Stable Transcription Activities Dependent on an Orientation of Tam3 Transposon Insertions into Antirrhinum and Yeast Promoters Occur Only within Chromatin¹

Takako Uchiyama, Kaien Fujino, Takashi Ogawa, Akihito Wakatsuki², Yuji Kishima*, Tetsuo Mikami, and Yoshio Sano

Laboratories of Plant Breeding (T.U., T.O., Y.K., Y.S.), Crop Physiology (K.F.), and Genetic Engineering (A.W., T.M.), Research Faculty of Agriculture, Hokkaido University, Sapporo 060–8589, Japan

Transposon insertions occasionally occur in the promoter regions of plant genes, many of which are still capable of being transcribed. However, it remains unclear how transcription of such promoters is able to occur. Insertion of the Tam3 transposon into various genes of Antirrhinum majus can confer leaky phenotypes without its excision. These genes, named Tam3-permissible alleles, often contain Tam3 in their promoter regions. Two alleles at different anthocyanin biosynthesis loci, niv-recurrens::Tam3 (niv-rec) and pallida-recurrens::Tam3 (pal-rec), both contain Tam3 at a similar position immediately upstream of the promoter TATA-box; however, these insertions had different phenotypic consequences. Under conditions where the inserted Tam3 is immobilized, the niv-rec line produces pale red petals, whereas the pal-rec line produces no pigment. These pigmentation patterns are correlated with the level of transcripts from the niv-rec or pal-rec alleles, and these transcriptional activities are independent of DNA methylation in their promoter regions. In niv-rec, Tam3 is inserted in an orientation that results in the 3′ end of Tam3 adjacent to the 5′ region of the gene coding sequence. In contrast, the pal-rec allele contains a Tam3 insertion in the opposite orientation. Four of five different nonrelated genes that are also Tam3-permissible alleles and contain Tam3 within the promoter region share the same Tam3 orientation as niv-rec. The different transcriptional activities dependent on Tam3 orientation in the Antirrhinum promoters were consistent with expression of luciferase reporter constructs introduced into yeast chromosomes but not with transient expression of these constructs in Antirrhinum cells. These results suggest that for Tam3 to sustain stable transcriptional activity in various promoters it must be embedded in chromatin.

¹ This work was supported by research fellowships of the Japan Society for the Promotion of Science for Young Scientists (to T.U.) and the Suhara Memorial Foundation (to Y.K.).

² Present address: Sakata Seed Corporation, 2-7-1 Nakamachidai, Tsuzuki-ku, Yokohama, Kanagawa 224-0041, Japan.

* Corresponding author; e-mail kishima@abs.agr.hokudai.ac.jp.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Yuji Kishima (kishima@abs.agr.hokudai.ac.jp).

¹[W] The online version of this article contains Web-only data: www.plantphysiol.org/cgi/doi/10.1104/pp.109.142356
the hAT transposon family (Calvi et al., 1991). Among the Tam3 copies in the genome, approximately 10 copies are known to be active and to retain an identical structure carrying the 3.6-kb sequence for the autonomous element encoding transposase (TPase; Kishima et al., 1999). The extremely homogeneous structure within the Tam3 copies has been preserved by arrest of gap repair after the excision of Tam3 (Yamashita et al., 1999). Tam3 has a remarkably potent ability to give rise to instability in Antirrhinum genes due to the nature of its low-temperature-dependent transposition (Hashida et al., 2003, 2006); low growing temperatures around 15°C permit transposition, but high growing temperatures around 25°C strongly inhibit this activity (Harrison and Fincham, 1964; Carpenter et al., 1987). The low-temperature-dependent transposition of Tam3 has facilitated the isolation and characterization of mutable lines of Antirrhinum (Schwarz-Sommer et al., 2003). In the mutable lines analyzed so far, Tam3 insertions have been mostly found in noncoding regions such as promoters, 5′ untranslated regions (UTRs), and introns (Table I). Interestingly, even with immobilized copies of Tam3, most of the genes with Tam3 insertions in noncoding regions were not completely suppressed but appeared to show permissible transcription (Table I). In this study, these alleles are termed Tam3-permissible alleles. For instance, in the cincinnata locus of Antirrhinum, each of three Tam3 insertions found in the 5′ UTR or promoter regions did not produce any abnormal phenotypes (Nath et al., 2003). Chatterjee and Martin (1997) described that Tam3 suppressed the mutant phenotype of the dag locus of Antirrhinum when Tam3 was inactivated. This is similar to the results described for Mu. An exception was found in the paladinasus::Tam3 (pa/prs) allele, with a Tam3 insertion in the promoter region that abolished pal transcription (Martin et al., 1985).

The nivora (niv) locus encodes the chalcone synthase (CHS) gene responsible for the first step of anthocyanin biosynthesis (Sommer et al., 1985). The nivora recurrens::Tam3 (niv rec) allele carries a Tam3 insertion at 70 bp upstream of the niv transcription start site (29 bp upstream of the TATA-box) and produces variegated spots due to Tam3 excision at low temperatures (Sommer and Saedler, 1986). Among the Tam3-permissible alleles in Antirrhinum, niv rec is the first allele that was described to show a leaky phenotype along with the occurrence of variegated spots even before the identification of Tam3 (Harrison and Carpenter, 1973). In stable conditions for Tam3, the flowers of niv rec show pale red color in the lobe that was designated as a "flushed" flower by Harrison and Carpenter (1973; Fig. 1). The niv rec allele reduces the amount of the transcript relative to the wild-type allele but is capable of producing anthocyanin pigment, while a null allele of the niv locus shows no background pigmentation (Lister and Martin, 1989). In the pal locus, which encodes dihydroflavonol-4-reductase responsible for a late step in the anthocyanin biosynthetic pathway (Almeida et al., 1989), the insertion of Tam3 at a promoter site similar to the niv rec allele prevents pal transcription and results in an ivory color in the petals (Martin et al., 1985; Fig. 1). The precise excision of Tam3 from niv rec or pal rec resulted in a revertant phenotype similar to the wild type (Martin et al., 1985; Sommer

Table I. Antirrhinum alleles associated with Tam3 insertions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Locus</th>
<th>Allele or Line</th>
<th>Tam3 Position</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permissible b</td>
<td>Nivea</td>
<td>niv rec</td>
<td>Promoter</td>
<td>Sommer et al. (1985)</td>
</tr>
<tr>
<td>Olive</td>
<td>oli-605</td>
<td>Promoter</td>
<td>→</td>
<td>Hudson et al. (1993)</td>
</tr>
<tr>
<td>Plena</td>
<td>ple-627</td>
<td>Intron</td>
<td>←</td>
<td>Bradley et al. (1993)</td>
</tr>
<tr>
<td>Fimbriata</td>
<td>fim-619</td>
<td>Promoter</td>
<td>→</td>
<td>Simon et al. (1994)</td>
</tr>
<tr>
<td>Centroradialis</td>
<td>cen-594</td>
<td>Intron</td>
<td>?</td>
<td>Ingram et al. (1997)</td>
</tr>
<tr>
<td>Dag</td>
<td>dag::Tam3</td>
<td>5′ UTR</td>
<td>→</td>
<td>Chatterjee and Martin (1997)</td>
</tr>
<tr>
<td>Hirz</td>
<td>Hirz-d153</td>
<td>Intron</td>
<td>?</td>
<td>Golz et al. (2002)</td>
</tr>
<tr>
<td>Ina</td>
<td>Ina-d153</td>
<td>5′ UTR</td>
<td>?</td>
<td>Golz et al. (2002)</td>
</tr>
<tr>
<td>Cincinnata</td>
<td>cin-757</td>
<td>Promoter</td>
<td>←</td>
<td>Nath et al. (2003)</td>
</tr>
<tr>
<td>cin-758</td>
<td>cin-758</td>
<td>5′ UTR</td>
<td>→</td>
<td>Nath et al. (2003)</td>
</tr>
<tr>
<td>cin-759</td>
<td>cin-759</td>
<td>Promoter</td>
<td>←</td>
<td>Nath et al. (2003)</td>
</tr>
<tr>
<td>Nonpermissible c</td>
<td>Palida</td>
<td>pal rec</td>
<td>Promoter</td>
<td>Martin et al. (1985)</td>
</tr>
<tr>
<td>Floricaula</td>
<td>fle-613</td>
<td>Coding region</td>
<td>?</td>
<td>Coen et al. (1990)</td>
</tr>
<tr>
<td>Plena</td>
<td>ple-625</td>
<td>Intron</td>
<td>→</td>
<td>Bradley et al. (1993)</td>
</tr>
<tr>
<td>Fimbriata</td>
<td>fim-620</td>
<td>Promoter</td>
<td>? (disrupted d)</td>
<td>Simon et al. (1994)</td>
</tr>
<tr>
<td>Lipless1</td>
<td>lip1</td>
<td>Coding region</td>
<td>←</td>
<td>Keck et al. (2003)</td>
</tr>
<tr>
<td>Lipless2</td>
<td>lip2</td>
<td>Coding region</td>
<td>→</td>
<td>Keck et al. (2003)</td>
</tr>
</tbody>
</table>

aIn cases where the arrowhead is directed to the right, Tam3 is oriented in the same direction as the host gene. A question mark indicates that the direction is unknown. bThe genes can be expressed in permissible alleles even when Tam3 is stable. cThe genes cannot be expressed in nonpermissible alleles when Tam3 is stable. dIn fim-620, a disrupted Tam3 copy is inserted 196 bp upstream of the translation start site and gives rise to aberrant transcription activity.

Downloaded from on November 11, 2017 - Published by www.plantphysiol.org Copyright © 2009 American Society of Plant Biologists. All rights reserved.
et al., 1985), confirming that the difference in petal pigmentation between nivrec and palrec is attributable to Tam3 insertion (Fig. 1). The effects of Tam3 on the two anthocyanin pigmentation loci lead to the question of how Tam3 controls proximal genes. This study analyzed the structural differences between the two Tam3-inserted alleles and constructed various mimic structures with reporter genes for expression in Antirrhinum and yeast. The results obtained from both Antirrhinum and yeast demonstrated that the different expression activities between the palrec and nivrec alleles were independent of DNA methylation and that the orientation of the Tam3 insertion was important for the control of downstream gene expression. Furthermore, analyses using yeast showed that the 3' region of Tam3 was required for the permissible expression, whereas this sequence itself did not retain the functional motifs of a strong promoter. When reporter constructs containing Tam3 sequence in the promoters similar to nivrec and palrec were transiently introduced into the cells of Antirrhinum, expression patterns did not follow those observed for the genes present within the chromosomes of Antirrhinum and yeast. The results suggest that Tam3 is needed to form a chromatin structure in order to accommodate various gene expression activities in Antirrhinum.

RESULTS

Structures of nivrec and palrec

In Antirrhinum, Tam3-permissible alleles were observed at 13 loci where gene expression levels were tolerant of Tam3 insertion in the gene promoters, 5' UTRs, or introns (Table I). Both the palrec and nivrec alleles carry 3.6-kb Tam3 insertions at similar sites in their promoter sequences, where Tam3 is inserted 43 and 29 bp upstream of the TATA-box, respectively (Sommer et al., 1985; Coen et al., 1986; Fig. 1). In contrast to the nivrec promoter sequences, the palrec promoter retains the Tam3 sequence in the opposite orientation: the 5' end of Tam3 in palrec is proximal to the gene coding sequence, whereas the 3' end of Tam3 in nivrec is adjacent to the coding sequence (Fig. 1). The Tam3 insertions of the nivrec and palrec alleles conferred different phenotypic consequences. In a state where Tam3 is immobilized, nivrec enabled the expression of pale red-pigmented petals (Harrison and Carpenter, 1973), whereas palrec showed ivory petal color due to the very low transcriptional activity (Martin et al., 1985; Fig. 1). Two motifs, the G-box (CACGTG) and the H-box (CCTACC), located near the TATA-box are known to be necessary for transcriptional activation of the phenylpropanoid pathway genes (Loake et al., 1992). The nivrec promoter contains G-box (CACGTG) and H-box (CCTACC), located near the TATA-box are known to be necessary for transcriptional activation of the phenylpropanoid pathway genes (Loake et al., 1992). The niv promoter contains G-box (CACGTG) and H-box (CCTACC) motifs around 150 and 50 bp upstream from the transcription start site, respectively (Sablowski et al., 1994), and the pal promoter also possesses G-box (CACGTG) and H-box (CCTACC) motifs around 80 and 60 bp from the transcriptional start site, respectively (Fig. 1). In the palrec allele, both the G-box and H-box were separated together 3.6 kb away from their authentic positions by the Tam3 insertion. In palrec, the H-box and G-box are separated by the Tam3 insertion, suggesting that the H-box alone is unable to activate pal expression, as shown by Almeida et al. (1989).
Expression of the \textit{niv}^{\text{rec}} Allele

Unlike \textit{pat}^{\text{rec}}, it has not been examined whether \textit{niv} transcription was influenced by Tam3 insertion in the promoter region. This study investigated the effect of Tam3 insertion on \textit{niv} expression. Different activities of the wild-type \textit{niv} and \textit{niv}^{\text{rec}} alleles were determined by examining pigmentation of the petal epidermal cells (Fig. 2). Compared with the wild-type \textit{niv} plant, \textit{niv}^{\text{rec}} plants grown at 25$^\circ$C showed a lower level of pigment accumulation in the petal cells, although uniform accumulation was observed throughout the pigmented cells (Fig. 2). The petal pigmentation appeared to be correlated with the \textit{niv} expression level in the two lines. In the \textit{niv}^{\text{rec}} line, the pigmentation pattern was different between lobe and tube in a petal, as shown in Figure 1. Northern blots with RNA samples prepared from lobe and tube in the wild-type and \textit{niv}^{\text{rec}} lines confirmed the parallel correlation between \textit{niv} transcriptional activity and petal pigmentation (Fig. 3A); tissue such as the lobe in the petal where the \textit{niv} allele was strongly expressed showed detectable pigmentation in plants carrying the \textit{niv}^{\text{rec}} allele, although tissues such as the tube in the petal where the \textit{niv} allele showed relatively low expression level had an undetectable level of transcript for the \textit{niv}^{\text{rec}} allele. A similar difference in blotting patterns was observed between the wild type and \textit{niv}^{\text{rec}} for both the young leaf and lobe in flower buds (Fig. 3B), although it was very difficult to evaluate in detail the tissue-specific differences in gene expression. Despite Tam3 insertion at the promoter core site, the \textit{niv}^{\text{rec}} allele was able to be transcribed, and although the expression was much lower, it did appear to follow a similar temporal pattern.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Anthocyanin pigment accumulation in the petal epidermal cells. Nomarski images show the petal epidermises from above (A) and microscopy-captured images for pigmented cells in the epidermises (B) and pigmentation in cone-shaped epidermal cells in petal sections (C). WT, Wild type. Bar = 100 $\mu$m.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{The \textit{niv} (CHS gene) expression in the \textit{niv} wild-type (WT) and \textit{niv}^{\text{rec}} lines. A, The blotting patterns represent differential \textit{niv} activities in the \textit{niv} wild-type and \textit{niv}^{\text{rec}} lines, and the differential expressions in the flower lobe and tube were observed similarly between the \textit{niv} wild-type and \textit{niv}^{\text{rec}} lines. The ubiquitin gene was used as a probe to see the amount of RNA in the blots, and no differences were observed among the intensities of the ubiquitin transcripts. B, The differential \textit{niv} expression levels in the wild-type and \textit{niv}^{\text{rec}} lines are also detected in young leaves, as shown in the lobes from flower buds. The \textit{niv} expression was examined using RNA prepared from each of the tissues in two \textit{Antirrhinum} lines with the wild-type and \textit{niv}^{\text{rec}} alleles. The ethidium bromide (EtBr) staining patterns are indicated for equal loading of rRNA. These plant materials were grown at 25$^\circ$C for at least 2 months.}
\end{figure}
To evaluate the possibility of nivrec-specific expression, an attempt was made to detect cis-elements of DNA-binding factors related to transcriptional activation from the 3’ end region of the Tam3 sequence using public databases (PLACE; http://www.dna.affrc.go.jp/PLACE/signalup.html). The database searches demonstrated that a number of binding sites of transcription factors distinct from the authentic niv promoter were present in the 3’ end region of the Tam3 sequence (data not shown), although the standard motifs for the induction of transcriptional activity were not found in the Tam3 sequence.

**Transcriptional Activity of the nivrec Promoter**

The Tam3 sequence is unlikely to recruit transcriptional factors required for niv expression because the sequences present in Tam3 are not standard promoter elements. Even if Tam3 itself has promoter activity, the transcription start sites might differ from the original site. Primer extension analysis was performed to identify the transcription start site of the nivrec allele. The nivrec allele in Tam3-inactive conditions initiated transcription at 93 bp upstream of the translation start site, which was the same as the wild-type niv allele (Fig. 4). This result showed that Tam3 insertion did not affect transcriptional initiation.

To verify the transcriptional activity of the nivrec promoter, two plasmid constructs containing different promoter sequences fused to the luciferase reporter gene and cauliflower mosaic virus (CaMV) terminator were generated for transient assays. As depicted in Figure 5, the promoter sequences of the niv-485/luc and niv-70/luc constructs were derived from the wild-type niv allele; niv-485/luc contained a 485-bp sequence upstream from the niv transcription start site, while niv-70/luc corresponded to the region between the Tam3 insertion site and the transcription start site in the nivrec allele. Both promoter sequences retained the TATA-box at the position found in the wild-type niv allele. These plasmids were introduced transiently into flower buds of the nivrec line using the particle bombardment method, and luciferase activity was measured with protein extracted from the bombarded materials. Because the niv-485/luc plasmid contained nearly the full promoter sequence of the wild-type niv allele, the relative luciferase activity of niv-485/luc was much higher than that of the niv-70/luc construct in both materials. Taking the relative value of niv-485/luc as 100, the niv-70/luc construct generated a signal of only 3.35 in the flower buds of Antirrhinum (Fig. 5). The 70-bp upstream sequence alone from the niv transcription start site was suggested to not represent an active promoter. The Tam3 sequence in the nivrec line could compensate the 70-bp downstream sequence for minimum gene expression.

**DNA Methylation States in the Promoter Regions**

In general, DNA methylation in promoter sequences suppresses transcription. The DNA methylation state of the nivrec and palrec promoter sequences and wild-type alleles was examined by a bisulfite sequencing method. DNA samples were isolated from the leaves of individual homozygous lines grown at 25°C in order to prevent Tam3 transposition. The regions in the four alleles examined by bisulfite sequencing analysis were across a 300-bp range, including the promoter region, 5’ UTR, and the coding region around the respective positions of the Tam3 insertion sites in the nivrec and palrec alleles. Although the four sequences were different, the proportion of methylated cytosine residues was indicative of the methylation states of the given genomic sequences. Sequences from 26 clones of niv, 15 clones of nivrrec, 23 clones of pal, and 16 clones of palrec were evaluated, and all were confirmed to be independent due to the presence of

---

**Figure 4.** Primer extension analysis to define the niv transcription start sites of the nivrrec and wild-type alleles. A fluorescently labeled antisense primer, CHS-pre-ex, was used for detection of the transcription start sites of the two niv alleles (W, niv wild type; R, nivrec) and T, G, C, and A ladder sequencing. The open arrow indicates that the two alleles shared a transcription start site. Nucleotides (left) correspond to the ladders of nucleotide positions from 54 to 88 bp upstream of the niv translation start site. The bottom of the ladder is proximal to the coding sequence. Note that this transcription start site is located 7 bp upstream of the site previously reported by Sommer and Saedler (1986).
Therefore, the silencing of the transcription of pal particular, showed a very low methylation state. The Transcriptional Activities of Promoters Containing Tam3 in Yeast

The above results suggested that the Tam3 sequence exerts an effect on promoter activity independent of the involvement of authentic transcription factors required for the expression of each gene. The possibility was considered that if Tam3 was present in a promoter region, the transcription of a given gene may be permitted in other eukaryotes. For this purpose, promoter assays were performed with various sequences containing Tam3-related fragments in yeast. The yeast system has several advantages for the current study: (1) no DNA methylation occurs; (2) the DNA sequences examined are targeted to a desired insertion site via homologous recombination; (3) a number of individual recombinants can be analyzed; and (4) plant-specific transcription factors are not present. A pGAPZ vector was used that contains the GAP promoter in the vector was modified with the insertion of various Tam3-related fragments, and these were fused to a luciferase reporter gene. These pGAPZ constructs with the luciferase reporter gene were inserted into the GAP promoter locus of the yeast genome through a single crossover event between the locus and the vector. Therefore, stable expression of each recombinant vector was expected in this system without chromosomal position effects.

As depicted in Figure 7, 18 plasmids constructed on the basis of pGAPZ were examined in this study. A Tam3 sequence lacking the TPase gene (dTam3; 1,368 bp in length, which consisted of 721 and 647 bp from the 5' and 3' ends of the element, respectively) was produced in order to inactivate the element (Hashida et al., 2003; Fig. 7B). Three deletion derivatives of the Tam3 sequence, dTam3:ΔI3′-TIR, dTam3:ΔR, and dTam3:ΔL, were prepared by PCR with the dTam3 sequence (Fig. 7; Supplemental Fig. S2A; Supplemental Table S1). These Tam3 and control sequences were inserted into the DraI or MunI sites in the GAP promoter sequence linked to a luciferase gene; the DraI site is located 9 bp upstream of the TATA-box, and the MunI site is 15 bp downstream of the TATA-box (Fig. 7C; Supplemental Fig. S2, B and C). Both directions of the dTam3 sequence at each insertion site in the GAP promoter were also tested (Fig. 7).

The luciferase activity was calculated as the relative volume of luciferase to the total amount of yeast proteins after 24 h of culture of the recombinant yeasts. The average and SE in the activities of each construct resulted from three or more independent replicate transformations. The luciferase activity obtained from the intact GAP promoter (clone 2) was standardized as 100%. Clones 3 and 5 contained dTam3 in reverse orientations at the same site upstream of the TATA-box, and both directions of the dTam3 sequence at each insertion site in the GAP promoter were also tested (Fig. 7).

The Transcriptional Activities of Promoters Containing Tam3 in Yeast

The above results suggested that the Tam3 sequence exerts an effect on promoter activity independent of the involvement of authentic transcription factors required for the expression of each gene. The possibility was considered that if Tam3 was present in a promoter region, the transcription of a given gene may be permitted in other eukaryotes. For this purpose, promoter assays were performed with various sequences containing Tam3-related fragments in yeast. The yeast system has several advantages for the current study: (1) no DNA methylation occurs; (2) the DNA sequences examined are targeted to a desired insertion site via homologous recombination; (3) a number of individual recombinants can be analyzed; and (4) plant-specific transcription factors are not present. A pGAPZ vector was used that contains the GAP promoter to constitutively express recombinant proteins in Pichia pastoris, a methylotrophic yeast. First, the unique methylated cytosine sites (Supplemental Fig. S1, A and B). Figure 6 shows the degree of methylation on the sense strands of the four alleles. Both the niv rec and pal rec alleles showed relatively low methylation states throughout the sense strands examined compared with their wild-type alleles (Fig. 6). The promoter region in pal rec, including the 5' end of Tam3 in particular, showed a very low methylation state. Therefore, the silencing of the transcription of pal rec was unlikely to be caused by DNA methylation of the promoter sequence. A previous study demonstrated that differences in excision activity at low temperature among Tam3 copies were associated with the degree of methylation at each insertion site (Kitamura et al., 2003). The high excision activity of Tam3 from the promoter region in compared with their wild-type alleles (Fig. 6). The promoter activity of the niv-485 construct was estimated as 100, the niv-70 construct was 3.35.

Figure 5. Luciferase (LUC) assay of niv promoter activities in Antirrhinum flower buds. The two plasmid constructs from the niv wild-type promoter sequence were fused to the luciferase gene and transiently introduced into Antirrhinum flower buds. The niv-485 construct had nearly the full promoter sequence, while niv-70 contained a 70-bp promoter sequence from the transcription start site, which was consistent with the sequence from the transcription start site to the Tam3 insertion site. When the luciferase activity of the niv-485 construct was estimated as 100, the niv-70 construct was 3.35.
upstream of the TATA-box (the DraI site) was found to be important for activation of the *luciferase* gene, as activation of the *luciferase* gene failed in cases where the Tam3 sequence was present at the MunI site downstream of the TATA-box (clones 4, 6, 8, 10, 12, 14, 16, and 18). Thus, it was inferred from these results that the Tam3 sequence per se does not retain a strong promoter activity. The other important factor for the activity was found to be the direction of Tam3, whose 3′ region should be oriented to the TATA-box, as clones 3, 7, and 15 showed higher luciferase activities compared with clones 5, 9, and 17, respectively. Although the 12-bp terminal inverted repeat (TIR) of the 3′ Tam3 end (647 bp) appeared to be unnecessary for the activity (clone 7), the 3′ region including the subterminal repeat was responsible for the high transcription activity (clone 15). On the other hand, the 5′ Tam3 region alone did not show any enhancement of luciferase activity (clones 11 and 13). Taken together, two conditions were considered essential to activate

**Figure 6.** DNA methylation patterns of the promoter sequences from the four alleles, *niv* wild type (WT), *niv*<sup>{rec}</sup>, *pal* wild type, and *pal*<sup>{rec}</sup>. Independent sequences were evaluated from a total of 80 clones (26 clones of *niv* wild type, 15 clones of *niv*<sup>{rec}</sup>, 23 clones of *pal* wild type, and 16 clones of *pal*<sup>{rec}</sup>). Two or more clones that showed the same methylated cytosine sites throughout the sequences were removed from the analyzed clones (Supplemental Fig. S1). A, The distribution and frequency of methylated cytosines in the sense strands of the 300-bp promoter sequences in the four alleles were analyzed by bisulfite sequencing. The positions of cytosine residues in each genomic sequence are indicated on the horizontal axis (white boxes, transcribed region; black boxes, TATA-boxes; gray boxes, Tam3 sequences; triangles, Tam3-TIR). The frequency of methylated cytosines in the three contexts is indicated on the vertical bars (black bars, CpG; blue bars, CpNpG; red bars, CpHpH). B, The overall proportions of methylated cytosines in every context are summarized (black bars, CpG; gray bars, CpNpG; white bars, CpHpH). The numbers above the columns indicate the total number of cytosine residues examined. The details of the sequence results are given in Supplemental Figure S1.
Figure 7. Luciferase (LUC) activities of GAP promoters carrying Tam3 segments in the yeast P. pastoris. A, Eighteen Tam3 segments, which were inserted into either the DraI (D) or MunI (M) site of the GAP promoter (left), and their relative activities are indicated (right). The average absolute values of luciferase activities from three independent trials and the relative values (percentage) are indicated to the right of the columns. Each value is estimated relative to the luciferase value of construct clone 2. B, Tam3 segments, dTam3:Δ3’-TIR (clones 7–10), dTam3:ΔR (clones 11–14), and dTam3:ΔL (clones 15–18) were modified from dTam3 (1,368 bp); see also Supplemental Figure S2A. The primers used are listed in Supplemental Table S1. C, The modified Tam3 segments were inserted into either DraI (immediately upstream) or MunI (immediately downstream) on either side of the TATA-box (T) in the GAP promoter. L, 721 bp of Tam3 5’ end region; R, 647 bp of Tam3 3’ end region.

The luciferase gene in recombinant yeast: the Tam3 sequence should be located upstream of the TATA-box, and the Tam3 3’ subterminal region should face the TATA-box. These conditions are consistent with the structural features accounting for the preference found in Antirrhinum genes carrying Tam3, such as the nivrec and other Tam3-permissible alleles. The opposite orientation of Tam3 observed in the palrec allele resulted in a very low level of luciferase expression in yeast. The outcomes of the yeast promoter database search did not show any strong promoter elements that contain the array of yeast transcription factor-binding
motifs in both of the subterminal regions of Tam3 (data not shown).

The Transient Expression of Tam3-Containing niv and pal Promoters

It is difficult to find commonality between Antirrhinum and yeast in the underlying mechanisms for the gene expression mediated by Tam3-containing promoters. Known mechanisms explainable by specific binding of transcription factors to the CAAT-box or its related cis-element motifs may not be responsible for transcriptional activation in Tam3 sequences. If the known mechanisms function in Tam3-containing promoters, transient introduction of luciferase reporter constructs with Tam3 in the promoter should also reflect the stable expression observed in Antirrhinum and yeast. To examine this possibility, six luciferase reporter constructs were prepared: four sequences corresponding to the promoter sequences of the niv, niv<sup>rec</sup>, pal, and pal<sup>rec</sup> alleles, and two with reverse orientations of the Tam3 sequence in the niv<sup>rec</sup> and pal<sup>rec</sup> alleles (Fig. 8). The six constructs were introduced into flower buds of Antirrhinum using the particle bombardment method, and luciferase activity was measured with protein extracted from the bombarded materials. The transient expression levels of these constructs showed different patterns from those of the niv and pal alleles in petals. The luciferase activity of niv-Tam3/luc, which shared the promoter sequence with the niv<sup>rec</sup> allele, was greater than that of niv-485/luc carrying wild-type niv promoter. The niv-3maT/luc construct, which contained Tam3 in the reverse orientation relative to niv-Tam3/luc, was not significantly lower than niv-485/luc. The pal-3maT/luc construct containing the promoter sequence of the pal<sup>rec</sup> allele showed considerably higher luciferase activity contrary to the very low expression of the pal<sup>rec</sup> allele per se, while pal-Tam3/luc with a reverse orientation of Tam3 in the promoter of pal<sup>rec</sup> showed significantly lower expression compared with the other two constructs carrying the pal promoter. The relative activities of these transient expression constructs did not reflect those of the niv<sup>rec</sup>, pal<sup>rec</sup>, and wild-type alleles residing in the plant genomes, and the presence of Tam3 in the promoters of the transiently introduced constructs gave rise to complicated transcriptional activity patterns. These observations are consistent with the possibility that Tam3-mediated gene expression is functionally regulated by chromatin structure.

DISCUSSION

Structural Features of Tam3-Permissible Alleles

The insertion of transposable elements (TEs) often incompletely represses gene expression or alters the expression pattern. One of the typical examples in Antirrhinum is the niv<sup>rec</sup> allele that was tolerant of Tam3 insertion in the promoter region for the initiation of transcription. However, unlike Spm-dependent, Spm-suppressible, and Mu-suppressible alleles (Fedoroff, 2002), the transcriptional initiation of the niv<sup>rec</sup> allele was not influenced by Tam3 activity. On the other hand, the pal<sup>rec</sup> allele, which has a similar Tam3 insertion but in the opposite orientation, interfered with transcription. Monitoring using yeast showed that the luciferase expression patterns of the GAP promoter with opposite orientations of a Tam3 element paralleled those of niv<sup>rec</sup> and pal<sup>rec</sup> in Antirrhinum. Comparison of the luciferase activities among the different promoter constructs demonstrated that the Tam3 sequence had to reside at a site upstream of the TATA-box for reporter gene expression, and the 3' region of

---

*Figure 8.* Luciferase (Luc) activities of the niv and pal promoter constructs containing Tam3 sequences of both orientations. The promoter sequences of the examined plasmids were derived from the niv, niv<sup>rec</sup>, pal, and pal<sup>rec</sup> alleles, and promoters with reverse orientations of the Tam3 sequence relative to the niv<sup>rec</sup> and pal<sup>rec</sup> alleles were also constructed as described in “Materials and Methods.” These constructs were fused to the luciferase gene and transiently introduced into Antirrhinum flower buds. The luciferase activities are shown as relative values when the activity of the niv-485/luc or pal-424/luc was calculated as 100.
Tam3 conferred stronger activity than the 5’ region, reflecting the situation with the niv<sup>rec</sup> and pal<sup>rec</sup> alleles.

**The cis-Regulatory Elements in Tam3**

Various promoter sequences in *Antirrhinum* are tolerant of Tam3 invasion, and their transcription is maintained (Table I). The 3’ end region in Tam3 seems to ubiquitously adapt any promoter sequence to permit expression of the gene, although it seems to be difficult to identify particular motifs participating in the transcriptional activity among these genes. In fact, a series of *niv* promoter mutants, which resulted from the imprecise Tam3 excision from the *niv<sup>rec</sup>* allele, showed various pigmentation patterns in the flower petals as determined by the *niv* expression level (Y. Kishima and C. Martin, unpublished data). Their pigmentation patterns were not explained merely by the presence or absence of G-box or H-box motifs, which are located in a number of phenylpropanoid pathway genes and involved in transcriptional activation (Loake et al., 1992). Indeed, these different *niv* promoter sequences did not allow the prediction of the degree of *niv* expression activity.

**Transcriptional Regulation by Transposable Elements**

DNA methylation in TEs is likely to be involved in the control of adjacent genes. Besides Mu-suppressive alleles, it has also been reported that the transcription of various genes in plants is influenced by changes in DNA methylation state in adjacent TEs (Kashkush et al., 2003; Yang et al., 2005; Kashkush and Khadsan, 2007; Kinoshita et al., 2007). This study demonstrated that Tam3 permits the transcription of adjacent genes independent of DNA methylation. The 3’ end sequence of Tam3 does not interfere with the function of TATA-boxes in either the *niv<sup>rec</sup>* allele of *Antirrhinum* or the modified GAP promoter of yeast. If epigenetic mechanisms are associated with Tam3-permissible alleles, the 3’ end sequence of Tam3 might ubiquitously facilitate the formation of a chromatin condition to initiate the transcription of Tam3-permissible alleles. This is assumed from counter evidence that the transient expression of the *luciferase* reporter gene (in plasmid constructs with nonchromatin structure) driven by the mimic promoters of the *niv<sup>rec</sup>* and pal<sup>rec</sup> alleles in *Antirrhinum* cells differed from the expression levels of the genes present in the chromosomes. The promoter sequences with Tam3 insertion, which can stably drive transcription, may facilitate the specific positioning of histones to organize nucleosomes (Mavrich et al., 2008a). Indeed, the 3’ subterminal region of Tam3 preferentially contains arrays of AA, TT, and TA dinucleotides that have been shown likely to contribute to nucleosome positioning in yeast, *Drosophila*, and human (Loshikhes et al., 2006; Segal et al., 2006; Gupta et al., 2008; Mavrich et al., 2008b; Jiang and Pugh, 2009).

An analogous phenomenon to that observed in the *niv<sup>rec</sup>* and pal<sup>rec</sup> alleles was reported in the *plena* locus in *Antirrhinum* (Bradley et al., 1993; Table I). Two alleles of the *plena* locus, which is responsible for the C function of flower development in *Antirrhinum*, containing Tam3 sequences in the same intron with opposite orientations conferred distinct transcriptional activities (deregulated and inhibitory expression) and resulted in complementary floral homeotic phenotypes (Bradley et al., 1993). The direction of Tam3 is commonly associated with the transcriptional regulation of genes for flower morphology and pigmentation, and similar incidents occurring in different genes do not seem to be either coincidental phenomena or inevitable consequences determined by specific DNA sequences, such as transcriptional cis-elements or splicing regulatory elements.

**Comparisons with the dag::Tam3 Allele**

Chatterjee and Martin (1997) reported that the *dag::* Tam3 allele caused abnormalities in the development of plastids in *Antirrhinum* and was similar to the Mu-suppressive system of maize. The transcriptional activity of *dag::Tam3* was negatively correlated with the activity of Tam3 transposition (Chatterjee and Martin, 1997). Although it is not known whether or not DNA methylation of Tam3 was involved in the expression of the *dag::Tam3* allele, the expression of the *niv<sup>rec</sup>* allele occurred irrespective of the Tam3 activity. Tam3 in the *dag::Tam3* allele was inserted only 3 bp upstream of the wild-type *dag* transcription start site (Chatterjee and Martin, 1997). The transcriptional initiation of *dag::Tam3* was found at 9 bp from the 3’ end of Tam3 (Chatterjee and Martin, 1997), while the transcriptional initiation site of *niv<sup>rec</sup>* coincided with that of the wild-type *niv* allele. As in the cases of Spm- and Mu-related alleles (Barkan and Martienssen, 1991; Fedoroff, 2002; Cui et al., 2003), the different patterns of gene expression among the Tam3-permissible alleles might be due to the locus and insertion site of the element. Alternatively, the 3’ region of Tam3 per se could have a promoter function, but its effect may be too small to be detected by the GAP promoter assay in yeast. A small amount of the *dag* transcript should be sufficient to give the normal phenotype (Chatterjee et al., 1996). The *niv<sup>rec</sup>* promoter was capable of producing transcript levels less than half that of the wild type, which should be relatively abundant compared with that of *dag*. Therefore, the transcriptional activities of the GAP promoters carrying Tam3 fragments in yeast could exhibit major transcriptional activities of the Tam3-permissible alleles in *Antirrhinum*.

**Evolutionary Implications of Tam3-Permissible Alleles**

Plant genomes possess various TEs, and most of the elements have been inherited stably over the genera-
tions. If TE families have contributed to adaptive evolution with their host plant genomes, the elements should be advantageous to the host irrespective of the induction of mutations (Dooner and Weil, 2007). In fact, substantial evidence has demonstrated that plant T\textit{Pa}se genes have turned into new genes for essential functions in their host plants (Bundock and Hooykaas, 2005; Lin et al., 2007; Roccavo et al., 2007). In another aspect, TEs have been occasionally found in the regions proximal to genes, and Jordan et al. (2003) provided substantial evidence that 25% of the promoter regions analyzed in the human genome contain TE-derived sequences. These facts represent the evolutionary benefit to the host plant genomes with the utilization of TE-derived sequences. Tam3 is affiliated with the hAT family, whose elements are distributed widely over eukaryotic organisms through vertical transfer (Rubin et al., 2001). \textit{Antirrhinum} has coordinately evolved with Tam3 (Kishima et al., 1999; Yamashita et al., 1999). Among the alleles tagged by Tam3, Tam3-permissible alleles (12 alleles) outnumber nonpermissible alleles (five alleles; Table I), indicating that Tam3-permissible alleles might be one of the strategies to accommodate the transposon in the host genome rather than cause interference with host functions.

MATERIALS AND METHODS

Plant Materials

Three \textit{Antirrhinum majus} lines were used, the \textit{niv}rec (stock line JI-98), \textit{pal}rec (stock line JI-2), and wild-type (stock line JI-7) lines, which originated from the John Innes Centre. Homozygous alleles for \textit{niv}rec and \textit{pal}rec give rise to low-temperature-dependent variegation due to Tam3 excision from the promoters of the respective genes. The wild-type line carries the \textit{niv} and \textit{pal} alleles and contains no active Tam3 copies at the sites corresponding to those of the other lines. Generally, plant material was grown continuously at 25°C to prevent Tam3 transposition. DNA was extracted from young leaves (3–4 cm in length) of \textit{Antirrhinum} plants. The procedure for DNA extraction was modified from that described by Hashida et al. (2003); to exclude RNA debris, RNase A was added and the mixture was incubated at 65°C for 20 min before chloroform extraction. RNA was extracted from the young leaves according to the method of Martin et al. (1989).

RNA Gel-Blot Analysis

Twenty micrograms of each RNA sample extracted from the plants was electrophoresed on a 1% agarose gel containing formaldehyde and transferred onto nylon membranes (Positively Charged; Roche). The

Microscopic Observations of Plant Epidermal Cells

The flower petals from two lines with the wild-type \textit{niv} allele or \textit{niv} rec allele were provided to observe the pigmentation of epidermal cells. The petal epidermal cells were observed using an optical microscope (Nikon E-600) with or without Nomarski imaging. Sections of petal tissues were produced using a microslicer (Dosaka EM DTK-1000).

Sodium Bisulfite Sequencing to Detect Methylcytosine

Prior to the sodium bisulfite reaction, leaf genomic DNA from the \textit{niv}rec, \textit{pal}rec, and wild-type lines was purified with the repeated use of phenol/
chloroform extraction. Sodium bisulphite modification was carried out using a MethylEasy Xceed Rapid DNA Bisulphite Modification Kit (Diagenode) in accordance with the manufacturer’s instructions. Bisulphite-modified DNA was amplified with specific primers for nested PCR. The locations and sequences of the primers used are listed in Supplemental Table S1. Each of the nested PCRs was performed as follows: one cycle of 95°C for 3 min; 30 cycles of 95°C for 1 min, 50°C for 2 min, and 72°C for 1 min; and then 72°C for 10 min. The amplified PCR products were cloned into pT7Blue T-Vector (Novagen). The clones were sequenced using an ABI377 Automated DNA Sequencer (Applied Biosystems). Among the sequences obtained from each allele, two or more clones that coincided with cytosine methylation sites in an overall sequence were evaluated as a single clone. In Figure 6, seven clones (5, 7, 9, 13, 14, 15, and 16) from pal showed the same cytosine methylation pattern, while each of these clones contained unique sites of cytosine methylation outside of the sequence displayed in Figure 6 (Supplemental Fig. S1).

Construction of Plasmids for Luciferase Assays in Yeast

The Pichia pastoris expression vector, pGAPZ B (Invitrogen), was used for the constitutive expression of luciferase proteins. The linear DNA of this plasmid can be introduced into yeast chromosomes at the GAP promoter locus, which arises from a single crossover event between the locus and the GAP region of the plasmid. First, the lucifere gene was amplified with P. Pastoria and GAP I. The linear DNA of this plasmid is located upstream of the multicloning sites of pGAPZ B containing EcoRI and XbaI. The GAP promoter sequence located upstream of the multicloning sites has a TATA-box between the Murl (downstream side) and Durl (upstream side) sites (Fig. 7C; Supplemental Fig. S2). The Tam3-related fragments were inserted into either Murl or Durl. Eighteen of the Tam3 fragments used here were derived from the plasmid pBS18-10E containing dTam3 (1,368 bp in length), which consists of 721 and 647 bp from the 5' and 3' ends of the element, respectively (Hashida et al., 2003). All of the Tam3 fragments were amplified with pBS18-10E as a template. The PCR primers used to amplify 18 Tam3 fragments are listed in Supplemental Table S1.

Transformation of P. pastoris and Luciferase Assay

The plasmids constructed with pGAPZ B were transformed into P. pastoris strain X-33 (Invitrogen) by electroporation as described by Becker and Guarente (1991). Growth media and conditions for the selection of transformants were as described by the manufacturer (Invitrogen). Overnight suspensions of transformed cells were added to 1 ml of selection medium at 30°C (upstream side) and 75°C (downstream side) and Durl (upstream side) sites (Fig. 7C; Supplemental Fig. S2). The Tam3-related fragments were inserted into either Murl or Durl. Eighteen of the Tam3 fragments used here were derived from the plasmid pBS18-10E containing dTam3 (1,368 bp in length), which consists of 721 and 647 bp from the 5’ and 3’ ends of the element, respectively (Hashida et al., 2003). All of the Tam3 fragments were amplified with pBS18-10E as a template. The PCR primers used to amplify 18 Tam3 fragments are listed in Supplemental Table S1.

Database Searches

The search for binding sites of transcription factors from plants was performed using the public Web site PLACE (http://www.dna.affrc.go.jp/PLACE/). Searches for binding sites of transcription factors from yeast were performed with Patch (http://www.gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/patch.cgi).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers X03710, X86945, and X86876 for nis alleles and M14756 for pal alleles.

Supplemental Data

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

Supplemental Figure S1. The mapping of methylated cytosine nucleotides of both strands in the promoter regions of nisDS, palDS, and their wild-type alleles.

Supplemental Figure S2. Schematic diagrams of the basic fragments for the plasmid constructs for luciferase assays in P. pastoris.

Supplemental Table S1. Primers used to construct the plasmids for the luciferase assay with P. pastoris.

Supplemental Table S2. Primers used for the bisulphite analysis in this study.

ACKNOWLEDGMENTS

We thank Cathy Martin and Lucy Copsey (John Innes Centre) for gifts of the Antirrhinum seeds, Hironori Nagano (Research Faculty of Agriculture, Hokkaido University) for technical advice, and Derek Goto (Research Faculty of Agriculture, Hokkaido University) for his critical reading of the manuscript.

Received June 4, 2009; accepted September 9, 2009; published September 16, 2009.

LITERATURE CITED


Barkan A, Martienssen RA (1991) Inactivation of maize transposon Mu suppresses a mutant phenotype by activating an outward-reading promoter near the end of Mu1. Proc Natl Acad Sci USA 88: 3502-3506


Supplemental Figure S1. The mapping of methylated cytosine nucleotides of both strands in the promoter regions of nisDS, palDS, and their wild-type alleles.

Supplemental Figure S2. Schematic diagrams of the basic fragments for the plasmid constructs for luciferase assays in P. pastoris.
Promoter Activities in Tam3-Permissible Alleles


