The Temperature-Dependent Change in Methylation of the Antirrhinum Transposon Tam3 Is Controlled by the Activity of Its Transposase

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The Antirrhinum majus transposon Tam3 undergoes low temperature–dependent transposition (LTDT). Growth at 15°C permits transposition, whereas growth at 25°C strongly suppresses it. The degree of Tam3 DNA methylation is altered somatically and positively correlated with growth temperature, an exceptional epigenetic system in plants. Using a Tam3-inactive line, we show that methylation change depends on Tam3 activity. Random binding site selection analysis and electrophoretic mobility shift assays revealed that the Tam3 transposase (TPase) binds to the major repeat in the subterminal regions of Tam3, the site showing the biggest temperature-dependent change in methylation state. Methylcytosines in the motif impair the binding ability of the TPase. Proteins in a nuclear extract from plants grown at 15°C but not 25°C bind to this motif in Tam3. The decrease in Tam3 DNA methylation at low temperature also requires cell division. Thus, TPase binding to Tam3 occurs only during growth at low temperature and immediately after DNA replication, resulting in a Tam3-specific decrease in methylation of transposon DNA. Consequently, the Tam3 methylation level in LTDTC is regulated by Tam3 activity, which is dependent on the ability of its TPase to bind DNA and affected by growth temperature. Thus, the methylation/demethylation of Tam3 is the consequence, not the cause, of LTDT.

INTRODUCTION

A large proportion of many eukaryote genomes is variably methylated during the lifetime of the organism. DNA methylation is an epigenetic mark mediated by the addition of a methyl group to cytosine in DNA, but methyl groups can also be removed from DNA by DNA demethylation. The erasure of epigenetic information, acquired initially through DNA methylation, plays a crucial role in renewing the environment of the genome. In mammals, genome reprogramming in early embryos and germ cells is initiated by a genome-wide DNA demethylation that is an intrinsic part of normal development (Reik et al., 2001). In contrast with methylation in mammalian genomes, methylation in plant genomes is usually inherited over several generations (Habu et al., 2001; Kakutani, 2002). However, several phenomena that are linked to decreases in DNA methylation have been reported, including tobacco (Nicotiana tabacum) pollen development (Oakeley et al., 1997) and vernalization in Arabidopsis thaliana (Finnegan et al., 1998) and winter wheat (Triticum aestivum) (Sherman and Talbert, 2002). A gene-specific decrease in methylation was also demonstrated in maize for a 1.8-kb fragment containing part of a retrotransposon-like sequence in chilled root tissue (Steward et al., 2002). The FWA locus in Arabidopsis endosperm shows an imprinting phenomenon in which maternal origin–specific demethylation occurs (Kinoshita et al., 2004). These phenomena illustrate the divergent biological consequences of demethylation in plant genomes. Furthermore, analyses of Arabidopsis mutants in which imprinting and gene silencing were affected led to the identification of Demeter (Choi et al., 2002) and Ros1 (Gong et al., 2002), which are genes that may indirectly lead to demethylation in the plant genome as a result of DNA glycosylase activity targeting methylcytosines. The existence of these genes emphasizes the functional importance of demethylation in plant genomes. Despite their importance, the processes involved in DNA demethylation in plants remain unclear.

DNA methylation seems to be a key factor in the repression of the transposition of transposable elements (TEs) (Yoder et al., 1997; Martienssen, 1998). There is abundant evidence for a link between DNA methylation and the inactive state of TEs. An increased level of DNA methylation of the promoter regions of autonomous elements such as Activator (Ac) (Schwartz and Dennis, 1986; Chomet et al., 1987), Suppressor-mutator (Spm) (Schnapp et al., 1994), and MuDR (Chandler and Walbot, 1986; Bennettzen, 1987; Lisch et al., 1995, 2002) tends to reduce the production of transposase (TPase) transcripts and the transposition.
frequency. Methylation at TPase binding sites adjacent to the terminal inverted repeats (TIRs) of Ac (Kunze and Starlinger, 1989) and Spm (Gierl et al., 1988) also inhibits TPase binding activity, which probably suppresses transposition. In Arabidopsis, hypomethylation of the genome resulting from a mutation in \textit{DECREASE IN DNA METHYLATION1} activated at least two different types of TEs, \textit{Mutator-like} elements (Singer et al., 2001) and CACTA family elements (Miura et al., 2001). These studies demonstrated that DNA methylation generally acts as a force to repress TE activity. However, few studies have examined whether there is a direct relationship between the degree of methylation and transpositional activity based on comparisons between individual transposon copies within a single genome. An exception was in the case of \textit{Spm} from maize (\textit{Zea mays}), in which the activity of individual copies was inversely correlated with the degree of methylation of the 5’ region of each element. A change of \textit{Spm} activity termed “phase change” involves demethylation that reactivates the element (Schlappi et al., 1994; Fedoroff et al., 1995). Demethylation is associated with \textit{Spm} encoded TnpA protein, which controls the methylation state of the 5’ region in the element (Bank et al., 1988). In this case, \textit{Spm} appears to be activated in its hypomethylated state, mediated by the \textit{Spm}-encoded TnpA protein (Cui and Fedoroff, 2002).

The transposon Tam3 in \textit{Antirrhinum majus} is activated at low growth temperatures of \(\sim 15^\circ\text{C}\) (permissive temperature), whereas the activity is strictly suppressed at high growth temperatures of \(\sim 25^\circ\text{C}\) (nonpermissive temperature) (Harrison and Fincham, 1964; Carpenter et al., 1987). In low temperature–dependent transposition (LTDT), there is a change in the methylation state of the Tam3 sequence that parallels Tam3 behavior: the methylation level at \(15^\circ\text{C}\) is markedly lower than that at \(25^\circ\text{C}\) (Hashida et al., 2003). The methylation state of the Tam3 sequence is reversibly altered and the temperature-dependent change can occur during the lifetime of a single plant (Hashida et al., 2003). Such a rapid change in methylation state in response to a change of temperature is a unique feature of Tam3 and provides an interesting system for analyzing the effects of changes of the methylation state, particularly the processes involved in decreasing DNA methylation.

Because the change of methylation of the Tam3 sequence appears to be correlated with its activity, we examined the properties of Tam3 TPase as a possible factor influencing DNA methylation. We found that the temperature-dependent change in methylation of the Tam3 sequence occurred only in the presence of the TPase activity, and the sites showing changes in methylation were coincident with Tam3 TPase binding sites in the subterminal repeats of the element. The temperature-dependent methylation change was correlated with the binding activity to Tam3 in nuclear extracts. Nuclear protein extracts from plants grown at low temperature included proteins that bound Tam3, but very little binding was observed with a nuclear protein extract from plants grown at high temperature. Decreases in methylation occurred only in the tissues that underwent cell division after the decrease in temperature; the methylation state was stable in old tissues even at permissive temperatures. We conclude that it is the ability of the Tam3 TPase to bind to the ends of the TE after cell division (or DNA replication) that regulates the methylation level of the element. Although methylation of the element will, in turn, influence the somatic transposition frequency caused by reduced binding by the TPase to methylated DNA, the influence of temperature on the ability of TPase to bind DNA must be the primary mechanism underpinning LTDT.

\section*{RESULTS}

\subsection*{Low Temperature–Dependent Hypomethylation Is Negated by the \textit{New Stabilizer} Allele}

We previously reported that the reversible methylation state of Tam3 paralleled the LTDT mechanism in an \textit{Antirrhinum} line with high Tam3 activity (Hashida et al., 2003). In this study, we compared the methylation state between a Tam3-inactive line (HAM3) and a Tam3-active line (HAM5) (Figure 1). Because HAM3 was derived from HAM5, the two lines are isogenic, except that HAM3 carries the semidominant allele of the \textit{New Stabilizer} (\textit{NSt}) locus, which strongly suppresses Tam3 transposition. \textit{NSt} is independent of the original Stabilizer locus that was described by Harrison and Fincham (1968) (see Supplemental Figure 1 online). These two isogenic lines harbor \(\sim 50\) copies of Tam3 in each genome (Kishima et al., 1999).

\begin{figure}[ht]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Pigmentation Phenotypes of Flowers Demonstrating LTDT and the Effect of the \textit{NSt} Locus on the Activity of the Tam3 Copy Inserted at the nivea Locus of \textit{A. majus}.}
\end{figure}

Both lines carry Tam3 inserted in the promoter of the \textit{nivea} gene that encodes chalcone synthase (Somm et al., 1985). When the TE is inserted, it greatly inhibits \textit{nivea} gene expression, giving rise to a very pale red/white phenotype in flowers. When the TE excises, wild-type gene function is restored, as indicated by the formation of sites and sectors producing dark red anthocyanin. The frequency of these sectors is a measure of the somatic excision frequency of Tam3 from this locus. At left are flowers from the line HAM5, which is homozygous for the \textit{nst} allele; at right are flowers from the HAM3 line, which are homozygous for the \textit{NSt} allele: HAM5 grown at \(21^\circ\text{C}\) \((\text{A})\), HAM5 grown at \(15^\circ\text{C}\) \((\text{B})\), HAM3 grown at \(21^\circ\text{C}\) \((\text{C})\), and HAM3 grown at \(15^\circ\text{C}\) \((\text{D})\). Comparison of \((\text{A})\) and \((\text{B})\) shows the increase in somatic transposition of Tam3 at low temperature in the form of increased frequency of somatic reversion sites at nivea. Comparison of \((\text{C})\) and \((\text{D})\) shows that the \textit{NSt} allele suppresses Tam3 transposition, an effect that cannot be reversed by growth at \(15^\circ\text{C}\).
many other TEs, Tam3 consists of nearly intact copies, and no nonautonomous copies have been identified in the *Antirrhinum* genome (Kishima et al., 1999). The uniform structure of Tam3 in the genome facilitated the analysis of the methylation state of the entire set of Tam3 copies based on genomic DNA gel blot hybridization. Methylation was analyzed using isoschizomeric restriction enzymes (*Hpa*II and *Msp*I have differential methylation sensitivity for CCGG sites). The methylation state was analyzed for NST (HAM3) and nst (HAM5) plants grown at 25 and 15°C at 2 months after the seeds were sown at 25°C. Seven *Hpa*II/*Msp*I sites within the 1.2-kb *Eco*RV fragment located in the middle of the Tam3 sequence were examined using a 360-bp probe (Figure 2A). *Msp*I digestion gave rise to several bands of different sizes as a result of the slight methylation sensitivity of the enzyme (*Msp*I is sensitive to CpNpG methylation but not to CpG methylation, whereas *Hpa*II is sensitive to methylation of both types), and the patterns of these bands were similar among the different samples examined (Figure 2B). In the *Hpa*II digests, the samples from the nst plants showed methylation changes dependent on the growth temperature, whereas the samples from the NST plants gave rise to similar patterns at the different growth temperatures and showed significant levels of the 1.2-kb *Eco*RV band, which resulted from heavy methylation at all seven *Hpa*II/*Msp*I sites (Figure 2B). This result implied that the temperature-dependent change in methylation of the TE is correlated with Tam3 transposition activity.

**Identification of Tam3 TPase Binding Sites**

We suspected that DNA binding of the Tam3 TPase might be responsible for the temperature-dependent methylation change. Identification of the Tam3 binding motif(s) within the Tam3 sequence was important to address this possibility. A method to identify *cis* elements for DNA binding proteins, namely random binding site selection (RBSS) (Martinez-Garcia et al., 1998), was applied for detection of sequences with high-affinity binding sites for the Tam3 TPase. Tam3 TPase peptides were expressed in an *Escherichia coli* expression system using a C-terminal fusion protein with glutathione S-transferase (GST). Unfortunately, we failed to obtain the entire Tam3 TPase polypeptide in the *E. coli* expression system. Consequently, we divided the putative Tam3 TPase gene into four segments and successfully purified the encoded peptides for use in the RBSS assay. When TPase55-230:GST (the partial Tam3 TPase with amino acid residues 55 to 230 fused to the GST protein) was used in the RBSS assay, DNA binding by the protein was detected. However, neither TPase227-511:GST nor TPase505-803:GST showed DNA binding activity (data not shown). The BED–zinc finger domain, which is a putative DNA binding motif found in other hAT family TPases, such as those of Ac, Tag1, and Tol2, is present at amino acid...
positions 181 to 234 in the Tam3 TPase (Aravind, 2000). Although TPase1-250:GST contains the BED–zinc finger domain, its DNA binding activity was weaker than that of TPase55-230:GST, and the TPase1-250:GST protein tended to be degraded in the E. coli lysate (data not shown).

For RBSS analysis, we prepared PCR-amplified 34-bp DNAs containing 16 random nucleotides (rsDNA) in the middle. Those PCR-amplified DNA molecules bound by the Tam3 TPase were selected using glutathione–Sepharose beads conjugated with TPase55-230:GST. The molecules were subsequently recovered by release from the beads. After nine repetitions of this DNA–protein affinity procedure, the purified DNA molecules were cloned and sequenced. The sequences obtained are listed in Figure 3A. Alignment of 24 independent sequences revealed a clear consensus. The consensus was found in the succession of six nucleotides, DGCHCG, which is the presumptive binding site for TPase55-230:GST (Figure 3B).

**Identification of Cytosines That Are Variably Methylated in Tam3**

The RBSS result showed that the DGCHCG motif is a candidate for the Tam3 TPase binding site and a possible target site for temperature-dependent changes in methylation. Sodium bisulfite sequencing analysis was performed to examine the latter possibility. In this analysis, we also addressed whether there were substantial differences in the methylated cytosine sites in Tam3 between the NSt and nst lines. The two lines are genetically identical except for the NSt locus, so they have common Tam3 insertion sites. These are at least eight Tam3 copies that excise

![Figure 3](image-url)

**Figure 3.** Sequences of Selected Tam3 TPase (TPase55-230:GST) Binding Sites Determined by RBSS Analysis.

**(A)** Random sequences of 24 independent clones are aligned, with a consensus motif shown in gray.

**(B)** Consensus sequences were determined from the frequency of bases present at each position in the oligonucleotides that corresponded to a binding site.
at different frequencies, depending on the position of the TE insertion in the nst genome (Kishima et al., 1999; Kitamura et al., 2001). Two Tam3 insertion loci, S-6 and S-99, were selected for this analysis. The excision frequencies for these copies at 15°C were estimated to be S-6:S-99 = 1:28 (Kitamura et al., 2001). A remarkable difference in methylation state was observed at the ends of the transposon, between the different copies of Tam3 (Hashida et al., 2003). In the sodium bisulfite sequencing analysis, eight clones were randomly selected from each of the 16 individual experimental plots, and the frequencies of methylcytosine were counted at 39 and 42 cytosine sites present in the 120 nucleotides of the 5’ and 3’ ends of Tam3, respectively (Figure 4). The proportions of methylcytosine as CpG, CpNpG, and asymmetric C at both ends of S-6 and S-99 were compared in the HAM3 (NST) and HAM5 (nst) lines (Figure 5). As shown by the DNA gel blot analysis, the methylation state in the NST line was not affected by the difference in growth temperature, whereas the methylation state of Tam3 in the nst line differed with growth temperature. Apparent differences in the methylation status were not observed in the NST line for either S-6 or S-99 Tam3 copies. However, the Tam3 copy at S-6 was consistently more methylated than that at S-99, and in each case, the 5’ end was preferentially methylated relative to the 3’ end of the TE (Kitamura et al., 2001).

By mapping the methylation frequency for each cytosine site at the ends of the Tam3 copies at S-6 and S-99 in the NST line (Figure 4), we attempted to deduce the motifs targeted by methylation. At most of the cytosine sites, high temperature induced more frequent methylation than low-temperature growth conditions. Figure 6 summarizes differences in the methylcytosine frequency in both Tam3 end sequences between 25 and 15°C. Several cytosine clusters showed markedly different methylation frequencies at 25 and 15°C. We focused on seven cytosine clusters. Three from the region at the 5’ end contained cytosine sites for which >50% of the total clones showed alteration of the methylation status after a temperature shift. The other four from the region at the 3’ end contained cytosine sites that exhibited >25% difference in their degree of methylation between plants grown at high versus low temperature. Interestingly, most of these cytosine clusters possessed a common consensus motif, GCHCG (H = not G). This GCHCG matches the binding motif of the Tam3 TPase identified by the RBSS analysis. In addition to the subterminal regions, cytosine sites in the TIR at the 3’ end of the TE showed considerable differences in methylation state. These results showed that a change in the methylation state of Tam3 is associated with the activity of the TE and that the change occurred predominantly around the GCHCG motifs.

The Tam3 TPase Binds to the GCHCG Motif

To verify whether the Tam3 TPase binds to the GCHCG motif, electrophoretic mobility shift assay (EMSA) analysis was performed using a 26-bp sequence from the 5’ end of Tam3 (nucleotides 79 to 104 [Tam3:79-104], which contained three of the GCHCG motifs) as a probe (Figure 7A). We used TPase55-230:GST to determine binding specificity. EMSA analysis showed that the DNA–protein complex of Tam3:79-104 and TPase55-230:GST resulted in three different bands without competitor DNA (Figure 7B). These three bands probably originated from differential binding of the TPase55-230 to each of the three GCHCG motifs within the probe sequence of Tam3:79-104 (Figure 7B). The addition of double-stranded competitor DNA for GCCGC, GCTCG, or GCACG impaired the formation of the binding complex with the probe (Figure 7B). The binding of TPase55-230:GST to the probe was not competed for by the addition of excess sequence from Tam3 (nucleotide positions 3446 to 3496) that did not contain the GCHCG motif (Figure 7B). These results confirmed that the GCHCG motif is a binding site for the Tam3 TPase.

We then performed EMSA analysis to analyze the ability of TPase to bind to the methylated GCHCG motif. As shown in Figure 7C, three different competitors that contained methylcytosine(s) instead of cytosine(s) were prepared: two hemimethylated DNAs (Hm1, GCCGC/GCGGCm; Hm2, GCMcMCmC/ CGGCG) and a fully methylated DNA (Fm, GCMcMCmcG/ CmGGGCm). Figure 7C shows that Hm1 was able to interfere with the binding of TPase55-230:GST to the probe, but Hm2 and Fm were not able to impair the binding affinity. The opposite strand of the GCHCG motif, CGDGC, was unlikely to be targeted by the Tam3 TPase, because binding of the TPase to the probe was inhibited by Hm1 but not by Hm2. These results confirmed that methylcytosine prevents the binding of the Tam3 TPase that is specifically targeted to the GCHCG motif but not to the opposite strand. This finding suggests that the temperature-dependent change in the methylation of Tam3 strongly influences the binding properties of the Tam3 TPase for its target DNA.

Binding of Extracts of Nuclear Proteins to Tam3

To investigate whether binding of the TPase in the nucleus influenced the methylation change in the Tam3 sequence or whether binding was influenced by the methylation change, we examined the binding of nuclear protein extracts to the Tam3 sequence. We conducted EMSA analysis with leaf nuclear protein extracts from nst plants grown at 15 and 25°C. As shown in Figure 8, the nuclear extract from the plants grown at 15°C clearly bound to the probe DNA of Tam3:79–104, as indicated by a low-mobility fragment, whereas the nuclear extract from the plants grown at 25°C barely showed any binding to the probe. In high-temperature-grown plants, the binding of the TPase to the Tam3 sequence was inhibited, whereas in low-temperature-grown plants, the Tam3 TPase was able to bind to the Tam3 sequence. Microscopic observation and dot immunobinding assay using the anti-histone H3 confirmed that the nuclear protein extracts were clearly enriched and distinct from total protein (see Supplemental Figure 2 online). These results strongly support the view that an increase in the binding activity of the TPase in the nucleus occurs in plants grown at low temperature and that this is followed by a decrease in the methylation level of the Tam3 sequence.

The Decrease in Methylation of Tam3 Occurs after Cell Division

Although methylation of Tam3 interfered with TPase binding, the changes in TPase binding and methylation are unlikely to
Figure 4. Mapping of Methylcytosines in the End Regions of Tam3 (120 Nucleotides) for the Two Identical Copies of Tam3 at S6 and S99.

DNA samples were isolated from the HAM3 (NST/NST) and HAM5 (nst/nst) plants grown at 15 or 25°C. Eight clones were randomly selected from each DNA sample, and sodium bisulfite sequencing was performed for the clones of the antisense strand from the 5’-end region and of the sense strand from the 3’-end region. The methylcytosines are distinguished as being in one of three contexts, CpG, CpNpG, and asymmetric C, which are indicated by diamonds, squares, and circles, respectively. The nucleotide sequences shown between the two columns identify the cytosine position, and the positions of the TIRs are indicated by red triangles at the end. The gray shows the position of the GCHCG motif, and the site of the sequence 5’-CCGG-3’ is marked as HpaII.
occur simultaneously. Because, generally, methylated motifs are not bound by TPase, TPase binding is usually considered to be the event that follows a decrease in methylation after DNA replication during cell division. In our analysis, to examine whether or not the decrease in methylation in the Tam3 sequence occurs after DNA replication, we prepared DNA samples from leaves of the nst plants (Figure 9A). The plants were grown at low temperature after they had been grown at high temperature for 2 months. The methylation state was compared between DNA samples isolated from the leaves that developed before the shift to the low temperature (old leaf) and the other leaves that had developed during the period of growth at low temperature (new leaf). First, DNA gel blot analysis with the same Tam3 probe used in Figure 2 confirmed that Tam3 copies were methylated in the plant leaves grown at high temperature for 2 months. Similarly, Tam3 copies were methylated in the genomic DNA from the old leaves, whereas Tam3 copies in the genomic DNA from the new leaves were unmethylated (Figure 9A). Tam3 transposition was also detected only in the new leaves while the plants were being grown at the low temperature (Figure 9B). The results revealed that the temperature-dependent decrease in methylation of the Tam3 sequence required cell division, implying that DNA replication is necessary for the Tam3-specific decrease in methylation. Therefore, the process of decreasing Tam3 methylation is likely to be based on the fact that Tam3 TPase binds to the GCHCG sites immediately after DNA replication and thereby prevents the maintenance of the methylated state.

**DISCUSSION**

The Methylation State of Tam3 Is Dependent on the Binding Ability of TPase

This study showed that the change in methylation of the ends of Tam3 that occurs during LTDT occurs at the GCHCG motif, which is also a binding site for the Tam3 TPase. Moreover, decreases in methylation of the Tam3 sequence were detected in developing tissues of *Antirrhinum* plants grown at low temperature, but not in developed tissues, nor in tissues of plants grown at high temperature. Based on these results, we propose that the change in the methylation of Tam3 that occurs during LTDT is caused by a switch in the balance of two activities: one that adds a methyl group to cytosine, and the other that involves the binding of the Tam3 TPase to the GCHCG motif, as illustrated in Figure 10. In the process of demethylation of Tam3 at the permissive temperature, the TPase binding activity is initially increased, such that the TPase can bind to Tam3 after cell division and thus protect the TPase binding sites from methylation, resulting in a preferential decrease in methylation of these specific sites. At the nonpermissive temperature, the TPase binding activity in the nucleus is strongly reduced or abolished, such that the TPase cannot bind to Tam3 and the ends of the element become methylated. Therefore, in the process of LTDT of Tam3, the methylation change is dependent on the activity of the TPase, and methylation is a secondary effect (consequence) that occurs in the absence of the Tam3 TPase activity, rather than a major force (cause) that suppresses Tam3 transposition at nonpermissive temperatures (Hashida et al., 2005). For the maize TE *Spm*, it has not yet been shown whether the TnpA-mediated decrease in methylation induces transposition activity or whether methylation is a secondary effect, as in Tam3 in *Antirrhinum* (Cui and Fedoroff, 2002). The Tam3 TPase gene is constitutively transcribed under both growth temperatures (Hashida et al.,

![Figure 5](image-url)
Thus, changes in TPase activity in response to growth temperature are likely to be determined by posttranscriptional processes. This model is also supported by the results from the EMSA analysis using the nuclear protein extracts shown in Figure 8. This is in contrast with the Spm element of maize, whose activity is controlled by TnpA transcription, which is promoted by a decrease in methylation of the TE (Fedoroff et al., 1995; Cui and Fedoroff, 2002). It seems likely that it is the DNA binding activity or the accessibility of the Tam3 TPase that is sensitive to growth temperature.

**Process of Demethylation of Tam3**

Passive demethylation involves protein binding to DNA, leading to decreases in methylation at the bound sites. This demethylation system was described for the E. coli lac repressor/operator system, which was used as an episome (Lin et al., 2000) or as a segment integrated into the chromosome in human cells (Lin and Hsieh, 2001). In these examples, decreases in methylation appeared to be limited to the lacO sites bound by the LacI protein. By contrast, active demethylation is independent of replication and requires an enzymatic activity referred to as demethylase. Cui and Fedoroff (2002) concluded that it is likely that the demethylation of Spm is caused by an active mechanism, because TnpA was presumed to have demethylation activity or to recruit a demethylase to the TE. We cannot exclude the possibility that the Tam3 TPase has demethylation activity. This is because the temperature shift does affect the degree of methylation change at non-GCHCG–associated HpaI/MspI sites within Tam3, although we found in an earlier study that HpaI/MspI sites in another repetitive sequence did not change in their methylation status during LTDT (Hashida et al., 2003). We consider it likely that the demethylation of Tam3 is a passive rather than an active process, because (1) the decrease in

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**Figure 6. Differences in the Quantities of Methylcytosine in the Tam3 End Regions (120 Nucleotides) of the HAM5 Plants Grown at 25 and 15°C.**

Sodium bisulfite sequencing was performed for the antisense strand of the 5’ end region and for the sense strand of the 3’ end region of the two identical Tam3 copies at S6 and S99. Vertical columns indicate the percentage of methylcytosine calculated based on the sums of S6 and S99. The red columns indicate the difference between the higher percentage and that at the other temperature at the same cytosine position. The nucleotide sequences are shown between the rows of columns to identify the cytosine positions, and the TIRs are indicated by green letters at the end. The arrows indicate the GCHCG motif, and the gray zones correspond to the cytosine clusters with the largest differences in methylation between the two temperatures. The gray zones mostly overlap with the clusters of the GCHCG motif. The detailed methylcytosine patterns and distributions in both the HAM3 and HAM5 lines are provided in Figure 4.
methylation of Tam3 requires DNA replication, (2) a major domain for the methylation change in Tam3 corresponds to the TPase binding site, and (3) it is unlikely from its primary structure that the Tam3 TPase possesses a DNA glycosylase domain like those found in *Demeter* (Choi et al., 2002) and *Ros1* (Gong et al., 2002).

**Binding Property of the Tam3 TPase**

According to previous studies on the binding properties of plant TPases, transposition requires binding of TPase to the subterminal repeats of the TE (Gierl et al., 1988; Bravo-Angel et al., 1995; Becker and Kunze, 1997; Raina et al., 1998; Mack and Crawford, 2001). In the case of Ac, effective binding by TPase was achieved by recognition of multiple binding motifs (Bravo-Angel et al., 1995; Becker and Kunze, 1996, 1997). We found that the sites that showed reduced methylation levels in the subterminal regions of Tam3 in plants grown at low temperature contain a contiguous cluster of GCHCG motif repeats on the same strand. EMSA analysis demonstrated that the Tam3 TPase bound to the GCHCG motif, 40 of which are located in the overall Tam3 sequence. In particular, 28 copies of the motif...
are present within the putative subterminal regions consisting of 200 bp from both 5' and 3' ends of Tam3. Although the TPase binding motifs are different among three hAT elements (i.e., in Ac, AGGG or TCGG; in Tag1, AAACCC or TGACCC) (Becker and Kunze, 1997; Liu et al., 2001), these TPases may share the function of having a bipartite DNA binding domain that binds to subterminal repeats and TIRs. The binding affinities of these TPases for their TIRs were reported to be weak (Becker and Kunze, 1997; Mack and Crawford, 2001). In this study, the affinity of the Tam3 TPase for the TIRs was also reduced from sodium bisulfite sequencing analysis (Figures 4 and 6). The binding by TPase occurred independently of that to the TIR (S.-N. Hashida, Y. Kishima, and T. Mikami, unpublished data), although the TIR motif of Tam3 was not found in our RBSS analysis because the binding affinity of the TPase for the TIR is very weak (Becker and Kunze, 1997; Mack and Crawford, 2001). In this study, the affinity of the Tam3 TPase for the TIRs was also deduced from sodium bisulfite sequencing analysis (Figures 4 and 6). The binding by TPase occurred independently of that to the TIR (S.-N. Hashida, Y. Kishima, and T. Mikami, unpublished data), although the TIR motif of Tam3 was not found in our RBSS analysis because the binding affinity of the TPase for the TIR is very weak. The TPase binding sites, including the TIR, are contiguously aligned within a 100-bp region from both ends of the TE. This contiguous array of the TPase binding sites may be important for the transposition reaction, as suggested for other TEs (Gierl et al., 1988; Becker and Kunze, 1996). Our observation that the methylation change in the end regions of Tam3 was more marked than that in the internal Tam3 sequence is consistent with the localization of the TPase binding sites (Kitamura et al., 2001).

Role of Methylation in the LTDT of Tam3

Methylation at the GCHCG motif in Tam3 suppressed the binding of the Tam3 TPase in vitro. As shown for many other TEs, methylation may function to reduce Tam3 transposition and stabilize the genome. The importance of methylation for Tam3 transposition was also revealed by the strong effect of genome position on the transposition of different copies of Tam3 within the genome (Kitamura et al., 2001). However, our results showed that methylation is not the main driving force behind LTDT, because the methylation change depends on Tam3 TPase activity. If methylation itself controlled the LTDT of Tam3, it is likely that the specific decreases in methylation of Tam3, observed at lower temperatures, would be performed by a specific demethylase that targets the Tam3 sequence. However, our data indicate that the decrease in methylation of Tam3 is strongly correlated with the binding of the TPase to the Tam3 sequence. At the nonpermissive (high) temperature, the completely inactive state of Tam3 copies was unlikely attributable to

Figure 8. DNA Binding Activity of Nuclear Protein Extracts Derived from Plants Grown at 15 and 25°C.

A high purity of the nuclear protein extracts was confirmed, as shown in Supplemental Figure 2 online. The nuclear proteins (1.1 μg) from the plants grown at 15°C bound to the Tam3 probe (right lane), but those (1.8 μg) from the plants grown at 25°C did not bind to the probe (middle lane). The probe without the proteins was loaded in the left lane. PAGE was performed using 10% polyacrylamide, which gave rise to a single fragment for the binding of the nuclear proteins to the probe DNA and was too high a concentration of acrylamide to resolve the binding complexes into the three bands observed in Figure 7.

Figure 9. Comparison of Methylation State and Excision Activity of Tam3 between Developed and Developing Leaves during Growth at 15°C.

(A) DNA gel blot hybridization analysis was conducted by the same methods described for Figure 2 with DNA from developing leaves at 25°C (new leaf, 25°C), leaves that had developed before the temperature shift to 15°C (old leaf, 15°C), and leaves developing during growth at 15°C (new leaf, 15°C). Methylated (M) fragments occurred in the 1.2-kb EcoRV fragment, whereas unmethylated or partially methylated (U) fragments were smaller than 1.2 kb. (B) PCR detected Tam3 excision at the nivea locus only in the sample from the developing leaves during growth at 15°C (new leaf, 15°C). No excision was detected in the other samples. A sequence in the pallida locus was used as a positive control for PCR.
the methylation states of Tam3 copies, because the methylation levels varied among the individual copies present in the same genome (Figure 5) (Hashida et al., 2003). In addition, treatment with methylation inhibitors (5-azacytidine or 5-azacytidine + ethionine) did not affect Tam3 excision frequency in Antirrhinum callus (Hashida et al., 2005). In the case of Ac/Ds, a hemimethylated state for the TPase binding sites promotes transposition, and even completely methylated Ds is able to transpose after replication (Wang and Kunze, 1998; Ros and Kunze, 2001). These results with Ac/Ds suggest that the arrest of Tam3 transposition at high temperature is not likely to be the direct result of methylation, because high temperature accelerates cell division and replication, which promotes (at least transient) reductions in methylation. Therefore, we do not consider methylation to be the factor determining the LTDT of Tam3; rather, it is a secondary effect of changes in the activity of the Tam3 TPase. Of course, it is possible that the LTDT of Tam3 is driven by other factor(s) that are obscured by methylation. By contrast, methylation may have caused Tam3 inactivation when it was introduced into tobacco (Martin et al., 1989), because, in this example, treatment with 5-azacytosine did result in further transposition of Tam3 copies that had been initially inactivated.

The Control of the Tam3 Transposition in LTDT

Our previous studies have shown that the Tam3 TPase gene is transcribed and that similar levels of TPase activity can be detected in vivo in plants grown at both the permissive and nonpermissive temperatures (Hashida et al., 2003). Thus, the expression of the TPase gene is unlikely to be the point of the modulation of TPase activity in LTDT. What does control Tam3 transposition in LTDT? Our results suggest that the methylation change of the TE in LTDT might reflect a difference of binding affinity or accessibility of the TPase to the Tam3 sequence; the permissive temperature enhances the binding affinity or accessibility of the Tam3 sequence to Tam3 TPase, whereas the non-permissive temperature abolishes such TPase properties, as shown Figure 8. One possible factor underpinning LTDT could be that the DNA binding properties of the Tam3 TPase in nucleus are temperature-sensitive. Alternatively, differential subcellular localizations of the TPase at permissive and nonpermissive temperatures could explain LTDT. Temperature effects on subcellular localization offer a likely mechanism, because analyses of Ac and Mutator in maize have shown that functional TPase might be localized in the nucleus, whereas the other TPase protein aggregates in the cytoplasm (Heinlein et al., 1994; Boehm et al., 1995; Ono et al., 2002).

METHODS

Plant Materials and Extraction of DNA

We used two isogenic lines, HAM3 (nivea acumens::Tam3/New Stabilizer) and HAM5 (nivea acumens::Tam3/new stabilizer), of Antirrhinum majus.
(Figure 1). HAM3 carries the NSt allele, which originated from a mutation of the HAM5 line. Both lines have nivea\textsuperscript{ecotropic}:Tam3, which encodes the enzyme chalcone synthase, the first committed step in flavonoid biosynthesis (Sommer and Saedler, 1986). The insertion of the TE causes a severe curtailment in the transcription of the nivea gene, resulting in very pale or acyanic flowers as a result of the reduced synthesis of anthocyanins (see Supplemental Figure 1A online). When the TE excises somatically from the nivea locus, transcription of the nivea gene is restored, which is observed as full red sites and sectors of pigment on the petals against a pale mutant background. The progenitor line HAM5 has a high frequency of somatic instability showing >1000 sites per flower, on average, in plants grown at ~20°C. The plant that gave rise to the NSt allele had been cultivated at 15°C in a growth cabinet, conditions that promote the transposition of Tam3 (LTDT). The new allele was recognized when it segregated as a very palely pigmented line (HAM3) showing a very low frequency of somatic sites (fewer than one per flower) even in individuals cultivated at 15°C (see Supplemental Figure 1B online). This phenotype was stably inherited through more than six generations. Molecular analysis showed this line to carry Tam3 at the nivea locus, exactly as in the progenitor line. Neither the Tam3 copy nor the nivea locus showed any changes in sequence compared with the insertion in HAM5 \textit{(nivea\textsuperscript{ecotropic}:Tam3)}, as shown by size fractionation of genomic DNA, cloning in \textit{N. nivea} \textit{XM1149}, and sequencing the Tam3 copy and regions of the nivea locus flanking the transposon.

The NSt allele is semidominant. A cross between an NSt homozygote (Nst/Nst), HAM3, and HAM5 (nst/nst) gave identical plants in the F1 showing a moderate level of somatic instability (~10 full red sites or sectors per flower). In an F2 population, four plants segregated with a low frequency of somatic reversion (<1 site per flower), eight plants segregated with a high frequency of reversion (1 to 10 sites per flower), and three plants segregated with a high frequency of reversion (>1000 sites per flower), as shown in Supplemental Figure 1D online. This fitted well with the 1:2:1 ratio predicted for a semidominant allele that suppresses the frequency of somatic excision of Tam3. This phenotype was very similar to that of the \textit{Stabilizer \textit{(St)} locus of \textit{A. majus}, described originally by Harrison and Fincham (1968) and for its effect on Tam3 at the nivea locus by Carpenter et al. (1987). Nst also suppressed Tam3 transposition at the \textit{dag locus} of \textit{Antirrhinum} and suppressed the phenotype caused by the Tam3 insertion in the same way as the \textit{St} locus, as described by Chatterjee and Martin (1997).

The HAM3 line showed some signs of somatic instability. On the flowers of Nst/Nst homozygous plants grown at low temperature, sectors could occasionally be observed in which there was a significantly greater frequency of revertant sites caused by Tam3 excision from nivea (see Supplemental Figure 1E online). This finding suggested that the Nst allele might have been generated as a result of transposon insertion into the niv locus.

to test whether NSt was allelic to \textit{St}, a cross was made between stock line Jt:558 and the HAM3 line (homozygous for the NSt allele and \textit{nivea\textsuperscript{ecotropic}:Tam3}). Line Jt:558 (Carpenter et al., 1987) carries \textit{nivea\textsuperscript{ecotropic}:Tam3} and is homozygous for \textit{St} (St/\textit{St}). The F1 plants were all identical, with pale background pigmentation to the flowers but with fewer than one revertant site per flower (see Supplemental Figure 1F online). Clearly, Nst and St did not complement, but because of the semidominant nature of both alleles, this did not mean that the two mutations were allelic. The segregation of somatic spotting frequency was scored in the F2. From 201 F2 progeny scored, 130 had a low spotting frequency of <1 site per flower, 50 had a medium spotting frequency of 1 to 10 sites per flower, and 21 had a high spotting frequency of >1000 sites per flower (see Supplemental Figure 1F online). This fitted to a segregation of 11:4:1, which could be obtained from independent segregation of two semidominant loci. When neither of the stabilizing alleles is present, flowers would have a high spotting frequency (which should arise in 1/16th of the F2 plants). When one stabilizing allele is present (either \textit{Nat} or \textit{St}), a medium spotting frequency should result (which should occur in 4/16th of the F2 plants). When more than one stabilizing allele is present, somatic reversion of Tam3 is very low (which should occur in 11/16th of the F2 plants). The genetic segregation data fitted this model, except that rather more high-spotting individuals were obtained than expected (21 observed compared with 13 expected). The overrepresentation of this class might be attributable to germinial reversion of NSt to nst as a result of transposon excision from the locus. These data showed that \textit{Nat} and \textit{St} are two different loci that segregate independently and therefore are unlinked.

Detection of DNA Methylation by DNA Gel Blotting

The methylation state of Tam3 was examined by the DNA gel blot hybridization method using a methylecystosine-sensitive enzyme, \textit{Hpall}, a partially sensitive enzyme, \textit{MspI} (isoschizomer of \textit{Hpall}), and an insensitive enzyme, \textit{EcoRV}. An \textit{EcoRV} fragment in Tam3 (nucleotide positions 814 to 2053 of the 3630-bp Tam3), which contains seven \textit{Hpall/MspI} sites, was targeted to examine the methylation state, and a 360-bp PCR fragment (nucleotide positions 1493 to 1799 of Tam3) was prepared as a probe with digoxigenin-labeling mix (Roche). The digested genomic DNA was separated by electrophoresis on a 1.0% (w/v) agarose gel and blotted onto a nylon membrane (Positively Charged; Roche). Hybridization was performed using a digoxigenin detection system (Roche). To verify that complete digestion by the enzymes was achieved, a chloroplast DNA fragment, 5.2-kb \textit{SmaI} fragment of buckwheat (\textit{Fagopyrum esculentum}) (Kishima et al., 1995), was used as a control.

Isolation of GST-Fused TPase Protein

The insert sequences for the expression plasmids included the sequences encoding the entire putative Tam3 TPase (amino acids 1 to 803) or partial Tam3 TPases (amino acid positions 1 to 250, 55 to 230, 227 to 511, and 505 to 803). These sequences were prepared by PCR amplification. The PCR products were subcloned into pGEX-4X-1 (Amersham Biosciences), which carries a GST gene. Using a plasmid (pS-\textit{CH5: Tam3} containing an entire Tam3 sequence in the nivea locus as a template, PCR amplification was performed with the following primer combinations: TPase1-803, 5′-CGCGAATTCTGGCAAGATTTTTAGG-3′; TPase5-250, 5′-CGCGGAATTCGGACTGATTATGCG-3′; TPase250-511, 5′-CGCGAATTCTGTCACCGATCACTTGGC-3′; TPase55-230, 5′-CGCGAATTCCCGACTACAGA-3′; TPase1-250, 5′-CGCGAATTCTTGGAACGAGAAAAAATC-3′; and 5′-CGCGAATTCGCCAAGTGATCGGT-3′. The expression plasmids were transformed into \textit{Escherichia coli} (BL21[DE3] strain; Novagen). The transformants were cultured overnight with shaking at 30°C in 3 mL of Luria-Bertani medium containing 100 \mu g/mL ampicillin. The culturing was continued in 30 mL of Luria-Bertani + ampicillin medium. Immediately after the cells were grown to an \textit{OD}_{600} of 0.6 to 0.8, expression of the fusion proteins was induced by adding isopropylthio-
β-galactoside at a final concentration of 0.1 mM for 1.5 h. All subsequent steps were performed on ice or at 4°C. Cells were pelleted by
centrifugation and resuspended in PBS buffer (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄, pH 7.4). The pellet collected after centrifugation was suspended in 10% Triton X-100 and sonicated with a UD-201 ultrasonic disruptor (TOMY) until the suspension was slightly translucent (two pulses, ~4 min each). To isolate the soluble fraction, the sonicated fraction was centrifuged at ~12,000 g for 20 min to remove cellular debris. The soluble fractions containing GST fusion proteins were incubated with 400 μL of 50% glutathione–Sepharose 4B (Amersham Biosciences) for 16 h. To elute the GST fusion proteins, the resin was incubated with an aliquot of GSH buffer (10 mM reduced glutathione [Sigma-Aldrich], 10 mM HEPES, pH 8.0, and 60 mM KCl) for 5 min, and the fusion proteins were recovered after centrifugation.

### RBSS Analysis

The oligonucleotide mixture rsDNA (5'-GGGAGATCTGTGTTGTTGNNN-NNNNNNNNNNNNCTCTCCCTCTGTGCAAG-3'), containing 16 random nucleotides flanked by 18 bases of defined sequence, was used as the source of Tam3 TPase binding sites in the binding site selection procedure. The oligonucleotide mixture was made double-stranded by annealing with primers (5’-GGGAGATCTGTGTTGTTGTTTCC-3’ and 5’-CCTTGATCAAGGGGTAG-3’) and using the Klenow fragment (Takara) to synthesize the opposite strand. The same primer combination was used for PCR amplification during RBSS.

The double-stranded rsDNA was incubated with glutathione–Sepharose beads conjugated with TPase55-230:GST or GST protein in the binding buffer (50 mM Tris-HCl, pH 8.0, 60 mM NaCl, 3 mM MgCl₂, 2 μM ZnCl₂, 10 mM 2-mercaptoethanol, 0.04% Triton X-100, and 4% glycerol). The protein–DNA complexes were collected by centrifugation after the final round of this procedure, the selected DNA fragments were cloned into pBluescript SK+ (Stratagene) and sequenced individually.

### Detection of Methylcytosine by the Sodium Bisulfite Reaction Method

Before the sodium bisulfite reaction, the genomic DNAs were digested with Mbol to facilitate the reaction. The sodium bisulfite modification procedures were adapted as described by Hashida et al. (2003). In brief, ~10 μg of the digested genomic DNA was denatured in 0.2 M NaOH for 10 min. A control, 100 ng of EcoRV-digested pBluescript SK− plasmid was added in the denaturing treatment. Thirty microliters of 10 mM hydroquinone (Sigma-Aldrich) and 270 μL of 3 M sodium bisulfite (Sigma-Aldrich) at pH 5, both freshly prepared, were added and mixed, and samples were incubated under mineral oil at 55°C for 16 h. Modified DNA was purified using the GeneClean spin kit (BIO 101) according to the manufacturer’s recommendations and eluted to 80 μL. Bisulfite-modified DNA was amplified with specific primers for five nested PCRs. The following primers were used: Tam3 5’-S-6 antisense strand first primer set, 5’-CGCCGCACTGGCCCGACGCTT-3’ and 5’-AACACATACATGACACATATTATTA-3’; Tam3 3’ of S-99 sense strand first primer set, 5’-TTCTAATTCAACACGTGAAATTCACAAAATTT-3’; Tam3 3’ of S-99 sense strand second primer set, 5’-GGTGGAATTTGAGTTGGAATAATTGTT-3’; Tam3 2’ of S-99 sense strand second primer set, 5’-TTTTTTTTTTATGCGAGGGTTTTTTTTT-3’ and 5’-AAATTTATACCACATATTTATTTA-3’; Tam3 2’ of S-99 sense strand second primer set, 5’-GGTTGAGAATTTGGAATAATTTT-3’; pBluescript SK− primer set, 5’-GGTTGAGAATTTTGGAGAAATTTG-3’ and 5’-AACACATACATGACACATATTATTA-3’.

### EMSA

An oligonucleotide (single stranded) and an oligonucleotide pair (double stranded) corresponding to the Tam3 subterminal sequence, Tam3:79-104 (nucleotide positions 79 to 104, 5’-AAGCGTGGCGCGCATGCGGCCATT-3’ and 5’-AATGGCCCGCATGGCCCGACGCTT-3’), were designed as DNA probes for EMSA. To test the binding specificity to this probe, we also designed an oligonucleotide that corresponded to the possible TPase binding motif (5’-CCCGC-3’ and 5’-CCGGC-3’) and also oligonucleotides in which some of the cytosines were substituted by adenine, thymine, or methylcytosine as competitors (Figure 7). The other competitor sequence was derived from nucleotide positions 3446 to 3469 of Tam3, which lacks the possible binding motif. Double-stranded oligonucleotide DNA was prepared by annealing complementary oligonucleotides as follows: 125 ng of each oligonucleotide was boiled for 5 min in TE buffer and allowed to cool to 37°C. Double-stranded DNA was labeled by filling in with [γ-32P]dATP for 1 h at 37°C using T4 polynucleotide kinase (5 units/100 ng DNA) in polynucleotide kinase buffer. The EMSA reaction was performed in a mixture containing 5 μL of the fusion protein (TPase55-230:GST) at 0.1 mg/mL, 100 pmol of the labeled DNA probe, 8 μL of GRA buffer (60 mM NaCl, 3 mM MgCl₂, 2 μM ZnCl₂, 10 mM β-mercaptoethanol, 0.04% Triton X-100, and 4% glycerol), with 2 μL of 5 mg/mL poly(dL-dC) (Amersham Biosciences) for 30 min at room temperature. Gel electrophoresis was performed with a 1% agarose gel at 4°C in 0.5× Tris-borate-EDTA at 8 V/cm for 1.5 to 2 h, followed by autoradiography.

### Preparation of Nuclear Protein Extract and EMSA

Approximately 3 g of young leaves was ground to powder in liquid nitrogen. Nuclei were released in 10 mL of extraction buffer A (50 mM HEPES, pH 6.0, 25 mM NaCl, 1.1 M sucrose, 25 mM EDTA, 1.1 mg spermine, 1.6 mM spermidine, 5 mM DTT, 2 mM phenylmethylsulfonyl fluoride [PMSF], 0.4 mM Pefabloc, 0.2% Triton X-100, 3.2% Dextran T500, and 0.6% polyvinylpyrrolidone). The slurry was filtered through two layers of Miracloth (Calbiochem) and stainless-steel mesh with 8-μm-square pores and centrifuged at 700g for 10 min. The pellet was resuspended in 3 mL of extraction buffer B (50 mM HEPES, pH 6.0, 25 mM NaCl, 0.3 M sucrose, 25 mM EDTA, 1.1 mg spermine, 1.6 mM spermidine, 5 mM DTT, 2 mM PMSF, 0.4 mM Pefabloc, and 0.2% Triton X-100). The suspension was loaded onto 30% Percoll (Amersham Biosciences) solution diluted with extraction buffer B and centrifuged at 700g for 10 min. Pelleted nuclei were resuspended in 800 μL of extraction buffer B and loaded onto a 70% Percoll solution for centrifugation (700g for 20 min). The middle layer, including nuclei, was collected, and fluorescence microscopy observation indicated that this layer contained many nuclei with debris but no chloroplasts as an indicator of contaminating cytoplasmic fragments (see Supplemental Figure 2 online). The
nuclear extract was resuspended with 500 μL of lysis buffer (25 mM HEPES, pH 7.9, 50 mM KCl, 20% glycerol, 0.1 mM EDTA, 5 mM DTT, 0.1 mM PMSF, and 0.4 mM Pefablock) followed by a rinse with extraction buffer B to exclude the Percoll solution. A supernatant was obtained by the addition of 0.5 volume saturated ammonium sulfate and centrifugation at 12,000g for 10 min. Proteins were precipitated from the supernatant by the addition of 0.5 volume saturated ammonium sulfate and resuspended in 50 μL of dialysis buffer (25 mM HEPES, pH 7.9, 50 mM KCl, 20% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 0.4 mM Pefablock). The nuclear proteins were treated with Benzonase nuclease (Takara). After overnight dialysis, the protein concentration was measured by the Bradford method (Bio-Rad). To examine the purity of the nuclear proteins, we conducted a dot immunobinding assay. Total protein was extracted from young leaves of Antirrhinum plants. Approximately 0.5 g of tissue was homogenized with 1 mL of protein sample buffer containing 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5 μg of aprotinin, and 35 μg of PMSG and then supplemented with 50 μL of 2-mercaptoethanol. The homogenate was centrifuged for 5 min at 15,000 rpm. The resulting supernatant was separated from the cell debris. The protein concentration in the extracts was measured using a protein assay kit (Bio-Rad). A dilution series of the initial protein concentration, 100 ng/μL, was spotted onto Hybond-P (Amersham Biosciences) in a semidry condition. After blocking with 4% (w/v) skim milk, the membrane was incubated overnight at 4°C with a 2500-fold-diluted primary antibody (anti-histone H3 from rabbit). The membrane was incubated for 1 h with a 5000-fold-diluted secondary antibody (anti-rabbit immunoglobulin antibody conjugated with horseradish peroxidase) and then stained with the ECL Plus Western Blotting Detection System (RPN2132; Amersham Biosciences) according to the manufacturer’s recommendations. Signals were detected using Fuji x-ray film.

Subsequently, nuclear protein extracts were used for EMSA. The probe DNA, Tam3:79-104, was end-labeled with 6-carboxyfluorescein. The EMSA reaction was performed by the addition of 1.1 or 1.8 μg of nuclear proteins instead of the fusion protein in the same EMSA reaction mixture described above. Electrophoresis was performed on 10% polyacrylamide gels at 4°C in 0.5× Tris–NaOAc–EDTA at 70 V for 2 h. The fragments were visualized by scanning the fluorescent signals on the gel plate using a Typhoon 8600 (Molecular Dynamics).

Detection of de Novo Tam3 Excision at nivea

To examine the Tam3 excision frequency by PCR analysis, we focused on the Tam3 copy inserted at nivea. The following primers were used:

\[
\text{TACC-3: TACC-3} \rightarrow \text{CCTATTGGCAAAATTAGG-5}\]

The reaction was performed under the same conditions as described (Hashida et al., 2003). To provide a quantitative standard for the genomic DNA, PCR was performed under the same conditions as described (Hashida et al., 2003). One of the primers from nivea, 5′-CCATTTGGCCAAATTAGG-TACC-3′ and 5′-AACCTCCTCAACAGTACCATT-3′; palida, 5′-TGCGATTGACACTTGCCGC-3′ and 5′-CGCATTCTTCTGCTCCCTGGC-3′.

Accession Numbers

Sequence data (Tam3 sequences) from this article can be found in the GenBank/EMBL/DDJB data libraries under accession number AB038406 (Tam3 from S-CHS), which is common to all of the Tam3 sequences mentioned here. The Tam3 TPase gene open reading frame is present in all of the Tam3 sequences.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Genetic Analysis of the Nst Allele.

Supplemental Figure 2. Examination of the Purity of the Extracts of Nuclear Proteins.

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The Temperature-Dependent Change in Methylation of the *Antirrhinum* Transposon Tam3 Is Controlled by the Activity of Its Transposase
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