Expression of the C-Terminal Domain of Mammalian TET3 DNA Dioxygenase in Arabidopsis thaliana Induces Heritable Methylation Changes at rDNA Loci

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Abstract

In plants, demethylation of 5-methylcytosine (5 mC) residues is controlled by DNA glycosylases, while in mammals it requires oxidation of 5 mC by TET proteins, a group of Fe(II)/2-oxoglutarate-dependent dioxygenases. We analysed the effects of expressing the C-terminal catalytic domain of the human TET3 gene (TET3c) in Arabidopsis thaliana, using an rDNA region as a methylation reporter. In TET3c transformants, epialleles with hypomethylation or hypermethylation patterns can be induced, which is each stably retained in progeny lines even after removal of the TET3c transgene. In TET3c transformants, 5-hydroxymethylcytosine (5 hmC) marks are detected, indicative of the oxidative activity of the transgenic enzyme. 5-formylcytosine (5 fC) is only detectable in TET3c transformants with a DNA glycosylase mutant background suggesting further oxidation of 5 hmC residues to 5 fC by TET3c, and efficient recognition and removal of 5 fC by plant glycosylases. The results suggest that TET3c can be employed to induce heritable locus-specific changes in DNA methylation, and that accumulation of 5 hmC can be used as a marker for TET3c target regions.

Keywords
Arabidopsis thaliana, DNA Methylation, DNA Demethylation, Ten-Eleven-Translocation (TET) Proteins, Dioxygenase, 5-Hydroxy-Methylcytosine (5 hmC), 5-Formyl-Cytosine

1. Introduction

In mammals and plants, DNA methylation is targeted to transposons, retrotransposons and other repetitive ele-

ments (repeat methylation) but also to actively transcribed genes (genic/body methylation) [1]. DNA methylation is critically important in silencing transposons and regulating plant development. Methylation in promoters appears to repress transcription [2] and severe loss of methylation results in a genome-wide massive transcriptional reactivation of transposons [3]. Methylated repeat regions in plants accumulate heterochromatic histone H3K9me2 methylation marks, which are closely linked to CNG methylation [4]. The plant-specific CHROMOMETHYLASE3 (CMT3) interacts with the H3 Lys9 dimethylation (H3K9me2) pathway to maintain DNA methylation at CNG sites whereas the de novo MTase DOMAINS REARRANGED METHYLASE 1 and 2 (DRM1/2) maintain DNA methylation at CNN sites requiring the active targeting of small interfering RNAs [5]. Like its mammalian homologue DNA-methyltransferase 1 (Dnm1), plant DNA METHYLTRANSFERASE 1 (MET1) acts as a maintenance methyltransferase for CG methylation [6]. In addition, MET1 is essential for maintenance of dense methylation patterns at certain loci, which contain 5 mC marks in CG and non-CG contexts [7].

Active DNA demethylation is a part of an essential dynamic that maintains the plasticity of the epigenome in response to developmental and environmental cues [8] [9]. Mammals and plants have developed alternative demethylation mechanisms. Demethylation in plants is based on demethylation of 5 mC through a base excision repair process by a family of DNA glycosylase domain-containing proteins represented by Arabidopsis DME (DEMERTER), ROS1 (REPRESSOR OF SILENCING 1) and DEMETER-LIKE proteins DML2 and DML3 [10] [11]. Members of the DME/ROS1 family are unusually large (1100 - 2000 amino acids) compared to typical DNA glycosylases and appear to be unique to plants [12]. In mammals, demethylation is mediated by Ten-Eleven-Translocation (TET) proteins, a group of Fe(II)/2-oxoglutarate-dependent dioxygenases, which catalyse sequential oxidation of 5 mC to 5-hydroxymethylcytosine (5 hmC), 5-formyl-cytosine (5 fC) or 5-carboxylcytosine (5 caC) [13]. The oxidation products 5 fC and 5 caC serve as substrates for thymine-DNA glycosylase (TdG), which mediates their removal and replacement by unmethylated cytosine via the base excision repair (BER) pathway [14] [15]. Plants have no identifiable homologues of TET proteins [16], there is no evidence for 5 hmC presence in plants [17] and it is unclear if oxidation products of 5 mC can be processed by plant glycosylases in vivo.

The differences between plant and mammalian DNA demethylation systems provide an opportunity to exploit plants to study mammalian epigenetic modifiers and to use mammalian demethylation functions to induce epigenetic changes in plants. Expression of the c-terminal domain of the human TET3 gene in Arabidopsis resulted in the locus-specific appearance of 5 hmC and 5fC marks and demonstrated the potential of TET3 to induce heritable epigenetic changes in plants.

2. Material and Methods
2.1. Vector Construction and Plant Transformation

The 3' region of cDNA, encoding an 858-1795 of NP_001274420.1 Homo sapiens methylycytosine dioxygenase TET3 (Figure 1), was amplified from a cDNA clone using primers TET3cF 5' ccaacacaggtgceccccagctgct and TET3cR 5' ecaagatcgtgagctctctcet, and was cloned as a HindIII/EcoRI fragment into the polylinker of the plant expression vector, 35S pGreen 0179 [18], producing vector p35S TET3c.

The p35STET3c vector was transferred into Agrobacterium [19] for transformation of a wildtype Columbia ecotype and a ros1-3; dml2-1; dml3-1 triple mutant [20].

Figure 1. Schematic map of the 937aa C-terminal catalytic domain of human TET3 encoded by the TET3c construct with a cysteine-rich region (CrR) and two dioxygenase domains separated by a spacer domain.
2.2. Plant Material

All plants were grown in a growth chamber under long-day conditions (16 hours light, 8 hours dark, temperature 22°C). Genomic DNA for bisulfite analysis and Southern blot analysis was extracted from the rosette leaves of 5-week-old plants as described [21].

2.3. Southern Blot analysis

An rDNA-specific probe was amplified by PCR of genomic DNA using primers rDNA-F 5’ gttgcggggaattgccgtga and rDNA-R 5’ atgcgtcgccagcacagagg, which amplifies a 328bp region within the 18S rRNA genes AT2G01010 and AT3G41768.

2.4. DNA Methylation Analysis

Bisulphite treated genomic DNA was amplified using primers BS-F 5’ gggagagntagctttaatttaataa and BS-R 5’BS cactctatcttttaartaaca, which amplifies a 311bp region within the 18S rRNA genes AT2G01010 and AT3G41768. For 5 hmC analysis, oxidative bisulphite sequencing (oxBS-Seq) data [22], which represent 5 mC values, were compared to BS-Seq data, which represent 5 mC and 5 hmC values. 5fC levels were measured by comparing reduced bisulphite sequencing (redBS-Seq) data [23], which comprise 5 mC, 5 hmC and 5fC values with BS-sequencing DNA. For each of the three BS-Seq experiments, between 20 and 40 clones were sequenced for each line.

3. Results

The 3’ region of a cDNA of the human TET3 gene, encoding the c-terminal regions of TET3, was cloned behind the 35S promoter in a plant expression vector, and the resulting TET3c construct was transferred into Arabidopsis thaliana. Transfomants expressing the TET3c transgene were selected using RT-PCR (Figure 2(d)) and used for Southern blot analysis with DNA methylation-sensitive enzymes. In mammals the overexpression of TET genes alter methylation-specific restriction sensitivity of distinct genomic regions, including rDNA loci [24]. We therefore hybridised restricted DNA to an rDNA probe to screen transformants for region-specific changes in Arabidopsis DNA methylation. Two lines were selected for further characterisation as they showed antagonistic DNA methylation changes.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Southern blot analysis of rDNA loci in TET3c transformants. (a) HpaII and MspI restricted DNA shows hypomethylation pattern for line A and hypermethylation pattern for line B; (b) Context-specific methylation analysis by DNA restriction with BstUI (C)G, EcoRII (CNG) and MnlI (CNN); (c) HpaII and MspI restriction patterns in two successive generations; (d) RT-PCR analysis of TET3c expression shows similar transgene expression levels in lines A and B.
Line A showed a hypomethylation pattern with enhanced restriction of its DNA by HpaII and MspI while line B showed a hypermethylation response with reduced digestion efficiency for both enzymes. These effects were most pronounced in MspI-digested DNA resulting in a loss of high molecular weight bands in line A and the appearance of large-size restriction fragments for line B (Figure 2(a)). The same patterns were observed when DNA of the two lines was restricted with BstUI and EcoRII, which are sensitive to C methylation at CG and CNG sites, respectively (Figure 2(b)). To assess the stability of these DNA methylation patterns, we examined HpaII and MspI restriction patterns over two generations. Both F2 and F3 lines retained the specific hypomethylation profile for line A and the hypermethylation profile for line B (Figure 2(c)).

To test if the changes in restriction sensitivity reflected altered 5 mC levels and if this involved the production of 5 mC oxidation products, we screened methylation marks in the rDNA by bisulphite sequencing in combination with oxidative bisulphite sequencing. To examine if methylation changes were heritable, plants from which the TET3c transgene had been segregated away (TET3c−) were selected using PCR (data not shown). Data from three plants that contained the TET3c transgene (TET3c+) were compared with data from three plants from TET3c−. In line A, overall 5 mC levels are reduced by about 50%, both in TET3c+ and TET3c− lines, indicative of a heritable hypomethylation effect that matches the Southern blot data for line A. In line B, overall 5 mC levels are increased by about 75% in TET3c−, which confirms the hypermethylation status observed for line B (Figure 3(a)). The TET3c+ line, however, did not show a significant increase in 5 mC content as would be expected to explain the observed reduced sensitivity of the DNA to digestion by HpaII or MspI. As, however, both enzymes are not only inhibited by 5 mC but also by 5 hmC, an alternative explanation for the increased resistance of the restriction of line B TET3c+ DNA could be an increase in 5 hmC levels. In contrast to the wildtype Arabidopsis, which contained no significant levels of 5 hmC, 5 hmC was detectable in TET3c+ plants of line B, and to a lesser extent also in line A (Figure 3(b)). For both lines, 5 hmC marks were only observed in plants that had retained the TET3c transgene, which suggests that 5 hmC production required TET3c activity.

To investigate if TET3c activity also leads to further oxidation of 5 hmC, we measured 5 fC rates via reduced bisulphite sequencing in TET3c+ plants of lines A and B. To assess the influence of plant DNA glycosylases in processing TET3c+ oxidation products, 5 hmC and 5 fC values were also measured in a ros1-3; dml2-1; dml3-1 triple mutant. As observed for lines A and B, 5 hmC production in the glycosylase mutant background required TET3c expression and was accompanied by a reduction in 5 mC levels as in line A (Figure 4(a)). 5 fC was not observed in any line except in the TET3c expression line with glycosylase mutant background, where it was restricted to cytosines in a CG-specific sequence context (Figure 4(b)).

Figure 3. 5mc (a) and 5hmC (b) levels of a18S rDNA region in plants derived from transformants that have retained (TET3c+) or lost (TET3c−) the transgene. (a) 5mc levels are reduced in Line A. Methylation levels are shown as a percentage of each cytosine context, calculated as 5 mC/C. The total percentage of 5 mC across all contexts is shown in purple. The significance of a change in total methylation from WT is indicated by asterisks; ns= not significant, * = P<0.05, *** = P<0.005 (b) 5 hmC can be detected in TET3c+ plants. Negative values for levels of 5 hmC suggest the range within which results can be attributed to inherent variability of the technique (±3% for total 5 hmC).
4. Discussion

Expression of the c-terminal domain of TET3 (TET3c) provides a new tool for the modification of DNA methylation states and gives us new insight into the presence and processing of 5 mC oxidation products in Arabidopsis thaliana.

Analysis of methylation marks in lines A and B illustrates that TET3c expression can induce both hyper- and hypomethylated rDNA epialleles. The increase in methylation observed in line B suggests that, despite the induction of 5 hmC marks, TET3c activity does not necessarily result in demethylation. This may reflect the influence of compensating DNA methylation activities, possibly via recruitment of DNA methyltransferases. Some of these enzymes may also act as dehydroxymethylases as demonstrated for the mammalian de novo DNA methyltransferases DNMT3A and DNMT3B, which, unlike the maintenance methyltransferase DNMT1, are redox-dependent DNA dehydroxymethylases [25]. The maintenance of hypo- and hypermethylation states in subsequent generations suggests that TET3c-mediated changes are induced during a relatively short time period, after which they are retained unaltered. Considering the susceptibility of epigenetic states to environmental stress [26], the T-DNA transfer may contribute to a temporary accessibility of genomic regions to TET3c and other methylation functions that cause epigenetic changes.

5 hmC marks are only detected in lines that contain the TET3c transgene. The lack of 5 hmC marks in lines that don’t contain or have lost the TET3c transgene supports previous data from a mass spectrometry study in different Arabidopsis thaliana tissue types and genetic backgrounds, which found no evidence for the presence of 5 hmC in Arabidopsis [17]. The absence of 5 hmC may be due to a lack of TET functions in Arabidopsis or it may be an indicator for the efficient processing of 5 hmC by plant enzymes. The latter explanation, however, becomes less likely in view of the presence of 5 hmC marks in TET3c containing lines. Plant DNA glycosylases have been shown to process 5 hmC in vitro [27]-[29] but our data argue against an efficient 5 hmC conversion rate in vivo. While 5 hmC levels are higher in the hypermethylated line B compared to the hypomethylated line A, both lines have comparable 5 hmC/5 mC ratios, with 33% for line A and 43% for line B. This may indicate a relatively stable conversion rate of 5 mC by TET3c but this interpretation remains speculative considering we only analysed two sample lines.

The lack of 5 fC marks in TET3c transformants and the exclusive presence of 5 fC marks in the TET3c-containing glycosylase mutant shows that TET3c can oxidise 5 hmC to 5 fC in plants, and suggests that 5 fC is efficiently recognised by one or several of the three mutated plant glycosylases and channelled into the excision repair pathway. This is surprising considering that in vitro assay has shown a lack of 5 fC-specific excision activity for ROS [29] and for DME and DML3 [28].
In mammals, TET-mediated oxidation causes demethylation via enhanced passive dilution of methylation marks as 5hmC is not recognised by DNMT1 [30] and is therefore lost after two rounds of replication, and also via removal of 5mC oxidation products in the base excision repair (BER) pathway. BER is induced via recognition of 5mC oxidation products, and, unlike in plants where glycosylases recognize and excise 5mC directly to initiate BER, no glycosylase enzymes have been identified in mammals that process 5mC [31]. The key enzyme in BER-mediated removal of 5mC is thymine-DNA glycosylase (TDG), which does not recognise 5hmC but rapidly removes its oxidation products 5fC and 5caC [15]. The conversion of 5hmC into 5fC has therefore been proposed as a distinct signal for active DNA demethylation [32]. Accumulation of 5hmC and lack of 5fC in Arabidopsis TET3c transfectants could also reflect a stronger affinity of plant glycosylases for 5fC marks compared to 5hmC marks.

In the glycosylase triple mutant, 5fC marks are only detected at CG targets. This suggests that while TET3c mediated conversion of 5mC to 5hmC is not influenced by sequence context, repetitive oxidation by TET3c could be restricted to CG regions. For mammals, it has been proposed that repetitive oxidation is mediated by specific binding of RING-finger type E3 ubiquitin ligases Uhrf1 and Uhrf2 to 5hmC marks, improving regional access for TET enzymes [32]. Considering a potential structural influence on TET-mediated repetitive oxidation in Arabidopsis, plant-specific CG binding factors may contribute to the specific accumulation of 5fC marks at CG sites. This would imply that while demethylation via passive dilution of 5hmC marks can occur at cytosines in all sequence contexts, in plants demethylation via 5fC excision preferentially affects cytosines in CG contexts.

Expression of TET3c or other mammalian TET constructs could provide a useful strategy to alter DNA methylation states in plants. We only examined DNA methylation changes at rDNA loci, and can therefore not specify if and how many other loci are affected by TET3c. It also remains to be seen if TET3c access is limited to specific loci, and if TET3c− mediated demethylation is retained at some loci and efficiently reverted at others. A genome-wide screen for 5hmC marks, which are not present in wildtype genomic DNA [17] may help to identify loci with TET3c− induced epi-alleles. TET3c expression may also help to investigate if plant species differ in their susceptibility to methylation changes. While even triple mutants of Arabidopsis ros1, dml2, and dml3 glycosylases show little or no developmental alterations [20], a null mutant of a rice ROS1 gene is not transmittable to the next generation [33], and mutations of the tomato ROS orthologue SlDML2 significantly affect fruit ripening [34]. It is therefore conceivable that TET3c expression in certain species can be used to link specific phenotypes with DNA methylation changes at individual loci. This may offer new strategies to target TET3c effects to individual genomic regions to generate specific phenotypes. Considering the successful adaptation of the CRISPR-Cas9 system to recruit protein domains to individual genomic loci [35], Cas9-TET3c fusion constructs may be used to target or enhance DNA methylation changes at individual loci in plants.

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Slow-Turnover Catalyst That Initiates DNA Demethylation in a Distributive Fashion

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