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RESEARCH PAPER

Light-regulated and cell-specific methylation of the maize *PEPC* promoter

Ben J. Tolley[1], Helen Woodfield[1], Samart Wanchana[2], Richard Bruskiewich[2] and Julian M. Hibberd[1,]*

[1] Department of Plant Sciences, Downing Street, University of Cambridge, Cambridge CB2 3EA, UK
[2] International Rice Research Institute (IRRI), DAPO Box 7777, Metro Manila, Philippines

* To whom correspondence should be addressed. E-mail: jmh65@cam.ac.uk

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Abstract

The molecular mechanisms governing *PEPC* expression in maize remain to be fully defined. Differential methylation of a region in the *PEPC* promoter has been shown to correlate with transcript accumulation, however, to date, investigations into the role of DNA methylation in maize *PEPC* expression have relied on the use of methylation-sensitive restriction enzymes. Bisulphite sequencing was used here to provide a single-base resolution methylation map of the maize *PEPC* promoter. It is shown that four cytosine residues in the *PEPC* promoter are heavily methylated in maize root tissue. In leaves, de-methylation of these cytosines is dependent on illumination and is coincident with elevated *PEPC* expression. Furthermore, light-regulated de-methylation of these cytosines occurs only in mesophyll cells. No methylation was discovered in the 0.6 kb promoter required for mesophyll-specific expression indicating that cytosine methylation is not required to direct the cell-specificity of *PEPC* expression. This raises interesting questions regarding the function of the cell-specific cytosine de-methylation observed in the upstream region of the *PEPC* promoter.

Key words: Bundle sheath, C_4 photosynthesis, maize, mesophyll, methylation, PEPC.

Introduction

In plants, phosho*enol*pyruvate carboxylase (PEPC) is a cytosolic enzyme that catalyses the conversion of phospho*enol*pyruvate (PEP) and bicarbonate (HCO_3^-) to the four carbon acid oxaloacetate (OAA) and inorganic phosphate (Chollet *et al.*, 1996). In all known C_4 plants, PEPC operates as the primary carboxylase enzyme; in two-celled systems fixing CO_2 as bicarbonate into OAA in mesophyll (M) cells prior to decarboxylation around Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase (RuBisCO) in the parenchymatous bundle sheath (PBS) cells.

The compartmentation of proteins between M and PBS cells is considered a key characteristic of the C_4 leaf (Brown *et al.*, 2005), and numerous mechanisms underlying cell specificity have been reported (Hibberd and Covshoff, 2010; Brown *et al.*, 2011). In the dicotyledonous C_4 plants *Flaveria trinervia* (Spreng.) C. Mohr. and *F. bidentis* (L.) Kuntze, the control of *PEPC* expression is primarily exerted at the level of transcription. Fusion of 2 kb of the *F. trinervia* promoter to the β-glucuronidase (GUS) reporter gene is sufficient to generate M-specific GUS accumulation in *F. bidentis* (Stockhaus *et al.*, 1997). A 41 nucleotide region of this promoter called the mesophyll enhancing module 1 (MEM1) containing a CACT tetranucleotide was shown to be capable of directing M-specific *PEPC* expression when integrated into the promoter of the C_3 plant *F. pringlei* (Gowik *et al.*, 2004; Akyildiz *et al.*, 2007). To date, however, *trans*-acting factors associated with this element remain to be identified. In maize, GUS reporter experiments have shown that 0.6 kb of the *PEPC* promoter is capable of driving M-specific expression (Taniguchi *et al.*, 2000; Kausch *et al.*, 2001). *ZmPEPC* transcription occurs in all cell-types (except xylem tissue) in very young leaves and is subsequently repressed in all but M cells (Langdale *et al.*, 1987, 1988; Kausch *et al.*, 2001) suggesting that developmental signals are important in regulating the transcriptional activity of the promoter. The C_4 *PEPC* promoter in maize has been shown to bind various

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protein complexes (Yanagisawa and Izui, 1990, 1992, 1993; Kano-Murakami *et al.*, 1991; Yanagisawa, 1995; Yanagisawa and Sheen, 1998). However, the identity and specific function of these proteins remains to be defined.

Epigenetic modifications have also been shown to correlate with maize *PEPC* expression as both histone and DNA methylation have been implicated in its regulation. While light induces histone H4 acetylation in both M and PBS cells (Offermann *et al.*, 2006) histone H3K4 tails are heavily tri-methylated in M compared with PBS cells. This pattern of histone modification remained unchanged in dark-grown leaves when *PEPC* expression was low, suggesting that this alone is not sufficient to account for the high amount of M-specific *PEPC* transcripts seen in maize leaves (Danker *et al.*, 2008). Interestingly, the maize C_4 *NADP-ME* gene that is expressed in PBS cells shows an inverse pattern in which tri-methylation of H3K4 occurs in PBS cells (Danker *et al.*, 2008).

Although chromatin patterns are important in regulating *PEPC* expression in maize, during cell division chromatin structures are removed from DNA (Lucchini and Sogo, 1995) and therefore must subsequently be re-established following replication, implying a further level of regulation. The selective methylation of DNA provides a mechanism for regulating gene expression in plants (Spena *et al.*, 1983; Hepburn *et al.*, 1987; Bianchi and Viotti, 1988; Bucherna *et al.*, 2001) and animals (Cedar, 1988) and has been shown to impact strongly on chromatin patterns (Lande-Diner *et al.*, 2007) forming a basal template for chromatin arrangements (Weber and Schubeler, 2007; Suzuki and Bird, 2008). Indeed, previous work has linked DNA methylation with the expression of *PEPC* in plants. For example, methylation of four cytosines located in the promoter region and de-methylation of four cytosines in the 5' UTR of the *McPPC1* gene from the facultative Crassulacean Acid Metabolism (CAM) plant *Mesembryanthemum crystallinum* is co-incident with an increase in expression associated with the switch from C_3 to CAM metabolism (Huang *et al.*, 2010). Furthermore, differential methylation of a *Pvu*II restriction site 3.1 kb upstream from the maize *PEPC* transcription start site in response to illumination was correlated with changes to *PEPC* expression (Langdale *et al.*, 1991). To provide additional insight into the extent to which DNA methylation of the maize *PEPC* promoter occurs, we used bisulphite sequencing. Treatment of DNA with sodium bisulphite results in deamination of unmethylated cytosines to uracil, however, 5-methylcytosines remain unconverted. Following sequencing of bisulphite-converted DNA unmethylated and methylated cytosines can be distinguished from one another because they appear as thymines and cytosines, respectively, in the amplified product (Frommer *et al.*, 1992). Therefore, sequencing of bisulphite-treated DNA can determine the methylation status of a given DNA sequence at single-nucleotide resolution. In this paper, to assess the extent to which regulation of maize *PEPC* is related to the methylation status of the promoter, methylation was examined at single base resolution.

Materials and methods

Plant material

Maize (B73) plant material was germinated after overnight imbibition in molecular grade biology water. It was then planted in Levington M3 potting compost (Scotts, Ohio, USA) treated with intercept (200 mg l^{-1}) (Scotts Miracle-Gro, Ohio, USA). All plant material was grown at a relative humidity (RH) of 50%, a constant temperature of 28 °C and an atmospheric CO_2 concentration of 400 μl l^{-1}. Etiolated seedling material was harvested above the mesocotyl under a dim green safelight after 7 d. The remaining dark-grown plants were then transferred to a 16/8 h light/dark regime (400 μmol m^{-2} s^{-1}) 2 h into the light period and second leaves were harvested after 72 h. Leaves exposed to 72 h light were used for cell separation. In all cases, material was flash-frozen in liquid nitrogen and stored at –80 °C for subsequent DNA and RNA extraction.

RT-qPCR

Total RNA for RT-qPCR was extracted from 7-d-old maize seedlings using the Qiagen RNeasy® Plant Mini Kit (Qiagen, Alameda, CA) according to the manufacturer's instructions. To remove contaminating genomic DNA, samples were treated with 10 U μl^{-1} RNase-free DNase (Qiagen Alameda, CA) for 30 min at 20 °C and 15 min at 65 °C. RNA quality was analysed using an Agilent 2100 Bioanalyser and an RNA nano-chip. All samples had RNA integrity numbers (RINs) above 6.60 indicating the RNA was high quality (Fleige and Pfaffl, 2006). 1 μg RNA was reverse transcribed using an oligo(dT) primer and Superscript II (InVitrogen Life Technologies, USA). The total cDNA volume of 20 μl was stored at –20 °C overnight. Real-time quantitative PCR (RT-qPCR) was carried out using SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich, Germany) and 5-fold dilution of the template and primers at 0.2 μM final concentration. Primers were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3) to have melting temperatures of 60 °C. Sequences of primers used to detect *ZmPEPC* and *ZmMAZ95* (Lin *et al.*, 2008) are listed in Supplementary Table S1 at *JXB* online. RT-qPCR was performed in a Rotor-Gene™ thermal cycler (Qiagen Alameda, CA). Cycling conditions were: 94 °C for 2 min, followed by 40 cycles of 94 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s, and 75 °C for 5 s. The fluorescence threshold was set to a constant value of 0.04, which was manually determined to be as early as possible into the exponential phase of fluorescence for all transcripts. The C_T values were calculated from means of three technical replicates for three independent biological replicates of each line. Relative abundance of transcripts (to *ZmMAZ95*) was calculated using the $2^{-\Delta\Delta CT}$ method after Livak and Schmittgen (2001). Standard errors were calculated from $2^{-\Delta\Delta C}$T values of each combination of (biological and technical) replicates.

Parenchymatous bundle sheath/mesophyll cell extraction

Leaves from a minimum of 20 plants exposed to 72 h light were used for cell separation. M and PBS cells were separated after the method described by Markelz *et al.* (2003). Details of each preparation are given below. Protoplast and PBS strand integrity was assessed by light microscopy.

M cell preparation

Second and third maize leaves (corresponding to 5 g of leaf tissue) were cut perpendicularly to remove the midrib and subsequently transversely into 1–2 mm strips. The leaf samples were subjected to enzymatic digestion in enzyme buffer (20 mM MES (pH 5.5), 1 mM $MgCl_2$, 0.6 M sorbitol, 2% (w/v) Cellulase Onazuka (Yakult Pharmaceuticals, Tokyo), and 0.1% (w/v) macerase (Calbiochem, San Diego) for 3 h at 21 °C. The strips were filtered through a 135 μm nylon mesh (Millipore, MA, USA) and resuspended in 50 ml

wash buffer (50 mM TRIS-HCl (pH 7.5), 1 mM $MgCl_2$, 0.6 M sorbitol, and 100 mM β-mercaptoethanol). Gentle pressure was applied with a stainless steel spoon for approximately 1.5 min to release the protoplasts. Removal of cellular debris was conducted by filtration through a 60 μm nylon mesh (Millipore, MA, USA). The filtrate containing protoplasts was subjected to centrifugation at 1200 *g* for 10 min, resuspended in wash buffer, and re-centrifuged. The pellet was resuspended in 500 μl of wash buffer solution and dropped into liquid nitrogen in peel-away cups (VWR Scientific, NJ, USA).

PBS cell preparation

To isolate PBS cells from maize leaves, second and third leaves were cut into 2×2 mm squares (4 g tissue) and disrupted by three 10 s pulses on 'low' setting in a blender (Waring Products, CT, USA) in 50 ml PBS buffer I (0.33 M sorbitol, 0.3 M NaCl, 0.01 M EGTA, 0.01 M dithiothreitol, 0.005 M diethyldithiocarbamic acid, and 0.2 M TRIS-HCl (pH 9.0)). The resulting buffer/tissue solution was filtered using 60 μm nylon mesh (Millipore, MA, USA) and subsequently blended for three 1 min pulses on 'high' setting, in PBS buffer II (0.35 M sorbitol, 0.005 M EDTA, 0.1% (v/v) β-mercaptoethanol, and 0.05 M TRIS (pH 8.0)) re-filtering through the mesh between each pulse. The blender was washed out with molecular biology grade water between each filtration. PBS strands retained on the nylon mesh were dried briefly by placing the mesh on paper towels to wick away excess moisture. PBS strands were then removed from the mesh and flash frozen in liquid nitrogen.

Bisulphite sequencing

Total cellular DNA was extracted from 100 mg fresh plant material (root, dark-grown and light-grown leaves, M, PBS) ground in liquid nitrogen. In the case of total leaf extractions three leaves from independent plants were ground together in liquid nitrogen at –80°C. DNA extraction was performed using the DNeasy© DNA extraction kit (Qiagen Ltd., West Sussex, UK) following the manufacturer's recommended protocol. The quantity of DNA was determined spectrophotometrically using a NanoDrop 1000™ spectrophotometer (Thermo Scientific, Wilmington, USA). The quality of the DNA sample was validated via agarose gel electrophoresis. 1 μg DNA and 5 μl DNA loading buffer (comprising 50% (v/v) glycerol, 1 mM ethylenediamine tetra-acetic acid disodium salt (EDTA), 0.4% (w/v) bromophenol blue (BPB), 0.005× TBE (TRIS-borate-EDTA) buffer, 48% (v/v) formamide) were heated to 60 °C for 10 min and subsequently run on a 1.5% (w/v) agarose gel containing 0.5 μg ml^{-1} ethidium bromide using 0.5× TBE buffer (44.5 mM *tris*-hydroxymethyl) aminomethane, 44.5 mM boric acid, and 1 mM EDTA at pH 8.0).

500 ng maize genomic DNA was treated with sodium bisulphite ($NaHSO_3$) using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, CA, USA) according to the manufacturer's protocol. Treatment of DNA with sodium bisulphite results in the selective deamination of non-methylated cytosines to uracil, whereas 5' methylated cytosines remain unconverted during the treatment (Wang *et al.*, 1980). The methylation status of the DNA can be determined by DNA sequencing of sodium bisulphite-treated and untreated controls following PCR amplification. Primers (see Supplementary Table S1 at *JXB* online) were used to amplify a region of the maize *PEPC* promoter (sense strand), 3.22–2.88 kb upstream of the transcription initiation site, surrounding the differentially-methylated *Pvu*II (–3.029 kb) restriction site identified by Langdale *et al.* (1991) in order to validate the sensitivity of bisulphite sequencing in this context. Primers for bisulphite sequencing (see Supplementary Table S1 at *JXB* online) were designed after Henderson *et al.* (2010) and were biased to amplify from bisulphite-converted template DNA.

PCR was performed in a total volume of 20 μl (9.2 μl molecular biology grade water, 4 μl 5× NH_4^+ BioTaq buffer, 0.2 μl dNTPs, 1 μM Forward primer, 1 μM Reverse primer, 200 ng DNA template, 0.2 μl BioTaq polymerase). PCRs were carried out using a Techne™ thermal cycler and BioTaq™ high-fidelity DNA polymerase (TaKaRa, Shiga, Japan). Taq polymerase was selected for non-proofreading activity since proofreading polymerases stall after incorporation of deoxyuracil, a base which is efficiently incorporated into amplified products by BioTaq. PCR began with a hot start before BioTaq polymerase was added (3 min at 95 °C) to reduce non-specific binding, followed by 35 cycles of; further denaturation (20 s at 95 °C), annealing (30 s at 50–55 °C), and extension (60 s at 62 °C). A final extension was carried out at 62 °C for 10 min. PCR products were examined on 1.5% agarose gel, by loading 5 μl PCR product and 5 μl loading buffer. A Hyperladder IV™ (Bioline, Ltd., London, UK) size marker was used to determine the molecular weight of the products. Amplified products were size-excluded and purified using the QIAquick™ gel extraction kit (Qiagen, Alameda, CA) according to the manufacturer's protocol. PCR products amplified this way were cloned into PJet 1.2 plasmid vector using the CloneJET™ PCR Cloning Kit (Fermentas, Germany) as per the manufacturer's instructions and transformed into *Escherichia coli* DH5α competent cells. Bacteria were plated on LB agar selective media containing 100 mg ml^{-1} ampicillin. Successfully transformed colonies were screened by PCR.

Cycle sequencing of cleaned PCR products was performed in a Techne™ thermal cycler (initial denaturation at 96 °C, followed by 25 cycles of 96 °C for 10 s; 50 °C for 5 s, and, finally, 60 °C for 4 min) using the following reagents: 200 ng PCR product (plasmid), PJet 1.2 Forward Primer (10 μM) 0.5 μl, ddH_2O (to 10 μl) BigDye v3.1 5X cycle sequencing buffer 2 μl and BigDye v3.1 1 μl (Applied Biosystems, Foster City, CA). DNA precipitation and removal of unincorporated terminators and sequencing was performed by the sequencing facility at the Department of Biochemistry, University of Cambridge, UK. Sequence analysis was performed using BioEdit™ v7.0.5 sequence alignment software for Windows™ on a Dell™ Optiplex 740 computer. Since all cytosines are converted to uracil as a consequence of bisulphite treatment, cytosine residues in amplified sequences (excluding primer sequences) were interpreted as methylated bases. Thymine residues occurring at the equivalent positions as cytosines in untreated controls were classified as unmethylated. Initially, the vector inserts of at least 10 independent clones were sequenced using forward primers against the PJet 1.2 vector backbone, followed by a further 10 independent clones per treatment in regions where cytosine methylation was detected. Care was taken to ensure that all sequences analysed varied at a minimum of one C/T base in order that the same amplicon was not sequenced multiple times. Results are expressed as percentages of clones with cytosine residues at the nucleotide position indicated.

Results

PEPC transcripts accumulate in maize leaves in response to illumination

To establish the amount of *PEPC* transcripts in each tissue type in maize, RNA was extracted from root tissue, leaves from 7-d-old plants grown in the dark or leaves transferred to the light for 72 h. After production of cDNA, Real-time quantitative Polymerase Chain Reaction (RT-qPCR) was used to quantify *PEPC* transcripts relative to maize actin (*ZmMaz95*). Very little *PEPC* expression was detected in roots and dark-grown leaves (Fig. 1). However, significant transcript accumulation was observed in light-grown leaves indicating that *PEPC* expression was responsive to illumination and that *PEPC* transcripts were more abundant in light-grown leaves relative to roots and dark-grown leaves (Fig. 1).

Four cytosine residues in the PEPC *promoter are heavily methylated in maize root tissue*

In order to define precisely the methylation status of the differentially methylated *Pvu*II restriction site (CAGCTG) identified in the maize *PEPC* promoter by Langdale *et al.* (1991), DNA extracted from 7-d-old root tissue was subjected to modification with sodium bisulphite to convert unmethylated cytosines to uracil. A 270 bp region of the sense strand DNA (–3178 to –2908) surrounding the *Pvu*II site was then amplified from this template via PCR, sequenced, and compared with an untreated control. The majority of cytosines in this region appeared as thymines in the amplified product validating this method in converting unmethylated cytosines to uracil. By contrast, four cytosine residues (CHG context) on the sense strand of the 270 bp region showed high methylation frequencies in root tissue samples taken from 7-d-old maize seedlings. Methylation of both cytosines in the *Pvu*II site (positions –3034 and –3031) was detected and at two previously unidentified cytosines further upstream (–3171 and –3165) (Fig. 2). 95% and 80% of cytosines (both CAG context) at positions –3171 and –3165 were found to be methylated in root samples (Fig. 2). Similarly, both cytosines in the *Pvu*II site –3034 and –3031 kb upstream from the transcription start site were methylated at a frequency of 85% and 95%, respectively (Fig. 2).

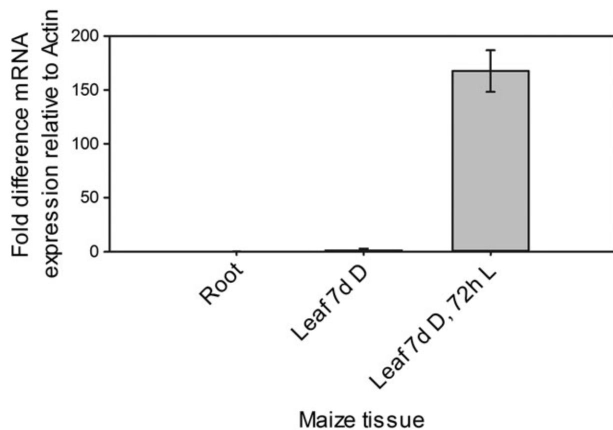

Fig. 1. *PEPC* transcripts accumulate in maize leaves in response to illumination. The $2^{-\Delta\Delta C}$T method was used to quantify the relative abundance of transcripts (Livak and Schmittgen, 2001). *ZmMaz95* (Lin *et al.*, 2008) was used as a reference. Results are expressed as mean C_T values calculated from a minimum of three biological and three technical replicates. Error bars represent one standard error of the mean calculated from $2^{-\Delta\Delta C}$T values of each combination of biological replicates.

De-methylation of the PEPC *promoter in maize leaves is dependent on illumination*

To determine whether the methylation status of the 270 bp region (–3178 to –2908) within the *PEPC* promoter was preserved in leaf as well as root tissue, it was amplified via PCR from leaves of 7-d-old etiolated seedlings. The four cytosines methylated in roots were found to be methylated at similar frequencies in leaves of etiolated seedlings, with 100% of cytosines at positions –3171 and –3031 and 83% at positions –3165 and –3034 methylated, respectively. This indicated that methylation of these cytosines is maintained in roots and dark-grown leaf tissue (Fig. 2).

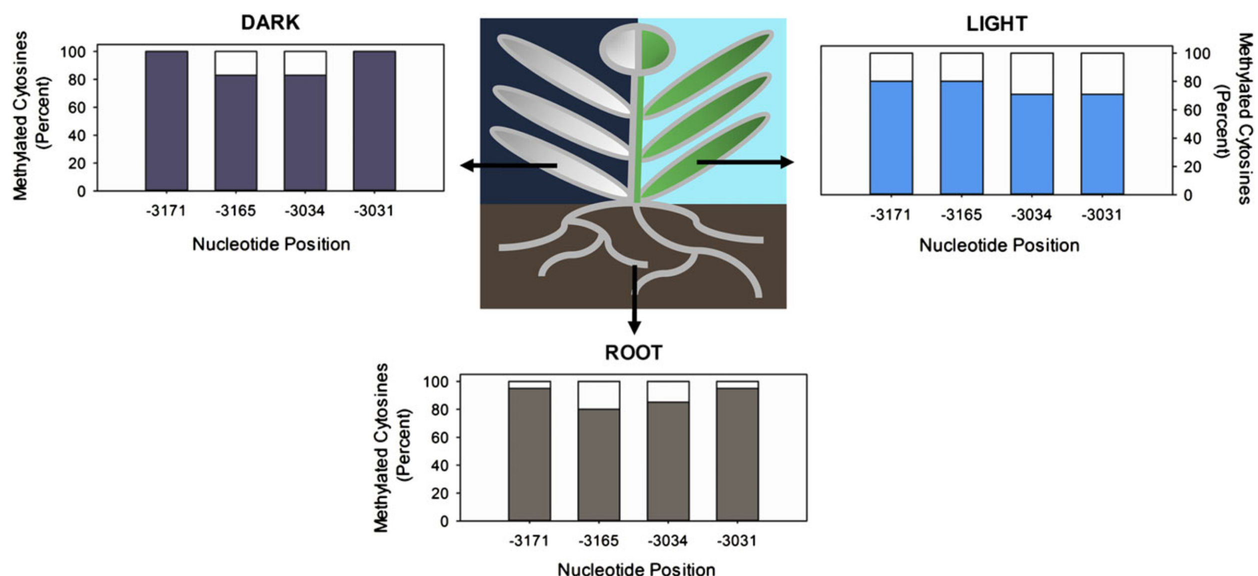

Fig. 2. Four cytosine residues in the *PEPC* promoter are de-methylated in maize leaves in response to illumination. The methylation status of *PEPC* promoter region –3178 to –2906 kb upstream of the transcription start site in maize roots, dark-grown leaves, and leaves transferred to light for 72 h. The extent of DNA methylation was determined by bisulphite sequencing; cytosine residues in amplified products indicated methylated bases, whereas thymines in the place of cytosines were classified as unmethylated. Percentages refer to the percentage of clones with cytosine residues at the nucleotide position indicated. At least 20 independent clones were sequenced per treatment and all sequences varied at a minimum of one C/T base.

However, in DNA from leaves of 7-d-old etiolated seedlings which were subsequently transferred to light for 72 h, these cytosines were less heavily methylated. Although cytosines at position –3165 were methylated at a similar level to those in roots, the methylation of cytosines at –3171 was reduced to 80% (Fig. 2). Both cytosines in the *Pvu*II site (positions –3034 and –3031) were de-methylated upon illumination in 29% of amplified products (Fig. 2). De-methylation of these cytosine residues in the *PEPC* promoter therefore coincides with an increase in *PEPC* expression in maize leaves in response to illumination.

Light induced de-methylation of the PEPC *promoter occurs predominantly in M cells*

In order to determine whether de-methylation of the cytosine residues was spatially coincident with *PEPC* expression in the maize leaf, the 270 bp region of DNA (sense strand –3178 to –-2908) surrounding the four sites was amplified and sequenced from bisulphite-treated DNA extracted from M and PBS cells of 3-week-old light-grown leaf tissue. PBS strands were assessed for contamination by M cells and we estimate this contamination as being less than 5% M. This agrees with previous work in which contamination of PBS or M cell preparations by the other cell type is typically lower than 5% (Sawers *et al.*, 2007). Representative images of the M and PBS preparations are shown in Fig. 3A and B. In M cells all four cytosines were de-methylated in response to light, while methylation of these residues was retained in PBS cells. Whilst only 67% and 72% of cytosines at positions –3171 and –3165 remained methylated in M cell extracts (Fig. 3C, E), 84% and 100% of cytosines at these positions were found to be methylated in PBS cells, respectively (Fig. 3D, F). De-methylation in cytosines at positions –3034 and –3031 was more pronounced with only 21% and 29% methylated in M cells (Fig. 3C, E), whereas 100% and 90% of cytosines, respectively, were methylated in PBS cells (Fig. 3D, F) corresponding to a 79% and 61% change in methylation at these nucleotides between the two cell types. The changes in methylation status of cytosine residues observed upon illumination of whole leaves can thus be attributed to a M-specific de-methylation of four cytosine residues, co-incident with *PEPC* transcript accumulation.

DNA methylation in 1 kb upstream of the transcription initiation site does not regulate PEPC *expression*

Despite the discovery of four cytosine residues ~3.1 kb upstream from the transcription start site (TSS) which are specifically de-methylated in M cells in response to light, several studies have shown that only –1212 to +88 of the *PEPC* gene is required for M-specific expression in maize (Taniguchi *et al.*, 2000; Kausch *et al.*, 2001). Indeed, maize *PEPC* and *PPDK* promoters also generate GUS expression in the appropriate cell-types in a light-dependent manner when placed in rice (Matsuoka *et al.*, 1993, 1994; Ku *et al.*, 1999; Nomura *et al.*, 2000) indicating that the relevant *trans*-acting factors required for the recognition of these genes are not only conserved in these species but also operate in the

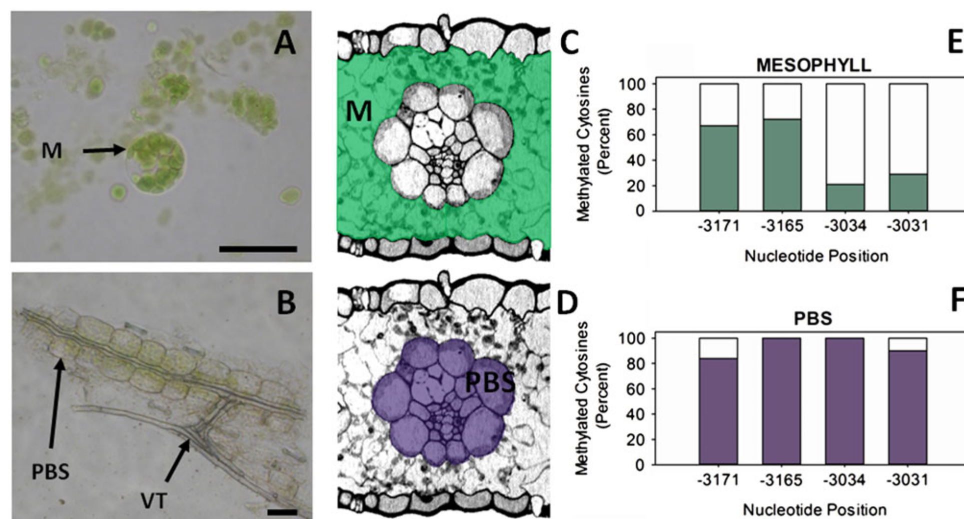

Fig. 3. Light induced de-methylation of the *PEPC* promoter occurs only in M cells. Representative pictures of two cell extracts from maize leaf tissue used for DNA extraction and subsequent bisulphite sequencing (A, B). The position of these extracts within the leaf is shown on transverse sections of maize leaf tissue in (C) and (D). The methylation status of *PEPC* promoter region –3178 to –2908 kb upstream of the transcription start site in M and PBS cells of light-grown maize leaves determined by bisulphite sequencing is shown in (E) and (F). The occurrence of cytosine residues in amplified products indicated methylated bases, whereas thymines in the place of cytosines were classified as unmethylated. Percentages refer to the percentage of clones with cytosine residues at the nucleotide position indicated. At least 20 independent clones were sequenced per treatment and all sequences varied at a minimum of one C/T base. Shaded areas indicate tissue types in each extract used for DNA extraction. Abbreviations are as follows: M, mesophyll; PBS, parenchyma bundle sheath; VT, vascular tissue. Scale bars=25 μm.

correct cell-types. Strong GUS accumulation in maize M cells when the *uidA* gene encoding β-glucoronidase are fused to 1.7 kb or 0.6 kb of the maize *PEPC* promoter indicates that the site ~3.1 kb upstream of the TSS is not necessary for M-specific expression in maize (Taniguchi *et al.*, 2000; Kausch *et al.*, 2001). Although studies using methylation-sensitive restriction enzymes have not shown evidence of methylation in the 'core' *PEPC* promoter (–1212 to +88) (Langdale *et al.*, 1991), it was hypothesized that methylated cytosines in this region may have gone undetected as a consequence of the limited resolution inherent in experiments predicated on the occurrence of appropriate restriction sites. To discover whether methylation of this region is involved in directing tissue- and/or cell-specificity of *PEPC* transcript accumulation, the sense strand of this region of genomic DNA (–158 to +155) of *PEPC* in roots and dark/light-grown leaves as well as in M and PBS of light-grown leaves was interrogated using bisulphite sequencing as described previously. No cytosine residues in either CG or CHG contexts in the cluster were found to be methylated/de-methylated in response to light or in a cell-specific manner (Fig. 4).

The regulatory protein complex PEP-I has been identified as an important regulator of *PEPC* expression in maize (Kano-Murakami *et al.*, 1991). PEP-I binding is sensitive to methylation interference at two guanine residues in the consensus binding sequence (Kano-Murakami *et al.*, 1991). It was therefore hypothesized that differential methylation of cytosines in the PEP-I binding sites could influence the binding affinity of PEP-I to this region of the promoter. To test this, after treatment with sodium bisulphite, a 328 bp region (–521 to –176) of sense strand DNA including the PEP-I binding sites was amplified and sequenced. None of the cytosines in root, dark-grown leaves or illuminated leaves were found to be methylated and no differences were found between M and PBS sequences for this region. Overall, this indicates that cytosine methylation is unlikely to regulate differential binding of PEP-I in maize roots or leaves. Similarly, when the remainder of the maize *PEPC* promoter region up to –1270 downstream of the TSS (regions B, C, and D) was screened for changes in cytosine methylation by bisulphite sequencing, no cytosine methylation was detected. These regions include binding sites for MNF1 and Dof, indicating that cytosine methylation at these sites does not regulate the binding of these proteins to the *PEPC* promoter in maize nor is it required for tissue- or cell-specificity of maize *PEPC* expression. The lack of methylation in this region of the promoter led to an examination of the distribution of localized concentrations of CpG dinucleotides (cytsoine and guanine nucleotides separated by a single phosphate) in the maize *PEPC* promoter. The –1270 to +155 bp region was screened for CpG clusters using the Methprimer™ software (urogene.com) (Li and Dahiya, 2002). Using the programme default settings (100 bp segments with 50% GC content, observed/expected ratio of CpG dinucleotides >0.6), three CpG islands were predicted within the *PEPC* promoter (–946 to –817; –782/–595; –200/+105) (Fig. 4). Increasing the stringency of the search parameters to 300 bp segments, observed/expected ratio >0.6 and >70% GC revealed one large CG cluster (–200 to +105) spanning the *PEPC* TATA box, TSS and ATG sequences.

Discussion

Regulatory protein binding is unlikely to be regulated by cytosine methylation

Several regulatory protein complexes have been found to bind the *PEPC* promoter. One in particular, PEP-I,

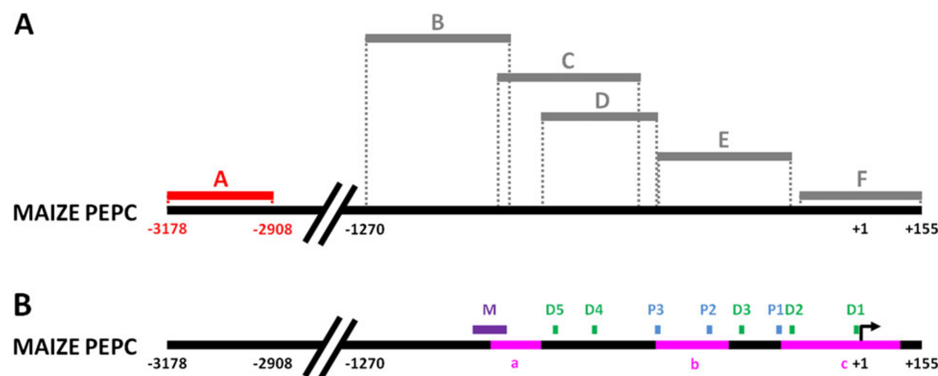

Fig. 4. Cytosine methylation in the ~1 kb upstream region of the TSS does not correlate with *PEPC* expression. The methylation status of *PEPC* promoter region +155 to –1270 in roots, dark-grown leaves, and M and PBS cells of maize leaves transferred to light for 72 h is shown in panel (A). Grey bars indicate amplicons where no methylation was detected. Red bars indicate differentially-methylated regions. Dashed lines show the position of amplified sequences on the maize *PEPC* promoter. Regions amplified from bisulphite treated DNA are as follows: (A) –3178/–2908; (B) –1270/–888; (C) –902/–551; (D) –817/–518; (E) –521/–176; (F) –158/+155. The sites of protein complex binding relative to the maize *PEPC* promoter sequence are indicated in panel (B). Abbreviations are as follows: M, MNF1; D, DOF; P, PEP-I. The positions of CpG islands in the *PEPC* promoter predicted by Methprimer™ software are depicted as pink bars in panel (B) (a, –946 to –817; b, –782/–595; c, –200/+105). The program settings were as follows: 100 bp segments, observed/expected CpG dinucleotides >0.6 and 50% GC content.

interacts with the promoter in a tissue-specific manner, binding in maize leaves but not roots. It has also been shown that PEP-I binding *in vitro* is sensitive to methylation interference at two guanine residues in the consensus binding sequence (Kano-Murakami *et al.*, 1991). However, differential methylation of cytosines between –521 and –176 did not differ between roots and dark- or light-grown leaves. Similarly, no differences in the methylation status of cytosine residues were observed between tissue- or cell types in the region from –905 to –818 corresponding to the binding site of MNF1 (Yanagisawa and Izui, 1992) or the DOF proteins (Yanagisawa and Izui, 1993; Yanagisawa, 1995; Yanagisawa and Sheen, 1998), indicating that cytosine methylation is unlikely to play a role in regulating the binding affinity of these proteins *in vivo*.

Cytosine methylation is not required for cell-specific expression of PEPC

Two cytosine residues in the upstream region of *PEPC* which undergo M-specific de-methylation in response to illumination have been identified. Two more cytosines are de-methylated at an adjacent *Pvu*II restriction site, previously discovered by Langdale *et al.* (1991); the extent of cytosine de-methylation at this site in different maize tissues and cell-types has now been quantified. As GUS accumulates in maize M cells when the *uidA* gene encoding β-glucoronidase is fused to 0.6 kb of the *ZmPEPC* promoter (Taniguchi *et al.*, 2000; Kausch *et al.*, 2001), neither of these sites at 3.1 kb that undergo de-methylation appear necessary for M-specific expression in maize. In fact, the *PEPC* promoter sequence from –389 upstream to the first ATG generated 75% reporter gene expression in transient assays of isolated maize M cells (Shäffner and Sheen, 1992). Similarly, when maize *PEPC* is transformed into rice under the control of maize 1.2 kb promoter sequence, *PEPC* transcripts accumulate faithfully in M cells (Matsuoka *et al.*, 1994). It is shown here that there is no cytosine methylation in the first 1.3 kb of the maize *PEPC* promoter in roots, etiolated leaves or M and PBS cells of light-grown leaves. This indicates that cytosine methylation in the minimal promoter of maize *PEPC* is not directly involved in regulating its cell-specific expression. Due to a high GC content, the availability of appropriate sites for bisulphite primer design was limited (Henderson *et al.*, 2010) and so a stretch of 16 nt (–175 to –157) was not analysed in this study. Although this region could contain differentially-methylated sites, it represents 1.1% of the total promoter sequence interrogated and contains only two CpG sites.

The discovery that ~1.3 kb of the *PEPC* promoter is not methylated is rather unexpected, given the cell-specific chromatin patterns observed in the promoter associated with its transcription in M cells. The discovery of three clusters of CG dinucleotides within the promoter sequence is also unusual. These regions, called CpG islands are typically found to be unmethylated, but, in animals, are normally associated with constitutive expression observed in genes with housekeeping functions (Cedar, 1988) rather than cell-specific gene expression. However, there is at least one example in maize where this is not the case. The expression of the maize gene encoding alcohol dehydrogenase (*Adh1*) is repressed in leaves (Okimoto *et al.*, 1980), yet a 900 bp CpG island in the promoter of the *Adh1* gene is not methylated in this tissue (Nick *et al.*, 1986). It is not known how CpG islands are maintained in an unmethylated state, although it has been hypothesized that proteins may bind these regions to protect them from methylation (Voo *et al.*, 2000; Bader *et al.*, 2003) and it has been shown that CXXC finger protein 1 (Cfp1) binds to over 80% of unmethylated CpG islands in mammals and directly influences local chromatin structures (Blackledge *et al.*, 2010; Thomson *et al.*, 2010). In the case of maize *PEPC* it could be that protein complexes binding the region between –0.6 kb and the TSS, containing two CpG islands, perform this function. It is particularly interesting in this context that some of the proteins that have been identified as binding the maize *PEPC* promoter also bind other promoters and that Cfp1 was shown to be closely associated with tri-methylation at H3K4 (Thompson *et al.*, 2010), which was shown to occur in the *PEPC* promoter in M cells (Danker *et al.*, 2008). With this in mind, a model is proposed whereby a protein with similar properties to Cfp1 binds unmethylated CpGs in the PEPC promoter in M cells and directs H3K4 trimethylation, maintaining an open chromatin conformation and permitting transcription to occur. Either the absence of this protein or competition for binding sites in PBS cells prevents H3K4 methylation and therefore transcription (Figure 5).

Our study demonstrates that methylation of the *PEPC* promoter is unlikely to be involved in directing cell-specific expression, and the question of how the differential methylation of four cytosines 3.1 kb upstream relates to *PEPC* expression remains unresolved. Preliminary *in silico* analysis of maize chromosome 9 suggests that the nearest predicted gene upstream (5') of this region on the same strand is approximately 12 kb away (see Supplementary Figs S1–S4 at *JXB* online), indicating that the site is unlikely to be regulating a gene upstream of *PEPC* in the opposite orientation. Another possibility is that the differentially-methylated region operates as an enhancer element to *PEPC* expression. Studies introducing maize *PEPC* into rice with a minimal promoter (–1212 to +88) show that the amount of expression was not identical to *PEPC* transcript abundance in maize (Matsuoka *et al.*, 1994) supporting this hypothesis. To test the effect of methylation at the *Pvu*II site more directly, we attempted to grow maize on methylation inhibitors. However, preliminary analysis indicates that in maize plants grown on media containing 5'-azacytidine or zebularine, cytosine analogues which inhibit methylation in actively dividing cells (Jones *et al.*, 1985; Christman, 2002; Baubec *et al.*, 2009), the amount of methylation in leaves remain unaffected (H Woodfield, unpublished results).

Recent developments in nanopore sequencing technology (Kasianowicz *et al.*, 1996; Astier *et al.*, 2006; Clarke *et al.*, 2009) include the ability to distinguish 5-methylcytosine from unmethylated nucleotide bases due to differences in

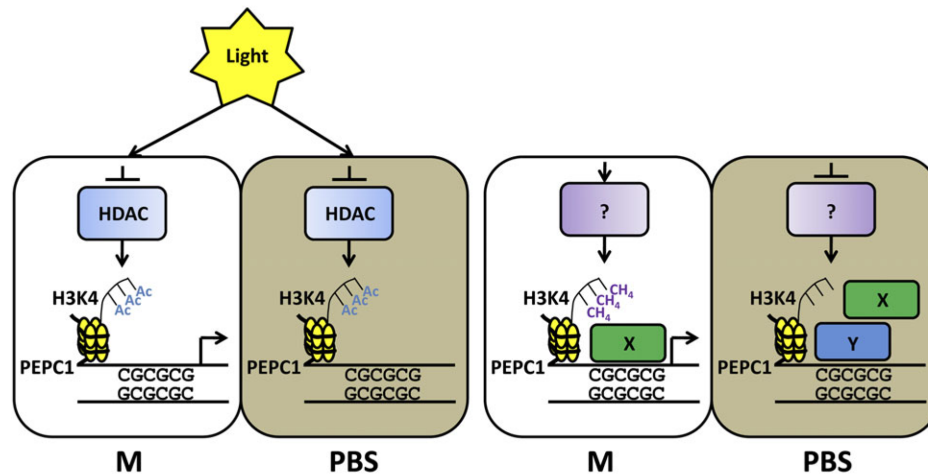

Fig. 5. Epigenetic interactions influencing *PEPC* transcription in maize leaves. Abbreviations: A, acetylation; CH_4^+, methylation; HDAC, histone deacetylase; M, mesophyll; PBS, parenchymatous bundle sheath; H3K4, histone 3 lysine 4; X, putative positive regulatory protein; Y, Putative negative regulatory protein.

its ionic conductivity (Clarke *et al.*, 2009) and promise to provide a high-throughput, affordable method of analysing the methylome of species with large genomes such as maize, to single base resolution. This will enable the methylation status of M and PBS cell genomes to be interrogated simultaneously and promises to greatly improve our understanding of the role of DNA methylation in regulating C_4 gene expression.

In summary, methylation marks within the *Pvu*II site identified by Langdale *et al.* (1991) have been defined and two additional cytosine residues have been identified in the *PEPC* promoter that are methylated in maize root tissue. In leaves, de-methylation of these cytosines is dependent on illumination and is coincident with elevated *PEPC* expression. Furthermore, light-regulated de-methylation of these cytosines occurs only in M cells. No evidence of cytosine methylation was found in the 0.6 kb promoter required for M-specific expression indicating that cytosine methylation does not play a direct role in directing cell-specificity. However, the abundance of unmethylated CpG sites in the *PEPC* promoter suggests that the epigenetic status of the *PEPC* promoter may be important in maintaining an open chromatin structure for transcription factor binding. The function of the four differentially-methylated cytosines in the upstream region of the *PEPC* promoter remains unclear: however, the possibility remains that this site exerts some distant regulatory control over the cell-specific expression of the gene. The identification of proteins binding to the 5' flanking region of maize *PEPC* gene is a priority and together with developments in nanopore sequencing, should provide a clearer picture of the regulatory mechanisms governing *PEPC* expression in maize.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Gene prediction in the region 15 kb upstream of *ZmPEPC1*.

Supplementary Fig. S2. Predicted protein coding sequences in the region 15 kb upstream of *ZmPEPC1*.

Supplementary Fig. S3. Gene prediction in the region 50 kb upstream of *ZmPEPC1*.

Supplementary Fig. S4. Predicted protein coding sequences in the region 50 kb upstream of *ZmPEPC1*.

Supplementary Table S1. Primer sequences used in bisulphite sequencing PCR to amplify regions of the maize *PEPC1* promoter.

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