Tag1 Is an Autonomous Transposable Element That Shows Somatic Excision in Both Arabidopsis and Tobacco

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Tag1 is a transposable element first identified as an insertion in the CHL1 gene of Arabidopsis. The chl1::Tag1 mutant originated from a plant (ecotype Landsberg erecta) that had been transformed with the maize transposon Activator (Ac), which is distantly related to Tag1. Genomic analysis of untransformed Landsberg erecta plants demonstrated that two identical Tag1 elements are present in the Landsberg erecta genome. To determine what provides transposase function for Tag1 transposition, we examined Tag1 excision in different genetic backgrounds. First, the chl1::Tag1 mutant was backcrossed to untransformed wild-type Arabidopsis plants to remove the Ac element(s) from the genome. F2 progeny that had no Ac elements but still retained Tag1 in the CHL1 gene were identified. Tag1 still excised in these Ac-minus progeny producing CHL1 revertants; therefore, Ac is not required for Tag1 excision. Next, Tag1 was inserted between a cauliflower mosaic virus 35S promoter and a β-glucuronidase (GUS) marker gene and transformed into tobacco. Transformants showed blue-staining sectors indicative of Tag1 excision. Transgenic tobacco containing a defective Tag1 element, which was constructed in vitro by deleting an internal 1.4-kb EcoRI fragment, did not show blue-staining sectors. We conclude that Tag1 is an autonomous element capable of independent excision. The 35SGUS::Tag1 construct was then introduced into Arabidopsis. Blue-staining sectors were found in cotyledons, leaves, and roots, showing that Tag1 undergoes somatic excision during vegetative development in its native host.

INTRODUCTION

Transposons are mobile genetic elements that can comprise a substantial part of a plant’s genome (SanMiguel et al., 1996) and create tremendous phenotypic diversity (reviewed in Fedoroff and Botstein, 1992; Fiavelli et al., 1994; Saedler and Gierl, 1996). They also serve as valuable tools for plant developmental studies and for plant genome analysis, in which they have been used as insertional mutagens that “tag” genes and as vectors that mobilize reporter genes (reviewed in Altmann et al., 1992; Bancroft et al., 1992; Fedoroff and Smith, 1993; Honma et al., 1993; Aarts et al., 1995). These systems are modeled after naturally occurring transposons that exist as (1) autonomous elements capable of independent transposition or (2) defective or nonautonomous elements that require transposase function.

Arabidopsis has a family of copia-like retrotransposons that includes Ta1-10 (Voytas and Ausubel, 1988; Voytas et al., 1990; Konieczny et al., 1991) as well as two DNA transposon-like elements, Tat1 (Peleman et al., 1991) and Limpet1 (Klimyuk and Jones, 1997). These elements were discovered by examining genetic polymorphisms among different ecotypes of Arabidopsis. An unrelated element called Tag1 was found while screening for new mutations in the nitrate transporter gene CHL1 (Tsay et al., 1993). Mutations in CHL1 confer resistance to chlorate, a chlorine analog of nitrate that is taken up and reduced to toxic chlorite (Crawford and Arst, 1993; Hoff et al., 1994; Crawford, 1995). New chl1 mutants were found among progeny from Arabidopsis plants that had been transformed with the maize Activator (Ac) element. The Ac element was active in these lines, which were of the Landsberg erecta ecotype (Dean et al., 1992). The chlorate-resistant mutants had an insertion in the fourth intron of the CHL1 gene. This insertion was a 3.3-kb transposable element that we called Tag1.

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Tag1 was originally identified in a line of Arabidopsis (ecotype Landsberg erecta) that had been transformed with the maize element Ac. DNA gel blot analysis of untransformed Landsberg erecta plants showed that several DNA fragments hybridized with Tag1. These fragments could correspond to Tag1 or Tag1-related elements in the genome. Analysis of two other ecotypes, Columbia and Wassilewskija (WS), showed no hybridization with an internal 1.4-kb Tag1 probe and indicated no Tag1 elements in these ecotypes. In the CHL7 revertants, new Tag1-hybridizing bands appeared, indicating that at least some of the Tag1 or Tag1-related elements had moved to new locations. Thus, Tag1 is mobile and has the potential to generate insertion mutations useful for gene tagging.

Sequence analysis of Tag1 has revealed that it is a member of the Ac (or hAT) superfamily of transposable elements (Warren et al., 1994; Essers and Kunze, 1995). This family includes Ac and Bg from maize, Tam3 from Antirrhinum, hobo from Drosophila, Hermes from the housefly, and Slide from tobacco (Calvi et al., 1991; Warren et al., 1994; Essers and Kunze, 1995; Grappin et al., 1996). All of these elements duplicate 8 bp of genomic sequence when inserted and have a conserved signature sequence in a 30-amino acid region near the C terminus of the transposase. Tag1 also contains this signature sequence (Warren et al., 1994; Essers and Kunze, 1995). The fact that Tag1 is distantly related to Ac and that Tag1 transposition was observed in an Ac-containing line could be coincidental or could mean that Ac is needed for Tag1 transposition. The sequence of Tag1 did not reveal whether it might be capable of encoding a functional transposase, so the genetic autonomy of Tag1, whether it is defective or autonomous, has yet to be determined. Therefore, we examined the dependency of Tag1 excision on Ac in the chl1::Tag1 mutant lines. We also cloned and characterized all Tag1 and Tag1-related elements present in the Landsberg erecta genome and determined whether Tag1 could move autonomously in a heterologous host, tobacco. A β-glucuronidase (GUS)-based assay system was used to monitor Tag1 excision in both Arabidopsis and tobacco and to reveal any somatic excision that might occur during vegetative development. The results of these experiments are provided below.

RESULTS

Two Tag1 Elements Exist in the Genome of Landsberg erecta

Tag1 was originally identified in a line of Arabidopsis (ecotype Landsberg erecta) that had been transformed with the maize element Ac. DNA gel blot analysis of untransformed Landsberg erecta plants (containing no Ac) revealed multiple bands that hybridized with an internal 1.4-kb EcoRI fragment from Tag1 (Tsay et al., 1993). We wished to identify the autonomous element in this small family of elements and began by cloning all Tag1 or Tag1-related elements present in the Landsberg erecta genome.

Digestion of genomic DNA from Landsberg erecta with Clal or BamH1 revealed two bands when hybridized with the entire Tag1 element (Figure 1A). Because neither enzyme cuts within Tag1, this result indicates that there are two Tag1 or Tag1-related elements in the Landsberg erecta genome. Digestion of genomic DNA with BgIII produced five bands (Figure 1A; Tsay et al., 1993). Because BgIII has two restriction sites within Tag1, the appearance of five bands, one from an internal fragment and four from flanking sequences, also indicates two elements. Subsequent screening of 60,000 plaques of a Landsberg erecta genomic library with a complete Tag1 element as a probe produced 14 positive clones. Restriction analysis revealed that these clones represent only two elements, each with unique flanking sequences, as seen in the restriction maps (Figure 1B), and an 8-bp target site duplication (Figure 1C). Sequence analysis showed that both elements are identical to Tag1.

We distinguished the elements at different loci by naming them Tag1-2 and Tag1-3 (Figure 1C), reserving Tag1-1 for the original insertion at chl1. The Tag1-2 clone has three BgIII fragments (3.5, 2.5, and 1.0 kb), as does the Tag1-3 clone (5.2, 2.5, and 1.0 kb) (Figure 1B). All BgIII bands on the DNA gel blot can be accounted for by these genomic clones, except for the 6-kb band. The 6-kb band is most likely due to partial protection of the BgIII site in Tag1-3 (denoted by an asterisk in Figure 1B), producing a 6.2-kb band from the 5.2- and 1.0-kb fragments) because repeated digests of Landsberg erecta genomic DNA isolated by different procedures gave the same band pattern, as seen in Figure 1A, and probing such gel blots with another radiolabeled DNA, a CHL7 genomic fragment, showed complete digestion products (data not shown). We conclude that there are only two Tag1 elements in the Landsberg erecta background. Tag1 has been mapped to position 106.3 centimorgans near the bottom of chromosome 1 (see Lister and Dean, 1993; http://nasc.nott.ac.uk/new_ri_map.html); therefore, both elements must be located in this region.

Ac Transposase Is Not Required for Tag1 Excision

Because the original Tag1 transposition event (into the chl1 gene) was found in a line that contained both Ac and Tag1, it is possible that the Ac element(s) present in the genome provided the transposase function for Tag1. To test this possibility, Ac elements were removed from the genome by backcrossing to wild-type plants, and then Tag1 excision was examined. If Tag1 excision is dependent on Ac transposase, no excision should be observed in Ac-minus segregants.
Figure 1. Analysis of Tag1 Elements in the Landsberg erecta Genome.

(A) DNA gel blot analysis of Tag1. Total DNA from Arabidopsis ecotype Landsberg erecta was digested with the restriction enzyme BglII (BII), Clal (Cl), or BamHI (BH) and analyzed on a gel blot using the radiolabeled 3.3-kb Tag1 element as a probe. DNA markers are indicated at left.

(B) Restriction analysis of two Tag1 elements. DNA clones containing Tag1 were isolated by screening a Landsberg erecta genomic library with a radiolabeled 3.3-kb Tag1 element. Overlapping clones were placed into two groups corresponding to two Tag1 elements designated Tag1-2 and Tag1-3. Restriction maps for these elements and their flanking sequences are shown. A restriction map of the Tag1 element itself is shown at top. Restriction sites are indicated as follows: RI, EcoRI; BII, BglII.

(C) Sequences flanking the Tag1 elements. Target site duplications are shown for Tag1-1, which originally was isolated from CHL1, and for Tag1-2 and Tag1-3. The gray bars represent Tag1.
The *chl7::Tag1* homozygous mutant (ecotype Landsberg erecta) was backcrossed to untransformed Arabidopsis plants of the Columbia ecotype. These plants contain no detectable *Tag1* elements (Tsay et al., 1993). Individual *F2* segregants (from selfing the *F1* plants) that contained no *Ac* elements were identified by polymerase chain reaction (PCR) analysis, using primers specific to the *Ac* transposase region (oligonucleotides O94 and O93; see Methods and Figure 2A), and verified in the *F3* generation (produced by selfing the *F2* plants) by DNA gel blot analysis, using an *Ac* fragment (0.9-kb HindIII-EcoRI; Figure 2A) as a probe (data not shown). Next, chlorate-resistant *F3* plants were selected to ensure that *Tag1* was present in both *chl7* alleles. Resistant *F3* plants were allowed to self, seed was planted, and the resulting *F4* plants were examined for *Tag1* excision by using PCR analysis. Three primers were selected so that two PCR fragments could be generated in a single reaction: a 600-bp product if *Tag1* were present and a 200-bp fragment if *Tag1* had excised (oligonucleotides O37, O38, and O68; see Methods and Figure 2B).

![Diagram](image)

Figure 2. Schematic Diagrams of *Ac* and *chl7::Tag1*.

(A) A diagram of the maize *Ac* element is shown, with solid arrowheads representing oligonucleotides used as primers for PCR analysis and a solid bar indicating the fragment used as a probe for DNA gel blot analysis. This diagram was adapted from Figure 2 in Kunze et al. (1987). B I, BamHI; H III, HindIII; RI, EcoRI.

(B) Diagram of *Tag1* inserted into the *chl7* gene (*chl7::Tag1*), showing the oligonucleotides used for PCR analysis, represented by solid arrowheads (adapted from Figure 1 of Tsay et al. [1993]). Exons of the *CHL7* gene are indicated by filled boxes. Empty boxes represent the 5' and 3' untranslated regions. The gradient bar represents the *Tag1* element.

(C) Sequences of *Tag1* excision sites at *CHL7*. Sequence analysis of *Tag1* footprints from *Ac*-minus Arabidopsis lines is shown. Primers specific for the *CHL7* gene (O37 and O38) were used to amplify DNA at the *Tag1* insertion site. At top, the genomic sequence in the *CHL7* gene immediately adjacent to *Tag1*, including the 8-bp duplication (in bold) in the *chl7::Tag1* allele, is shown. Below are shown the sequences of the DNA remaining after *Tag1* excision. The gaps were introduced to allow alignment of the duplicated target site and are not present in the actual sequence.
Using this procedure, we examined 172 F₂ segregants and found 44 to have no Ac elements. F₃ seed was collected from plants that contained at least one Tag1 element in the CHL1 gene (29 of the 44 Ac-minus lines). These seeds were planted, and 16 F₂ chlorate-resistant plants were selected, each from a distinct F₂ family. F₃ seed was collected from these selfed plants. DNA from the F₃ seedlings was analyzed by PCR, and excision events were detected in 14 plants (from a total of 164 plants analyzed) that were obtained from four F₃ families (data not shown). The 200-bp PCR products (indicative of an excision event) from six of these revertants were cloned and sequenced. The sequences revealed characteristic footprints found in excision alleles (Figure 2C). Thus, Tag1 excision from the chl1 locus does not require Ac transposase.

**Tag1 is an Autonomous Element**

Because Tag1 is not dependent on Ac for excision and is the only element we could identify in the Landsberg erecta genome, we surmised that Tag1 is either autonomous or dependent on another unidentified element that does not cross-hybridize with Tag1. To resolve this issue, we introduced Tag1 into a heterologous host. Tobacco was selected because it is easy to transform and has been used extensively for studying heterologous transposons (Baker et al., 1986; Masson and Fedoroff, 1989; Pereira and Sauder, 1989). For easy detection of Tag1 excision, Tag1 was inserted between a cauliflower mosaic virus 35S promoter and the GUS reporter coding sequence (Figure 3A). GUS-based vectors have been used successfully to follow excision of Ac and Suppressor-mutator (Spm) in both tobacco and Arabidopsis (Finnegan et al., 1989; Masson and Fedoroff, 1989; Lawson et al., 1994). In our constructs, the Tag1 element blocks GUS expression; when it excises, a functional 35S–GUS gene is regenerated and GUS expression is restored. Plant cells inheriting the restored GUS gene will stain blue in the presence of the GUS substrate X-gluc.

The 35S–GUS::Tag1 construct was introduced into tobacco by Agrobacterium-mediated transformation, and transgenic plants were selected with kanamycin. Leaves from the primary transformants (T₁) were assayed for GUS activity. Nine independent primary transformants showed patches of blue-staining tissue in the presence of X-gluc (Figures 4C and 4D). In contrast, untransformed tobacco leaves showed no blue staining (Figure 4A). The size of the sectors varied within a leaf but was never larger than one-eighth of a leaf, and the number of sectors varied among the lines (Figures 4C and 4D). These sectors indicate that Tag1 is excising and is thus active. To confirm that the blue staining was due to Tag1 excision, PCR analysis of genomic DNA from leaves of the transgenic tobacco was performed using primers specific for the cauliflower mosaic virus 35S promoter and the GUS gene, which would generate a 300-bp product if Tag1 excised. In six plants analyzed, a 300-bp PCR fragment was found. PCR products that were cloned and sequenced showed small footprints (Figure 3C) characteristic of excision. These results confirm Tag1 excision from the 35S–GUS::Tag1 construct in tobacco.

To demonstrate that Tag1 itself and not an endogenous tobacco element was providing transposase function for Tag1 excision, tobacco plants were transformed with a defective Tag1 element (dTTag1). This defective element was constructed in vitro by deleting an internal 1.4-kb EcoRI fragment producing a 1.9-kb dTag1. The dTag1 element was inserted between the 35S promoter and GUS reporter genes at the same location used for the intact Tag1 (Figure 3B). When 35S–GUS::dTTag1 was introduced into tobacco and 50 leaves from 12 independent T₁ lines containing 35S–GUS::dTTag1 were analyzed, no blue-staining sectors were found (Figure 4B), with one exception. The exception was a single leaf that had a few blue-staining cells in the vascular tissue, which could be accounted for by a recombination or splicing event that removed most or all of the dTag1 element. PCR analysis of all 12 transgenic plants confirmed that each still contained the 35S–GUS::dTTag1 construct (data not shown). To be certain that dTag1 is capable of excision if an autonomous element is present in the genome, 35S–GUS::dTTag1 constructs were introduced into Arabidopsis plants of the Landsberg erecta ecotype, which contains two Tag1 elements. These transgenic plants had numerous blue-staining sectors indicative of excision (data not shown). From the above analysis, we conclude that Tag1 must be providing its own transposase in tobacco and therefore must be an autonomous element.

**Analysis of Tag1 Excision in Arabidopsis**

We next examined the excision of Tag1 in its native host Arabidopsis by using the 35S–GUS::Tag1 construct described above. Two ecotypes, Columbia and Nossen (No-O), were used for this experiment, with each ecotype receiving a construct with different sequences flanking the Tag1 insert (see Methods). Neither of these ecotypes has an endogenous Tag1 element, as shown by DNA gel blot hybridization analysis (Tsai et al., 1993; data not shown). Several transgenic plants were selected with kanamycin, and tissue was then assayed for GUS expression. Analysis of roots, cotyledons, and leaves revealed blue-staining sectors in all three organs indicative of Tag1 excision events that restored GUS expression (Figure 5). Untransformed plants showed no staining in roots or leaves, as was expected (Figure 5A). In true leaves and cotyledons, blue-staining cells were restricted to small sectors (Figure 5B), except for a few rare larger sectors that covered more than one-fifth of a true leaf (Figure 5C). In roots, blue sectors varied from a few cells (Figure 5E) to a complete root branch (Figure 5F). In No-O transgenic plants, the chance of observing a sector in at least one cotyledon or leaf was 44% for one transgenic line and 85% for another. In Columbia, the chance of observing a sector(s) in
Figure 3. Diagram of the 35S-GUS::Tag1 Constructs.

(A) Schematic diagram of the GUS::Tag1 construct. Tag1 was cloned into the untranslated region between a cauliflower mosaic virus (CaMV) 35S promoter (P) and a GUS gene in the pBI210 plasmid, as shown (see Methods). The restriction enzymes indicated are as follows: BII, BglII; PI, PstI; RI, EcoRI, and XI, XbaI. Solid arrowheads indicate the direction and position of the primers used for PCR. Nos-P is the nopaline synthase promoter; Nos-t is the nopaline synthase terminator; Kan R is the kanamycin resistance gene; LB and RB are the left and right border sequences, respectively.

(B) Schematic diagram of the GUS::dTTag1 construct. Defective Tag1 (dTTag1) was inserted in the same location as described in (A) and in the same orientation. Abbreviations are as given in (A).

(C) Sequences of Tag1 excision sites from 35S-GUS in tobacco. Sequence analysis of revertant alleles from the 35S-GUS::Tag1 constructs is shown for tobacco. Primers specific for the CaMV 35S promoter (0127) and the GUS gene (0128) were used to amplify the empty sites left after Tag1 excision. At top is the sequence of the 35S-GUS::Tag1 construct immediately adjacent to the Tag1 element. Below are the sequences of three revertant alleles with duplicated flanking sequences. Gaps were introduced for alignment and are not present in the actual sequence.
at least one leaf or cotyledon was 77% for one line and 100% for the other.

Sometimes a leaf or cotyledon would stain completely blue (Figure 5D), indicating a possible germinal revertant. We verified that these were germinal revertants by examining the subsequent generation. Examination of the progeny from 12 putative revertants showed a 3:1 segregation of the revertant phenotype (data not shown). We also confirmed that GUS expression was due to an excision of Tagl from the 35S-GUS::Tagl gene by sequence analysis of PCR products. From six independent revertants, PCR amplification of genomic DNA immediately adjacent to the Tagl insertion site produced 300-bp fragments that contained footprints left behind by an excision event (Figure 3D). In the Columbia ecotype, the average frequency of revertants was 6% (39 of 614 from three independent lines); for No-0, the average was 1% (6 of 653 from two independent lines).

**DISCUSSION**

Our initial analysis of Tagl in the chl1::Tagl mutant indicated that Tagl is an active element, able to excise from the chl1 locus to produce chlorate-sensitive revertants in the Landsberg erecta background. Left unanswered by this study are the following questions: (1) How many Tagl elements are present in the genome? (2) Are there any related elements in the genome? (3) What provides the transposase function for Tagl excision? and (4) Can Tagl undergo somatic excision to produce sectors in vegetative organs? Answering these questions is complicated by the fact that the chl1::Tagl lines were transgenic plants containing the maize element Ac, which is related to Tagl. It is possible that Ac could be providing transposase function for Tagl. The other likely candidates were Tagl itself or other Tagl-related elements detected by DNA gel blot analysis (Tsay et al., 1993). The experiments described here address these issues and identify the autonomous element.

DNA gel blot and sequence analysis revealed that the genome of Landsberg erecta has two identical Tagl elements with distinct flanking sequences (designated Tagl-2 and Tagl-3). Only one map position for Tagl has been found in the Landsberg erecta background (see Lister and Dean, 1993; http://nasc.nott.ac.uk/new_ri_map.html); thus, Tagl-2 and Tagl-3 must be tightly linked. We have been unable to find any bacterial artificial chromosome clones that hybridize to sequences flanking both elements, suggesting that these two elements are not immediately adjacent (data not shown). We have found that both elements can excise in the chl1::Tagl mutant to produce empty sites, as visualized by PCR analysis (data not shown). One of these elements must have transposed to the top of chromosome 1 to produce the chl1 insertion mutation.

Our results show that Tagl excision is not dependent on Ac because Ac was not needed to maintain Tagl excision. Even though Tagl transposition was initially observed in an Ac-containing line, it is clear the Tagl is active on its own. However, because Tagl and Ac share some properties in that they duplicate 8 bp upon insertion and have similar signature sequences found in the Ac superfamily (Calvi et al., 1991; Warren et al., 1994; Essers and Kunze, 1995), we cannot rule out the possibility that there is some interaction between these two elements, such as enhancement of Tagl excision by Ac transposase. Additional experiments are needed to address this possibility.

The results described above strongly suggest that Tagl itself is an autonomous element. This hypothesis was confirmed when Tagl was found to excise in tobacco and in two Arabidopsis ecotypes containing no Tagl elements. Given this conclusion, it should be possible to find additional Tagl insertion mutants in the Landsberg erecta ecotype. Recently, just such a mutant (cup-shaped cotyledon) was found while screening progeny from the chl1::Tagl mutant. This mutant is defective in shoot apical meristem formation and in organ separation and has a Tagl insertion in the CUC2 gene (Aida et al., 1997). Hence, any unstable mutants in the Landsberg erecta background (especially in lines that have an Spm or Ac element) could be due to Tagl insertion.

To show that Tagl is autonomous, we used a 35S-GUS marker construct that would detect those cells that had inherited a Tagl excision event. This construct allowed us to score both vegetative sectors and germinal revertants. In our previous experiments, we scored only germinal revertants, that is, chlorate-sensitive plants among progeny from a chlorate-resistant plant (chl1::Tagl) (Tsay et al., 1993). With our 35S-GUS::Tagl lines, we found revertant sectors in cotyledons, leaves, and roots, which is indicative of somatic excision, and completely blue-stained seedlings, which is indicative of germinal excision. The frequency of germinal revertants from selfed individuals (using constructs...

**Figure 3.** (continued).

(D) Sequences of Tagl excision sites from 35S-GUS in Arabidopsis. As described in (C), empty sites remaining after Tagl excision in Arabidopsis were amplified using primers specific for the CaMV 35S promoter and the GUS gene. At top is the sequence immediately adjacent to Tagl in the 35S-GUS::Tagl construct. At bottom are the sequences from six independent revertants. Three of the revertants had the same sequence remaining after Tagl excision, indicated as revertant 1. Revertant 3 had a perfect repair, so no target site duplication was present. Revertant 4 had the extra sequence GTTTAGAC inserted between the imperfect target site duplication. The sequences duplicated upon Tagl insertion are shown. Gaps were introduced for alignment and are not present in the actual sequence.
with different flanking sequences in each ecotype) was ~1% for the No-0 lines and 6% for the Columbia lines. These frequencies are a little higher than those reported for Ac in Arabidopsis (Van Sluys et al., 1987; Schmidt and Willmitzer, 1989; Dean et al., 1992) but lower than those that we reported from our chl1::Tag1 mutant (Tsay et al., 1993). We feel that the germinal reversion frequencies obtained from the 35S-GUS::Tag1 lines are more reliable than those from the chl1::Tag1 mutant, because chlorate selections can overestimate the number of revertants (chlorate-sensitive plants), due in part to the presence of revertant sectors in roots that permit the uptake of enough chlorate to cause toxicity. Further analysis of Tag1 germinal revertant frequencies is needed to determine the full extent of the variation and to establish how the frequency depends on such factors as Tag1 copy number. Such detailed analysis of the excision behavior of Tag1 in Arabidopsis has been conducted (see Liu and Crawford, 1997).

In summary, the study of Tag1 has provided several important findings about this family of transposons. Tag1 is a member of the Ac superfamily of transposons (Warren et al., 1994; Essers and Kunze, 1995) and is an autonomous element that can transpose in Arabidopsis and tobacco. Tag1 insertion mutations have been found in at least two genes of Arabidopsis—CHL1, which encodes a nitrate transporter and confers sensitivity to chlorate (Tsay et al., 1993), and CUC2, which is involved in formation of shoot apical meristems and in organ separation in Arabidopsis (Aida et al., 1997). Tag1 is found in Landsberg erecta plants but not in Columbia, WS, and No-0 ecotypes. Tag1 excision produces revertant sectors in leaves, cotyledons, and roots and in this study produced germinal revertants at a frequency from 1 to

**Figure 4.** Phenotypes of Variegated Tobacco Plants Containing 35S-GUS::Tag1 Constructs.

Histochemical staining of plants, as described in Methods, is shown. All photographs were taken using the same magnification (×0.8).

(A) Untransformed tobacco leaf.

(B) Leaf from a T1 transgenic tobacco plant containing 35S-GUS::dTtag1.

(C) and (D) Leaves from plants of two independent T1, transgenic tobacco lines containing 35S-GUS::Tag1.
Figure 5. Phenotypes of Variegated and Revertant Arabidopsis Plants Containing 35S-GUS::Tag1 Constructs.

Histochemical staining of plants, as described in Methods, is shown.

(A) A 14-day-old control seedling from a chl1::Tag1 plant lacking a GUS gene. Control plants were generally colorless, except for a pale green hue that occasionally was seen where some of the chlorophyll was not completely bleached with the ethanol.

(B) to (F) Seedlings, a cotyledon, and roots from transgenic plants containing the 35S-GUS::Tag1 constructs and having the following phenotypes: (B) a 14-day-old seedling showing variegated phenotype; (C) a seedling showing both small sectors and a rare large sector; (D) a completely blue-staining cotyledon from a seedling that is a possible germinal revertant; (E) roots from a seedling showing small dark blue-staining sectors; and (F) roots from a seedling showing a blue-staining lateral root.

6%. Germinal revertants (from excision events at the chl1 locus) have new Tag1 insertions in the genome, indicating that Tag1 can transpose from one site to another (Tsay et al., 1993). These results indicate that Tag1 can produce new mutations in Landsberg erecta, which is commonly used for genetic experiments, and if introduced into other ecotypes of Arabidopsis or into tobacco, should generate additional insertion mutations.

METHODS

Screening of an Arabidopsis thaliana Genomic Library

An Arabidopsis genomic library made from Landsberg erecta plants was kindly provided by J. Chory (Salk Institute, La Jolla, CA). The DNA was partially digested with Mbol and ligated into the Xhol sites of λ Fix (Stratagene, La Jolla, CA). Sixty thousand plaques were screened using the 3.3-kb Tag1 element as a probe. The nitrocellulose filters were hybridized at 65°C in a solution of 5 x SSPE (1 x SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4), 5 x Denhardt's solution (1 x Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 0.5% SDS, and 0.025 mg/mL single-stranded herring sperm DNA. The filters were rinsed once at 22°C in 2 x SSPE for 30 min, once at 65°C in 2 x SSPE and 0.1% SDS for 30 min, and once at 65°C in 1 x SSPE and 0.1% SDS for 30 min. The filters were exposed to Kodak x-ray film at -80°C with an intensifying screen.

Arabidopsis Growth and Chlorate Selections

Plant growth and chlorate selections were performed as described previously (Wilkinson and Crawford, 1991), except that 5 mM KH2PO4, pH 5.5, 2.5 mM NH4NO3, and 0.5 mM CaCl2 were used in the nutrient medium. Plants were irrigated twice with chlorate in nutrient medium.
(3 and 6 days after placing pots at 22°C in the light), followed by nutrient medium without chlorate.

**Plant Transformation**

For the construct used for the Nossen (No-0) ecotype, the ends of Tagl in the CHL1 gene were generated by polymerase chain reaction (PCR) amplification using primers 5’-CGCTCTAGAACTAGTGGATCC-3’ and 5’-CAATGTTTTCAGCTCC-3’ to amplify the left end of Tagl and primers 5’-TCGAGTCGAGTCGATCC-3’ and 5’-CCCTCTAGACTCTCGGAATTAGTCGG-3’ to amplify the right end. PCR reactions were done with Taq polymerase (Pharmacia) in 10 mM Tris, pH 8.3, 50 mM KCl, 2 to 4 mM MgCl₂, 0.001% gelatin, and 0.2 mM deoxynucleotide triphosphates for 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min per cycle. The correct sequences of the amplified regions were verified by the deoxynucleotide chain termination method, as described previously (Sanger et al., 1977), using Sequenase 2.0 (U.S. Biochemical). The complete Tagl element was reconstructed by ligating the two end fragments to the internal EcoRl fragment of Tagl and then subcloned into the XbaI and Smal sites of the pBluescript II KS+ vector (Stratagene, Palo Alto, CA), with the orientation shown in Figure 3. For the construct used for tobacco and the Columbia ecotype of Arabidopsis, Tagl-2 was amplified by PCR, using the following primers specific to the flanking sequence: 5’-CCCTCTAGAATTGATTCCTTACGTCTWC-3’ and 5’-CAATGTTTTCAGCTCC-3’. PCR conditions were for 30 cycles at 90°C for 1 min, 55°C for 1 min, and 72°C for 5 min. During amplification, XbaI and BamHI sites, which are part of the synthesized primers, were added to the 5’ and 3’ ends of the PCR product, respectively. The PCR product was digested with XbaI and BamHI and cloned into the corresponding sites on pBluescript II KS+ (Stratagene). This PCR product, which also contains the target site duplication, was sequenced to verify that no errors had occurred during PCR amplification. This Tagl element was then cloned into the XbaI and BamHI sites on the pBluescript II KS+ plasmid in the same orientation as described above. Defective Tagl (dTagl) was constructed by digesting Tagl with EcoRI and then ligating the end fragments together minus the internal 1.4-kb fragment in pBluescript II KS+. This element was cloned into pBluescript II KS+ by using the same strategy (Figure 3).

35S-GUS::Tagl and 35S-GUS::dTagl were independently transformed into Agrobacterium tumefaciens C58 AGL-O (Lazo et al., 1991). Leaf discs from Nicotiana tabacum Xanthi were incubated with Agrobacterium containing either 35S-GUS::Tagl or 35S-GUS::dTagl, and transgenic plants were produced as described previously (Voelker et al., 1987). Transgenic Arabidopsis plants (ecotype No-0) were produced by incubating root explants with Agrobacterium containing 35S-GUS::Tagl (Valvekens et al., 1988). Transgenic Arabidopsis plants (ecotype Columbia or Landsberg erecta) were produced by vacuum infiltrating 4-week-old plants in Agrobacterium culture containing the appropriate construct (Buchtil et al., 1993). Seeds from treated plants were collected and screened for kanamycin resistance. Transgenic plants identified at this generation were classified as T₁ plants.

**Plant Genomic DNA Isolation and Gel Blot Hybridization**

For PCR analysis, genomic DNA was isolated, as described previously (Edwards et al., 1991), except that a phenol-chloroform extraction was performed before isopropanol precipitation. For gel blot analysis, Arabidopsis DNA was isolated as described previously (Wilkinson and Crawford, 1991), except that grinding was done on ice. Approximately 2 μg of DNA was digested with the indicated restriction enzyme in the presence of 2 mM spermine, separated by agarose gel electrophoresis, blotted to Hybond N membranes (Amersham), and probed with the indicated radiolabeled fragment. The fragments were radiolabeled by the random priming method (Feinberg and Vogelstein, 1983). The 0.9-kb HindIII-EcoRI fragment of Activator (Ac) was a gift from C. Dean (John Innes Laboratory, Norwich, UK). The filters to identify Tagl or Tagl-related elements were hybridized at 42°C in a solution of 50% formamide, 6 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5 × Denhardt’s solution, 0.5% SDS, and 0.1 mg/mL single-stranded salmon sperm DNA. The filters were rinsed twice at 22°C in 2 × SSC and 0.5% SDS for 20 min, once at 22°C in 0.1 × SSC and 0.1% SDS for 30 min, and once at 42°C in 0.1 × SSC and 0.1% SDS for 45 min. The filters were exposed to Fuji X-ray film at ~80°C with an intensifying screen.

**Kanamycin Screens and Histochemical Staining**

Seeds were sterilized first in 70% ethanol for 2 min and then in 1% SDS and 5% bleach for 15 min. Seeds were then washed three times with sterile water. Seeds collected from vacuum-infiltrated Arabidopsis plants were sterilized and then plated using 0.1% agarose on GM media (Sigma) plates containing 50 μg/mL kanamycin and 30 μg/mL kanamycin. Progeny seed from transgenic plants were sterilized and then plated on GM media containing 30 to 50 μg/mL kanamycin. After a 1- to 3-day cold treatment, plates were incubated at 22°C under continuous light. Ten days after germination, plants were scored as resistant (fully green) or sensitive (fully white). Whole seedlings or tissue from kanamycin-resistant plants was incubated in a GUS staining buffer consisting of 25 mM sodium phosphate buffer, pH 6.8, with chloramphenicol and 0.5 mM X-gluc, 0.5 mM KFe(CN)₆, 0.5 mM KFe(CN)₆, and 10 mM EDTA for 1 or 2 days at room temperature or 37°C in the dark. Tobacco leaves were incubated in the same GUS staining buffer, except for the addition of 20% methanol. The GUS staining solution was removed, and tissue was incubated in 70% ethanol for 2 min and then in 1% SDS and 5% bleach for 15 min. Seeds were then washed three times with sterile water. Seeds collected from vacuum-infiltrated Arabidopsis plants were sterilized and then plated using 0.1% agarose on GM media (Sigma) plates containing 50 μg/mL kanamycin and 30 μg/mL kanamycin. Progeny seed from transgenic plants were sterilized and then plated on GM media containing 30 to 50 μg/mL kanamycin. After a 1- to 3-day cold treatment, plates were incubated at 22°C under continuous light. Ten days after germination, plants were scored as resistant (fully green) or sensitive (fully white). Whole seedlings or tissue from kanamycin-resistant plants was incubated in a GUS staining buffer consisting of 25 mM sodium phosphate buffer, pH 6.8, with chloramphenicol and 0.5 mM X-gluc, 0.5 mM KFe(CN)₆, 0.5 mM KFe(CN)₆, and 10 mM EDTA for 1 or 2 days at room temperature or 37°C in the dark. Tobacco leaves were incubated in the same GUS staining buffer, except for the addition of 20% methanol. The GUS staining solution was removed, and tissue was incubated in 70% ethanol for “bleaching” the tissue and for long-term storage.

**PCR Analysis and Sequencing**

To check F₂ segregating lines for the presence of an Ac element, primers O93 (5’-GTACAGCTACAGATCCG-3’) and O94 (5’-CATGATTGAGTAAAGG-3’) were used to amplify a region that encodes the Ac transposase. To determine whether Tagl was inserted into the chI1 gene or had excised in the Ac-minus lines, primers O37 (5’-CCCTCTAGAGCTGGTAAGTCG-3’) and O38 (5’-CCCTGCTGGTGTTTATTTCGCG-3’) surrounding the chI1::Tagl junction and O68 (5’-CCCTCAGCTGGCATACAAATCGAGACG-3’) specific to Tagl were used. A 200-bp PCR product indicates that Tagl excised; a 600-bp PCR product indicates that Tagl is inserted into chI1. To check for excision of Tagl or dTagl from the 35S-GUS marker gene, the region between the 35S promoter and GUS gene was amplified by PCR using the following primers: O127 (5’-GCTTCAAGATCTCATCAGATCCCG-3’) and O128 (5’-CATGATTGAGTAAAGG-3’) and O128 (5’-CCATCGCATCAAAGAAGGC-3’) specific to Tagl were used. A 200-bp PCR product indicates that Tagl is inserted into chI1. To check for excision of Tagl or dTagl from the 35S-GUS marker gene, the region between the 35S promoter and GUS gene was amplified by PCR using the following primers: O127 (5’-GCTTCAAGATCTCATCAGATCCCG-3’) and O128 (5’-CCATCGCATCAAAGAAGGC-3’) specific to Tagl were used. A 200-bp PCR product indicates that Tagl is inserted into chI1.
0.01% gelatin, and 0.2 mM deoxynucleotide triphosphates under the following conditions: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and 30 sec for 35 cycles. Subcloned fragments were sequenced using the dideoxynucleotide method, as previously described (Sanger et al., 1977), using Sequenase 2.0.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Science Foundation (No. MCB-9219374).

Received July 11, 1997; accepted August 13, 1997.

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Plant Cell 1997;9:1745-1756
DOI 10.1105/tpc.9.10.1745

This information is current as of November 7, 2017