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SHORT COMMUNICATION

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.

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

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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 *A. 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 647–1493: 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