetic changes of grafted plants and their sexual self-crossing offspring after grafting is of great significance in explaining the phenotypic changes that occur during grafting. The changes in methylation levels and states of grafted plants and their sexual self-crossing offspring after grafting are also important aspects of grafting epigenetic inheritance.

This experiment used two different genera of materials, pumpkin (rootstock) and cucumber (scion), for distant grafting. To study the changes in methylation levels and patterns of self pollinated sexual offspring of cucumber grafted plants, laying a foundation for the epigenetic research of sexual offspring of grafted plants.

Materials and Methods

Cucumber ‘Zhenghuang 1409’, a high-generation inbred line of cucumber was grafted onto the rootstock ‘Liangzhen No. 1’, a first-generation hybrid of pumpkin (*C. moschata*). From May-July 2019, the cucumber scions were grafted at the Pumpkin Breeding Test Base of Henan University of Science and Technology and 10 selected plants (G0) were self-pollinated to obtain the first self-pollinated generation of seeds (G1-1–G1-8). Subsequent experiments were undertaken in a plastic greenhouse in Zhuzhuangtun Village, Muye District, Xinxiang City, Henan (35°18′N 113°54′E). From March to June 2020, G1 seeds were cultivated and self-pollinated to obtain the second self-generation seeds (G2), from which 9 lines were cultivated. The other strain was a special variant type, marked as SV, over the period March to June, 2021. Thereafter, genetic and phenotypic variations in the different G2 lines were investigated and analyzed. The cucumber scion inbred line ‘Zhenghuang 1409’ was used as the experimental control, termed S1, and its inbred progeny was termed S2.

From March to June of 2020 and 2021, the G1 generation and G2 generation cucumber materials were planted and cultivated, respectively. In an incubator set at 28°C, cucumber seeds

are allowed to germinate. The buds are seeded into a 50-hole tray when they are 1-2 mm long. The nursery substrate was peat soil: garden soil: perlite = 4:4:2. During the seedling stage, the temperature was maintained at 25°C in the daytime and 18°C at night, with a light/dark cycle of 16/8 hours and humidity of 50-60%. When the seedlings achieved the three-leaf stage and one heart, they were transplanted to a plastic greenhouse with a row spacing of 40 cm × 125 cm. The seedlings were pruned to a single vine, covered with a plastic film, and watered every 5-7 days. The greenhouse temperature was 28-30°C with a humidity of about 8%.

Genomic DNA was isolated from young leaves from grafted cucumber inbred offspring and cucumber inbred line materials, according to the instructions of TaKaRa MiniBEST Plant Genomic DNA Extraction Kit (Takara Biotechnology Co., Ltd., Dalian, China). The DNA concentration and purity were determined by micro spectrophotometer (Thermo Scientific Nanodrop 2000c, Chicago, USA). Integrity of DNA samples was also checked on 1.0% agarose gel.

To detect methylation variations in grafted cucumber inbred offspring and cucumber inbred line, MSAP technique was employed following the procedure of Cao *et al.* (2011). Two reactions were set up at the same time. Isoschizomers *Hpa* II and *Msp* I and restriction endonuclease *Eco*R I (Takara Biomedical Technology, Beijing) were used for two-step digestion of genomic DNA. The first step was performed for *Hpa* II/*Msp* I enzyme digestion at 37°C for 4 hours followed by enzyme deactivation at 80°C for 20 minutes in a 25 µl, reaction volume containing sample DNA 500 ng, *Hpa* II/*Msp* I 10 U, 10 × Cutsmart buffer 2.5 µl,and 20.5 µl ddH_2O. The second step was*Eco*R I digestion performed at 37°C for 5 hours, followed by deactivation at 65°C for 20 minutes, in a 30 µl reaction volume containing 25 µL of the first step enzyme digestion product, *Eco*R I 15 U, 10 × Cutsmart buffer 3 µl, and 1.0 µl ddH_2O .

The digested DNA fragments (21 µl) were ligated with the double-stranded *Eco*RI adapter and the *Hpa*II/*Msp*I adapter simultaneously using T4 DNA ligase (Takara Biomedical Technology, Beijing) according to the manufacturer's instructions. Subsequently, the ligation products were used as templates in the pre-amplification reaction. The adapters, pre-amplification primers, and selective amplification primers are listed in Table 1.

A pre-amplification reaction was carried out in a total volume of 25 µl, containing 2 µl of 10 mM dNTPs, 2.5 µl of 10 × buffer, 3 U Taq polymerase (Takara Biomedical Technology, Beijing), 0.5 µl of 10 µM E0-primer, 0.5 µl of 10 µM MH0-primer, and 2 µl of the ligation products. The pre-amplification PCR reaction protocol consisted of 20 cycles at 94°C for 30 sec, 56°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 5 min. The pre-amplification products were checked by agarose gel electrophoresis, and the fragments were 100-900 bp in length. The pre-amplification products were diluted 1:120 with sterilized ultra-pure water for further selective amplification.

Selective amplification primers were based on pre-amplification primers with three selective bases. The selective amplification PCR protocol consisted of 13 cycles for the touchdown program at 94°C for 30 sec, dropping 0.7°C per cycle from 65 to 56°C for 30 sec, 72°C for 1 min. This procedure was followed by another 23 cycles of PCR amplification, denaturing at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

Selective amplification products were mixed with loading buffer and denatured at 94°C for 10 min. The samples were then resolved by electrophoresis on a 6% denaturing polyacrylamide gel (PAGE, 6% polyacrylamide, 8 M urea). The polyacrylamide gel was stained according to the silver staining method and photographed (Bassam *et al.* 1991).

Table 1. List of primers for methylation sensitive amplification polymorphism analysis.

Primer type	Name of primer and oligonucleotide sequences (5′-3′)	
	EcoR I (E)	*Msp* I, *Hpa* II (MH)
Primer joint	EA1 CTCGTAGACTGCGTACC	MHA1 GATCATGAGCCTGCT
	EA2 AATTGGTACGCAGTC	MHA2 CGAGCAGGACTCATGA
Pre-amplified primer	E0 GACTGCGTACCAATTCA	MH0 ATCATGAGTCCTGCTCGG
Select-amplified primer	E1 GACTGCGTACCAATTCACA	MH1 ATCATGAGTCCTGCTCGGTCAA
	E2 GACTGCGTACCAATTCACG	MH2 ATCATGAGTCCTGCTCGGTCT
	E3 GACTGCGTCCAATTCAGG	MH3 ATATGAGTCCTGCTCGGTCG
	E4 GACTGCGTACCAATTCAAC	MH4 ATCATGAGTCCTGCTCGGTCC
	E5 GACTGCGTACCAATTCAAG	MH5 ATCATGAGTCCTGCTCGGTTG
	E6 GACTGCGTACCAATTCAGC	MH6 ATCATGAGTCCTGCTCGGTGT
	E7 GACTGCGTACCAATTCACT	MH7 ATCATGAGTCCTGCTCGGTC
	E8 GACTGCGTACCAATTCACC	MH8 ATCATGAGTCCTGCTCGGTT
	E9 GACTGCGTACCAATTCATC	MH9 ATCATGAGTCCTGCTCGGTCA

81 pairs of primer combinations were preliminarily screened on agarose gel, and the primer combinations with good repeatability, polymorphism, and clear bands were selected for verification analysis of denatured polyacrylamide gel electrophoresis of the MSAP system. Only clear and reproducible bands were scored. The scored MSAP bands were transformed into a binary character matrix, 1 for presence and 0 for absence of a band at a particular position in the MSAP profiles.

After the MSAP assay, the specific bands were selected and recovered by a denatured polyacrylamide gel recovery kit (Beijing Solarbio Science & Technology Co., Ltd) for sequencing to identify the genes related to the changes in DNA methylation. The eluted DNA was amplified with the same selective primers under the same conditions as the selective amplification. The PCR products were ligated to the pMD18-T vector (Takara Biotechnology Co., Ltd., Dalian, China) and transformed into the competent *E. coli* DH5α. The recombinants were screened by sequencing at Sangon Biotech Co., Ltd. (Shanghai, China). The sequences were analyzed by NCBI BLAST (www.ncbi.nlm.nih.gov).

Results and Discussion

The genomic DNA of cucumber leaves extracted by using commercial DNA isolation kit was resolved through 1% agarose gel electrophoresis, and it was found that the main band was clear, without RNA, meeting the requirements of MSAP for templates (Fig. 1).

Methylation differences between the grafted cucumber inbred offspring and cucumber inbred line were detected by MSAP. In total, 2928 5′-CCGG-3′ site bands were amplified in the samples. Among the MSAP amplified bands, the fragments could be divided into four types: type I fragments, which were unmethylated sites, indicated by the presence of bands for both enzyme combinations; type II fragments, indicated by the presence of bands for only *Eco*RI/*Msp*I, which is a semi-methylated band; type III fragments, indicated by the presence of bands for *Eco*RI/*Hpa*II; and type IV fragments, indicated by the absence of a band for either enzyme combination (Wang *et al.* 2011). In this study, types IV was detected (Table 2).

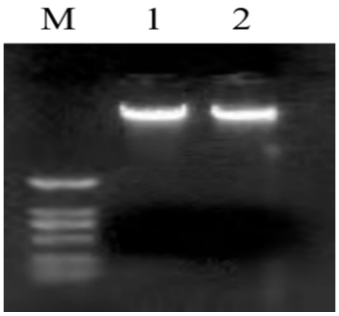

Fig. 1. Agarose gel electrophoresis of the genomic DNA isolated from two representative samples. Lane M is DL2000 and lanes1-2 were two representative samples.

Table 2. Methylation-sensitive restriction bands of *Hpa* II & *Msp* I and the classification of the banding pattern.

Type	Restriction enzyme activity and restriction bands		State of CCGG site	Methylation state of CCGG site
	Hpa II(H)	*Msp* I(M)		
I	1	1	CCGG	unmethylated
II	0	1	C5mCGG	hemi-methylated
III	1	0	5hmCGG C5hmCGG	hemi-methylated
IV	0	0	5mC5mCGG 5mCCGG	fully methylated

From Table 3, it can be seen that the number of bands detected by each pair of primer combinations among the 6 pairs of primers in each group of materials is 26-30, and the DNA methylation of each group of materials is mainly double stranded cytosine methylation. Compared with S1, the DNA methylation level of grafted cucumber inbred generation G1-VI significantly increased, mainly with an increase in single-stranded extracellular cytosine methylation level and a slight increase in double stranded intracellular cytosine methylation level. Compared with S2, G1-VII showed a slight increase in methylation levels, showing an increase in double stranded cytosine methylation levels and a slight decrease in single stranded extracellular cytosine methylation levels.

There was a slight increase in DNA methylation level in grafted cucumber inbred second-generation G2-VI compared to S2, with a decrease in double stranded cytosine methylation level and an increase in single stranded extracellular cytosine methylation level. G2-VII exhibits a similar phenomenon compared to S2, but the increase in methylation level of G2-VII is greater than that of G2-VI, and the decrease in double chain cytosine methylation level and the increase in single chain cytosine methylation level are also greater than those of G2-VI.

The methylation levels of two types of mutant materials, the first and second generations of grafted cucumber inbred lines, were both higher than those of scion inbred lines, but the changes in double chain cytosine methylation levels or single chain extracellular cytosine methylation levels showed an almost opposite trend from the first to second generations of inbred lines.

Table 3. Methylation levels of two variation materials of self-bred of grafted cucumbers.

Materials	Type of amplified bands						
	Type I	Type II	Type III	Total amplified bands [a]	Methylation of the inner cytosine [b]	Methylation of the outer cytosine [c]	MSAP (%)
S1	360	68	34	462	14.72	7.36	22.08
G1-VI	332	73	54	459	15.90	11.76	27.67
G1-VII	356	73	33	462	15.80	7.14	22.94
S2	409	82	40	531	15.44	7.53	22.98
G2-VI	378	75	39	492	15.24	7.93	23.17
G2-VII	395	71	56	522	13.60	10.73	24.33

[a]:Total amplified bands=I+II+III, [b]: Methylation of the inner cytosine loci ratio = II/total amplified bands. [c]:Methylation of the outer cytosine loci ratio = III/ total amplified bands; MSAP = (II + III)/(I + II + III).

As shown in Table 4, there were a total of 12 bands in the methylation sensitive amplification of grafted cucumber inbred offspring. Fig. 2 showed the main band patterns of grafted cucumber inbred offspring, which represented changes in methylation status. There are two types of methylation patterns: polymorphism and monomorphism. Polymorphism referred to a change in methylation status compared to the control, a change in methylation status at the CCGG site. Conversely, it was a monomorphism pattern (Avramidou *et al.* 2015). There were three types of polymorphisms: methylation (A), demethylation (B), and amorphous (C). A1 and A2 were remethylated (both the scion inbred lines H and M have bands, while the grafted cucumber inbred offspring only have bands H or M), while A3 and A4 belonged to hypermethylation (the scion inbred lines H or M have a band, while the grafted cucumber inbred offspring H and M do not have bands). Type A indicated that the grafting of pumpkin and cucumber induced an increase in the methylation level of genomic DNA in the offspring of cucumber selfing. Type B was a demethylation type, which was opposite to type A, indicating that the grafting of pumpkin and cucumber induced a decrease in methylation levels in the genomic DNA of the self bred offspring of the grafted cucumber. Type C was an indefinite type, which means that the difference in methylation degree between grafted cucumber inbred offspring and scion inbred lines cannot be determined. Monomorphism, which means that the self crossing offspring of grafted cucumber have the same band type as the self crossing offspring of scion, indicated that there was no change in the methylation status of the CCGG site between the self crossing offspring of grafted cucumber and the self crossing line of scion. D1 indicated no methylation, while D2 and D3 indicated hemimethylation. The methylation pattern bands A, B, C, and D of grafted cucumber inbred lines and scion inbred lines, as well as the corresponding number of loci, were shown in Table 4.

As shown in Fig. 2, through MSAP analysis, fragments with different methylation states between grafted cucumber inbred offspring and scion inbred lines were recovered, cloned, and sequenced. Finally, 9 sequences of different fragments were successfully obtained (Table 5). Homology analysis results showed that, there were 7 fragments highly homologous to the genes corresponding to known proteins in the cucumber genome (Chinese Long) v3 of the Cucurbitaceae Genomics Database (E value in Table 5). The homologous genes of these fragments mainly corresponded to protein structures, proteins containing specific structural domains, enzyme proteins, and carrier proteins. The homologous gene of M1 corresponded to the ternary complex

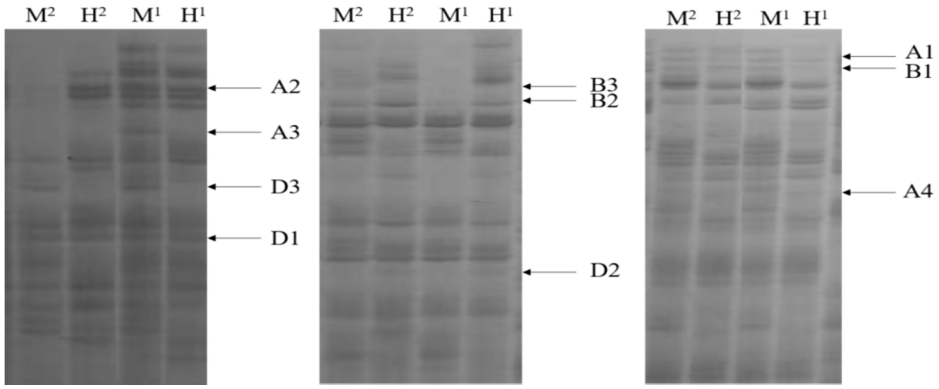

Fig. 2. Schematic diagram of genome MSAP band pattern changes of grafted cucumber self-bred progeny compared with cucumber scion inbred line.

Table 4. Patterns of DNA methylation in the self-progeny grafted cucumber.

Digestion [a]				Changes of methylation status		Numbers of sites				Band pattern [b]
H	M	H	M	S1/S2	G1/G2	G1-VI	G1-VII	G2-VI	G2-VII	
1	1	0	1	CCGG GGCC	C<u>C</u>GG GG<u>C</u>C	23	8	10	9	A1
1	1	1	0	CCGG GGCC	<u>CC</u>GG <u>CC</u>GG GGC<u>C</u> CCGG	6	10	10	18	A2
0	1	0	0	C<u>C</u>GG GG<u>C</u>C	<u>CC</u>GG GG<u>CC</u>	7	3	9	12	A3
1	0	0	0	<u>CC</u>GG <u>CC</u>GG GGC<u>C</u> CCGG	<u>CC</u>GG GG<u>CC</u>	1	6	1	9	A4
0	1	1	1	C<u>C</u>GG GG<u>C</u>C	CCGG GGCC	9	12	10	3	B1
1	0	1	1	C<u>C</u>GG GG<u>C</u>C	<u>CC</u>GG <u>CC</u>GG GGC<u>C</u> CCGG	3	6	12	13	B2
0	0	0	1	<u>CC</u>GG GG<u>CC</u>	C<u>C</u>GG GG<u>C</u>C	2	6	6	6	B3
0	0	1	1	<u>CC</u>GG GG<u>CC</u>	CCGG GGCC	13	18	11	6	B4
0	1	1	0	C<u>C</u>GG GG<u>C</u>C	<u>CC</u>GG <u>CC</u>GG GGC<u>C</u> CCGG	2	3	1	3	C
1	1	1	1	CCGG GGCC	CCGG GGCC	266	278	381	334	D1
1	0	1	0	<u>CC</u>GG <u>CC</u>GG GGC<u>C</u> CCGG	<u>CC</u>GG <u>CC</u>GG GGC<u>C</u> CCGG	14	13	25	26	D2
0	1	0	1	C<u>C</u>GG GG<u>C</u>C	C<u>C</u>GG GG<u>C</u>C	47	45	44	59	D3

[a]: H and M represent digestion with *EcoR* I/ *Hpa* II与*EcoR* I/ *Msp Msp* I, respectively; [b]: Band patterns are referred to Fig. 2; <u>C</u> and <u>CC</u> represent methylated cytosine; 1: Presence of band; 0: Absence of band.

M1P1, or leucine zipper, which can bind to DNA in the form of externally charged amino acids. The homologous genes of M2 and M7 corresponded to proteins containing the Sec 1 domain and proteins containing the NAC domain, respectively. The former participates in vesicle mediated transport, while the latter has transcriptional regulation functions. The homologous genes of M4 and M5 were paired with O-glucosyltransferase rumi homologues and aspartate kinase, respectively. The former was involved in lipid modification, while the latter was involved in the synthesis of cellular amino acids and the metabolism of glycine and L-serine. M6 homologous genes correspond to flowering time control proteins, M9 corresponds to carrier protein 4 or mitochondria, which can participate in the redox process and redox stress response. The homologous genes of M3 and M8 correspond to unknown proteins. These MSAP specific fragments had homologous genes that participate in biological processes, molecular functions, and cellular components in GO functional classification. MSAP specific fragments indicated variations in the methylation state of their homologous genes, which may affect the functional expression of their homologous genes and, in turn, affect the growth and development of grafted cucumber inbred offspring. It even affected the phenotypic traits of grafted cucumber inbred offspring.

Table 5. Sequence analysis of MSAP distinctive fragments.

Fragment [a]	Primer combination [b]	Pattern [c]	E Value	Homologous DNA ID	Description
M1	E3MH2	IV to III	4.88E-65	CsaV3_7G010180.1	Triple composite factor MIP1, leucine zipper
M2	E1MH8	I to II	3.45E-40	CsaV3_5G012530.1	Sec1 Domain Protein
M3	E3MH5	II to I	1.4E-101	CsaV3_UNG208680.1	Unknown protein
M4	E1MH7	II to I	4.25E-81	CsaV3_3G017110.1	O-glucosyltransferase rumi homolog
M5	E5MH5	III to I	3.99E-41	CsaV3_5G024880.1	Aspartokinase (AK)
M6	E4MH5	IV to II	1.81E-48	CsaV3_6G009800.1	Flowering time control protein FPA
M7	E6MH6	I to III	4.1E-153	CsaV3_4G008680.1	NAC Domain Protein
M8	E7MH8	II to IV	2.92E-46	CsaV3_6G017070.1	Unknown protein
M9	E6MH8	I to II	2.86E-46	CsaV3_2G035140.1	Carrier Protein 4, Mitochondria

[a]:MSAP different fragments between grafted cucumber self-progeny and scion inbred lines, [b]: Combination of primers used in amplification of the MSAP different fragment, [c]: Transition of band patterns between grafted cucumber self-progeny and scion inbred lines

DNA methylation plays an important regulatory role in the expression of important functional genes, genome defense, and cell development and differentiation during plant growth (Richards 1997, Yoder *et al.* 1997). Avramidou and his partners used MSAP methylation detection technology in their experiment on allogeneic grafting in the gourd family and found that the genome methylation levels of grafted cucumbers, watermelons, and muskmelon changed and increased after grafting (Avramidou *et al.* 2015). This experiment used Cucurbitaceae plant pumpkin and cucumber as grafting and used MSAP technology to detect the changes in methylation levels of grafted cucumber selfing offspring. It was found that the methylation level of the first generation of grafted cucumber selfing increased, and the methylation level of the second generation of grafted cucumber selfing still increased, indicating that grafting can lead to an increase in methylation level of grafted cucumber selfing offspring. In addition, Wu and his partners also used MSAP methylation detection in the grafting research of Solanaceae plants and found that there were changes in the genomic DNA methylation levels of grafted tomatoes, eggplants, and chili peppers, and this change in genomic DNA methylation levels induced by grafting was inherited to the self-crossing offspring of the grafted plants (Wu *et al.* 2013). The

results of this study indicate that changes in methylation levels induced by grafting exhibit genetic phenomena in the self-bred offspring of grafted cucumber, similar to their research findings.

Lewsey and his partners found that 24-nt sRNA can complement sequences by targeting DNA methylation specific sites (CG, CHG and CHH) mediated by RNA to achieve gene silencing (Lewsey *et al*. 2016). This means that changes in the methylation state of genomic DNA may cause gene silencing and affect gene expression, affecting plant growth and development, and even causing changes in phenotypic traits. The experimental results on the grafting between *B. juncea* and *B. oleracea* showed that the DNA methylation changes induced by grafting may play a role in the phenotypic variations observed during the grafting process. The DNA methylation changes induced by grafting can be guided by changes in siRNA, which provides a basis for exploring the genetic mechanism of phenotypic variations induced by grafting (Cao *et al.* 2011).

This study found that a large amount of remethylation and demethylation changes occurred in the offspring of grafted cucumber inbred lines, and the two types of mutant materials were opposite in the first and second generations of inbred lines compared to scion inbred lines. In the homology analysis of fragments with changes in methylation status, it was found that the functional proteins corresponding to these homologous genes of different fragments have functions such as participating in DNA binding, transcriptional regulation, and cellular amino acid metabolism. It plays a role in the biological processes and molecular functions of plants, affecting their growth and development. The MSAP specific fragment indicates a change in the methylation status of its homologous gene, which may affect its functional expression. This indicates that the grafting of pumpkin and cucumber induces a change in the genomic DNA methylation status of grafted cucumber self-crossing offspring. These changes can affect the growth and development of grafted cucumber self-crossing offspring and even affect the growth and phenotype performance of grafted cucumber self-crossing offspring. The changes in methylation status of two types of mutant materials may be one of the reasons for phenotypic trait variation.

Acknowledgement

The author thanks the project Henan province science and technology development plan project (202102110202), Henan province science and technology research and development orogram project (232102110233\242102110301) for providing support for this research and sincerely thanks the College of Horticulture and Landscape Architecture at Henan University of Science and Technology for providing all the land and laboratory facilities.

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(Manuscript received on 10 November 2023; revised on 30 May 2025)