The Plant Cell, Vol. 12, 357–368, March 2000, www.plantcell.org © 2000 American Society of Plant Physiologists

# Silencing of Retrotransposons in Arabidopsis and Reactivation by the *ddm1* Mutation

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**Gene silencing associated with repeated DNA sequences has been reported for many eukaryotes, including plants. However, its biological significance remains to be determined. One important function that has been proposed is the suppression of transposons. Here, we address transposon suppression by examining the behavior of the tobacco retrotransposon *Tto1* and endogenous retrotransposons in Arabidopsis. After an initial increase in copy number because of active transposition in the Arabidopsis genome, *Tto1* became silent. The amount of transcript was reduced, and the inactivated *Tto1* became methylated. This silencing correlated with an increase in copy number. These phenomena mimic repeat-induced gene silencing. The homozygous *ddm1* (for decrease in DNA methylation) mutation of Arabidopsis results in genomic DNA hypomethylation and the release of silencing in repeated genes. To investigate the role of DNA methylation and the gene-silencing machinery in the suppression of *Tto1*, we introduced the *ddm1* mutation into an Arabidopsis line carrying inactivated *Tto1* copies. In the homozygous *ddm1* background, *Tto1* became hypomethylated and transcriptionally and transpositionally active. In addition, one of the newly isolated endogenous Arabidopsis retrotransposon families, named *Tar17*, also became hypomethylated and transcriptionally active in the *ddm1* mutant background. Our results suggest that the inactivation of retrotransposons and the silencing of repeated genes have mechanisms in common.**

## INTRODUCTION

Suppression of repeated genes is found in several eukaryotes and is referred to as homology-dependent gene silencing. The silencing of repeated transgenes has been investigated extensively in plants (reviewed in Flavell, 1994; Matzke et al., 1996; Meyer and Saedler, 1996; Vaucheret et al., 1998; Grant, 1999; Selker, 1999). Silencing results from a decrease in the amount of mRNA at steady state and is often, but not always, accompanied by cytosine methylation of the affected genes. The DNA methylation associated with silencing is not restricted to repeated transgenes but is also found in repeated endogenous genes, such as the tryptophan biosynthetic gene *PAI* of Arabidopsis (Bender and Fink, 1995) and *R* of maize (Ronchi et al., 1995).

The biological significance of the silencing of repeated genes, however, remains to be determined. Silencing repeated genes should confer a selective advantage, given that it is a well-conserved biological phenomenon seen in plants, mammals, fungi, and other eukaryotes (Pal-Bhadra et al., 1997; Garrick et al., 1998). One possible function of DNA methylation and inactivation of repeated sequences is suppression of transposons within the genome (Yoder et al., 1997; Martienssen, 1998). Transposons induce detrimental changes in the host genes in several ways, one of which is the disruption of genes by insertion mutations. For Drosophila, in which DNA methylation does not occur, 80% of spontaneous mutations are caused by the insertion of retrotransposons (Green, 1988). In plants, a large part of the genome is occupied by retrotransposons. For example, 50 to 80% of the 2400-Mb maize genome and at least 40% of the 12,500-Mb fava bean genome are occupied by retrotransposons (Pearce et al., 1996; SanMiguel et al., 1996; SanMiguel and Bennetzen, 1998). However, only a small portion of spontaneous mutations in plants has been shown to be caused by retrotransposons (Wessler et al., 1995). In contrast to the retrotransposons of Drosophila, most plant retrotransposons are highly methylated, and they are almost always not transcribed (Bennetzen et al., 1994; Hirochika, 1997). Together, these results suggest that methylation is an effective strategy in retrotransposon suppression.

Because retrotransposons transpose by way of an RNA, inhibiting transcription by DNA methylation should be the most effective way to suppress the transposition of retrotransposons. In addition, because retrotransposons carry relatively strong promoter–enhancer elements (Pouteau et al., 1991; Hirochika et al., 1996a), the presence of a large

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number of retrotransposons in the genome could have a marked influence on the transcription of flanking genes. Thus, suppression of the promoter–enhancer activity by methylation should also be important for ensuring proper transcription of the nearby genes. Suppression of transcription may be more important than suppression of transposition, because most plant retrotransposons have already lost the ability to transpose, given an accumulation of mutations (Flavell et al., 1992; Voytas et al., 1992; Hirochika and Hirochika, 1993).

For genetic dissection of gene silencing and DNA methylation, Arabidopsis provides a good model system. Two types of Arabidopsis mutants with decreases in DNA methylation have been isolated: *ddm1* mutants isolated from a population mutagenized by exposure to ethyl methanesulfonate (Vongs et al., 1993; Kakutani et al., 1995), and transgenic plants that express the DNA methyltransferase gene (*MET1*) in the antisense orientation (Finnegan and Dennis, 1993; Finnegan et al., 1996; Ronemus et al., 1996). Recently, several mutants modifying the silencing of repeated transgenes were also isolated, including modifiers of gene silencing such as the *egs1* and *egs2* mutations, which enhance silencing of *rolB* transgene (Dehio and Schell, 1994); suppressors of transgene silencing such as the *sgs1* and *sgs2* mutations, which release cosuppression (Elmayan et al., 1998); somniferous (*som*) mutations, which release the silencing of repeated hygromycin phosphotransferase (*HPT*) genes driven by the cauliflower mosaic virus 35S promoter (Mittelsten Scheid et al., 1998); and the altered transgene-silencing *hog1, sil1*, and *sil2* mutants, which reduce the silencing of repeated chalcone synthase (*CHS*) transgenes (Furner et al., 1998).

For both repeated *HPT* and repeated *CHS* systems, the *ddm1* mutation also releases transgene silencing (Furner et al., 1998; Mittelsten Scheid et al., 1998). Moreover, this mutation impairs silencing of the repeated endogenous *PAI2* gene (Jeddeloh et al., 1998). Some of the *som* mutations are allelic to *ddm1*, and all the *som* mutants and the *hog1* mutant cause hypomethylation of the genomic DNA as well as of the transgenes, although methylation is not affected by the *sil1* or *sil2* mutations (Furner et al., 1998; Mittelsten Scheid et al., 1998). Interestingly, *sgs1* and *sgs2* distinctly modify methylation of the transgene but not methylation of the host centromere (Elmayan et al., 1998).

In this study, we tested the hypothesis that both methylation of genomic DNA and the gene-silencing machinery are necessary for suppression of retrotransposons. An impaired gene-silencing system could be induced by the *ddm1* mutation of Arabidopsis. Because no active endogenous retrotransposon has thus far been isolated from Arabidopsis, we used the tobacco retrotransposon *Tto1* (Hirochika, 1993). After an initial increase in copy number, *Tto1* became silent in the wild-type Arabidopsis genome. In the *ddm1* mutant background, however, the *Tto1* transcript accumulated, and the *Tto1* copy number increased. In addition, a family of endogenous retrotransposons was transcriptionally reactivated in a *ddm1* mutant background. These results strongly suggest that the gene-silencing machinery is effective in defending the genome against retrotransposons.

## RESULTS

### Transposition of *Tto1* in Arabidopsis

The tobacco retrotransposon *Tto1* is one of a few active plant retrotransposons (Hirochika, 1993). *Tto1* is almost inactive in unstressed tissues but can be activated by tissue culture. Because transposition of *Tto1* occurs concomitantly with a marked increase in *Tto1* RNA, it appears to be primarily regulated at the transcriptional level. Being an autonomous element known to be active in the distantly related heterologous host rice (Hirochika et al., 1996a), the *Tto1-1* clone was introduced into Arabidopsis by way of Agrobacterium-mediated transformation. Because the silencing of *Tto1* was observed clearly in transgenic lines that carried a high copy number of transposed *Tto1* copies, the main focus of this study was on transgenic lines with high copy numbers. Characterization of lines with low copy numbers will be described elsewhere (H. Okamoto and H. Hirochika, unpublished data).

Transposition of *Tto1* was assayed by using DNA gel blot analysis. As shown in Figure 1A, the copy number of *Tto1* ranged from ~10 to 15 in five independent $T_0$ transgenic plants characterized in this study. The original copy number of *Tto1* introduced into Arabidopsis was estimated by counting the copy number of the *HPT* gene placed downstream of *Tto1* in the Ti plasmid. Only one or two copies of the *HPT* gene were detected in the five transgenic lines (Figure 1C), supporting the transposition of *Tto1.*

To further confirm transposition, the following experiments were performed. As shown in Figure 2A, the original *Tto1* retrotransposon introduced into Arabidopsis carries a 36-bp deletion at the 5′ end. If transposition occurs, the 5′ end should be recovered (Boeke et al., 1985) and integrated into the genome. This experimental strategy has been used to demonstrate the transposition of *Tnt1* (Lucas et al., 1995) and *Tto1* (Hirochika et al., 1996a) in Arabidopsis and rice, respectively. The recovery of the deleted sequence was examined by polymerase chain reaction (PCR), using a primer pair in which one primer (primer 1) was complementary to the deleted sequence. A fragment of the expected length was amplified from all of the *Tto1* transgenic plants (Figure 2B). From control plants transformed with the β-glucuronidase (*GUS*) gene or with the plasmid carrying *Tto1* with the 36-bp deletion (pSKTto1[−36]), no fragment was amplified. These data, along with the data from DNA gel blot analysis, indicate that *Tto1* had transposed in Arabidopsis. Presumably, the transposition of *Tto1* occurred mainly in callus tissues during the transformation process, because the transcription of *Tto1* is induced by tissue culture in Ara-

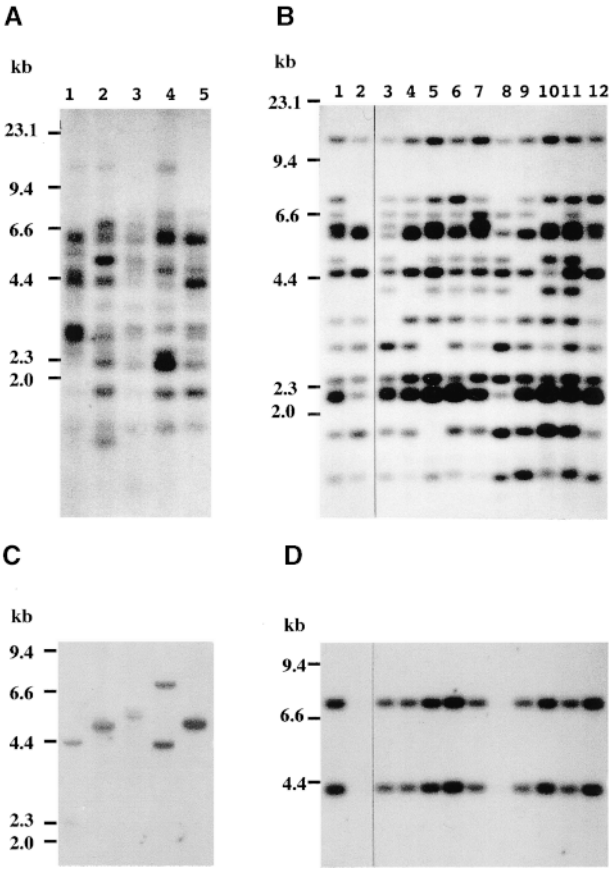


**Figure 1.** DNA Gel Blot Analysis of Transposition of *Tto1* in Transgenic Arabidopsis Lines.

**(A)** and **(C)** Analyses of five independent $T_0$ transgenic lines.
**(B)** and **(D)** Analyses of the $T_1$ progeny of one $T_0$ line (lane 4 in **[A]**).
Genomic DNA was digested with EcoRV and hybridized with a *Tto1-gag* probe (**[A]** and **[B]**) or an *HPT* probe (**[C]** and **[D]**). DNA length markers are shown at left in kilobases.

bidopsis, as is the case with tobacco (H. Okamoto and H. Hirochika, unpublished data). The *Tto1* copies detected in the $T_0$ plant are stably inherited in the next generation, as shown in Figure 1B, and no new transposed copy was detected in the progeny. The latter result is consistent with the tissue culture–specific expression of *Tto1* in tobacco.

### *Tto1* Copies in the Transgenic Plants Are Silenced and Highly Methylated

The above results suggest that the transposition of *Tto1* can be reactivated in transgenic Arabidopsis plants by tissue culture, as is the case with the original host, tobacco. To test this hypothesis, the progeny of $T_1$ plants were cultured to activate *Tto1* and regenerated to measure the increase in copy number. The DNA of $T_1$ plants derived from one transgenic line and of regenerated plants from cultured $T_2$ plants was analyzed by gel blot analysis. If transposition is induced during tissue culture, new bands not found in the parental $T_1$ plants should be detected. Contrary to our initial expectation, no new band was observed in the regenerated plants (Figure 3). Minor variations in the banding pattern can be

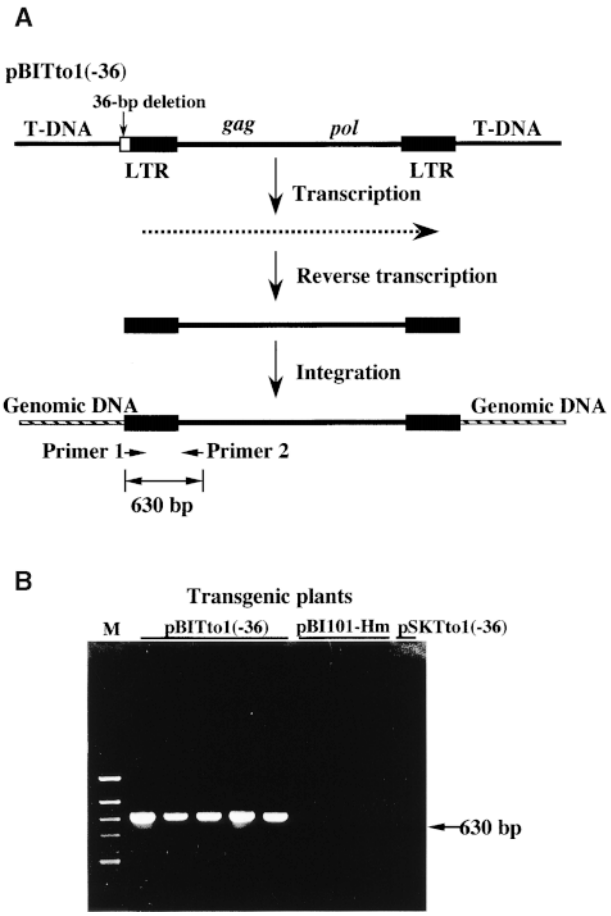


**Figure 2.** PCR Analysis of Transposition of *Tto1* in $T_0$ Transgenic Lines.

**(A)** Process of transposition and recovery of the 5′ deleted sequence expected from transposition. A derivative of *Tto1-1* carrying a 36-bp deletion at the 5′ end (*Tto1*[−36]) was inserted into the T-DNA region of the vector plasmid, resulting in pBITto1(−36). In transgenic lines, the 5′ deleted sequence is expected to be recovered during transposition by means of an RNA. A 630-bp fragment was amplified only from transposed *Tto1* copies by using primers 1 and 2, because primer 1 is homologous to the deleted sequence. LTR, long terminal repeat.
**(B)** Results of PCR analysis. Five $T_0$ transgenic lines used in Figure 1A and control transgenic lines transformed with the vector plasmid (pBI101-Hm) were subjected to PCR analysis. The plasmid carrying *Tto1*(−36), pSKTto1(−36), was also used as a control. M, HincII-digested ϕX174 as a length marker.

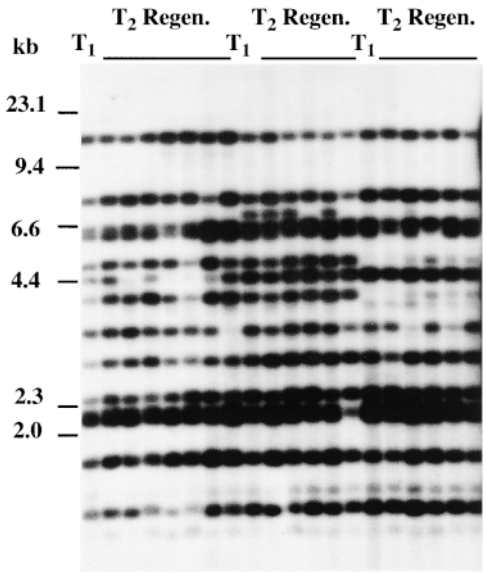


**Figure 3.** Effect of Tissue Culture on Transposition in the Progeny of Transgenic Lines.

Hypocotyl sections of pooled progeny (10 $T_2$ plants from each $T_1$ plant) of three $T_1$ plants derived from one $T_0$ line (Figure 1B, lanes 2, 4, and 5) were cultured on callus-inducing medium. Genomic DNA prepared from $T_1$ plants ($T_1$) and from regenerated plants ($T_2$ Regen.) from induced calli was digested with EcoRV and subjected to DNA gel blot analysis with the *Tto1 gag* probe. DNA length markers are shown at left in kilobases.

explained by the segregation of *Tto1* copies, because the regenerated plants were derived from pooled progeny. These results indicate that *Tto1* in the $T_2$ generation has become inactive.

DNA methylation of inactivated *Tto1* was then examined by DNA gel blot analysis with the methylation-sensitive restriction endonucleases HpaII and MspI. HpaII cleaves CCGG sites only when the C residue is not methylated, whereas MspI, an isoschizomer of HpaII, cleaves C5mCGG but not 5mCCGG. DNAs from three regenerated $T_2$ plants, in which the *Tto1* copies were silenced, were treated with either HpaII or MspI and subjected to DNA gel blot analysis with a *Tto1 gag* probe. If *Tto1* is not methylated, three bands of 1532, 427, and 256 bp should be visible (Figure 4A). As shown in Figure 4B, only a weak band of 1532 bp was detected in the HpaII-digested samples. Other major bands were longer than expected, indicating that all of the *Tto1* copies present in the genome of $T_2$ generations are highly methylated. Even in the MspI-digested samples, major bands were longer than expected, indicating that the first C residue in the CCGG sequence is also methylated. Using a *pol* probe gave the same results (data not shown), indicating that the DNA methylation is not confined to a specific region of *Tto1.* Accordingly, the inactivation of *Tto1* is associated with DNA methylation.

To determine when the DNA methylation had been induced, DNA from $T_0$ and $T_1$ plants was analyzed. As shown in Figure 4B, *Tto1* copies in $T_0$ and $T_1$ plants were already highly methylated, although the extent of methylation in $T_0$ plants was less than that in $T_1$ plants. These results indicate that DNA methylation increased progressively through the generations. On the other hand, in the transgenic plants carrying a low copy number of *Tto1* (one to two copies), *Tto1* was almost unmethylated (Figure 4C), suggesting that increased copy number is a major factor in DNA methylation. In the transgenic line carrying six copies of *Tto1*, methylation of *Tto1* was extensive (Figure 4C). The increase in copy number correlates with both the DNA methylation and the inactivation of *Tto1.*

### Demethylation of Silenced *Tto1* by the *ddm1* Mutation

The results mentioned above suggest that the multiple-copy *Tto1* was suppressed by a mechanism similar to repeat-induced gene silencing associated with DNA methylation. To test this possibility, the *ddm1* mutation of Arabidopsis, which is known to cause genomic DNA hypomethylation (Vongs et al., 1993) and release of gene silencing (Furner et al., 1998; Mittelsten Scheid et al., 1998), was introduced into the transgenic line carrying silenced *Tto1* copies by crossing one $T_2$ plant with the *ddm1* homozygous mutant. Because the *ddm1* mutation is recessive (Vongs et al., 1993; Kakutani et al., 1999), homozygous mutant lines (119 and 120) were screened among $F_2$ plants as described in the Methods, and $F_3$ plants of these two lines were used in the subsequent experiments. Controls were $T_3$ plants (plants 121 and 122) originating from the transgenic plant by selfing, and $F_3$ sibling lines (123 and 124) in which the *ddm1* mutation was not introduced. DNA methylation of *Tto1* in these lines was examined by using HpaII. As shown in Figure 5, three clear bands of 1.8, 1.5, and 0.4 kb were detected in the two *ddm1* homozygous lines but not in control plants. These results indicate that the *ddm1* mutation causes the loss of methylation in most HpaII sites in *Tto1.*

### Reactivation of Transcription and Transposition of Silenced *Tto1* by the *ddm1* Mutation

Transcription is the first step in the transposition of retrotransposons and has been shown to be the major regulatory step for the well-characterized plant retrotransposons, such as *Tto1* (Hirochika, 1993), *Tnt1* (Pouteau et al., 1991), and *Tos17* (Hirochika et al., 1996b). Because DNA methylation within promoters represses the transcription of cellular genes (Kass et al., 1997), transcripts of the inactivated and the demethylated *Tto1* were compared (Figure 6) by using $F_3$

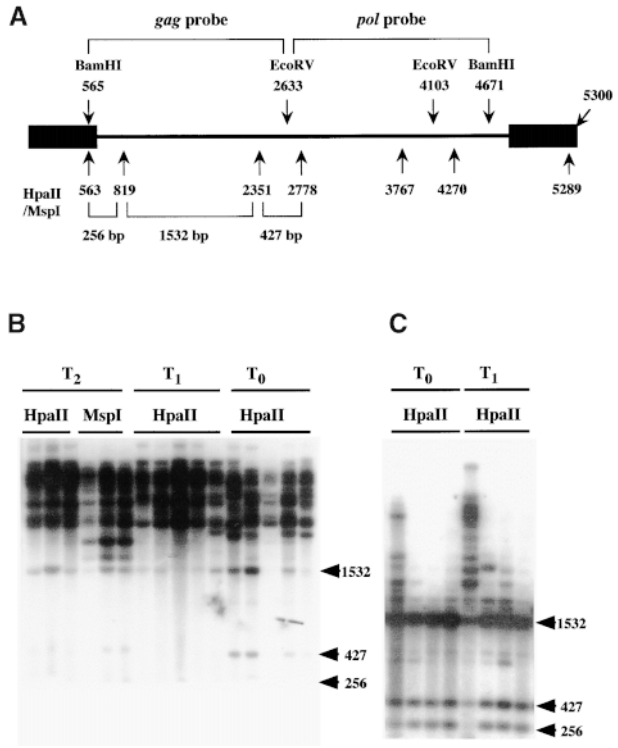


**Figure 4.** Analysis of DNA Methylation of *Tto1* in $T_0$, $T_1$, and $T_2$ Transgenic Lines.

**(A)** Restriction maps of *Tto1-1* and probes used for the analysis. Black rectangles denote the long terminal repeat.

**(B)** DNA gel blot analysis of methylation of *Tto1* in transgenic lines having high copy numbers. Genomic DNA was digested with HpaII or MspI and analyzed by DNA gel blotting with the *Tto1 gag* probe. $T_2$ DNA was prepared from the pooled progeny (four regenerated $T_2$ plants from each $T_1$ plant used in Figure 3). $T_1$ DNA was prepared from the pooled progeny (five $T_1$ plants) derived from each $T_0$ line.

**(C)** DNA gel blot analysis of methylation of *Tto1* in transgenic lines having low or medium copy numbers. Genomic DNA was digested with HpaII and analyzed by DNA gel blotting with the *Tto1 gag* probe. $T_1$ DNA was prepared from the pooled progeny (10 $T_1$ plants) derived from each $T_0$ line. The copy numbers of *Tto1* in transgenic lines (from left to right) are six, two, one, and one (data not shown). Only the left-most transgenic line carries transposed copies (four copies).

All of the DNA samples analyzed in **(B)** and **(C)** were shown to be equally well digested with the restriction enzyme by reprobing the blot with a single-copy sequence (m105; Pruitt and Meyerowitz, 1986) (data not shown). Lengths and positions of fragments expected from digestion of unmethylated *Tto1* are shown at right in **(B)** and **(C)** in kilobases.

plants derived from mutant lines (119 and 120), sibling lines (123 and 124), and wild-type lines (121 and 122). Because the transcription of *Tto1* is activated in tissue culture in tobacco (Hirochika, 1993; Takeda et al., 1999) and also in Arabidopsis (H. Okamoto and H. Hirochika, unpublished data), normal plant tissues and cultured cells were analyzed for its presence. Cultured cells of the *ddm1* mutants showed clear induction of *Tto1* RNA having the expected length, but in quantities two- to threefold more than that induced in tobacco. However, the methylated *Tto1* in the wild-type background was transcribed very little in both cultured cells and normal plant tissues. The *ddm1*-induced strong RNA signal was observed in both callus and normal plant tissues, correlating with the *Tto1* hypomethylation in both tissues.

To examine the transpositional activity in tissue culture, calli were induced from $F_3$ plants derived from mutant lines (119 and 120) and wild-type lines (121 and 122) and then were cultured for 3 months. Weak, smeared bands detected by DNA gel blot analysis were induced in the mutant lines but not in the wild type (data not shown). Smeared bands could be explained by transposition occurring independently in each cell of the mutant calli. To show newly transposed *Tto1* copies individually, calli were divided into small pieces, and each piece was cultured for one more month

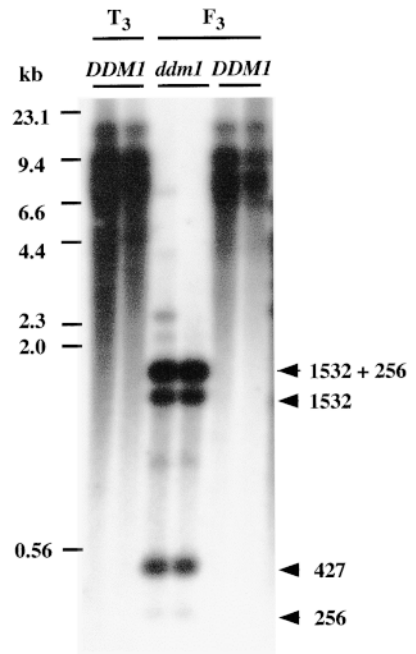


**Figure 5.** Analysis of DNA Methylation of *Tto1* in the Wild Type and *ddm1* Mutant.

Genomic DNAs prepared from two $T_3$ plants ($T_3$; lines 121 and 122), and from $F_3$ *ddm1*/*ddm1* (*ddm1*; lines 119 and 120) and *DDM1*/*DDM1* (*DDM1*; lines 123 and 124) families (from a cross $T_2$ plant [*Tto1 DDM1*] × [*ddm1*]) were digested with EcoRV and analyzed by DNA gel blotting with the *Tto1 gag* probe. All of the DNA samples were found to be equally well digested by the restriction enzyme by reprobing the blot with the single-copy sequence discussed in the legend to Figure 4 (data not shown). DNA length markers are shown at left in kilobases. Expected lengths of fragments are shown at right in kilobases.

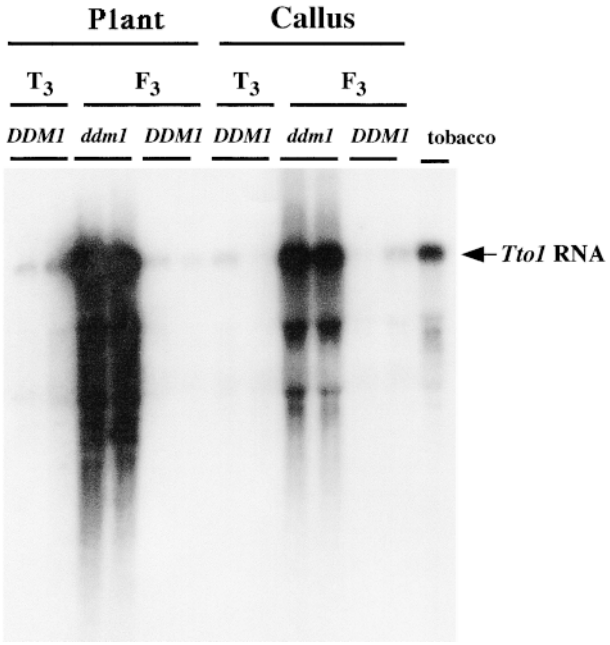


**Figure 6.** Analysis of *Tto1* RNA in the Wild Type and *ddm1* Mutant.

Total RNA was prepared from whole plants or calli of $T_3$ plants ($T_3$; lines 121 and 122) and $F_3$ *ddm1*/*ddm1* (*ddm1*; lines 119 and 120) and *DDM1*/*DDM1* (*DDM1*; lines 123 and 124) families. As a control, total RNA from tobacco BY2 cells (Nagata et al., 1981) was analyzed. Twenty micrograms of total RNA was loaded on the gel.

separately from the others—a process that reduced heterogeneity.

Analysis for the copy of *Tto1* in these partially cloned calli (Figure 7) showed no new bands in calli derived from the wild type. Some minor differences observed among calli were also observed in the original plants. In *ddm1* calli derived from lines 119 and 120, new bands that had not been detected in the original plants were visible. In addition, a 2.6-kb band that was present in wild-type calli was much stronger in the *ddm1* calli. This length of band, which is comparable to that of the left end fragment of the linear *Tto1* molecule generated by digestion with EcoRV (Figure 4A), suggests the presence of a *Tto1* linear molecule.

To test for the possibility of linearity, undigested genomic DNA was analyzed (Figure 8). A strong DNA band of the size of full-length linear *Tto1* (5.3 kb) was detected for the *ddm1* calli, but only trace amounts of it were evident for wild-type calli, indicating that the linear molecule accumulated in the *ddm1* calli. Comparing the intensity of the band with that of the copy number control yielded an estimate of four to eight copies of the linear molecule per cell (data not shown). The linear molecule is believed to be a precursor for the transposition of the *Ty1* retrotransposon of yeast (Eichinger and Boeke, 1988), of retroviruses (Fujiwara and Mizuuchi, 1988), and of the tobacco retrotransposon *Tnt1* (Feuerbach et al., 1997). The structural analysis of the linear *Tto1* DNA molecule is under way.

Although the finding was not directly comparable with the above data in tissue culture, we could not detect retrotransposition in normal plant tissue. The copy number of *Tto1* in $F_3$ plants of lines 119 and 120 was assayed by DNA gel blotting. Despite assaying >80 plants, no evidence of transposition was obtained (data not shown), even though *Tto1* RNA accumulated in normal plant tissues (Figure 6). A tissue-specific control mechanism in steps after transcription (e.g., translation and integration) may also be involved.

### Reactivation of Transcription of an Endogenous Retrotransposon by the *ddm1* Mutation

The above results demonstrate that DNA methylation and gene-silencing machinery are effective against exogenous retrotransposons. This raises the possibility that endogenous retrotransposons may have been silenced by similar mechanisms. In fact, transcripts of endogenous retrotransposons were generally not detected (Konieczny et al., 1991; H. Hirochika, unpublished data). To test whether the gene-silencing mechanism also functions for endogenous transposons, the expression of retrotransposons was surveyed by reverse transcription–PCR (Hirochika, 1993) with the

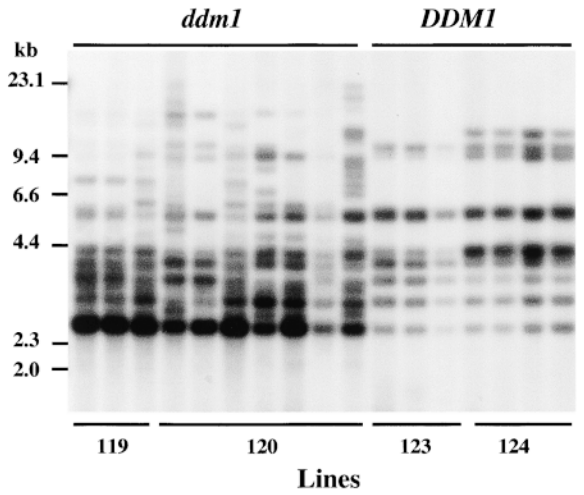


**Figure 7.** Reactivation of *Tto1* Transposition by the *ddm1* Mutation in Calli.

Calli were induced from $F_3$ *ddm1*/*ddm1* (*ddm1*; lines 119 and 120) and *DDM1*/*DDM1* (*DDM1*; lines 123 and 124) families and cultured for 3 months. Induced calli were smashed into pieces, and each piece was cultured separately for one more month. DNA from each callus was digested with EcoRV and analyzed by DNA gel blotting with the *Tto1 gag* probe. DNA length markers are shown at left in kilobases.

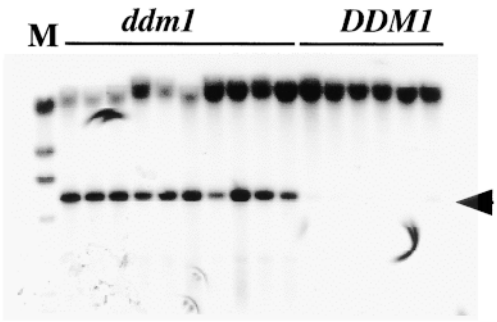


**Figure 8.** Analysis of the Linear Molecule of *Tto1* in the *ddm1* Mutant.

Total DNAs prepared from calli of $F_3$ *ddm1*/*ddm1* (*ddm1*; lines 119 and 120) and *DDM1*/*DDM1* (*DDM1*; lines 123 and 124) families were analyzed without restriction digestion by DNA blotting with the *Tto1-gag* probe. The linear *Tto1* molecule is indicated by an arrow. M, HindIII-digested λ DNA as a length marker.

*ddm1* mutant. cDNA prepared from cultured cells of the *ddm1* mutant was used as a template for PCR amplification with degenerate primers corresponding to the conserved *pol* region. Analysis of 18 sequences determined 10 families of retrotransposons: *Ta3* and *Ta4* (Konieczny et al., 1991), *Tar1* (Hirochika and Hirochika, 1993), *Tar8*, *Tar13*, *Tar17*, and *Tar26* to *Tar29. Tar1* to *Tar3* (Hirochika and Hirochika, 1993) and *Tar4* to *Tar25* (H. Hirochika, unpublished data) have been isolated by PCR with use of genomic DNA as a template. Using RNA gel blot analysis to examine the transcriptional activation of these retrotransposons in the *ddm1* mutant, we detected the RNA band only with the *Tar17* probe. The *Tar17*-specific RNA band of ~7 kb was detected in RNA from leaf tissues of the *ddm1* mutant but not callus tissue (Figure 9A). In the DNA databases, the complete *Tar17* sequence was found (GenBank accession number AC006841, the bacterial artificial chromosome sequence of chromosome 2). The total length of *Tar17* is 7788 bases. The 5′ and 3′ long terminal repeat (LTR) sequences are 724 bases long and differ at only five base positions. The internal region encodes one uninterrupted open reading frame of 1333 amino acids. These features indicate that *Tar17* was transposed relatively recently. To show that the RNA detected by gel blot analysis is not due to read-through from the flanking sequence, we used primer extension analysis (Figure 9B). The signal was detected only for the *ddm1* mutant, and transcription was shown to start within the LTR (Figure 9C).

DNA gel blot analysis showed that *Tar17* is demethylated in the *ddm1* mutant (data not shown). DNA gel blot analysis using a methylation-insensitive enzyme showed two other weakly hybridizing signals. By using recombinant inbred lines from Lister and Dean (1993), their position was mapped to chromosomes 2 (*Tar17c*) and 5 (*Tar17b*) (see Methods). The mapping information is available in the database at Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk/new_ri_map.html). From the map position and sequence similarity, *Tar17c* is likely to be T16I21.11 in a sequenced bacterial artificial chromosome (accession number AC006570). Expression of T16I21.11 was also *ddm1*-specific (data not shown). These results indicate that the *DDM1* gene product is necessary for suppressing *Tar17* members in the wild type. Despite the transcriptional activation, no evidence for transpositional activation was obtained (data not shown), perhaps because of the accumulation of mutations, which led to loss of function of the encoded proteins.

## DISCUSSION

### Regulation of Transposons and Gene Silencing

In most plant species, a large part of the genome consists of retrotransposons (Pearce et al., 1996; SanMiguel et al., 1996; SanMiguel and Bennetzen, 1998). The results presented in this report indicate that *DDM1* gene function is necessary to suppress retrotransposons, both the endogenous *Tar17* family and *Tto1* introduced from tobacco.

Two types of gene-silencing mechanisms have been observed in plants: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). TGS affects transcriptional initiation, whereas PTGS affects the stability of the transcript. TGS is meiotically heritable, whereas PTGS is reset after meiosis and recurs every generation at some stage of plant development (reviewed in Vaucheret et al., 1998). The *DDM1* gene is necessary for TGS, whereas the *EGS1*, *EGS2*, *SGS1*, and *SGS2* genes affect PTGS.

We have shown here that the increase in copy number correlates with DNA methylation and inactivation of *Tto1.* This suggests that the increased copy number is a primary cause for silencing and methylation of *Tto1.* A similar correlation was previously observed in the case of *Mutator* elements of maize (Bennetzen, 1987), although the regulatory mechanism involved there is still unclear. Several lines of evidence suggest that the inactivation of multiple-copy *Tto1* is similar to TGS. The inactivation of *Tto1* was observed in both callus and plant tissues. During propagation, the methylation spread progressively, and the silencing strengthened. Finally, the silencing was released by the *ddm1* mutation. The increasing methylation progressing through generations and being associated with silencing has been reported for TGS in Arabidopsis (Luff et al., 1999). It should be noted that increased methylation during a progression of generations and even during the development of an individual plant was also observed in the case of *Mutator* (reviewed in Bennetzen et al., 1993).

Much evidence suggests that PTGS is effective in defending plants against RNA viruses (Covey et al., 1997; Ratcliff et al., 1997; Al-Kaff et al., 1998; reviewed in Vaucheret et al., 1998). Similarly, the results presented here suggest that TGS is effective in protecting the plant genome against

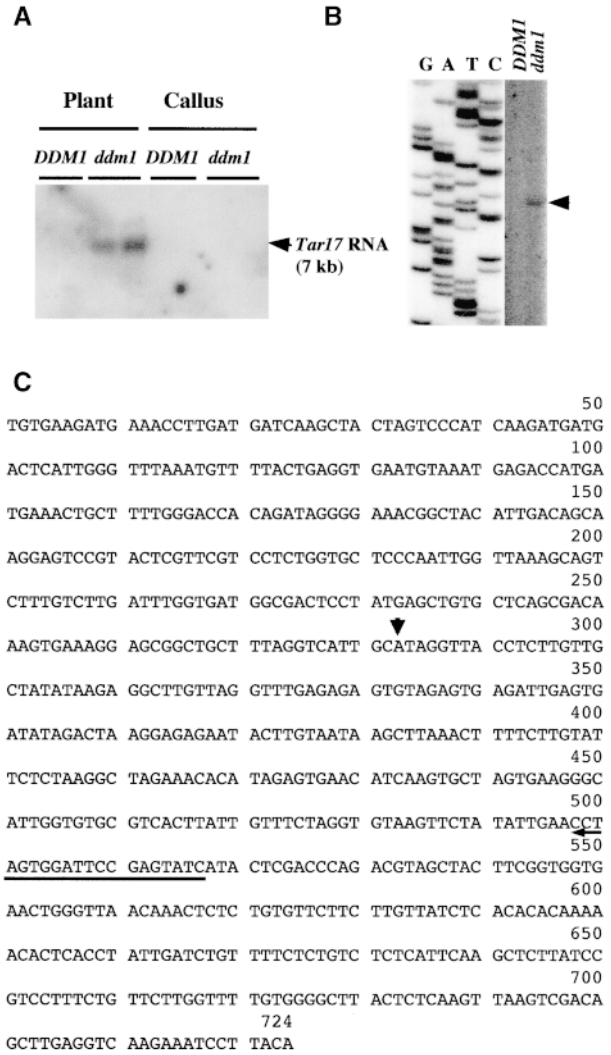


**Figure 9.** Activation of Transcription of the Endogenous Retrotransposon *Tar17* by the *ddm1* Mutation.

**(A)** RNA gel blot analysis. Total RNAs prepared from whole plants or calli of *ddm1*/*ddm1* (*ddm1*) and *DDM1*/*DDM1* (*DDM1*) were analyzed by using the *Tto1 gag* probe.

**(B)** Primer extension analysis. Ten micrograms of total RNA extracted from whole plants was hybridized with the 5′ end-labeled oligonucleotide. The hybrids were extended with reverse transcriptase, and the cDNA products were electrophoresed on a sequencing gel alongside a sequencing reaction using the same primer, as described previously (Hirochika, 1993). One band (indicated by an arrowhead) was detected in the *ddm1* mutant only.

**(C)** Nucleotide sequence of the LTR. The 5′ end of *Tar17* RNA, determined by primer extension, is indicated by an arrowhead. The position of the oligonucleotide used for primer extension analysis is indicated by an arrow.

transposons. Several properties of TGS are useful for defense against retrotransposons. If TGS were a defense against parasites in the genome, for example, its meiotically heritable property would be important; indeed, the silenced state would be maintained at every stage of development to maintain genome structure and proper gene expression.

To date, release of TGS by the *ddm1* mutation has been reported in three systems: clustered *p35S*::*HPT* (Mittelsten Scheid et al., 1998), clustered *CHS* genes (Furner et al., 1998), and *PAI* genes (Jeddeloh et al., 1998). In all of these, silencing was induced by a tandem repeat or inverted repeat at one locus. In the *Tto1* system reported here, all or most copies are scattered throughout the genome (data not shown). If the primary function of TGS is to defend against retrotransposons, silencing the induction of multiple unlinked copies would be important because retrotransposons generally jump to an unlinked position, resulting in dispersed repeats. In this context, one should note the propagation of the silenced state and DNA methylation in *trans* from the *PAI1*-*PAI4* inverted repeat to single-copy *PAI* genes (Luff et al., 1999) and from the *CHS* transgene repeat to the endogenous *CHS* gene (Furner et al., 1998).

## Control of Retrotransposons

We previously showed that the tobacco retrotransposon *Tto1* can transpose in rice (Hirochika et al., 1996a). Here, we show that *Tto1* can transpose in another heterologous plant, Arabidopsis. After the initial active transposition in the Arabidopsis genome, however, *Tto1* became hypermethylated and silent. We provide here direct evidence of repeat-induced silencing of a plant retrotransposon; Lucas et al. (1995) have suggested that the tobacco retrotransposon *Tnt1* in Arabidopsis is also silenced.

Although we have shown here that the *Tar17* family became transcriptionally active in a *ddm1* mutant background, the other nine families of endogenous retrotransposons did not show transcriptional activation, or at least not to a detectable amount when RNA gel blot analysis was used. One possibility is that in most retrotransposons, the LTR function may have become inefficient because of the accumulation of mutations. Alternatively, even if the endogenous retrotransposons are released from silencing, the conditions for induction of transcription may not have been appropriate. In fact, transcription of the tobacco retrotransposons *Tnt1* and *Tto1* is activated only under stress conditions or in specific tissues (Pouteau et al., 1991; Grandbastien, 1998; Takeda et al., 1998, 1999). Interestingly, the amount of expression of *Tar17* was greater in normal plant tissue than in callus tissue.

Unlike most other plant species, only a small part of the genome in Arabidopsis consists of retrotransposons, and most of the endogenous retrotransposon families are present in only a few copies in the genome. How then is a retrotransposon of low copy number silenced? Because ret-

rotransposons have two identical promoter–enhancer elements, we can speculate that a single-copy retrotransposon is also a target for gene silencing, even if an increase in copy number makes silencing more efficient. In fact, tandemly arrayed two transgene copies were shown to be silenced and methylated in Arabidopsis (Assaad et al., 1993). In the original tobacco hosts, the copy number of *Tto1* is ~30, and *Tto1* copies are not silenced (Hirochika, 1993). Even in the cell line BY2, in which the copy number was increased up to 300, *Tto1* copies are actively transcribed. We do not know how *Tto1* copies escape gene silencing in tobacco. One possible explanation is that silencing is not induced due to the heterogeneity of sequences in each member of *Tto1*, which has been reported for *Tnt1* (Casacuberta et al., 1995). Alternatively, the suppression of retrotransposons may not be as efficient in tobacco as in Arabidopsis, reflecting its large genome.

## Plant Gene Expression, DNA Methylation, and Suppression of Repeat Elements

In addition to the release of gene silencing, the *ddm1* mutation induces several kinds of developmental abnormalities by causing heritable change at other loci (Kakutani et al., 1996; Kakutani, 1997). Similar developmental abnormalities were induced in *MET1* antisense lines (Finnegan et al., 1996; Ronemus et al., 1996). At least some of the *ddm1*-induced phenotypes seem to result from epigenetic change, because all of four independently established late-flowering traits were mapped to the same chromosomal location near the *FWA* locus (Kakutani, 1997). These results could be interpreted to mean that DNA methylation is important for tissue-specific expression of plant genes during development. Alternatively, the change in genomic DNA methylation might cause activation of endogenous repeat elements, which disturbs the normal expression of nearby plant genes.

Although some of the *ddm1*-induced developmental abnormalities are inherited as dominant traits, others are inherited as recessive traits (Kakutani et al., 1996; Kakutani, 1997). Activation of repeat elements could cause not only overexpression but also suppression of nearby plant genes. In fact, in the *hcf106-mum1* allele of maize, in which a nonautonomous *Mutator1* (*Mu1*) transposable element is inserted in the promoter region, the gene becomes epigenetically silent only when *Mu1* becomes active and hypomethylated in the presence of the autonomous *MuDR* elsewhere in the genome (i.e., in "Mu-on" plants) (Barkan and Martienssen, 1991; Martienssen, 1996). Examining the activity of DNA-type transposons in the Arabidopsis genome (Surzycki and Belknap, 1999) under the *ddm1* mutant background should also be interesting.

The transcript level of retrotransposon-related endogenous retroviruses, which are normally transcriptionally silent, has recently been shown to be increased in mouse embryos that are deficient in DNA methyltransferase-1 (Walsh et al., 1998). Because the *DDM1* gene encodes a protein similar to the chromatin-remodeling factor SWI2/SNF2 (Jeddeloh et al., 1999), the primary effect of the *ddm1* mutation possibly involves the chromosomal epigenetic state rather than DNA methylation. Interestingly, retrotransposon *I* of Drosophila is also suppressed by homology-dependent gene silencing, despite the lack of detectable genomic DNA methylation (Chaboissier et al., 1998; Jensen et al., 1999). The results of Walsh et al. (1998) mentioned above, however, suggest that DNA methylation is directly involved in suppression of retroelements, at least in the mouse system.

In summary, TGS, and possibly DNA methylation, may have evolved to support symbioses of the plant genes with the transposons. Comparative studies of repeat-induced gene silencing and control of transposons would lead to a better understanding of the plant genome.

## METHODS

### Construction of Plasmids

The plasmid used in transformation of the plants (*Arabidopsis thaliana*) was constructed as follows. The deleted form of *Tto1-1*, *Tto1*(−36), was derived from the plasmid pSKTto1(−36) (Hirochika et al., 1996a). The HindIII-PstI fragment carrying the 5′ part of *Tto1-1* (−36) and the PstI-XhoI fragment carrying the 3′ part of *Tto1-1*(−36) were mixed and ligated with the largest HindIII-SalI fragment of pBI101-Hm (Akama et al., 1992) to construct pBITto1(−36). pBI101-Hm is a derivative of pBI101 (Jefferson et al., 1987) and carries the hygromycin phosphotransferase (*HPT*) gene in addition to the neomycin phosphotransferase (*NPTII*) gene as a selective marker gene. The construct was introduced into *Agrobacterium tumefaciens* EHA101 (Hood et al., 1986).

### Transformation and Tissue Culture

Agrobacterium-mediated transformation of Arabidopsis (ecotype Wassilewskija) was performed as described (Akama et al., 1992). Transformed calli ($T_0$ generation) were selected in the presence of kanamycin (50 μg/mL) and hygromycin (20 μg/mL). Calli were induced from transgenic lines by culturing hypocotyl sections on callus-inducing medium (Valvekens et al., 1988) at 22°C and transferred onto fresh media every month. To ensure cell activity, calli were transferred twice (1 week apart) onto fresh media before extraction of RNA and DNA. For production of regenerated plants, hypocotyl sections of pooled $T_2$ transgenic plants were cultured on callus-inducing medium for 12 days and transferred onto shoot-inducing medium to regenerate plants.

### Extraction of Nucleic Acids and RNA and DNA Gel Blot Hybridization

Extraction of total RNA and DNA, blotting, preparation of probes, and hybridization were performed as described previously (Hirochika et al., 1996a).

### Introduction of *ddm1* Mutation into a Transgenic Arabidopsis Line with Silenced *Tto1*

A transgenic line with silenced *Tto1* was crossed with a decreased DNA methylation *ddm1* plant that had been backcrossed six times (Kakutani et al., 1996). From the $F_2$ progeny, homozygous *ddm1* mutants were selected by examining the methylation status of the genomic DNA, using gel blot analysis with the methylation-sensitive restriction enzyme HpaII. Of the 21 $F_2$ progeny, two plants showed hypomethylation in all copies of *Tto1* sequences and endogenous rDNA sequences (Vongs et al., 1993), indicating that they were *ddm1* homozygotes. *DDM1* plants from this segregating family were used as controls.

### Polymerase Chain Reaction Analysis of Transposition

The oligonucleotide primers *LTR-11* (5′-TGTTAGTTTTTCCAACAA-TTATGGT-3′, corresponding to nucleotides 1 to 25 of *Tto1-1*) and *Tnt2-gag* (5′-GGATGAATAGTACTCGTACGTATG-3′, corresponding to nucleotides 630 to 606; Hirochika et al., 1996a) were used to amplify the 5′ end recovered after transposition of *Tto1*(−36) carried on the plasmid pBITto1(−36).

### Amplification and Cloning of Reverse Transcriptase Domain of Retrotransposons

The genomic DNA or the first-strand cDNA was amplified by using degenerate primers corresponding to QMDVKT and YVDDM, as described previously (Hirochika, 1993). The cDNA was generated from poly(A)$^+$ RNA isolated from callus tissues of the *ddm1* mutant by using random primers and a cDNA cycle kit (Invitrogen, Chatsworth, CA). Polymerase chain reaction (PCR) products were cloned into the HincII site of M13mp18.

### Restriction Fragment Length Polymorphism Mapping of *Tar17* Family Members by Using Recombinant Inbred Lines

During DNA gel blot filter analysis using EcoRV, the *Tar17* probe detected one strong signal (*Tar17a*) and two weak signals (*Tar17b* and *Tar17c*). All of them showed polymorphism between Columbia and Landsberg *erecta* ecotypes (the *Tar17a* signal was not detected in the Landsberg *erecta* ecotype). Map positions were determined by using 96 recombinant inbred lines constructed by Lister and Dean (1993).

## ACKNOWLEDGMENTS

We thank the Nottingham Arabidopsis Stock Centre (Colney, UK) and the Arabidopsis Biological Stock Center (Ohio State University, Columbus) for recombinant inbred mapping and the bacterial artificial chromosome clones. This study was supported by a project grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan and a grant for the enhancement of Center-of-Excellence, the special coordination funds for promoting science and technology in Japan.

Received November 16, 1999; accepted January 6, 2000.

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