Plant Science 178 (2010) 41–46

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Cytosine methylation changes in the ice plant *Ppc1* promoter during transition from C_3 to Crassulacean acid metabolism

Nu-Chuan Huang, Chang-Hua Li, Jun-Yi Lee, Hungchen Emilie Yen *

Department of Life Sciences, National Chung Hsing University, 250 Kuo-Kwang Rd, Taichung 40227, Taiwan

ARTICLE INFO

Article history:
Received 4 August 2009
Received in revised form 11 October 2009
Accepted 15 October 2009
Available online 27 October 2009

Keywords:
CAM induction
DNA methylation
Mesembryanthemum crystallinum
Phosphoenolpyruvate carboxylase

ABSTRACT

The induction of Crassulacean acid metabolism (CAM) in *Mesembryanthemum crystallinum* is characterized by an increase in the expression of CAM cycle-related genes. The expression of *Ppc1*, a CAM-specific member of the phosphoenolpyruvate carboxylase gene family, increases rapidly during transition from C_3 carbon assimilation to CAM. We used bisulfite sequencing to detect the changes in the cytosine methylation of two GC-rich regions of *Ppc1* in C_3 and CAM leaves. In the 800 bp 5′-flanking sequence of *Ppc1*, four cytosines located at the promoter region were unmethylated in C_3 leaves but methylated in CAM leaves and four out of five cytosines found in the 5′ untranslated region were methylated in C_3 leaves yet demethylated in CAM leaves. Analysis of the sequence contexts of 9 cytosines revealed that 2 were GC, 2 were CTT, and 5 were C(A/T)G methylations. Within 72 h of salt treatment in 6-week-old plants, the increase in methylation of a CTG site at the TATA box of the *Ppc1* promoter coincided with the increase in the expression of *Ppc1*. The sequence-specific and stress-responsive cytosine methylation of *Ppc1* indicates that epigenetic regulation is involved in the activation of *Ppc1* during CAM transition.

1. Introduction

Inducible CAM plants are capable of switching from the C_3 mode of photosynthesis to Crassulacean acid metabolism (CAM). The transition from C_3 to CAM is regulated by several developmental and environmental cues [1]. Some examples include the induction of CAM by photoperiod in *Kalanchoe* [2,3] and by water availability in *Mesembryanthemum* [4] and *Clusia* [5,6]. During this transition from C_3 to CAM mode, the activities of phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) and many CAM-related enzymes increase several folds [7]. Increased activity of *Ppc1*, a CAM-specific isoform of PEPC in *M. crystallinum*, is in part regulated at the transcriptional level: the transcriptional activity of *Ppc1* begins to increase 1 day following salt stress and reaches a sixfold increase by day 3 [8]. Several salt-responsive *cis*-acting elements [9] and corresponding *trans*-acting factors [10] involved in *Ppc1* regulation have been identified.

DNA methylation plays important roles in gene activation, gene silencing [11], gene imprinting [12], chromatin structure [13], and many other cellular activities. Cytosine is the major methylated nucleotide and methylcytosines are found in the sequence contexts of CG, CNG (N is any nucleotide), and other asymmetric sites. Using bisulfite sequencing technology, the methylome of the Arabidopsis genome has been determined at the single-base resolution [14,15]. Analysis of such genome scale data has shown that sequence context has major effects on methylation state. For example, CTG and CAG sites are more frequently methylated than CCG sites and CTH or CAH (H is A, T, or C) sites are more commonly methylated than CCH sites [15]. Furthermore, the correlation between gene expression and cytosine methylation has been studied in depth in the Arabidopsis genome using microarray [16–18] and direct sequencing [14,15] approaches. These studies have shown that a large number of genes are methylated in the transcribed and flanking regions and that gene methylation is correlated with transcription level.

Developmental stages and environmental factors have a substantial effect on the patterns of cytosine methylation across the plant genome [19]. Chilling stress, for example, causes a genome-wide demethylation in roots of maize seedlings. Specifically, cold-induced demethylation was found in the core nucleosomes regions surrounding *ZmMI1*, a gene only expressed under chilling conditions [20]. In another example, Dyachenko et al. [21] used methylation-sensitive restriction endonucleases to observe a two-fold increase in the level of CC(A/T)GG methylation in the ice plant genome during CAM transition. They observed no change in CC(A/T)GG methylation in the promoter region of *Ppc1*,

* Corresponding author. Tel.: +886 4 22840416x520; fax: +886 4 22874740.
E-mail address: heyen@dragon.nchu.edu.tw (H.E. Yen).

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doi:10.1016/j.plantsci.2009.10.005

but did notice an increase in CNG methylation in the satellite DNA. Dyachenko et al. suggested that hypermethylation of satellite DNA may induce the formation of a specialized chromatin structure responsible for regulating expression of a large set of CAM-related genes [21].

In this report, we used bisulfite sequencing [22] to determine the methylation status of the 5′-flanking region of *Ppc1* at single-base resolution in *M. crystallinum* in either C_3 or CAM modes. Changes in methylation status of a CTG site in the vicinity of the TATA box were further monitored using primer sets specific for the methylated or unmethylated cytosine [23]. The results showed that cytosine methylation/demethylation participates in the regulation of *Ppc1* expression during CAM transition.

2. Materials and methods

2.1. Plant materials

Ice plant (*Mesembryanthemum crystallinum* L.) was germinated in a soil matrix (humus:vermiculite:sand, 3:1:2, v/v/v) under 16-h light (600–700 μmol quanta m^{-2} s^{-1})/8-h dark at 30 °C daytime temperature and 16 °C at night. Fertilizer (No. 2, Hyponex Corporation, USA) was applied twice a week. To impose salt stress, 6-week-old plants were irrigated with 200 mM NaCl everyday for seven consecutive days. After 1 week of salt treatment, plants were kept in the same growth conditions irrigated tap water twice a week until seeds set. Samples were collected from 3-day-old seedlings, 3rd and 4th pairs of primary leaves of 5- and 6-week-old plants, and secondary leaves on the side branches of 10-week-old plants.

2.2. Isolation of genomic DNA and bisulfite treatment

Leaf samples from different growth stages and different days of salt stress were homogenized in extraction buffer containing 5% (v/v) phenol, 0.5% SDS, 0.5% Triton X-100, 0.5 mM EDTA, 1 M NaCl, 8 M urea, and 50 mM Tris–HCl (pH 8.0). After addition of an equal volume of chloroform–isoamyl alcohol (49:1, v/v), the extract was heated at 65 °C for 10 min with gentle shaking. Nucleic acid was precipitated by adding an equal volume of 100% isopropyl alcohol. RNA was removed by RNase digestion at 37 °C for 1 h and DNA was purified using phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) extraction following ethanol precipitation. For bisulfite treatment, genomic DNA was alkaline-denatured and then treated with sodium bisulfite for 16 h at 50 °C. The modified DNA was purified by Zymo-spin column (EZ DNA Methylation™, Zymo Research Corporation, USA).

2.3. Cloning and sequence determination of bisulfite-treated DNA

Under denaturing conditions, sodium bisulfite ($NaHSO_3$) converts cytosines, but not methylcytosines, into uracil. After PCR amplification, unmethylated cytosines appear as thymines. Therefore, bisulfite-treated genomic DNA from 5- and 10-week-old plants was PCR-amplified followed by DNA sequencing. The primer set was Ppc-5′ (5′-GCTACATCTACAACTAGTTTATTGGACCCACTTTATA-3′, forward primer) and Ppc-3′ (5′-TGACCCAACTAATCTCACAGGA-3′, reverse primer) covering the 5′ non-coding region of *Ppc1* from −540 to +309. Each PCR reaction contained 250 ng bisulfite-treated DNA in a total volume of 25 μL. PCR was performed at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The PCR products were cloned into pGEM T-easy vector (Promega, USA). At least 10 independent clones for each sample were subjected to sequence analysis (ABI PRISM™ 310 Genetic Analyzer, Applied Biosystems, USA).

2.4. Detection of cytosine methylation at TATA sequence

We designed an "M" primer (5′-**G**ACTATTATAAAAGCCACACA**G**-3′) and Ppc-3′ (shown above) to amplify methylated cytosines. Corresponding unmethylated cytosine fragments were amplified using a "U" primer (5′-**A**ACTATTATAAAAGCCACACA**A**-3′) and Ppc-3′. The M and U primers differ at their 5′- and 3′-ends, respectively and include a TATAAAA motif. When using these primer sets, each PCR reaction contained 250 ng of bisulfite-treated DNA in a total volume of 25 μL. PCR was performed at 95 °C for 5 min, 30 cycles at 95 °C for 1 min, *X* °C for 30 s (*X* is the annealing temperature indicated in the text), 72 °C for 1 min, and a final extension at 72 °C for 7 min (TGradient, Biometra, Germany). The PCR products were separated on 1% agarose gels, stained with ethidium bromide, and directly visualized under UV illumination. TINA software (version 2.09e) (Raytest, Germany) was used to quantify band intensity.

3. Results

In order to examine the possible effects of DNA methylation on *Ppc1* transcription, we first analyzed the GC composition of the ice plant *Ppc1* using MethPrimer program (www.urogene.org) [24]. The 9.1 kb region of the *Ppc1* including a 1.4 kb promoter region and a 7.7 kb coding region was subjected to CpG island prediction. Using the default settings of this program (200-bp segments with 50% GC content, observed/expected ratio of CpG dinucleotides >0.6), no CpG island was predicted within the entire 9.1 kb region of *Ppc1*. However, once we lowered the detection setting to 100-bp segments with 30% GC content, we found 7 predicted GC-rich regions (Fig. 1). The 5′-flanking region of *Ppc1* was mostly AT-rich as illustrated by the presence of only one GC-stretch region at −407 to −287. Another GC-stretch region was located in the 5′-UTR at +83 to +183. The other five GC-rich regions in the gene body were located towards the 3′ terminus ranging from +4000 to +7000. Results showed a low GC content in the 5′ non-coding region of *Ppc1* with only two relatively long GC-stretch regions, both located close to the transcription start site.

Initially we used methylation-sensitive restriction digestion to detect the methylation patterns of GC-rich regions in the *Ppc1* promoter. HpaII was used to detect possible changes of methylation status of two CCGG sites in the 5′-flanking sequence. However, the methylation pattern of these two CpG sequences did not change during development or under salt stress (Supplementary Fig. 1). We found a similar result in the 5′-UTR region using another methylation-sensitive restriction enzyme SalI (Supplementary Fig. 2). These results are similar to those of Dyachenko et al. [21], which failed to detect changes of CC(A/T)GG methylation in the *Ppc1* promoter. Together these data suggest that the methylation status of cytosines located in the HpaII or SalI recognition sites of the *Ppc1* promoter did not change during development.

In order to elucidate the exact methylation pattern of the *Ppc1* 5′ region, we performed bisulfite-modified DNA sequencing.

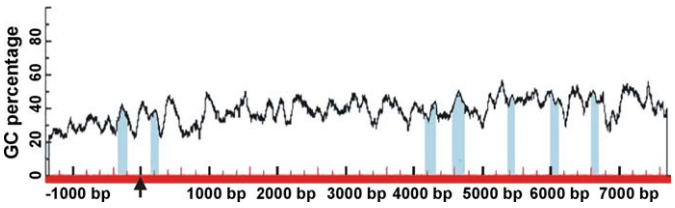

Fig. 1. Distribution of GC-rich regions in ice plant *Ppc1*. Blue areas indicate GC-rich regions predicted by the MethPrimer program (www.urogene.org; criteria used: Island size >100, GC Percent >30.0, Obs/Exp >0.6). The transcription start site is indicated by an arrow. The input sequences of ice plant *Ppc1* were obtained from Genbank under accession numbers: coding region, X14587; 5′-flanking region, X63774.