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Activation of Tag1 transposable elements in *Arabidopsis* dedifferentiating cells and their regulation by CHROMOMETHYLASE 3-mediated CHG methylation

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ABSTRACT

Dedifferentiation, that is, the acquisition of stem cell-like state, commonly induced by stress (*e.g.*, protoplasting), is characterized by open chromatin conformation, a chromatin state that could lead to activation of transposable elements (TEs). Here, we studied the activation of the *Arabidopsis* class II TE Tag1, in which two copies, situated close to each other (near genes) on chromosome 1 are found in Landsberg erecta (Ler) but not in Columbia (Col). We first transformed protoplasts with a construct in which a truncated Tag1 (ΔTag1 non-autonomous) blocks the expression of a reporter gene AtMBD5-GFP and found a relatively high ectopic excision of ΔTag1 accompanied by expression of AtMBD5-GFP in protoplasts derived from Ler compared to Col; further increase was observed in *ddm1* (*decrease in DNA methylation1*) protoplasts (Ler background). Ectopic excision was associated with transcription of the endogenous Tag1 and changes in histone H3 methylation at the promoter region. Focusing on the endogenous Tag1 elements we found low level of excision in Ler protoplasts, which was slightly and strongly enhanced in *ddm1* and *cmt3* (*chromomethylase3*) protoplasts, respectively, concomitantly with reduction in Tag1 gene body (GB) CHG methylation and increased Tag1 transcription; strong activation of Tag1 was also observed in *cmt3* leaves. Notably, in *cmt3*, but not in *ddm1*, Tag1 elements were excised out from their original sites and transposed elsewhere in the genome. Our results suggest that dedifferentiation is associated with Tag1 activation and that CMT3 rather than DDM1 plays a central role in restraining Tag1 activation *via* inducing GB CHG methylation.

1. Introduction

Dedifferentiation signifies the capability of cells to revert from a given differentiated state into a stem cell-like state that confers pluripotency – a process preceding trans-/re-differentiation, reentry into the cell cycle, and even the commitment for cell death [1]. In plants, dedifferentiation characterizes the transition of differentiated leaf cells to protoplasts (plant cells devoid of cell walls) [2,3]. Accordingly, protoplasts acquire stem cell-like state with open chromatin conformation [3,4] - a described feature of animal stem cells [5] as well as of plant meristematic cells [6] – and have the capability to differentiate into various cell types, depending on the type of stimulus applied [3,7–9].

The process of cellular dedifferentiation has drawn attention in recent years owing to its role in switching cell fate *via* the formation of pluripotent stem cells. This is exemplified in applications such as somatic cell nuclear transfer (SCNT) or induced pluripotent stem cells (iPSCs) [10,11]. Generally, however, the success of these applications is very low due to genetic variation/genomic instability occurring in cells during reprogramming and time in culture, at least partly *via* activation and transposition of retroelements [12,13]. Similarly, asexual regeneration of plants by means of tissue culture often display phenotypic perturbations, commonly known as somaclonal variation, which is induced *de novo*, at least partly, by genetic variation driven by DNA transposition [14]. Barbra McClintock [15] has recognized the dramatic effect that stress (such as tissue culturing and pathogen infection) might impose on the genome predicting that aberrant genome responses to stress, bringing about genetic variation, are likely to be induced by mobilization of transposable elements (TEs).

Activation of TEs was extensively studied in plants following exposure to various biotic and abiotic stresses, including protoplasting, tissue culturing, heat and pathogen infection [16–18]. However, most studies related to transposon activation following exposure to stress focused on the class I LTR retrotransposons [16]. Accordingly, protoplasting as well as exposure to multiple biotic and abiotic stresses were shown to

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enhance the transcription and transposition of Tnt1 retroelement in tobacco and in heterologous systems including *Arabidopsis*, tomato and alfalfa [16,19]. Also, Tto1 retroelement was activated in tobacco protoplasts, which was often accompanied by increase in copy number in established cell lines as well as in plants regenerated from tissue culture [20]. However, activation and excision of endogenous class II transposable elements (TEs) in dedifferentiating protoplasts and following exposure to stress is limited. Activation of class II TEs and transposition takes place by the cut-and-paste mechanism, which involves the activities of multiple factors that cut the TE at its flanking inverted repeats followed by rejoining of the broken ends and reinsertion of the TE element elsewhere in the genome. The maize Ac (autonomous)/Ds (non-autonomous) transposons are the prototype of the class II transposons of the hAT (hobo-Ac-Tam3) superfamily, first discovered by McClintock [21], whose members were identified in a variety of organisms ranging from plants to animals [22,23]. The maize Ac/Ds transposons were found active in many plant species and are widely used as a tool in functional genomics [24].

Generally, silencing of TEs is mediated epigenetically by complementary mechanisms that include DNA methylation and histone modification often mediated by small RNA [25,26]. Accordingly, activation of TEs was reported in plants carrying mutations in genes involved in DNA and histone methylation. TEs are methylated and silenced by three methylation contexts/pathways, CG, CHG, and CHH (where H = A, T or C). Mutation in the *DDM1* (*DECREASE IN DNA METHYLATION1*) gene, encoding a SWI/SNF chromatin remodeling factor, and in *MET1* (*METHYLTRANSFERASE1*) gene, encoding for a DNA methyltransferase maintaining CpG methylation that significantly reduce cytosine methylation [27,28] were reported to strongly activate the expression and mobilization of TEs [29–31]. Genome-wide DNA methylome analysis of *ddm1* mutant revealed that DDM1 facilitates asymmetric cytosine methylation of TEs, which is mediated by CHROMOMETHYLASE2 (CMT2) independently of small RNAs [32]. It has been suggested that DDM1 and RdDM (RNA-dependent DNA Methylation) pathways are responsible for almost all transposon methylation and silencing [32]. Also, mutation of the CMT3 gene that affects methylation almost entirely in CHG context [33,34], led to activation of TEs but to a much lesser extent compared to mutation of DDM1 [35,36]. CMT3 was found to mediate methylation in the bodies of long TEs enriched with H3K9me2 to which CMT3 binds. In contrast, DRM1/2 (DOMAIN REARRANGED METHYLASES1/2) mediate *de novo* methylation *via* RdDM pathway, and are responsible for methylation of TE edges as well as small TEs located proximal to promoters of genes; mutation of *DRM1/2* genes led to a relatively low level expression of TEs [32,35].

The *Arabidopsis* Tag1 is a low copy number, autonomous transposable element of the *hAT* (*hobo, Ac, Tam3*) superfamily found in *Arabidopsis* Landsberg erecta (Ler) but not in the Columbia (Col) ecotype [37]. It was first identified in a screen for Ac element-carrying *Arabidopsis* lines resistant to chlorate as a result of Tag1 integration into the *CHL1* (*NTR1*) gene, whose product required for chlorate and nitrate uptake [37]. The *Arabidopsis* Ler ecotype possesses two Tag1 elements designated Tag1-2 and Tag1-3 [38] located close to each other on chromosome 1. These elements were previously shown to undergo somatic excision in *Arabidopsis* and tobacco plants [37,38]. The transcriptional activity of Tag1 promoter-GUS fusion construct was examined in transgenic plants demonstrating that Tag1 expression prevails in the reproductive organs of flower buds leading to the hypothesis that the intrinsic activity of Tag1 is restricted to germinal excision [39].

We wanted to test the assumption that activation of endogenous Tag1 transposons can occur in somatic leaf cells following protoplasting that brings about dedifferentiation and acquisition of open chromatin conformation [1]. In addition, we wanted to gain insight into the epigenetic mechanisms underlying their regulation. To this end, we generated a construct to evaluate the extent of Tag1 activation upon protoplasting. Accordingly, the expression of a reporter gene AtMBD5-GFP is blocked by ΔTag1 – a truncated element [38] carrying a deletion in the transposase gene. This deletion renders it inactive (non-autonomous), yet containing all the necessary information for excision and transposition *in trans* [38]. We showed a high level of ectopic excision in protoplasts derived from Ler compared to protoplasts derived from Columbia ecotype. Further increase in ectopic excision events was observed in protoplasts derived from *ddm1* mutant (Ler background). Further study of endogenous Tag1 elements showed low level of excision followed by canonical end joining in wild type protoplasts, which was significantly enhanced in *ddm1* and particularly in *cmt3* mutant concomitantly with increased Tag1 transcription. In *cmt3* mutant, but not in *ddm1*, Tag1 elements were excised out from their original sites and transposed elsewhere in the genome. Our data highlighted the significance of CHG methylation mediated by CMT3 in silencing of class II low copy number Tag1 element.

2. Materials and methods

2.1. Plant material

All *Arabidopsis* lines [wild type Col and Ler, mutants *ddm1* (Ler background CSHL- GT24941), *cmt3* (provided by D. Autran, University of Montpellier, Montpellier, France) were grown in a controlled growth room under long day photoperiod (16 h light and 8 h dark, light intensity 200 mmol photons m-2 s-1) at 22 °C ± 2 and 70% humidity.

2.2. Preparation of ΔTag1 construct

The ΔTag1 was amplified by PCR, using as a template GUS-ΔTag1 construct described previously (kindly provided by N.M. Crawford) [38] using Tag1IR-FR as forward and reverse primer (all primers used are given in supplemental Table S1) in PCR reactions under the following conditions: 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. The PCR product was cloned into a pGEM-T Easy vector. This plasmid was digested with *Bam*HI and the ΔTag1 transposon was cloned into the *Bgl*II site downstream from the 35S promoter of pUC19-35S-MBD5-GFP [40] to generate pUC19-35S-ΔTag1-MBD5-GFP. pUC19-35S-MBD5-RFP was previously described [41].

2.3. Protoplasts preparation and transient transformation

Transient expression in protoplasts was performed essentially as described [42]. *Arabidopsis* rosette leaves were incubated in a cell wall degrading solution containing 1–1.5% cellulase, 0.3–0.5% macerozyme, 0.4 M mannitol, 20 mM KCl, 20 mM MES, 10 mM $CaCl_2$ and 0.1% BSA, placed in a vacuum for 20 min, and then gently shaken for 90–120 min at 50 rpm. The protoplasts were then filtered through a 180 μm mesh, diluted with 1 volume of W5 (150 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2 mM MES) and pelleted by centrifugation (Room temperature, 2 min at 300 ×*g*). The protoplasts were re-suspended in W5 solution and incubated for 30 min on ice, before being centrifuged again and re-suspended in 100 μl of MMg solution containing, 0.4 M mannitol and 15 mM $MgCl_2$. Plasmid DNA (5–20 μg) was added to protoplasts together with equal volume of 40% PEG solution (in 0.2 M mannitol and 0.1 M $CaCl_2$) and the mixture was incubated for 30 min. Two volumes of W5 were added to each sample, centrifuged for 2 min, re-suspended in 1 ml of W5 and then incubated at room temperature for 24 h and inspected under a confocal microscope (Zeiss LSM 510 Meta). To count the number of cells with GFP or RFP fluorescence, a 200 μm area (of the transformed protoplasts) was selected and number of cells with RFP (pUC19-35S-MBD5-RFP) or GFP (pUC19-35S-ΔTAG-MBD5-GFP) signals was counted manually using a mechanical tally counter. The relative ratio between GFP-positive cells (indicative for excision) and RFP-positive cells (indicative for transformed cells) was then determined using MS-excel software. Image processing and