DNA methylation polymorphism in a set of elite maize inbred lines revealed by methylation-sensitive ISSR analysis

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Summary

DNA methylation polymorphism among nine elite maize inbred lines was assessed by inter-simple sequence repeat (ISSR) fingerprinting on intact DNA and DNA digested by either of a pair of methylation-sensitive isoschizomers, *Hpa*II and *Msp*I. It was found that, along with distinct genetic differentiation, extensive DNA methylation polymorphism exists among the nine inbred lines. The line-specific methylation patterns are homogeneous within each line, indicating their usefulness as molecular markers for cultivar identification. DNA sequences underlying the DNA methylation variations include a high proportion of coding genes. Cluster analysis, however, indicates the existence of incongruence between DNA methylation polymorphism and known-pedigree of the maize inbred lines.

Index Words: Maize inbred lines, DNA methylation polymorphism, ISSR fingerprinting, Molecular marker

Introduction

Covalent modification of nuclear DNA by adding a methyl group to the 5-position of normal cytosines is common in most eukaryotes, and particularly prevalent in higher plants, with up to 30% of their cytosine residues being modified in this way (Richards 1997). In contrast to animals wherein the methylation patterns are reset in each generation, in plants the methylation patterns are usually stably inherited through generations (Messeguer *et al.* 1991; Kovarik *et al.* 1997; Colot & Rossignol 1999; Tariq & Paszkowski 2004), and hence, may serve as useful epigenetic markers. Cytosine DNA methylation has been implicated to play important roles in controlling development-associated gene expressions, and in maintaining genomic integrity by repressing activity of invasive nucleic acids, like mobile elements and viruses (Colot & Rossignol 1999; Kato *et al.* 2003).

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The usefulness of DNA markers in plant breeding is being increasingly appreciated by plant breeders. This includes assessment of genetic diversity, parental selection, cultivars development and authentication etc (Morgante & Salamini 2003). Given the important functions of DNA methylation, its variability among cultivars or lines may represent a novel and useful addition to conventional genetic markers based on variations in nucleotide sequence. Indeed, it has been found in several plant species, including rice (Ashikawa 2001; Xiong *et al.* 1999; Wang *et al.* 2004), *Pisum* (Knox & Ellis 2001), *Arabidopsis* (Cervera *et al.* 2002; Riddle & Richards 2002) and cotton (Keyte *et al.* 2005) that DNA methylation polymorphism is abundant and potentially exploitable as molecular markers for cultivar identification and breeding purposes. More interestingly, it was found in maize that the epigenetic states (e.g., DNA methylation patterns) in inbred lines appeared to associate with heterosis of the F1 hybrids under normal or stressful conditions (Hollick & Chandler 1998).

The present study was aimed to further explore characteristics of DNA methylation polymorphism in maize inbred lines, and the usefulness of this kind of polymorphism as molecular markers. By using nine maize inbred lines that are currently being extensively used for hybrid production in the "corn belt" of China, we investigated the extent of within-line stability and inter-line variation in DNA methylation polymorphism, as well as the relationship between DNA methylation polymorphism and genetic relatedness of the maize inbred lines.

Materials and methods

Plants: A set of nine elite maize inbreed lines (Mo17, Ji842, 7922, Ji818, Longkang11, Ji853, 1037, M67 and 444) currently being extensively employed in hybrid seed production in the "corn belt" of China, were used in this study. The inbred nature of the lines was ensured by strict bagging in each generation.

DNA Isolation and ISSR fingerprinting: Genomic DNA was isolated from expanded leaves of individual plants by a modified CTAB method (Kidwell & Osborn 1992). Each DNA sample was divided into three portions: undigested, digested with *Hpa*II and digested with *Msp*I. Being a pair of isoschizomers, *Hpa*II and *Msp*I are differentially sensitive to methylation of cytosines at the CCGG sites, a predominant methylation sites in both animals and plants (Gruenbaum *et al.* 1981). To ensure complete digestion, two successive rounds of incubations were performed. That is, the DNA samples were first incubated with 3-fold enzymes (1μg/3U) overnight, then the same amount of enzymes were added and incubated again for another overnight. The uncut and cut DNA samples were then subjected to inter-simple sequence repeat (ISSR) analysis (Zietkiewicz *et al.* 1994) with 29 pre-selected primers. The PCR products were resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide-staining under a UV-transilluminator.

Cluster analysis of the maize inbreed lines based on the ISSR banding patterns: ISSR bands generated from uncut and cut DNA of the nine maize inbred lines were transformed into a binary character matrix ("1" for presence and "0" for absence of a band at a particular position). Pairwise distance (similarity) matrices were computed based on Jaccard's coefficient of similarity using the NTSYS-pc version 2.02a software package (Rohlf 1993). Dendrograms were created with the UPGMA (unweighted pair group method with arithmetic averaging) cluster analysis (Swofford & Olsen 1990).

Sequence analysis of the bands showing methylation polymorphism: A subset of bands that showed clear methylation polymorphism among the inbred lines in the ISSR profiles were isolated, reamplified by the same ISSR primer and ligated into the TA-cloning vector (The Shengong Biotech. Inc, Shanghai, China). The clones were sequenced by using vector primers and queried against the Genbank database for similarity at the NCBI website (http://www.ncbi.nlm.nih.gov/).

Result and discussion

ISSR fingerprinting implicates extensive methylation polymorphism among the studied maize inbreed lines: It was noted that there are many polymorphic bands in the ISSR patterns among the nine maize inbred lines in the undigested DNAs, which indicate primary sequence variations at, and/or internal of, the ISSR priming sites among these lines (Fig. 1, marked by empty arrowheads). In addition to the genetic variations, many bands are monomorphic among all or some of the lines in the undigested samples; nevertheless, these bands showed marked variation in either of the *Hpa*II/*Msp*I digestions (Fig.1, marked by solid arrowheads). Because *Hpa*II and *Msp*I are a pair of isoschizomers recognizing the same sequence CCGG but have different sensitivity to the methylation states of either cytosines, the later type of polymorphism should reflect DNA methylation polymorphism among the maize lines. Specifically, *Hpa*II will not digest if either the inner or outer cytosines are fully methylated but will digest if the outer cytosine is hemi-methylated, whereas *Msp*I will not digest if the outer cytosines are fully or hemi-methylated, but will digest if the inner cytosines are methylated (McClelland *et al.* 1994). Hence, a band amplifiable in *Hpa*II but not in *Msp*I denotes methylation of the inner cytosine(s) of the CCGG site(s) internal of a given ISSR primer, while its amplification in *Hpa*II but not in *Msp*I indicates hemi-methylation of the outer cytosine(s). Apparently, both types of DNA methylation polymorphism exist among the nine maize inbred lines in nearly all the ISSR primers studied (Table 1 and Fig. 1). The degree of methylation polymorphisms among the maize inbreed lines is much higher than those detected in rice (Ashikawa 2001) and cotton (Keyte *et al.* 2005), which may correlate to the exceptionally high level of within species sequence diversity in maize (Walbot 2004), as genomic structural divergence may interlace with methylation alterations (reviewed in Liu & Wendel 2003). For the methylation polymorphism to be useful as molecular markers, it is essential that patterns be conserved within a given line. To test this, randomly chosen

individual plants for each of the nine inbred lines were analyzed by five selected ISSR primers that showed the highest level of methylation polymorphism among the lines (Table 1). It was found that in all cases, the line-specific methylation patterns are perfectly conserved in all individual plants of a given line (e.g., Fig. 2), indicating stability of the line-specific methylation patterns. This result is in accord with the data on rice(Ashikawa 2001; Wang *et al.* 2004) and cotton (Keyte *et al.* 2005).

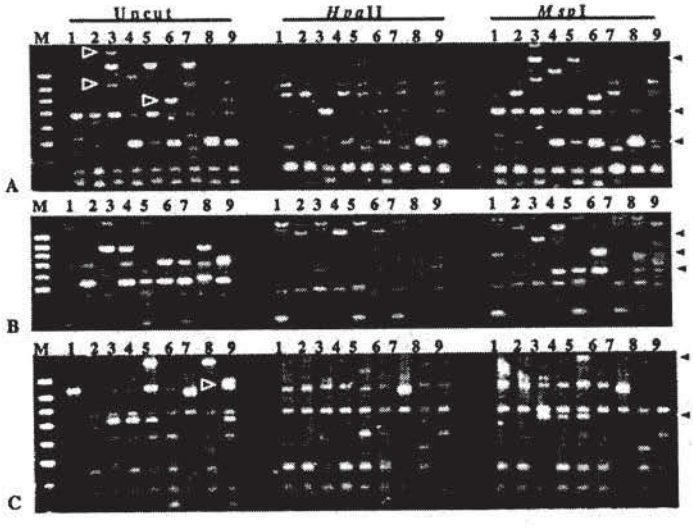

Fig. 1. Examples of ISSR amplification profiles [primers $(CT)_8RG$, $(GA)_8C$ and $(AC)_8T$ respectively for A, B and C] on intact and *HpaII*/*MspI*-digested genomic DNAs of the nine maize inbred lines (lanes 1 through 9 are respectively Mo17, Ji842, 7922, Ji818, Longkang11, Ji853, 1037, M67 and 444). Polymorphism among the lines due to genetic variation are indicated by empty triangles in the amplifications from uncut DNA, whereas polymorphisms due to differential cytosine methylation at the CCGG sites (as being reflected by banding pattern changes only in amplifications from *Hpa*II or *Msp*I digested DNA not from uncut DNA) are indicated on the right side by filled triangles. The amplification products were resolved in 2% agarose gels and stained by ethidium bromide. The 100 bp DNA ladder (Fermentas Inc.) was used as a molecular size marker.

Sequences underlying DNA methylation polymorphism among the maize inbred lines: To get some insight into the nature of sequences underlying the differential methylation patterns among the maize inbred lines, we isolated and sequenced 16 bands from the ISSR gels. It was found that all 16 sequences contain one or more CCGG site(s) internal of the ISSR primers, confirming that their amplification on *Hpa*II- and/or *Msp*I-digested **DNA of** some inbred lines was due to cytosine methylation of the relevant CCGG sites, whereas the failure of their amplification in other lines was resulted from hypomethylation at these sites. Homology querying showed that three bands are highly homologous to known-function genes of maize or rice, while another five bands had significant homology with maize cDNAs (Table 1). The

differential methylation of such a high proportion of coding sequences underscored the possible functional significance of the methylation polymorphism among the maize inbred lines (Portis *et al.* 2003; Tariq, Paszkowski 2004; Wada *et al.* 2004). The rest 10 bands showed no homology.

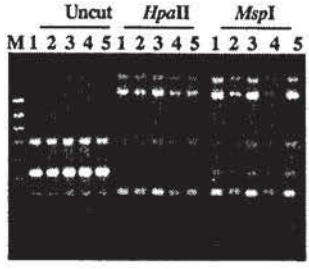

Fig. 2. An example [primer $(GA)_8C$] of the uniformity (stability) of both genetic (ISSR amplifications on uncut DNA) and methylation polymorphisms (ISSR amplifications on *Hpa*II- or *Msp*I-digested DNA) among randomly chosen individual plants within a given maize inbred line. It was found that the line-specific methylation patterns are perfectly conserved in each of the nine maize inbred lines of all 5 primers tested. The 100 bp DNA ladder (Fermentas Inc.) was used as a molecular size marker.

Grouping of the nine maize inbred lines based on the genetic and methylation polymorphisms: Based on the dendrogram generated on the ISSR patterns of uncut DNA, the nine maize inbred lines were classified into four distinct groups, which is in complete agreement with the known pedigree of these lines (Fig. 3A; Zhao *et al.* 2004). This showed that the ISSR fingerprinting is a suitable marker for pedigree analysis of maize inbred lines. Based solely on methylation polymorphisms inferred from ISSR amplifications on DNAs cut with *Hpa*II or *Msp*I (after excluding the polymorphism existed in the uncut DNAs) and combination of the two, three more dendrograms were constructed, namely, *Hpa*II alone (Fig. 3B), *Msp*I alone (Fig. 3C), and *Hpa*II together with *Msp*I (Fig. 3D). It was notable that none of the methylation polymorphism-based dendrogram was identical with the one generated on uncut DNA, indicating that DNA methylation similarity was not fully dependent on genetic relationships. Similar finding was also made in rice (Ashikawa 2001), but not in cotton (Keyte *et al.* 2005) where an apparent association between DNA methylation polymorphism and the known phylogeny of a set of accessions was observed. This suggested that the origins and/or perpetuation of the cultivar-specific DNA methylation patterns might be different in different plants species. It seemed that, as in the case of rice (Ashikawa 2001), the DNA methylation polymorphism probably should not be used for pedigree analysis in maize, nevertheless, this would not preclude it from being used for the cultivar identification purposes given stability of the cultivars-specific patterns (Fig. 1 and 2).

Table 1 Characterization of 16 isolated ISSR bands that showed alterations in DNA methylation among the maize inbred lines

ISSR band	Amplified by ISSR primer	Size (bp)	No. of CCGG sites within sequenced region	BAC clone ID based on maize EST[1]	Genbank Accession & E-value	Protein function predicted by Blast X[2]	Accession number & E-value
Y42i	BDB$(CA)_6$	650	1	MEST386-H11	BM266852 3.0e-119	None	
Y43a	BDB$(CA)_6$	500	1	None		None	
Y91c	$(CT)_8$RG	1500	1	Maize Endosperm cDNA (g-007)	CK720527 4.0e-20	Putative cinnamoyl-CoA reductase [*Oryza sativa* L.]	BAD35675 9e-12
Y93e	$(CT)_8$RG	500	4	None		None	
Y311d	$(AG)_8$T	1000	4	None		None	
Y331a	$(GA)_8$T	550	1		BM4999351 2e-08	None	
Y332i	$(GA)_8$T	900	1	Maize endosperm cDNA (g-007)	CK720527 4.0e-20	Putative cinnamoyl-CoA reductas [*Oryza sativa* L.]	BAD35675 1e-11
Y341e	$(GA)_8$C	850	4	None		None	
Y342h	$(GA)_8$C	700	6	None		None	
Y343d	$(GA)_8$C	500	3	None		None	
Y351d	$(CT)_8$A	1000	2	MEST613-H12	BQ539238 1.1e-11		
W82b	$(CT)_8$G	550	2	None		None	
W142c	$(AC)_8$T	650	7	None		None	
W161a	$(GA)_8$YT	1250	2	Zmsp6610	CF075560 1.0e-41	Prpol [*Zea mays*]	AAL76007 2e-35
W242b	$(AC)_8$YA	400	1	Maize ear cDNA(3529)	CB603740 3.3e-28		
W251b	$(AC)_8$YG	450	1	None		None	

[1]Conducted at the Gramene website: http://www.gramene.org/

[2]Conducted at the NCBI website: http://www.ncbi.nlm.nih.gov/

In conclusion, we have reported in this communication that there existed extensive DNA methylation polymorphism among maize inbred lines. Sequences underlying the methylation variations included a high proportion of coding genes. The line-specific methylation patterns were homogeneous within a line, and hence could be used as molecular markers for cultivars

identification purposes. On the other hand, there existed apparent incongruence between DNA methylation polymorphism and known pedigree of the maize inbred lines.

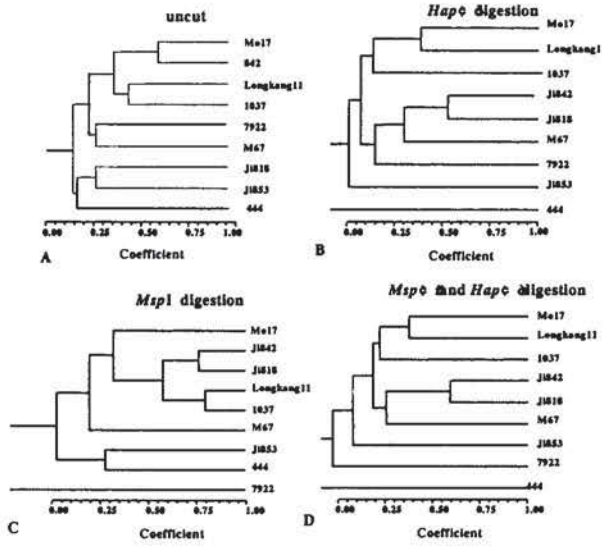

Fig. 3. Dendrograms derived from UPGMA cluster analysis using the Jaccard's coefficient of similarity based on genetic polymorphism generated by ISSR markers on uncut DNA (**A**), based on cytosine DNA methylation polymorphism (after excluding genetic polymorphism) generated on *HpaII*-digested DNA (**B**), based on cytosine DNA methylation polymorphism (after excluding genetic polymorphism) generated on *MspI*-digested DNA (**C**), and based on cytosine DNA methylation polymorphism produced on both *Hpa*II- and *Msp*I-digested DNA (**D**).

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