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ORIGINAL ARTICLE

Effect of male sterile and fertile cytoplasm on nuclear DNA methylation in hybrid rice

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Abstract
Nucleus-controlled fertility restoration and cytoplasmic male sterility are important mechanisms to exploit heterosis. However, the effect of DNA methylation on cytoplasmic-nuclear interaction is not well understood yet. The current study used a methylation-sensitive amplified polymorphism to characterize polymorphism in nuclear DNA methylation among cytoplasmic male sterile line (D62A), corresponding maintainer line (D62B), and two F1 hybrids (D62A × R527 and D62B × R527). In results, 495 fragments were amplified between the parental D62A and D62B lines. The total methylation (double + single-stranded) and full methylation (double-stranded) rates of D62A (33.13%, 24.24%) both were found to be lower than that of corresponding maintainer D62B (33.94%, 24.85%). Analysis of methylation revealed that male sterile line D62A was less methylated than that of corresponding maintainer line D62B in all methylation types I, II and III. A total of 516 fragments were amplified between two F1 hybrids (D62A × R527 and D62B × R527). The total methylation in both hybrids (D62A × R527 and D62B × R527) was identical (34.69%). While full methylation rates for D62A × R527 and D62B × R527 were 25.78% and 25.58%, respectively, that is non-significant. Moreover, polymorphism in DNA methylation was found higher in F1 hybrids (5.43%) than parents (4.24%). These results implied that different cytoplasm leads to changes in nuclear DNA methylation and sterile cytoplasm has reduced the effect on nuclear methylation than non-sterile cytoplasm. Current study explains the interaction between cytoplasmic male sterility and DNA methylation which may contribute to further research.

Keywords CMS · F1 hybrid · Methylation level · MSAP · Polymorphism

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited character, in which plant cannot produce viable or fertile pollens (Traven et al. 2001). Cytoplasm causes pollen infertility by retrograde signaling that is originated from cytoplasm but control nuclear gene expression (Fujii and Toriyama 2008). Utilization of heterosis relies on three-line system including CMS, maintainer and restorer lines that are currently being used in hybrid seed production in rice. It is reported that cytoplasmic male sterility (CMS) is categorized into three types: wild abortion type (WA), the package table type (BT) and the red lotus type (HL), of which wild type relatively plays a major role (90%) in commercial production of hybrid rice in China (Xu et al. 2013). CMS is not only important for hybrid seed production, but also provides basis for genetical research of cytoplasmic inheritance and genetic distance among wild relatives (Ba et al. 2014). Therefore, the studies of CMS have always been concerned in molecular biology fields.

CMS line is a good source of nuclear-cytoplasmic interaction studies as its infertility is only attributed to cytoplasm. Whereas, A line and maintainer line (B) constitute same nucleus but heterogeneous body of cytoplasm. Therefore, A and B lines are considered to be an excellent material for

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studying the cytoplasmic effect (He et al. 2010). Previous studies were also carried to study cytoplasmic effect with homo-nuclear but hetero-cytoplasmic platform (Li et al. 2016). It was further reported that cytoplasm may lead to short plant height, delayed heading stage, shortened ear length and reduced yield per plant (Tao et al. 2004). Cytoplasm has also significant effects on filled-grain ratio and 1000-grain number per spike (Li et al. 2016; Tao et al. 2011). The genotypic effects of male sterile cytoplasm in rice are not found similar as reported in phenotypic studies, as genotype is largely influenced by environment, which shows limitations in the estimation of apparent epistatic effects (Xu et al. 2013).

DNA methylation may regulate plant growth and development by intervening regulation of gene transcription, silencing, transposon activity and gene expression (Law and Jacobsen 2010; Zhang et al. 2010). DNA methylation is an epigenetic event that occurs more frequently in CpG dinucleotide, where it alters gene expression and also affects cellular function (Ng and Adrian 1999). DNA methylation has been studied in many plants in response to biotic and abiotic stresses including cotton (Xu et al. 2000), Arabidopsis (Cervera et al. 2002), barley (Li et al. 2008), rapeseed (Guzywrobelska et al. 2013) and rice (Dowen et al. 2012; Shi et al. 2015). DNA methylation affects gene expression (Yan et al. 2010), genetic diversity (Yinging and Daming 2010), plant development (Dowen et al. 2012), cell differentiation (Ba et al. 2014), biotic and abiotic stress responses (Wang et al. 2011), inheritance to offspring (Xiong et al. 1999), and gene dosage (Hongyu et al. 2006). However, the effect of male sterile cytoplasm on nuclear DNA methylation in hybrid rice is not reported yet.

Epigenetic mechanisms such as methylation modifications (addition or removal of methyl groups to cytosine and adenine) can alter biological function of some traits, e.g. flowering time and gametophyte development (Du et al. 2015; Gazzani et al. 2003; Zemach et al. 2010). Although the male sterile and maintainer line have only a difference of male sterility, it showed different plant height, growth period and some other differences in phenotypic traits (Schmitz et al. 2013; Shi et al. 2015). To test the hypothesis, whether nuclear DNA methylation modifications are the causing factor for these differences, MSAP analysis was employed to detect methylation-sensitive restriction sites in D62A, D62B, D62A × R527, and D62B × R527. The objective of the current study was to explore effects of sterile and fertile cytoplasm on nuclear DNA methylation in CMS line A, corresponding maintainer line B, and F1 hybrids in rice.

Materials and methods

Plant materials

The experimental materials D62A (CMS line) and D62B (maintainer line) had been used for many years and backcrossed up to 30 or more generations in China for commercial hybrid seed production. D62A, D62B and R527 are conventional A, B and R lines, respectively, that were completely homozygous. Germplasm was obtained from Rice Research Institute, Sichuan Agricultural University (N: 30.67° E: 104.06°), Sichuan Province, China. F_1 generations D62A × R527 and D62B × R527 were obtained by hybridizing A and B lines with restorer line Shuhui 527 (R527), respectively. Crosses were grown in the paddy field of Chengdu (N: 30.67° E: 104.06°), Sichuan Province, to get the seed of F1 hybrids.

Seeds of each line (D62A and D62B, R527, two F1 hybrids D62A × R527 and D62B × R527) were planted in a flat tray filled with sand for germination and growth. Seedlings were grown under controlled conditions in an artificial climate chamber (Fuma Test Equipment, Tianjin, China Co. limited) as genomic methylome is affected by environment and stage (Xiong et al. 1999). Micro-conditions were maintained as following: temperature of 30/20 °C (day/night), 80% relative humidity, artificial light with a 12/13-h day/night photoperiod and about 180 μmol m^{-2} s^{-1} light intensity. After, the plants have reached four-leaf stage; first leaf was taken from each of line as a pool of five plants and used for DNA extraction.

DNA extraction

A CTAB method was used to extract the genomic DNA of leaves with multiple extractions with chloroform/isoamyl alcohol as the method described by Murray and Thompson (1980).

Polymorphism analysis in parents and hybrids

420 pairs of uniformly distributed SSR markers on 12 chromosomes were used to detect the DNA polymorphic sites among male sterile line (D62A), maintainer line (D62B), a restorer line (R527) and two F1 hybrids (D62A × R527 and D62B × R527). SSR markers were used as anchors that show polymorphism among male sterile line (D62A), maintainer line (D62B), a restorer line (R527) and two F1 hybrids (D62A × R527 and D62B × R527). The PCR amplification was performed according to (Xu et al. 2013) with following specifications. Pre-denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The PCR products were separated by 3% agarose gels, stained with ethidium bromide (EB) and photographed.

MSAP assay

Method described by (Xiong et al. 1999) was applied to get DNA methylation polymorphism among male sterile

line (D62A), maintainer line (D62B), and two F1 hybrids (D62A × R527 and D62B × R527). MSAP analysis consisting of two enzymatic digestions of *Eco*RI + *Msp*I and *Eco*RI + *Hpa*II was applied for each sample. The digestion, ligation, pre-amplification and selective amplification reactions were carried as reported in mentioned protocol. Primer and linker sequences of *Eco*RI+ *Msp*I and *Eco*RI + *Hpa*II (NEB) are given in Table 1. The amplified products were electrophoresed on 6% polyacrylamide gel and photographed after silver staining. Thus, genomic DNA with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I was ligated after the selective amplification; according to the presence or absence of both enzyme activities, methylation polymorphism was divided into four types (Ba et al. 2014), as shown in Table 2.

Types of methylation

The absence of bands from *Hpa*II and *Msp*I is recognized as Type I methylation that indicates the external cytosines on both DNA strands are methylated. The presence of bands from *Msp*I and absence from *Hpa*II is recognized as Type II methylation, which indicates the internal cytosines on both strands are methylated. The presence of bands from *Hpa*II but absence from *Msp*I is recognized as Type III methylation, which indicates the methylation on one of the either strands. The presence of bands from both *Hpa*II and *Msp*I is recognized as Type IV methylation that is representing either no methylation occurs or internal cytosines on one of the both strands are methylated.

Scoring of bands

The PAGE bands were calculated using the "1 and 0" assignment method. According to the presence or absence of bands in the lane, the electrophoretogram was marked with a clear band as "1"; the same position without band was labeled as "0". The total number of bands amplified and the methylation ratio of each pair of primers were calculated in MS Excel 2010.

Table 1 Primers and adapters used in MSAP assay of D62A, D62B and corresponding hybrids with R527

Primers and linkers	*Eco*RI	*Msp*I/*Hpa*II
Adapter 1	5′-CTCGTAGACTGCGTACC-3′	5′-GACGATGAGTCCTGAG-3′
Adapter 2	3′-CTGACGCATGGTTAA-5′	3′-TGCTACTCAGGACTCAT-5′
Pre-amp. primer	E00: 5′-GACTGCGTACCAATTC-3′	MH00: 5′-GACGATGAGTCCTGAGTAAA-3′
Selective primer		
Primer 1	E1: 5′-GACTGCGTACCAATTCAAC-3′	MH1: 5′-GATGAGTCCTGAGCGGCAA-3′
Primer 2	E2: 5′-GACTGCGTACCAATTCAAG-3′	MH2: 5′-GATGAGTCCTGAGCGGCAC-3′
Primer 3		MH3: 5′-GATGAGTCCTGAGCGGCAG-3′
Primer 4		MH4: 5′-GATGAGTCCTGAGCGGCAT-3′
Primer 5		MH5: 5′-GATGAGTCCTGAGCGGCTA-3′
Primer 6		MH6: 5′-GATGAGTCCTGAGCGGCTC-3′
Primer 7		MH7: 5′-GATGAGTCCTGAGCGGCTG-3′
Primer 8		MH8: 5′-GATGAGTCCTGAGCGGTCT-3′
Primer 9		MH9: 5′-GATGAGTCCTGAGCGGTCG-3′
Primer 10		MH10: 5′-GATGAGTCCTGAGCGGTCC-3′

Table 2 The activity and restrictive bands of *Hpa*II and *Msp*I

Type	Methylation		Restriction enzyme		Restriction band	
			*Hpa*II	*Msp*I	H	M
I	Double-stranded methylation	$^{5m}C^{5m}CGG\ GG^{5m}C^{5m}C$	Inactive	Inactive	−	−
II	Double-stranded methylation	$C^{5m}CGG\ GG^{5m}CC$	Inactive	Active	−	+
III	Single-chain methylation	$^{5m}CCGG\ GGCC$	Active	Inactive	+	−
IV	No methylation	CCGG GGCC	Active	Active	+	+

− band absent, + band present

Results

Nuclear genome purity testing

To ensure consistency in nuclear genome of five studied materials (a male sterile line D62A, a maintainer line D62B, a restorer line R527, two F1 hybrids D62A × R527 and D62B × R527), 420 SSR markers uniformly distributed on 12 chromosomes were applied to verify polymorphism in nuclear DNA. Among them, 324 pairs of markers were successfully amplified and showed clear bands. Results revealed that sterile line D62A, and maintainer line D62B showed same band position. F_1 hybrid bands for both D62A × R527 and D62B × R527 as shown in were also consistent in band position (Fig. 1). These results showed that parents (D62A, D62B) have same nuclear DNA. F1 hybrids (D62 A × R527, and D62 B × R527) which were derived from same restorer but different A and B lines also have same nuclear DNA that came from A or B line. This laid a solid foundation for studying DNA methylation polymorphism in A, B, A × R and B × R. RM150, RM152, RM153, RM154 and RM156 were SSR markers that showed the amplification of bands from D62A, D62B, D62A × R527, D62B × R527 and R527.

Difference in DNA methylation was attributed to cytoplasm

The selective amplified E_1 and E_2 primers were paired with HM1–HM10, respectively, to form 20 pairs of primers. Using these 20 pairs of primers, 495 bands were amplified between D62A and D62B line, and 516 bands were amplified between their corresponding hybrids A × R and B × R. Difference in the number of bands was based on homonuclear background of D62A and D62B, coupled with consistent background of A × R and B × R hybrids. Therefore, differences in the number of bands for methylation types, e.g. I, II and III, are reflecting the differences of methylation caused from different cytoplasm as they share same nuclear genetic backgrounds.

Analysis of DNA methylation type and level in parents and hybrids

A total of 495 bands were amplified between parents D62A and D62B lines (Table 3). Among methylation types, type I was found to be lowest than type II and III. The number of total methylation sites (including full methylation and only one strand methylation, i.e. type I, type II and type III) and methylation amplification polymorphism were 164 and 168 that account for 33.13% and 33.94%, respectively. The total methylated numbers of bands were 120 and 123 in D62A and D62B lines, respectively. Their full methylation rates (double-stranded methylation, i.e. type I and type II) were 24.24% and 24.85%, respectively. Male sterile line D62A was less methylated than that of corresponding maintainer line D62B in all methylation types I, II and III. These results revealed that D62A was lower in total methylation and full methylation level than its corresponding maintainer D62B line.

516 fragments were amplified between corresponding F1 hybrids of A and B lines, D62A × R527 and D62B × R527 as shown in (Table 3). Unlike the parents, both, F1 hybrids showed identical total methylation (34.69%) but methylation rates were relatively found higher in hybrids than the corresponding parents.

Polymorphism of DNA methylation between parents and hybrids

DNA methylation polymorphism between parents and hybrids was analyzed based on the presence or absence of amplification of both (*Hpa*II and *Msp*I) isoschizomers (Fig. 2). At each locus, of *Eco*RI + *Hpa*II digestion or *Eco*RI + *Msp*I digestion, differences in polymorphism were detected between parents and hybrids, and each position was considered as a polymorphic locus. Therefore, the degree of polymorphism at methylation level between parents and hybrids can be calculated. Results revealed that overall polymorphic loci and polymorphism were 21 and 28 that account

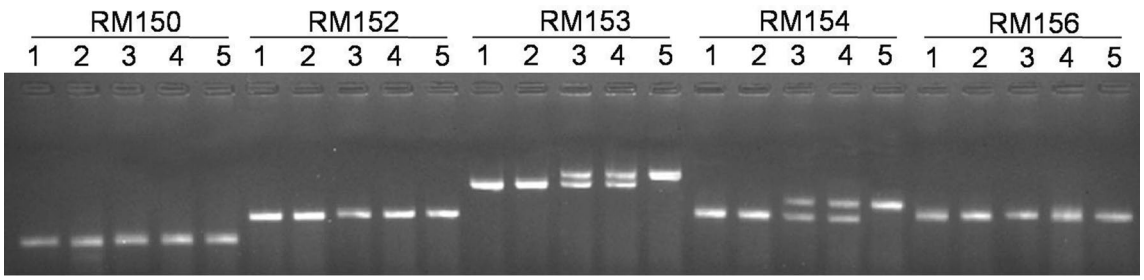

Fig. 1 The amplified bands with SSR primers among parents and their corresponding hybrids. Lanes from 1 to 5 are sample of D62A, D62B, D62A × R527, D62B × R527 and R527, respectively

Table 3 Types of DNA methylation among parents and corresponding hybrids

Types of methylation	Parents		F_1	
	D62A	D62B	D62A × R527	D62B × R527
Methylation type I	5	7	9	8
Methylation type II	115	116	124	124
Methylation type III	44	45	46	47
Methylation type IV	331	327	337	337
The total number of bands	495	495	516	516
Total methylation band	164	168	179	179
MSAP %	33.13%	33.94%	34.69%	34.69%
Full methylation band number	120	123	133	132
Full methylation rate %	24.24%	24.85%	25.78%	25.58%

Total methylated bands = I + II + III

MSAP (%) = (I + II + III)/(I + II + III + IV) × 100%

Full methylated bands = I + II

Ratio of fully methylated sites = (I + II)/(I + II + III + IV) × 100%

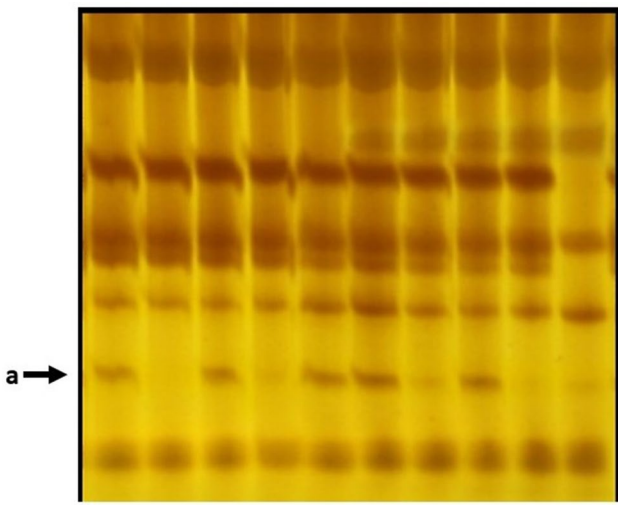

Fig. 2 Example of DNA methylation polymorphism detected in parents and corresponding hybrids. Lanes from 1 to 5 are sample of D62A, D62B, D62A × R527, D62B × R527 and R527, respectively. Arrow means a polymorphism site found between D62A and D62B, and also between D62A × R527 and D62B × R527

Table 4 Polymorphism of DNA methylation in parents vs. hybrids

Types of methylation	Parents	F_1
Polymorphic loci	21	28
Total recognition site	495	516
Polymorphism (%)	4.24%	5.43%

for 4.24% and 5.43%, respectively, between parents and F1 hybrids (Table 4).

The results shown in Tables 3 and 4 revealed polymorphisms in DNA methylation between male sterile line D62A and maintainer line D62B based on their two polymorphic loci on each position, as sterile line D62A and maintainer line D62B have same nucleus, and are only different in cytoplasm. Therefore, changes in the level of methylation are attributed only due to different cytoplasm. Furthermore, methylation level between D62A × R527 and D62B × R527 was consistent, but polymorphism was higher in hybrids (5.43%) than parents (4.24%).

Discussion

Reliability of detection of DNA methylation

Current study employed a universally accepted method for the detection of methylation, that is highly efficient for large-scale detection of cytosine methylation in plants (Chakrabarty et al. 2003; Dong et al. 2006; Sha et al. 2005; Xiong et al. 1999). Current study used MSAP to analyze differences of methylation in nuclear DNA between iso-nuclear but allo-cytoplasmic A and B lines, and studied polymorphism under intervention of a restorer line. *Hpa*II is insensitive to the full methylation site (double-stranded methylation), while *Msp*I is insensitive to the semi-methylation site (single-stranded methylation) (Sha et al. 2005). It is possible, that level of detected methylation may be lower than actual, but data suggest that both enzymes do not recognize off-mentioned methylation types that appeared to be low in frequency (Cervera et al. 2002). In current results, total methylation (MSAP %) that was calculated from number of methylated restriction sites could be assigned as the relative degree of DNA methylation (Zhang et al. 2006). In present experiment, a relative degree of DNA methylation in different material (D62A, D62B, D62A × R527 and D62B × R527) was ranged between 33.13 and 34.69%. Results of present study suggested that genomic DNA of hybrids (D62A × R527 and

D62B × R527) was more methylated than their parents (D62A and D62B), which is different from previous studies (Xiong et al. 1999). As polymorphism difference is based on the influence of two homo-nuclear but different cytoplasmic lines (D62A and D62B), changes in nuclear methylation level are only attributed to cytoplasm. Therefore, the selection of appropriate cytoplasm is important in hybrid rice breeding. Using this relationship between DNA methylation and cytoplasm–nucleus interaction, we may design our future studies on mechanisms of nuclear methylation regulation associated with hybrid rice.

Sterile cytoplasm affects nuclear DNA methylation status

A high-throughput sequencing technology in the progeny of reciprocal crosses of Nipponbare and 9311 was used (He et al. 2010). Although the phenotypes of reciprocal hybrid did not show significant differences in field, the whole-genome methylation analysis revealed differences in histone modification and small RNA quantity. Methylation modification and gene expression revealed highly significant differences among the reciprocal hybrid that has same nuclear background except cytoplasm. Current study also predicts different cytoplasmic effects on nuclear DNA methylation were possible reasons for differences. Mitochondrial and chloroplast DNA also vary among different cytoplasm (Miyashita et al. 1994). Further studies should be focused on the role of methylation of chloroplast and mitochondrial DNA in male sterile and fertile line to understand the mechanism of sterility induction. Once the mechanism is fully understood, artificial engineering of methylation can be manipulated to induce sterility for developing efficient CMS lines.

Cytoplasmic male sterility and fertility restoration may regulate the level of DNA methylation

D62A belongs to wild-type cytoplasmic male sterility system. The wild-type sterile cytoplasm can produce a cytotoxic protein that accumulates in microspores and results in male sterile sporophytes (Ahmadikhah and Karlov 2006). Restoration lines allow fertility of sterile line to recover, which involves complex mechanism of cytoplasmic-nuclear interactions (Jing et al. 2001). The restorer line R527 used in current study is also a strong restorer line containing two restoring genes (*RF3* and *RF4)*. *RF4* functions at the transcriptional level, whereas *RF3* works at translational level (Chen and Liu 2014), which indicates that these restoring genes can act upon sterile cytoplasm at transcriptional and translational levels. The data in this study are indicating that intervention of restorer line can affect cytoplasm by modifying the level of methylation. This observation opens a new way to study the interaction between sterile cytoplasm and restorer genes. Moreover, results of Table 4 also suggest that sterile and fertile cytoplasm could buffer the change in methylation partially after crossing with restorer line, but restorer line cannot completely offset the effect of the sterile cytoplasm. Moreover, it was further confirmed that the methylation level could be modified in the presence of restorer stimulus. But to prove this prediction, more research is needed to be done. Subsequent studies can be carried out by sequencing of recovery fragments of polymorphic methylation. Gene function analysis is required for in-depth discussion of possible regulatory mechanisms of methylation.

Author contribution statement AA, XW, HC, and PX conceived and designed the experiments. AA and WK performed the experiments. AA, LY, MI, MF, CX, and HZ contributed to the writing of the manuscript. All authors read and approved the manuscript.

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