Unorthodox mRNA start site to extend the highly structured leader of retrotransposon Tto1 mRNA increases transposition rate

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ABSTRACT

Retroelement RNAs serve as templates for both translation and reverse transcription into extrachromosomal DNA. DNA copies may be inserted into the host genome to multiply element sequences. This transpositional activity of retroelements is usually restricted to specific conditions, particularly to conditions that impose stress on the host organism. In this work, we examined how the mRNA initiation point, and features of primary and secondary structure, of tobacco retrotransposon Tto1 RNA influence its transpositional activity. We found that the most abundant Tto1 RNA is not a substrate for reverse transcription. It is poorly translated, and its 5′-end does not contain a region of redundancy with the most prominent 3′-end. In contrast, expression of an mRNA with the 5′-end extended by 28 nucleotides allows translation and gives rise to transposition events in the heterologous host, *Arabidopsis thaliana*. In addition, the presence of extended hairpins and of two short open reading frames in the 5′-leader sequence of Tto1 mRNA suggests that translation does not involve ribosome scanning from the mRNA 5′-end to the translation initiation site.

Keywords: ***Arabidopsis thaliana*; retrotransposon; short upstream reading frame; translation efficiency**

INTRODUCTION

Retrotransposons are (usually nonessential) genetic entities that can replicate more often than the host DNA in which they reside (for reviews, see Boeke and Stoye 1997; Kunze et al. 1997; Grandbastien 1998; Kumar and Bennetzen 1999; Bennetzen 2000; Feschotte et al. 2002; Peterson-Burch and Voytas 2002; Kazazian Jr. 2004). The accepted model for overreplication involves transcription by RNA polymerase II, and subsequent translation of retrotransposon-encoded proteins. Reverse transcriptase, a key component among these proteins, converts the element's RNA into complementary DNA (cDNA). Retrotransposon-encoded integrase catalyzes insertion of the cDNA into a new site of the host genome. Retrotransposons occur in all eukaryotes and are the major constituents of large genomes, forming a matrix into which genes with more obvious roles for the organism's well-being are embedded. Among the two major types of retroelements, poly(A) elements and long terminal repeat (LTR) containing elements, the poly(A) class is more primordial (Eickbush and Malik 2002). LTR retrotransposons, in turn, are believed to be progenitors of the more complex retroviruses, with which they share basic features of the replication cycle. In particular, the LTR present on both ends of LTR retroelements and retroviruses allows, via two cDNA strand transfer reactions, regeneration of a complete copy of the element from its mRNA, which contains only a short sequence of redundancy, the R region, on both ends (Boeke and Stoye 1997).

Most retroelements found in eukaryotic genomes are deficient and therefore cannot transpose. However, even those elements that carry all functions necessary for transposition, are usually inactive. Studies during recent years have demonstrated that mechanisms that detect repeated sequences by the occurrence of double-stranded RNAs lead to silencing of

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retrotransposons, and thereby contribute to genome integrity by keeping retrotransposition at a low level (Hirochika et al. 2000; Miura et al. 2001; Tijsterman et al. 2002; Matzke and Birchler 2005). In addition, retroelement-encoded proteins may also negatively influence the transposition frequency (Meignin et al. 2003). Nonetheless, some retroelements can be highly active in the germ line (Lecher et al. 1997), or under stressful conditions (Mhiri et al. 1997; Takeda et al. 1999). In the latter case, the nature of stimulating stress conditions varies among the elements and their hosts (Grandbastien 1998; Pourtau et al. 2003).

The Tto1 element of tobacco studied in this work is an LTR retrotransposon that belongs to the Ty1/*copia* class of elements, and encodes a single long open reading frame (ORF) (Hirochika 1993). In addition to its native host, tobacco, Tto1 can transpose in the dicot plant *Arabidopsis*, and in the monocotyledonous rice (Hirochika 1993; Hirochika et al. 1996; Okamoto and Hirochika 2000). Tto1 is activated in response to tissue culture conditions and pathogen stress (Takeda et al. 1999). We investigated which features of the element prevent high activity under nonstress conditions and found that control of transposition can be exerted by choice of transcript initiation site. We also show that activity may be controlled by RNA secondary structure, and by two short upstream ORFs. Consideration of these features allowed the use of a heterologous promoter to induce Tto1 transposition in *Arabidopsis thaliana*.

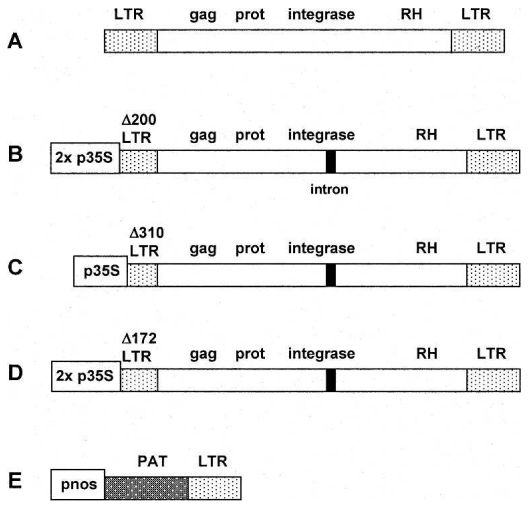

FIGURE 1. Constructs used for in planta analysis. (*A*) Schematic drawing of retrotransposon Tto1. (*B*) Derivative of Tto1, in which the long terminal repeat (LTR) was truncated to nt 200 and linked to the strong 2x CaMV promoter. In addition, the construct contains an intron in the integrase domain. (*C*) Construct similar to *B*, but LTR was deleted to nt 310. (*D*) Construct identical to *B*, but with LTR deletion spanning nt 1–171. (*E*) Construct used to determine transcription termination sites in the LTR. (gag) Protein domain homologous to group-specific antigens of retroviruses; (prot) protease domain; (RH) ribonuclease H domain; (2x p35S) promoter of the 35S transcript of Cauliflower Mosaic Virus (CaMV) with duplicated enhancer region; (p35S) CaMV 35S promoter without enhancer duplication; (pnos) promoter of the nopaline synthase gene of T-DNA; (PAT) phosphinothricin acetyl transferase. For further explanations, see text.

RESULTS

Construction of marked Tto1 elements

In order to better monitor transposition activity of retrotransposon Tto1 in the model plant *A. thaliana* accession Wassilewskija (Ws), we introduced an intron into its single ORF. The 94-nt intron sequence was copied from the gene encoding the largest subunit of RNA polymerase II (At4g35800; nt 3372–3465 of accession X52954) (Nawrath et al. 1990). In this manner, the modified Tto1 constructs introduced into plants by the T-DNA of *Agrobacterium* (Fig. 1B–D) were labeled by the intron. However, reverse-transcribed RNA in its extrachromosomal form, or after integration at a new location in the genome, has lost the intron marker and can thereby be distinguished from the T-DNA-encoded copy. In addition, all constructs used in this work have a deletion at the beginning of the 5′-LTR sequence. During the process of reverse transcription, this part of the 5′-LTR (called U3 region because it is uniquely present at the 3′-end of the RNA) is copied from the 3′-end of the mRNA, thus restoring the 5′-LTR irrespective of deletions in the mother element (Luciw and Leung 1992; Boeke and Stoye 1997). These modifications allowed us to distinguish input DNA from reverse-transcribed cDNA copies by PCR-based assays.

In vivo expression of marked elements

In order to study transposition without relying on the activity of the stress-inducible Tto1 promoter, we used the constitutive 35S RNA promoter of Cauliflower Mosaic Virus (CaMV 35S promoter) to drive Tto1 transcription in *Arabidopsis*. Two constructs were generated that carried the CaMV 35S promoter fused to either nucleotide (nt) position 200 (Fig. 1, construct B), or 310 (Fig. 1, construct C) of the Tto1 sequence. These constructs were integrated into the *Arabidopsis* genome by *Agrobacterium* T-DNA-mediated transformation. Using PCR with primers flanking the intron marker, activity of Tto1 was monitored in more than 40 transgenic calli and, later on, in leaves from several regenerated plant lines containing construct B and one line containing construct C. RT-PCR control experiments as shown in Figure 2A confirmed that both constructs B and C are competent for transcription. Figure 2B shows the representative result of a PCR-based assay to detect intron-less cDNA. Whereas only trace amounts of cDNA can be detected with construct B, expression of a properly spliced, reverse-transcribed cDNA was readily demonstrated for construct C. This was somewhat surprising, because the in vivo mRNA start had been previously mapped to nt position 200 of Tto1 (Hirochika 1993). Thus, construct B was expected to allow the production of an mRNA identical to the one transcribed from

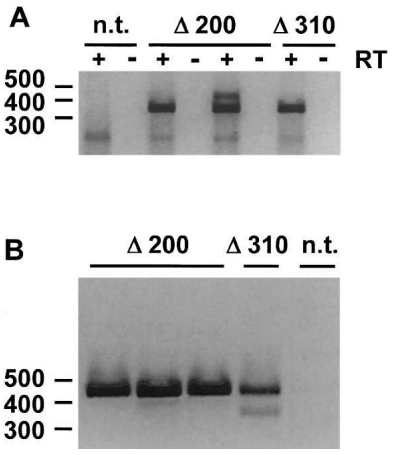

FIGURE 2. PCR reaction products demonstrating presence or absence of Tto1 mRNA and cDNA. (*A*) RT-PCR to demonstrate transcription of the Tto1 transgene constructs B and C in *Arabidopsis thaliana*. PCR products of (spliced) mRNA are ~340 nt long, and those from genomic DNA, or from unspliced mRNA, are ~430 nt in length. (Δ200) RNA from two independent transgenic lines expressing construct B was isolated and subjected to reverse transcription followed by PCR amplification, using oligonucleotides that flank the intron. (Δ310) RNA was isolated from one plant transformed with construct C. (n.t.) RNA was isolated from nontransformed plant. Plus and minus signs *above* the lanes indicate whether reverse transcriptase enzyme was included in the reaction. The position of DNA markers (size in base pairs) is indicated to the *left*. (*B*) PCR to detect cDNA. Designations are as in panel *A*. DNA was isolated from three independent lines containing construct B (two of which were also used for panel *A*), and from one line expressing construct C (same line as used in panel *A*). Note the presence of a spliced, reverse-transcribed product (~340-nt band) with construct C, but not with construct B.

unmodified Tto1, which can transpose in *Arabidopsis*. Nonetheless, neither expression of mRNA from construct B, nor from construct C, permitted detection of a new Tto1 copy in all cells of a progeny plant (so-called germinal transposition), suggesting low transpositional activity.

RNA secondary-structure prediction

The low capacity of construct B to produce intron-less cDNA prompted us to use RNA secondary structure prediction programs to analyze the structure of the mRNA 5′-region. As shown in Figure 3, the leader sequence of Tto1 mRNA may form extended base-paired regions. In particular, the 5′-end of the mRNA previously detected in vivo, which starts at position 200 of Tto1, is engaged in a stable stem structure (Fig. 3A). In contrast, a shorter mRNA starting at position 310 of Tto1 was predicted to form an accessible 5′-end (Fig. 3B). This prediction correlated with the activity observed by the intron-PCR assay. Therefore, the secondary structure analysis also indicated that the accessibility of the Tto1 mRNA 5′-end might control the efficiency of translation.

The Tto1 leader contains two short ORFs

Sequence analysis of the Tto1 5′-region also indicates the presence of three ATG codons, at positions 269, 413, and 424 of Tto1, which are upstream of the long continuous reading frame that starts at position 716 and encodes Tto1 protein. Figure 4 highlights the two short ORFs starting at ATG 269 and at ATG 413, respectively (the two-amino-acid ORF starting at nt 424 is contained within the second short ORF, and was not further considered). Site-specific mutagenesis studies indicated that introduction of another upstream ATG (introduced by a G-to-A change at nt 341) into the Tto1 wild-type element, does not abolish transposition activity (data not shown). Together, these findings strongly suggest that Tto1 mRNA is not translated by a ribosome-scanning mechanism (Jackson 2000). This deviation from the general structural requirements for normal translation initiation pinpointed features that might influence the efficiency of Tto1 transposition (see Discussion).

Determination of Tto1 mRNA 3′-end sequences

Although Tto1 construct C yielded detectable, presumably extrachromosomal cDNA, it did not support transposition (data not shown). This raised the question whether sequences upstream of Tto1 position 310 might be necessary for transposition. One possible reason for the apparent inability of construct C to transpose is that mRNA transcribed from construct C does not have sequence redundancy between the 5′- and 3′-ends. The redundancy (R) region of LTRs usually encompasses a stretch of at least 10 bases that are present at both ends of the RNA. After cDNA synthesis stops in the R region of the 5′-end, the cDNA is transferred to its counterpart at the 3′-end of the RNA, where cDNA synthesis can resume (Luciw and Leung 1992). Without sequence redundancy, mRNA transcribed from construct C could not support an orderly first-strand transfer, and any cDNA detected might, in that case, be the result of aberrant priming of cDNA synthesis, for instance, after transient fold-back of the RNA 3′-end. To test this possibility, we examined the 3′-end of Tto1 mRNA. We created a construct in which the 3′-LTR of Tto1 was used as a transcriptional terminator in a gene cassette containing nopaline synthase promoter, Basta (Phosphinothricin acetyl transferase, PAT) resistance gene, and Tto1 3′-LTR (Fig. 1E). RNA was isolated from transgenic plants containing construct E, and cDNA was made with a special oligonucleotide dT-containing primer and AMV reverse transcriptase. Subsequently, the cDNA was PCR-amplified, cloned, and sequenced (see Materials and Methods). Most transcripts end around position 4914 of Tto1 (which corresponds to position 188 of the 5′-LTR). A minority of transcripts extend to position 5230 (corresponding to position 504 of the 5′-LTR) (see Fig. 4). Additional, rare 3′-ends were mapped to positions 4839, 4857, and 5174.

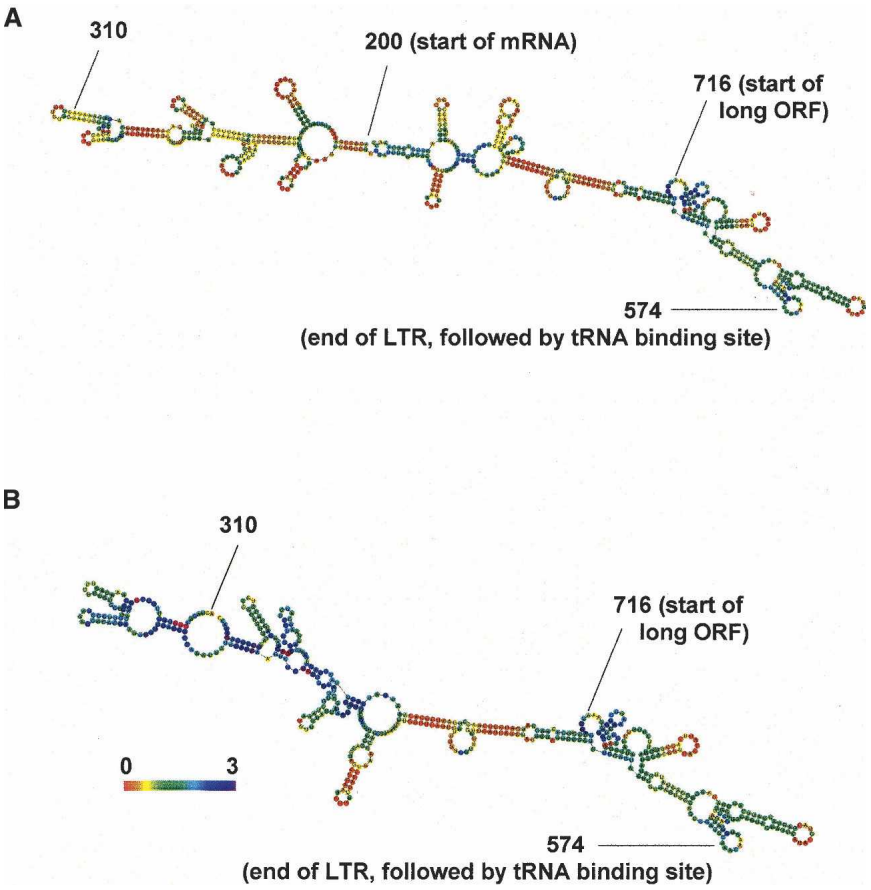

FIGURE 3. Predicted secondary structure of the 5′ part of different Tto1-derived mRNAs. (*A*) Sequence starting at position 200 of Tto1, the in vivo mapped transcription initiation site. Note tight base-pairing of the 5′-end. Other features indicated are (310) position used as mRNA 5′-end in construct C (see Fig. 1 and panel *B*); (574) end of LTR, which is followed by the iMet-tRNA primer binding site (the sequence following position 574 is therefore not base-pairing with adjacent sequences in the intermediate competent for reverse transcription); (716) first ATG of the long Tto1 reading frame. (*B*) Predicted folding of mRNA starting at nt 310. The mRNA 5′-end is apparently accessible for ribosomes. Other designations are as for panel *A*. Both sequences are color coded on a logarithmic scale to indicate "well-definedness" of the displayed folding (see color bar in the *bottom left* corner). Sequences in red have no additional option to form similarly stable structures by alternative base-pairing, or remain single stranded in alternative folds (number 0 *above* the red color indicates $2^0 = 1$ possibilities). Sequences in purple can form several similarly stable structures by alternative base-pairing (number 3 *above* the purple color indicates $\sim 2^3 = 8$ possibilities).

Tto1 RNAs ending at position 4914 do not have sequence redundancy at the 5′-end for start points at positions 200 (produced by construct B; position 200 is the mapped transcription initiation site of Tto1 in tobacco callus) (Hirochika 1993), or position 310 (as produced by construct C). Therefore, mRNAs with these combinations of start and end point cannot participate in the first-strand transfer reaction (Luciw and Leung 1992), and therefore do not support transposition. In addition, the absence of transposition events with construct C, where some mRNAs initiating at nt 310 extend to position 5230, suggested that sequence redundancy alone may be insufficient to guarantee orderly first-strand transfer (the latter mRNAs have ample sequence redundancy between their 5′- and 3′-ends) (see Fig. 4).

Secondary structure at the 3′-ends of Tto1 mRNAs

As suggested above, the strand transfer reaction of the Tto1 retrotransposition cycle probably has stringent structural and sequence requirements (see Discussion section). Therefore, we examined whether Tto1 RNA terminating at position 4914 was folded in a way permissive for orderly first cDNA strand transfer, whereas RNA terminating at position 5230 (see Fig. 4) was not. We calculated secondary structures of these ends. The predicted structure of the longer mRNA end is shown in Figure 5. Interestingly, while there are numerous hairpin structures, a region in proximity to the poly(A) tail cannot pair with the local sequence environment. Instead, sequences close to position 3570 are complementary to this region (gray background in Fig. 5). Thus, annealing of the 3′-end (at position 5230) of the longer RNA to internal sequences could contribute to aberrant priming of cDNA synthesis, for instance, by aligning the poly(A) tail's free 3′-end with U residues upstream of nt 3570. Because the intron monitored by PCR in Figure 2 was inserted at position 2466, the sequence amplified would be present on single-stranded cDNA pieces formed by such a hypothetical mechanism. Synthesis of a complete second strand, however, would predictably not be supported by these templates. RNAs ending at position 4914 have no similarly suggestive features. Characteristically, however, the shorter and the longer mRNA 3′-regions can form entirely distinct secondary structures (data not shown). In summary, the structure prediction data supported our hypothesis that the cDNAs detected by PCR in plants containing construct C did not arise by ordered strand transfer, as would be necessary for transposition. In general, these data indicated that variations at both the 5′-end and the 3′-end of Tto1 mRNA could significantly influence transposition frequency.

Tto1 mRNA starting at position 172 is translated and supports transposition

In order to test whether—in spite of the unusual leader sequence—an accessible 5′-end of Tto1 mRNA is necessary for translation, we designed and tested another construct (Fig. 1D).

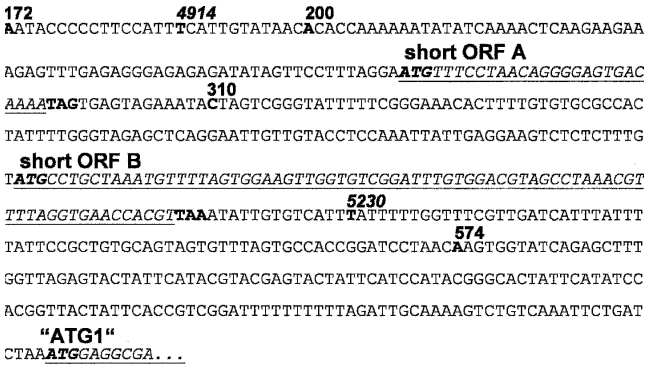

FIGURE 4. Features of the Tto1 mRNA 5′- and 3′-region. The Tto1 sequence is shown starting at nt position 172. Numbers 172, 200, and 310 *above* bold-type bases correspond to transcription start sites used in this work. The Tto1 leader contains two short ORFs A and B, turning ribosome scanning from the mRNA start to "ATG1" into an inefficient mode of translation. Nucleotide 574 is the last base of the 5′-LTR. It is followed by a two-base spacer and the tRNA primer-binding site. Numbers 4914 and 5230 (both in italics) indicate position of termination sites within the 3′-LTR, highlighting the sequence redundancy between the 5′- and 3′-region of different mRNAs.

In this construct, the CaMV 35S promoter was used to synthesize Tto1 mRNAs that start at position 172. According to secondary structure prediction and 3′-end determination, these mRNAs were expected to have an accessible 5′-end, and 17-nt sequence redundancy between the 5′-end and the most abundant 3′-end at position 4914 (see Fig. 4). Upon introduction of construct D into plants, we observed newly transposed copies of Tto1 (germinal transposition events). In order to unambiguously demonstrate transposition activity, newly transposed Tto1 copies were separated from the T-DNA-linked construct D by outcrossing the transgenic plants with wild type. The segregation of two newly inserted Tto1 elements, and their mother copies located on the T-DNA is shown in Figures 6 and 7. Both PCR (Figs. 6A, 7A), and Southern hybridization (Figs. 6C, 7C) demonstrate a transposition event. As expected, PCR reaction using primers specific to the hygromycin-resistance (HPT) gene present on the T-DNA fails to detect a specific band with DNA from those plants that have lost the intron-containing mother copy of Tto1 (Figs. 6B, 7B). This finding was confirmed by Southern hybridization with an HPT probe (Figs. 6D, 7D). Finally, in order to confirm the expectation that we observed true transposition events, the new insertion shown in Figure 7 was characterized by sequence determination (Fig. 8). Tto1 had inserted into the coding region of an expressed gene, At4g15420. As expected from the known replication cycle of retroelements (Luciw and Leung 1992; Kazazian Jr. 2004), the newly transposed copy, which had lost the intron because of the reverse-transcription step (see PCR assay of Fig. 7), did not contain the 35S promoter at the 5′-end, but a complete LTR sequence instead. Furthermore, the transposed Tto1 copy is flanked by a 5-bp target site duplication (Fig. 8).

Taken together, these data demonstrated that expression of a modified Tto1 RNA, carrying its 5′-end at position 172, from a heterologous promoter facilitates transposition in *A. thaliana*.

DISCUSSION

In this work, we identified features of the plant retrotransposon Tto1 that negatively influence its transposition activity. Tto1 belongs to the Ty1/*copia* group of elements, and contains a single long ORF. It inserts preferentially into actively transcribed genes (Okamoto and Hirochika 2000). The necessity to avoid killing of the host plant by high activity suggests that Tto1 transposition should be under tight control. We used expression of various Tto1-derived mRNAs in the heterologous host *A. thaliana* (Fig. 1) and

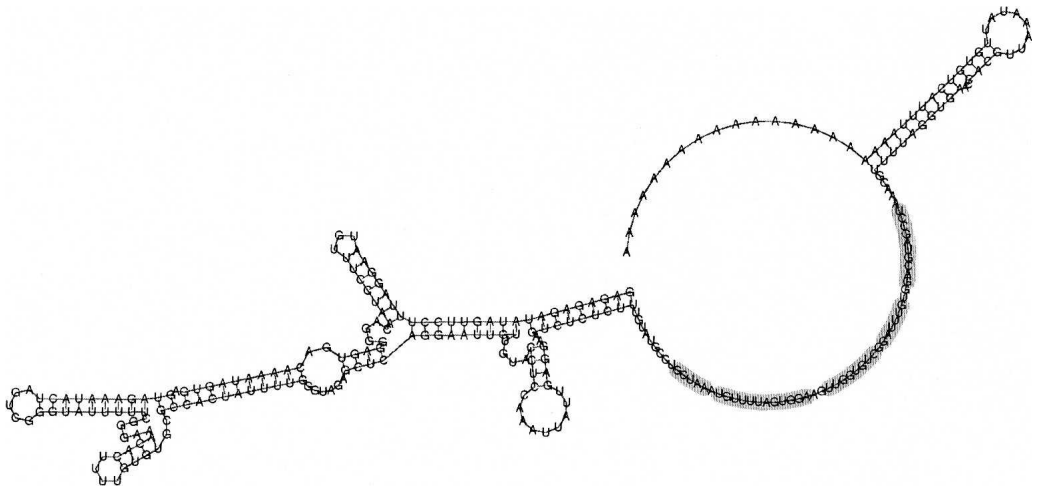

FIGURE 5. Calculated structure of a Tto1 mRNA 3′-end. mRNAs ending at position 5230 can form a stable hairpin close to the poly(A) addition site, but another stretch of bases remains unpaired to the local environment. This region (gray background) is complementary to sequences around position 3570. Binding to this internal sequence might allow generation of truncated single-stranded cDNA molecules, thereby undermining the orderly reverse transcription process necessary for transposition.

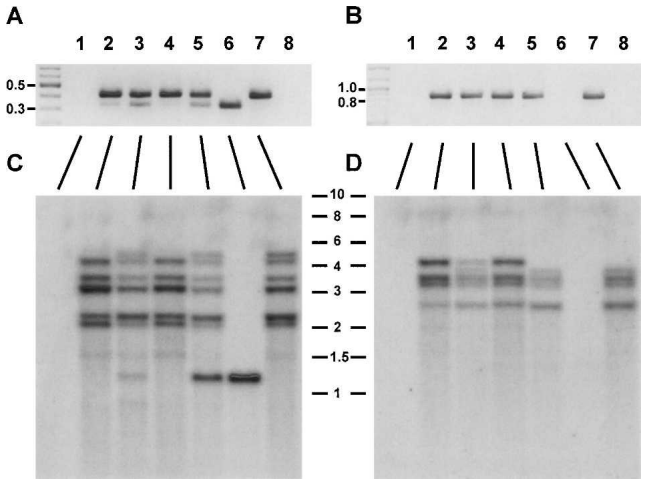

FIGURE 6. Characterization of a transposition event. A plant line containing construct D of Figure 1 at a complex T-DNA locus, and a transposed Tto1 copy, was grown to the T4 generation. (*A*) Progeny plants were tested using PCR primers that flank an artificially introduced intron. The transposed Tto1 copy, product of reverse transcription from the spliced mRNA, gives the smaller band detected in plants of lanes *3*,*5*,*6*. A weak band can also be observed in lanes *2*,*4*, which is ascribed to ongoing reverse transcription to produce extrachromosomal Tto1 cDNA, new integration events, or both. The plant of lane *6* has lost the T-DNA locus by random segregation, whereas plants of lanes *2*,*4*,*7* contain only the T-DNA locus. (Lane *1*) Control with DNA from nontransformed plants; (lane *8*) no DNA control. (*C*) Confirmation of panel *A* PCR results by DNA gel blot, using an internal sequence of Tto1 as a probe. (*B*,*D*) Same DNA as used in panels *A* and *C* was tested with PCR primers specific for the T-DNA marker gene (HPT), and with an HPT-specific probe, respectively. Marker sizes are indicated in kilobases.

found that choice of the mRNA 5′-end, of the mRNA 3′-end, as well as the secondary structure of the Tto1 mRNA leader are relevant to down-regulation of transposition (summarized in Table 1). Based on these data, we succeeded in constructing an active Tto1 element driven by a heterologous promoter.

Like most retrotransposons, Tto1 transcription is largely, but not completely, restricted to stressful conditions (Takeda et al. 1999). Apart from promoter activity, Tto1 transposition is controlled by several additional regulatory mechanisms. We showed that the Tto1 mRNA is capable of extensive base-pairing in its 5′-region (Fig. 3). This was somewhat surprising, because double-stranded RNA is a known inducer of RNA-based defense mechanisms (Tijsterman et al. 2002; Matzke and Birchler 2005). It is therefore likely that high transpositional activity of the element is only possible when RNA-based defense mechanisms are overloaded or down-regulated. This feature therefore constitutes another possible constraint on Tto1 activity.

Tto1 mRNA starting at position 200, which is most abundant in the native host, tobacco, and results from transcription initiation at a prominent TATA-box (Hirochika 1993; Takeda et al. 1999), was not biologically active in our experiments. More than 50 calli transformed with construct B (mRNA start point 200) showed neither reverse transcripts, nor transposition events. Plant lines regenerated from three of these calli are shown in Figure 2B. A PCR-based assay showing presence of intron-free cDNA in plant cells was used as a proof of protein expression from the mRNAs investigated. In contrast to construct B, both constructs C and D, which differ from construct B by different choice of transcription initiation site, generate reverse transcripts in planta (Figs. 2, 6, 7). In order to understand this strong dependence of reverse transcriptase activity on the mRNA initiation site, we investigated structural features of the Tto1 mRNA leader sequence. RNA structure prediction data indicate that the 5′-end of mRNA starting at position 200 is not accessible for the translation initiation complex (Figs. 2, 3). In contrast, mRNAs that were shortened by 110 nt, or elongated by 28 nt at their 5′-end, both with a 5′-end not engaged in a stable stem structure (Fig. 3), are translated, as shown by production of reverse transcripts (Figs. 2, 6, 7). These data suggest that the biologically active Tto1 mRNA species either does not start at position 200, or, alternatively, the highly folded, naturally occurring mRNA species starting at this position is subject to RNA unwinding or to other conformational changes under activating stress conditions. Strictly, we have shown this conundrum only for the *Arabidopsis* host, but it may hold for other plants as well, because accessibility of the

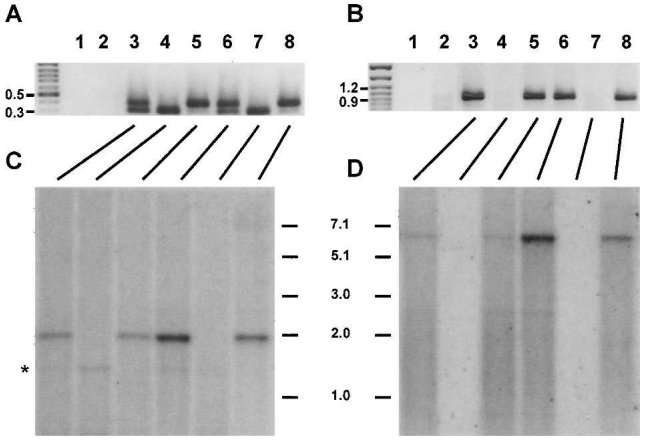

FIGURE 7. Characterization of a transposition event similar to Figure 6, but lines arose from a different transgene integration event. A plant line containing construct D of Figure 1, and a transposed Tto1 copy, was crossed to nontransformed wild-type plants. (*A*) F_2 segregants were tested using PCR primers that flank an artificially introduced intron. The transposed Tto1 copy, product of reverse transcription from the spliced mRNA, gives the smaller band detected in plants of lanes *3*,*4*,*6*,*7*. Plants of lanes *4*,*7* have lost the mother copy of the T-DNA by random segregation, whereas plants of lanes *5*,*8* contain only the T-DNA-localized mother copy. (Lane *1*) No DNA control; (lane *2*) control with DNA from nontransformed plants. (*C*) Confirmation of panel *A* PCR results by DNA gel blot, using an internal sequence of Tto1 as a probe. (*B*,*D*) Same DNA as used in panels *A* and *C* was tested with PCR primers specific for the T-DNA marker gene (HPT), and with an HPT-specific probe, respectively. The asterisk to the *left* indicates the position of the new Tto1 copy. Marker sizes are indicated in kilobases.

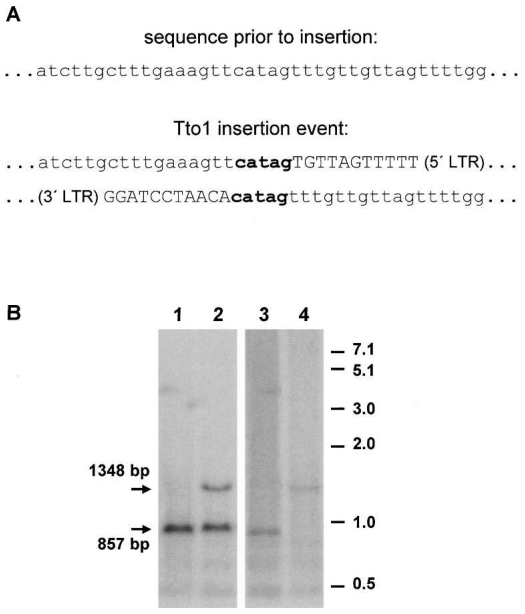

FIGURE 8. Characterization of an integration site. The transposition event of Figure 7 was characterized by isolation of DNA flanking the Tto1 insert and by sequence determination. (*A*) Sequence of the gene At4g15420 and its interruption by Tto1. The retrotransposon is flanked by a 5-bp target site duplication. The target site duplication is printed in bold; Tto1 sequences are in capital letters. (*B*) DNA gel blot using a probe from gene At4g15420 indicates the expected size difference compared to wild-type DNA. (Lane *1*) DNA from line containing Tto1 as part of a T-DNA locus, but no insertion in At4g15420. (Lane *2*) Line contains Tto1 insertion into At4g15420 (heterozygous state). (Lane *3*) DNA of wild-type (nontransgenic) plant line was used for digestion and blotting. (Lane *4*) DNA from line with insert in At4g15420 in homozygous state was used. Molecular weight marker positions are indicated to the *right*, and the expected size of fragments as calculated from DNA sequence information is indicated to the *left*.

cap for the cap-binding complex is generally a strong determinant of translation efficiency (Cormier et al. 2003; Cougot et al. 2004). Because we were unable to identify transposition events or reverse transcripts when the "native" mRNA (start position 200) was expressed with a heterologous promoter under tissue culture stress conditions, we favor the model that a change in transcription initiation site is a necessary step in the Tto1 life cycle. If this is correct, the Tto1 native promoter may allow initiation at a position different from position 200 under stress conditions. This change at the 5′-end of the mRNA then facilitates expression of Tto1 proteins.

Mapping of Tto1 RNA 3′-ends indicated that the major transcription termination site is located at position 4914 (corresponds to position 188 in the 5′-LTR) (see Fig. 4). mRNAs ending at this position do not have sequence redundancy with the 5′-end of the canonical Tto1 transcript in tobacco (initiation at position 200). Because such a sequence overlap is essential for the first-strand transfer of the reverse transcription process, this combination of 5′- and 3′-ends cannot support reverse transcription. Some transcripts, however, end at position 5230 (corresponding to position 504 of the 5′-LTR) (see Fig. 4) and have therefore sequence redundancy with 5′-ends starting either at position 200, or at position 310 (which is produced by construct C) (Figs. 1, 2). Whereas transcripts starting at position 200 probably do not allow protein expression, those starting at position 310 are apparently permissive for translation (Figs. 1C, 2B, 3B). Nonetheless, no transposition events were detected with construct C of Figure 1. We tentatively concluded that mRNA 3′-ends at position 5230 do not support all steps of the life cycle.

Interestingly, RNA polymerase II (Pol II) is not the only DNA-dependent RNA polymerase known to generate transcripts from retrotransposons. The recently described, plant-specific Pol IV has the reported function to produce long RNAs from repeat sequences such as retrotransposons, that are subsequently channeled into an siRNA-based silencing pathway (Herr et al. 2005; Onodera et al. 2005). It remains to be investigated whether, under stress conditions, Pol II generates biologically competent transcripts (e.g., starting upstream of nt 200), or whether some Pol IV transcripts have permissive 5′- and 3′-ends and can escape cleavage by Dicer-type ribonucleases under stress conditions, so that they serve as substrates for translation and reverse transcription.

It is generally believed that first-strand transfer of retroelements is a catalyzed, highly ordered process, so that the

TABLE 1. Features of Ttol RNA with potential relevance to retrotransposition

Hypothesis	Experimental support
Naturally occurring mRNA with 5′-end at position 200 is not translated and does not support transposition	mRNA 5′-end is engaged in secondary structure (Fig. 3); no activity of construct B as compared to construct C (Fig. 2)
mRNA 3′-end at position 5230 allows aberrant reverse transcription, but not orderly first-strand transfer	Sequence at 3′-end of mRNA can base-pair with internal region (Fig.5); cDNA formation by construct C (Fig. 2), but no transposition
mRNA 3′-end at position 4914 allows orderly cDNA first-strand transfer (together with 5′-end at position 172)	Transposition activity of construct D (Fig. 6), but not of construct C
Tto1 mRNA is translated by a mechanism that requires ribosome entry at the 5′-end, but does not proceed by ribosome scanning (mechanism related to those proposed by Hohn et al. [2001] and Yaman et al. [2003])	Importance of accessible 5′-end for translatability (Figs. 2, 3) rules out internal ribosome entry; presence of two short ORFs before long ORF (Fig. 4) rules out scanning mechanism

strand transfer reaction does not depend on random alignment of the redundant sequences from the 5′- and 3′-ends. For instance, retroviruses form noncovalent dimers of their mRNA to facilitate strand transfer *in trans* (Paillart et al. 1996). In the yeast retrotransposon Ty3, an extended, base-paired structure encompassing two mRNAs and two tRNAs has been identified (Gabus et al. 1998). RNA of yeast Ty1 also displays base-pairing interactions, both between 5′- and 3′-sequences, and within the 5′-region, whose likely effect is to align sequences for reverse transcription and strand transfer (Cristofari et al. 2002; Bolton et al. 2005). Even the formation of a covalent linkage between the 5′-end and a base close to the 3′-end of Ty1 RNA has been discussed in the literature (Cheng and Menees 2004; Coombes and Boeke 2005). Structures that facilitate strand transfer in the more primordial Tto1 element are not known at present. However, knowing precisely the 3′-ends of Tto1 mRNA in *Arabidopsis*, we asked the question whether any structural feature at these 3′-ends puts a constraint on alignment of the mRNA 5′- and 3′-ends. This was also interesting in light of the fact that expression of construct C leads to reverse transcription (Fig. 2), but not to transposition. Figure 5 shows the structure modeling data with the last 250 nt of Tto1 mRNA ending at position 5230. Interestingly, a region not engaged in local double-strand formation has the potential to bind to an internal Tto1 site around position 3570. A fold-back structure mediated by this self-complementarity could facilitate priming of a shortened cDNA by the mRNA poly(A) end. However, a complete copy of Tto1 as necessary for transposition cannot be formed in this way. Because such a shortened cDNA can be detected by the intron removal assay shown in Figure 2, this hypothesis offers an explanation for the features of construct C, and is consistent with the finding that mRNA ends at position 5230 do not detectably support transposition.

Construct D of Figure 1 was designed to produce an mRNA with an accessible 5′-end that lies upstream of position 200. The choice of position 172 was made to include all sequences up to, but exclusive of, the TATA-box. Incidentally, this 5′-end provides a sequence redundancy of 17 bases with the most abundant 3′-end of Tto1, which lies at position 4914 (corresponding to position 188 in the 5′-LTR) (see Fig. 4). Presence of transposed Tto1 copies in the progeny of some construct-D-expressing plants (Figs. 6, 7, 8) shows that an mRNA 5′-end at position 172 is both permissive for translation, and can participate in the first-strand transfer, possibly with the 3′-end at position 4914. In summary, two of 13 plant lines regenerated from calli expressing the "active" construct D (mRNA start point 172) showed transposition in the germ line (as opposed to no event detected among more than 50 calli transformed with construct B, which initiates transcription at position 200) (Figs. 6, 7, 8). The available data also indicate that the integration characteristics of construct D correspond to those of unmanipulated Tto1: Sequence analysis of one new insertion event showed integration into a gene (At4g14520) and generation of a 5-bp pair target site duplication (Fig. 8), characteristics also found with native Tto1 (Okamoto and Hirochika 2000).

Finally, another remarkable feature of Tto1 mRNA discussed in this work, but not directly addressed by experimentation, concerns the presence of two short ORFs in its long leader sequence (Fig. 4). The analysis presented strongly suggests that the mRNA 5′-end must be accessible for ribosome entry, in order to allow biological activity. Nonetheless, it is unlikely that Tto1 mRNA is translated by a normal scanning mechanism (Jackson 2000). Most ribosomes passing through an ORF are expected to disassemble at its end. Passage through two ORFs would therefore restrict translation of the large Tto1 reading frame to very low levels. Interestingly, a similar structure is present in the plant pararetrovirus Cauliflower Mosaic Virus (CaMV). The CaMV RNA is believed to be translated by "ribosome shunting," a process in which the ribosome assembles at the cap, but dissociates from the mRNA and re-enters at a downstream position, in order to skip over a region of extended secondary structure, which also contains short ORFs (Fütterer et al. 1993; Hohn et al. 2001). An alternative to this ribosome shunting model (in which one and the same ribosome "jumps" over a part of the mRNA) was recently proposed for the mammalian arginine/lysine transporter (Yaman et al. 2003; Fernandez et al. 2005). According to that model, a ribosome that enters the mRNA at the 5′-end and starts translating short reading frames can create transiently an internal ribosome entry site, so that a second ribosome can bind and initiate translation at the major, long ORF. An interesting facet of both potential modes of translation is that no ribosome would pass across the region where initiator-Met tRNA binds to Tto1 mRNA as a primer for reverse transcription (i.e., shortly after the end of the LTR, which is at position 574) (Figs. 3, 4). This would allow annealing of the tRNA, and translation, on the same RNA molecule.

In summary, we have identified several features of Tto1 RNA structure that influence Tto1 activity (listed in Table 1). The identification allowed us to induce Tto1 transposition using a heterologous promoter. Further understanding of the transposition process will build on the results obtained so far, allowing more detailed analysis, and experimental control, of Tto1 transposition.

MATERIALS AND METHODS

Transgenic plants and microbes

Agrobacterium-mediated transformation of *A. thaliana* (ecotype Wassilewskija) was carried out by the root (Valvekens et al. 1988) transformation method with *Agrobacterium tumefaciens* C58C1 (pGV2260) carrying the Tto1 expression constructs in

pBIN-based binary vectors. *Escherichia coli* strain XL1-blue was used for construction of the Tto1 vectors described in this work.

Tto1 vector constructs

An SpeI (site filled in by DNA polymerase Klenow fragment)–XbaI fragment extending from position 310 to position 4285 of the Tto1 sequence (accession number D83003) was excised from a pSK^+-based Tto1 clone (Hirochika 1993) and transferred into MscI–XbaI-cleaved expression vector pRT103 (Töpfer et al. 1987) to obtain construct pRTSpX. A HindIII–XbaI fragment of pRTSpX was moved into the HindIII–XbaI-cleaved vector pBIB Hyg (Becker 1990) to obtain construct pBCSpX. An XbaI–HindIII fragment, encompassing part of the Tto1 reading frame and 3′-LTR (starting at position 4285 of Tto1 and extending into adjacent tobacco DNA of the original Tto1 clone) was cloned in pSK, then partially digested with BamHI, treated with DNA polymerase Klenow fragment, and re-ligated in the presence of a NotI linker (sequence AGCGGCCGCT; New England Biolabs) to obtain pTtoXN, which carried the NotI linker 6 bp from the 3′-end of Tto1 (position 5294). Tto1 sequences carried by a NotI–XbaI fragment of pTtoXN were inserted into Asp718–XbaI-digested pBCSpX by filling in the NotI and Asp718 sites to restore the Tto1 reading frame, resulting in construct C without introns.

An AatII–EcoRV (positions 1995–2630 of Tto1) fragment was cloned into AatII–SmaI-cut vector pUC19 to give pUCAD. Using oligonucleotides CCCCAGTTGAATGGCTTGGCTGCGAGGATG AACCGTACGTTGATC (Tto1 positions 2441–2485) and AATCTT TTCTGGAGCCT (complementary to Tto1 positions 2601–2617), and Tto1 DNA as a template, a fragment with an additional BsiWI site (but encoding wild-type Tto1 protein sequence) was amplified, digested with BstXI and DraIII, and inserted between BstXI and DraIII sites of pUCAD to obtain pUCABD. Oligonucleotides CCCCAGTT GAATGGCTTGGCTGAGAGGTAGCAATTTCATCAGCGG (carrying Tto1 sequence from positions 2441–2466, followed by positions 3372–3390 of accession X52954; the latter sequence encodes an intron of the RNA polymerase II largest subunit gene At4g35800) and CCC GTACGGTTCATCCTGAAAATGGATAAG (encoding a BsiWI site, followed by complementarity to Tto1 positions 2473–2467, followed by complementary to 3465 to 3451 of accession X52954), and *Arabidopsis* (ecotype Col-0) template DNA were used for PCR to amplify a fragment that contains a 94-nt intron from the largest subunit of RNA polymerase II (Nawrath et al. 1990). After BsiWI, BstXI digest, the fragment was cloned into BstXI–BsiWI-digested pUCABD. An AatII–DraIII fragment (positions 1995–2580 of Tto1) from this clone was transferred, via intermediate vectors, into construct C minus intron to give construct C.

To obtain construct B, construct C was digested with EcoRI and after filling in the site, a NotI linker (sequence AGCGGCCGCT; New England Biolabs) was inserted. After XbaI, partial NotI digestion, an XbaI–NotI fragment from pTtoXPN was inserted. pTtoXPN was obtained by using oligonucleotides AAATGGATCGGACATGTT and GAAGATCTGTTTAAACTAACAATTCTCCCCCTTC, together with Tto1 DNA as a template, for PCR reaction and subsequent digestion with BglII and PstI. The ensuing fragment was inserted between PstI and BamHI sites of pTtoXN. To obtain a unique site at the 3′-end of Tto1, the Not I site at the 3′-end of Tto1 was converted, via filling in and linker addition (sequence CCCCGGGG; New England Biolabs), into an SmaI site to obtain vector pBTtoP+S. Vector p2RT103 (Schlögelhofer and Bachmair 2002) was used to insert an XhoI–MscI fragment from pRTSpX (Tto1 positions 310–1851). Using the oligonucleotides GCTCGAGACACCAAAAAATA TATC and CGTGGATATGAATAGTGCCCGT, a fragment (Tto1 positions 200–568) was PCR-amplified, digested with XhoI and BamHI, and inserted into the p2RT103-based vector to obtain p2RT200T. Finally, a HindIII–SalI fragment was inserted into pBTtoP+S to generate construct B.

Construct D was obtained in a similar manner as construct B, but the fragment inserted into the p2RT103-based clone started at nt 172 (the oligonucleotides used for the PCR reaction were ACGCGTC GACAAGCTTCTCGAGAATACCCCCTTCCATTTCAT [contains Tto1 positions 172–191] and CGTGGATATGAATAGTGCCCGT).

Construct E contains Tto1 sequence nt 4700–5294, which is the same 3′-region present in constructs B–D.

PCR reactions for cloning steps were carried out with Platinum Pfx polymerase (Invitrogen) as recommended by the manufacturer. All PCR-generated DNA segments incorporated into constructs were subjected to sequence confirmation.

DNA analysis

Plant leaf tissue was ground to a fine powder in liquid nitrogen and processed using the Qiagen DNeasy Plant Maxi Kit (QIAGEN) as recommended by the manufacturer. DNA gel blot analysis was carried out according to Ausubel et al. (1987).

To obtain a hybridization probe specific for Tto1, oligonucleotides CATCGCAGCAACGGAGGCTTGC and CCACTATCCCTGCAGC TTCTC were used to amplify nt 4390–4697 of Tto1 via PCR, using plasmid-encoded Tto1 sequence as a template. A probe for detection of T-DNA vector contained sequences from hygromycin phosphotransferase HPT. A probe for detection of At4g15420 was generated by PCR using oligonucleotides GTTTTGGTAGCTTGATGTGAAA CT and GTCAGTCTCCAGAACCGAAATGCT with genomic DNA, and subsequent digestion of the obtained fragment with HindIII to obtain a 426-bp fragment. Probes were labeled using random hexanucleotides according to the manufacturer's recommendations (Roche).

PCR genotyping of plant material

For rapid genotyping, 50 mg of leaf or callus material from *Arabidopsis* was frozen in a 1.5-mL reaction tube. After addition of quartz sand and 400 μL of isolation buffer (200 mM Tris at pH 7.5, 250 mM NaCl, 0.5% SDS, 25 mM EDTA), the material was homogenized with a glass pestle. The supernatant of a 5-min centrifugation step was mixed with 400 μL of *iso*-propanol, kept at room temperature for 5 min, and centrifuged for another 5 min. The pellet was washed with 70% ethanol, dried, and resuspended in 50 μL of TE buffer (10 mM Tris, 1 mM EDTA at pH 8.0). After incubation at 65°C for 5 min and resuspension of the residual pellet, the supernatant of a 2-min centrifugation step was transferred to a fresh 1.5 mL reaction tube. Then 2 μL from this solution was used for PCR. Primers GGTGGAAA GAGAGACTGGTAA (homologous to Tto1 positions 2314–2334) and CCCGTAATTGATCATAAGAGA (complementary to Tto1 positions 2634–2654) served to monitor intron excision from Tto-derived DNA in transgenic plants. Primers GGGTTTCTGGAGT TTAATGAGCT (homologous to a sequence of the nopaline synthase promoter) and GAACATCGCCTCGCTC CAGT (complementary to a region in the hygromycin phosphotransferase gene) were used for

detection of the hygromycin-resistance gene. PCR reactions were performed according to Ausubel et al. (1987).

Isolation of DNA flanking Tto1 insert in At4g15420

DNA from plants containing a new insertion, but no T-DNA-borne copy of Tto1, was isolated using the Qiagen DNeasy Plant Maxi Kit (QIAGEN) as recommended by the manufacturer. Then 30 μg of DNA was digested with EcoRI and HindIII and size-fractionated by agarose gel electrophoresis. DNA in the size range of 1.5 kb was isolated in several fractions and tested for the presence of Tto1 sequences by PCR. The best fraction was cloned into EcoRI- and HindIII-digested vector pSK (Stratagene). After transformation into One Shot competent cells (Invitrogen), candidate clones were identified by colony hybridization (Ausubel et al. 1987). Sequence determination identified the insertion site. Oligonucleotides CTGTATGGTAAATGCTCTCCTAGGTT (chosen from the *Arabidopsis* genome sequence to allow isolation of the sequence flanking the Tto1 5′-end) and CGTGGATATGAA TAGTGCCCGT (complementary to a Tto1 sequence adjacent to the 5′-LTR) were used for PCR reaction using a DNA sample as above. The ensuing fragment was digested with BamHI and MfeI and cloned into BamHI- and EcoRI-digested vector pSK. Sequence determination allowed assignment of the target site duplication.

RT-PCR

RNA from plant material was prepared using the RNeasy Plant Mini Kit (QIAGEN). Reverse transcription with AMV reverse transcriptase (Roche) and oligonucleotide CCCGTAATTGATCATAAGAGA was carried out according to the manufacturer's recommendations in a 20 μL reaction volume. Then 1 μL was amplified by 25 PCR reaction cycles, using primers and conditions as applied for plant genotyping.

Mapping of Tto1 mRNA 3′-ends

RNA was prepared from young seedlings grown for 3 wk on agar medium using the Qiagen RNeasy Plant Mini Kit (QIAGEN). Poly(A) RNA was enriched using Dyna Beads Oligo$(dT)_{25}$ (Dynal) according to the manufacturer's recommendations. The RNA-loaded beads were rinsed once with 1× AMV buffer and resuspended in 30 μL of 1× AMV buffer. Then 15 μL of the suspension was used in a reverse transcription reaction with 25 U of AMV reverse transcriptase (Roche) for 3 h at 42°C in a final volume of 20 μL. An aliquot of 2 μL was subjected to PCR using oligonucleotides CGGACGCTCA GCCAGGTTTTTTTTTTTTTTTTTTTTTTTTTTT and ACAGGGCT TCAAGAGCGTGGTC. The PCR conditions included five cycles with annealing temperature 42°C, followed by 35 cycles with 55°C annealing temperature. Of this reaction, 1/50 was used for PCR with nested primer ATGCACGAGGCGCTCGGATATGCC and clamp primer CGGACGCTCAGCCAGGTTT, keeping the annealing temperature at 55°C. Ensuing DNA fragments were separated by agarose gel electrophoresis, isolated, and cloned into vector pCR2.1 (Invitrogen).

RNA secondary-structure prediction

RNA secondary structures were predicted using the RNAfold program from the Vienna RNA package version 1.5 (Hofacker 2003), with the energy parameters of Mathews et al. (1999).

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REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.F., Moore, D.O., Seidman, J.G., Smith, J.A., and Struhl, K. 1987. *Current protocols in molecular biology.* John Wiley, New York.

Becker, D. 1990. Binary vectors which allow the exchange of plant selectable markers and reporter genes. *Nucleic Acids Res.* **18:** 203.

Bennetzen, J.L. 2000. Transposable element contributions to plant gene and genome evolution. *Plant Mol. Biol.* **42:** 251–269.

Boeke, J.D. and Stoye, J.P. 1997. Retrotransposons, endogenous retroviruses, and the evolution. In *Retroviruses* (eds. J.M. Coffin et al.), pp. 343–435. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Bolton, E.C., Coombes, C., Eby, Y., Cardell, M., and Boeke, J.D. 2005. Identification and characterization of critical *cis*-acting sequences within the yeast Ty1 retrotransposon. *RNA* **11:** 308–322.

Cheng, Z. and Menees, T.M. 2004. RNA branching and debranching in the yeast retrovirus-like element Ty1. *Science* **303:** 240–243.

Coombes, C.E. and Boeke, J.D. 2005. An evaluation of detection methods for large lariat RNAs. *RNA* **11:** 323–331.

Cormier, P., Pyronnet, S., Salaün, P., Mulner-Lorillon, O., and Sonenberg, N. 2003. Cap-dependent translation and control of the cell cycle. *Prog. Cell Cycle Res.* **5:** 469–475.

Cougot, N., van Dijk, E., Babajko, S., and Seraphin, B. 2004. Cap-tabolism. *Trends Biochem. Sci.* **29:** 436–444.

Cristofari, G., Bampi, C., Wilhelm, M., Wilhelm, F.-X., and Darlix, J.-L. 2002. A 5′–3′ long-range interaction in Ty1 RNA controls its reverse transcription and retrotransposition. *EMBO J.* **21:** 4368–4379.

Eickbush, T.H. and Malik, H.S. 2002. Origins and evolution of retrotransposons. In *Mobile DNA II* (eds. N.L. Craig et al.), pp. 1111–1144. ASM Press, Washington, DC.

Fernandez, J., Yaman, I., Huang, C., Liu, H., Lopez, A.B., Komar, A.A., Caprara, M.G., Merrick, W.C., Snider, M.D., Kaufman, R.J., et al. 2005. Ribosome stalling regulates IRES-mediated translation in eukaryotes, a parallel to prokaryotic attenuation. *Mol. Cell* **17:** 405–416.

Feschotte, C., Jiang, N., and Wessler, S.R. 2002. Plant transposable elements: Where genetics meets genomics. *Nat. Rev. Genet.* **3:** 329–341.

Fütterer, J., Kiss-László, Z., and Hohn, T. 1993. Nonlinear ribosome migration on Cauliflower Mosaic Virus 35S RNA. *Cell* **73:** 789–802.

Gabus, C., Ficheux, D., Rau, M., Keith, G., Sandmeyer, S., and Darlix, J.L. 1998. The yeast Ty3 retrotransposon contains a 5′–3′ bipartite primer binding site and encodes nucleocapsid protein NCP9 functionally homologous to HIV-1 NCP7. *EMBO J.* **17:** 4873–4880.

Grandbastien, M.-A. 1998. Activation of plant retrotransposons under stress conditions. *Trends Plant Sci.* **3:** 181–187.

Herr, A.J., Jensen, M.B., Dalmay, T., and Baulcombe, D.C. 2005. RNA polymerase IV directs silencing of endogenous DNA. *Science* **308:** 118–120.

Hirochika, H. 1993. Activation of tobacco retrotransposons during tissue culture. *EMBO J.* **12:** 2521–2528.

Hirochika, H., Otsuki, H., Yoshikawa, M., Otsuki, Y., Sugimoto, K., and Takeda, S. 1996. Autonomous transposition of the tobacco retrotransposon Tto1 in rice. *Plant Cell* **8:** 725–734.

Hirochika, H., Okamoto, H., and Kakutani, T. 2000. Silencing of retrotransposon in *Arabidopsis* and reactivation by the *ddm1* mutation. *Plant Cell* **12:** 357–368.

Hofacker, I.L. 2003. Vienna RNA secondary structure server. *Nucleic Acids Res.* **31:** 3429–3431.

Hohn, T., Corsten, S., Dominguez, D., Fütterer, J., Kirk, D., Hemmings-Mieszczak, M., Pooggin, M., Schärer-Hernandez, N., and Ryabova, L. 2001. Shunting is a translation strategy used by plant pararetroviruses (Caulimoviridae). *Micron* **32:** 51–57.

Jackson, R.J. 2000. A comparative view of initiation site selection mechanisms. In *Translational control of gene expression* (eds. N. Sonenberg et al.), pp. 127–183. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Kazazian Jr., H.H. 2004. Mobile elements: Drivers of genome evolution. *Science* **303:** 1626–1632.

Kumar, A. and Bennetzen, J.L. 1999. Plant retrotransposons. *Annu. Rev. Genet.* **33:** 479–532.

Kunze, R., Saedler, H., and Lönnig, W.-E. 1997. Plant transposable elements. *Adv. Bot. Res.* **27:** 332–470.

Lecher, P., Bucheton, A., and Pelisson, A. 1997. Expression of the *Drosophila* retrovirus *gypsy* as ultrastructurally detectable particles in the ovaries of flies carrying a permissive flamenco allele. *J. Gen. Virol.* **78:** 2379–2388.

Luciw, P.A. and Leung, N.L. 1992. Mechanisms of retrovirus replication. In *The retroviridae* (ed. J.A. Levy), Vol. 1 pp. 159–298. Plenum Press, NY.

Mathews, D.H., Sabina, J., Zuker, M., and Turner, D.H. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* **288:** 911–940.

Matzke, M. and Birchler, J.A. 2005. RNAi-mediated pathways in the nucleus. *Nat. Rev. Genet.* **6:** 24–35.

Meignin, C., Bailly, J.-L., Arnaud, F., Dastugue, B., and Vaury, C. 2003. The 5′ untranslated region and gag protein of *Idefix*, a long terminal repeat-retrotransposon from *Drosophila melanogaster*, act together to initiate a switch between translated and untranslated states in the genomic mRNA. *Mol. Cell. Biol.* **23:** 8246–8254.

Mhiri, C., Morel, J.-B., Vernhettes, S., Casacuberta, J.M., Lucas, H., and Grandbastien, M.-A. 1997. The promoter of the tobacco Tnt1 retrotransposon is induced by wounding an abiotic stress. *Plant Mol. Biol.* **33:** 257–266.

Miura, A., Yonebayashi, S., Watanabe, K., Toyama, T., Shimada, H., and Kakutani, T. 2001. Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. *Nature* **411:** 212–214.

Nawrath, C., Schell, J., and Koncz, C. 1990. Homologous domains of the largest subunit of eukaryotic RNA polymerase II are conserved in plants. *Mol. Gen. Genet.* **223:** 65–75.

Okamoto, H. and Hirochika, H. 2000. Efficient insertion mutagenesis of *Arabidopsis* by tissue culture-induced activation of the tobacco retrotransposon Tto1. *Plant J.* **23:** 291–304.

Onodera, Y., Haag, J.R., Ream, T., Costa Nunes, P., Pontes, O., and Pikaard, C.S. 2005. Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation. *Cell* **120:** 613–622.

Paillart, J.C., Marquet, R., Skripkin, E., Ehresmann, C., and Ehresmann, B. 1996. Dimerization of retroviral genomic RNAs: Structural and functional implications. *Biochimie* **78:** 639–653.

Peterson-Burch, B.D. and Voytas, D.F. 2002. Genes of the Pseudoviridae (Ty1/*copia* retrotransposons). *Mol. Biol. Evol.* **19:** 1832–1845.

Pourtau, N., Lauga, B., Audéon, C., Grandbastien, M.-A., Goulas, P., and Salvado, J.-C. 2003. The promoter of the Tnt1A retrotransposon is activated by ozone air pollution in tomato, but not in its natural host tobacco. *Plant Sci.* **165:** 983–992.

Schlögelhofer, P. and Bachmair, A. 2002. A test of fusion protein stability in the plant, *Arabidopsis thaliana*, reveals degradation signals from ACC synthase and from the plant N-end rule pathway. *Plant Cell Rep.* **21:** 174–179.

Takeda, S., Sugimoto, K., Otsuki, H., and Hirochika, H. 1999. A 13-bp *cis*-regulatory element in the LTR promoter of the tobacco retrotransposon Tto1 is involved in responsiveness to tissue culture, wounding, methyl jasmonate and fungal elicitors. *Plant J.* **18:** 383–393.

Tijsterman, M., Ketting, R.F., and Pasterk, R.H.A. 2002. The genetics of RNA silencing. *Annu. Rev. Genet.* **36:** 489–519.

Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J., and Steinbiss, H.-H. 1987. A set of plant vectors for transcriptional and translational fusions. *Nucleic Acids Res.* **15:** 5890.

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. 1988. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci.* **85:** 5536–5540.

Yaman, I., Fernandez, J., Liu, H., Caprara, M., Komar, A.A., Koromilas, A.E., Zhou, L., Snider, M.D., Scheuner, D., Kaufman, R.J., et al. 2003. The zipper model of translational control: A small upstream ORF is the switch that controls structural remodeling of an mRNA leader. *Cell* **113:** 519–531.