Temperature Shift Coordinately Changes the Activity and the Methylation State of Transposon Tam3 in *Antirrhinum majus*

Shin-nosuke Hashida, Ken Kitamura, Tetsuo Mikami, and Yuji Kishima*

Laboratories of Genetic Engineering (S.-n.H., K.K., T.M., Y.K.) and Plant Breeding (Y.K.), Graduate School of Agriculture, Hokkaido University, Sapporo 060–8589, Japan

The transposition frequency of Tam3 in *Antirrhinum majus*, unlike that of most other cut-and-paste-type transposons, is tightly controlled by temperature: Tam3 transposes rarely at 25°C, but much more frequently at 15°C. Here, we studied the mechanism of the low-temperature-dependent transposition (LTDT) of Tam3. Our results strongly suggest that LTDT is not likely to be due to either transcriptional regulation or posttranscriptional regulation of the *Tam3 TPase* gene. We found that temperature shift induced a remarkable change of the methylation state unique to Tam3 sequences in the genome: Higher temperature resulted in hypermethylation, whereas lower temperature resulted in reduced methylation. The methylation state was reversible within a single generation in response to a temperature shift. Although our data demonstrate a close link between LTDT and the methylation of Tam3, they also suggest that secondary factor(s) other than DNA methylation is involved in repression of Tam3 transposition.

Tam3 in *Antirrhinum majus* is exceptional among cut-and-paste-type transposons (TEs) in that it is the only known TE whose transpositional behavior can be strictly controlled by environmental influence. Tam3 transposition is strongly affected by temperature: It is active at low temperatures (around 15°C) and stable at high temperatures (around 25°C; Harrison and Fincham, 1964). Shifting the temperature controls the activity mitotically and probably meiotically. Based on a study of variegation spots, Carpenter et al. (1987) reported that the Tam3 excision rate was approximately 1,000-fold greater at 15°C than at 25°C. Thus, Tam3 is remarkably sensitive to the growth temperature. This trait has provided a great advantage for the isolation and analysis of a variety of genes involved in pigmentation and development in *A. majus* (Coen et al., 1989).

Among the regulatory factors associated with TE activity, DNA methylation is widely involved in the inhibition of transpositional events in plant TEs. DNA methylation can regulate TE transposition at the levels of both transposase (*TPase*) gene expression and the TPase binding process (Schlappi et al., 1994; Ros and Kunze, 2001). Martin et al. (1989) suggested that Tam3 inactivation is also associated with DNA methylation. Kitamura et al. (2001) showed that at high temperatures, the repression of transposition occurs simultaneously for all Tam3 copies in the genome, whereas at low temperatures, the repression is released to various degrees depending on the location of the copies. The degree of the Tam3 transposition activity was found to be influenced by chromosomal position and related to the degree of methylation of the element ends.

Here, we attempted to find differences between the active and inactive states of Tam3 in plants grown at different temperatures. We compared the amounts of the transcript of the *TPase* gene and compared the enzymatic activity of Tam3 TPase between plants grown at 25°C and 15°C. The results showed that neither the transcription of the *TPase* gene nor its enzymatic activity were markedly influenced by the temperature shift. Interestingly, the methylation state of the genomic Tam3 elements at different temperatures changed specifically in a manner that paralleled the low-temperature-dependent transposition (LTDT), and the original methylation state could be restored within a single generation after a temperature shift. The mechanism of LTDT of Tam3 appeared to be profoundly linked with the methylation state of the end regions of the element. However, our results do not prove that methylation is a direct cause of Tam3 inactivation, but they suggest that there is secondary factor(s) that suppresses Tam3 transposition in the LTDT process.

RESULTS

Expression of the *TPase* Gene

The HAM5 line of *A. majus*, which is homozygous for the *nivea*$^{\text{recurrens:Tam3}}$ (*niv* $^{\text{rec:Tam3}}$) allele, showed a number of flower variegation spots, which resulted from the somatic reversion of *niv* expression, at 15°C, whereas only a few spots were observed at 25°C due to the relative stability of Tam3 at the higher temper-

* Corresponding author; e-mail kishima@abs.agr.hokudai.ac.jp; fax 81–11–706–4934.

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ature (Fig. 1). This effect of temperature on Tam3 excision was previously described by Harrison and Fincham (1964) and Carpenter et al. (1987). One possible mechanism of LTDT is that the transcriptional level of the *Tam3 TPase* gene depends on the temperature. Hehl et al. (1991) reported that northern analysis revealed a fairly large (about 5 kb) band that was larger than expected (2.7–3.0 kb) based on the TPase open reading frame. This 5-kb transcript containing Tam3 sequence is unlikely to be translated to a proper Tam3 TPase. We also detected this band in leaf RNA samples isolated from plants grown at either 25°C or 15°C (data not shown). No obvious difference in the 5-kb transcript was seen when the 15°C and 25°C RNA samples were compared.

To detect the transcriptional start sites of the *TPase* gene within the Tam3 sequence, primer extension analysis, which is rather more sensitive than northern analysis, was carried out using the same RNA samples employed in northern analysis. The adenine of the putative first ATG of the *Tam3 TPase* gene is present at nucleotide 690 of the Tam3 sequence, and the TATA box and transcription initiator element (INR) were predicted to be located between nucleotides 480 and 530 of Tam3 (Fig. 2). Primer Tam3/587 (nucleotides 587–613 of Tam3) generated several extension products in the predicted promoter region of the Tam3 *TPase* gene (Fig. 2). Corresponding signals were also obtained using a different primer (primer Tam3/825; nucleotides 825–847 of Tam3; data not shown). The multiple signals might have originated from the transcription of different sequences of various Tam3 copies in the genome. The same signals were detected in the 25°C and 15°C RNA samples (Fig. 2). These results indicated that the *Tam3 TPase* gene was similarly transcribed from the sites proximal to its putative promoter sequence at the two temperatures.

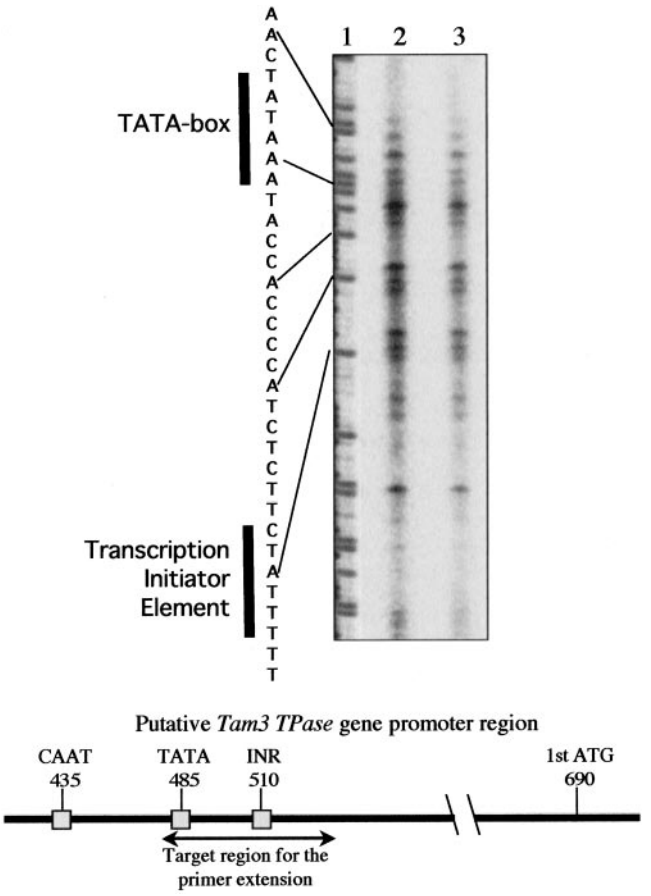

Figure 2. Primer extension analysis to detect transcriptional start sites of the *TPase* genes. The fluorescent-labeled antisense primer, primer-Tam3/587, corresponding to nucleotides 587 to 609 from the Tam3 5′ end, was used for detection of transcriptional start sites of the *TPase* genes (lanes 2 and 3) and "A" ladder sequencing (lane 1). Lane 2, Leaf RNA sample extracted from a plant grown at 15°C. Lane 3, Leaf RNA sample extracted from the same plant grown at 25°C. The sequence of nucleotides 483 to 515 from the Tam3 5′ end is shown on the left of the "A" ladder. The TATA box and INR are indicated by bold vertical lines, and the positions of the CAAT-box, TATA-box, INR, and first ATG on the Tam3 sequence are indicated on the horizontal bar.

In Vivo Activity of the Tam3 TPase

To analyze the Tam3 TPase activity, we prepared plasmid constructs containing a Tam3 element in

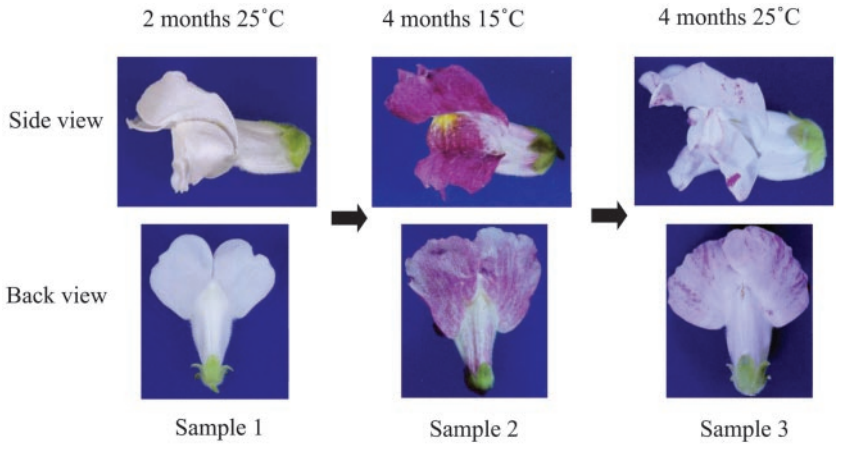

Figure 1. Changes of flower phenotype in HAM5 after temperature shift. Left, Flower of a plant grown at 25°C has white petals (sample 1). Middle, Flower of the same plant grown at 15°C for 4 months after rearing of seedlings for 2 months at 25°C shows spots of variegation at the lobe (sample 2). Right, Pigmentation of the flowers of the same plant returned to the 25°C condition and grown for 4 months has reverted to that of sample 1, i.e. white petals with a small number of pigmented spots (sample 3). Most of the Tam3 excision events at $niv^{rec:Tam3}$ result in the reversion (dominance) of the flower pigmentation because the element is present upstream of the transcription start site. RNA or genomic DNA was prepared from plants at each stage, and in further experiments, the genomic DNAs were designated by the sample numbers shown here.

which there was a deletion of the internal sequence. If Tam3 TPase activity is present in a plant cell, the Tam3 element is expected to be excised from the plasmid DNA after integration into the cell. A transient assay for Ac activity used to demonstrate Ds excision after bombardment of transgenic barley (*Hordeum vulgare*) callus lines containing Ac has been reported (McElroy et al., 1997). Using this assay, we delivered the plasmid pBS18–10E, carrying a 1.6-kb partial Tam3 DNA sequence, into leaf cells through particle bombardment. After rescue of the plasmids, the size was checked by agarose gel electrophoresis. The frequencies of Tam3-excised plasmids from randomly selected colonies are shown in Table I. We could not statistically distinguish difference in the frequencies of Tam3-excised plasmids between the leaves of plant grown at 25°C and the leaves of plant grown at 15°C. On the other hand, no Tam3-excised plasmid was obtained from tobacco, which possesses no Tam3 TPase activity. The HAM3 line, which has the *Stabilizer* gene (which represses Tam3 activity), had a very low frequency of excision of Tam3 from the plasmid (Table I). These results showed that the TPase activity is not influenced by temperature. As a consequence, we concluded that LTDT of Tam3 is not associated with the control of *TPase* gene expression. In addition, our data interestingly suggested that *Stabilizer* might function to suppress Tam3 TPase activity (perhaps via posttranscriptional gene silencing).

Methylation of Tam3 Sequences in Genomic DNA

Next, to examine the methylation state of the *Tam3* sequences in the HAM5 genome, we performed hybridization analysis using a methylation-sensitive enzyme, *Hpa*II, and a partially methylcytosine-sensitive isoschizomer, *Msp*I. We examined plants exposed to different temperature conditions: The seeds were reared at 25°C for 2 months (sample 1), then the plants were shifted to 15°C and grown for 4 months (sample 2), and then they were shifted back to 25°C for 4 months (sample 3; Fig. 1). DNA sampling of the HAM5 leaves was performed before the end of the growth period at each temperature. The three probes employed were designed from the region containing the first ATG (probe A), the middle region (Tam3 nucleotides 1,419–1,799), and the region containing the stop codon of the putative *Tam3 TPase* gene (Fig. 3). The blotting results represent the whole Tam3 family in the *A. majus* genome, which contains about 50 copies of Tam3. A chloroplast DNA fragment (Kishima et al., 1995) was used as a control probe to confirm the completeness of digestions (data not shown).

The blotting patterns obtained using the three Tam3 probes showed a common trend (Fig. 3). The *Msp*I (first lane) and *Hpa*II (second lane) digests of DNA from sample 1 showed different digestion patterns, indicating that the Tam3 sequences were considerably methylated in the HAM5 genomic DNA at 25°C. Preferential digestion of DNA from sample 2 (third lane) compared with DNA from sample 1 was observed with *Hpa*II, indicating that the Tam3 sequences reduced the methylated level at 15°C relative to 25°C. The digestion patterns obtained for sample 3 had reverted to those of sample 1 (fourth lane). These results showed that the methylation state of Tam3 changes reversibly depending on the temperature.

The methylation state varied depending on the region within Tam3. As shown in each panel of Figure 3, the results obtained with probes B and C revealed relatively heavier methylation of the Tam3 TPase coding sequence at both the temperatures, compared with the 5′ region of the gene as revealed by the hybridization of probe A with the smallest possible *Hpa*II fragment (0.7 kb). This implies that the TPase coding sequence is highly methylated at 25°C despite production of the transcript.

Methylation of the End Regions in Representative Copies

We then investigated the methylation state of the end regions of the Tam3 copies inserted in the *S-6*, *S-78*, and *S-99* loci (Kishima et al., 1999). These copies have identical structures but show very different ex-

Table I. *Frequency of Tam3 excision from plasmid p18-10E after the bombardment*

Trial	Bombarded Plants[a]											
	HAM5 25 °C			HAM5 15 °C			HAM3 25 °C			Tobacco (*Nicotiana tabacum*)		
	Re[b]	Ex[c]	Fr[d]	Re	Ex	Fr	Re	Ex	Fr	Re	Ex	Fr
1	60	3	5.0	27	1	3.7	96	0	0	48	0	0
2	48	4	8.3	48	3	6.3	96	1	1.0	48	0	0
3	42	4	9.5	48	4	8.3	96	0	0	–	–	–
4	48	7	14.6	48	1	2.1	–	–	–	–	–	–
5	56	3	5.4	48	3	6.3	–	–	–	–	–	–
6	–	–	–	48	4	8.3	–	–	–	–	–	–
Total	254	21	8.3[e]	267	16	6.0[e]	267	1	0.03	96	0	0

[a] All target materials for the bombardment were leaves of the respective plants. [b] No. of rescued clones. [c] No. of Tam3-excised clones. [d] Frequency (%). [e] Both the averages of the frequencies were calculated as $t = 2.26^{ns}$, which indicates no statistically difference.

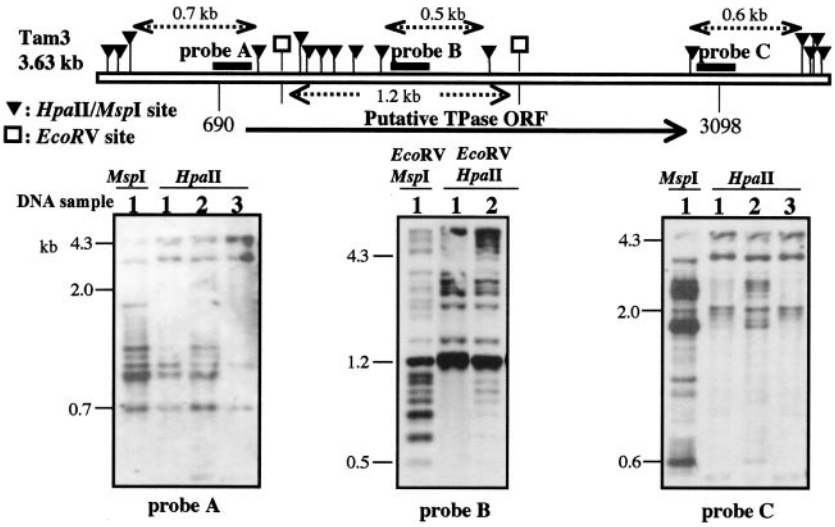

Figure 3. Methylation state of the Tam3 end regions in the genomic DNAs extracted from the plants grown at different temperatures. The upper map corresponds to a representative Tam3 sequence with the indicated *Hpa*II/*Msp*I (black triangle) sites. The regions of the three amplified PCR probes are shown in the physical map by thick lines (probes A–C). The putative *TPase* gene is marked, and the start and stop codons are indicated as Tam3 positions 690 and 3,098, respectively. The DNA samples were extracted from the leaves of the plants designated by numbers that correspond with those of the flower samples in Figure 1 (sample 1, first 2 months/25°C; sample 2, next 4 months/15°C; and sample 3, last 4 months/25°C). Due to accidental loss of the genomic DNA of sample 3, the fourth lane for probe B was not present. The DNA was electrophoresed on a 1.5% (w/v) agarose gel. For the blot with probe B, DNAs were first digested with *Eco*RV (a methylation-insensitive enzyme) to restrict *Hpa*II/*Msp*I sites within the TPase coding region, and then a secondary digestion was performed with *Hpa*II or *Msp*I. The left-most lane shows the *Msp*I digestion pattern, and the other lanes show the *Hpa*II digestion patterns. The minimum lengths of the hybridized bands generated are indicated above the map.

cision frequencies in the active condition: Tam3: *S-6* is relatively inactive, Tam3: *S-99* has modest excision activity, and Tam3: *S-78* is very active (relative excision frequencies: S-6:S-99:S-78 = 1:28:125; Kitamura et al., 2001). These Tam3 transposition frequencies were clearly correlated with the methylation state of the end regions in plants grown at 15°C. DNA samples from plant samples 1 to 3 described in the above section were examined (see Fig. 1). PCR fragments from both flanking regions of each copy were used as probes (see maps in Fig. 4). First, DNA was digested with *Mbo*I, and then a secondary digestion was performed with *Hpa*II. All the probes gave rise to a single band or a few bands when the 15°C sample was digested with *Mbo*I (insensitive to cytosine methylation; Fig. 4, left-most lane in each panel). Some of the *Hpa*II sites are clustered in the end regions, with the outermost sites located 33 and 31 bp from the 5′ and 3′ ends, respectively. There are no *Hpa*II sites in the sequences immediately flanking these three Tam3 copies (see maps in Fig. 4).

The hybridization patterns in Figure 4 revealed the methylation states of the end regions in the three copies. In each panel, the *Mbo*I/*Hpa*II digestion patterns of samples 1 and 3 (the two 25°C samples) were similar, whereas the digestion pattern of the 15°C sample (sample 2) was different except in the case of the inactive Tam3 at *S-6*. With *Mbo*I/*Hpa*II double digestion, most of the 25°C samples produced the same largest sized bands as produced by *Mbo*I single digestion, but in the 15°C samples, these bands disappeared in the active copies at the *S-78* and *S-99* loci. The largest bands in the 25°C samples resulted from extensive and heavy methylation of *Hpa*II sites in the *Mbo*I fragment. The methylation occurred in the initial 25°C growth condition, and it recurred when the temperature was shifted back to 25°C after growth at 15°C. The reversion to the high degree of methylation within single generation is a unique phenomenon for the plant genome.

Smaller sized bands, which indicated a less methylated state at the Tam3 end, arose in the active Tam3 copies at the *S-78* and *S-99* loci in the 15°C condition. In the 3′ region of Tam3: *S-78*, the most active copy (extra bands due to Tam3 excision reflect the activity at *S-78*; see Fig. 4), the temperature-dependent alteration of the methylation pattern was limited in the outermost sites of the *Hpa*II cluster. The results showed that methylation of the Tam3 end regions also parallels the temperature change, like methylation of the Tam3 internal regions, and is a somatically reversible reaction. Taken together, the results showed that the degree of methylation state of the Tam3 sequences of the high-temperature samples was much heavier than that of the low-temperature sample and that the change of the Tam3 methylation state was well correlated with the LTDT response.

However, except in the case of the 5′ end of *S-6*, the smaller bands, indicative of hypomethylation, did not disappear in the 25°C samples, implying that

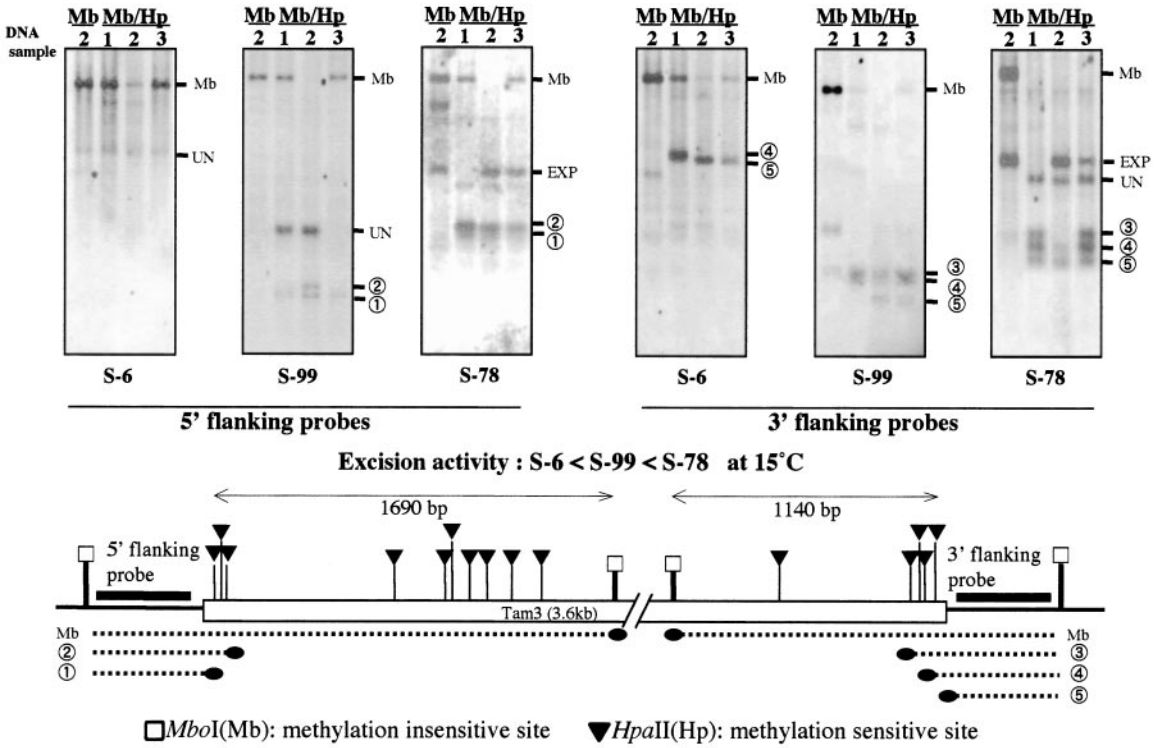

Figure 4. Methylation state of Tam3 at three representative loci in the genomic DNAs extracted from plants grown at different temperatures. The end regions of Tam3 at three loci, S-6, S-99, and S-78, were analyzed. These copies have identical sequences. The transposition activity of the three copies varies: the order of the activity is S-6 < S-99 < S-78 in the active condition, as noted in the middle of the figure. The leaf DNA samples are designated by numbers that correspond to those of the flower samples in Figure 1 (sample 1, first 2 months/25°C; sample 2, next 4 months/15°C; and sample 3, last 4 months/25°C). We used the restriction enzymes *Mbo*I (left-most lane) and *Mbo*I+*Hpa*II (the other three lanes). Clusters of *Hpa*II sites are present in both end regions of the Tam3 sequence, and *Mbo*I sites flank these clusters. The amplified probes hybridized with the two flanking regions and are marked by thick lines. The DNA was electrophoresed on a 1% (w/v) agarose gel. The hybridized fragments are indicated by dotted lines with the respective numbers under the map. The probes also hybridized with several bands that were unrelated to the target sites of the probes: These are marked "UN" (unknown fragment). In the blots of S-78, intense bands were detected at sizes that were not related to those of the methylated bands: These were due to de novo excision products of Tam3 and are marked "EXP" (excision products). Extensive methylation at *Hpa*II sites present within the *Mbo*I fragment gave rise to a fragment of the same size as the *Mbo*I fragment (Mb).

methylation at the terminal sites was not complete in the inactive condition of Tam3. This leads us to suppose that methylation in the end regions might not be the sole determinant of the suppression of Tam3 transposition at the higher temperature, although the varying transposition activities among the copies appeared to be related to the methylation level at the terminal sites of each locus.

Methylation Detected by Sodium Bisulfite Sequencing

We performed 5-methylcytosine analysis using sodium bisulfite sequencing to confirm the above results. The genomic DNAs from plant samples 1 and 2 (see Fig. 1) were examined, and primers were designed to amplify the 3′ regions of Tam3 at the *S-6* and *S-99* loci (unfortunately, we were unable to amplify the 5′ regions of the two Tam3 loci). The sense strands of 120 nucleotides from the 3′ end of Tam3 that contains six sites of CpG, 15 sites of CpNpG (where N is either A, C, G, or T), and 22 asymmetric (CpHpH, where H is either A, C, or T) cytosine sites were analyzed. As expected in the above results, the 3′ regions of both the Tam3 loci in the 25°C sample exhibited the hypermethylation state compared with that of each locus in the 15°C samples (Fig. 5). The difference in the methylation levels at the two loci also reflected the results obtained by Southern hybridization analysis (Fig. 4) where the methylation state of Tam3 of *S-6* was heavier than that of *S-99*. In the *S-6* locus, about 65% of the total cytosines in the 3′ end region were methylated in the plant grown at 25°C, and the methylcytosines in the plant grown at 15°C were reduced to one-half of those in the plant grown at 25°C. In the Tam3 3′ end region of *S-99*, 20% of the overall cytosines in the 25°C sample were methylated, whereas in the 15°C sample, only 10% of cytosines were detected as methylcytosine. These results also show that the methylation level remarkably varies among Tam3 copies, which inserted into dif-

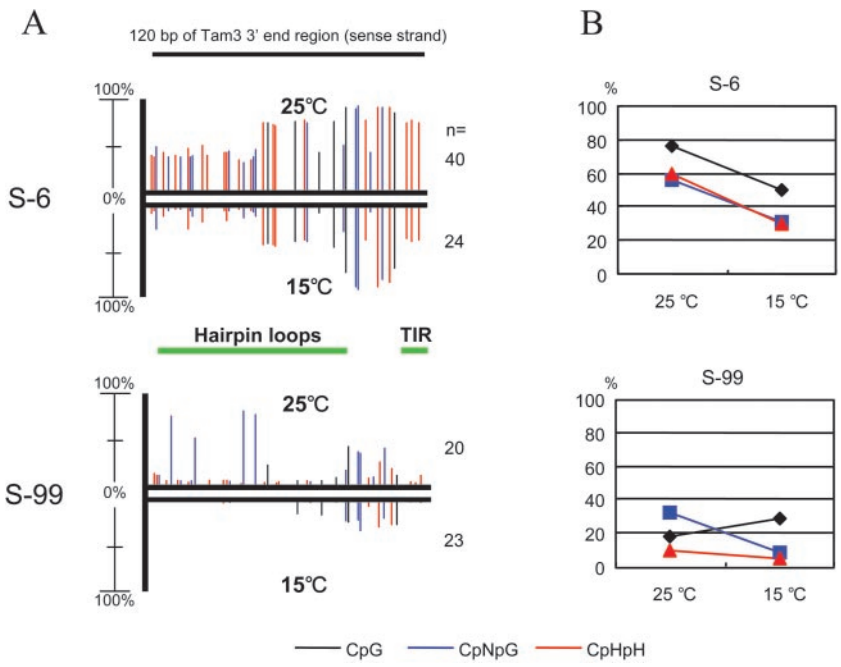

Figure 5. Distribution and quantity of methylcytosine in the Tam3 3′ end regions of *S-6* and *S–99* loci at the two temperatures. A, Sodium bisulfite sequencing was performed for the sense strand of the Tam3 3′ end regions (120 nucleotides) at the *S-6* and *S-99* loci. Vertical lines indicate the positions of methylcytosines, with the height of each line representing the percentage of the methylcytosine at the position. The upper pattern exhibits the methylation state of the sample 1 (see Fig. 1) genomic DNA isolated from the plant grown at 25°C, and the lower pattern exhibits the methylation state of the sample 2 (see Fig. 1) genomic DNA isolated from the plant grown at 15°C. n, Clone number analyzed for each plot. The green horizontal bars correspond with the terminal inverted repeat (TIR) and the hairpin loop cluster region in the Tam3 3′ subterminal sequence. The black, blue, and red lines indicate cytosines in the context of CpG, CpNpG, and CpHpH, respectively. B, Quantitative change of methylcytosines in the context of CpG, CpNpG, and CpHpH resulted from the temperature shift. Based on A, the percentages of the methylcytosines in CpG, CpNpG, and CpHpH were estimated. The two different temperatures affected quantity of methylcytosine of these sequences.

ferent genomic locations. As shown in Figure 5A, the regions where the methylation levels were affected by a temperature change corresponded between *S-6* and *S-99*. However, no specific relationships seem to be present between the change of methylation level and CpG, CpNpG, and CpHpH sites (Fig. 5B). The methylcytosines that responded to the temperature shift were localized on the terminal inverted repeat and the hairpin loop cluster in the 3′ subterminal region of Tam3 (Yamashita et al., 1999). These regions correspond with sequence forming secondary structure that may also contain possible motifs for the TPase-binding sites.

Methylation of Other Repetitive Sequences in the Genome

To examine whether the methylation state of other repetitive sequences in the *A. majus* genome changes depending on the temperature, we used the Southern-blot method using two different repetitive sequences as probes (Fig. 6). A TE-like short sequence in the *A. majus* genome, Tam661 (Yamashita et al., 1998), and the 25S-17S rDNA region, which has often been used as a standard to evaluate the overall methylation state of the genome (Ellis et al., 1990), were employed as probes. Both of these repetitive sequences contain *Hpa*II sites. As indicated in Figure 6, each repetitive sequence was methylated in the genome, as indicated by the different hybridization patterns produced by digestion of the samples with *Msp*I versus *Hpa*II, but the repetitive probes did not produce different digestion patterns for the genomic DNAs isolated from plants grown at 25°C versus 15°C. This suggests that there was no significant difference in the methylation state of the repetitive sequences at the two temperatures. Although analysis of additional repetitive sequences would be necessary to assess the overall methylation state in various regions of the genome, the present results show that the methylation state of Tam3 in the genome

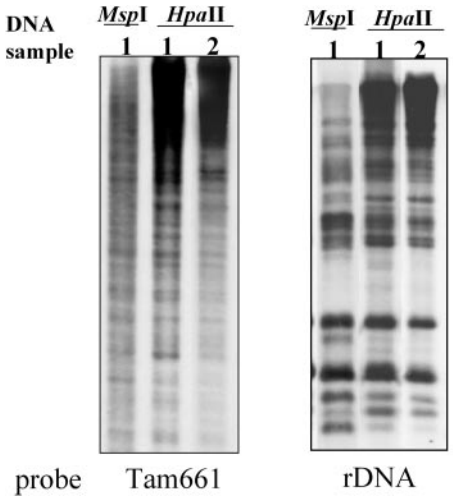

Figure 6. Methylation state of two repetitive sequences in the genomic DNAs extracted from plants grown at different temperatures. DNAs from samples 1 and 2 (see Fig. 1) were probed with two repetitive sequences, Tam661 and rDNA. Tam661 is a transposon-like sequence (Yamashita et al., 1998). The DNA samples were digested with *Hpa*II, for which restriction sites are present in both of the sequences. The DNA was electrophoresed on a 1% (w/v) agarose gel. The blotting patterns of the two repetitive sequences showed little difference between the samples.

changes specifically in response to a temperature shift.

DISCUSSION

We showed here that LTDT of Tam3 was coordinated with a change of the methylation level of the Tam3 sequence. One striking finding of our study was that shifting the temperature affected the DNA methylation level of a specific sequence during a single generation of plant growth. Although genomic methylation in mammals undergoes resetting early in development, the methylation state of plant DNA is generally well maintained throughout the lifespan of the organism, and methylation at some loci has been reported to be heritably stable over a number of generations (Cubas et al., 1999). Particular interesting phenomenon observed here is a rapid reversion from the reduced methylation state after the rising of the temperature. Although the number of studies concerning methylation in the plant genomes have analyzed the aspect of the reducing level, the studies on increasing level of methylation were few. In Arabidopsis, hypomethylation induced by *ddm1* mutation did not readily revert even in the dominant *DDM1* background, and the methylation level slowly restored through several generations (Kakutani et al., 1999). The rapid reversion of the methylation level of Tam3 implicates that Tam3 is a preferential target for methylation and demethylation.

DNA methylation seems to be a key factor for repressing the transposition of plant TEs. A number of studies have addressed the relationship between DNA methylation and *TPase* gene expression. An increased level of DNA methylation of the promoter regions of autonomous elements such as Ac, Spm, and MuDR tends to reduce the production of TPase transcripts and the transposition frequency (Chandler and Walbot, 1986; Schwartz and Dennis, 1986; Bennetzen, 1987; Chomet et al., 1987; Kunze et al., 1987, 1988; Brown and Sundaresan, 1992; Brutnell and Dellaporta, 1994; Schlappi et al., 1994). On the other hand, the methylation at TPase-binding sites has been addressed by in vitro binding assays (Kunze and Starlinger, 1989), which showed that the Ac TPase specifically binds to AAACGG, of which multiple copies are present in both ends of Ac, and that cytosine methylation of this sequence almost completely inhibits binding of the TPase. Recently, in vivo assays demonstrated that transposition of hemimethylated Ac/Ds occurred more frequently than transposition of the fully methylated element (Ros and Kunze, 2001). The Tam3 transposition activity at the low temperature depends on the chromosomal position, and this effect is also related to the degree of methylation of the copy's ends (Kitamura et al., 2001). The position effect seems also to be determined, in part, by the binding affinity of TPase to the ends of the element, including the binding affinity to the terminal inverted repeat motifs.

Several possible mechanisms of regulating the level of methylation in the Tam3 ends can be suggested, including the following: (a) Tam3 DNA contains a total of 16 firm hairpin structures with high GC content in sequences that lie within 500 bp from each end of the element (Yamashita et al., 1999). These hairpins might be targets for DNA methylation. The human (*Homo sapiens*) fragile X DNA triplet repeats with hairpin structures cause hypermethylation resulting in the suppression of the FMR-1 gene (Chen et al., 1995; Godde et al., 1996). It has been suggested that the hairpin formation could account for the specific methylation of the CpG Island in the fragile X DNA repeats. Similarly, it is reasonable to predict that the hairpins in Tam3 may facilitate recognition of a temperature-sensitive DNA methyltransferase because there are locus-specific methyltransferase genes (Cao and Jacobsen, 2002). (b) Alternatively, considering that no recognition sequences (neither for symmetrical nor for asymmetrical sites such as CpG, CpNpG, and so on) of DNA methyltransferases have been identified so far, protein(s) that are expressed at low temperature might prevent interaction of a DNA methyltransferase with the Tam3 end regions or might trap a DNA methyltransferase. Gorbunova et al. (2000) reported that some plant nuclear proteins bind to motifs that are included in the terminal regions of Ac/Ds and Mu elements but that these host proteins did not appear to be essential at least for excision of Ds; thus, the function of the binding proteins remains unknown. Similar proteins might compete with DNA methyltransferase for binding to the Tam3 terminal regions at low temperature. (c) At present, we cannot rule out the presence of an enzyme with DNA demethylation activity (Wolffe et al., 1999) in *A. majus*. (d) Finally, factor(s) could modulate the local chromatin structures containing Tam3 in a temperature-dependent manner, and these factor(s) might simultaneously influence both the transposition and methylation state. Mammalian histone deacetylases HDAC1 and 2, whose activities alter the chromatin state, resulting in transcriptional repression, are recruited by the methyl-CpG-binding protein MeCP2 (Nan et al., 1998). In addition, HDAC1 has been found to associate with a DNA methyltransferase, Dnmt1 (Fuks et al., 2000; Robertson et al., 2000). The possible involvement of these factors associated with DNA methyltransferases in LTDT of Tam3 should be considered.

The arrest of Tam3 transposition at the high temperature might not be determined solely by methylation. This possibility is suggested by two features of the stable condition of Tam3: The transcriptional and posttranscriptional activities of the *Tam3 TPase* gene were detectable (Figs. 2 and 3), and methylation was not complete at the end regions of the active Tam3 copies (Figs. 4 and 5). Although the correlation between the Tam3 activity and the methylation state accords well with the fact that DNA methylation is

often found in inactive transposons, our results raise the question of whether methylation is a direct cause of LTDT or an event that occurs simultaneously with LTDT. Evidence that methylation is not essential for repression of the TE activity was reported for the retrotransposon MAGGY of *Magnaporthe grisea* (Nakayashiki et al., 2001). We must take into account the other possibility that DNA methylation alone is not responsible for Tam3 inactivation in the LTDT process.

There is evidence that temperature affects the transposition of plant TEs in several descriptions of unstable pigmentation phenotypes: petal flaking frequency in *Portulaca grandyflora* (Beale and Faberge, 1941), speckled red-on-white sectors at the *v* locus in *Nicotiana* spp. (Sand, 1957), dot frequency in the Dotted-a1 system of maize (*Zea mays*; Rhoades, 1941), and the frequency of purple sectors determined by the mutable allele *e* in *Primula sinensis* (Harrison and Fincham, 1964). Although all these examples show a much weaker effect of temperature on the variegation frequency than examples involving Tam3, in all of these cases, an increase in temperature tends to decrease the frequency of pigmented cells. A common mechanism might underlie these LTDTs if they are due to TEs.

MATERIALS AND METHODS

Plant Materials and Extraction of DNA and RNA

We used the HAM5 and HAM3 lines of *Antirrhinum majus*, which were kindly provided by Dr. Cathie Martin (John Innes Center, Norwich, UK). HAM5, derived from the *nivea*$^{\text{recurrens:Tam3}}$/*stabilizer*− line, was initially grown at 25°C for 2 months, and subsequently shifted to a 15°C growth chamber and grown for 4 months, then shifted back to 25°C and grown for 4 months. The HAM5 isogenic line HAM3, which carries the stabilizer+ allele, was grown at 25°C. DNA was extracted from young leaves (3–4 cm in length) of *A. majus* plants. The procedure of the DNA extraction was modified from the one described by Murray and Thompson (1980), i.e. to exclude RNA debris, aliquot of RNase A was added and incubated at 65°C for 20 min before chloroform extraction. RNA was extracted from the young leaves according to Martin et al. (1989).

Northern-Blot Hybridization

Twenty micrograms of each RNA sample was electrophoresed on a 1% (w/v) agarose gel containing formaldehyde and transferred onto a nylon membrane (Positively Charged, Boehringer Mannheim/Roche, Basel). A 1,300-bp (nucleotide positions 1,700–3,000 in the Tam3 sequence) region of the Tam3 TPase coding region was employed as the template for the probe. The Tam3 probe was prepared using a PCR-based labeling system with PCR DIG labeling mix (Roche) and the following primers: Tam3–1700F, 5′GTTGCATTACCGCACATTGG3′; and Tam3-C, 5′TCTCTATATTGTTGGTCGAGCATGTCT3′. Hybridization was carried out overnight at 65°C, and detection was performed using a DIG Nucleic Acid Detection Kit (Roche).

Primer Extension

For determination of the transcription initiation sites of the *Tam3 TPase* gene, primer extension experiments were performed using polyadenylated poly(A$^+$) RNA isolated from the leaves. Poly(A$^+$) RNA was isolated using the PolyATtract mRNA isolation systems (Promega, Madison, WI). Oligonucleotide primers 5′-CACCGTGGAGGTATGAC-3′ (primer-Tam3/587), and 5′-GTGAATCTTCATATGGTGTATC-3′ (primer-Tam3/825), corresponding to sequences starting at nucleotide positions 587 and 825 of Tam3, respectively, were end labeled with IRD800 and IRD700 (LI-COR, Lincoln, NE), respectively. Poly(A$^+$) RNA (2 μg) was hybridized with the labeled primers in a solution containing 250 mM KCl, 10 mM Tris/HCl (pH 8.3), and 1 mM dithiothreitol at 85°C for 10 min and then at room temperature for 90 min. The extension reaction was carried out in 50 mM Tris/HCl (pH 8.3), 75 mM MgCl2, 1 mM dithiothreitol, each dNTP at 0.25 mM, 1 unit of RNAsin (Takara, Kyoto), and 5 μL of reverse transcriptase (100 units μL^{-1} MMLV Reverse Transcriptase RNaseH−, TOYOBO, Tokyo, Japan) at 42°C for 1h. After RNase treatment (16 μg mL^{-1}, 37°C for 30 min), the cDNA products were extracted with phenol and chloroform and precipitated with ethanol. The DNA was analyzed by electrophoresis on a 4.5% (w/v) polyacrylamide sequencing gel. Dideoxy sequencing products primed with the corresponding primers were electrophoresed in parallel for size comparison. The fluorescent signals were detected using a DNA Sequencer model LIC400 (LI-COR).

In Vivo Assay of Tam3 Excision by the Plasmid Rescue Method

A plasmid, pBS18–10E, carrying a 1,370-bp partial Tam3 sequence with an internal deletion of the 2,260-bp region from the *Bal*I to the *Tth*HB81 site was used for monitoring Tam3 excision. This plasmid was constructed by combining the vector pBluescriptSK with the Tam3 5′ region from AG1400 (SK; from the *Xba*I to the *Bal*I site) and the Tam3 3′ region from Tam3:S-18 (SK; from the *Tth*HB81 to the *Eae*I site). The Tam3 5′ and 3′ regions were ligated to each other and then treated by filling in with T_4 DNA polymerase. This plasmid does not have 8-bp duplications at the ends of Tam3 and, therefore, does not undergo loop-out recombination between the target site duplications. Thus, the plasmid produced pBS18–10E, which was delivered via the bombardment method into leaves isolated from plants grown at different temperatures. For the bombardment delivery, surface-sterilized *A. majus* leaves were placed directly on agar plates containing 0.8% (w/v) agar in water. Gold particles (2 mg, 1.0-μm diameter, Bio-Rad Laboratories, Hercules, CA) were coated with a 6:1 (w/v) ratio of the reporter plasmid carrying the internally deleted Tam3 to the reference plasmid carrying the renilla luciferase reporter gene. The plant material was bombarded with 1 μg of DNA-coated gold particles using a helium-driven Biolistic PDS 1000 System (Bio-Rad Laboratories) with a 28-mmHg vacuum. The distance between the rupture disc and the macrocarrier was 1 cm. The bombarded leaves were incubated for 16 h at 15 or 25°C. Leaves from HAM5, HAM3, and tobacco (*Nicotiana tabacum* cv SR1) were obtained from plants grown at 15°C or 25°C. The leaf DNA was extracted after incubation at 15°C or 25°C for 16 h. To recover the plasmid, an aliquot of the DNA solution was used for transformation of *Escherichia coli* (DH5α strain, TOYOBO), and the transformed *E. coli* were grown in Luria-Bertani medium containing 50 μg mL^{-1} ampicillin. Two hundred colonies were randomly selected, and the plasmids were collected from them. To examine whether the plasmid had lost Tam3, its size was checked by 1.5% (w/v) agarose gel electrophoresis after *Sac*I digestion. The relative activity of Tam3 TPase was calculated as the ratio of the number of colonies with the altered-size plasmid to the total number of plasmid-carrying colonies.

Southern Blotting to Detect Methylation

The methylation state of Tam3 and the other repetitive elements was investigated by Southern-blot analysis using the C-methylation-sensitive enzyme *Hpa*II, partially sensitive enzyme *Msp*I (isoschizomer of *Hpa*II), and insensitive enzymes *Eco*RV and *Mbo*I. HAM5 genomic DNA was isolated from the plants as described above. The following probes were prepared using a PCR-based labeling system with PCR DIG labeling mix (Roche): Tam3 probe A, CCTCACATTTTTATTTCTTAGTG + GGGTCGGTACTTGGAACTCC; Tam3 probe B, CACCGTGGAGGTATGAC + GAGGACATTGTGGCATCGCG; Tam3 probe C, CTAACCCCTGTCTTGGC + ACGCGTCGACGCAACTACAACAAAGGTGC; 5′-flanking sequence of (5′ fla.) S-6, CAAACAGGTTCAGCTCTCC + GTATCTACACCAATAACTGCG; 3′ flanking sequence of (3′ fla.) S-6, AAAATATGTCATCTTGGTCACTGGTTGC + GTGTACTTACTGCATAGCGTTCCTT; 5′ fla. S-78, CTTGTTCGTGGATTGGTTGGTGGTCGCCTG + GTTGTAGCATAGTGTAGTTAG; 3′ fla. S-78, GCAATAGATACAACAATAGCAGG + GATCATGAACCAATTTCAAAACTCTCC; 5′ fla. S-99, AACTTCCTCCTACGATATTGCTC +

CCCTTTAATTGAGTGGTCATCTCTC; and 3′ fla. S-99, ACAGTGGACTATGTCTCCTAGTATAC + AATTCGCGGCCGCT. The DNA templates (the plasmid clones; Kitamura et al., 2001) and primers were denatured at 94°C for 30 s, annealed at a few degrees above the melting temperature calculated for each primer and extended at 72°C for 30 s for a total of 35 cycles. Fifteen micrograms of each digested DNA sample was electrophoresed on 1% or 1.5% (w/v) agarose gel and transferred onto a nylon membrane (Positively Charged, Roche). The probes for Tam661 and rDNA (a rice [*Oryza sativa*] 17S-25S rDNA fragment) were prepared from plasmid clones (Sano and Sano, 1990; Yamashita et al., 1998). Hybridization was performed using the ECL gene detection system (Amersham, Buckinghamshire, UK). To verify that complete digestion was achieved by the enzymes, a chloroplast DNA fragment, 5.2-kb Sma-8 of buckwheat (*Fagopyrum esculentum*; Kishima et al., 1995), was used as a control probe.

Sodium Bisulfite Sequencing to Detect Methylcytosine

Before the sodium bisulfite reaction, the genomic DNAs of samples 1 and 2 (see Fig. 1) were digested with *Mbo*I to facilitate the reaction. The sodium bisulfite modification procedures were adapted as described by Frommer et al. (1992). In brief, approximately 10 μg of the digested genomic DNA was denatured in 0.2 M NaOH for 10 min. As a control, 100 ng of *Eco*RV-digested Bluescript plasmid was added in the denature treatment. Thirty microliters of 10 mM hydroquinone (Sigma, St. Louis) and 270 μL of 3 M sodium bisulfite (Sigma) 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, Vista, CA) according to the manufacturer's recommendations and eluted into 80 μL. Bisulfite-modified DNA was amplified with specific primers for five nested PCR reactions. Primers used here were as follows: Tam3 5′ of S-6 antisense strand first primer, TCTTCTTCARCTCAAAGCCCCTATTA+AATTGAAAAATGTTATGAAGGTATTGTGTG; Tam3 5′ of S-6 antisense strand second primer, CCTAATAAAACAACAATTARCACACCTA+GGCTAGAAGTAGATTAGAATTGG; GGGGTTGGAATAGGAGAGTGTATGAGGGAG+CCATAAACTCCACCCCCCTAACAAACATC; Tam3 5′ of S-99 antisense strand first primer, AACTTCCTCCTACRATATTRCTCTA+AATTGAAAAATGTTATGAAGGTATTGTGTG; Tam3 5′ of S-99 antisense strand second primer, AAARTCAAACRCACACRAR+GGCTAGAAGTAGATTAGAATTGG; Tam3 3′ of S-6 sense strand first primer, TTTTTTTTTTTATGCAGGGGTTTTTTTTTT+ATACACTTCACCTCARACCCAAAARCAAAAC; Tam3 3′ of S-6 sense strand second primer, GGGTTGAATTGGTTGGAGAAATGG+TTCTAATTCACAAACGTAATACAAAAAATT; Tam3 3′ of S-99 sense strand first primer, TTTTTTTTTTTATGCAGGGGTTTTTTTTTT+AATATTTATTAAACACATATTTTA; Tam3 3′ of S-99 sense strand second primer, GGGTTGAATTGGTTGGAGAAATGG+AACACATATACTAACAAACACATATTTTAT; and pBluescript primer, GGGTTGAATTGGTTGGAGAAATGG+AACACATATACTAACAAACACATATTTTAT. PCR reactions were performed as follows: two cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min; two cycles of 95°C for 30 s, 58°C for 1 min, 72°C for 1 min, 40 cycles of 95°C for 30 s, 56°C for 1 min, 72°C for 1 min; and termination at 72°C for 7 min. Amplified PCR products were cloned by pBluescript SK (Stratagene, La Jolla, CA) vector. Individual clones were sequenced by an ABI377 Automated DNA Sequencer (PE-Applied Biosystems, Foster City, CA).

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