Advances in Bioscience and Biotechnology, 2016, 7, 243-250
Published Online May 2016 in SciRes. http://www.scirp.org/journal/abb
http://dx.doi.org/10.4236/abb.2016.75023

Expression of the C-Terminal Domain of Mammalian *TET*3 DNA Dioxygenase in *Arabidopsis thaliana* Induces Heritable Methylation Changes at *rDNA* Loci

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Received 10 February 2016; accepted 28 May 2016; published 31 May 2016

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 *TET*3 gene (*TET*3*c*) in *Arabidopsis thaliana*, using an *rDNA* region as a methylation reporter. In *TET*3*c* transformants, epialleles with hypomethylation or hypermethylation patterns can be induced, which is each stably retained in progeny lines even after removal of the *TET*3*c* transgene. In *TET*3*c* 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 *TET*3*c* transformants with a DNA glycosylase mutant background suggesting further oxidation of 5 hmC residues to 5 fC by *TET*3*c*, and efficient recognition and removal of 5 fC by plant glycosylases. The results suggest that *TET*3*c* 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 *TET*3*c* 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-

How to cite this paper: Hollwey, E., Watson, M. and Meyer, P. (2016) Expression of the C-Terminal Domain of Mammalian *TET*3 DNA Dioxygenase in *Arabidopsis thaliana* Induces Heritable Methylation Changes at *rDNA* Loci. *Advances in Bioscience and Biotechnology*, **7**, 243-250. http://dx.doi.org/10.4236/abb.2016.75023

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 (Dnmt1), 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 (DEMETER), 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-hydroxy-methylcytosine (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 *TET*3 gene in *Arabidopsis* resulted in the locus-specific appearance of 5 hmC and 5fC marks and demonstrated the potential of *TET*3 to induce heritable epigenetic changes in plants.

2. Material and Methods

2.1. Vector Construction and Plant Transformation

The 3' region of cDNA, encoding aa 858-1795 of NP_001274420.1 *Homo sapiens* methylcytosine dioxygenase *TET*3 (**Figure 1**), was amplified from a cDNA clone using primers *TET*3*cF* 5' ccaaccaagcttggccccacggtcgcctctat and *TET*3cR 5' ccagaattctgaggtacgctggctccct, and was cloned as *HindIII/EcoRI* fragment into the polylinker of the plant expression vector, 35S pGreen 0179 [18], producing vector p35S *TET*3*c*.

The p35S*TET*3c vector was transferred into *Agrobacterium* [19] for transformation of a wildtype Columbia ecotype and a *ros*1-3; *dml*2-1; *dml*3-1 triple mutant [20].

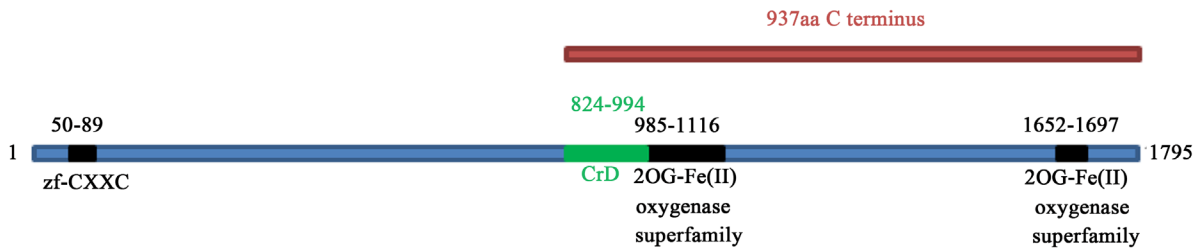

Figure 1. Schematic map of the 937aa C-terminal catalytic domain of human *TET*3 encoded by the *TET*3*c* construct with a cysteine-rich region (CrR) and two dioxygenase domains separated by a spacer domain.

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’ gtggcggggaattgccgtga and *rDNA*-R 5’ atgcgtcgccagcacagagg, which amplifies a 328bp region within the 18S rRNA genes *AT*2G01010 and *AT*3*G*41768.

2.4. DNA Methylation Analysis

Bisulphite treated genomic DNA was amplified using primers BS-F 5’ ggggaggtagtgayaataaataa and BS-R 5’BS cactctaatttcttcaaartaaca, which amplifies a 311bp region within the 18S rRNA genes *AT*2*G*01010 and *AT*3*G*41768. 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 *TET*3 gene, encoding the c-terminal regions of *TET*3, was cloned behind the 35S promoter in a plant expression vector, and the resulting *TET*3*c* construct was transferred into *Arabidopsis thaliana*. Transformants expressing the *TET*3*c* 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 regionspecific changes in *Arabidopsis* DNA methylation. Two lines were selected for further characterisation as they showed antagonistic DNA methylation changes.

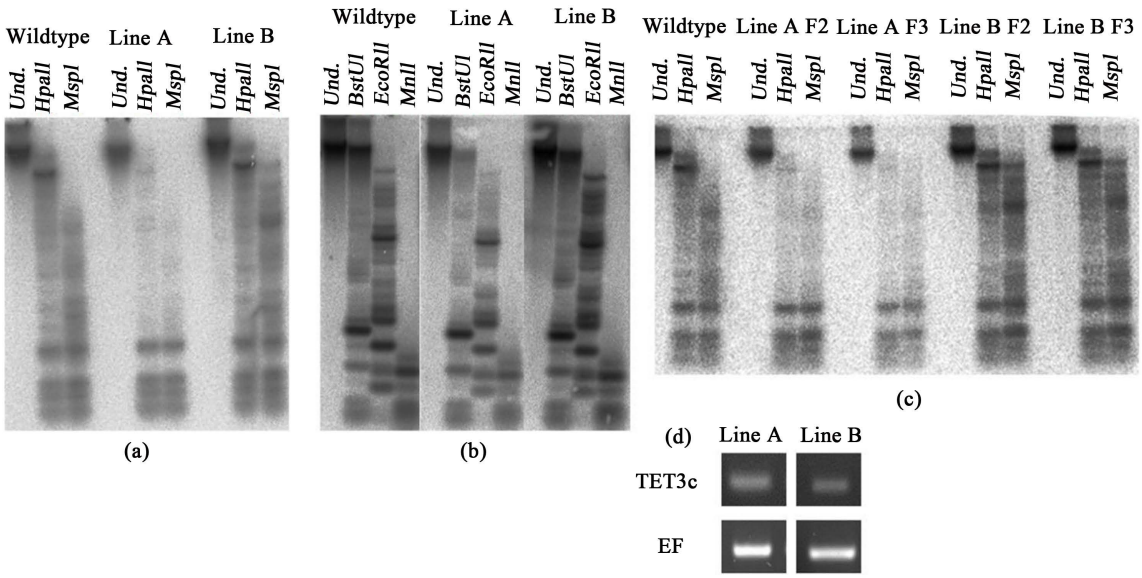

Figure 2. Southern blot analysis of *rDNA* loci in *TET*3*c* transformants. (a) *HpaII* and *MspI* restricted DNA shows hypomethylation pattern for line A and hypermethylation pattern for line B; (b) Context-specific methylation analysis by DNA restriction with *BstUI* (*CG*), *EcoRII* (CNG) and *MnlI* (CNN); (c) *HpaII* and *MspI* restriction patterns in two successive generations; (d) RT-PCR analysis of *TET*3*c* 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 *TET*3*c* transgene had been segregated away (*TET*3*c*−) were selected using PCR (data not shown). Data from three plants that contained the *TET*3*c* transgene (*TET*3*c*+) were compared with data from three plants from *TET*3c−. In line A, overall 5 mC levels are reduced by about 50%, both in *TET*3*c*+ and *TET*3*c*− 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 *TET*3-, which confirms the hypermethylation status observed for line B (**Figure 3(a)**). The *TET*3*c*+ 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 *TET*3*c*+ 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 *TET*3*c*+ 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 *TET*3*c* transgene, which suggests that 5 hmC production required *TET*3*c* activity.

To investigate if *TET*3*c* activity also leads to further oxidation of 5 hmC, we measured 5 fC rates via reduced bisulphite sequencing in *TET*3*c*+ plants of lines A and B. To assess the influence of plant DNA glycosylases in processing *TET*3*c*+ oxidation products, 5 hmC and 5 fC values were also measured in a *ros*1-3; *dml2-1; dml*3-1 triple mutant. As observed for lines A and B, 5 hmC production in the glycosylase mutant background required *TET*3*c* 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 *TET*3*c* expression line with glycosylase mutant background, where it was restricted to cytosines in a CG-specific sequence context (**Figure 4(b)**).

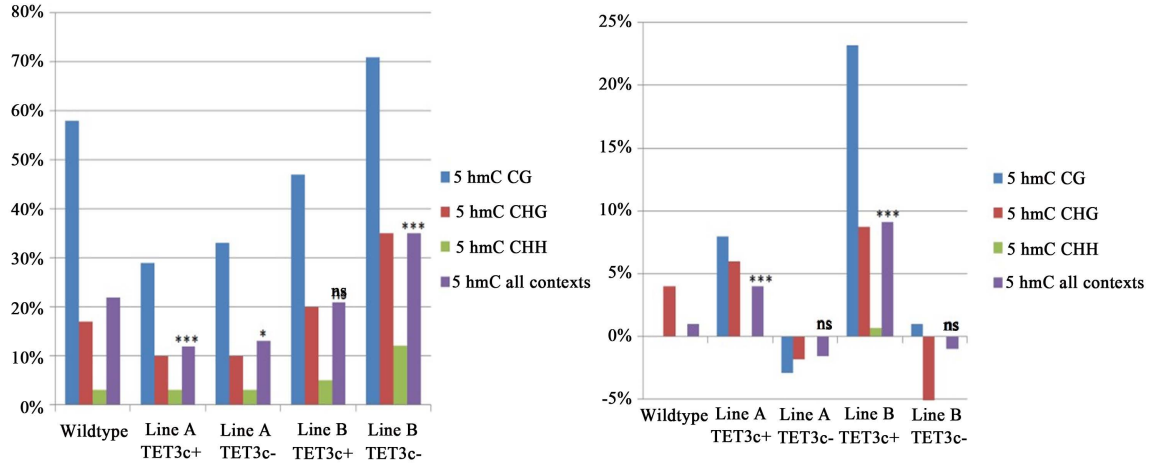

Figure 3. 5mc (a) and 5hmC (b) levels of a18*S rDNA* region in plants derived from transformants that have retained (*TET*3*c*+) or lost (*TET*3*c*−) the transgene. (a) 5mc levels are reduced in Line A. Methylation levels are shown as a percentage of each cytosine context, calculated as 5 mC/C. The total 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 *TET*3c+ 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).

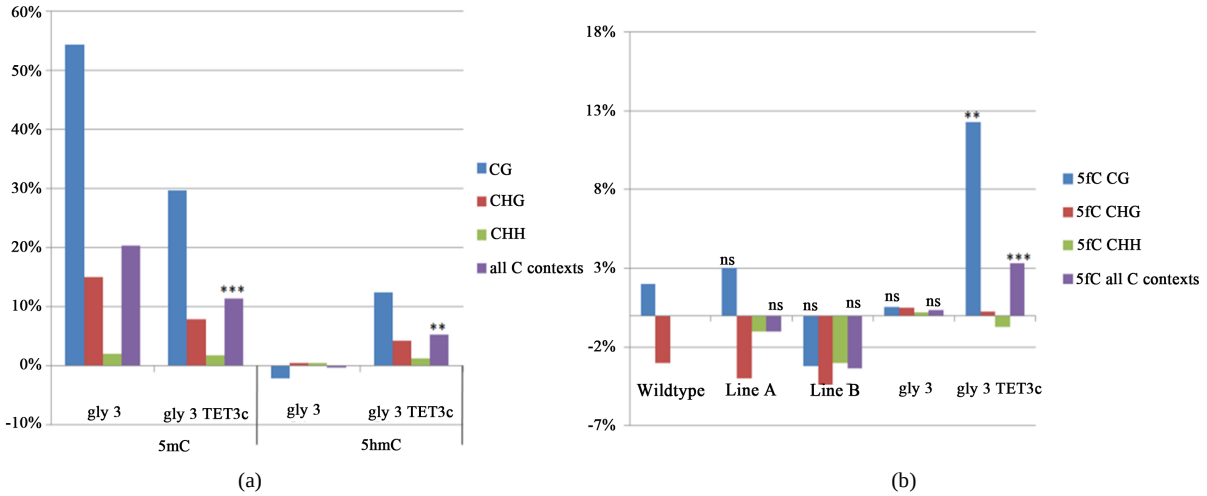

Figure 4. (a) 5mC and 5hmC levels in a *gly*3; *ros*1-3; *dml*2-1; *dml*3-1 triple mutant (*gly*3) and in *gly*3 plants containing *TET*3*c* (*gly*3 *TET*3*c*). Methylation levels are shown as a percentage of each cytosine context, calculated as 5 mC/C or 5 hmC/C. The significance of a change in total methylation from *gly*3 is indicated by asterisks; $^{**} = P < 0.01$, $^{***} = P < 0.005$; (b) 5 fC values in wildtype, *TET*3 Line A and B, *gly*3 and in a *gly*3 mutant expressing *TET*3*c*. The *gly*3 mutant contains 5 fC in the CG context. The significance of any change from WT in 5 fC CG and total 5 fC is indicated by asterisks; ns= not significant, $^{**} = P < 0.01$, $^{***} = P < 0.005$.

4. Discussion

Expression of the c-terminal domain of *TET*3 (*TET*3*c*) 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 *TET*3*c* 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, *TET*3*c* 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 *TET*3*c*-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 *TET*3*c* and other methylation functions that cause epigenetic changes.

5 hmC marks are only detected in lines that contain the *TET*3*c* transgene. The lack of 5 hmC marks in lines that don't contain or have lost the *TET*3*c* 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 *TET*3*c* 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 *TET*3*c* but this interpretation remains speculative considering we only analysed two sample lines.

The lack of 5 fC marks in *TET*3*c* transformants and the exclusive presence of 5 fC marks in the *TET*3*c*− containing glycosylase mutant shows that *TET*3*c* 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 5 hmC is not recognised by DNMT1 [30] and is therefore lost after two rounds of replication, and also via removal of 5 mC oxidation products in the base excision repair (BER) pathway. BER is induced via recognition of 5 mC oxidation products, and, unlike in plants where glycosylases recognize and excise 5 mC directly to initiate BER, no glycosylase enzymes have been identified in mammals that process 5 mC [31]. The key enzyme in BER-mediated removal of 5 mC is thymine-DNA glycosylase (TDG), which does not recognise 5 hmC but rapidly removes its oxidation products 5 fC and 5 caC [15]. The conversion of 5 hmC into 5 fC has therefore been proposed as a distinct signal for active DNA demethylation [32]. Accumulation of 5 hmC and lack of 5 fC in *Arabidopsis TET*3*c* transformants could also reflect a stronger affinity of plant glycosylases for 5fC marks compared to 5 hmC marks.

In the glycosylase triple mutant, 5 fC marks are only detected at CG targets. This suggests that while *TET*3*c* mediated conversion of 5 mC to 5 hmC is not influenced by sequence context, repetitive oxidation by *TET*3*c* 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 5 hmC 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 5 hmC marks can occur at cytosines in all sequence contexts, in plants demethylation via 5fC excision preferentially affects cytosines in CG contexts.

Expression of *TET*3*c* 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 *TET*3*c*. It also remains to be seen if *TET*3*c* access is limited to specific loci, and if *TET*3*c*− mediated demethylation is retained at some loci and efficiently reverted at others. A genome-wide screen for 5 hmC marks, which are not present in wildtype genomic DNA [17] may help to identify loci with *TET*3*c*− induced epi-alleles. *TET*3*c* expression may also help to investigate if plant species differ in their susceptibility to methylation changes. While even triple mutants of *Arabidopsis ros*1, *dml*2, and *dml*3 glycosylases show little or no developmental alterations [20], a null mutant of a rice *ROS*1 gene is not transmittable to the next generation [33], and mutations of the tomato *ROS* orthologue *SlDML*2 significantly affect fruit ripening [34]. It is therefore conceivable that *TET*3*c* 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 *TET*3*c* 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-*TET*3*c* fusion constructs may be used to target or enhance DNA methylation changes at individual loci in plants.

Acknowledgements

We thank Richard Meehan, MRC IGMM University of Edinburgh, for providing the *TET*3 cDNA and Robert Fischer, University of California, Berkeley for providing the *ros1*-3; *dml*2-1; *dml*3-1 triple mutant. EH and MW were supported by BBSRC Case studentships.

References

[1] Henderson, I.R. and Jacobsen, S.E. (2007) Epigenetic Inheritance in Plants. *Nature*, **447**, 418. http://dx.doi.org/10.1038/nature05917

[2] Zhang, X. and Jacobsen, S.E. (2006) Genetic Analyses of DNA Methyltransferases in *Arabidopsis thaliana*. Cold Spring Harbor Symposia on Quantitative Biology, **71**, 439-447. http://dx.doi.org/10.1101/sqb.2006.71.047

[3] Zilberman, D., Gehring, M., Tran, R.K., Ballinger, T. and Henikoff, S. (2007) Genome-Wide Analysis of *Arabidopsis thaliana* DNA Methylation Uncovers an Interdependence between Methylation and Transcription. *Nature Genetics*, **39**, 61. http://dx.doi.org/10.1038/ng1929

[4] Bernatavichute, Y.V., Zhang, X., Cokus, S., Pellegrini, M. and Jacobsen, S.E. (2008) Genome-Wide Association of Histone H3 Lysine Nine Methylation with CHG DNA Methylation in *Arabidopsis thaliana*. *PLoS ONE*, **3**, e3156. http://dx.doi.org/10.1371/journal.pone.0003156

[5] Chan, S.W. (2004) RNA Silencing Genes Control *de Novo* DNA Methylation. *Science*, **303**, 1336. http://dx.doi.org/10.1126/science.1095989

[6] Goll, M.G. and Bestor, T.H. (2005) Eukaryotic Cytosine Methyltransferases. Annual Review of Biochemistry, **74**, 481-514. http://dx.doi.org/10.1146/annurev.biochem.74.010904.153721

[7] Watson, M., Hawkes, E. and Meyer, P. (2014) Transmission of Epi-Alleles with MET1-Dependent Dense Methylation in *Arabidopsis thaliana*. *PLoS ONE*, **9**, e105338. http://dx.doi.org/10.1371/journal.pone.0105338

[8] Gehring, M. and Henikoff, S. (2007) DNA Methylation Dynamics in Plant Genomes. Biochimica et Biophysica Acta, **1769**, 276-286. http://dx.doi.org/10.1016/j.bbaexp.2007.01.009

[9] Morgan, H.D., Santos, F., Green, K., Dean, W. and Reik, W. (2005) Epigenetic Reprogramming in Mammals. *Human Molecular Genetics*, **14**, R47-R58. http://dx.doi.org/10.1093/hmg/ddi114

[10] Choi, Y., Gehring, M., Johnson, L., Hannon, M., Harada, J.J., Goldberg, R.B., Jacobsen, S.E. and Fischer, R.L. (2002) DEMETER, a DNA Glycosylase Domain Protein, Is Required for Endosperm Gene Imprinting and Seed Viability in Arabidopsis. *Cell*, **110**, 33. http://dx.doi.org/10.1016/s0092-8674(02)00807-3

[11] Ponferrada-Marín, M.I., Roldán-Arjona, T. and Ariza, R.R. (2009) ROS1 5-Methylcytosine DNA Glycosylase Is a Slow-Turnover Catalyst That Initiates DNA Demethylation in a Distributive Fashion. *Nucleic Acids Research*, **37**, 4264-4274. http://dx.doi.org/10.1093/nar/gkp390

[12] Denver, D.R., Swenson, S.L. and Lynch, M. (2003) An Evolutionary Analysis of the Helix-Hairpin Helix Superfamily of DNA Repair Glycosylases. *Molecular Biology and Evolution*, **20**, 1603-1611.

[13] Ito, S., Shen, L., Dai, Q., Wu, S.C., Collins, L.B., Swenberg, J.A., He, C. and Zhang, Y. (2011) Tet Proteins Can Convert 5-Methylcytosine to 5-Formylcytosine and 5-Carboxylcytosine. *Science*, **333**, 1300-1303. http://dx.doi.org/10.1126/science.1210597

[14] He, Y.-F., Li, B.-Z., Li, Z., Liu, P., Wang, Y., Tang, Q., Ding, J., Jia, Y., Chen, Z., Li, L., *et al*. (2011) Tet-Mediated Formation of 5-Carboxylcytosine and Its Excision by TDG in Mammalian DNA. *Science*, **333**, 1303-1307. http://dx.doi.org/10.1126/science.1210944

[15] Maiti, A. and Drohat, A.C. (2011) Thymine DNA Glycosylase Can Rapidly Excise 5-Formylcytosine and 5-Carboxylcytosine: Potential Implications for Active Demethylation of CpG Sites. *Journal of Biological Chemistry*, **286**, 35334-35338. http://dx.doi.org/10.1074/jbc.C111.284620

[16] Iyer, L.M., Tahiliani, M., Rao, A. and Aravind, L. (2009) Prediction of Novel Families of Enzymes Involved in Oxidative and Other Complex Modifications of Bases in Nucleic Acids. *Cell Cycle*, **8**, 1698-1710. http://dx.doi.org/10.4161/cc.8.11.8580

[17] Erdmann, R.M., Souza, A.L., Clish, C.B. and Gehring, M. (2015) 5-Hydroxymethylcytosine Is Not Present in Appreciable Quantities in Arabidopsis DNA. *G3*: *Genes*, *Genomes*, *Genetics*, **5**, 1-8.

[18] Hellens, R., Edwards, E.A., Leyland, N., Bean, S. and Mullineaux, P. (2000) pGreen: A Versatile and Flexible Binary Ti Vector for Agrobacterium-Mediated Plant Transformation. *Plant Molecular Biology*, **42**, 819-832. http://dx.doi.org/10.1023/A:1006496308160

[19] Shen, W.J. and Forde, B.G. (1989) Efficient Transformation of *Agrobacterium* spp. by High Voltage Electroporation. *Nucleic Acids Research*, **17**, 8385. http://dx.doi.org/10.1093/nar/17.20.8385

[20] Penterman, J., Zilberman, D., Huh, J.H., Ballinger, T., Henikoff, S. and Fischer, R.L. (2007) DNA Demethylation in the *Arabidopsis* Genome. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 6752-6757. http://dx.doi.org/10.1073/pnas.0701861104

[21] Vejlupkova, Z. and Fowler, J. (2003) Maize DNA Preps for Undergraduate Students: A Robust Method for PCR Genotyping. *MNL*, **77**, 24-25.

[22] Booth, M.J., Ost, T.W.B., Beraldi, D., Bell, N.M., Branco, M.R., Reik, W. and Balasubramanian, S. (2013) Oxidative Bisulfite Sequencing of 5-Methylcytosine and 5-Hydroxymethylcytosine. *Nature Protocols*, **8**, 1841-1851. http://dx.doi.org/10.1038/nprot.2013.115

[23] Booth, M.J., Marsico, G., Bachman, M., Beraldi, D. and Balasubramanian, S. (2014) Quantitative Sequencing of 5-Formylcytosine in DNA at Single-Base Resolution. *Nature Chemistry*, **6**, 435-440. http://dx.doi.org/10.1038/nchem.1893

[24] Guo, J.J.U., Su, Y.J., Zhong, C., Ming, G.-L. and Song, H.J. (2011) Hydroxylation of 5Methylcytosine by TET1 Promotes Active DNA Demethylation in the Adult Brain. *Cell*, **145**, 423-434. http://dx.doi.org/10.1016/j.cell.2011.03.022

[25] Chen, C.-C., Wang, K.-Y. and Shen, C.-K.J. (2012) The Mammalian *de Novo* DNA Methyltransferases DNMT3A and DNMT3B Are Also DNA 5-Hydroxymethylcytosine Dehydroxymethylases. *Journal of Biological Chemistry*, **287**, 33116-33121. http://dx.doi.org/10.1074/jbc.C112.406975

[26] Mirouze, M. and Paszkowski, J. (2011) Epigenetic Contribution to Stress Adaptation in Plants. *Current Opinion in Plant Biology*, **14**, 267-274. http://dx.doi.org/10.1016/j.pbi.2011.03.004

[27] Jang, H., Shin, H., Eichman, B.F. and Huh, J.H. (2014) Excision of 5-Hydroxymethylcytosine by DEMETER Family

DNA Glycosylases. *Biochemical and Biophysical Research Communications*, **446**, 1067-1072. http://dx.doi.org/10.1016/j.bbrc.2014.03.060

[28] Brooks, S.C., Fischer, R.L., Huh, J.H. and Eichman, B.F. (2014) 5-Methylcytosine Recognition by Arabidopsis thaliana DNA Glycosylases DEMETER and DML3. *Biochemistry*, **53**, 2525-2532. http://dx.doi.org/10.1021/bi5002294

[29] Hong, S., Hashimoto, H., Kow, Y.W., Zhang, X. and Cheng, X. (2014) The Carboxy-Terminal Domain of ROS1 Is Essential for 5-Methylcytosine DNA Glycosylase Activity. *Journal of Molecular Biology*, **426**, 3703-3712. http://dx.doi.org/10.1016/j.jmb.2014.09.010

[30] Valinluck, V. and Sowers, L.C. (2007) Endogenous Cytosine Damage Products Alter the Site Selectivity of Human DNA Maintenance Methyltransferase DNMT1. *Cancer Research*, **67**, 946-950. http://dx.doi.org/10.1158/0008-5472.CAN-06-3123

[31] Xu, G.-L. and Wong, J.M. (2015) Oxidative DNA Demethylation Mediated by Tet Enzymes. *National Science Review*, **2**, 318-328. http://dx.doi.org/10.1093/nsr/nwv029

[32] Spruijt, C.G., Gnerlich, F., Smits, A.H., Pfaffeneder, T., Jansen, P.W.T.C., Bauer, C., Münzel, M., Wagner, M., Müller, M., Khan, F., *et al.* (2013) Dynamic Readers for 5-(Hydroxy)Methylcytosine and Its Oxidized Derivatives. *Cell*, **152**, 1146-1159. http://dx.doi.org/10.1016/j.cell.2013.02.004

[33] Ono, A., Yamaguchi, K., Fukada-Tanaka, S., Terada, R., Mitsui, T. and Iida, S. (2012) A Null Mutation of *ROS1a* for DNA Demethylation in Rice Is Not Transmittable to Progeny. *The Plant Journal*, **71**, 564-574. http://dx.doi.org/10.1111/j.1365-313X.2012.05009.x

[34] Liu, R., How-Kit, A., Stammitti, L., Teyssier, E., Rolin, D., Mortain-Bertrand, A., Halle, S., Liu, M., Kong, J., Wu, C., *et al.* (2015) A DEMETER-Like DNA Demethylase Governs Tomato Fruit Ripening. *Proceedings of the National Academy of Sciences of the United States of America*, **112**, 10804-10809. http://dx.doi.org/10.1073/pnas.1503362112

[35] Sander, J.D. and Joung, J.K. (2014) CRISPR-Cas Systems for Editing, Regulating and Targeting Genomes. *Nature Biotechnology*, **32**, 347-355. http://dx.doi.org/10.1038/nbt.2842