Functional Dissection of the cis-Acting Sequences of the Arabidopsis Transposable Element Tag1 Reveals Dissimilar Subterminal Sequence and Minimal Spacing Requirements for Transposition

Dong Liu, Alyson Mack, Rongchen Wang, Mary Galli, Jason Belk, Nan I. Ketpura and Nigel M. Crawford

Section of Cell and Developmental Biology, Division of Biology, University of California, San Diego, California 92093-0116

Manuscript received April 10, 2000
Accepted for publication October 12, 2000

ABSTRACT

The Arabidopsis transposon Tag1 has an unusual subterminal structure containing four sets of dissimilar repeats: one set near the 5’ end and three near the 3’ end. To determine sequence requirements for efficient and regulated transposition, deletion derivatives of Tag1 were tested in Arabidopsis plants. These tests showed that a 98-bp 5’ fragment containing the 22-bp inverted repeat and four copies of the AAACX5 (X = C, A, G) 5’ subterminal repeat is sufficient for transposition while a 52-bp 5’ fragment containing only one copy of the subterminal repeat is not. At the 3’ end, a 109-bp fragment containing four copies of the most 3’ repeat TGACCC, but not a 55-bp fragment, which has no copies of the subterminal repeats, is sufficient for transposition. The 5’ and 3’ end fragments are not functionally interchangeable and require an internal spacer DNA of minimal length between 238 and 325 bp to be active. Elements with these minimal requirements show transposition rates and developmental control of excision that are comparable to the autonomous Tag1 element. Last, a DNA-binding activity that interacts with the 3’ 109-bp fragment but not the 5’ 98-bp fragment of Tag1 was found in nuclear extracts of Arabidopsis plants devoid of Tag1.

A 3.3-kb DNA transposable element of Arabidopsis thaliana, Tag1, was discovered as an insertion in the nitrate transporter gene NRT1 (CHL1) (Tsay et al. 1993). Tag1 is the only known mobile element native to the Arabidopsis genome to date although sequences or elements with similarity to a large variety of retrotransposons and transposons including Ac, En/Spm, MuDR, MITEs, foldbacks and copia have been identified (Voytas and Ausubel 1988; Voytas et al. 1990; Casacuberta et al. 1998; Wright and Voytas 1998; Ade and Belzile 1999; Copenhaver et al. 1999; Henk et al. 1999; Lin et al. 1999; Mayer et al. 1999). Tag1 is an autonomous element (Frank et al. 1997) and has the interesting feature that its transposition is controlled during shoot development (Liu and Crawford 1998a). Using 35S-Tag1-GUS constructs, it was found that Tag1 excision is restricted with a few exceptions to late events as manifested by tiny sectors in leaves, flowers, and siliques (Liu and Crawford 1998a), reminiscent of the late excision behavior of the maize Mutator element (reviewed in Bennetzen 1996). The late excision of Tag1 also carries through to the gametes and leads to independent germlinal revertants, which appear at frequencies from 0 to 27% (Liu and Crawford 1998a). Late somatic excision has also been observed in rice that has been transformed with Tag1 (Liu et al. 1999).

Analysis of Tag1 has revealed that it belongs to the Ac or hAT superfamily of elements (Warren et al. 1994; Essers and Kunze 1995; Liu and Crawford 1998b). Tag1 produces one major (2.3 kb) and several minor (between 1.0 and 1.2 kb) transcripts in both Arabidopsis and rice (Liu and Crawford 1998b; Liu et al. 1999). The major transcript encodes a putative transposase, which contains a signature sequence shared with other members of the hAT family including Ac and Bg from maize, Tam3 from snapdragon, hobo from Drosophila, Hermes from housefly, Slide from tobacco, and restless from fungus (Liu and Crawford 1998b and references therein). Surrounding the transcribed regions of Tag1 are 22-bp terminal repeats and subterminal repeat elements (Liu and Crawford 1998b). Unlike other plant transposons, the subterminal repeats of Tag1 are different at the 5’ and 3’ ends. The 5’ end has 12 copies of a repeat with the consensus AAACCC in direct and inverse orientation. The 3’ end has three nonoverlapping sets of repeats that differ from each other and the 5’ repeat.

For many transposons, the transposase binds to a target sequence at or near the ends of the element. Trans-
posase binding results in a synaptic complex that brings the ends of the element together for subsequent DNA cleavage (reviewed in Saedler and Gierl 1996). For the Caenorhabditis elegans Tc1 and Tc3 elements, transposase binding occurs within the terminal inverted repeats (Colloms et al. 1994; Vos and Plasterk 1994). For P elements binding occurs at two sites with the sequence A-T-A/C-C-A-C-T-T-A-A, located very near the 5’ and 3’ ends but outside the inverted terminal repeats (Kaufman et al. 1989). For the most-studied plant transposons, sequences in either the terminal inverted repeats or the subterminal repeat region serve as transposase binding sites. For the maize Mutator element, a 32-bp sequence within the 210-bp inverted repeat binds the MURA transposase protein (Benito and Walbot 1997). The maize Ac transposase binds specifically and cooperatively to repetitive ACG and TCG trinucleotides, which are found in >20 copies in both 5’ and 3’ subterminal regions usually within the sequence AAACGG (Kunze and Starling 1989; Becker and Kunze 1997). The Ac transposase also weakly interacts with the terminal repeats (Becker and Kunze 1997). The TNPA transposase protein of the maize En/Spm element binds a 12-bp sequence found in multiple copies within the 5’ and 3’ 300-bp subterminal repeat regions (Gierl et al. 1988; Trentmann et al. 1993). What is similar about all these elements is that the transposase binding sites are identical or very similar at each end. Tag1, however, has three sets of repeats within the 3’ subterminal regions that are different from each other and from the repeated sequence within the 5’ subterminal region. This novel organization suggests that Tag1 excision or transposase binding may involve some unique mechanisms.

This article describes our work to determine what role, if any, the subterminal repeat regions of Tag1 play in developmentally regulated transposition by establishing the minimal cis-acting sequences required for excision and reinsertion of Tag1 in Arabidopsis. Such studies are typically done by analyzing defective elements native to the host genome or by introducing mutant forms of the element into cells containing a transposase source. Because no defective Tag1 elements (dTag1) have been reported in the Arabidopsis genome, dTag1 elements were constructed and introduced into plants containing Tag1 transposase. The initial dTag1 construct had a deletion of a 1.4-kb internal EcoRI fragment (Frank et al. 1997). This dTag1 (2 kb) element was found not to transpose in the commonly used Columbia ecotype of Arabidopsis but did transpose in the Landsberg (erecta) ecotype (Frank et al. 1997; Liu and Crawford 1998a). Analysis of these ecotypes revealed that Columbia has no Tag1 elements but Landsberg has two, which are 0.3 cM apart at position 106 cM near the bottom of chromosome 1 (Frank et al. 1997, 1998; Bhatt et al. 1998). Normally the endogenous elements in Landsberg are dormant and produce no mRNA transcripts (Bhatt et al. 1998; Liu and Crawford 1998b). However, transforming this ecotype using Agrobacterium activates the transcription of the native autonomous elements, resulting in the production of transposase, which can mobilize endogenous elements or an introduced defective 2-kb dTag1 element (Bhatt et al. 1998; Liu and Crawford 1998b). This approach of transforming Landsberg plants was then applied to the study of other dTag1 elements to identify the minimal sequences required for excision. Unfortunately, this approach did not work as smaller dTag1 elements failed to activate the endogenous elements. Another method for providing transposase was therefore developed and used successfully to reveal which sequences are sufficient and required for efficient and developmentally regulated transposition. During the course of this work, it was discovered that high excision frequencies of dTag1 are limited by size restrictions on the element.

**MATERIALS AND METHODS**

**Plasmid construction:** All Tag1 deletion derivatives (dTag1 elements) were generated from plasmid pBT1, which has the entire 3.3-kb Tag1 element cloned into the XbaI and BamHI sites in pBluescript (SK) (Stratagene, San Diego; Liu and Crawford 1998a). This Tag1 clone contains an additional 8 bp of duplicated target sequences at each end along with 12 and 23 bp of flanking genomic sequences from Arabidopsis (excluding 8-bp target sequence) at the 5’ and 3’ ends, respectively. All constructs were assembled first in the pBluescript (SK) vector, which was modified to delete the EcoRI site, and then transferred to the XbaI/BamHI sites between CaMV 35S promoter and GUS gene, encoding β-glucuronidase of the plant expression vector pBI121 (CLONTECH, Palo Alto, CA). Details for each dTag1 element follow.

pTG3: pBT1 was digested with EcoRI to remove the 1.4-kb internal Tag1 sequence to produce a 2-kb dTag1 element (1.1-kb left fragment and 0.9-kb right fragment) in Bluescript to make pTBS33. The 2-kb dTag1 element was then transferred to pBI121 to make pTG3 (Figure 1).

pTG-A to pTG-E and pTG29 to pTG36: Specific Tag1 sequences were produced by PCR amplification and then ligated to the 1.1-kb left end fragment or 0.9-kb right fragment of dTag1 in pTBS33 (Figures 3 and 4). All these dTag1 elements have the same genomic flanking sequences as found on pBT1 and pTBS33. To generate the 2-kb dTag1 element without the 8-bp target sequences, primers directed to the 22-bp terminal inverted repeat were used to PCR amplify the left 1.1-kb and right 0.9-kb fragments of Tag1 element.

pTG0.5, pTG0.9, and pTG5.0: DNA fragments from randomly picked Arabidopsis genomic DNA clones of 0.5, 0.9, and 5 kb in length (corresponding to clones ALC030, ALC119, and ALC093, respectively) were inserted into the EcoRI site between the 98-bp 5’ end and 109-bp 3’ end fragments of Tag1 (see Figure 5).

pTG-5F and pTG-3F: The 5’ and 3’ subterminal fragments (270 and 262 bp, respectively) of Tag1 were amplified by PCR (see Figure 5). These fragments were used to replace the 1.1- or 0.9-kb end fragments of pTBS33 as shown in Figure 5.

35S-Tag1-SPT (pTS): Tag1 was excised from plasmid pBT1 by SacI/XhoI digestion and cloned into the same sites on plasmid pCL0111 (a kind gift from Caroline Dean; Jones et al. 1989). In this construct the Tag1 element is between the 35S promoter and streptomyacin phosphotransferase gene.
were resuspended in 15 ml Honda buffer and then centrifuged genic lines (data not shown). These results suggested to final concentration of 5 mM DTT, and 5 mM Hepes, pH 8.0, 80 0.2
44 ml 2 al.
Arabidopsis plant leaves as described (Raghothama 2B). tallied regulated transposition.

Crawford streptomycin (100 1992). Seeds were germinated on GM medium containing and very similar to that observed for the autonomous

Vacuum infiltration procedures were used for plant transfor-
dation, transgenic plants were selected on GM medium (Val-
vekens et al. 1988) with kanamycin at 30 μg/ml. Isolation of
genomic DNA and Southern blot analysis were performed as
described (Liu and Crawford 1998a). Flanking sequences were
amplified by TAIL PCR as described (Tsugeki et al. 1996).
PCR products were cloned into pGEM-T PCR vector (Promega, Madison, WI) and sequenced. The degenerate
primers used for the PCR were:

AD1: 5'-ATCTGASTWTSWGTTT-3'
AD2: 5'-STTGNTASTACTTNGC-3'
AD4: 5'-XTGTGASHGAAVMGAGA-3'
AD11: 5'-CTTCTTCGCACTTTGGA-3'

(S = C/G; W = A/T; N = A/G/C/T). The anchored primers
used were:

ALC 119-1: 5'-CGACTGTGATCATCTTCATGC-3'
ALC 119-2: 5'-GGAATTCATTATGGTGTAACCC-3'
ALC 119-3: 5'-GGATATGATCCATACGTTGCG-3'

Phenotypic assays for Tag1 excision frequencies: Histo-
chemical assays for GUS expression were performed as de-
excisions of dTag1 elements were visualized as blue sectors on
a white background. Germinal excision frequency of dTag1
elements was scored by counting the number of completely
blue-staining seedlings from a progeny population of 100
seedlings for each line.

The phenotypic assays for transgenic plants carrying 35S-
Tag1-SPT construct were performed as described (Dean et al. 1992). Seeds were germinated on GM medium containing
streptomycin (100 μg/ml). Ten days after germination, germi-
nal revertants were identified as fully green streptomycin-resis-
tant seedlings (Figure 2A). Somatic sectors on cotyledons
are observed as green spots on a bleached background (Figure
2B).

Gel mobility-shift assays: Nuclear extracts were prepared
from Arabidopsis plant leaves as described (Raghothama et al. 1993). Briefly, leaves (25 g) were ground to a fine powder in
liquid nitrogen and added to an extraction buffer contain-
ing 149 ml Honda buffer (3.3% Ficoll, 6.6% Dextran T40, 33
mM Tris-HCl, pH 8.5, 3.3% Triton X-100, 6.6 mM MgCl2, 44
mM 2 M sucrose, 140 μM 2-mercaptoethanol and 1.89
mM 0.2 M spermine). Material was homogenized in a Polytron
twice for each 20 sec. Homogenates were filtered through
nytex, which was pre-wet with Honda buffer. Filtered homoge-

nate was centrifuged at 5000 rpm for 5 min at 4°C. The pellets
were resuspended in 15 ml Honda buffer and then centrifuged
again. The pellets were resuspended in 10 ml nuclei wash
buffer (50 mM Tris-HCl, pH 8.5, 5 mM MgCl2, 1 mM 2-mercap-
toethanol, and 20% glycerol) and centrifuged as above. After
two washes the pellet was resuspended in 1.5 ml nuclei resus-
pension buffer (10 mM Hepes, pH 8.0, 50 mM NaCl, 0.5 M
sucrose, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM dithiothreitol
(DTT), and 5 mM MgCl2). Spermidine and NaCl were added
to final concentration of 5 mM and 0.5 M to lyse nuclei on
ice for 30 min. After lysis, the sample was centrifuged for 10
min at 4°C. The supernatants were dialyzed into 10 mM Hepes,
PH 8.0, 1 mM MgCl2, 1 mM DTT, 50% glycerol, 50 mM NaCl,
0.8 mM phenylmethylsulfonyl fluoride overnight with several
changes. After dialysis, the nuclei extracts were aliquoted and
stored at –70°C.

The DNA-protein binding reactions (15 μl) contained 5000
cpm of 32P-labeled target DNA, 2 μg of poly(dI-dC), 5 μg of
nuclear protein, 25 mM Hepes, pH 8.0, 40 mM KCl, 5 mM
MgCl2, 1 mM DTT, 1 mM EDTA, and 8% glycerol. The binding
reaction was incubated at 4°C for 30 min. Reaction mixtures
were then separated by electrophoresis on 8% polyacrylamide
gels in 0.5× TBE buffer. Gels were run at 10 V per cm for
2 hr and exposed to X-ray film at –80°C overnight.

RESULTS

Establishing a system for analysis of Tagl cis-acting
sequences: We wished to determine which Tagl se-
quenences are sufficient for efficient and regulated exci-
sion of Tag1 in Arabidopsis. Our initial strategy was to
test a series of Tag1 deletion derivatives (dTag1 ele-
ments) in Arabidopsis plants of the Landsberg (erecta)
ecotype (hereafter referred to as Landsberg). Lands-
berg has two endogenous Tag1 elements that provide
transposase function but only after activation by trans-
formation of plants with dTag1 DNA (Liu and Crawford
1998b). We introduced dTag1 elements into Landsberg via Agrobacterium-mediated transformation
of whole plants. As described above, the first construct
tested was a 2-kb dTag1 element inserted between a
CaMV 35S promoter and GUS marker gene (see Figure
1 for a schematic diagram of this construct, pTG3). This
element excises in Landsberg to produce small GUS
sectors indicative of late excision (Frank et al. 1997; Liu
and Crawford 1998a). The size of these sectors showed
that the timing of excision for the 2-kb element is late
and very similar to that observed for the autonomous
Tag1 element in the same 35S-GUS plasmids (Liu and
Crawford 1998a). Thus, the 2-kb dTag1 element con-
tains sufficient sequence information for development-
ally regulated transposition.

We next made a 532-bp dTag1 element (Figure 1, con-
struct pTG-A) containing only 262-bp sequences from the
5′ end and 270 bp from the 3′ end of Tag1, which includes
the inverted and subterminal repeats. This dTag1 was transformated into Landsberg using the
35S-GUS marker construct (construct pTG-A in Figure
1). Eighteen transgenic plants were generated, but none
showed any GUS sectors. Northern blot hybridizations
failed to detect Tag1 transcripts in any of the 18 trans-
genic lines (data not shown). These results suggested
that the inability of the 532-bp dTag1 to excise in these
lines was due to a failure to activate the endogenous
Tag1 elements. However, a lack of a required cis-acting
sequence in the dTag1 element could also explain the
results. To test these possibilities, we developed another
method for supplying transposase activity for our Tag1
defecton mutants.

Instead of relying on the endogenous elements of
Landsberg to provide transposase function, we intro-

Arabidopsis Transposon Tag1 819
duced an intact Tag1 element into Arabidopsis plants of the Columbia background, which have no endogenous Tag1 elements (Tsay et al. 1993). The Tag1 element was inserted between a 35S promoter and an SPT (construct pTS in Figure 1) so that excision of the intact Tag1 could be monitored independently of dTag1 elements in the GUS vectors. The SPT system has been used to monitor germinal and somatic excision of Ac in Arabidopsis (Dean et al. 1992). Germinal excision of Tag1 should lead to streptomycin-resistant plants appearing as fully green seedlings on agar plates containing streptomycin while untransformed seedlings become completely bleached (Figure 2A). One can also detect green somatic sectors in cotyledons using this system (Figure 2B). Eleven transgenic plants in the Columbia background carrying the 35S-Tag1-SPT construct were generated. Evidence for both somatic (Figure 2B) and germinal (Figure 2A) excision events was observed in the progeny of these plants. Northern blot analysis showed that 35S-Tag1-SPT transgenic plants produced the typical pattern of Tag1 mRNA transcripts (data not shown). These results demonstrated that the 35S-Tag1-SPT plants could now be used as a source of transposase for testing our dTag1 constructs.

One line (TS28) was selected that was homozygous Landsberg plants is due to its inability to activate the two endogenous Tag1 elements, rather than to its lacking for four copies of the 35S-Tag1-SPT construct at a single locus (based on analysis of Southern blots and segregation ratios of kanamycin-sensitive to kanamycin-resistant progeny, data not shown). This line has a germinial excision frequency of 5%. This line was crossed with four plants carrying the 2-kb dTag1 construct pTG-3 in the Columbia background. Recall that the 2-kb dTag1 element can be mobilized when transformed directly into Landsberg plants, but by itself shows no GUS sectors in Columbia plants (Figure 2C). The F1 plants from the crosses showed GUS sectors in all plant organs examined (roots, cotyledons, leaves, flowers, and siliques; Figure 2, D–G, and data not shown) indicating that insertional mobilization of the dTag1 element. As evidenced by the tiny sectors, the timing of dTag1 excision in shoot organs was late in shoot development. The F1 plants were selfed to produce F2 seeds, and germinal revertants staining completely blue were identified. These results indicate that Tag1 in the 35S-Tag1-SPT transgenic plants will mobilize a defective element leading to developmentally controlled somatic excision.

The transposase activity in the TS28 35S-Tag1-SPT plants was tested next with the 532-bp dTag1 element (pTG-A, Figure 1, which showed no activity when transformed directly into Landsberg plants). When five plants carrying this dTag1 element were crossed to TS28, the F1 progeny from four of the crosses showed GUS sectors that were very small (Figure 2J and data not shown). This line has a germinial excision frequency of 5%. This line was crossed with four plants carrying the 2-kb dTag1 construct pTG-3 in the Columbia background. Recall that the 2-kb dTag1 element can be mobilized when transformed directly into Landsberg plants, but by itself shows no GUS sectors in Columbia plants (Figure 2C). The F1 plants from the crosses showed GUS sectors in all plant organs examined (roots, cotyledons, leaves, flowers, and siliques; Figure 2, D–G, and data not shown) indicating that insertional mobilization of the dTag1 element. As evidenced by the tiny sectors, the timing of dTag1 excision in shoot organs was late in shoot development. The F1 plants were selfed to produce F2 seeds, and germinal revertants staining completely blue were identified. These results indicate that Tag1 in the 35S-Tag1-SPT transgenic plants will mobilize a defective element leading to developmentally controlled somatic excision.

The transposase activity in the TS28 35S-Tag1-SPT plants was tested next with the 532-bp dTag1 element (pTG-A, Figure 1, which showed no activity when transformed directly into Landsberg plants). When five plants carrying this dTag1 element were crossed to TS28, the F1 progeny from four of the crosses showed GUS sectors that were very small (Figure 2J and data not shown). This line has a germinial excision frequency of 5%. This line was crossed with four plants carrying the 2-kb dTag1 construct pTG-3 in the Columbia background. Recall that the 2-kb dTag1 element can be mobilized when transformed directly into Landsberg plants, but by itself shows no GUS sectors in Columbia plants (Figure 2C). The F1 plants from the crosses showed GUS sectors in all plant organs examined (roots, cotyledons, leaves, flowers, and siliques; Figure 2, D–G, and data not shown) indicating that insertional mobilization of the dTag1 element. As evidenced by the tiny sectors, the timing of dTag1 excision in shoot organs was late in shoot development. The F1 plants were selfed to produce F2 seeds, and germinal revertants staining completely blue were identified. These results indicate that Tag1 in the 35S-Tag1-SPT transgenic plants will mobilize a defective element leading to developmentally controlled somatic excision.

The transposase activity in the TS28 35S-Tag1-SPT plants was tested next with the 532-bp dTag1 element (pTG-A, Figure 1, which showed no activity when transformed directly into Landsberg plants). When five plants carrying this dTag1 element were crossed to TS28, the F1 progeny from four of the crosses showed GUS sectors that were very small (Figure 2J and data not shown). This line has a germinial excision frequency of 5%. This line was crossed with four plants carrying the 2-kb dTag1 construct pTG-3 in the Columbia background. Recall that the 2-kb dTag1 element can be mobilized when transformed directly into Landsberg plants, but by itself shows no GUS sectors in Columbia plants (Figure 2C). The F1 plants from the crosses showed GUS sectors in all plant organs examined (roots, cotyledons, leaves, flowers, and siliques; Figure 2, D–G, and data not shown) indicating that insertional mobilization of the dTag1 element. As evidenced by the tiny sectors, the timing of dTag1 excision in shoot organs was late in shoot development. The F1 plants were selfed to produce F2 seeds, and germinal revertants staining completely blue were identified. These results indicate that Tag1 in the 35S-Tag1-SPT transgenic plants will mobilize a defective element leading to developmentally controlled somatic excision.

The transposase activity in the TS28 35S-Tag1-SPT plants was tested next with the 532-bp dTag1 element (pTG-A, Figure 1, which showed no activity when transformed directly into Landsberg plants). When five plants carrying this dTag1 element were crossed to TS28, the F1 progeny from four of the crosses showed GUS sectors that were very small (Figure 2J and data not shown). This line has a germinial excision frequency of 5%. This line was crossed with four plants carrying the 2-kb dTag1 construct pTG-3 in the Columbia background. Recall that the 2-kb dTag1 element can be mobilized when transformed directly into Landsberg plants, but by itself shows no GUS sectors in Columbia plants (Figure 2C).
These constructs were cloned into the 35S-GUS vector and then transformed into Columbia plants. These plants (eight primary transgenic lines per construct) were then crossed to TS28, which provides Tag1 transposase, and GUS sectors were analyzed in the F1 plants.

The result from these crosses was that no GUS sectors were observed in any of the F1 plants (data not shown). To verify that no excision was occurring, primers flanking the dTag1 insertion sites were used for PCR analysis of DNAs from F1 hybrid seedlings containing both Tag1 and dTag1. No excision products were detected (data not shown). These results indicate that dTag1 elements smaller than about 500 bp cannot be mobilized by an autonomous Tag1 element.

These experiments had the complication that when these smaller dTag1 elements were tested in the 35S-GUS vectors, significant background or diffuse GUS staining was observed in primary transformants, which

**Figure 2.**—Transposition behavior of Tag1 and dTag1 elements in transgenic plants. (A) Seedlings of plant TS28 with 35S-Tag1-SPT construct grown on streptomycin-containing medium. Tag1 germinal revertants are fully green seedlings. Plants retaining the Tag1 element are bleached. (B) A close-up of a bleached seedling showing somatic sectors (green spots indicated by arrows). (C) Seedling containing pTG3 construct (35S-dTag1-GUS) in Columbia stained for GUS activity. (D–G) TS28 plants (containing transposase) were crossed to plants with pTG3 [35S-dTag1 (2 kb)-GUS], and F1 progeny were stained for GUS activity to assay for dTag1 excision. Various organs of F1 are shown: (D) seedling, (E) leaf, (F) flower, and (G) silique. (H and I) Leaves from two primary transgenic plants containing the construct pTG-A [35S-dTag1 (532 bp)-GUS] were stained for GUS activity and showed background staining. (J) An F1 hybrid seedling from a cross between TS28 and the pTG-A containing plant was stained for GUS activity and showed dTag1 sectors on a blue background.

**Figure 3.**—Schematic diagram of small dTag1 constructs that were inactive. Top line shows the 5' and 3' ends (about 300 bp each) of Tag1 with the position of the subterminal repeats shown using the following symbols: (□) repeat AAACCC, (△) repeat TTATT, (○) repeat TATATA, (■) repeat TGACCC. The dTag1 constructs are shown using the same symbols as in Figure 1 and with the size of the element shown in parentheses and its excision activity shown at far right. The bottom shows the T-DNA vector used for plant transformation. Symbols are as described in Figure 1 legend.
TABLE 1
Summary of diffusive GUS staining phenotype of transgenic plants carrying 35S-dTag1-GUS constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Size of dTag1</th>
<th>No. of lines examined</th>
<th>No. of lines showing diffusive GUS staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTG-A</td>
<td>532 bp</td>
<td>14</td>
<td>5 (36%)</td>
</tr>
<tr>
<td>pTG-B</td>
<td>444 bp</td>
<td>24</td>
<td>22 (91%)</td>
</tr>
<tr>
<td>pTG-C</td>
<td>268 bp</td>
<td>22</td>
<td>19 (81%)</td>
</tr>
<tr>
<td>pTG-D</td>
<td>438 bp</td>
<td>24</td>
<td>17 (70%)</td>
</tr>
<tr>
<td>pTG-E</td>
<td>371 bp</td>
<td>24</td>
<td>19 (79%)</td>
</tr>
<tr>
<td>pTG29</td>
<td>1.28 kb</td>
<td>24</td>
<td>4 (16%)</td>
</tr>
<tr>
<td>pTG30</td>
<td>1.21 kb</td>
<td>24</td>
<td>2 (8%)</td>
</tr>
<tr>
<td>pTG32</td>
<td>1.07 kb</td>
<td>24</td>
<td>5 (13%)</td>
</tr>
<tr>
<td>pTG33</td>
<td>1.00 kb</td>
<td>24</td>
<td>4 (16%)</td>
</tr>
<tr>
<td>pTG34</td>
<td>0.95 kb</td>
<td>24</td>
<td>1 (4%)</td>
</tr>
<tr>
<td>pTG35</td>
<td>1.12 kb</td>
<td>24</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>pTG36</td>
<td>1.16 kb</td>
<td>24</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>pTG-0.5</td>
<td>0.70 kb</td>
<td>24</td>
<td>5 (21%)</td>
</tr>
<tr>
<td>pTG-0.9</td>
<td>1.10 kb</td>
<td>24</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>pTG-5.0</td>
<td>5.20 kb</td>
<td>20</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>pTG-5F</td>
<td>1.40 kb</td>
<td>24</td>
<td>5 (21%)</td>
</tr>
<tr>
<td>pTG-3F</td>
<td>1.40 kb</td>
<td>24</td>
<td>3 (13%)</td>
</tr>
</tbody>
</table>

could be due to a number of factors including “read through” translation. Fortunately, the background staining did not usually obscure visualization of GUS sectors, as can be seen in plants containing the 532-bp dTag1 element (Figure 2J). In our crosses with the TS28 line, we tested 19 dTag1-containing plants with diffusive staining and 13 with no staining. In all cases, no GUS sectors were observed. Therefore, all our data indicate that no excision was occurring for elements <445 bp.

Mineral sequence and spacing requirements for dTag1 excision: The failure of Tag1 to mobilize dTag1 elements smaller than 445 bp could be due to one of two reasons: a necessary sequence is missing in these constructs or all the necessary sequences are present but the distance between the 5’ and 3’ ends is not sufficient. We examined these possibilities by making two additional sets of deletion derivatives that contained a spacer sequence between the two subterminal repeat regions of Tag1. Figure 4 shows the various constructs and the relative positions of repetitive sequence motifs. Within the 5’ subterminal region, a motif with a consensus sequence of AAACCC is repeated 12 times in both direct and inverse orientations. Within the 3’ subterminal region, the first motif (TTATT) is repeated 14 times; the second (TATATA) and third (TGACCC) are repeated 4 times each in same orientation. The first set of deletion mutants we made retained the 0.9-kb Tag1 sequence at the 3’ end fused to various deletion fragments of the 5’ end (Figure 4, construct pTG32, pTG33, and pTG34). Twenty-four independent primary trans-

![Figure 4](image-url)

**Figure 4.**—Schematic diagram of larger dTag1 constructs. A 0.9- to 1.1-kb fragment from either the 5’ or 3’ end of Tag1 was ligated with various deletion derivatives and inserted into a T-DNA vector and transformed into Columbia. The activity of the dTag1 elements in these constructs was tested by crossing plants to TS28 (containing transposase). Whether or not excision activity was observed is given on the right of the figure. Positions of subterminal repeats are shown schematically above each set of deletion derivatives. Symbols are as described in Figure 1 legend.
TABLE 2

Somatic and germinal excision frequencies of F1 hybrids from various 35S-dTag1-GUS lines crossed with 35S-Tag1-SPT plants

<table>
<thead>
<tr>
<th>Line</th>
<th>KanR:KanS (selfed progeny)</th>
<th>F1 hybrid seedling GUS(+) GUS(−)</th>
<th>Excision frequency in leaves</th>
<th>Germinal excision frequency (%)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTG29-3</td>
<td>3:1</td>
<td>9</td>
<td>M, M, M</td>
<td>6, 6, 5</td>
<td>5.6</td>
</tr>
<tr>
<td>pTG29-4</td>
<td>50:1</td>
<td>10</td>
<td>L, L, L</td>
<td>11, 8, 7</td>
<td>8.6</td>
</tr>
<tr>
<td>pTG29-12</td>
<td>3:1</td>
<td>12</td>
<td>L, L, L</td>
<td>11, 7, 6</td>
<td>8.0</td>
</tr>
<tr>
<td>pTG29-13</td>
<td>3:1</td>
<td>10</td>
<td>L, L, L</td>
<td>0, 0, 0</td>
<td>0</td>
</tr>
<tr>
<td>pTG29-14</td>
<td>3:1</td>
<td>13</td>
<td>M, M, H</td>
<td>1, 4, 3</td>
<td>2.6</td>
</tr>
<tr>
<td>pTG29-16</td>
<td>40:1</td>
<td>13</td>
<td>H, H, H</td>
<td>13, 11, 11</td>
<td>12.3</td>
</tr>
<tr>
<td>pTG29-23</td>
<td>3:1</td>
<td>25</td>
<td>H, H, M</td>
<td>5, 7, 8</td>
<td>6.3</td>
</tr>
<tr>
<td>pTG29-24</td>
<td>5:1</td>
<td>28</td>
<td>L, L, L</td>
<td>0, 0, 0</td>
<td>0</td>
</tr>
<tr>
<td>pTG30-3</td>
<td>10:1</td>
<td>14</td>
<td>H, H, M</td>
<td>5, 4, 5</td>
<td>4.6</td>
</tr>
<tr>
<td>pTG30-5</td>
<td>3:1</td>
<td>19</td>
<td>M, M, M</td>
<td>4, 7, 6</td>
<td>5.6</td>
</tr>
<tr>
<td>pTG30-9</td>
<td>3:1</td>
<td>7</td>
<td>M, M, M</td>
<td>11, 12, 12</td>
<td>11.6</td>
</tr>
<tr>
<td>pTG30-12</td>
<td>5:1</td>
<td>26</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>pTG30-13</td>
<td>3:1</td>
<td>6</td>
<td>L, L, M</td>
<td>5, 6, 6</td>
<td>5.6</td>
</tr>
<tr>
<td>pTG30-17</td>
<td>3:1</td>
<td>9</td>
<td>M, M, H</td>
<td>6, 0, 4</td>
<td>3.3</td>
</tr>
<tr>
<td>pTG30-21</td>
<td>3:1</td>
<td>5</td>
<td>M, M, M</td>
<td>5, 5, 4</td>
<td>4.6</td>
</tr>
<tr>
<td>pTG29-23</td>
<td>8:1</td>
<td>9</td>
<td>L, M, M</td>
<td>10, 9, 9</td>
<td>9.6</td>
</tr>
<tr>
<td>pTG30-3</td>
<td>30:1</td>
<td>36</td>
<td>H, H, M</td>
<td>13, 10, 11</td>
<td>11.3</td>
</tr>
<tr>
<td>pTG30-7</td>
<td>3:1</td>
<td>5</td>
<td>H, M, M</td>
<td>15, 16, 13</td>
<td>14.6</td>
</tr>
<tr>
<td>pTG30-10</td>
<td>20:1</td>
<td>11</td>
<td>L, L, L</td>
<td>9, 12, 16</td>
<td>12.3</td>
</tr>
<tr>
<td>pTG30-15</td>
<td>150:1</td>
<td>23</td>
<td>L, M, M</td>
<td>0, 9, 2</td>
<td>3.6</td>
</tr>
<tr>
<td>pTG30-19</td>
<td>4:1</td>
<td>15</td>
<td>L, L, L</td>
<td>4, 7, 6</td>
<td>5.6</td>
</tr>
<tr>
<td>pTG30-23</td>
<td>3:1</td>
<td>18</td>
<td>L, L, L</td>
<td>12, 12, 11</td>
<td>11.6</td>
</tr>
<tr>
<td>pTG30-27</td>
<td>3:1</td>
<td>12</td>
<td>L, L, L</td>
<td>6, 5, 6</td>
<td>5.6</td>
</tr>
<tr>
<td>pTG33-2</td>
<td>7:1</td>
<td>20</td>
<td>M, M, H</td>
<td>5, 6, 3</td>
<td>4.6</td>
</tr>
<tr>
<td>pTG33-3</td>
<td>3:1</td>
<td>15</td>
<td>L, L, M</td>
<td>0, 1, 3</td>
<td>1.3</td>
</tr>
<tr>
<td>pTG33-4</td>
<td>25:1</td>
<td>15</td>
<td>M, M, H</td>
<td>5, 8, 3</td>
<td>5.3</td>
</tr>
<tr>
<td>pTG33-6</td>
<td>6:1</td>
<td>16</td>
<td>L, L, L</td>
<td>3, 0, 0</td>
<td>1.0</td>
</tr>
<tr>
<td>pTG33-14</td>
<td>5:1</td>
<td>9</td>
<td>M, M, H</td>
<td>7, 5, 10</td>
<td>7.3</td>
</tr>
<tr>
<td>pTG33-18</td>
<td>2:1</td>
<td>15</td>
<td>H, H, M</td>
<td>0, 0, 2</td>
<td>0.6</td>
</tr>
<tr>
<td>pTG33-19</td>
<td>16:1</td>
<td>17</td>
<td>M, M, M</td>
<td>10, 12, 12</td>
<td>11.3</td>
</tr>
</tbody>
</table>

H, >300 sectors/leaf; M, 50–300 sectors/leaf; L, <50 sectors/leaf; ND, none detected.

formants were prepared for each construct, and 7 with no background staining were selected for crossing to TS28 plants. The second set of derivatives retained the 1.1-kb sequence at the 5′ end fused to various fragments of the 3′ end (Figure 4, construct pTG29, pTG30, pTG35, and pTG36). Sixteen to 24 independent transgenic plants were prepared for each construct, and then 6 to 8 with no background staining were selected for crossing to TS28, which contains the Tag1 transposase. After crossing, the GUS activity in all F1 seedlings was examined.

The results of this experiment (Table 2) show that the smallest fragments capable of supporting excision are the 98-bp fragment at the 5′ end and the 109-bp fragment at the 3′ end (see Figure 4, constructs pTG33 and pTG30). The next smallest fragments tested, 52 bp at the 5′ end and 55 bp at the 3′ end, did not show any activity in vivo (constructs pTG34 and pTG35, Figure 3). The functional 98-bp fragment at the 5′ end contains four copies of the repetitive sequence AAACCX (X = C, G, A). The nonfunctional 55-bp 5′ end fragment contains only a single copy of the repetitive sequence. At the 3′ end, the functional fragment contained all four copies of the last repeat sequence TGACCC. The two nonfunctional fragments contained no 3′ repeat sequence and one had only the 22-bp inverted repeat sequence (pTG35 and pTG36). These results show that the inverted repeat alone is not sufficient for excision.

The results shown in Figure 4 also indicate that the small, inactive dTag1 elements shown in Figure 3 do have all the cis-acting sequences needed for excision. Therefore, it is likely that a minimal spacing between the two end fragments is required for excision. To test this idea, three constructs were made that had increasing lengths of non-Tag1 spacer DNA inserted between the 98- and 109-bp end fragments of Tag1 (Figure 5). Transgenic plants containing these DNAs cloned into the 35S-GUS vector were crossed to the TS28 line. All
Constructs showed excision activity (Figure 5). These results, including those from Figure 3, demonstrate that a minimal spacer of 238–325 bp separating the two end fragments is required for excision. A 5-kb spacer also supports excision (Figure 5). Consistent with results described above, the length of the spacer also affected the percentage of primary transformants showing diffuse, background GUS staining. The larger the spacer was, the lower the percentage of background staining plants (Table 1).

5’ and 3’ minimal sequences of dTag1 are not interchangeable: Most plant transposable elements, including Ac, Spm, and Mutator, have the same or very similar sequences at their 5’ and 3’ ends; nevertheless, the 5’ and 3’ end fragments are not functionally interchangeable (Coupland et al., 1989; Gierl, 1996). Tag1 has two different repeated sequences at its 5’ and 3’ ends. We tested if the 5’ and 3’ end fragments of Tag1 were interchangeable. Two constructs were made (Figure 5). The first (pTG-5F) contained duplicated 5’ ends with the 1.1-kb 5’ fragment fused to the 270-bp 5’ fragment. The second (pTG-3F) had duplicated 3’ ends using the 270-bp 3’ fragment and the 0.9-kb 3’ fragment. Six independent transformants for each construct were crossed to TS28 plants containing the Tag1 transposase. GUS staining of F1 plants showed no sectors. These results indicate that 5’ and 3’ sequences are not functionally interchangeable.

Somatic and germinal excision properties of dTag1 elements: The distinctive behavior of Tag1 excision from 35S-Tag1-GUS constructs during shoot development, which produced tiny somatic sectors and independent germinal revertants, indicates that excision is developmentally regulated in these lines (Liu and Crawford, 1998a). In contrast, the frequency of Tag1 excision was quite variable, with germinal excision rates varying from 0 to 27% (Liu and Crawford, 1998a). We wished to determine if the dTag1 derivatives also displayed developmentally regulated excision and a similar range of excision frequencies to that of Tag1. Plants containing each dTag1 derivative were crossed to TS28 plants containing Tag1 transposase. To examine somatic excision frequency, GUS sector number was determined on leaf 3 or 4 in three F1 plants from each TS28 cross and then classified as high (>300 sectors per leaf), medium (50–300 sectors per leaf), and low (<50 sectors per leaf) as described previously (Liu and Crawford, 1998a). To determine germinal excision frequency, three F1 plants showing somatic sectors were allowed to self, and then the percentage of whole blue staining seedlings were counted in the F2 generation.

Our data (Tables 2 and 3) show that dTag1 elements with the minimal end sequences and within a specific size range show very similar somatic excision frequencies compared with the autonomous Tag1 element (Liu and Crawford, 1998a). The number of somatic sectors for the simple deletion derivatives is variable from line to line, but the range and average are typical for Tag1 (Table 2). However, as described above, if the size of the dTag1 element is 440 bp or less, no excision was observed (Figure 3). For the dTag1 elements containing spacer DNA, typical ranges and averages of excision frequencies for the pTG0.5 and pTG0.9 elements (0.7 and 1.1 kb in size, respectively) were observed (Table 3). For the 5.2-kb element pTG5.0, however, the somatic excision frequency was much lower (Table 3). This reduction could be due to the size of the spacer or to an inhibitory sequence within the DNA. In conclusion, there appears to be no correlation between somatic excision frequency and the length or sequence of the dTag1 elements for elements between 0.7 and 1.2 kb. If the autonomous 3.3-kb Tag1 element is included, the range of lengths that accommodate typical excision rates is from 0.7 to 3.3 kb.

When germinal excision of dTag1 was examined, similar ranges and averages of excision rates were found for dTag1 elements between 0.7 and 1.2 kb in length (0–16%; Tables 2 and 3), and these values matched those for the autonomous 3.3-kb Tag1 element (Liu and Crawford, 1998a). The average rate for all the active dTag1 elements tested (minus the pTG5.0 element) was 5.8%. This value is very close to the 5.3%
TABLE 3

Somatic and germinal excision frequencies of F1 hybrids resulting from plants with 35S-dTag1-GUS lines with spacers crossed with 35S-Tag1-SPT plants

<table>
<thead>
<tr>
<th>Line</th>
<th>KanR:KanS (selfed progeny)</th>
<th>F1 hybrid seedling</th>
<th>Excision frequency in leaves</th>
<th>Germinal excision frequency (%)</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTG0.5-4</td>
<td>25:1</td>
<td>5</td>
<td>M, H, M</td>
<td>2, 0, 3</td>
<td>1.6</td>
</tr>
<tr>
<td>pTG0.5-6</td>
<td>30:1</td>
<td>17</td>
<td>M, M, M</td>
<td>3, 5, 2</td>
<td>3.3</td>
</tr>
<tr>
<td>pTG0.5-8</td>
<td>3:1</td>
<td>20</td>
<td>H, H, H</td>
<td>10, 11, 12</td>
<td>11.0</td>
</tr>
<tr>
<td>pTG0.5-10</td>
<td>3:1</td>
<td>8</td>
<td>H, M, H</td>
<td>10, 4, 6</td>
<td>6.6</td>
</tr>
<tr>
<td>pTG0.5-21</td>
<td>2:1</td>
<td>12</td>
<td>H, M, H</td>
<td>2, 4, 2</td>
<td>2.6</td>
</tr>
<tr>
<td>pTG0.9-3</td>
<td>150:1</td>
<td>17</td>
<td>L, L, M</td>
<td>6, 4, 5</td>
<td>5.0</td>
</tr>
<tr>
<td>pTG0.9-4</td>
<td>40:1</td>
<td>15</td>
<td>L, M, M</td>
<td>12, 8, 3</td>
<td>5.6</td>
</tr>
<tr>
<td>pTG0.9-6</td>
<td>65:1</td>
<td>21</td>
<td>M, M, M</td>
<td>13, 2, 4</td>
<td>6.3</td>
</tr>
<tr>
<td>pTG0.9-8</td>
<td>170:1</td>
<td>21</td>
<td>M, M, M</td>
<td>9, 0, 0</td>
<td>3.0</td>
</tr>
<tr>
<td>pTG0.9-9</td>
<td>3:1</td>
<td>12</td>
<td>M, M, M</td>
<td>5, 4, 7</td>
<td>5.3</td>
</tr>
<tr>
<td>pTG0.9-11</td>
<td>3:1</td>
<td>13</td>
<td>L, M, M</td>
<td>8, 5, 4</td>
<td>5.6</td>
</tr>
<tr>
<td>pTG0.9-12</td>
<td>1:1</td>
<td>15</td>
<td>M, M, M</td>
<td>1, 3, 5</td>
<td>3.0</td>
</tr>
<tr>
<td>pTG5.0-1</td>
<td>3:1</td>
<td>1</td>
<td>L, L, L</td>
<td>0, 1, 0</td>
<td>0.3</td>
</tr>
<tr>
<td>pTG5.0-2</td>
<td>3:1</td>
<td>0</td>
<td>0, 0, 0</td>
<td>0, 1, 2</td>
<td>1.0</td>
</tr>
<tr>
<td>pTG5.0-3</td>
<td>3:1</td>
<td>16</td>
<td>L, L, L</td>
<td>0, 0, 1</td>
<td>0.3</td>
</tr>
<tr>
<td>pTG5.0-4</td>
<td>95:1</td>
<td>9</td>
<td>L, L, L</td>
<td>3, 2, 0</td>
<td>1.7</td>
</tr>
<tr>
<td>pTG5.0-5</td>
<td>165:1</td>
<td>2</td>
<td>0, 0, 0</td>
<td>1, 0, 1</td>
<td>0.7</td>
</tr>
<tr>
<td>pTG5.0-6</td>
<td>3:1</td>
<td>1</td>
<td>L, L, L</td>
<td>1, 2, 1</td>
<td>1.3</td>
</tr>
</tbody>
</table>

H, >300 sectors/leaf; M, 50–300 sectors/leaf; L, <50 sectors/leaf; ND, none detected.

average value determined for Tag1. The latter value is based on the germinal excision rates observed for the original 47 Tag1 transgenic lines reported previously (Liu and Crawford 1998a) as well as an additional 170 Tag1 lines constructed more recently (data not shown). As was the case for somatic excision, no correlation was observed between germinal excision frequency and the sequence or length of the dTag1 element within the size range given above (Tables 2 and 3). The 5.2-kb element pTG5.0 and elements of length 440 bp or less showed no or much reduced germinal excision. We conclude that there is no obvious difference in germinal excision frequencies between the 3.3-kb Tag1 and the 0.7- to 1.2-kb dTag1 elements.

The timing of dTag1 excision in all F1 plants was examined next. For the simple deletion dTag1 constructs pTG29, 30, 32, and 33 and for the dTag1 elements with spacers pTG0.5, 0.9, and 5.0, excision timing was similar to that of Tag1. In roots and cotyledons, GUS cis sequence still retain their ability to reinsert into the genome after excision. Thirteen germinal revertants were selected from an F2 population produced by crossing TS28 with plants containing the dTag1 construct pTG-0.9. The 0.9-kb spacer sequence within this dTag1 element was used as probe. The 0.9-kb spacer was a randomly selected fragment from the Arabidopsis genome. When the spacer DNA is hybridized with genomic DNA from the TS28 parent digested with XbaI, which does not cut the dTag1 element but does cut once in the T-DNA, five bands appear (Figure 6, lane 2). The top band corresponds to the spacer DNA in its native

The 8-bp duplicated target sequence flanking Tag1 does not affect excision frequency and timing: Tag1 generates an 8-bp duplication when it inserts into the genome. All the dTag1 constructs tested in this study were flanked by an 8-bp direct repeat of target sequence. To test if these flanking sequences have any impact on dTag1 excision frequency and timing, a derivative of the pTG3 2-kb element (Figure 1) was made that lacked the 8-bp repeat. This construct was introduced into Columbia plants and four independent transgenic lines were crossed to TS28 line. The resultant F1 plants showed excision timing and range of frequencies similar to those of plants containing pTG3 (data not shown). Thus, the target duplication has no apparent impact on dTag1 excision similar to what was shown for Ac (Dooner et al. 1988).

Reinsertion of dTag1 elements into the plant genome: Southern blot and sequence analyses were performed to determine if dTag1 elements with minimal 5' and 3' cis sequence still retain their ability to reinsert into the genome after excision. Thirteen germinal revertants were selected from an F2 population produced by crossing TS28 with plants containing the dTag1 construct pTG-0.9. The 0.9-kb spacer sequence within this dTag1 element was used as probe. The 0.9-kb spacer was a randomly selected fragment from the Arabidopsis genome. When the spacer DNA is hybridized with genomic DNA from the TS28 parent digested with XbaI, which does not cut the dTag1 element but does cut once in the T-DNA, five bands appear (Figure 6, lane 2). The top band corresponds to the spacer DNA in its native
the dTag1 elements introduced into TS28. When DNA from the 13 germlinal revertants was examined, 8 were found to contain a new band indicative of a reinsertion event (Figure 6, lanes 4, 5, 7, 10–12, 14, and 15; in lane 15 the third “band” is a doublet on the original) and 5 had no new bands (Figure 6, lanes 3, 6, 8, 9, and 13). Based on the unique position of the new bands, most of the reinsertions appear to be independent. Those lines that did not have a new visible band could still have undergone a reinsertion event that was hidden under one of the other bands. To confirm that dTag1 was indeed reinserting into the genome, DNA flanking dTag1 reinsertions was amplified by TAIL PCR and then analyzed as described in materials and methods. DNA from 11 independent germlinal revertants was cloned and sequenced. Nine of these 11 sequences had flanking DNAs that had matches to Arabidopsis genomic clones deposited in GenBank (Table 4); the other two gave sequences identical to the T-DNA vector pBI121. The identified genomic clones (usually bacterial artificial chromosome clones) have all been mapped on the Arabidopsis genome (Table 4) and were found to be scattered on all five chromosomes (Figure 7). These results indicate that a dTag1 element with only 98 bp of 5’ DNA, 109 bp of 3’ DNA, and spacer DNA is capable of reinserting into the genome after its excision at about the same frequency observed for Tag1, which is approximately 75% as reported in Liu and Crawford (1998a).

Evidence for a host-encoded DNA-binding activity

position in the Arabidopsis genome (this band is also present in Columbia plants not containing the dTag1 construct, lane 1), and the bottom four correspond to

![Figure 6](image_url)

**Figure 6.**—Southern blot analysis of dTag1 reinsertions into the Arabidopsis genome. Genomic DNA was prepared from (lane 1) untransformed plants of Columbia ecotype (single band in lane is hybridization of probe to endogenous sequence present in Arabidopsis genome); (lane 2) the parental transgenic line (pTG0.9-11 Table 3) containing dTag1 elements in the 35S-GUS construct; and (lanes 3–15) germlinal revertants of pTG0.9-11 that inherited a dTag1 excision event (new bands represent reinsertions of dTag1). The revertants were identified among a pool of F2 progeny from a cross between line TS28 (containing Tag1 transposase) and a line identified genomic clones (usually bacterial artificial chromosome clones) have all been mapped on the Arabidopsis genome (Table 4) and were found to be scattered on all five chromosomes (Figure 7). These results indicate that a dTag1 element with only 98 bp of 5’ DNA, 109 bp of 3’ DNA, and spacer DNA is capable of reinserting into the genome after its excision at about the same frequency observed for Tag1, which is approximately 75% as reported in Liu and Crawford (1998a).

**TABLE 4**

Summary of pTG 0.9 reinsertion sites

<table>
<thead>
<tr>
<th>Revertant no.</th>
<th>Clone</th>
<th>Chromosome no. (position)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>T24P13</td>
<td>I (10.1 Mb)</td>
<td>5’-CATTGCTTGTTGCTTTTTTTGTCTTGTCTGGGA AAAGTTGATGAACTTTTTTTGCCTTAA-3’</td>
</tr>
<tr>
<td>R2</td>
<td>T31E10</td>
<td>II (15.6 Mb)</td>
<td>5’-CATTGTTATCACCTATTTTTTTTTTTTTTGGAGAAGT</td>
</tr>
<tr>
<td>R4</td>
<td>F2G14</td>
<td>V (7.4 Mb)</td>
<td>5’-CATTGCCCTTGGCTTTGGCTAGTGCTTTAAA-3’</td>
</tr>
<tr>
<td>R5</td>
<td>F16L2</td>
<td>III (17.5 Mb)</td>
<td>5’-CATTGTAATATCTAGTTATTTGAAAAACGACT</td>
</tr>
<tr>
<td>R8</td>
<td>T15K4</td>
<td>I (14.5 Mb)</td>
<td>5’-CATTGGTTATAGCTGATTTCTCTATATTGAATATAACCAGCTTTTTTGTCTGCTATT-3’</td>
</tr>
<tr>
<td>R9</td>
<td>AP21</td>
<td>IV (22.3 Mb)</td>
<td>5’-CATTGTTATATCCTCACTCTATAGCTTGGGCATTAATGCTTACATGCGTTGGAAAAGCATT</td>
</tr>
<tr>
<td>R11</td>
<td>F25I18</td>
<td>II (15.1 Mb)</td>
<td>5’-CATTGGTTATATCCTCACTCTATAGCTTGGGCATTAATGCTTACATGCGTTGGAAAAGCATT</td>
</tr>
<tr>
<td>R12</td>
<td>F316</td>
<td>I (9.4 Mb)</td>
<td>5’-CATTGGTTATATCCTCACTCTATAGCTTGGGCATTAATGCTTACATGCGTTGGAAAAGCATT</td>
</tr>
<tr>
<td>R14</td>
<td>F24P17</td>
<td>III (2.7 Mb)</td>
<td>5’-CATTGGTTATATCCTCACTCTATAGCTTGGGCATTAATGCTTACATGCGTTGGAAAAGCATT</td>
</tr>
</tbody>
</table>

*Clone position determined using TAIR map viewer and AGI maps located at www.arabidopsis.org/servlets/mapper.

*Nucleotides in plain text are genomic flanking sequences. Nucleotides in boldface text correspond to the last five nucleotides of Tag1 terminal inverted repeats according to sequencing results.

*The R8 insertion is located 80 bp 5’ of the start of the Arabinogalactan-protein (AGP5) mRNA sequence.
that interacts with the 3' but not 5' region of Tag1: Our above analysis shows that approximately 100 bp at both 5' and 3' ends of Tag1 element is sufficient for Tag1 transposition in a developmentally regulated manner. To begin examining the mechanism for the regulated excision, DNA-binding activities were assayed in protein extracts from Arabidopsis plants. Nuclear extracts were first prepared from leaves of untransformed Columbia plants, which contain no Tag1 elements. DNA binding was assayed by gel retardation methods using radiolabeled fragments from each end of Tag1. When these nuclear extracts were incubated with radiolabeled 109-bp 3' end fragment or 98-bp 5' end fragment, a DNA-protein complex was observed for the 3' fragment (Figure 8A, lane 2) but not with the 5' fragment (Figure 8A, lanes 4 and 5). Competition experiments confirmed that this DNA-binding activity is specific (Figure 8B). Next, the DNA-binding activity in the nuclear extracts made from line TS28, which contains active Tag1 elements, was tested. For the 5' fragment no binding activity could be detected. For the 3' fragment a DNA-protein complex with similar mobility to that from Tag1-minus nuclear extracts was observed, but its intensity was dramatically increased (Figure 8A, lane 3). Whether this stronger signal indicates another complex or a simple enhancement of the binding activity already existing in Tag1-free plants is unknown. These experiments provide evidence that a host-encoded factor(s) binds to the 3' end of Tag1. Because both 5' and 3' DNA probes contain the same 22-bp terminal inverted repeat and 8-bp duplicated target sequence, these results suggest that the DNA-binding factor does not simply bind to these sequences but must at least include unique sequence present in the 3' subterminal fragment.

DISCUSSION

The data from our deletion analysis show that end fragments of 98 bp at the 5' end and 109 bp at the 3' end are sufficient and required for excision and reinsertion of dTag1 in Arabidopsis plants at rates typical for the autonomous Tag1 element. These end fragments include the 22-bp inverted repeat and four copies of either the 5' subterminal repeat (AAACCX, where X = C, A, G in direct or inverse orientation) or the 3' subterminal repeat (TGACCC). Smaller end fragments of about 50 bp containing either one copy of the 5' subterminal repeat or no copies of the 3' subterminal repeat support no excision in Arabidopsis. To be active, the 98- and 109-bp end fragments must be separated by spacer DNA.

These findings shed light on the unusual repeat structure of Tag1. At the 3' end, which contains three sets of unrelated subterminal repeats, the most 3' repeat region (containing TGACCC) is sufficient for transposition. The other two 3' repeat regions are dispensable for dTag1 but may play a role for the intact element, such as serving as signals for processing the transposase.
mRNA. At the 5′ end a region with only 4 copies (out of 12) of the AAACCX repeat is required for efficient excision and reinsertion. The 5′ and 3′ end fragments are not interchangeable as duplication of either end leads to an inactive element as is the case for Ac (COPPLAND et al. 1989) and Spm (GIERL 1996). Our functional dTag1 elements displayed developmentally controlled timing of excision indicating that any cis-acting sequences involved in regulation of excision are embedded in the short ends of Tag1. This result is similar to what has been found for other elements where key methylation and regulatory sites of En/Spm reside in the first 0.55 kb of the element including the terminal 0.2-kb sequence (reviewed in FEDOROFF et al. 1995) and binding sites of the KP repressor in P elements overlap those of the transposase within the 31-bp inverted repeat and the 11-bp transpositional enhancer sequence (LEE et al. 1996, 1998).

Special emphasis is placed here on the 5′ and 3′ subterminal repeats of Tag1. On the basis of our in vivo data, we cannot state that they are the sequences to which the transposase binds, yet they are the most likely binding sites for the Tag1 transposase based on what is known about other eukaryotic transposons. As described above, Ac and En/Spm rely on subterminal repeats for transposase binding (GIERL et al. 1988; KUNZE and STARLINGER 1989; TRENTMANN et al. 1993; BECKER and KUNZE 1997). We have found for Tag1, which is related to Ac, that the parts of the subterminal repeat regions are critical for transposition. These regions contain repeats that are not identical, which is unusual. These repeats do, however, share the tetranucleotide sequence ACCC. Studies of Ac have shown that a repeated trinucleotide sequence can serve as a binding site (BECKER and KUNZE 1997); therefore, it is certainly possible that the Tag1 transposase may recognize the ACCC sequence. Alternatively, there may be two distinct recognition sequences for the Tag1 transposase: AAACCC at the 5′ end and TGACCC at the 3′ end. Further experiments testing the DNA-binding properties of the Tag1 transposase in vitro are required to determine the exact binding sequences of the Tag1 transposon.

We have shown that dTag1 elements have a strict minimal size requirement for efficient excision. At least 298–325 bp of DNA must be present between the 98-bp 5′ end and 109-bp 3′ end fragments of Tag1 for excision to occur. A dTag1 element of 532 bp showed normal excision rates while an element of 444 bp showed no excision even though it contained all the required cis-acting sequences. In contrast, a slightly larger construct, pTG0.5, which had the 98-bp 5′ end and 109-bp 3′ end fragments separated by 0.5-kb spacer DNA, displayed excision frequencies typical of the 3.3-kb element. If Tag1 excision occurs upon the formation of a synaptic complex held together by oligomers of transposase proteins, our results suggest that Tag1 has critical length requirements for efficient formation or resolution of such complexes. Perhaps a minimal length is required for binding DNA to form the synaptic complex. We are not aware of any reports describing minimal length requirements for eukaryotic transposons; however, DNA bending for formation of transposase-
DNA complexes has been shown, for example, for To3 (van Pouderoyne et al. 1997).

We have also found that there is an activity in crude nuclear extracts from Arabidopsis plants devoid of Tag1 and Tag1 mRNA. Binding activity is higher in plants that have active Tag1 elements. This higher activity could be due to (1) an increase in the plant-encoded activity that was induced by transformation or introduction of Tag1 or (2) the presence of Tag1 transposase-DNA complexes, which happen to migrate to the same position. Interestingly, this activity is specific for the 3′ 109-bp fragment and does not show any binding to the 5′ 98-bp fragment, which indicates that it is not binding exclusively to the inverted terminal repeat. In comparison, plant-encoded factors in both maize and tobacco have been found that bind to the subterminal repeat regions of Ac but not the transposase binding sites themselves (Becker and Kunze 1996; Levy et al. 1996). Two other host-encoded proteins have been described that bind to the ends of transposons but only within the inverted repeats: a Drosophila-encoded inverted repeat binding protein that binds to P elements DNA (Rio and Rubin 1988) and a maize-encoded protein that binds the ends of Mutator (Zhao and Sundaresan 1991). The function of these proteins has yet to be determined, but they are candidate regulatory factors that might control excision.

This work was supported by a grant from the National Science Foundation (MCB-9808215).

LITERATURE CITED


Liu, D., and N. M. Crawford, 1998b Characterization of the putative transposase mRNA of Tagl, which is ubiquitously expressed in Arabidopsis and can be induced by Agrobacterium-mediated transformation with dTagl DNA. Genetics 149: 693–701.


Raghothama, K. G., D. Liu, D. E. Nelson, P. M. Hasegawa and R. A. Bressan, 1993 Analysis of an osmotically regulated patho-


Communicating editor: V. L. Chandler