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Full Length Article

Epigenetic Regulation during Internode Elongation of Maize (*Zea mays*)

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Abstract

DNA methylation, plays a vital role in regulation of gene expression that involves in modulation of plant development, and organ and tissue differentiation. Here, methylation-sensitive amplified polymorphisms (MSAP) were adopted to study the spatial and temporal profiling of DNA methylation between non-elongating internode and elongating internode cells of maize. Overall, approximately 34.97%~39.52% cytosines of CCGG context in two repetitions were methylated, and the average level of DNA methylation was significantly increased in those elongating internode cells. In addition, both demethylation and methylation events were observed, of which demethylation dominated, most sites of the stage-specific methylated DNA were conserved, although the level of methylated DNA was dynamic in the process of internode elongation. A subset of 31 differentially methylated fragments (DMR) was identified within genic and inter-genic regions. These differentially methylated fragments were involved in hormone regulation, photosynthesis, and transposon activation. Our results demonstrated that MSAP technique is a powerful technique to detect large-scale DNA methylation in the maize genome, and that DNA methylation is involved in epigenetic regulation of maize internode elongation.

Keywords: Internode cells; DNA methylation; Gene expression; Maize; MSAP

Introduction

The internode length of maize plants, one of the most important agronomic traits, is highly associated with the vertical distribution of leaves, and also is closely related to resistance to lodging. The elongation pattern of stalk is a sigmoidal model, which arises from the variable growth rates of individual internode comprising the maize stalk (Morrison *et al.,* 1994). The elongation of individual internode of maize was divided into four phases on the basis of the spatial distribution of the component cell length and of the segmental elongation rates (Martin, 1988; Morrison *et al.,* 1994; Robertson, 1994; Fournier and Andrieu, 2000a, b). In phase I, the rate of elongation rises exponentially, and then increases sharply during a short period (phase II), which is followed by a linear period of appreciably constant growth rate (phase III) and a shorter period in which the elongation rate declines (phase IV). Previous studies indicated that the elongation of internode is regulated by genetic factors and endogenous factors. Up to now, a few internode elongation-associated genes have been identified in several plant species, such as *EUI1* (Luo *et al.,* 2006), *OsGLU1* (Zhou *et al.,* 2006), *CENL1* (Ruonala *et al.,* 2008), *SNORKEL1* and *SNORKEL2* (Hattori *et al.,* 2009), *ACO1* (Iwamoto *et al.,* 2010), *SSD1* (Asano *et al.,* 2010), most of which are involved in regulation of endogenous hormone pathway. Many investigations also revealed that gibberellin and ethylene play an important role in regulating the internodal growth of submerged floating rice (Raskin and Kende, 1984a, b; Azuma *et al.,* 1990). So far, genetics and physiological studies of internode elongation have been widely conducted, however, relatively little is known about the epigenetic regulation during the process of internode elongation in maize.

DNA methylation, one of the first discovered epigenetic variations, could generate phenotypic variations through tuning expression of genes in a tissue-specific or a development stage-specific pattern (Angers *et al.,* 2010; Zhang *et al.,* 2010). In general, methylated cytosine account for 20 ~ 30% in plant genome (Richards *et al.,* 1997). Moreover, most of methylated cytosine primarily occurs in CG, CHG and CHH (H=A, T, C) contexts (Finnegan *et al.,* 1998; Zhang *et al*, 2006). In *Arabidopsis*, the heterochromatic regions containing more transposon were frequently hyper-methylated (Hirochika *et al.,* 2000; Lippman *et al.,* 2004), the ratio of expressed genes with methylated transcription region is more than 33%, and the ratio of expressed genes with methylated promoter regions is less than 5%, moreover, the genes containing methylated promoters are more likely to exhibit tissue specificity (Zhang *et al.,* 2006). Genes expressed moderately are the most likely to be methylated, and the extremely expressed genes are least methylated; DNA methylation can repressed the expression of genes but demethylation of genes led to increasing levels of transcription (Zilberman *et al.,* 2006). Thus, the variation of DNA methylation would affect gene expression during plant development.

Several methods have been reported for detecting

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DNA methylation in organism genome, among which the methylation-sensitive amplified polymorphisms (MSAP) technique was highly efficient for large-scale detection of cytosine methylation. Using the MSAP method, Lu *et al.* (2008) have estimated the extent of DNA methylation in three tissues of maize and found that DNA methylation distributed widely in the maize genome and are tissue-specific. Wang *et al.* (2011a) have analyzed the drought-induced DNA methylation in rice, the result of which indicated that drought could induce genome-wide changes in DNA methylation/demethylation, accounting for 12.1% of site-specific methylation differences in the rice. Shan *et al.* (2013) have assessed the effect of cold stress on the DNA methylation in maize seedlings and discovered cold-induced DNA methylation polymorphisms accounted for 32.6 to 34.8% of the total bands at the different treatment time-points, demethylation of fully methylated fragments was the main contributor of the DNA methylation alterations. MSAP is extensively applied to investigate associations between methylation and plant phenotypic variation under various conditions (Labra *et al.*, 2004; Tan, 2010).

Maize is the world's first leading cereal crop, its stalk is developed as lignocellulosic feedstocks for biofuel production, for which genetic diversity, construction of the genetic linkage map, gene cloning and functional analysis had been widely studied in previous works (Cook *et al.*, 2012; Hu *et al.*, 2012; Molin *et al.*, 2013; Teng *et al.*, 2013), while little attention has been paid to the alteration of epigenetic modification during the internode development. Given that DNA methylation is related to most growth and development processes through regulation of gene expression, we suspected that this epigenetic modification may involved in the modulation of maize internode elongation. The major objective of the present study, therefore, is to explore the characterization and regularity of DNA methylation during internode elongation and development, we investigated the spatial and temporal profiling of DNA methylation between non-elongating internode and elongating internode of maize, also we isolated, sequenced, and verified some differentially methylated fragments in different stages. The results of this study will provide valuable information for a better understanding of the role of DNA methylation in internode elongation and development.

Materials and Methods

Plant Material and Data Analysis

A well characterized maize (*Zea mays* L.) inbred line, NX531, was used in this study. The methylation profiles of NX531 were evaluated in two replicated experiments consecutively from May to August of 2011 at the experimental farm of Baoding. In the first replication, seeds of the inbred line NX531 were sown in a three-row plot with 36 plants per plot at a spacing of 60×25 cm between rows and plants within each plot on 13 May 2011. The supply of fertilizer and water was sufficient and regular, and pests and weeds were rigorously controlled by field management. Before elongation stage, one plant of each row, that is, three plants bearing six to seven expanded leaves were harvested, the leaves, leaf sheath were removed, and piled-up internodes were sampled. After elongation stage, another three plants bearing thirteen to fourteen expanded leaves were collected, the basal internode (IN1) and the internode just below the node bearing the ear (IN7) were excised, and leaves, leaf sheath and nodes were removed likewise (Fig. 1). Once sampled, internodes were stored at -80°C freezer for DNA extraction. In the second replication, seeds of the inbred line NX531 were also sown at the experimental farm of Baoding on the 10 June 2011, the experimental design and samples collection were the same with the first replication. The mean number of three samples from each of the two biological repetitions was adopted to conduct statistical analysis. Analysis of variance and *F* tests were used to compare the differences of methylation levels between non-elongating internode and elongating internode of maize from two repetitions using the SAS program GLM (SAS Institute, 1999).

Analysis of Methylation-sensitive Amplified Polymorphism (MSAP)

The total DNA of maize genome was extracted by the modified CTAB method. The MSAP analysis was conducted as described in previous researches (Sha *et al.*, 2005; Dong *et al.*, 2006) with a certain degree improvements. Firstly, several primers were randomly selected to verify the repeatability of MSAP for the two replicates of every sample. It turned out that the MSAP bands between two replicates were uniform (data not shown). Briefly, double enzyme combinations *EcoR* I/*Msp* I and *EcoR* I/*Hpa* II were used to digest DNA samples. To minimize discrepancy due to experiment procedure, double enzyme digestion and ligation were implemented in one step. The total volume of digestion-ligation reaction was 25 μL which including 500 ng genomic DNA, 3 U of *EcoR* I, 3 U of *Hpa* II (or *Msp* I), 1.5 U of T4 DNA ligase (TaKaRa, Dalian, China), 5 pmol of EcoR I adapter, 50 pmol of Hpa II/Msp I adapter, and 2.5 μL of 10×T4-ligase buffer. The mixture was incubated at 37°C for 6 h and then stored at 4°C overnight, inactivated at 65°C for 10 min, and stored at -20°C. After that two successive PCR amplification were performed to identify specific DNA fragments, the final volume for pre-amplified reactions was 20 μL including 2 μL of ligation products, 2 μL of 10 × PCR buffer, 40 ng of E00 and HM00 pre-amplified primer, 1 U of Taq polymerase, 1.6 μL of dNTPs (2.5 mmol/L each), 1.2 μL of $MgCl_2$ (25 mmol/L). The pre-amplification reaction system were as follows: 94°C for 90 s, 56°C for 30 s, and 72°C for 1 min, 25 cycles, after detecting the quality of the pre-

amplified products, we diluted the pre-amplification products to 1: 20 (v:v) with ddH_2O, and 1.5 μL of diluted products was used as template to mixed with the PCR buffer, 40 ng of E00+3, and HM00+3-5 selective primer, which had 3 ~ 5 additional selective nucleotide bases compared to the pre-amplified primer, the selective amplification reaction system was the same with pre-amplification system. The selective amplification procedure consisted of 13 cycles at 94°C for 30 s, 65°C for 30 s (reduced by 0.7°C each cycle), 72°C for 1 min; 25 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The selective amplification products were denatured, resolved by electrophoresis on 6% polyacrylamide gels and finally the patterns and quantity of MSAP bands were analyzed.

Characterization of the differentially amplified DNA fragments

Total of 31 polymorphic methylated bands were separated with a sterilized scalpel blade, these bands were redissolved with 20 μL of ddH_2O, incubated at 95°C for 10 min. As templates, the eluted DNA was reamplified. Sequence information was obtained through cloning the reamplified bands to pMD19-T vector (Takara, Dalian, China) and the clones were sequenced. Homology search and sequence analysis performed at NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MaizeGDB (http://www.maizegdb.org).

Results

We used 40 pairs of primers to detect methylated cytosine in the non-elongating internode and elongating internode cells. The sensitivity of *Hpa* II and *Msp* I to DNA methylation in CCGG context are different. *Hpa* II is sensitive when either cytosine is fully methylated (methylation of both strands), whereas *Msp* I is inactive when the external cytosine is methylated, therefore, different band patterns from PCR amplification can reflect the methylation patterns. Based on the characteristic of bands for specific enzyme combination, we divided patterns of DNA methylation into four classes (Table 1, Fig. 2): class I, bands for *Hpa* II and *Msp* I are both present; class II, band for *Hpa* II is present, but band for *Msp* I is absent; class III, band for *Msp* I is present, but band for *Hpa* II is absent; and class IV indicates the bands of *Hpa* II and *Msp* I are absent. Here, class II represents semi-methylation of CCGG sites while class III and IV indicate that the CCGG sites are full methylated.

Methylation Profiles in Non-elongating and Elongating Internode Cells

We used forty primer combinations to analyze the dynamic of methylated cytosines between non-elongating and elongating internode in two repetitions. Two-way ANOVA (analysis of variance) identified that, the DNA methylation

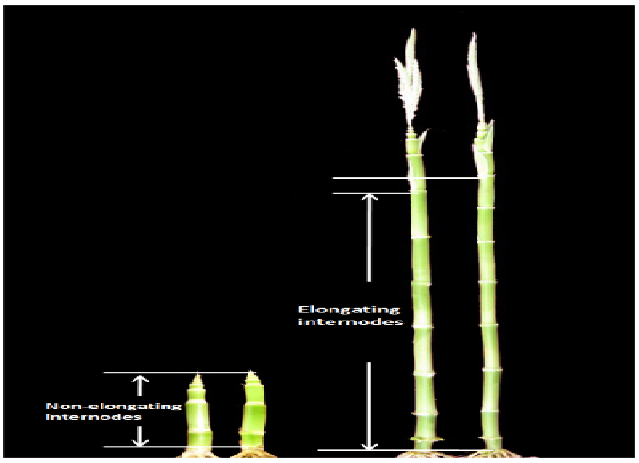

Fig. 1: Non-elongating internodes and elongating internodes

Table 1: Methylation sensitivity of isoschizomers and restriction patterns of CCGG

Type	Methylation status	*Hpa*II	*Msp*I	H	M
Class I	CCGG CCGG GGCC GGCC	Active	Active	1	1
Class II	CCGG GGCC	Active	Inctive	1	0
Class III	CCGG GGCC	Inctive	Active	0	1
Class IV	CCGG CCGG GGCC GGCC	Inactive	Inactive	0	0

Note: H and M indicate the enzyme combinations of Hpa II+EcoR I and Msp I+EcoR I; C: the methylated cytosine; 0: band absent; 1: band present

Table 2: Two-way ANOVA of different repetitions and elongation stages

Difference source	SS	df	MS	*F*	P-value	*F* crit
Stages	18.5761	1	18.5761	322.5017	0.035413	161.4476*
Repetitions	0.0036	1	0.0036	0.0625	0.844042	161.4476
Error	0.0576	1	0.0576			
Total	18.6373	3				

Note: * indicated DNA methylation levels of two elongation stages were significant difference in the same repetition

levels between two elongation stages were significantly different in the same repetition, while the levels of different repetitions were not significantly different in the same stage (Table 2). In summary, 1673 ~ 1739 fragments were amplified in non-elongating and elongating internode cells of two repetitions (Table 3). The ratios of methylated bands in total amplified bands were counted, the methylation level in non-elongating and elongating internode cells were 34.97%, 39.52% in repetition 1, and 35.15%, 39.22% in repetition 2, of which the corresponding fully methylated ratios were 20.58%, 23.44% in repetition 1, and 21.22%, 22.83% in repetition 2 respectively (Table 3). Hence, we concluded that the average level of methylation was significantly increased in those elongating internode cells, not only at the methylated levels, but also at the full

Table 3: Methylation levels of different elongation phages in inbred line NX531s

Samples	I	II	III	IV	Total bands amplified	Total methylated bands (%)	Full methylated bands (%)
Repetition 1 Non-elongating internodes	1205	245	251	99	1701	595（34.97%）	350（20.58%）
Repetition 2 Non-elongating internodes	1183	233	257	98	1673	588（35.15%）	355（21.22%）
Repetition 1 Elongating internodes	1135	273	290	108	1698	671（39.52%）	398（23.44%）
Repetition 2 Elongating internodes	1157	245	297	100	1739	682（39.22%）	397（22.83%）

Note: Total methylated bands＝II+III+IV; Fully methylated bands=III+IV

Table 4: The DNA methylation patterns in non-elongating and elongating internode cells

Class	Methylation band pattern				Bands number	
	Non-elongating internodes		Elongating internodes		Repetition 1	Repetition 2
	H	M	H	M		
A1	0	0	1	1	240	129
A2	1	0	1	1	46	65
A3	0	1	1	1	34	78
B1	1	1	0	1	92	141
B2	1	1	0	0	132	67
B3	1	1	1	0	13	27
C1	0	0	1	0	6	19
C2	1	0	0	0	19	10
D1	0	1	0	0	12	16
D2	0	0	0	1	12	11
E1	1	1	1	1	1027	932
E2	0	1	0	1	78	196
E3	1	0	1	0	7	4
Total amplified bands					1718	1695
Total polymorphic bands					606	563
Polymorphism (%)					35.27	33.22
Monomorphism (%)					64.73	66.78
Type A ratio in total polymorphic bands (A%)					52.81	48.31
Type B ratio in total polymorphic bands (B%)					39.11	41.74
Type C ratio in total polymorphic bands (C%)					4.12	5.15
Type D ratio in total polymorphic bands (D%)					3.96	4.80

Note: 1: band present,0: band absent, H and M indicate the enzyme combinations of *Hpa* II+*Eco*R I and *Msp* I+*Eco*R I

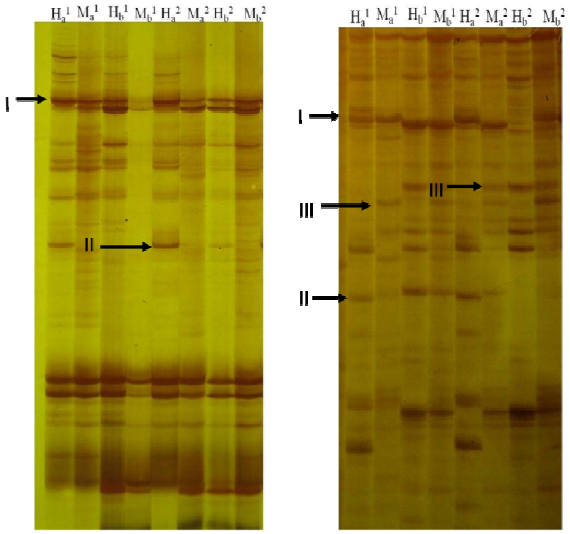

Fig. 2: The MSAP pattern of maize internode cells
H and M indicate the enzyme combinations of *Hpa*II+*Eco*RI and *Msp*I+*Eco*RI; a and b indicate the non-elongting and elongating internodes; 1 and 2 indicate the repetition 1 and repetition 2, respectively

methylated levels. Meanwhile, we found that type III (inner methylation of double-stranded DNA) had the most bands. The number of bands in non-elongating internode and elongating internode cells were 251, 290 in repetition 1, and 257, 297 in repetition 2. The corresponding ratios to total methylated site were 42.18%, 43.22% in repetition 1, and 43.71%, 46.26% in repetition 2. These data indicated that full methylation of internal cytosine occurred most frequently in all the four types at CCGG context in the maize genome.

Alteration of Methylation Patterns in Two Development Stages of Internode Cell

To investigate the alteration of methylated patterns between two stages of internode cell development, all possible patterns were compared, of which thirteen different patterns were observed in the non-elongating and actively elongating internode cells (Table 4). The patterns E1-E3 belonged to the monomorphic site, in which the methylation pattern was identical between non-elongating and elongating internode cells. The patterns A1-A3 represented cytosine demethylation and the patterns B1-B3 indicated cytosine methylation events respectively. The patterns C1-C2 and D1-D2 represented the converting pattern of external methylation and internal methylation of double-stranded DNA. The band numbers of monomorphism for repetition 1 and repetition 2 were 1112 and 1133, respectively, indicating that 64.7% ~ 66.8% of the methylation pattern remained indentical in the process of internode elongation. Compared with the non-elongating internode cells, the ratios of demethylation pattern were 52.81%, 48.31%, the ratios of methylation patterns were 39.11%, 41.74% in two repetitions and the converting patterns occurred least in these elongating internode cells. These findings indicated that both DNA methylation and demethylation events occurred in the process of internode elongation, among which demethylation dominated.

BLAST Results for the Polymorphic Fragments Sequences

To realize more information about the sequences that were differentially methylated in the non-elongating and elongating internode cells, 31 polymorphic methylation fragments were cloned and sequenced (Table 5). All the sequences were relatively short in length, ranging from 90

Table 5: The BLAST results of polymorphic methylated DNA fragments

Code	Primer	Size (bp)	Chr.	Methylated patterns	Sequence homology	E value	Identity (%)
M1	E2/H4	226	2	A1	Exon: GRMZM2G162724// Zea mays uncharacterized LOC100383328	2.e-111	99
M2	E6/H3	172	8	A1	Exon: GRMZM2G359952// MADS-box transcription factor 3	6.e-50	98
M3	E6/H5	160	1	A2	Exon: GRMZM2G019991// Zea mays Uncharacterized protein LOC100192998	3.e-77	100
M4	E1/H3	159	3	A3	Exon: GRMZM2G030814// Hypothetical protein ZEAMMB73_129410	1.e-83	99
M5	E2/H4	150	4	A2	Exon: GRMZM2G079491// Zea mays uncharacterized LOC100383195	9.e-48	96
M6	E6/H5	144	9	A3	Exon: GRMZM2G361699// Zea mays acylphosphatase (LOC100280604)	2.e-63	98
M7	E6/H5	135	2	A2	Exon: GRMZM2G104546// Zea mays aspartate kinase homoserine dehydrogenase2	1.e-25	100
M8	E1/H1	126	1	A1	Exon: GRMZM2G134450// Sorghum bicolor hypothetical protein	2.e-48	95
M9	E1/H2	99	10	A3	Exon: GRMZM5G866734// Regulator of chromosome condensation family protein	1.e-10	100
M10	E6/H5	90	2	A2	Exon: GRMZM2G337532// Leucine-rich repeat receptor-like protein kinase protein	1.e-13	92
M11	E2/H4	183	1	A3	Exon: GRMZM5G842537// Ribosomal protein S4 (RPS4A) family protein	1.e-34	96
M12	E6/H3	173	5	A1	Exon: GRMZM2G122654// Cytochrome P450 superfamily protein	3.e-29	100
M13	E4/H2	145	2	B2	Exon: GRMZM2G050131// Uncharacterized protein LOC100194135	5.e-18	100
M14	E2/H4	102	5	B2	Intron: GRMZM2G124416// Hypothetical protein ZEAMMB73_234730	2.e-20	93
M15	E7/H8	279	1	B1	48bp at 5' side: GRMZM2G556075//Hypothetical protein ZEAMMB73_042820	2.e-87	99
M16	E6/H5	132	1	B2	46bp at 5' side:GRMZM2G083551//Transmembrane emp24 domain-containing protein	2.e-67	99
M17	E3/H4	154	8	B3	875bp at 5' side: GRMZM2G136252//AP2/EREBP transcription factor superfamily protein	1.e-37	92
M18	E6/H5	128	10	B1	629bp at 5' side:GRMZM2G436779// DNA binding protein-like protein	2.e-20	87
M19	E6/H3	136	9	B1	627bp at 5' side:GRMZM2G022275//Serine-threonine kinase receptor-associated protein	2.e-42	100
M20	E2/H4	108	4	B2	394bp at 5' side:GRMZM2G553922// HLH DNA-binding domain superfamily protein	2.e-18	100
M21	E5/H1	185	4	B2	2213bp at 5' side:GRMZM2G525085//Alpha-L-arabinofuranosidase family protein	2.e-101	100
M22	E6/H3	173	5	B1	3072bp at 5' side: GRMZM2G45293 //Presenilin	3.e-94	100
M23	E2/H4	126	5	C1	5579 bp at 5' side: GRMZM2G077588 //Cell division protein ftsZ	10.e-60	98
M24	E5/H1	112	2	C1	2091bp at 5' side: GRMZM2G564571 //Hypothetical protein ZEAMMB73_105580	6.e-24	100
M25	E6/H5	106	4	C2	7095bp at 5' side: AC208838.3_FGT002 // Protein kinase superfamily protein	2.e-54	100
M26	E2/H4	223	5	C2	3775bp at 5' side :GRMZM2G449327// Hypothetical protein ZEAMMB73_234450	6.e-40	91
M27	E2/H4	172	8	A1	Zea mays strain B73 clone MMPa6 endosperm-specific maternally demethylated fragment	8.e-84	100
M28	E2/H4	156	5	D1	Milt1a Retrotransposon: Zea mays cultivar Coroico bz locus region	1.e-56	93
M29	E2/H4	96	5	D2	Zea mays gypsy-like retrotransposon, Romani-Zm1 (left)	1.e-24	91
M30	E3/H4	93	B	D1	Zea mays clone pBK118-1 LL repeat sequence and retrotransposon zeon1	6.e-27	94
M31	E2/H4	153	7	D2	Zea mays rust resistance protein rp3-1 (rp3-1)	1.e-46	90

Note: A1-A3: demethylated patterns; B1-B3: methylated patterns; C1-C2 and D1-D2: converting pattern of external methylation and internal methylation; Chr.: chromosome; 5'side: 5' upstream sequence

to 279 bp (the average size of which was 148 bp) and distributed widely in the maize genome except for chromosomes 6, indicating a genome wide alteration in DNA methylation pattern in the development of internode cell. Among which, 15 sequences were located in genic regions, and 16 sequences in uncoding regions, respectively. According to the results of sequence alignment, four of the differential methylated sequences involved genes encoding acylphosphatase, aspartate kinase homoserine dehydrogenase, leucine-rich repeat receptor-like protein kinase family protein, ribosomal protein; two were highly homologous to the transcription factors. These results showed that differentially methylated fragments sequenced may be related to genes involved a wide range of physiological and biochemical activities which manipulate the growth and development processes in plants.

Discussion

Epigenetic modification, for instance, DNA methylation, is known to involved in plants response to environmental stimuli in numerous studies (López-Maury *et al.*, 2008; Cao *et al.*, 2011), but the relationship between the methylated DNA and internode elongation is known little in maize. Here, the dynamics of DNA methylation in non-elongating and actively elongating internode cells under the normal conditions are described using the MSAP technique. The results showed that methylation levels range from 34.9% to 39.5% in maize internodes, and full methylation of internal cytosine occurred most frequently in all types at CCGG context in the maize genome. The methylation level obtained in our study is similar to that of Arabidopsis seedlings, for which the methylation level is 35% ~ 43% (Yi *et al.*, 2005a), but slightly lower than methylation level of 39.7% ~ 44.6% in seedling of maize inbred lines (Yi *et al.*, 2005b). Meanwhile, we discovered that about 65% of the methylation remained consistent between the two stages of internode elongation, and the amount of DNA methylation was obviously increased in those elongating internode cells. These phenomena provided evidence that methylation levels are distinct in different development stages, and change with plant growth and development. Such a variation in the DNA methylation may be related to the differential expression of genes regulating developmental stage specificity (Aceituno *et al.*, 2008; Lu *et al.*, 2008).

More detailed comparisons regarding the patterns of DNA methylation revealed that events of methylation and demethylation were simultaneously observed in the process of internode elongation, with a predominance of demethylation. It is known to all that DNA methylation may

regulate gene expression by hypermethylation and demethylation during plant development (Siroky *et al.*, 1998). Demethylation of cytosine can reactivate some silent genes to express in different development stages (Wassenegger, 2000), conversely, methylated transcribed regions or promoters within genes would antagonizes the transcription and expression of related genes according to the demand of plant growth (Grunau *et al.*, 2001). Compared with the non-elongating internode cells, numerous demethylation events (48.31%~52.81%) were detected in elongating internode cells, which was consisted with the prospective result that a mass of genes initiated to express when internode began to elongate. Simultaneously, less hypermethylation events (39.11%~41.74%) occurred, indicated that some genes are transcriptionally suppressed after internode elongation. Therefore, the mechanism of DNA methylation and demethylation may play a critical role in moldulating biological development of plant.

In 31 fragments sequenced, approximately half were homologous to particular functional genes, suggesting that methylation patterns of some function genes were altered in order to regulate their expression with demand of plant development. This observation is very similar to the studies performed in Arabidopsis and rice (Cervara *et al.*, 2002; Wang *et al.*, 2011a, b). The differentially methylated fragments can be identified to many categories of genes involved plant hormone regulation, plant development, and transcription factors. The MADS-box transcription factor, AP2/EREBP transcription factor super family protein were reported to involved cell cycle regulating, plant hormone signal transduction, flowering time and meiosis (Paul and Andrew, 1995; Gutterson and Reuber, 2004). Usually, internode elongation is correlated with cell division and elongation, in our study, two sequences (M22 and M23) were identified to genes encoding presenilin and cell division protein ftsZ, which are involved in the plant growth and cell expansion. As a comprehensive target for DNA methylation, transposable elements had frequently been associated with transcription silencing of genes (Cheng *et al.*, 2006). In this study, three MSAP sequences (M28-M30) were shown to be homologous to retrotransposons, which might modulate the activity of neighboring genes. The identification of a diverse category of genes with altered DNA methylation patterns in our study indicated that epigenetic modification may play a vital role in the process of internode elongation, though the regulation mechanism remains to be elucidated.

In conclusion, our results indicated that the levels and patterns of DNA methylation is different in non-elongating internode and elongating internode of maize, and the average methylated level was significantly increased in elongating internode. Both demethylation and methylation events occurred during the internode elongation, of which demethylation dominated. These differentially methylated fragments were identified to a diverse category of genes involved in growth and development processes in plant. Our results demonstrated that DNA methylation is involved in epigenetic regulation of maize internode elongation. Further study should explore such aspects that link methylation change with phenotype at the single gene and genome scales.

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