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Developmental information but not promoter activity controls the methylation state of histone H3 lysine 4 on two photosynthetic genes in maize

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Summary

We have investigated the establishment of histone H3 methylation with respect to environmental and developmental signals for two key genes associated with C_4 photosynthesis in maize. Tri-methylation of histone H3 lysine 4 (H3K4) in roots and leaves was shown to be controlled by autonomous cell-type-specific developmental signals that are independent of illumination and therefore independent of the initiation of transcription. Di- and mono-methylation of H3K4 act antagonistically to this process. The modifications were already established in etiolated seedlings, and remained stable when genes were inactivated by dark treatment or pharmaceutical inhibition of transcription. Constitutive di-methylation of H3K9 was concomitantly detected at specific gene positions. The data support a histone code model whereby cell-type-specific signals induce the formation of a chromatin structure that potentiates gene activation by environmental cues.

Keywords: C_4 photosynthesis, mesophyll, bundle sheath, epigenetics, histone modification.

Introduction

C_4 plants improve the efficiency of carbon fixation by separating primary and secondary carboxylation into two leaf tissues, the mesophyll (M) and the bundle sheath (BS) (Von Caemmerer and Furbank, 2003). Primary carboxylation is catalysed in M cells by phosphoenolpyruvate carboxylase (PEPC) to generate oxaloacetate, which is reduced to malate and diffuses into the BS, where decarboxylase malic enzyme (ME) releases CO_2 and effectively concentrates it for final fixation. The genes encoding PEPC and ME are expressed in a tissue-specific manner and controlled by multiple additional stimuli, including strong light induction (Sheen, 1999).

Histone methylation plays a pivotal role in the regulation of eukaryotic transcription. Complex methylation patterns involving at least six lysine residues in the N-terminal tails of histones H3 and H4 have been implicated in transcriptional activation and repression. Each of these residues can carry between one and three methyl groups (Martin and Zhang, 2005). Tri-methylation of histone H3 lysine 4 (H3K4me3) in the promoter and the proximal transcribed region correlates strongly with transcriptional activity in yeast and mammals (Bernstein *et al.*, 2005; Pokholok *et al.*, 2005), and seems to be involved in anchoring the transcription apparatus to promoters (Vermeulen *et al.*, 2007). The roles of H3K4me2 and H3K4me1 are less clear, and vary between species. In yeast, H3K4me2 is distributed throughout active and poised genes. H3K4me1 is mostly found at the end of genes, with little correlation to the rate of transcription (Li *et al.*, 2007; Pokholok *et al.*, 2005). In mammals, H3K4me2 tends to co-localize with H3K4me3, although additional, exclusively di-methylated regions are also found (Ruthenburg *et al.*, 2007). Little is known about the role of H3K4me1 in eukaryotes. In green algae, H3K4me1 is associated with silenced genes and transposons (van Dijk *et al.*, 2005), but in humans it is enriched in enhancer regions (Heintzman *et al.*, 2007) and involved in chromosome stability (Seol *et al.*, 2006).

Other types of histone methylation identify repressive chromatin. Histone H3 lysine 9 has been widely studied, and its modification varies among species. In mammals, H3K9me3 is enriched at pericentric heterochromatin. H3K9me2 and H3K9me1 are usually found in silent euchromatic regions (Rice *et al.*, 2003), but both modifications have also been detected in actively transcribed genes (Barski

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et al., 2007; Vakoc *et al.*, 2005). The distribution of H3K9 methylation in plants appears to depend on genome size. In species with small genomes such as Arabidopsis, H3K9me1 and H3K9me2 are usually confined to heterochromatic regions, but H3K9me3 is associated with transcriptionally repressed euchromatin (Pfluger and Wagner, 2007; Turck *et al.*, 2007). In plants with larger genomes, H3K9me2 is more evenly dispersed and perhaps involved in silencing of repetitive elements (Houben *et al.*, 2003). A recent immunolocalization study in maize confirmed that H3K9me1 is confined to heterochromatin, but H3K9me2 and H3K9me3 are distributed within euchromatic chromosome regions (Shi and Dawe, 2006).

We have previously reported that the chromatin of the *Pepc* gene is acetylated in response to light, and that this modification neither requires promoter activity nor induces the promoter (Offermann *et al.*, 2006). Here we show that a developmentally controlled switch from mono- and di- to tri-methylation of H3K4 is associated with the potentiation of two key genes in C_4 photosynthesis for cell-type-specific activation. This pattern is established independently of gene activity, and maintained even after prolonged promoter inactivation. H3K9 di-methylation is found at specific gene positions but not regulated during gene activation and repression.

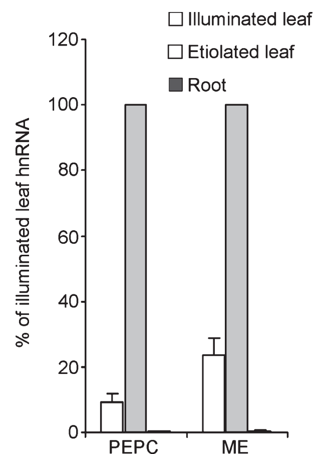

Figure 1. Quantification of promoter activity in the investigated tissues. Values are a percentage of the maximum hnRNA abundance. Data points are based on four independent experiments. Bars indicate SE.

Results

Selective depletion of H3K4 tri-methylation in roots

Transcription of the *Pepc* and *Me* genes is light-inducible and leaf-specific (Sheen, 1999). We confirmed these results by measuring the amount of corresponding unspliced heterogenous nuclear RNAs (hnRNAs), a sensitive direct indicator of promoter activity (Delany, 2001; Elferink and Reiners, 1996; Offermann *et al.*, 2006; see also Discussion). As shown in Figure 1, both genes were transcribed at a low basal level in leaves from etiolated plants, but were strongly induced by illumination. There was no promoter activity in roots. Three levels of transcription were thus represented: basal, induced and repressed.

Chromatin immunoprecipitation (ChIP) was used to detect the various H3K4 methylation states and H3K9 di-methylation on the two genes, and an antibody against the invariant C-terminus of histone H3 was used as a marker for nucleosome occupancy (Pokholok *et al.*, 2005). In each gene, we analysed the distal and proximal promoter sites (P1, P2), three sites within the transcribed region (C1–C3), and an intergenic site just beyond the polyadenylation site (I). Precipitation efficiencies for the promoter of the housekeeping gene *Actin-1* were used to correct the data for possible variations in the quality of chromatin preparations from different tissues (Haring *et al.*, 2007). The levels of *Actin-1* mRNA were comparable in all three tissues (data not shown). As shown in Figure 2, *Actin-1* ChIP signals from etiolated and green leaves were very similar with all antibodies, while signal intensities were reduced by up to 50% in root chromatin depending on the antibody. This pattern was reproducible with a primer pair specific for the coding region of the same gene (data not shown). Because H3K9me2 was not detected in the *Actin-1* gene, we instead used a primer system specific for a *Copia*-like retrotransposon, in which high levels of H3K9me2 have been identified (Haring *et al.*, 2007). Again, the signal obtained from roots was lower than that in leaves, suggesting that standardization is required for comparison of tissue-specific histone modifications.

The results for the *Pepc* and *Me* genes are shown in Figure 2. For each modification, we observed a gene-specific distribution that was mostly independent of the activation state. H3K4me3 was detected on the proximal promoters and transcribed regions, but not on the distal promoters or in the intergenic regions. The H3K4me3 signals peaked at the end of the transcribed region in *Pepc* (Figure 2e), but nearest to the promoter in *Me* (Figure 2f). H3K4me2 was distributed similarly to H3K4me3 in the *Me* gene (Figure 2i), but was clearly restricted to the proximal promoter and the end of the transcribed region in the *Pepc* gene (Figure 2h). H3K4me1 signals were more evenly distributed (Figure 2k,l), but peaked at the end of the transcribed regions. Conversely, strong H3K9me2 signals, comparable in intensity to those of the retrotransposon sequence, were detected in the intergenic regions, and, unexpectedly, at the C1 site of the *Pepc* gene (Figure 2n). Surprisingly, H3K9me2 was found constitutively in both roots and leaves.

The only clear difference between the tissues was the amount of H3K4me3 (Figure 2e,f): signals obtained from roots (inactive promoters) were very low or below the

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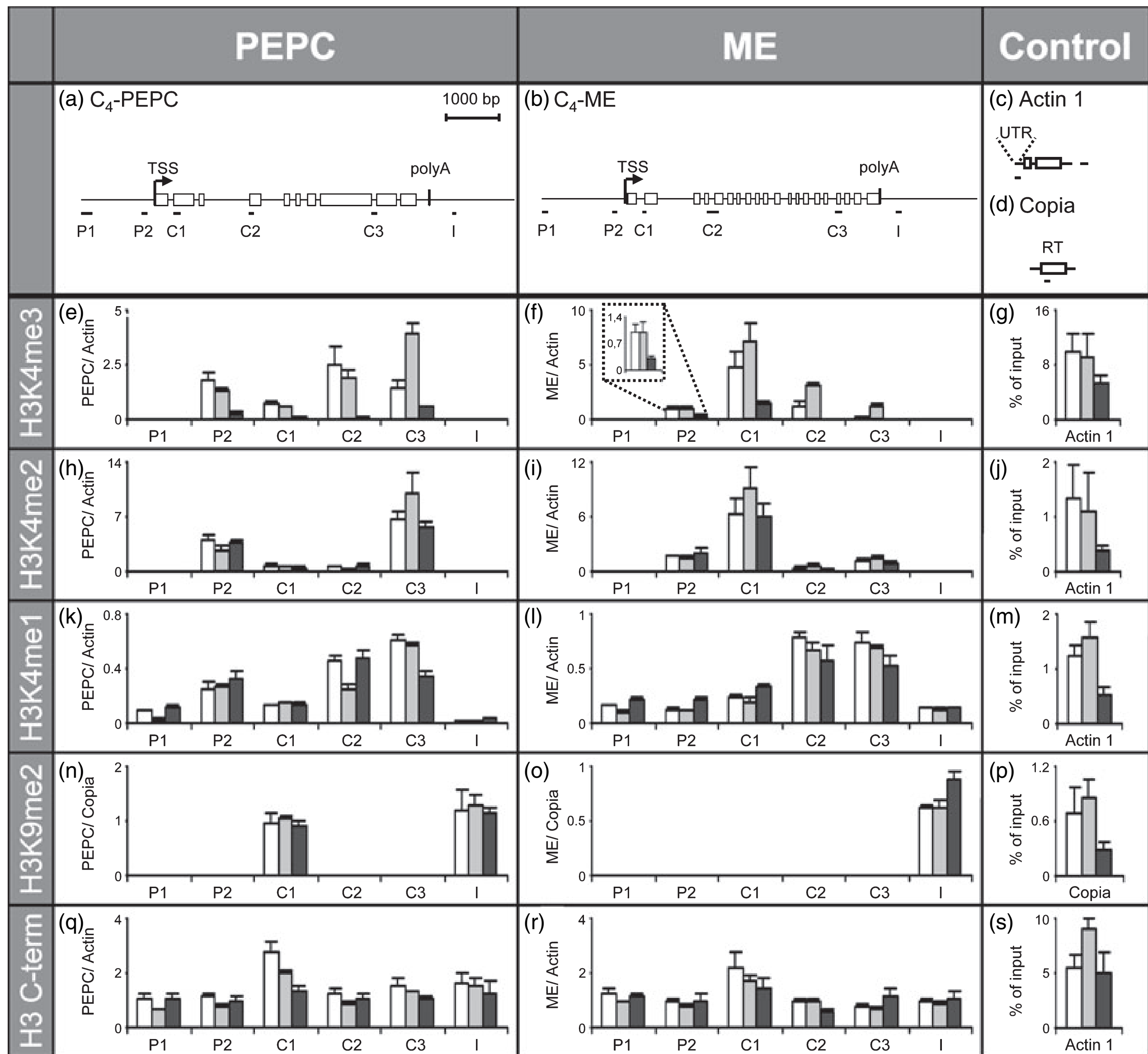

Figure 2. Histone methylation in roots (black), etiolated (white) and illuminated leaves (grey).
(a)–(d) Amplified regions used in this study (line, intron; block, exon; TSS, transcription start site; polyA, polyadenylation site; UTR, untranslated region; RT, reverse transcriptase coding sequence).
(e)–(s) Histone methylation on *Pepc*, *Me* and control regions. Amounts of chromatin precipitated with antibodies specific for the tri- (me3), di- (me2) and mono-methylation (me1) of histone H3 lysine 4 (H3K4), di-methylated histone H3 lysine 9 (H3K9me2) and an invariant C-terminal epitope on histone H3 (H3 C-term). Data points are based on at least three independent experiments. Bars indicate SE.

detection threshold, but strong signals were detected in etiolated leaves (basal activity) and illuminated leaves (induced activity). On both genes, H3K4me3 levels at the end of the transcribed regions increased with activity, but remained constant on the promoters. Thus, the promoter tri-methylation signal did not follow gene activity.

H3K4 tri-methylation and leaf cell-type specificity

We observed only minimal differences when comparing etiolated and illuminated whole leaves. However, the maize leaf is made up from M and BS cells, and the tested genes are only highly expressed in one or the other of the two cell types. We accordingly hypothesized that the histone modifications observed are also derived from either M or BS cells. We therefore isolated the cell types from etiolated and illuminated leaves and subjected the chromatin to ChIP analysis (Figure 3). *GAPDH-1* was used as the control (Hahnen *et al.*, 2003) because *Actin-1* is induced during mesophyll purification. Normalization was unnecessary because the signal intensities from both tissues were very similar. For the *Pepc* gene, H3K4me3 was almost exclusively detected in M cells,

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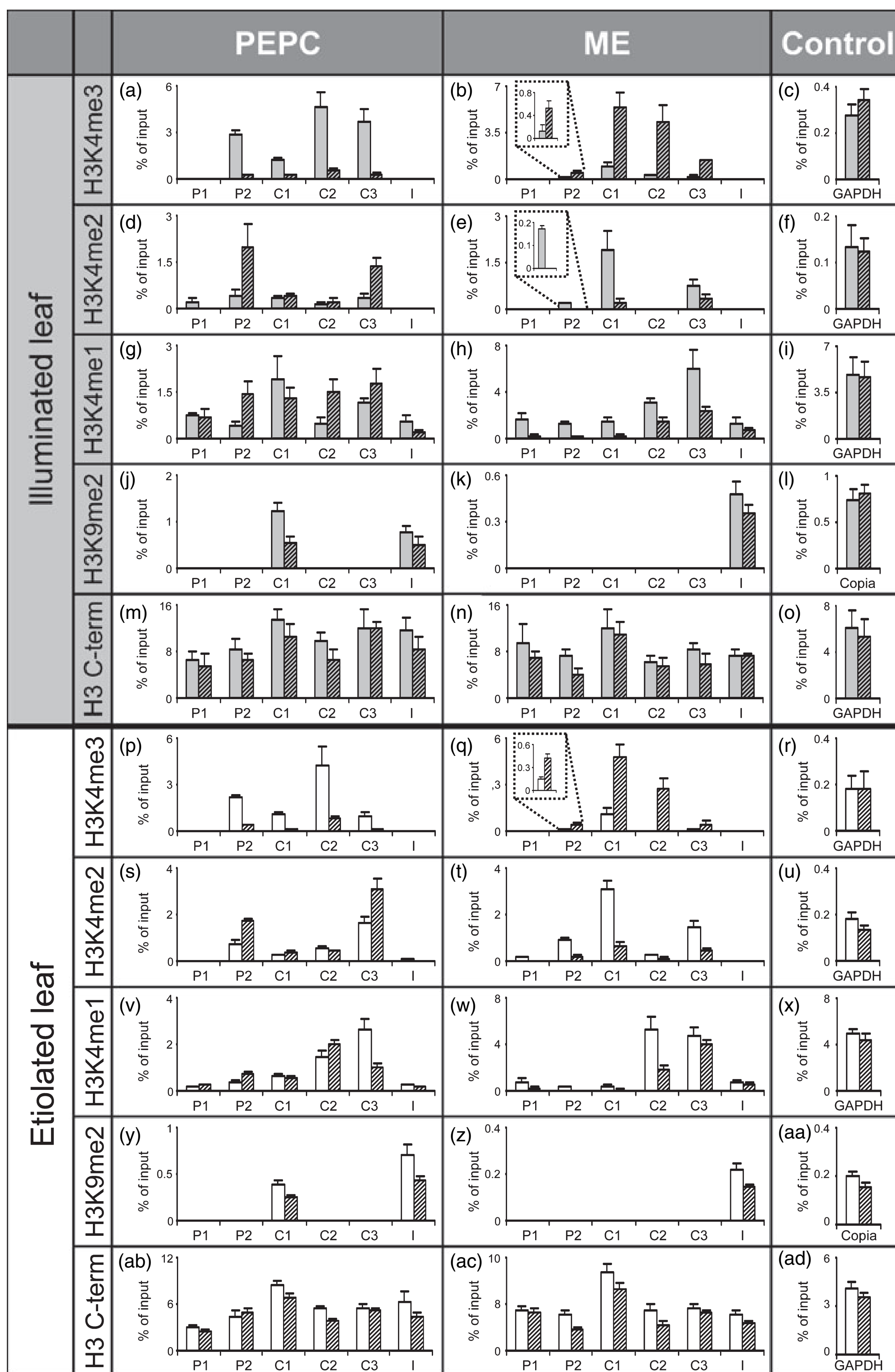
PEPC
ME
Control
Illuminated leaf
Etiolated leaf
H3K4me3
H3K4me2
H3K4me1
H3K9me2
H3 C-term
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
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(m)
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(o)
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(q)
(r)
(s)
(t)
(u)
(v)
(w)
(x)
(y)
(z)
(aa)
(ab)
(ac)
(ad)
% of input
P1 P2 C1 C2 C3 I
GAPDH
Copia

Figure 3. Histone methylation in mesophyll (open bars) and bundle sheath cells (striped bars) isolated from leaves of etiolated (white) and illuminated plants (grey). Histone methylation of *Pepc, Me* and *GAPDH-1.* Amounts of chromatin precipitated with antibodies specific for the tri- (me3), di- (me2) and mono-methylation (me1) of histone H3 lysine 4 (H3K4), di-methylated histone H3 lysine 9 (H3K9me2) and an invariant C-terminal epitope on histone H3 (H3 C-term). Values are a percentage of the amount of chromatin subjected to ChIP. Data points are based on at least three independent experiments. Bars indicate SE.

whereas H3K4me2 was clearly enriched in BS cells (Figure 3a,d). For the *Me* gene, tri-methylation was evident in BS cells and di-methylation in M cells (Figure 3b,e). Importantly, this pattern was observed not only in cells derived from illuminated leaves, but also in etiolated plants in which the genes had never been induced (Figure 3p–t). Thus, reciprocal patterns of histone methylation are established in the leaf cell types before gene activation, at least for the two genes tested in this study.

The distribution of H3K4me1 was less clear. In illuminated plants, the distribution was mostly similar to that observed for H3K4me2, i.e. strong signals for *Pepc* in BS cells and for *Me* in M cells (Figure 3g,h). However, we observed no cell-type specificity in chromatin from etiolated leaves (Figure 3v,w). Neither H3K9me2 markers just beyond the gene or at position C1 (for *Pepc*) nor nucleosome occupancy showed obvious differences between the two cell types and physiological situations. The results from whole leaves and isolated cell types indicate that H3K4me2 and H3K4me1 are generally antagonistic to H3K4me3. Cell-type-specific H3K4me3 patterns are controlled independently of the transcriptional state of the genes.

To ensure that the results obtained from isolated cell types were not influenced by the preparation method, we compared the signals obtained from whole leaves with the sum of the signals obtained from the isolated M and BS cells (Supplementary Figure S1). Very similar distribution profiles were observed at all tested positions, indicating that the patterns described for isolated tissues accurately represent the histone modification pattern *in vivo*.

BS-specific tri-methylation of H3K4 on the *Me* gene (Figure 3) is not in accordance with previous run-on assays that suggested high *Me* promoter activity in both M and BS cells and post-transcriptional regulation of mRNA abundance (Sheen, 1999). We re-assessed these results by measuring the abundance of the C_4-specific *Me* hnRNA in whole leaves and M cells. As shown in Figure 4, *Me* hnRNA levels in M cells fell to approximately 10% of the value in whole leaves, and were not light-inducible. In contrast, the light-inducible activity of the *Pepc* promoter could be reproduced in M cells, although transcription was partially reduced by the protoplasting procedure used for mesophyll isolation. We propose that *Me* shows only basal activity in mesophyll cells.

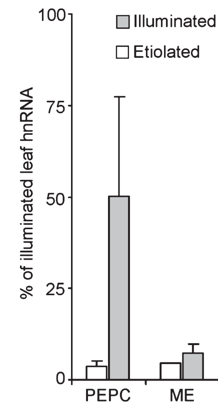

Figure 4. Quantification of *Pepc* and *Me* promoter activity in mesophyll protoplasts from etiolated and illuminated maize seedlings.
Values are a percentage of the maximum hnRNA abundance. Data points are based on at least three independent experiments. Bars indicate SE.

Conservation of H3K4 tri-methylation on genes inactivated after prior activity

The previous experiments showed that cell-type-specific H3K4 tri-methylation precedes promoter activation. This was unexpected because this modification has been mechanistically linked to elongating RNA polymerase II (Ng *et al.*, 2003). We wished to further test whether the tri-methylation signal was also stable if the gene was rendered inactive either by keeping previously illuminated seedlings in the dark for a prolonged period or treatment of detached leaves with the RNA polymerase II inhibitor α-amanitin. The treatments reduced the activity of the *Pepc* and *Me* promoters below detection levels. For *Me*, strong inactivation was also observed in detached leaves without α-amanitin, whereas the *Pepc* gene remained fully active under these conditions (data not shown). With the exception of a tendency for reduced signals at coding position C2 on the *Pepc* gene, H3K4me3 signals remained remarkably stable under all tested conditions (Figure 5). The results substantiate the developmental control of H3K4 tri-methylation, uncoupled from transcription of the genes.

Discussion

Developmental regulation of C_4 gene expression

All C_4-specific genes evolved from existing C_3 paralogues with functions in basal metabolism or unknown functions (Häusler *et al.*, 2002; Ku *et al.*, 1996; Miyao, 2003). Adoption of new regulatory elements for high transcription levels in a light-induced and cell-type-specific manner was therefore crucial for the development of C_4 photosynthesis. Studies in maize and *Flaveria* species defined *cis*- and *trans*-acting elements that are important for light induction (Yanagisawa

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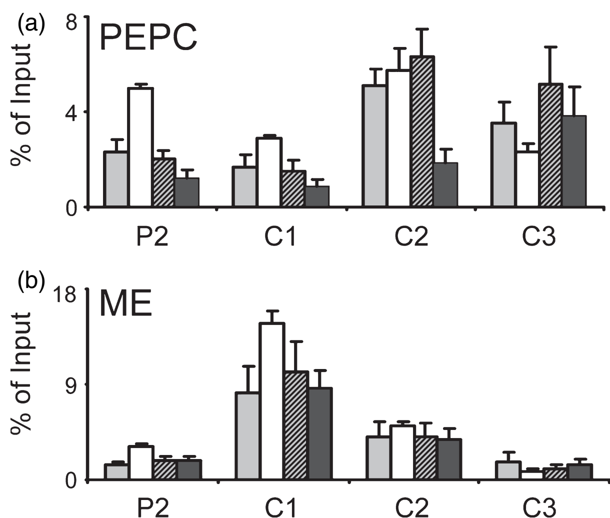

Figure 5. H3K4 tri-methylation of *Pepc* (a) and *Me* (b) genes after dark-induced or pharmaceutical down-regulation of transcription in control illuminated leaves (grey), leaves from plants kept in darkness for 72 h (white), detached leaves (striped), and detached leaves treated with α-amanitin (black).
Values are percentages of the amount of chromatin subjected to ChIP. Data points are based on at least three independent experiments ($n = 2$ for leaves kept in darkness). Bars indicate SE.

and Sheen, 1998) and cell-type specificity (Gowik *et al.*, 2004) of the C_4-specific *Pepc* gene. At the epigenetic level, two studies identified regulated DNA methylation sites on the *Pepc* gene in maize. Ngernprasirtsiri *et al.* (1988) described a site near to the transcription start that is methylated independently of illumination in BS cells but not in M cells. In contrast, Langdale *et al.* (1991) identified a methylation signal 3 kb upstream of the transcription start that was only de-methylated in illuminated M cells, and therefore probably linked to transcriptional activity. We recently described histone hyperacetylation as a light-induced modification of maize *Pepc* that is not influenced by cell-type-specific signals or transcriptional activity (Offermann *et al.*, 2006).

The results of this study suggest that cell-type specificity of C_4 photosynthetic genes in maize is controlled by a differentiation signal that is independent of light induction. Although transcriptional activity may contribute to H3K4me3 signal intensities at the end of the coding regions (Figures 2 and 5), the major pattern observed is limitation of this modification to the leaf cell type that exhibits potential for high-level expression of the gene. This pattern is already established in etiolated leaves (Figure 3), and is stable after gene inactivation from the highest activity state, and therefore is most probably under developmental control.

Bundle sheath-specific regulation of RNA abundance has been best studied for the gene encoding the small subunit of Rubisco, and appears to be more complex than M-cell-specific regulation. In maize, transient expression of promoter constructs in bombarded leaves revealed two independent M repressor and BS enhancer elements that are both also light-regulated (Bansal *et al.*, 1992; Purcell *et al.*, 1995; Xu *et al.*, 2001). Studies in *Flaveria* proposed post-transcriptional RNA degradation as an important control element of cell-type specificity (Patel *et al.*, 2006). The same has been suggested for maize *Me* based on run-on analyses with isolated M and BS cells (Sheen, 1999), although these assays probably did not discriminate between the BS-specific photosynthetic *Me* gene and a recently identified highly homologous non-photosynthetic paralogue in M cells (Tausta *et al.*, 2002). Consequently, the run-on results could not be reproduced using our gene-specific hnRNA detection system (Figure 4). Instead, we detected very low amounts of *Me* hnRNA in mesophyll protoplasts from both etiolated and illuminated plants. Given that the vast majority of introns are already spliced from the nascent transcript during transcription (Aguilera, 2005; Neugebauer, 2002), measurement of hnRNA abundance is a direct, quantitative and sensitive estimate of promoter activity. However, we cannot formally exclude the possibility that the low *Me* hnRNA abundance in mesophyll cells is caused by rapid degradation of nascent transcripts before splicing. Even this unlikely case (that could misleadingly be interpreted as low promoter activity) does not interfere with the main interpretation that tri-methylation of H3K4 on the *Me* gene is restricted to BS cells in which the *Me* gene is inducible by light.

Uncoupling of histone modifications and transcriptional activity

The results obtained also provide information about the functions of histone modifications in plants. For our model genes, dissimilar patterns were observed for the various modifications, although both genes show similar transcriptional regulation. Whereas H3K4me3 and H3K4me2 signals peaked at the proximal promoter and the end of the coding region for *Pepc*, both modifications were highly enriched at the start of the coding sequence on *Me*. The presence of H3K4me1 coincided with the higher methylation states on *Pepc*, but showed a reciprocal enrichment on *Me*. The distribution on *Me*, but not on *Pepc*, is in accordance with the 'standard' histone methylation profiles in mammals, showing a gradient from tri- to mono-methylation of H3K4 in coding regions (Xiao *et al.*, 2007, see Introduction).

Positional differences between histone methylation and acetylation profiles were evident in the distal promoter regions of both genes. Here, we observed high H4 and H3 acetylation (Offermann *et al.*, 2006; Dreesen *et al.*, B.D. and C.P., unpublished data), but tri- and di-methylation signals were not detected. Moreover – dependent on the tested cell type and the illumination regime – high histone acetylation is observed in the absence of H3K4me3 (e.g. *Pepc* in

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illuminated BS cells) and vice versa (e.g. *Pepc* in etiolated M cells or *Me* in etiolated BS cells). The tight coupling of H3K4 methylation and histone hyperacetylation at defined chromatin positions (Kurdistani *et al.*, 2004; Schubeler *et al.*, 2004) and even on single histone tails (Taverna *et al.*, 2007) was only reproduced in fully illuminated whole leaves. The same applies for the association of these modifications with high transcriptional activity (Pokholok *et al.*, 2005; Schubeler *et al.*, 2004). In our study, full activity was only observed when both classes of modification were present, but both histone acetylation and H3K4 tri-methylation can be uncoupled from transcription.

Histone modifications coupled to gene activation have been well studied, but little is known about the re-establishment of a repressive chromatin structure on previously fully active genes. Kouskouti and Talianidis (2005) described the persistence of activity markers on genes through mitotic down-regulation. Mitotic regulation might play a minor role in monocotyledonous leaves, where cell division is mostly restricted to basal meristematic regions (Tardieu *et al.*, 2000). Neither pharmaceutical inhibition of RNA polymerase II nor physiological down-regulation by prolonged dark treatment had a significant impact on HeK4me3 signals on the promoters, although incubation times were orders of magnitude beyond the half-life of transcription-induced H3K4me3 signals observed in yeast (Ng *et al.*, 2003). The transcription-induced enhancement of tri-methylation signals at the end of the coding regions (Figure 2) was partially verified by a corresponding decrease in the re-repressed state of *Pepc*, but not *Me*. Such quantitative differences might be controlled by multiple stimuli, and cannot be assigned to development, illumination or gene transcription alone. This is consistent with our observation that this effect showed the largest variation between independent experiments (data not shown).

Surprisingly few changes in histone modifications were observed when comparing leaves and roots (Figure 2). Although *Pepc* and *Me* genes are constitutively inactive in roots, the modifications tested so far do not discriminate the constitutively inactive state from the basal activity state (as observed in etiolated leaves). The only role that can be assigned to di- and mono-methylation of H3K4 at the present is acting antagonistically to tri-methylation at the level of tissue and cell-type specificity, which is different from functions described in non-plant systems (Ruthenburg *et al.*, 2007). The most obvious interpretation of the current results would be that the default di- or mono-methylated state is changed to tri-methylation by a methyltransferase that is recruited to the respective promoters in a cell-type-specific manner. H3K4 methylation in yeast and mammalian systems is catalysed by the SET1 methyltransferase in COMPASS/MLL complexes, and the switch from di- to tri-methylation appears to be controlled by associated factors (Crawford and Hess, 2006). The majority of plant SET domain proteins do not have animal homologues and are transcribed in a tissue-specific manner (Springer *et al.*, 2003), suggesting plant-specific mechanisms in the establishment and regulation of chromatin states. Further analyses in mutants may show whether depletion of SET domain proteins or associated factors can disturb the cell-type specificity of C_4 gene expression.

H3K9me2 signals, which are typically associated with transcriptionally inactive genome regions in maize (Houben *et al.*, 2003), are not increased in roots, but instead define the end of the transcribed regions on *Pepc* and *Me* in both leaves and roots. We speculate that this marker may act as a signal for dissociation of RNA polymerase II from chromatin, in accordance with the selective detection of this modification in maize euchromatic chromosome regions by immunofluorescence (Shi and Dawe, 2006). The function of the additional H3K9me2 site at the beginning of the *Pepc* coding region remains enigmatic. At this position, all markers associated with active genes, e.g. H3K4 methylation and histone acetylation (Offermann *et al.*, 2006), fell to their lowest levels. A high-resolution mapping approach with overlapping PCR systems indicated that the signal is restricted to an 800 bp DNA region, i.e. roughly four nucleosomes, directly downstream of the transcription start site (Dreesen *et al.*, B.D. and C.P., unpublished data). A similarly located signal is set during vernalization at the FLC locus in Arabidopsis to suppress transcription (He and Amasino, 2005), but the inactivity marker is removed from this gene upon full activation. Co-existence of histone modifications generally associated with gene activity and inactivity, respectively, has been also described for bivalent domains on mammalian genes that are poised for later activation (Bernstein *et al.*, 2006; Mikkelsen *et al.*, 2007). H3K9 methylation and heterochromatin protein HP1γ have even been linked with RNA polymerase II elongation on active genes (Vakoc *et al.*, 2005). However, a constitutive H3K9 methylation signal that is not influenced by and seemingly does not have an impact on gene activity has not been described so far to our knowledge.

In summary, our data indicate that H3K4me3 is a differentiation-induced marker that enables subsequent promoter activation by light. Under illumination, further epigenetic marks are added in the form of histone hyperacetylation (Offermann *et al.*, 2006). This resembles the stepwise addition of chromatin modifications on the β-phaseolin promoter during potentiation and activation (Ng *et al.*, 2005). However, the methylation markers are maintained on *Pepc* and *Me* during activation and repression, whereas, on the β-phaseolin promoter, modifications associated with potentiation are removed during activation. Our results support the existence of a 'histone code' (Jenuwein and Allis, 2001) or a more complex 'histone language' (Berger, 2007), whereby combinations of distinct histone modifications

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provide genes with information about specific developmental and environmental stimuli.

Experimental procedures

Sequence assembly

Assembly of the *Pepc* gene sequence has been described by Offermann *et al.* (2006). The *Me* gene sequence was assembled from the overlapping genome sequences ZmGSSTucs 11-12-04.9463.1, 11-12-04.763.1, 11-12-04.163608.1 and 11-12-04.29936.1 (http://www.plantgdb.org), using the corresponding cDNA (gi168527) as a starting point. Genomic continuity of the sequence was verified by genome walking and alignment with BAC sequences gi148807038 and gi115292543. Primers for *Actin-1* were derived from gi168403, those for *GAPDH-1* from gi22302, and those for the *Copia* retrotransposon from gi4234853.

Plant material and growth conditions

Maize cultivar Montello was grown under 16 h of illumination at 25°C and 8 h of darkness at 20°C. The photon flux density was between 120 and 180 μmol m^{-2} sec^{-1}. Seedlings were grown in soil for 10 to 12 days. Leaves were harvested 4 h after the onset of illumination. Etiolated plants were grown and harvested in complete darkness.

Tissue preparation

Bundle sheath strands for ChIP analysis were cross-linked and isolated according to the method described by Offermann *et al.* (2006). Mesophyll cells were isolated as described by Hahnen *et al.* (2003). Purities were always >80% based on microscopical evaluation and measurement of the relative abundance of *Pepc* and *Me* mRNAs (Sheen and Bogorad, 1987). Mesophyll protoplasts were cross-linked for 10 min in extraction buffer 1 (Bowler *et al.*, 2004), containing 3% v/v formaldehyde and 1× PBS instead of Tris–HCl. Reactions were stopped by addition of glycine to a final concentration of 160 mM. Roots were isolated from soil and extensively washed before cross-linking on a rotating wheel without vacuum infiltration.

α-amanitin treatment

Four hours after onset of illumination, 10- to 12-day-old leaves were detached under water 1 cm above the laminar joint and incubated for 8 h under illumination in a solution containing 5 mM *trans*-zeatin (Sigma, http://www.sigmaaldrich.com/), 16 mM KNO_3^- (Sugiharto *et al.*, 1992) and 10 μM α-amanitin (Sigma). Control leaves were incubated in the same solution without α-amanitin.

Chromatin immunoprecipitation (ChIP)

ChIP was performed according to the method described by Bowler *et al.* (2004), with modifications as described by Offermann *et al.* (2006) and Haring *et al.* (2007). The following antibody amounts were used: 7.5 μl anti-H3K4me1 (ab8895), 5.5 μl anti-H3K4me2 (ab7766), 2.5 μl anti-H3K4me3 (ab8580), 1 μl anti-H3 C-term (ab1791) (all from Abcam; http://www.abcam.com), and 10 μl anti-H3K9me2 (07-441, Millipore). The control serum was derived from rabbits immunized with an unrelated protein from potato (*Solanum tuberosum*).

RNA preparation and reverse transcription

RNA isolation and reverse transcription were performed as described by Offermann *et al.* (2006). hnRNAs were amplified from cDNA using primer systems specific for introns. A dilution series of illuminated leaf cDNA was used as a standard.

Quantitative real-time PCR

Quantitative PCR was performed on an ABI PRISM 7000 (Applied Biosystems, http://www.appliedbiosystems.com/) using SYBR green fluorescence (Platinum SYBR Green qPCR Mix, Invitrogen, http://www.invitrogen.com/) for detection. Oligonucleotide sequences and conditions are given in Supplementary Table S1. All PCR products were sequenced. For both genes, the hnRNA system and primer systems P2, C1, C2 and C3 exclusively amplify DNA from the C_4-specific paralogue. For P1 and I, single products were amplified, but sequence information from other paralogues is not available.

Data normalization and threshold

Measured ChIP signals were first corrected for the amount of DNA precipitated using the negative control serum (NCS). The NCS signal was always less than 10% of the signal obtained with a specific antibody. Data were normalized for chromatin input into the precipitation. Results lower than 0.1% of the input were set to zero. For comparison of root and leaf data, results were additionally normalized for the signal obtained using *Actin-1* as specified in Results.

Acknowledgements

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Comparison of data derived from whole leaves and isolated cell types.

Table S1 Oligonucleotides used in this study.

This material is available as part of the online article from http://www.blackwell-synergy.com

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