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Possible involvement of DNA methylation in regulating *ZmGRP3* tissue-specific expression in maize

Y.-G. SONG and W. DONG*

School of Life Science, Qufu Normal University, Qufu, 273165, Shandong, P.R. China

Abstract

Cytosine methylation (C meth) is a ubiquitous regulator of gene transcription. The maize gene *ZmGRP3* is a notable marker for root initiation, but how its root-specific transcription is regulated is not understood. Here, bisulfite sequencing and a C meth sensitive Southern blot assay were used to show that the transcription of *ZmGRP3* was promoted by a reduction in the extent of C meth both in its promoter and in the vicinity of its translation start site. The result was validated by revealing the effect of 5-aza-2-deoxycytidine on the C meth status in various seedling organs. An analysis of methylation patterns indicates that the C meth of cytosine-guanine dinucleotides was the most important determinant of transcription, however, the C meth of CHG or CHH trinucleotides had little influence.

Additional key words: cytosine methylation, gene expression, root initiation, *Zea mays*.

Introduction

Cytosine methylation (C meth) is a conserved epigenetic silencing mechanism implicated in the regulation of transcription of many genes (Song *et al.* 2012). In higher plant genomes, it affects predominantly CG dinucleotides but also CHG (where H could be A, C, or T) or CHH trinucleotides (Zhang *et al.* 2006, Li *et al.* 2008). C meth is present in both the promoter and coding sequences of many genes (Zilberman *et al.* 2007, Cokus *et al.* 2008). The former is well recognized as inducing gene silencing, but methylation in the coding sequence is also known to exert this effect (Lister *et al.* 2008).

Glycine rich proteins (GRPs) are ubiquitous (Sachetto-Martins *et al.* 2000), exhibiting a wide range of specificity with respect to both time (developmental stage) and space (organ/tissue) (De Oliveira *et al.* 1990). Some plant GRPs are preferentially deposited in fruits (Santino *et al.* 1997), others in inflorescences (Murphy and Ross 1998), roots (Sakuta *et al.* 1998, Matsuyama *et al.* 1999), root nodules (Kevei *et al.* 2002), and elsewhere (Lin *et al.* 2005). A number of plant GRPs are known to be involved in the development of the vascular system or the epidermis (Showalter 1993, Parsons and Mattoo 1994). The maize gene *ZmGRP3* is exclusively transcribed in young roots and its restriction to root growing tips has been exploited in the form of a biomarker for root initiation (Goddemeier *et al.* 1998, Woll *et al.* 2006).

The genome wide mapping of C meth in the model plant *Arabidopsis thaliana* has indicated that the time and/or location of specific transcription of genes is often associated with its occurrence in either the promoter or around the translation start site, whereas its presence in the coding region is more frequently associated with constitutive expression (Zhang *et al.* 2006). The mechanism whereby *ZmGRP3* transcription is confined to the root tip has not yet been identified. Here, the goal was to define the pattern of C meth in the *ZmGRP3* sequence in an attempt to determine whether a relationship between the methylation status of *ZmGRP3* and its expression occurs.

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Abbreviations: 5-aza-CdR - 5-aza-2-deoxycytidine; BSP - bisulfite sequencing PCR; C meth - cytosine methylation; G - guanine; GRP - glycine-rich proteins; HpaII/MspI - a pair of isoschizomers.

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* Corresponding author; fax: (+86) 537 4456221, e-mail: dongwei0207@163.com

Materials and methods

Growing conditions and 5-azacytidine treatment: Seedlings of maize (*Zea mays* L.) inbred line B73 were grown in pots with soil and *Vermiculite* (3:2, m/m) under a 16-h photoperiod, an irradiance of 300 μmol m^{-2} s^{-1}, day/night temperatures of 25/20 °C, and a relative humidity of 55 % for 14 d. They were then transferred into a Hoagland's solution (Hogland and Arnon 1950) containing either 0 mM (control) or 50 mM 5-aza-2-deoxycytidine (5-aza-CdR; a potent demethylating agent of genomic DNA) for 7 d (the solutions were replaced every day). Plants treated with 5-aza-CdR experience altered patterns of gene expression, including the reactivation of some genes silenced *via* DNA methylation. Some of these changes lead to phenotypic changes (Sano *at al.* 1990). Root tips, stems, and leaves were snap-frozen in liquid nitrogen and stored at -80 °C until required.

Transcription analysis: RNA was extracted from the frozen untreated and 5-aza-CdR treated tissues using a *TRIzol* reagent (*Invitrogen*, Carlsbad, USA). After spectrophotometric quantification of total RNA, the first cDNA strand was synthesized by reverse transcription, and this was then used as template for a real time quantitative PCR directed at *ZmGRP3*. The RT-qPCRs were based on the *SYBR Green I* real-time PCR detection system using the maize *tubulin* gene (Acc. No. Q41784) as reference sequence (for details see Song *et al.* 2012); relevant primer sequences are shown in Table 1 Suppl. A relative *ZmGRP3* transcript abundance was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

DNA extraction and bisulfite sequencing: Genomic DNA was extracted with a *DNeasy* plant mini kit (*Qiagen*, Hilden, Germany) and bisulfite sequencing was carried out following Song *et al.* (2012). The sequence of the maize amplicon amplified using a primer pair SC-*ZmGRP3* (Table 1 Suppl.) comprised 45 % of CG/CHG/CHH. The amplicon was inserted into the pMD18-T vector (*TaKaRa*, Dalian, China) which was subsequently transferred into *Escherichia coli* strain JM110 (*TaKaRa*). The plasmid was released using a plasmid extraction kit (*TaKaRa*) and treated with bisulfite in parallel with the maize genomic DNA to monitor the efficiency of converting C into T. Bisulfite sequencing primers, designed using the *Methyprimer 5.0* software (www.urogene.org/methprimer/) (sequences given in Table 1 Suppl.), directed the amplification of the genomic *ZmGRP3* sequence from its promoter to the end of its coding region. The PCR program consisted of 34 - 37 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s. The amplicons were purified using a *Wizard*® DNA clean-up system kit (*Promega*, Madison, USA), ligated into the PMD-18 vector and transferred into *E. coli* for sequencing. Ten clones per each biological sample were sequenced.

Methylation-sensitive Southern blot analysis: Genomic DNA was extracted from root tips, stems, and leaves of control plants and plants subjected to the 5-aza-CdR treatment and digested by *Hind*III (*TaKaRa*) for 6 h. The digested DNA was phenol/chloroform extracted and divided into two equal aliquots, one of which was digested with *Hpa*II (*TaKaRa*) and the other with its isoschizomer *Msp*I (Jackson *et al.* 2002). Subsequent Southern blotting procedures were performed following Song *et al.* (2012). A positive control consisted of a 100× dilution of the amplicon derived from the same region which supplied the hybridization probe, whereas a negative control was double distilled (dd) H_2O.

Statistical analysis: Data points represent the means of three independent biological replicates. All data were statistically analyzed by the *t*-test using the *STATISTICA 6.0* software, and values of P lower than 0.05 were interpreted as indicating statistically significant differences.

Results

Sequences corresponding to the *ZmGRP3* promoter and coding region were subjected to bisulfite sequencing (Fig. 1*A*). The efficiency of the sodium bisulfite treatment to convert C to T was estimated as 99.5 % (Fig. 1*B*). In the control plants, the highest methylation was observed in region IV (+252 to +447) in leaves (34.9 % of C was methylated), whereas in stems, the proportion was 30.2 %, and in root tips 28.8 %. Methylation patterns in regions II and III were similar: in region II (-741 to -470), the proportion of methylated C was about 26 % in both leaves and stems but only about 6 % in root tips (Fig. 1*D*, Table 1). Similarly, in region III (-186 to +102), about 28 % of C was methylated in leaves and stems but only 7 % in root tips (Fig. 1*E*, Table 1). In regions I (-1257 to -980) and V (+631 to +825), most C nucleotides were not methylated (Fig. 1*C*,*G*, Table 1). The effect of the 5-aza-CdR treatment was to reduce C meth in all five regions in leaves, stems, and root tips (Table 1). Methylation in regions I and V was only marginally reduced, whereas in regions II, III, and IV was substantially affected (Table 1).

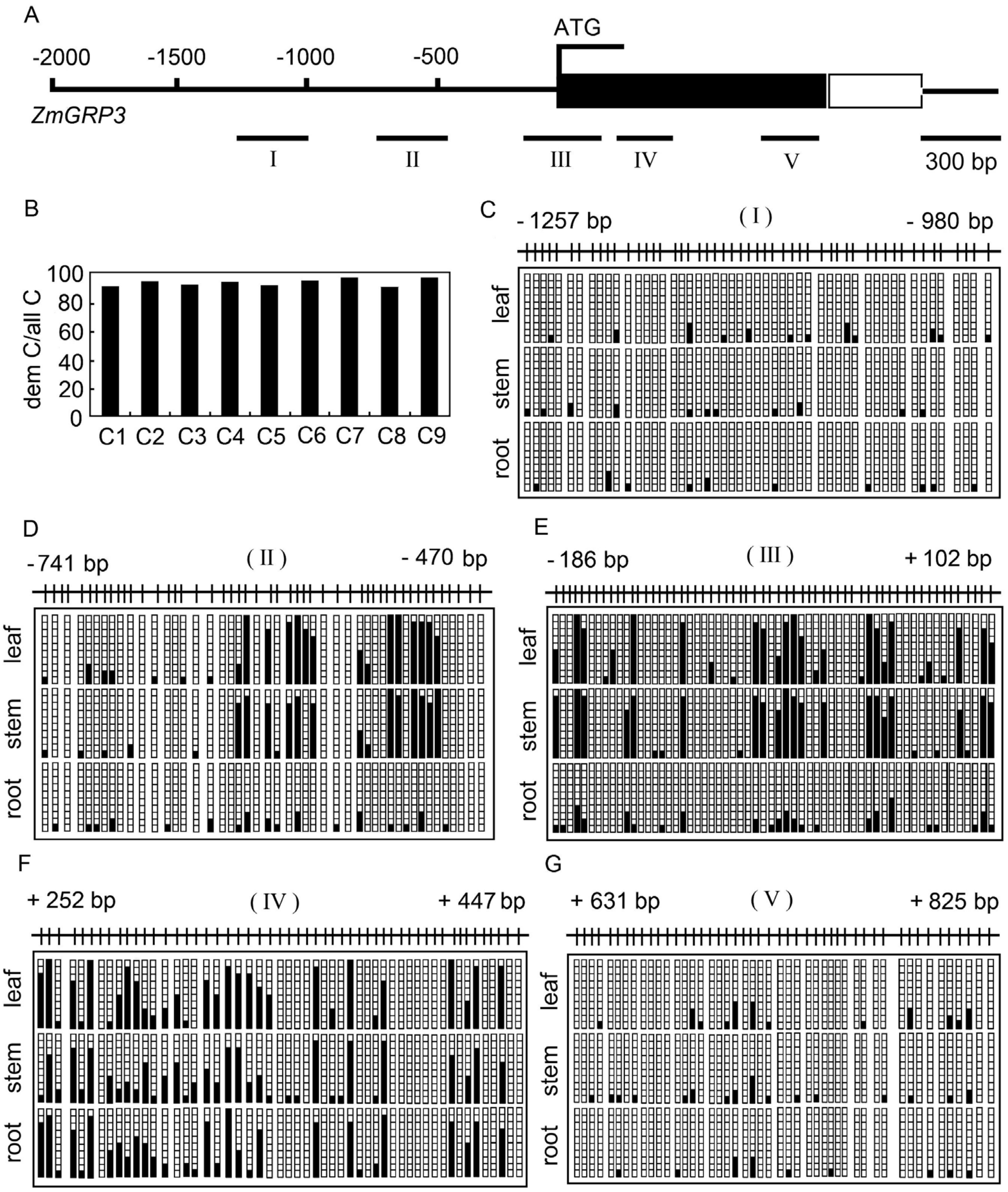

Fig. 1. The *ZmGRP3* C methylation in DNA of leaves, stems, and root tips of control seedlings. *A* - The structure of *ZmGRP3*: the *black* and *white boxes* represent the coding region and 3' UTR, respectively. I - V indicate the regions subjected to bisulfite sequencing. *B* - The efficiency of bisulfite treatment to convert non-methylated C to T. A maize genome fragment harbouring numerous cytosins was introduced into JM110 strain of *Escherichia coli* (*Agilent, Beijing, China*) and the plasmid was treated with bisulfite in parallel with maize genomic DNA. All clones processed showed a conversion efficiency > 99.5 %. *C* to *G* - The C meth of the *ZmGRP3* promoter and coding regions. The *vertical bars* indicate the distribution of CG dinucleotides, and CHG or CHH trinucleotides. Ten individual clones of the sequence were analyzed by bisulfite sequencing. The *black and white dots* indicate methylated and non-methylated C nucleotides, respectively.

Both the transcription and C-meth of *ZmGRP3* were assayed in both control and 5-aza-CdR treated leaves, stems, and root tips. In leaves and stems of the control seedlings, transcription was repressed; the promoter region (region II) and the vicinity of the translation start site (region III) were hypermethylated. In root tips, transcription was up-regulated and regions II and III were hypomethylated (Tables 1, 2). This behaviour suggests a negative relationship between C meth in regions II/III and transcription, but the relationship did not extend to region IV, since in root tips, gene transcription was up-regulated even though the region was highly methylated (Tables 1, 2). A negative relationship between C meth and transcription was confirmed by the outcome of the 5-aza-CdR treatment. In leaves and stems of the control seedlings, regions II and III were hypermethylated, and *ZmGRP3* transcript abundance was low. Following the 5-aza-CdR treatment, the methylation in these regions

Table 1. The DNA methylation of *ZmGRP3* in control and 5-aza-CdR treated leaves, stems, and root tips of maize. All groups of data show the percentage of methylated cytosine/total cytosine in *ZmGRP3* different regions. Means of three assays ± SE. Values followed by different letters are significantly different at $P < 0.05$.

Treatments	Organs	Region I	Region II	Region III	Region IV	Region V
Control	leaf	3.9±0.010a	26.3±0.04b	28.2±0.23b	34.9±0.03b	5.5±0.002a
	stem	2.7±0.004a	26.3±0.03b	28.1±0.43b	30.2±0.04b	4.5±0.003a
	root tip	2.5±0.020a	6.1±0.01a	7.5±0.016a	28.3±0.03b	3.1±0.004a
5-aza-CdR	leaf	3.5±0.013a	9.8±0.02b	13.3±0.019b	15.8±0.022b	2.2±0.006a
	stem	2.4±0.002a	9.7±0.016b	10.7±0.014b	11.9±0.016b	2.7±0.005a
	root tip	2.4±0.004a	4.0±0.007a	4.0±0.003a	10.0±0.004b	2.0±0.003a

Table 2. The real-time quantitave RT-PCR based assessment of *ZmGRP3* expression in control and 5-aza-CdR treated leaves, stems, and root tips of maize. Means of three assays ± SE. Values followed by different letters are significantly different at $P < 0.05$.

Treatment	Leaves	Stems	Root tips
Control	0.13±0.03a	0.11±0.21a	1.0±0.09b
5-azaCdR	0.62±0.07b	0.56±0.11b	1.0±0.17b

was markedly reduced (Fig. 1 Suppl., Table 1), and *ZmGRP3* transcript abundance in leaves and stems was higher than in the control seedlings (Table 2).

DNA methylation-sensitive Southern blotting was applied to verify above observations. First, genomic DNA was digested with restriction enzymes *Hind* III to generate a large fragment containing the target sequences detected by bisulfite sequencing, followed by digestion with a methylation-sensitive enzyme, either *Hpa*II or *Msp*I, both of which recognize the same sequence CCGG (Fig. 2*A*). *Hpa*II is sensitive to methylation of either an outer or inner cytosine, whereas *Msp*I is sensitive only to the outer cytosine. In DNA extracted from leaves and stems of the control seedlings, a 4.95 kbp fragment (Fig. 2*A*) was digested into several smaller fragments (3.4, 1.2, and 0.35 kbp) by *Msp*I treatment but was not digestible by *Hpa*II (Fig. 2*B*). For DNA extracted from root tips of the control seedlings, the 4.95 kbp fragment was digestible by both *Hpa*II and *Msp*I indicating that the sequence was less C-methylated in the root tips than in the other parts of the plants. Following the

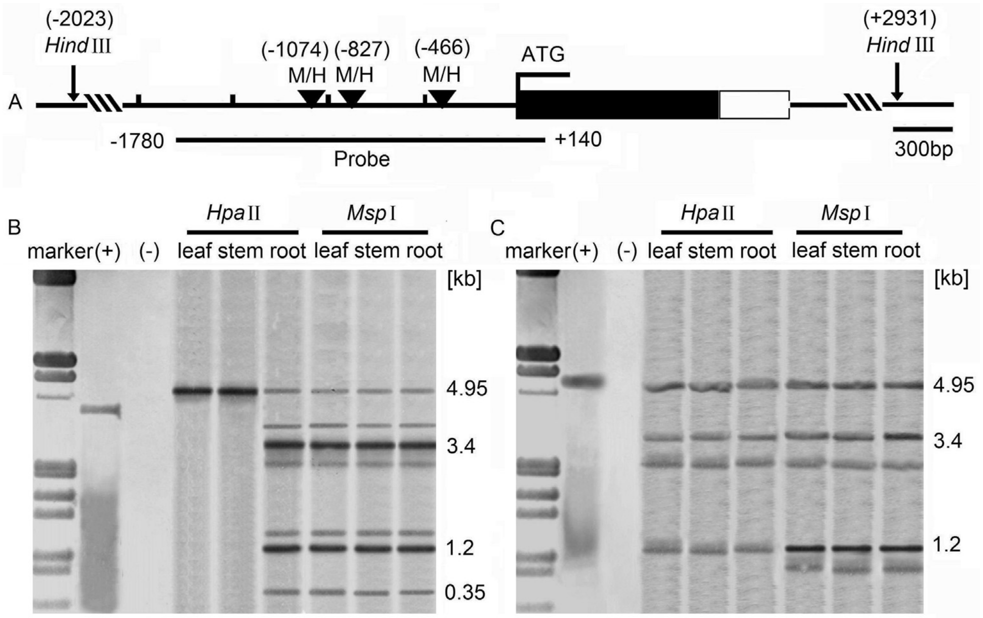

Fig. 2. The methylation-sensitive Southern blot analysis of the *ZmGRP3* genomic sequence in control and 5-aza-CdR treated seedlings. The structure of *ZmGRP3* and the location of *Msp*I/*Hpa*II recognition sites (*A*). The Southern blot analysis of DNA extracted from leaves, stems, and root tips of control (*B*) and 5-aza CdR treated (*C*) seedlings. Genomic DNA was first digested with *Hind*III and then with either *Hpa*II or *Msp*I. The probe sequence was a labelled segment of *ZmGRP3*. A positive control (+) was provided by the probe sequence, and a negative control (-) contained no DNA.

5-aza-CdR treatment, the 4.95 kb fragment was digestible by either *Hpa*II or *Msp*I suggesting that some of the CCGG sites were demethylated in leaves and stems of the 5-aza-CdR treated seedlings (Fig. 2*C*). The overall conclusion is that many C methylated sites in the *ZmGRP3* promoter and in the vicinity of the translation start site (ATG) were more demethylated in root tips than in stems and leaves. It is consistent with C meth being an important determinant of *ZmGRP3* organ-specific transcription.

Discussion

In plants, a substantial number of genes are expressed in an organ- or even in tissue-specific manner (Zhang *et al.* 2006). Dynamic C-meth contributes to this mode of regulation of gene expression (Melquist *et al.* 1999, Berdasco *et al.* 2008). It has been shown repeatedly that DNA methylation in the promoter sequence around the transcription start site or within the coding sequence is associated with tissue-specific gene expression (Song *et al.* 2005, Li *et al.* 2011, Vining *et al.* 2012). Here, in the I - V *ZmGRP3* regions analyzed, regions II (from -741 to -470) and III (-186 to +102, spanning part of the promoter and part of the coding sequence), were hypermethylated in leaves and stems, but hypomethylated in root tips implying that C meth in these two regions had a regulatory role over organ/tissue-specific expression. A similar scenario has been described for the carrot gene *C-LEC1* and the *Arabidopsis thaliana* gene *WUS* (Shibukawa *et al.* 2009, Li *et al.* 2011). Region IV (from +252 to +447, covering solely the coding sequence) was hypermethylated in leaves, stems, and root tips, which is consistent with an idea that C meth in the part of the coding sequence lying at a distance from the transcription start site tended to promote constitutive expression.

Although CG methylation is a common cause of gene silencing, sometimes CHG and/or CHH methylation also have this effect (Finnegan *et al.* 1998). The DNA methylation pattern revealed here indicates that within the methylated regions of *ZmGRP3*, C meth mainly affected CG dinucleotides, and only rarely CHG or CHH trinucleotides (Fig. 1*D*,*E*). In leaves and stems, most of the CG dinucleotides within regions II and III were hypermethylated (Fig. 1*D*,*E*), and *ZmGRP3* was not transcribed; whereas in root tips, these CG sites were demethylated and the gene was transcribed. The implication is that the methylation status of the CG dinucleotides in regions II and III was an important determinant of the gene transcription, with C meth of the CHG or CHH trinucleotides having little influence.

In leaves and stems of the 5-aza-CdR treated seedlings, C meth in regions II and III was reduced by about 2.6-fold (Table 1), and the abundance of *ZmGRP3* transcript raised by about 5-fold (Table 2). In root tips, although C meth in regions II and III was also reduced (Table 1), *ZmGRP3* transcription did not respond to the 5-aza-CdR treatment. The inference is that when regions II and III were hypermethylated, their demethylation tended to promote gene transcription (such as in leaves and stems of the 5-aza-CdR treated plants), but when they were hypomethylated, a further reduction had little effect (such as in root tips of the 5-aza-CdR treated plants).

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