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Variation of DNA Cytosine Methylation Patterns among Parent Lines and Reciprocal Hybrids in Hot Pepper

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DNA methylation plays an important role for regulation of gene expression in plants. Heterosis has been widely explored in *Capsicum* breeding to improve yield and quality, but the genetic and molecular mechanism underlying the phenomenon remains elusive. In present study, the genomic cytosine methylation of two *Capsicum* genotypes (the purple and green cotyledon hot pepper) and their reciprocal hybrids were analyzed using the methylation sensitive amplified polymorphism (MSAP). Results showed that the levels of DNA methylation in $F_{1\ D85\times D34}$ (67.0%) was higher, but $F_{1\ D34\times D85}$ (64.36%) was slightly lower, than the mid-parent value (MPV, 64.83%). Moreover, the characteristics of DNA methylation status were significant different among the reciprocal hybrids and their parental lines. Four classes of MSAP patterns, A, B, C and D, were identified. In pattern B, the de-methylation ratio, the proportion of de-methylated loci in the total polymorphic methylation loci, was 36.2% and 41.0% in $F_{1\ D85\times D34}$ and $F_{1\ D34\times D85}$. The hyper-methylation ratio (pattern C), the proportion of hyper-methylation loci in the total polymorphism methylation loci, was 51.1% and 41.6% in $F_{1\ D85\times D34}$ and $F_{1\ D34\times D85}$. This study has demonstrated that in *Capsicum* heterosis involves adjustment of DNA methylation, especially changes in DNA methylation patterns.

1. Introduction

Heterosis is a phenomenon in which hybrids exhibit superior phenotypes, such as enhanced biomass production, development rate, grain yield, and stress tolerance, relative to their parents. Heterosis has been effectively utilized to increase crop production in the world (Cheng et al., 2007). In recent years, the understanding of molecular mechanisms in plant heretorsis has made significant progress owing to the development of molecular biology. However, Our knowledge of the genetic mechanisms of heterosis has lagged behind its wide application. Several studies have reported that changes in epigenetic properties such as DNA methylation have occurred during the heterosis formation process in plants (Zhao et al., 2008).

Plant DNA methylation is easier to detect, and it is closely associated with regulation of gene expression and thus has received a great deal of attention. The DNA methylation level and pattern in the genomes of hybrids and parental lines have been studied extensively in maize (Lauria et al., 2004; Zhao et al., 2004), sorghum (Yi et al., 2005; Zhang et al., 2007), rice (Xiong et al., 1999; Dong et al., 2006; Takamiya et al., 2008), potato (Sanetomo and Hosaka, 2011), beans (Abid et al., 2011) and cotton (Zhao et al., 2008; Wei et al., 2012). These studies have identified significant changes in the genome DNA methylation level and patterns in hybrid offspring from their parents.

Pepper (*Capsicum* spp.) is an important vegetable crop in the Solanaceous family. During the past 50 years researches have been performed on the molecular mechanisms of hetersosis for phenotypes and physio-chemical properties, and the genetics and molecular markers associated with those traits. The introduction of various molecular marker technologies has led to significant progress in the fields of population classification and genetic distance for particular traits. The most popular molecular marker technologies include RFLP (Prince et al., 1992), RAPD (Sanatombi et al., 2010；Li et al., 2011), AFLP (Lefebvre et al., 2001), SSR (Chen et al., 2012), SRAP (Chen et al., 2012), and ISSR (Xu et al., 2013). But there is a void of information on heterosis and epigenetics such as DNA methylation in pepper.

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Purple pepper is a rare germplasm species found in China. Cotyledons, true leaves, stem, flowers, and young fruits are all purple color. It has comprehensive tolerance properties to fairly high temperature, drought, and diseases (Xu et al., 2011). Meanwhile, the purpose cotyledon trait can be used as a marker traits in early stage of hybridization screening, and thus increase the selection efficiency at early breeding stage. Because of these properties, this study used the purple pepper D85 and D34 with green cotyledons ad the parents, and the reciprocal crosses, using the methylation-sensitive amplified polymorphism (MSAP), to analyse genome DNA methylation properties from the parents and offspring. The objective was to determine the pepper genome DNA methylation changing properties and trend during heterosis formation process, this providing bases for the understanding of epigenetics regulation for the molecular mechanism of heterosis in pepper.

2. Materials and methods

2.1 Materials

Purple pepper 'D85' producing purple cotyledons, and 'D34' producing green cotyledons were used as parental lines. Both parental lines were self-pollinated for 6-8 generations to generate pure inbred lines. In spring, 2009, in Baiyun experimental station, Vegetable Institute, Guangdong Agricultural Academy of Sciences, the following two crosses were made: D85×D34 and D34×D85. F1 seeds were harvested. In spring, 2010, seeds from D85, D34, D85×D34, D34×D85 were sown in potting mix and seedlings were raised in 9 cm×9 cm pots in a greenhouse. A complete randomized block design was used to set up the greenhouse experiment. Plants were managed following regular schedules.

2.2 DNA extraction and purification

Genomic DNA was first isolated from expanded leaves at the 7-8th leaf-stage, of pooled hot pepper plants of the two inbred lines and hybrids by a modification CTAB method. . Additionally, the RNA enzyme was added to the DNA liquor to digest residual RNA; DNA quality, purity, and its content were tested by using the 0.8% agarose gel electrophoresis and ultraviolet spectrophotometer.

2.3 MSAP analysis

The methylation-sensitive amplified polymorphism analysis (MSAP) method essentially as reported (Li et al., 2014) was used. MSAP method was mainly described as follows: at first, genomic DNA of hot pepper was divided into two groups, for the first group, 20 units *Eco*RI (Takara, P.R. China) and 20 units *Hpa*II (Takara, P.R. China) were used to digest 200 ng genomic DNA in 20 µl of reaction mixture at 37°C for 2 h. For the second group, instead of *Hpa*II, the *Msp*I (Takara, P.R. China) was used in combination with *Eco*RI to digest 200 ng genomic DNA with the same condition. The enzyme digestion reaction was terminated by incubation at 65°C for 10 min. Then, the ligation reaction was proceeded in a final volume of 40 µl mixture, which contains 1 unit T4 DNA ligase, 0.2 mM ATP, 5 pm *Eco*RI adapters and 50 pm *Hpa*II/*Msp*I adapters for additional 6 h at 20°C (Table 1). Subsequently, the ligation mixture was used as the template for the preselected amplification reaction with *Eco*RI+A and *Hpa*II / *Msp*I+T primers (Table 1). The polymerase chain reactions were carried out in a 25 µl reaction mixture with 1 µl of ligation reaction mixture, 50 ng of E+A primer, 50 ng of HM +T primer, 0.5 unit *Taq* DNA polymerase (Biocentury transgene, P.R. China), 0.2 mM dNTP (Biocentury transgene, P.R. China) and 2.5 µl of 10× polymerase buffer (Biocentury transgene, P.R. China) for 21 cycles with 1 min denaturation at 94°C, 1 min annealing at 56°C, and 1 min extension at 72°C. The preselected amplification products were used as the template for the selective amplification reaction. The primers were the *Eco*RI and HpaII/*Msp*I primers with two added selective nucleotides (Table 1). The polymerase chain reactions were carried out in total volumes of 25 µl, containing 0.3 µl of pre-amplification product, 50 ng of *Eco*RI primer, 50 ng of *Hpa*II/*Msp*I primer, 1 unit Taq polymerase, 0.5 mM dNTP and 2.5 µl of 10× PCR buffer. The PCR procedure was performed according to the standard amplified fragment polymorphism touchdown protocol. The products of selective amplification were mixed with 8 µl of denaturating buffer (98% formamide, 10 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol), then denatured at 95°C for 5 min and separated on 6% polyacrylamide gel in 1× 44.5 Mm Tris/Borate, 0.5 Mm EDTA, pH 8.0 (TBE) buffer at 80 watts for 1 h. Gels were stained according to the silver staining method.

After staining, the bands that appeared in the electrophoretogram were detected. The scored MSAP bands were transformed into a binary character matrix, using "1" to define the presence of a band and "0" to define the absence of a band, respectively.

Using the two-dimensional matrix, the following information was recorded for each plant: I, not cut with *Hpa*II, but with *Msp*I (0, 1): II, cut with *Hpa*II, but not with *Msp*I (1, 0); and III, cut with *Hpa*II and *Msp*I (1, 1).

3. Results and analysis

3.1 DNA methylation level in pepper hybrids and parental lines

Table 1: The MSAP adaptors and primers

	*Eco*RI primers	*Hpa* II *Msp* I primers
Adaptor-1	CTCGTAGACTGCGTACC	GATCATGAGTCCTGCT
Adaptor-2	AATTGGTACGCAGTC	CGAGCAGGACTCATGA
Primers for pre-amplification	GACTGCGTACCAATTCA(E00)	ATCATGAGTCCTGCTCGG(H/M00)
Primers for selective amplification	GACTGCGTACCAATTCAAC(E1)	ATCATGAGTCCTGCTCGGTCG(H1)
	GACTGCGTACCAATTCAAG(E2)	ATCATGAGTCCTGCTCGGTTA(H2)
	GACTGCGTACCAATTCACA(E3)	ATCATGAGTCCTGCTCGGTGA(H3)
	GACTGCGTACCAATTCACT (E4)	ATCATGAGTCCTGCTCGGTGT(H4)
	GACTGCGTACCAATTCACG(E5)	ATCATGAGTCCTGCTCGGTGC(H5)
	GACTGCGTACCAATTCAGC(E6)	ATCATGAGTCCTGCTCGGTCC(H6)
	GACTGCGTACCAATTCATC(E7)	ATCATGAGTCCTGCTCGGTCT(H7)
		ATCATGAGTCCTGCTCGGTTC(H8)
		ATCATGAGTCCTGCTCGGTAC(H9)

From the 63 primer pairs (Table 1), ten primer pairs produced clear and distinct banding patterns. These ten pairs of MSAP primers, H1E2, H2E3, H2E4, H2E7, H3E7, H4E7, H5E2, H7E3, H7E6 and H7E7, were used in the amplification reactions of methylation bands from four pepper lines. In D85, D34 and the two reciprocal hybrid crosses, 2792 loci were identified. Data analysis indicates the total methylation ratio is 63.51% ~67.0% in the whole population examined in this study (Table 2).

Table 2: DNA methylation level of genomes from reciprocal crosses and parental lines of pepper

Methylation band type		D85	D34	Mid-parental value	$F_{1D85\times D34}$	$F_{1D34\times D85}$
*Hpa*II	*Msp*I					
	+	253	264	259	264	257
+		156	223	189	207	198
+	+	235	251	243	232	252
Number of loci		644	738	691	703	707
Methylation ratio (%)		63.51	65.99	64.83	67.0	64.36

Single strand methylation loci (0, 1) is more dominant than the double-strand methylation loci (1, 0). Further analysis found that in the direct-cross hybrid $F_{1\ D85\times D34}$, the total methylation level (67.0%) was higher than the mid-parental value (64.83%). For the back-cross hybrid, F_1D34×D85, the total methylation level was 64.36%, which is slightly lower than the mid-parental value.

3.2 DNA methylation patterns in parental lines and hybrids

DNA methylation at specific sites were compared among reciprocal crosses and parental lines to identify the types of cytosine methylation. Based on the inheritance and changes in cytosine methylation from parents to hybrid offspring, the four types of methylation sites were grouped into 32 subtypes.

Type A has a monomorphic band, which indicates that the same CCGG locus was found in the two parental lines and their crosses. A1 is the non-methylated subtype where the reciprocal crosses, $F_{1\ D85\times D34}$ and $F_{1D34\times D85}$, each has 64 and 85 loci, respectively. A2 is the completely methylated subtype. The reciprocal crosses, $F_{1\ D85\times D34}$ and $F_{1D34\times D85}$, each has 47 and 69 loci. A3 is semi-methylated subtype where only one strand is methylated during the reciprocal crossing process. $F_{1\ D85\times D34}$ and $F_{1D34\times D85}$, each has 12 and 11 loci under the A2 subtype. It is very clear that the number of monomorphic loci (111) in the direct cross, $F_{1\ D85\times D34}$ (111), is less than in the back-cross hybrid, $F_{1D34\times D85}$ (165).

Type B contains all the demethylated loci, which concurs with a reduction of methylation in the hybrid offspring than the parental lines. Type B was divided into 13 subtypes. From the reciprocal crosses, $F_{1\ D85\times D34}$ and $F_{1D34\times D85}$, 224 and 251 methylated loci belong to the type B, which accounts for 36.2% and 41% of the total number of methylated polymorphic loci. The demethylation level in the back cross, $F_{1D34\times D85}$, is higher than the direct cross, $F_{1\ D85\times D34}$.

Of the 13 subtypes, B13 is the dominant subtype in the reciprocal crosses, and 86 and 39 loci were amplified in F1 $_{D85\times D34}$ and $F_{1D34\times D85}$, respectively. The B1 type has the greatest level of variation between the two hybrids, where the direct cross, $F_{1D34\times D85,}$ is 16-fold more in loci number than the direct cross, $F_{1\ D85\times D34}$ (Table 3).

Table 3: The types of methylation in parental and hybrid lines

Methylation types	MSAP band pattern						Number of loci and percentage	
	Maternal parent		Paternal parent		F_1		$F_{1\ D85\times D34}$	$F_{1\ D34\times D85}$
	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I		
A1	+	+	+	+	+	+	64	85
A2		+		+		+	47	69
A3	+		+		+		12	11
Number of monomorphic loci							111	165
B1	+	+			+	+	2	32
B2		+				+	14	42
B3				+		+	32	25
B4			+	+	+	+	27	10
B5	+				+		6	40
B6		+		+	+	+	13	14
B7		+			+	+	3	6
B8		+	+		+	+	4	1
B9	+	+	+		+	+	19	10
B10	+		+	+	+	+	11	22
B11	+		+		+	+	3	6
B12	+				+	+	4	4
B13						+	86	39
Total number of de-methylated loci							224(36.2%)	251(41%)
C1			+	+			24	15
C2	+		+	+			4	10
C3	+						76	64
C4	+	+	+	+		+	25	14
C5		+		+			19	7
C6		+					68	76
C7				+			81	54
C8	+	+	+	+	+		16	7
C9	+	+				+	3	13
Total number of hyper-methylated loci							316(51.1%)	260(42.6%)
D1		+	+	+	+	+	25	10
D2	+	+		+	+	+	12	35
D3	+	+		+		+	5	14
D4		+	+	+		+	10	8
D5	+		+	+	+		4	21
D6	+	+	+		+		12	4
D7	+	+			+		10	8
							78(12.6%)	100(16.4%)
Total number of polymorphic methylation loci							618	611

Type C refers to the loci where the methylation level in the hybrids is higher than the parental lines, they are also known as hype-rmethylation loci. In the reciprocal crosses, $F_{1\ D85\times D34}$ and $F_{1D34\times D85}$, each has amplified 31 and 260 loci, which accounts for 51.1% and 42.6% of the total number of amplified polymorphic methylated loci.

It thus can be concluded that the hype-rmethylation level of the direct cross, $F_{1\ D85\times D34,}$ is higher than the back-cross $F_{1D34\times D85}$. The C-type was divided into 9 subtypes, and the C3, C6 and C7 represent the major methylation patterns in this group.

Type D has polymorphic methylation loci in hybrids and parents, and those polymorphism is inherited following the Mendelian's rules. In $F_{1\ D85\times D34}$ and $F_{1D34\times D85}$, each amplified 78 and 100 loci, which is 12.6% and 16.4% of the total number of polymorphic methylation loci. Type D was divided into 7 sub-types. From Table 3, it can be seen that type D loci are distributed diffusely across those subtypes.

4. Discussion

Recent studies on heterosis have demonstrated that epigenetic mechanisms play a significant role in regulating expression of specific genes in a hybrid genome, and it is feasible to enhance the growth vigor of hybrids by regulating the epigenetic pathways (Zhao et al., 2008). Several previous studies have demonstrated that the MSAP method is highly efficient for larger-scale detection of cytosine methylation in plant genomes (Ezio et al., 2004; Li et al., 2011).

Changes in methylation level occur during the processes of inter-specific hybridization as well as intra-specific crossing. A study of high-performance liquid chromatography analysis of DNA 5-methylcytosine in maize hybrids and parental lines found that the F_1 hybrid genome contains a lower methylation level than both parents (Tsaftaris et al., 1998). However in sorghum (Yi et al., 2005), cotton (Zhao et al., 2008), rice (Takamiya et al., 2008) and larix (Li et al., 2012), analysis using the MSAP method found that the methylation level in hybrid offspring and parental genome DNA is significantly lower than the mid-parental value. Furthermore, those results suggest that the reduced methylation in the hybrid genome DNA affects expression of genes conferring heterosis. But in several other studies, it was found that F_1 offspring show a higher methylation level than both parents. For instance, MSAP analysis of rice hybrids and parental genomes found that cytosine methylation is slightly higher in the hybrid offspring than the parental lines (Xiong et al., 1999). .

Results from this study on hot pepper revealed differential DNA methylation level among reciprocal crosses and their parental lines. In the direct cross, $F_{1\ D85\times D34}$, the total methylation level was higher than the mid-parental value, but in the back-cross offspring, $F_{1D34\times D85}$, it became slightly lower than the mid-parental value. It seems that heterosis in pepper may not involve changes in DNA methylation, however, the DNA methylation level in the hybrid offsprings was different from the parental lines.

During the process of hybridization in plants, it is accompanied by changes in DNA methylation patterns. This study on peppers has revealed alteration in methylation banding patterns from parental lines to the offspring of reciprocal crosses. Those changes can be summarized into three major types, and for each type over 10 methylation patterns were identified. The B type methylation variation follows very diffuse patterns, but more loci were found in the B13 sub-type. The C type methylation variation was clustered on a few patterns. D type is also more diffuse with methylation loci spreading out across nearly all the patterns. Correlation analysis on sorghum also found (Yi et al., 2005) that heterosis phenotypes are closely associated with changing methylation banding patterns from parents. In this study, only one pair of reciprocal crosses of pepper was compared. More diverse genetic materials should be analyzed to establish the relationship between heterosis and DNA methylation in pepper.

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