Running title:

Tam3 transposition and methylation

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DNA methylation is not necessary for the inactivation of the Tam3 transposon at non-permissive temperature in *Antirrhinum*.

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Summary

It has been proposed that DNA methylation plays an important role in the inactivation of transposons. This view stems from a comparison of the degree of methylation of transposons in the active and inactive state. However, direct evidence for the degree of methylation required for the suppression of transposition has not been reported. Transposon Tam3 in *Antirrhinum majus* undergoes somatic reversal of its transposition activity, which is tightly controlled by temperature: low temperature around 15°C permits transposition, high temperatures around 25°C strongly inhibits it. Our previous study had shown that the methylation state of the Tam3 end regions is negatively correlated with the Tam3 transposition frequency. The results of the present study reveal that the inactive state of Tam3 copies at high temperature is unlikely to be directly coupled to the methylation state. Treatment with methylation inhibitors (5-azacytidine or 5-azacytidine+ethionine) does not affect Tam3 excision frequency in calli derived from *Antirrhinum* hypocotyls. The results suggest that methylation is not essential for the suppression of Tam3 transposition at high temperature, but rather that some other mechanism(s) involved in the control of Tam3 transposition may be obscured by methylation.

Key Words

Antirrhinum majus, DNA methylation, Low-temperature-dependent transposition, Methylation inhibitors, Transposon Tam3,

Abbreviations

AzaC, 5-azacytidine; COBRA, combined bisulfite restriction assay; Ethi, ethionine; LTDT, low-temperature-dependent transposition; TE, transposable element.

Introduction

DNA methylation is believed to be a key factor in the repression of the transposition of transposable elements (TEs) (Yoder et al., 1997; Martienssen, 1998), but the degree of methylation that is necessary for the inhibition is not known. Few studies have shown a direct relationship between the degree of methylation and transpositional activity based on a comparison of individual copies. Therefore, it is possible that other factors involved in the repression of transpositional activity may be obscured by methylation.

Tam3 is a cut-and-paste-type transposon of *Antirrhinum majus* acting through the unique mechanism of low-temperature-dependent transposition (LTDT) (Harrison and Fincham, 1964; Carpenter et al., 1987). In all genotypes that have been examined, the Tam3 copies were always active at around 15°C and stable at around 25°C (Kitamura et al., 2001). Low temperature activates Tam3 transposition in a manner dependent on the chromosomal position, and this effect is also related to the degree of methylation of the copy's ends (Kitamura et al., 2001). In contrast, at high temperature, the degree of methylation of the Tam3 ends increases (Hashida et al., 2003). Therefore, the methylation state of the Tam3 ends changes temperature-dependingly and is negatively correlated with transposition activity (Hashida et al., 2003). These results led us to assume that methylation causes the suppression of Tam3 transposition. However, even in the stable condition of Tam3 in plants grown at high temperature, the transposase (TPase) gene was transcribed and *in vivo* TPase activity was detected at a level similar to the instable condition of Tam3 (Hashida et al., 2003). Moreover, some copies are not fully methylated at the ends in the stable condition of Tam3 (Hashida et al., 2003). These results raise the question of whether methylation is a direct cause of LTDT or merely an event that occurs

simultaneously with LTDT. To investigate this question we analyzed Tam3 transposition activity and methylation level in *Antirrhinum* calli treated with DNA demethylation agents.

Materials and methods

Callus induction and treatment with methylation inhibitors

We used the HAM5 line of *Antirrhinum majus* L. (kindly provided by Dr. Cathie Martin, John Innes Center, Norwich, UK). HAM5, derived from the *nivea*$^{recurrens:Tam3}$/*stabiliser*- line, was raised either at 25°C or at 15°C in growth-chambers. The HAM5 seeds were sterilized with 70% ethanol for 1 min and 0.6% sodium hypochlorite for 15 min, followed by several rinses in sterilized water. The seeds were germinated on 1% agar for 2 weeks and the hypocotyls excised and transferred to Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 2 mg/L 2,4-D for 4 weeks. The resultant callus was divided into several pieces and transferred to the same medium but with methylation inhibitors 5-azacytidine (azaC at 200 μM) and/or ethionine (Ethi at 300 μM). After 10 weeks, DNA was isolated from the calli as described by Kishima et al. (1997). DNA gel blot and PCR analyses were carried out as described by Kishima et al. (1999) and Kitamura et al. (2001), respectively.

Combined bisulfite restriction assay (COBRA)

To assay the effect of the methylation inhibitors, we performed the combined bisulfite restriction assay (COBRA) (Xiong and Laird, 1997). Callus DNA was subjected to sodium bisulfite modification treatment as described by Hashida et al. (2003). Subsequently, PCR amplification of an internal Tam3 sequence (nucleotide positions 647 to 1493: accession no. AB013982) was

performed with the primer combination [ATAATATTTTTTAATTATGGTAAA] + [AAACAACTCATTTTAAACTATAAA]. This region contains three potential *Dra*I sites (TTTAAA) after bisulfite treatment by which unmethylated cytosine is changed to uracil as indicated in Fig. 1B. Thus, the fact that a PCR product becomes susceptible to *Dra*I indicates the presence of unmethylated cytosines in the corresponding genomic DNA.

Quantitative real-time PCR assay

For the quantitative measurement of Tam3 excision in calli, real-time PCR was performed using a Light-Cycler (Roche) with SYBR Green I (Roche) (Wickert et al., 2002), which was covalently bound to double-stranded DNA according to the manufacturer's instructions (Roche). For the construction of standard curves, 10-fold serial dilutions (from 10^{-3} to 10^{-7}) of *Antirrhinum* genomic DNA were made in water as the template to amplify the *pallida* coding region, a Tam3-unlinked site. A straight line was obtained in every trial from the cycle threshold (C_T), which is the point where the concentration of the amplified DNA reaches a plateau. The C_T of each sample examined was determined at a certain F value during the growth period of the S-shaped curve. C_T was substituted by relative concentration using the straight line obtained from the amplification of the *pallida* coding region in the *Antirrhinum* genomic DNA. The PCR primers were designed in the Tam3 flanking sequences at the *nivea* locus as follows: [CCTATTGGGCAAAATTAGGTACC] + [AACCTCCTCAACAGTCACCATTT]. In order to normalize the total amount of DNA from each callus, we amplified the *pallida* coding region with the primer combination [TGCGATTGACACTTGCCGC] + [CGCATTTTCTTGTCCCTTGGC]. The PCR reaction mixture contained 4 mM $MgCl_2$, 20 pmol of each of the two PCR primers, 1-10 ng of template DNA, and 2.5 μL of SYBR Green I in a

final volume of 20 μL. The PCR mixtures were subjected to denaturation at 95°C for 0 sec, annealing at 60°C and extension at 72°C for 5 sec for a total of 40 cycles.

Results and discussion

If LTDT is controlled by methylation, a reduced level of DNA methylation may activate the transposition of Tam3 and abolish the LTDT mechanism. To test this hypothesis, we attempted to induce hypomethylation of the *Antirrhinum* genome using DNA demethylating agents, 5-azacytidine (azaC) and ethionine (Ethi). Kovarik et al. (1994) reported that the methylation inhibitors azaC and Ethi target different cytosines: the former affects methylation at CG dinucleotides and the latter at CNG trinucleotides. Prior to treatment of the *Antirrhinum* calli with azaC (200 μM) and Ethi (300 μM), we observed in preliminary experiments that these agents caused several abnormalities in *Antirrhinum* seedlings such as early senescence, lack of apical dominance, multi-inflorescence branches at the first node and semi-viability (data not shown). Calli cultured in media containing azaC or azaC+Ethi showed reduced growth. To examine the methylation state, genomic DNA gel blot analysis was performed using the *Hpa*II/*Msp*I restriction enzyme assay with the 25S-17S rDNA region as probe (Ellis et al.,1990). A comparison of the gel blot patterns revealed that the *Hap*II digests of the azaC treatment samples were more strongly hypomethylated than the *Msp*I digest of the control sample (Fig. 1A), indicating that the azaC treatments effectively converted the entire genomic DNAs of both the seedlings and calli into hypomethylation states. In calli grown without azaC, the Tam3 internal sequence was strongly methylated at 25°C, and this methylation level slightly declined at 15°C (Fig. 1B). The methylation change in the internal regions is less than that in the end

regions (Hashida et al., 2003). On the other hand, azaC treatment enhanced the reduction of the methylation level (Fig. 1B). We also tested the methylation levels of calli grown at 15°C and 25°C by means of the combined bisulfite restriction assay (COBRA) (Xiong and Laird, 1997) (Fig. 1C). In an internal sequence of Tam3 (between nucleotide positions 700 and 1260), three possible sites may become *Dra*I recognition sites (TTTAAA) after bisulfite treatment due to modification of C to U in cases where cytosine was not methylated (Fig. 1C). We designed primers that bracketed these three possible *Dra*I recognition sites, and performed PCR amplification after bisulfite treatment. The 820-bp PCR products were obtained for all the experimental plots. The PCR products from the calli cultured in media containing azaC or azaC+Ethi were susceptible to digestion with *Dra*I, indicating the presence of unmethylated cytosine(s) in the three sites. The digestion patterns showed that the treatment with azaC+Ethi effectively reduced the methylation level. These results suggest that the methylation inhibitors markedly impaired methylation in the entire Tam3 sequences.

To accurately quantify the Tam3 transposition activity in each type of callus described above, the degree of amplification of the excision products of Tam3 located in the promoter region of the *nivea* locus was measured by quantitative real-time PCR analysis. When Tam3 is excised, the two Tam3 flanking sequences become joined to each other. Thus, Tam3 excision can be measured by PCR amplification with primers located in the two Tam3 flanking sequences. For this analysis, we performed the Light-Cycler (Roche)-assisted approach using SYBR-Green as described in Materials and methods (Wickert et al., 2002). To normalize the total DNA amount in individual samples, the *pallida* locus of the HAM5 line was used, since this single-copy genomic region is not linked with Tam3 in HAM5 (Fig. 2). The Tam3 excision activity at *nivea*

was clearly different between the growth temperatures 15°C and 25°C. Treatment of the calli with azaC or azaC+Ethi did not have any significant effect on the Tam3 excision activity at 25°C (Fig. 2). These results suggest that LTDT of Tam3 is not dependent on the methylation level and that the change in methylation that occurs in parallel with the temperature change is not a direct cause of the suppression of the Tam3 transposition. On the other hand, the methylation inhibitors promoted a slightly but significantly higher excision frequency of Tam3 at 15°C, suggesting that DNA methylation inhibits the TPase activity in the active condition as described by Kitamura et al. (2001).

A number of phenomena associated with methylation have been reported to be influenced by methylation inhibitors. For instance, treatment of non-vernalized *Arabidopsis* plants with azaC induced the same effect on the flowering time as vernalization, which is required for flowering (Burn et al., 1993). It was found that vernalization promotes a reduced level of methylation in the *Arabidopsis* genome. In a phenomenon known as reduced expression of endogenous duplications (Reed), azaC restored anthocyanin gene expression suppressed by methylation due to duplication of the endogenous gene (Ronchi et al. 1995). The fact that Tam3 inactivation at high temperature was not overcome by the methylation inhibitors implies that there is no direct relationships between LTDT and methylation. Taken together, the above results lead us to conclude that methylation is not a determinant of the complete arrest of Tam3 transposition at high temperature. Therefore, LTDT of Tam3 is certainly driven by other factor(s) obscured by methylation.

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Figure legends

Figure 1: Decrease of DNA methylation in HAM5 calli through methylation inhibitors 5-azacytidine (azaC) and ethionine (Ethi).

A: DNA gel blot analysis showing the reduction of methylation of the 25S-17S rDNA sequence in seedlings and calli grown on medium containing azaC. We prepared 48 calli derived from

HAM5 hypocotyl for each experimental plot. The calli were grown at 25°C or 15°C, and they were cultured on MS medium with 200 μM azaC or without azaC. The DNA samples were extracted from individual seedlings and calli grown at 25°C (H) or 15°C (L). Each blot had a combined DNA with 6 individual calli. *Msp*I digestion was used as a control, which represents the hypomethylation state.

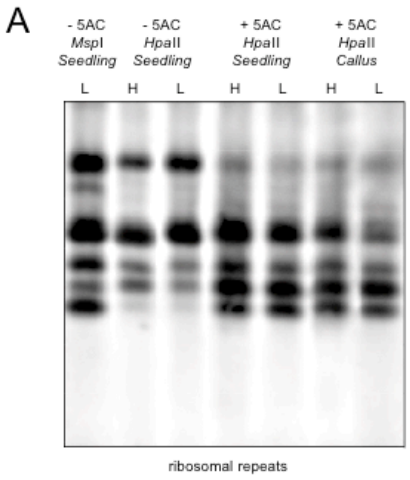
B: DNA gel blot analysis showing the reduction of the methylation level of an internal Tam3 sequence in calli grown on medium containing azaC. Genomic DNA isolated from individual calli was digested with *Eco*RV (methylation-insensitive enzyme) and *Hpa*II (methylation-sensitive enzyme). There are two *Eco*RV sites (nucleotide positions at 814 and 2053: accession no. AB013982) and seven *Hpa*II sites in Tam3. The probe was located within this internal Tam3 sequence. Therefore, in the case where Tam3 was fully methylated (FM), a band appeared with a size corresponding to the 1.2-kb *Eco*RV fragment of Tam3. Bands smaller than 1.2 kb indicate a partially methylated (PM) or unmethylated (0.52 kb) (UM) state of Tam3.

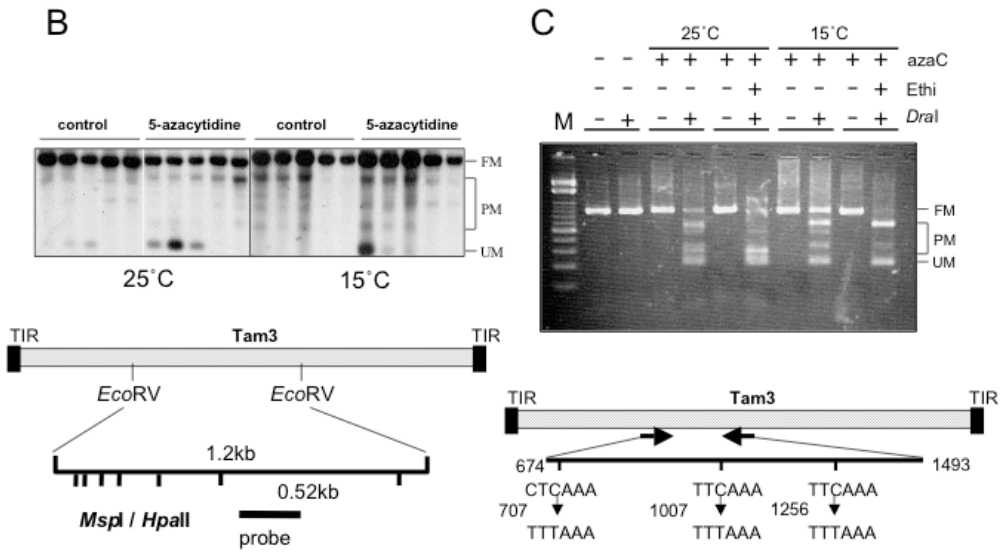
C: Combined bisulfite restriction assay (COBRA) also confirming the reduction of the methylation level of the internal Tam3 sequence in calli grown on medium containing 200 μM azaC or 200 μM azaC + 300 μM Ethi. After bisulfite treatment of genomic DNAs combined with six calli for each plot, the Tam3 internal sequence was amplified by PCR. Marks (+ or -) above the panel indicate the presence or absence of azaC or azaC+Ethi in the medium and *Dra*I digestion. The digestion of the PCR product by *Dra*I indicates that the genomic DNA contained an unmethylated sequence.

Figure 2: Tam3 excision activity at *nivea* and *pallida* loci.

Each experimental plot consists of at least 48 calli. The calli were grown at 25°C or 15°C, and

were cultured on MS medium with 200 μM azaC or 200 μM azaC + 300 μM Ethi or without inhibitors. The relative activity was measured by real-time PCR using SYBR Green I as described in Materials and methods. The percentages indicate relative C_T values for *nivea* (PCR amplification of Tam3 excision product) versus *pallida* (Tam3-unlinked region).




B
control
5-azacytidine
control
5-azacytidine
FM
PM
UM
25˚C
15˚C
TIR
Tam3
TIR
EcoRV
EcoRV
1.2kb
0.52kb
MspI / HpaII
probe
C
25˚C
15˚C
azaC
Ethi
M
DraI
FM
PM
UM
TIR
Tam3
TIR
674
1493
CTCAAA
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TTCAAA
707
1007
1256
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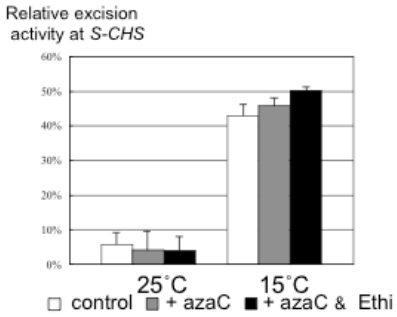
Relative excision
activity at S-CHS
60%
50%
40%
30%
20%
10%
0%
25˚C
15˚C
control
+ azaC
+ azaC & Ethi