Invasion of the Arabidopsis Genome by the Tobacco Retrotransposon Tnt1 Is Controlled by Reversible Transcriptional Gene Silencing[1][W]

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Long terminal repeat (LTR) retrotransposons are generally silent in plant genomes. However, they often constitute a large proportion of repeated sequences in plants. This suggests that their silencing is set up after a certain copy number is reached and/or that it can be released in some circumstances. We introduced the tobacco (*Nicotiana tabacum*) LTR retrotransposon Tnt1 into Arabidopsis (*Arabidopsis thaliana*), thus mimicking the horizontal transfer of a retrotransposon into a new host species and allowing us to study the regulatory mechanisms controlling its amplification. Tnt1 is transcriptionally silenced in Arabidopsis in a copy number-dependent manner. This silencing is associated with 24-nucleotide short-interfering RNAs targeting the promoter localized in the LTR region and with the non-CG site methylation of these sequences. Consequently, the silencing of Tnt1 is not released in *methyltransferase1* mutants, in contrast to *decrease in DNA methylation1* or *polymerase IVa* mutants. Stable reversion of Tnt1 silencing is obtained when the number of Tnt1 elements is reduced to two by genetic segregation. Our results support a model in which Tnt1 silencing in Arabidopsis occurs via an RNA-directed DNA methylation process. We further show that silencing can be partially overcome by some stresses.

Transposable elements, first discovered in plants, account for a large proportion of some plant genomes (Morgante, 2006). Long terminal repeat (LTR) retrotransposons, such as the tobacco (*Nicotiana tabacum*) Tnt1 element, are retrovirus-like elements that represent the most widespread class of mobile elements in plants. Most retrotransposons are silenced in plants (Zaratiegui et al., 2007). However, their amplification (by a ''copy and paste'' mechanism) has been allowed at some stage(s) during evolution, accounting for the presence of highly amplified families. Proliferation of retrotransposons was indeed recently shown to be involved in the genome expansion of some sunflower (*Helianthus annuus*, *Helianthus deserticola*, and *Helianthus paradoxus*) hybrids (Ungerer et al., 2006). Two major hypotheses can be proposed to explain the presence of a high number of copies of some LTR retrotransposon families: (1) silencing occurs only when a certain number of copies is reached, or (2) silencing is released under certain circumstances. To better understand the mechanisms regulating the amplification of LTR retrotransposons in plant genomes, we introduced, by transgenesis, the autonomous tobacco retrotransposon Tnt1 into Arabidopsis (*Arabidopsis thaliana*; Lucas et al., 1995). Arabidopsis is devoid of Tnt1-related elements. Hence, the introduction of Tnt1 into Arabidopsis mimics the horizontal transfer of an element into a new species.

The genome of Arabidopsis contains relatively few retrotransposons (Arabidopsis Genome Initiative, 2000; Feschotte et al., 2002), most of them being methylated and inactive, including LTR retrotransposons (Hirochika et al., 2000; Miura et al., 2001; Singer et al., 2001; Tompa et al., 2002; Kato et al., 2003, 2004; Lippman et al., 2003, 2004; Zhang et al., 2006). Transposable elements are inactivated by silencing mechanisms implicating DNA and histone modifications. In plants, the maintenance of DNA methylation at CG and non-CG sites is controlled by different mechanisms (Chan et al., 2005; Zaratiegui et al., 2007). The Arabidopsis ortholog of the mammalian Dnmt1 methyltransferase, METHYLTRANSFERASE1 (MET1), is responsible for the maintenance of CG methylation. The redundant methyltransferase enzymes of the Dnmt3 family, DOMAINS REARRANGED METHYLTRANSFERASE1 (DRM1) and DRM2, in addition to the plant-specific CHROMOMETHYLASE3 (CMT3), control non-CG methylation. Silencing of transposable elements relies mostly on MET1-mediated CG methylation, as they are massively reactivated in *met1* mutants but not in the triple *drm1 drm2 cmt3* mutant

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(Zhang et al., 2006). The maintenance of non-CG methylation is triggered by 24-nucleotide short-interfering RNA (siRNA)-directed DNA methylation and requires the activity of RNA interference-associated enzymes such as DICER-LIKE3 (DCL3), RNA-DEPENDENT RNA POLYMERASE2 (RDR2), ARGONAUTE4 (AGO4), AGO6, or subunits of the newly discovered Polymerase IV (PolIVa and PolIVb) associated with SNF2 domain-containing proteins like DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1 (DRD1) and CLASSY1 (CLSY1; Matzke et al., 2007). *DECREASE IN DNA METHYLATION1* (*DDM1*), encoding a chromatin-remodeling factor, is involved in the maintenance of both CG and non-CG methylation (Vongs et al., 1993). Several mutants in genes involved in methylation, like *met1* (Kankel et al., 2003; Saze et al., 2003), *ddm1* (Kakutani et al., 1996; Kakutani, 1997; Jeddeloh et al., 1999), *dcl2 dcl3* and *dcl3 dcl4* double mutants (Gasciolli et al., 2005), and the *drm1 drm2 cmt3* triple mutant (Chan et al., 2006), accumulate lethal mutations after several generations, partly due to uncontrolled transposition events.

Tnt1 structure is characteristic of the *Copia* LTR retrotransposons, with two 610-bp LTRs in direct orientation flanking the element and a central region containing the *gag* and *pol* genes. The *gag* gene encodes a capsid-like protein, and the *pol* gene encodes a polyprotein with protease, integrase, reverse transcriptase, and RNase H domains. The Tnt1 promoter is located in the U3 region of the LTR (Casacuberta and Grandbastien, 1993). Tnt1 transcription starts at the junction between the U3 and R regions in the 5′ LTR and terminates at the junction between the R and U5 regions in the 3′ LTR (Fig. 1A). In Arabidopsis, Tnt1 undergoes the three major steps of retrotransposition: transcription, reverse transcription, and integration of the double-stranded cDNA copies into the host genome (Lucas et al., 1995; Feuerbach et al., 1997). Tnt1 transposition events are induced at high frequency during the in vitro transformation and regeneration process in Arabidopsis, allowing the production of transformed plants carrying one to more than 30 transposed copies of the element integrated with no site specificity (Courtial et al., 2001). Analysis of Arabidopsis transgenic plants carrying a single Tnt1-GUS translational fusion showed that Tnt1-GUS expression is restricted to roots, cotyledons, mature rosette leaves, and some sporophytic floral organs. This expression pattern reflects that of Tnt1 in Arabidopsis plants carrying few copies of the element (Lucas et al., 1995). The absence of expression of Tnt1 in germ cells could explain the absence of germinal transposition events in the progeny of Tnt1-containing plants (Lucas et al., 1995). Analysis of transgenic Arabidopsis plants containing Tnt1 or Tnt1-GUS also showed that Tnt1 expression is induced by several stresses, such as wounding and $CuCl_2$ (Moreau-Mhiri et al., 1996; Mhiri et al., 1997).

In this study, we show that Tnt1 and Tnt1-GUS are regulated by transcriptional gene silencing dependent on the number of Tnt1 copies per plant. Silencing of Tnt1 is associated with non-CG methylation of its promoter and with small RNAs targeting the LTR regions, suggesting that an RNA-dependent DNA methylation (RdDM) process directed to the Tnt1 promoter is involved. Silencing is reset at each generation, allowing the reactivation of the element when the number of copies declines by genetic segregation. In addition, we show that Tnt1 silencing is locally released by mechanical stresses like wounding.

RESULTS

Tnt1 Is Silenced in Arabidopsis Plants Containing Numerous Tnt1 Elements

Introduction of the tobacco retrotransposon Tnt1 in the heterologous host Arabidopsis has been described in previous papers (Lucas et al., 1995; Courtial et al., 2001). Briefly, Arabidopsis root explants are cocultivated with *Agrobacterium tumefaciens* carrying Tnt1 in a binary vector. In vitro transformation and regeneration induce Tnt1 transposition at high frequency in Arabidopsis. As a result, primary transformants contain various numbers of transposed unlinked copies of the element, fixed in the genome and transmitted to the following generations. To determine whether Tnt1 copy number influenced its transcription activity, we analyzed the levels of Tnt1 mRNA in plants carrying high and low copy numbers of Tnt1. The S15, S17, T2, and S14 primary transformants (Courtial et al., 2001) were self-pollinated, and DNA from plants obtained after two generations was extracted, digested by *Nde*I (present as a unique site in Tnt1), and hybridized with a Tnt1 Gag-specific probe (Fig. 1A). Southern-blot analyses revealed that progeny derived from the S15, S17, and T2 primary transformants contained 14 or more fragments hybridizing with the Gag probe (Fig. 1B). Each of these fragments corresponds to at least one insertion locus of Tnt1. The difference of intensity between the fragments can be explained, on the one hand, by the fact that different insertion loci generate fragments of similar size, and, on the other hand, by the hemizygosity (one Tnt1 copy) or homozygosity (two Tnt1 copies) of each insertion locus. Plants derived from the S14 primary transformant contained two homozygous insertion loci of Tnt1 (four copies of the element), as determined by the analysis of S14 selfed progeny (Lucas et al., 1995). Tnt1 mRNA could be detected in mature rosette leaves of S14-derived plants, but was almost undetectable in S15-, S17-, and T2-derived plants containing high numbers of Tnt1 (Fig. 1B). These results suggested that Tnt1 mRNA was silenced in plants carrying a high copy number of Tnt1.

The transcriptionally active copies of Tnt1 present in S14 progeny were introduced by crossing into a T2-derived plant carrying a high copy number of Tnt1. In the resulting F1, containing 22 copies of Tnt1 as determined by Southern-blot analysis (data not shown), Tnt1 mRNAs were undetectable (Fig. 1C), demonstrat-

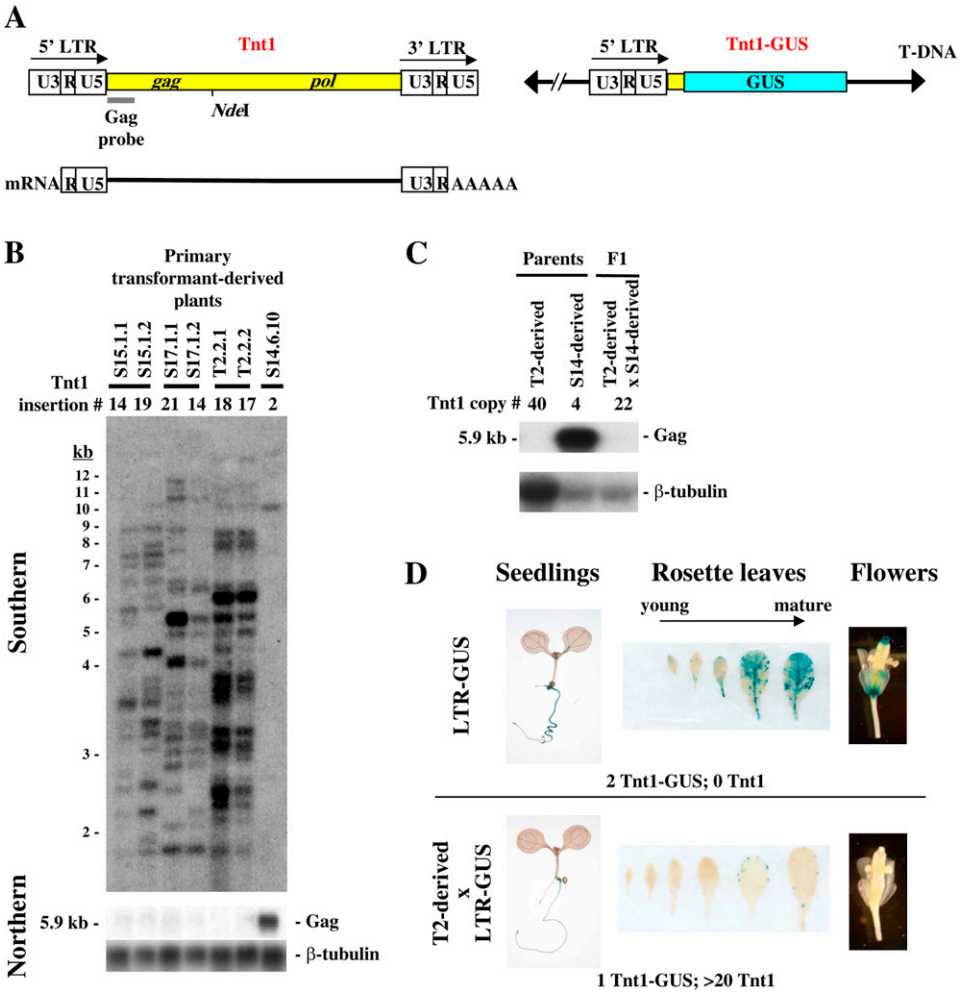

Figure 1. Tnt1 is silenced in plants containing numerous elements. A, Schematic representation of Tnt1 and Tnt1-GUS T-DNA. The *Nde*I restriction site present in the Tnt1 coding sequence is unique. B, DNA and RNA gel-blot analyses of 2 and 10 μg of DNA and total RNA, respectively, extracted from mature rosette leaves of S15, S17, T2, and S14 primary transformants self-pollinated for two generations. DNAs were digested with *Nde*I. The Southern blot was probed with DNA complementary to the Gag region of Tnt1, and the northern blot was successively probed with the same DNA and DNA corresponding to a β-tubulin gene for a loading control. Each fragment of the Southern blot corresponds to at least one Tnt1 insertion locus (Tnt1 insertion #) in the hemizygous or homozygous state. C, RNA gel-blot analysis of 10 μg of total RNA extracted from mature rosette leaves of a T2-derived transformant (genotype T2.2.4.28.20; see Supplemental Fig. S2 for a genealogy of the plants used in this study), a S14-derived transformant (genotype S14.6.10), and a F1 hybrid resulting from a cross between the two. The blots were successively probed with DNA complementary to the Gag region of Tnt1 and a β-tubulin gene for a loading control. D, Histochemical staining of seedlings, leaves, and flowers of plants expressing a Tnt1-GUS fusion (genotype LTR-GUS; homozygous for the Tnt1-GUS insertion) or resulting from a cross between a T2-derived plant and LTR-GUS (genotype T2.2.52 $\times$ LTR-GUS). Similar staining patterns were observed for the following F1 lines: T2.2.53, T2.2.60, and T2.2.61 $\times$ LTR-GUS (data not shown).

ing that transcriptionally active copies of Tnt1 are silenced when placed in a background containing multiple copies of the element.

To further demonstrate that Tnt1 is silenced when present at high numbers in Arabidopsis, we used plants carrying a T-DNA with a translational fusion between Tnt1 and the *GUS* reporter gene (Tnt1-GUS; Fig. 1A; Lucas et al., 1995) to monitor GUS activity in the presence of diverse numbers of Tnt1 elements. In the LTR-GUS line containing one homozygous copy of the T-DNA insert, GUS activity could be detected in mature rosette leaves, roots, and flowers, revealing the expression pattern of Tnt1 in Arabidopsis (Fig. 1D), consistent with results published earlier with the same line (Lucas et al., 1995; Moreau-Mhiri et al., 1996). Crosses between this line and T2-derived plants carrying numerous copies of Tnt1 resulted in F1 hybrid seedlings in which GUS activity was undetectable, except in the crown, at the junction between the roots and the hypocotyl (Fig. 1D). This indicates that Tnt1-GUS, like Tnt1, is silenced in plants containing multiple Tnt1 copies.

Together, these results suggest that Tnt1-GUS and Tnt1 are silenced when placed in a genome containing

many Tnt1 elements. Run-off analyses were performed to determine whether Tnt1 and Tnt1-GUS silencing was transcriptional or posttranscriptional. Tnt1 and Tnt1-GUS transcripts were detected in nuclei from plants containing few copies of the element but not in plants carrying more than 20 Tnt1 copies (Fig. 2A), demonstrating that both Tnt1 and Tnt1-GUS are silenced by transcriptional gene silencing. As Tnt1 and Tnt1-GUS share the same LTR sequences (Fig. 1A), it is likely that silencing occurs via these sequences.

Silencing of Tnt1 Is Associated with siRNAs Specific to the LTR Sequence

Silencing of transposable elements can be associated with the presence of siRNAs that direct DNA methylation. We thus tried to determine by northern-blot analyses whether siRNAs corresponding to Tnt1 could be detected in plants containing various copy numbers of Tnt1 or Tnt1-GUS. Small RNAs were extracted from plants carrying the Tnt1-GUS insert (LTR-GUS line), four copies of Tnt1 (S14-derived plants), or more than 20 silent copies of the element (T2-derived plants), blotted on a membrane, and hybridized with an RNA probe corresponding to the full-length LTR sequence. LTR-siRNAs accumulated as 24-nucleotide species in plants containing more than 20 silent Tnt1 copies (Fig. 2B) but not in plants containing the T-DNA insert carrying Tnt1-GUS or plants with transcriptionally active Tnt1 copies. The 24-nucleotide siRNA species are usually associated with transposable element silencing (Qi et al., 2006; Rajagopalan et al., 2006; Fahlgren et al., 2007), and 24-nucleotide siRNAs homologous to Tnt1 LTR were detected in tobacco (Hamilton et al., 2002). This suggests that siRNAs are the vectors of Tnt1 inactivation in Arabidopsis plants carrying high numbers of the element. We were unable to detect siRNAs using an RNA probe corresponding to the Gag region of Tnt1 (data not shown).

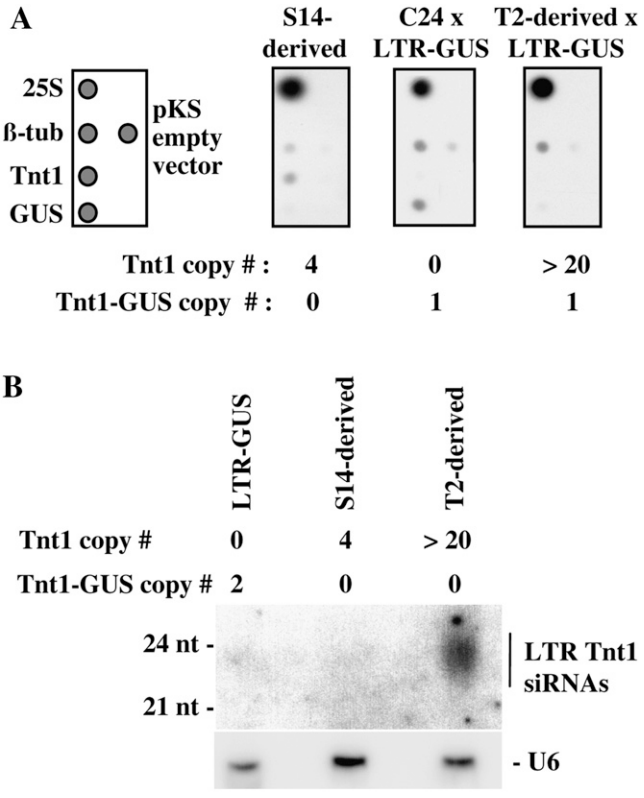

Figure 2. Tnt1 is inactivated by transcriptional gene silencing. A, Run-off experiments (see "Materials and Methods") performed on 10^6 to 10^7 isolated nuclei of roots from Tnt1-expressing plants (genotype S14.6.10), F1 hybrids resulting from crosses between the C24 ecotype and Tnt1-GUS-expressing plants (genotype C24 × LTR-GUS), and a F1 hybrid resulting from crosses between a T2-derived plant and Tnt1-GUS-expressing plants (genotype T2.2.61 × LTR-GUS). In vitro labeled RNAs were hybridized with dot-blotted DNAs corresponding to *Tnt1*, *GUS*, *25S*, and *β-tubulin* genes. The pKS vector DNA used to clone the different DNA fragments was used as a control. B, RNA gel-blot analysis of 10 μg of total RNAs prepared from mature rosette leaves of plants expressing Tnt1-GUS (LTR-GUS line), S14-derived plants, and T2-derived plants containing zero, four, or more than 20 Tnt1 copies, respectively. The blots were probed with an antisense radiolabeled RNA probe transcribed in vitro complementary to the LTR region of Tnt1. U6 hybridization was used as a loading control. nt, Nucleotides.

Silencing of Tnt1-GUS Depends on the Presence of a Minimal Copy Number of Tnt1

Thereafter, we tried to determine the minimal number of Tnt1 copies per plant necessary to inactivate Tnt1 and Tnt1-GUS transcription in Arabidopsis. Series of crosses were performed between plants carrying the Tnt1-GUS T-DNA insert in the homozygous state (LTR-GUS line) and T2-derived plants containing various numbers of Tnt1, obtained after backcrosses with the wild-type C24 ecotype (plants T2C24#1 to T2C24#3; Table I). The number and identity of Tnt1 insertion loci present in the resulting F1 plants were determined by Southern-blot analysis after restriction with *Nde*I and hybridization with the Gag probe (data not shown). The 12 DNA fragments revealed by the probe, corresponding to at least 12 insertion loci, were designated by a lowercase letters (a–l in Table I). Northern-blot analysis indicated that Tnt1 was silenced in the T2-derived plants used as female parents (data not shown). Tnt1-GUS expression was monitored in roots, mature leaves, and flowers. Out of the 55 F1 plants analyzed, 34 carried four or more copies of Tnt1 and showed very little or no expression of *GUS* in roots, mature leaves, and flowers (see Table I for a selection of these plants). On the contrary, the 21 plants carrying less than four copies of Tnt1 did show expression of Tnt1-GUS in these organs. In plants containing three copies of Tnt1, detection of GUS activity by staining was variable between the different organs analyzed. These results showed that Tnt1-GUS is silenced in the presence of four or more copies of Tnt1. The six Tnt1 copies present in the (T2C24#2 × LTR-GUS)10 F1 hybrid led to Tnt1-GUS silencing (Table I). However, none of these copies, when present

Table I. *Expression of Tnt1 and Tnt1-GUS in plants carrying various numbers of Tnt1 elements*

Crosses were performed between T2-derived plants (T2C24#1 to T2C24#3; see Supplemental Fig. S2 for the genotype of these plants) carrying various numbers of Tnt1 and plants carrying the Tnt1-GUS T-DNA insert in the homozygous state (LTR-GUS line). a to l, Tnt1 transposed copy insertion locus, corresponding to a particular restriction fragment on a Southern blot obtained after restriction of T2-derived plant DNA with *NdeI* and hybridization with a Gag probe. +, Presence of a particular Tnt1 insertion or detection of Tnt1 or Tnt1-GUS expression (determined by northern blot or GUS staining). −, Absence of a particular Tnt1 insertion or no detection of Tnt1 or Tnt1-GUS expression (determined by northern blot or GUS staining). +/−, Weak expression of Tnt1-GUS.

						F1 (T2-Derived × Tnt1-GUS)													
Type of Plant:		Tnt1-GUS Parent	T2-Derived Parents			(T2C24#1 × LTR-GUS)			(T2C24#2 × LTR-GUS)								(T2C24#3 × LTR-GUS)		
Plant Genotype:		LTR-GUS	T2C24#1	T2C24#2	T2C24#3	22	16	25	10	4, 5, 11	8	3	6	9	7	1, 2, 12	4, 5, 9	1, 3, 6, 7, 10	2, 8, 11, 12
Tnt1 insertion locus	a	−	+	+	−	+	+	−	+	+	+	+	−	−	−	−	−	−	−
	b	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	c	−	+[a]	+	+	+	+	+	+	+	−	−	+	−	−	−	+	−	−
	d/d′/d″[b]	−	+ + +	−	−	+ + +	+	+ + +	−	−	−	−	−	−	−	−	−	−	−
	e	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	f	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
	g	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	−	+	−
	h	−	+[a]	+	+	+	+	+	+	+	−	−	+	−	−	−	+	−	−
	i	−	+	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−
	j	−	+	+	−	+	+	−	+	+	+	+	−	−	+	−	−	−	−
	k	−	+	+	−	+	+	−	+	+	+	+	−	+	−	−	−	−	−
	l	−	+	+	−	+	+	−	+	−	+	−	+	+	+	+	−	−	−
Tnt1 copy no.		0	16	6	3	14	9	7	6	5	4	3	3	2	2	1	2	1	0
Tnt1-GUS copy no.		2	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
GUS staining	Roots	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+
	Flowers	+	−	−	−	−	−	−	−	−	−	+/−	+	+	+	+	+	+	+
	Mature leaves	+	−	−	−	−	−	−	−	−	−	+/−	+/−	+	+	+	+	+	+
Tnt1 transcription		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a]Segregation analysis showed that, among the 14 insertion loci present in plant T2C24#1, only c and h were homozygous and that all insertion sites segregated independently except for those two (data not shown). [b]Analysis of the intensity of the d fragment in 20 selfed progeny of T2C24#1 indicated that it corresponded to three fragments of similar size, and therefore to three insertion loci, thereafter designated d, d′, and d″.

alone or in combination with one or two others [Table I; F1 plants (T2C24#2 × LTR-GUS)3, -6, -9, -7, -1, -2, and -12], was able to silence Tnt1-GUS. This indicates that Tnt1-GUS silencing is linked to the number of Tnt1 elements in F1 plants rather than to one specific insertion locus.

Silencing of Tnt1 Is Reversed after a Decrease of Tnt1 Copy Number

Surprisingly, Tnt1 mRNAs were undetectable (data not shown) in all F1 plants analyzed (Table I), even those carrying low numbers of Tnt1 and expressing Tnt1-GUS. We thus tried to determine whether Tnt1 silencing was maintained across generations when less than four copies of the element were present. Tnt1 expression was analyzed by northern blot in plants derived from selfing a T2-derived F1 hybrid (T2C24#4; Supplemental Fig. S2) containing the c, e, and h hemizygous insertion loci (three copies) of Tnt1. We obtained F2 plants (T2C24#5; Supplemental Fig. S2) containing only the e insertion (two copies of Tnt1) in the homozygous state, as shown by Southern-blot analysis of the F3 generation. Expression of Tnt1 at the e insertion locus was restored in F3 and maintained to various extents in two successive selfed generations (Fig. 3A). Tnt1 expression was variable, as is usually observed in plants expressing Tnt1. In contrast, plants derived from another F2 plant (T2C24#6; Supplemental Fig. S2) containing the c and h insertion loci (four copies of Tnt1), both in the homozygous state, did not express Tnt1 in F4 (Fig. 3A). To determine whether reactivation of Tnt1 was specific of the e insertion locus or a general feature, we obtained another T2-derived F1 hybrid (T2C24 #7; Supplemental Fig. S2) containing only the h insertion locus in the hemizygous state. Transcription of Tnt1 was detected in F4 plants containing the homozygous h insertion locus (Fig. 3B). Together, our data indicate that Tnt1 silencing is reversible when the number of Tnt1 elements is reduced by genetic segregation.

Promoter Sequences of Silenced Tnt1 Are Methylated

Since methylation is associated with transposable element silencing, the patterns of methylation of both Tnt1 and Tnt1-GUS were analyzed by South-

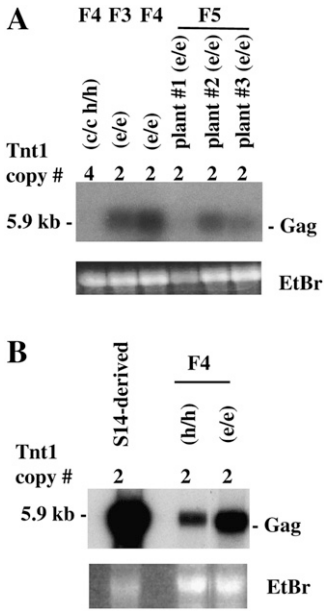

Figure 3. Tnt1 silencing is reversed after segregation of the element. RNA gel-blot analyses of 10 μg of total RNA extracted from mature rosette leaves of plants derived from selfing T2-derived plants backcrossed several times to the C24 ecotype or from S14-derived plants crossed to LTR-GUS [S14-derived, containing only one homozygous insertion of Tnt1; genotype (S14.6.10 × LTR-GUS)3.17.1]. *F3*, *F4*, and *F5* indicate the number of generations after segregation from high to low copy numbers of the element, as determined by Southern-blot analyses of DNAs digested by *Nde*I and probed with DNA complementary to the *Gag* gene of Tnt1 (data not shown). c, h, and e designate insertion loci as mentioned in Table I. The RNA blots were probed with DNAs complementary to the *Gag* gene of Tnt1. Ethidium bromide (EtBr) staining of the tRNA and 5S rRNA species is shown as a loading control. In A, genotypes of the plants are, from left to right, T2C24#6.1.n, T2C24#5.n, T2C24#5.1.n, and T2C24#5.1.1.n (three different plants); in B, genotypes are, from left to right, (S14.6.10 × LTR-GUS)3.17.1, T2C24#7.5.4.3, and T2C24#5.1.n. The origins of these plants are described in Supplemental Figure S2.

ern blot using methylation-sensitive restriction enzymes (Fig. 4A). First, we assessed Tnt1 methylation in its LTR region, which contains only non-CG sites. Southern-blot analyses were performed on a S14-derived plant containing four Tnt1 elements (Fig. 4B, S14-derived), a T2-derived plant containing more than 20 silenced copies of the element (Fig. 4B, T2-derived), a plant containing the reactivated e insertion locus of Tnt1 (Fig. 4B, reactivated), and a hybrid plant obtained by crossing S14- and T2-derived plants and containing numerous silenced Tnt1 elements (Fig. 4B, T2- × S14-derived), as shown by northern-blot analysis (Supplemental Fig. S1). DNAs from mature rosette leaves were extracted, digested with both *Hin*cII (revealing non-CG methylation) and *Nde*I (insensitive to methylation), blotted on a gel, and hybridized with a Gag probe. In plants expressing Tnt1, only a single fragment of 1,500 bp could be detected, indicating that neither of the two *Hin*cII sites tested, located in the promoter region and the coding region (Fig. 4A), was methylated (Fig. 4B, *Hin*cII *Nde*I digestions, Gag probe). In contrast, fragments larger than 1,904 bp were detected in plants carrying at least 20 Tnt1 copies, indicating that both *Hin*cII sites were methylated. Methylation of the U3 promoter region of Tnt1 in plants containing silenced elements was further confirmed by restriction of the same DNAs with both *Hin*dIII (revealing non-CG methylation) and *Nde*I enzymes and hybridization with the Gag probe. Detection of fragments larger than 1,785 bp shows that the *Hin*dIII site present in the promoter region is methylated when Tnt1 is silent but not when it is transcribed (Fig. 4B, *Hin*dIII *Nde*I digestions, Gag probe). Thus, two non-CG sites localized in the promoter region and revealed by *Hin*dIII and *Hin*cII are methylated in plants containing more than 20 copies, in contrast to plants with low copy numbers. The methylation state of the *Hin*cII site located in the Tnt1 coding sequence was then analyzed more carefully to determine whether non-CG sites of the coding sequence were methylated. The DNAs digested by *Hin*cII and *Nde*I were rehybridized with an internal Tnt1 probe (Fig. 4C, *Hin*cII *Nde*I digestions, B probe). In plants expressing Tnt1, detection of a single 404-bp fragment shows that the internal Tnt1 *Hin*cII site is not methylated, in contrast to plants with silenced elements in which larger DNA fragments are present (Fig. 4C, *Hin*cII *Nde*I digestions, B probe). Therefore, the coding sequence of Tnt1 is methylated at non-CG sites in plants with high numbers of elements. The intensity of the 404-bp fragment detected in these plants is a lot stronger than that of larger fragments, indicating that most elements are not methylated in their coding regions. The methylation state of cytosines located in the coding sequence was further analyzed in the same plants. After digestion with *Hpa*II and hybridization with a probe corresponding to a central *Hpa*II fragment (Fig. 4A, A probe), we detected a single 272-bp fragment in plants carrying active copies of Tnt1 (S14-derived plants), demonstrating that the Tnt1 coding region is unmethylated at both *Hpa*II sites (Fig. 4C, *Hpa*II digestion, A probe). This fragment was undetectable in plants with silenced Tnt1 (T2-derived plants) and was replaced by larger fragments, showing that at least one of the *Hpa*II sites analyzed is methylated. In the F1 hybrid resulting from a cross between S14- and T2-derived plants and containing more than 20 Tnt1 copies, the 272-bp fragment was undetectable. Thus, the coding region of the two Tnt1 elements originating from S14-derived plants becomes methylated in one generation when placed in a background with a high Tnt1 copy number. Interestingly, the coding sequence of the reactivated e element remained methylated, as shown by the absence of the short 272-bp fragment in the reactivated plant (Fig. 4C, *Hpa*II digestion, A probe). Thus, methylation of the coding sequence of Tnt1 does not interfere with its transcription.

Similarly, we compared the methylation state of cytosines present in Tnt1-GUS in mature rosette leaves of F1 hybrids derived from crosses between the

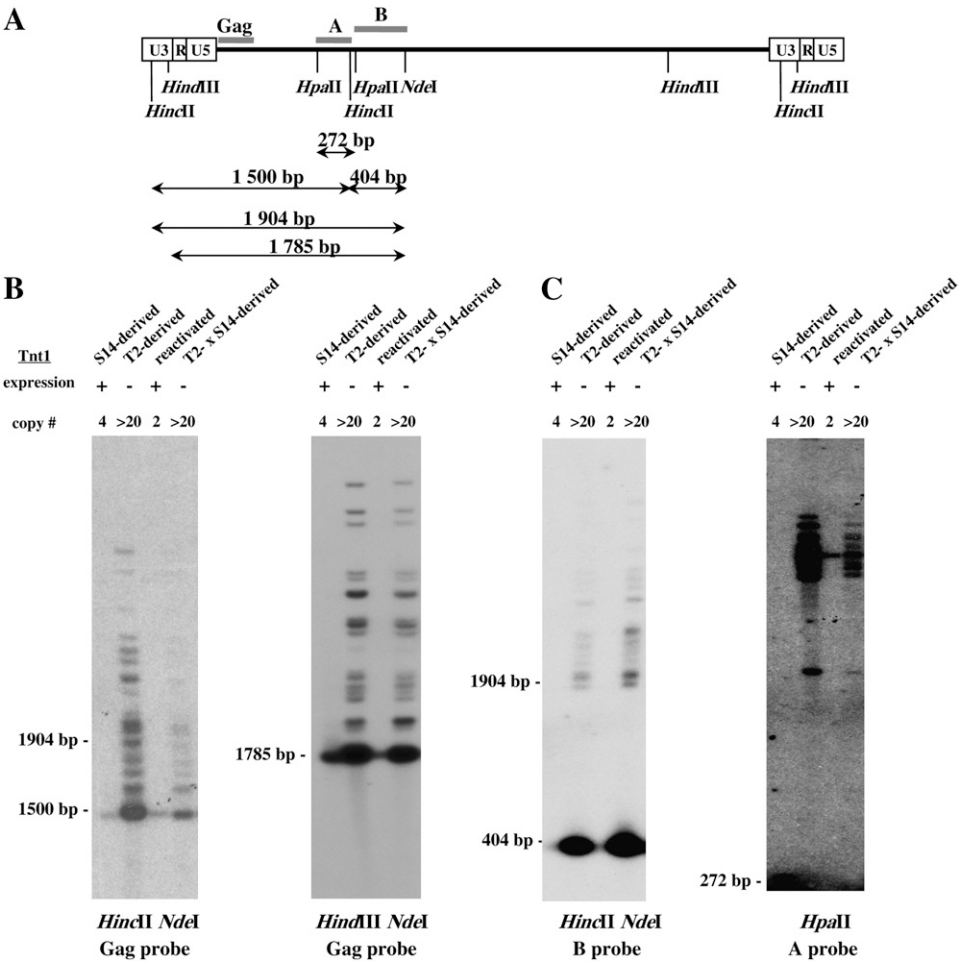

Figure 4. Methylation states of Tnt1 cytosine sites. A, Localization of Tnt1 restriction sites and probes used. *Hinc*II (restriction site, GTPyPuAC) does not cleave when the 3′ C is methylated; the restriction site in Tnt1 is GTTGAC. *Hin*dIII (restriction site, AAGCTT) does not cleave when the C is methylated. *Hpa*II (restriction site, CCGG) does not cleave when the second C is methylated. *Nde*I (restriction site, CATATG) is insensitive to methylation. *Hinc*II and *Hin*dIII both reveal non-CG methylation, while *Hpa*II reveals both CG and non-CG methylation. B and C, DNA gel-blot analyses of 2 μg of DNA extracted from mature rosette leaves of S14-derived (genotype S14.6.10) and T2-derived (genotype T2.2.4.28.20) plants, a plant containing two reactivated copies of Tnt1 derived from backcrossing T2-derived plants to the C24 ecotype followed by selfing (reactivated; genotype T2C24#5.1.1), and a hybrid between T2- and S14-derived plants (genotype T2.2.4.28.20 × S14.6.10). DNAs were digested with the restriction enzymes indicated (*Hinc*II, *Hin*dIII, *Hpa*II, and *Nde*I) to reveal the methylation state of non-CG sites in the Tnt1 promoter (B) or the methylation states of both CG and non-CG sites in the coding sequence of Tnt1 (C). Blots were probed with DNAs complementary to the regions indicated in A (Gag probe, A probe, B probe). The expression levels of Tnt1 (noted as + or −) were determined by northern blot (Supplemental Fig. S1).

LTR-GUS line (containing a homozygous Tnt1-GUS T-DNA) and T2-derived plants (containing one to more than 20 Tnt1 copies). Tnt1-GUS expression was assessed by histochemical staining for GUS activity in the hybrid plants. Mature rosette leaf DNAs were extracted from the same plants, digested by *Hinc*II, and hybridized with a probe corresponding to *GUS* (Fig. 5A, C probe). Methylation of the *Hinc*II site located in the Tnt1-GUS promoter increases with the number of Tnt1 copies, as demonstrated by the intensity of the 1,508-bp DNA fragment (Fig. 5B). Methylation of this *Hinc*II site was confirmed in both young and mature leaf tissues with the C+D probe (Fig. 5C, *Hinc*II digestion, probe C+D). Because fragments larger than 1,508 bp were not detected, the *GUS* coding sequence is not methylated in plants in which Tnt1-GUS is silent. This was confirmed by digesting the same DNAs with *Hpa*II and hybridizing them with the D probe located between two *Hinc*II sites in the central part of *GUS* (Fig. 5A). No fragment larger than 427, 227, or 83 bp could be detected (Fig. 5C, *Hpa*II digestion, D probe), demonstrating that the Tnt1-GUS coding sequence was not methylated.

Together, our data demonstrate that Tnt1 and Tnt1-GUS silencing is associated with the methylation of non-CG sites located in the U3 region of their

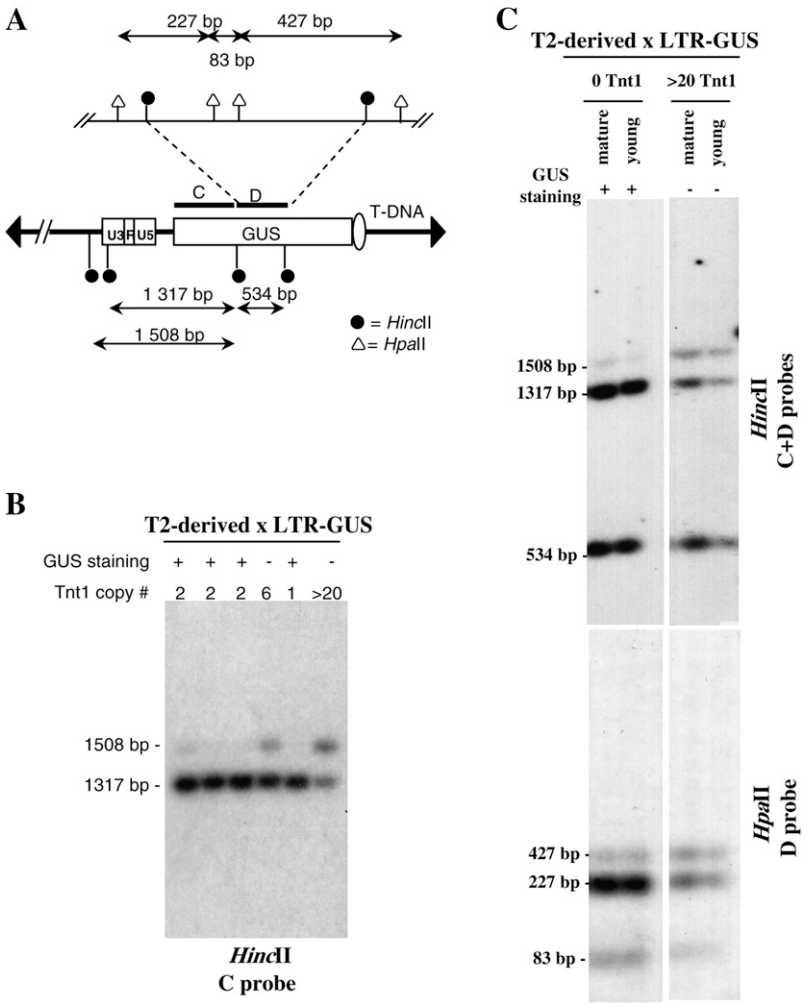

Figure 5. Methylation state of Tnt1-GUS cytosine sites. A, Localization of Tnt1-GUS restriction sites and probes used. The enzymes are described in the legend to Figure 4A. B, DNA gel-blot analyses of 2 μg of DNA extracted from rosette leaves of plants resulting from crosses between T2-derived plants and LTR-GUS plants (containing a homozygous Tnt1-GUS T-DNA). Genotypes of the plants are, from left to right, (T12C24#3 × LTR-GUS)4, (T12C24#2 × LTR-GUS)7, -9, and -10, (T2C24#5.1.1 × LTR-GUS)1, and (T2.2.4.28.20 × LTR-GUS)1. DNAs were digested with *Hinc*II, and blots were hybridized with the C probe (A) to reveal the methylation states of non-CG sites present in the LTR region. Expression of Tnt1-GUS (noted as + or −) was determined by histochemical staining (data not shown). C, DNA gel-blot analyses of 2 μg of DNA extracted from mature and young rosette leaves of a plant resulting from a cross between a T2-derived plant and LTR-GUS [genotype (T2.2.4.28.20 × LTR-GUS)13]. DNAs were digested with *Hinc*II or *Hpa*II, and blots were hybridized with the C and D probes (A) to reveal the methylation state of non-CG sites in both LTR and coding sequences of Tnt1-GUS (*Hinc*II digestion; C+D probes) or CG and non-CG sites in the coding sequence of Tnt1-GUS (*Hpa*II digestion; D probe). Expression of Tnt1-GUS (noted as + or −) was determined by histochemical staining (data not shown).

promoters. The methylation state of cytosine sites present in Tnt1 and Tnt1-GUS coding sequences is unrelated to their expression.

MET1 and DDM1 Are Differently Involved in Tnt1 Silencing

DDM1, encoding a chromatin-remodeling factor, and the cytosine methyltransferase MET1 both influence DNA methylation and chromatin organization (Chan et al., 2005; Zaratiegui et al., 2007). *MET1* encodes the major Arabidopsis CG maintenance methyltransferase (Kankel et al., 2003). Antisense suppression of the *MET1* gene causes a reduction of global cytosine methylation levels, particularly at CG sites. In plants containing an active antisense (AS)-*MET1* T-DNA, centromeric repeats are demethylated (Finnegan et al., 1996). We introduced a T-DNA carrying a *MET1* antisense construct (Finnegan et al., 1996) in T2-derived plants via *Agrobacterium* transformation to assess the role of MET1 on Tnt1 methylation. Efficiency of the antisense *MET1* construct was tested by analyzing the levels of methylation in centromeric repeats. DNAs from T2 AS-*MET1* and T2-derived plants, both containing more than 20 Tnt1 copies, were digested with *Hpa*II, blotted on a gel, and hybridized with a probe specific to the silenced centromeric repeats (Vongs et al., 1993). Most of the *Hpa*II sites in these regions were not methylated in the plant carrying the *MET1* antisense construct compared with the T2-derived plants (Fig. 6A), demonstrating that *MET1* was efficiently silenced. Tnt1 cytosine methylation was studied in the T2 AS-*MET1* plant by Southern blot after restriction with *Hpa*II and *Hinc*II/*Nde*I enzymes and hybridization with the A and B probes (Fig. 4A), respectively. Methylation of the Tnt1 coding sequence was almost completely released in T2 AS-*MET1* plants, as shown by the presence of the 272-bp fragment (Fig. 6B). Methylation at the *Hinc*II site present in the promoter region was similar between plants carrying the *MET1* antisense construct and the T2-derived plant (Fig. 6B). Thus, the Tnt1 promoter, containing only non-CG methylation sites, remains methylated in plants with reduced MET1 content, consistent with the role of MET1 in maintaining CG but not non-CG site methylation

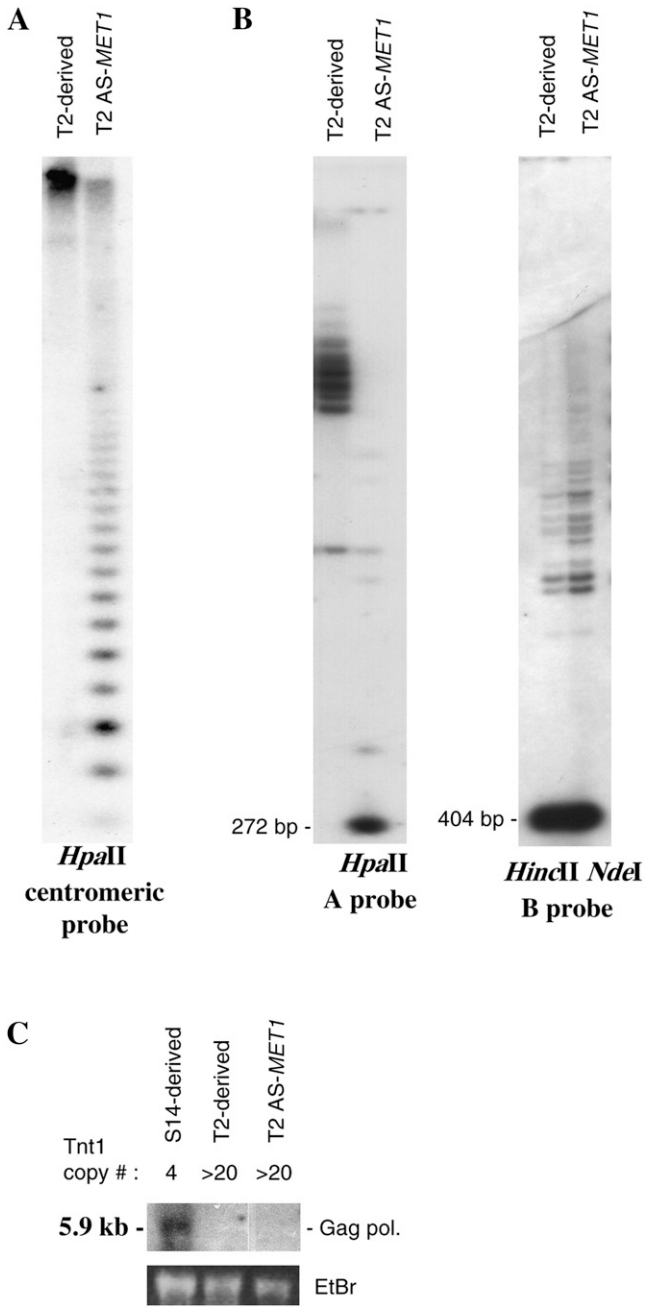

Figure 6. MET1 is not involved in Tnt1 silencing. DNA and RNA gel-blot analyses of 2 μg of DNA and 10 μg of total RNA extracted from S14-derived plants (S14-derived; genotype S14.6.10), a T2-derived plant (T2-derived; genotype T2.2.4.28), and a T2-derived plant transformed with a *MET1* antisense construct (T2 AS-*MET1*; genotype T2.2 AS-*MET1*). A and B, DNAs were restricted with *Hpa*II and probed with the 180-bp centromeric probe (Finnegan et al., 1996; A) or digested with *Hpa*II or *Hin*cII and *Nde*I and then hybridized with the probes indicated (B). C, The northern blot was probed with DNAs complementary to the Gag region of Tnt1. Ethidium bromide (EtBr) staining of the tRNA and 5S rRNA species is shown as a loading control.

(Chan et al., 2005; Zaratiegui et al., 2007). Tnt1 steady-state mRNA levels were analyzed in mature rosette leaves of T2 AS-*MET1* plants by northern blot (Fig. 6C), showing that T2 AS-*MET1* plants did not express Tnt1, confirming that methylation of Tnt1 CG sites in the coding sequence was not required for Tnt1 silencing.

We then tested the effect of DDM1 on Tnt1 silencing. A T2-derived plant containing more than 20 insertion loci of Tnt1 was crossed with plants heterozygous for the *ddm1-2* mutation (Jeddeloh et al., 1999). F2 plants were genotyped for the presence of *ddm1-2*, and the number of Tnt1 insertions was determined by Southern-blot analysis (data not shown). Tnt1 expression was monitored by northern blot in both F2 plants homozygous for *ddm1-2* and wild-type sibling plants, revealing that Tnt1 silencing was released in *ddm1* mutants only (Fig. 7A). We determined the methylation state of Tnt1 elements in both types of plants. Methylation of the LTR regions of Tnt1 in plants containing numerous elements was analyzed by restriction of their DNAs with both *Hin*dIII and *Nde*I enzymes and hybridization with the Gag probe (Fig. 1A). Detection of fragments larger than 1,785 bp shows that the *Hin*dIII site present in the promoter of Tnt1 is methylated in both *ddm1* plants and their wild-type siblings (Fig. 7B, *Hin*dIII *Nde*I digestions, Gag probe). As a control, we verified that Tnt1 promoter sequences were not methylated in plants containing transcribed copies of the element (Fig. 7B, S14-derived), in contrast to plants containing more than 20 Tnt1 copies (Fig. 7B, T2-derived). The methylation state of cytosines located in the Tnt1 coding sequence was also analyzed. After digestion with *Hpa*II and hybridization with a probe corresponding to a central *Hpa*II fragment (Fig. 4A, A probe), we detected a 272-bp fragment in *ddm1* mutants but not in *DDM1/DDM1* plants (Fig. 7B, *Hpa*II digestion, A probe). This showed that cytosines of the *Hpa*II sites present in Tnt1 coding sequences were demethylated in *ddm1* mutants. Together, our data indicate that Tnt1 silencing is released in *ddm1* mutants. Surprisingly, the Tnt1 methylation state remains apparently unchanged at non-CG sites of Tnt1 LTR sequences in *ddm1* mutants, while *Hpa*II sites revealing both CG and non-CG methylation sites of the coding sequence are demethylated.

Tnt1 Silencing Is Released in *polIVa* Mutants

NRDP1a, a subunit of the DNA-dependent RNA polymerase IV, is required for the production and/or amplification of siRNAs derived from transposons (Matzke et al., 2007). We introduced the three c, h, and l insertions at the homozygous state in a *nrpd1a-2* mutant (Herr et al., 2005) by subsequent crossing and genotyping steps. The presence of the *Tnt1* mRNA was assessed by reverse transcription (RT) and PCR amplification using primers specific for the *Tnt1* cDNA. The *Tnt1* mRNA could not be detected in total RNA extracted from c/c h/h l/l plants containing six copies of Tnt1, in contrast to *nrpd1a-2* mutants carrying the same Tnt1 insertions (Fig. 8, Tnt1 primers). This result

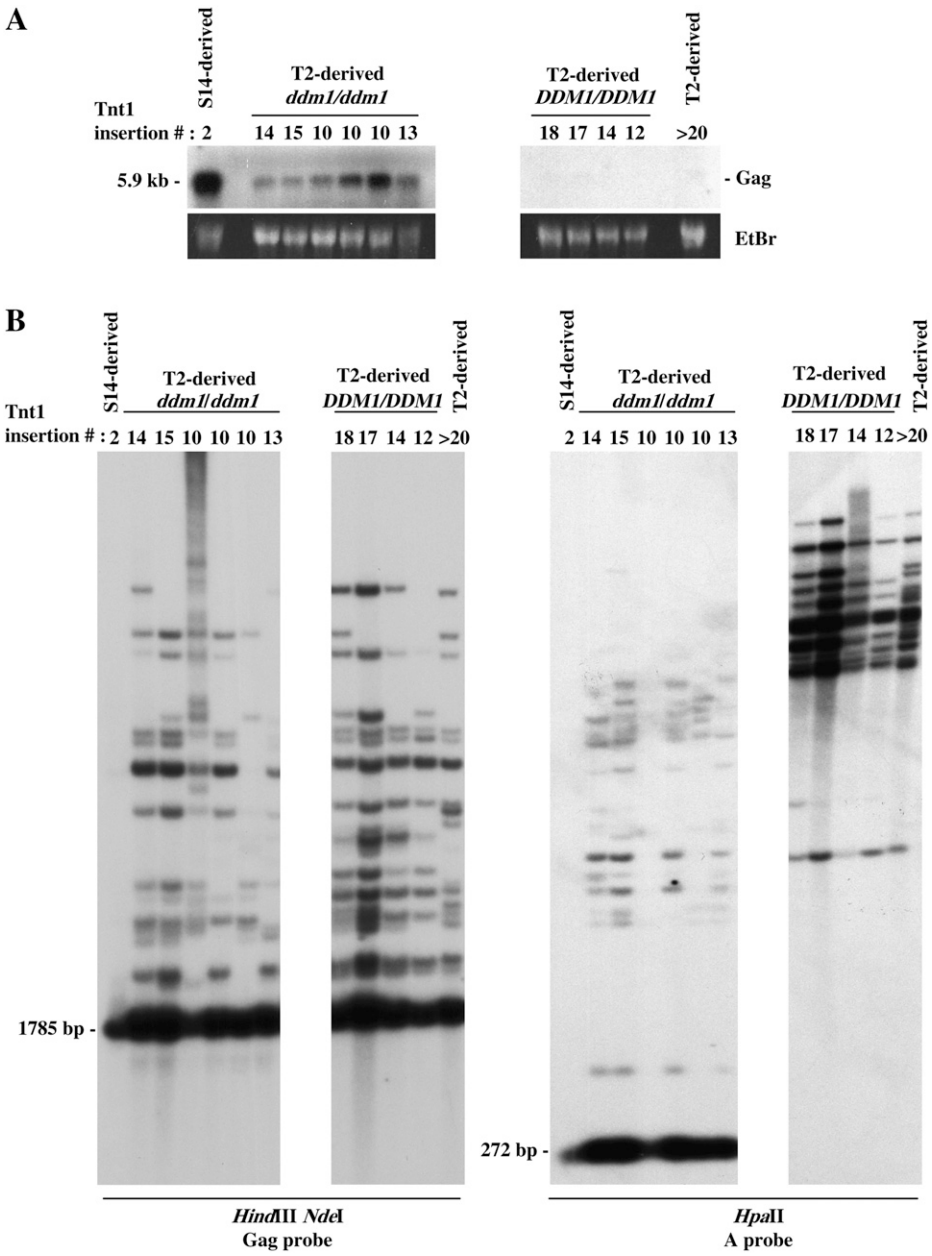

Figure 7. Silencing of Tnt1 is released in *ddm1* mutants. RNA and DNA gel-blot analyses of 10 μg of total RNA and 2 μg of DNA extracted from mature rosette leaves of F2 plants (T2-derived *ddm1/ddm1* or *DDM1/DDM1*) obtained by crossing a T2-derived plant (genotype T2.2.52) and a *ddm1-2/DDM1* plant. The number of Tnt1 insertion loci (Tnt1 insertion #) was determined by Southern-blot analysis (data not shown). S14-derived plants (S14-derived; genotype S14.6.10.6.10) and T2-derived plants (T2-derived; genotype T2.2.4.28.23.10 in A and T2.2.4.28.20.3.1 in B) were used as controls. A, Northern blots were probed with DNAs complementary to the Gag gene of Tnt1. Ethidium bromide (EtBr) staining of the tRNA and 5S rRNA species is shown as a loading control. B, DNAs were digested by *Hin*dIII and *Nde*I to reveal the methylation states of non-CG sites or by *Hpa*II to reveal the methylation states of both CG and non-CG sites. Blots were probed with DNAs complementary to the regions indicated in Figure 4A (Gag and A probes).

indicates that Tnt1 silencing is released in *polIVa* mutants. Amplification of an elongation factor mRNA-worked equally well on all RNA templates (Fig. 8, control primers + RT).

Silencing of Tnt1 Is Partially Released by Stress

We have previously shown that Tnt1-GUS expression is induced by abiotic stresses such as freezing, wounding, and treatment with $CuCl_2$ (Mhiri et al., 1997) or by biotic stresses such as infection by the bacteria *Pseudomonas syringae* (Moreau-Mhiri et al., 1996). Therefore, we analyzed the effect of leaf wounding on Tnt1-GUS silencing. In leaves of plants containing two copies of Tnt1-GUS (LTR-GUS line), GUS activity could be detected at the site of the injury 48 h after wounding (Fig. 9, LTR-GUS), in agreement with previous observations (Mhiri et al., 1997). Surprisingly, we also detected GUS activity at 48 h after wounding in leaves of F1 plants resulting from crosses between T2-derived and LTR-GUS plants (Fig. 9, T2-derived $\times$ LTR-GUS). In these plants, Tnt1-GUS was silenced by the presence of multiple Tnt1 copies, as no GUS staining could be detected immediately after wounding (Fig. 9). The difference of GUS staining between plants containing an active or a silent Tnt1-GUS reveals that leaf wounding is able to partially reverse Tnt1-GUS silencing.

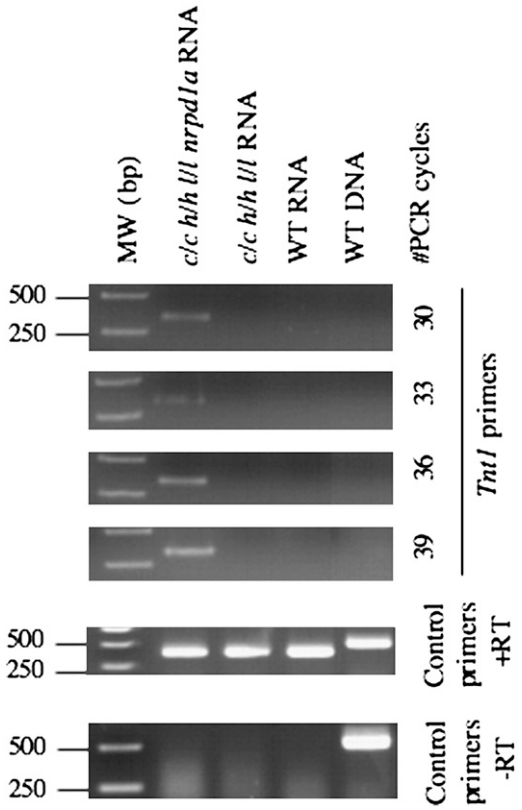

Figure 8. Silencing of Tnt1 is released in *nrpd1a* mutants. Expression analysis of *Tnt1* mRNA by RT-PCR in the *nrpd1a-2* mutant containing six copies of Tnt1. Total RNAs were isolated from 5-week-old plants and used as templates for RT. A 336-bp DNA fragment corresponding to a region of the *Tnt1* mRNA was amplified with specific primers in c/c h/h l/l *nrpd1a-2* plants but not in c/c h/h l/l or wild-type (WT; ecotype Columbia) plants. Control primers were used to amplify a region of the Arabidopsis mRNA (after RT) encoding elongation factor 1α (GenBank accession no. AY039583). −RT designates negative control experiments in which reverse transcriptase was omitted before the final PCR amplification step. cDNA synthesis and RT-PCR were repeated 10 times on three different batches of plants grown independently, and identical results were obtained.

DISCUSSION

Most plant retrotransposons are heavily methylated (Okamoto and Hirochika, 2001), and it is now widely accepted that transposable elements are silenced by epigenetic mechanisms. In Arabidopsis, most endogenous transposable elements are silent, methylated, and marked by histone modifications characteristic of heterochromatin (Zaratiegui et al., 2007). Silencing of transposable elements is suppressed in several mutants for genes involved in epigenetic regulation pathways (Lippman et al., 2003), and CACTA and Mu-like elements transpose efficiently in DNA methylation mutant backgrounds (Miura et al., 2001; Singer et al., 2001; Kato et al., 2003). Moreover, the majority of Arabidopsis endogenous siRNAs correspond to transposable elements (Qi et al., 2006; Rajagopalan et al., 2006; Fahlgren et al., 2007), suggesting that small RNAs and the RNA interference machinery are widely involved in their control. Still, the presence of a vast amount of amplified LTR retrotransposons in plants with large genomes suggests that silencing is either nonefficient in some species or for some elements or can be overcome under certain circumstances. Most studies performed on transposable element silencing were aimed at endogenous elements, for which silencing was probably set up many generations ago and that results from both genome defense and evolution. The introduction of the tobacco LTR retrotransposon Tnt1 into Arabidopsis by transgenesis mimics the horizontal transfer of a transposable element into a new host, providing a useful tool to study the first stages of the defense reaction of a host genome against invasive DNA elements.

Tnt1 Silencing Occurs via RdDM

Our results show that, in Arabidopsis, Tnt1 is transcriptionally silenced in a copy number-dependent manner, as shown by run-off analysis (Fig. 2A). This is in keeping with several lines of evidence suggesting that transposable elements are controlled by transcriptional gene silencing. Tnt1 silencing depends on Tnt1 copy number per plant, similar to what has been demonstrated for the Tto1 tobacco LTR retrotransposon (Hirochika et al., 2000) introduced into Arabidopsis and for the I element in *Drosophila melanogaster* (Chaboissier et al., 1998).

Small RNAs corresponding to Tnt1 LTR sequences were detected in plants containing more than 20 silenced Tnt1 copies but not in plants carrying active copies of the element (Fig. 2B). In a similar way, Tnt1 LTR-derived siRNAs were detected in tobacco, its original host (Hamilton et al., 2002; Andika et al., 2006). As in tobacco, the Arabidopsis Tnt1 siRNAs are 24 nucleotides in length (Fig. 2B), like the vast majority

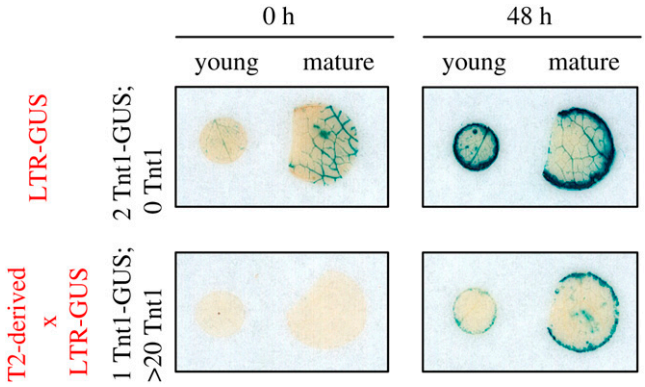

Figure 9. Leaf wounding partially releases Tnt1 silencing. Histochemical staining performed on leaf discs from young and mature leaves of plants containing a homozygous Tnt1-GUS construct (LTR-GUS) or resulting from a cross between a T2-derived plant and LTR-GUS (T2-derived × LTR-GUS; genotype T2.2.52 × LTR-GUS). Leaves were stained immediately after wounding (0 h) or 48 h later (48 h).

of siRNAs targeting transposable elements and repeated sequences (Qi et al., 2006; Rajagopalan et al., 2006; Fahlgren et al., 2007); thus, it is very likely that these siRNAs are DCL3 and RDR2 dependent. Production of small RNAs homologous to Tnt1 promoter sequences located in the U3 region leads probably to the methylation of non-CG sites and transcriptional silencing of the element. Recent studies have demonstrated the role of PolIV subunits associated with the SNF2-like proteins DRD1 and CLSY1 in the establishment and maintenance of RdDM (Herr et al., 2005; Kanno et al., 2005a, 2005b; Onodera et al., 2005; Pontier et al., 2005; Smith et al., 2007). Hence, these proteins, in combination with the non-CG methyltransferases DRM1, DRM2, and CMT3, might be involved in Tnt1 RdDM, triggered by 24-nucleotide siRNAs targeting the LTR. We have demonstrated that PolIVa is necessary for the silencing of Tnt1 in Arabidopsis (Fig. 8). The histone H3K4 methylation pathways might also be implicated, as shown for endogenous Arabidopsis retrotransposons and developmental genes (Chan et al., 2006; Huettel et al., 2006).

Tnt1 Can Escape Silencing in Arabidopsis

In plants, the methyltransferase MET1 maintains CG methylation, while DRM1, DRM2, and CMT3 are responsible for methylation at non-CG sites (Chan et al., 2005; Zaratiegui et al., 2007). MET1 maintains the methylation at CG sites over meiosis (Jones et al., 2001), while non-CG methylation necessitates the constant biosynthesis of siRNAs to be maintained. The Tnt1 promoter located in the U3 region of the LTR contains only non-CG sites and is totally devoid of CG sites. This could account for the nontransmissibility of the Tnt1 and Tnt1-GUS silenced state through meiosis, when the number of Tnt1 elements decreases (Fig. 3). It also explains why MET1 is not involved in maintaining Tnt1 silencing (Fig. 6), in contrast to the vast majority of endogenous transposable elements of Arabidopsis (Zhang et al., 2006). Having no CG sites in the promoter region might be a selective advantage for Tnt1, explaining why it was maintained throughout evolution, in spite of the high mutation rate during LTR retrotransposon replication (Gabriel et al., 1996). In addition, non-CG methylation may have evolved in plants as a supplementary level of control to silence regions escaping CG methylation. Supporting this hypothesis, CACTA elements transpose at high frequency in the double *met1 cmt3* mutant but not in single *met1* and *cmt3* mutants, implying that CG and non-CG methylation are both implicated in the silencing of this class of transposable elements (Kato et al., 2003). Although not involved in Tnt1 silencing, methylation of the Tnt1 coding sequence is observed in plants containing high numbers of the element, whereas the *GUS* coding region remains unmethylated when Tnt1-GUS is silenced. Thus, methylation of Tnt1 transcribed sequences has no obvious impact on their transcription. Tnt1 is reactivated in plants containing only a few copies (Fig. 3). Epigenetic marks other that DNA methylation (e.g. histone modifications) involved in Tnt1 silencing could be maintained over the first meiosis but suppressed after a few generations when the number of Tnt1 copies decreases. Still, multiple copies appear to be more efficient in maintaining silencing than single or few copies.

DDM1 is a chromatin-remodeling ATPase that seems to act indirectly on DNA methylation. For instance, DDM1 binds proteins that specifically bind methylated CG dinucleotides (Zemach et al., 2005). Mutations in the *DDM1* gene cause severe loss of DNA methylation, resulting in reactivation of transposable elements and finally leading to severe developmental abnormalities (Kakutani et al., 1996; Jeddeloh et al., 1999; Miura et al., 2001). Transgenes silenced by transcriptional gene silencing are also reactivated in *ddm1* (Morel et al., 2000). Here, we show that Tnt1 silencing is released in *ddm1* mutants (Fig. 7), similar to what was observed for Tto1 in Arabidopsis (Hirochika et al., 2000) and for Arabidopsis endogenous transposons such as Mutator-like elements (Singer et al., 2001), Tar17 (Hirochika et al., 2000), or CACTA transposons (Miura et al., 2001, 2004; Kato et al., 2004). Thus, DDM1 seems to be involved in both Tnt1 and endogenous Arabidopsis transposon silencing. Our results show that *Hpa*II site methylation of the Tnt1 coding sequence is lost in a *ddm1* background, similar to what was observed for the *Hpa*II sites of Tto1 (Hirochika et al., 2000). Surprisingly, the methylation of non-CG sites present in the Tnt1 promoter sequences is apparently unaffected by the *ddm1* mutation. This suggests either that only one or a few elements is reactivated in some cells of the F1 hybrids analyzed or that DDM1 acts downstream of non-CG methylation. However, this would be in contrast to what has been observed previously, with DDM1 affecting methylation at both CG and non-CG sites (Finnegan et al., 1996).

Tnt1 Silencing and Abiotic Stresses

The results presented here (Fig. 9) as well as some previously published results (Mhiri et al., 1997) show that wounding and treatment with $CuCl_2$ overcome, at least partially, Tnt1 and Tnt1-GUS silencing. These two stresses also activate Tnt1-GUS transcription in Arabidopsis plants carrying only the Tnt1-GUS insert without any Tnt1 element (Mhiri et al., 1997), possibly through the interaction of a MYB transcription factor with a 31-bp repeated sequence (the BII box) located in the Tnt1 U3 region (Casacuberta and Grandbastien, 1993; Vernhettes et al., 1997; Sugimoto et al., 2000). Interestingly, Tnt1 and Tnt1-GUS expression are also induced by biotic and abiotic stresses in tobacco, which contains several hundred Tnt1 elements (Pouteau et al., 1994; Mhiri et al., 1997). In *Drosophila*, Polycomb group-induced heterochromatic structures can prevent activators from binding to their target sequences.

However, this antagonistic effect can be overcome by large amounts of transcription factors (Zink and Paro, 1995). Consequently, the reactivation of Tnt1-GUS expression observed after wounding could be explained by a massive production of transcription factors able to overcome the silencing mechanisms. Studying the effect of other stresses on Tnt1 silencing would help to determine whether silencing escape is induced by effectors binding the Tnt1 promoter or whether stresses have a more general effect on genome regulation, as suggested by several authors (Kashkush et al., 2003; Sunkar and Zhu, 2004). In both cases, it is tempting to speculate that the stress activation observed for a number of class I and class II transposable elements (Walbot, 1992; Grandbastien, 1998) is the result of their escape from silencing rather than of (or in combination with) the specific induction of their regulatory sequences by stress. This escape from silencing would lead to the amplification of LTR retrotransposons in circumstances in which plants are subjected to changes of environment and to potentially adaptive mutations. Nevertheless, it remains to be determined whether this occurs by active demethylation of transposable element promoters. In such a case, DNA glycosylases like REPRESSOR OF SILENCING1 or proteins of the DEMETER family, responsible for the demethylation of certain promoters in Arabidopsis (Gong et al., 2002; Penterman et al., 2007), could be involved in the process and play an active role in plant responses to environmental stress.

CONCLUSION

In conclusion, Tnt1 is transcriptionally silenced when its copy number increases in Arabidopsis. We propose that Tnt1 silencing is due to RNA-directed DNA methylation and is triggered by small RNAs homologous to the Tnt1 promoter region and directing its methylation. Tnt1 transcription can be released, either when the copy number per plant decreases or after a stress. This provides new insights into the way LTR retrotransposons invade their host genomes despite active silencing mechanisms.

MATERIALS AND METHODS

Plants and Growth Conditions

S14, S15, S17, and T2 primary transformants and the tobacco (*Nicotiana tabacum*) LTR-GUS line, described by Lucas et al. (1995), Moreau-Mhiri et al. (1996), and Courtial et al. (2001), were obtained by in vitro transformation of the C24 Arabidopsis (*Arabidopsis thaliana*) ecotype. Transgenic T2.2 AS-*MET1* plants were obtained by in planta transformation (Bechtold and Pelletier, 1998) of T2.2.4.28 progeny with an *Agrobacterium tumefaciens* strain (Lazo et al., 1991) carrying the pMLBART vector (Finnegan et al., 1996). The genealogy of all plants analyzed in this study is given in Supplemental Figure S2. For Tnt1 and Tnt1-GUS expression analysis, plants were grown under short-day conditions (8 h of light at 20°C and 16 h of dark at 15°C). For self-pollination and crosses, plants were grown in the greenhouse.

Tnt1 insertions in plants used in this study were identified by Southern-blot analysis after DNA restriction by the *Nde*I and hybridization with a probe corresponding to the Gag region of Tnt1 (Fig. 1A). Segregation analysis of each fragment in the selfed progeny of a given plant indicated its homozygosity/heterozygosity in that plant. In addition, we identified the insertion sites of three independent Tnt1 transposed copies (c, h, and l) that can be genotyped using the primers described in Supplemental Table S1.

The *ddm1-2* mutation was genotyped using the CAPSF/CAPSR primers (Supplemental Table S1) and the *Rsa*I enzyme. The *nrpd1a-2* mutant was genotyped as described (Herr et al., 2005).

DNA and RNA Blot Analysis

Genomic DNA and total RNA were extracted as described (Lucas et al., 1995; Gasciolli et al., 2005). DNAs (2–3 μg) were digested with restriction enzymes, fractionated on 0.8% agarose gels, and blotted on membranes. Small RNAs were separated by electrophoresis on 15% polyacrylamide gels and blotted on membranes (Bouché et al., 2006). DNA and RNA blots were probed with radiolabeled DNAs complementary to the coding sequence of Tnt1 (accession no. X13777) and corresponding to nucleotides 613 to 1,056 for the Gag probe, nucleotides 1,341 to 1,558 for the A probe, and nucleotides 1,577 to 1,982 for the B probe. C and D probes correspond to the *GUS* region (accession no. S69414) spanning nucleotides 1 to 604 (ATG starting codon-*Hin*cII fragment) and 604 to 1,138 (*Hin*cII-*Hin*cII fragment), respectively. Radiolabeled RNA probes transcribed in vitro were used to detect siRNAs corresponding to the promoter of Tnt1 (accession no. X13777, nucleotides 1–610). The β-tubulin probe corresponds to the full-length cDNA coding for β-tubulin (accession no. ATTS0538). The 180-bp Arabidopsis centromeric probe corresponds to silenced centromeric repeats (Vongs et al., 1993). RNA and DNA hybridization signals were quantified using a Fuji phosphorimager or after exposure to x-ray films for 24 h to 15 d.

Nuclear Run-Off Transcription Assay

The Tnt1 *Bgl*II/*Bam*HI fragment located in the endonuclease region of Tnt1, the *Eco*RI 3′ terminal fragment of *GUS*, the rDNA 25S fragment, and the β-tubulin cDNA sequence were cloned in pKS vector. One milligram of plasmid DNAs was linearized by enzymatic restriction, denatured, and immobilized on Hybond N^+ membranes (Amersham) by vacuum slot blotting. Ten grams of Arabidopsis roots grown in aeroponic culture for 8 weeks was ground in liquid nitrogen and homogenized in a buffer containing 0.25 mM Suc, 10 mM NaCl, 10 mM KCl, 2% dextran T40, 10 mM MES, pH 5.4, 5 mM EDTA, 0.1 mM spermine, 0.5 mM spermidine, and 0.6% Triton X-100. Filtration and nuclei purification were performed as described (Elmayan et al., 1998) without a Percoll/Suc gradient. Isolated nuclei (10^6–10^7) were used for each in vitro transcription reaction following the previously described protocol (Elmayan and Vaucheret, 1996). Incorporation of [U-^{32}P]triphosphate after in vitro RNA synthesis was determined by probing replicates of the dot-blotted target DNAs with labeled RNAs. Hybridizations were performed in 1.5 mL of hybridization buffer containing 0.25 M Na_2HPO_4, pH 7.4, 7% SDS, 2 mM EDTA, and 50% formamide for 48 h at 65°C. Blots were washed twice in 2× SSC, 0.01% SDS, once in 0.2× SSC, 0.01% SDS at 42°C for 20 min, and once in 0.2× SSC, 0.01% SDS at 65°C for 30 min. Different exposure times were assayed to obtain similar β-tubulin transcription rates.

Analysis of *Tnt1* mRNA Expression by RT-PCR

Total RNA was isolated from leaves of 5-week-old plants grown in short-day conditions, and RNA extraction was performed with the RNeasy plant kit (Qiagen) as indicated by the manufacturer. Reverse transcriptase was used to prepare the corresponding cDNA templates from the total RNA extracts. PCR amplification of a 336-bp *Tnt1* cDNA-specific sequence was performed with Tnt1 4489+/Tnt1 TR1 primers (Supplemental Table S1) amplifying a region spanning nucleotides 4,489 to 4,825 of the *Tnt1* cDNA. PCR amplification of the cDNA encoding the elongation factor 1α of Arabidopsis (GenBank accession no. AY039583) with EFf/EFr primers (Supplemental Table S1) served as a control.

Histochemical Staining

Leaf discs were vacuum infiltrated with 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid in 0.1 M phosphate buffer, pH 7, containing 0.5 mM

potassium ferricyanide and ferrocyanide and incubated overnight at 37°C immediately after wounding or after 48 h of incubation in the dark on wet filter paper at room temperature. Plant tissues were destained and stored in 70% ethanol.

Supplemental Data

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

Supplemental Figure S1. Northern-blot analysis of plants containing active, silenced, and reactivated Tnt1.

Supplemental Figure S2. Genealogy of the plants used in this study.

Supplemental Table S1. Primers used in this study.

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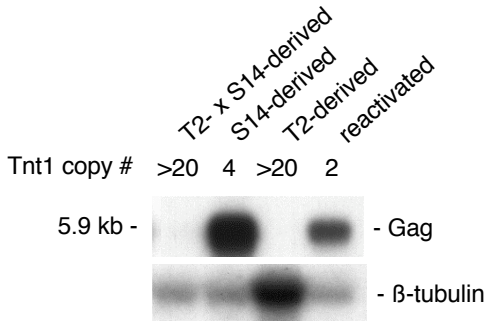


Supplemental Figure S1. Northern blot analysis of plants containing active, silenced and reactivated Tnt1

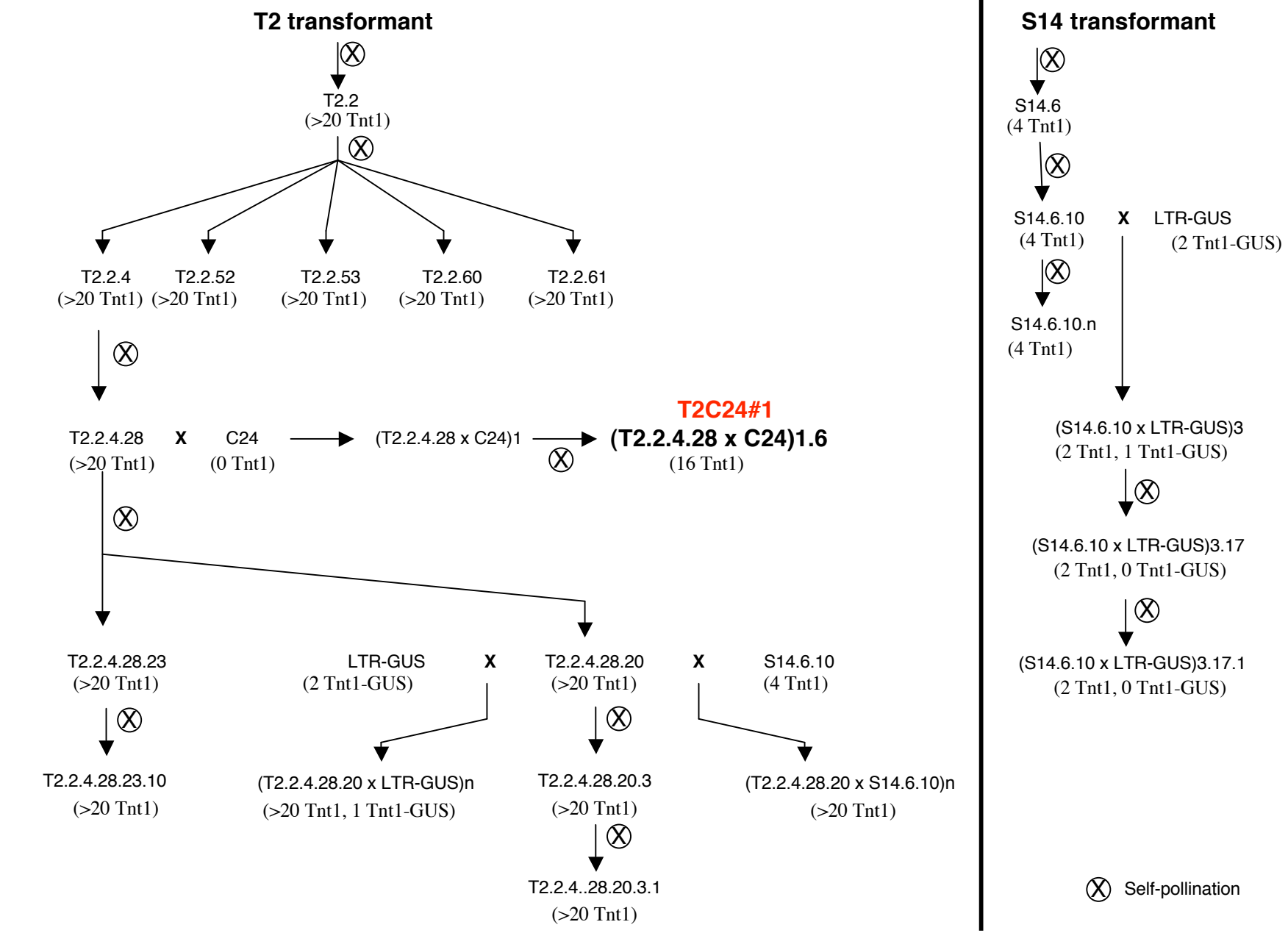
RNA gel blots analysis of 10 μg RNA extracted from mature rosette leaves of S14-derived plants (genotype: S14.6.10), a T2-derived plant (genotype: T2.2.4.28.20), a plant containing two reactivated copies of Tnt1 (genotype: T2C24#5.1.1) and of a hybrid between T2- and S14-derived plants (genotype: T2.2.4.28.20 x S14.6.10). Blots were probed with DNA complementary to the *Gag* gene (Fig. 4A; Gag) and to a β-tubulin gene used as a loading control.

Supplemental Figure S2. Genealogy of the plants used in this study.

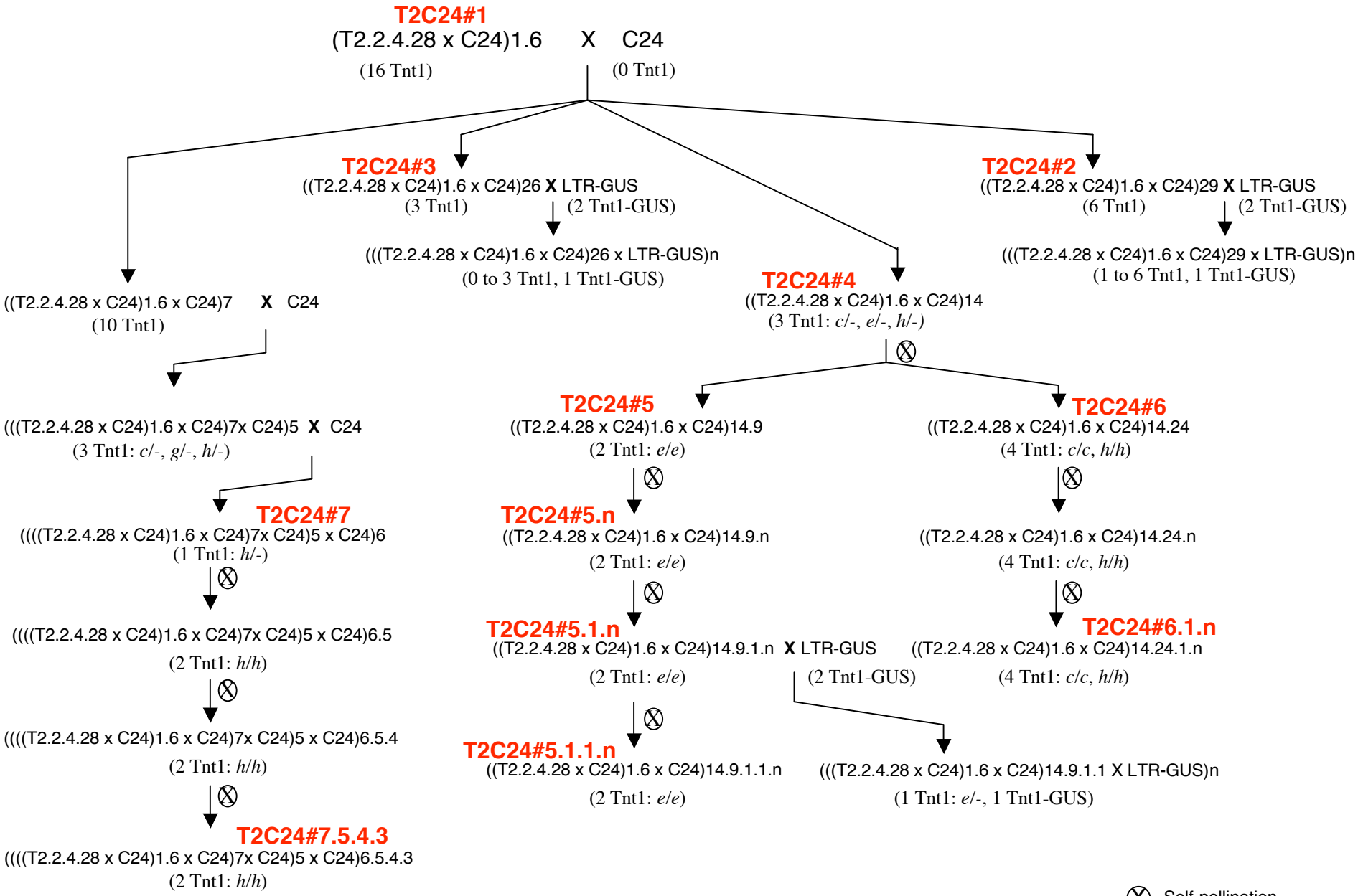
A, T2- and S14-derived plants

B, Progenies of plant T2C24#1 (T2.2.4.28 x C24)1.6, carrying 16 Tnt1 elements.

A


T2 transformant
T2.2
(>20 Tnt1)
T2.2.4
(>20 Tnt1)
T2.2.52
(>20 Tnt1)
T2.2.53
(>20 Tnt1)
T2.2.60
(>20 Tnt1)
T2.2.61
(>20 Tnt1)
T2.2.4.28
(>20 Tnt1)
X
C24
(0 Tnt1)
(T2.2.4.28 x C24)1
T2C24#1
(T2.2.4.28 x C24)1.6
(16 Tnt1)
T2.2.4.28.23
(>20 Tnt1)
T2.2.4.28.23.10
(>20 Tnt1)
LTR-GUS
(2 Tnt1-GUS)
X
(T2.2.4.28.20 x LTR-GUS)n
(>20 Tnt1, 1 Tnt1-GUS)
T2.2.4.28.20
(>20 Tnt1)
T2.2.4.28.20.3
(>20 Tnt1)
T2.2.4..28.20.3.1
(>20 Tnt1)
X
S14.6.10
(4 Tnt1)
(T2.2.4.28.20 x S14.6.10)n
(>20 Tnt1)
S14 transformant
S14.6
(4 Tnt1)
S14.6.10
(4 Tnt1)
S14.6.10.n
(4 Tnt1)
X
LTR-GUS
(2 Tnt1-GUS)
(S14.6.10 x LTR-GUS)3
(2 Tnt1, 1 Tnt1-GUS)
(S14.6.10 x LTR-GUS)3.17
(2 Tnt1, 0 Tnt1-GUS)
(S14.6.10 x LTR-GUS)3.17.1
(2 Tnt1, 0 Tnt1-GUS)
Self-pollination

B


T2C24#1
(T2.2.4.28 x C24)1.6 X C24
(16 Tnt1) (0 Tnt1)
T2C24#3
((T2.2.4.28 x C24)1.6 x C24)26 X LTR-GUS
(3 Tnt1) (2 Tnt1-GUS)
(((T2.2.4.28 x C24)1.6 x C24)26 x LTR-GUS)n
(0 to 3 Tnt1, 1 Tnt1-GUS)
T2C24#2
((T2.2.4.28 x C24)1.6 x C24)29 X LTR-GUS
(6 Tnt1) (2 Tnt1-GUS)
(((T2.2.4.28 x C24)1.6 x C24)29 x LTR-GUS)n
(1 to 6 Tnt1, 1 Tnt1-GUS)
((T2.2.4.28 x C24)1.6 x C24)7 X C24
(10 Tnt1)
T2C24#4
((T2.2.4.28 x C24)1.6 x C24)14
(3 Tnt1: c/-, e/-, h/-)
(((T2.2.4.28 x C24)1.6 x C24)7x C24)5 X C24
(3 Tnt1: c/-, g/-, h/-)
T2C24#5
((T2.2.4.28 x C24)1.6 x C24)14.9
(2 Tnt1: e/e)
T2C24#6
((T2.2.4.28 x C24)1.6 x C24)14.24
(4 Tnt1: c/c, h/h)
T2C24#7
((((T2.2.4.28 x C24)1.6 x C24)7x C24)5 x C24)6
(1 Tnt1: h/-)
T2C24#5.n
((T2.2.4.28 x C24)1.6 x C24)14.9.n
(2 Tnt1: e/e)
((T2.2.4.28 x C24)1.6 x C24)14.24.n
(4 Tnt1: c/c, h/h)
((((T2.2.4.28 x C24)1.6 x C24)7x C24)5 x C24)6.5
(2 Tnt1: h/h)
T2C24#5.1.n
((T2.2.4.28 x C24)1.6 x C24)14.9.1.n X LTR-GUS
(2 Tnt1: e/e)
(2 Tnt1-GUS)
T2C24#6.1.n
((T2.2.4.28 x C24)1.6 x C24)14.24.1.n
(4 Tnt1: c/c, h/h)
((((T2.2.4.28 x C24)1.6 x C24)7x C24)5 x C24)6.5.4
(2 Tnt1: h/h)
T2C24#5.1.1.n
((T2.2.4.28 x C24)1.6 x C24)14.9.1.1.n
(2 Tnt1: e/e)
(((T2.2.4.28 x C24)1.6 x C24)14.9.1.1 X LTR-GUS)n
(1 Tnt1: e/-, 1 Tnt1-GUS)
T2C24#7.5.4.3
((((T2.2.4.28 x C24)1.6 x C24)7x C24)5 x C24)6.5.4.3
(2 Tnt1: h/h)
Self-pollination

Supplemental Table S1. Primers

Name	**Sequence**
CAPSF	5'-ACGAAGCAACCAAGGAAGAA-3'
CAPSR	5'-GAGCCATGGGTTTGTGAAACGTA-3'
c#1[1]	5'-CCGATTGAGACCTGTG-3'
c#2[1]	5'-AATTCTGACCTAAACAGAG-3'
h#1[1]	5'-ATCCATTAAAGTTACGAGTTC-3'
h#2[1]	5'-GCATCACTAACGACGAAG-3'
l#1[1]	5'-CCGTTACCAGAAATCAAC-3'
l#2[1]	5'-CAAAGGCAACCTCGT-3'
Tnt1 625-[1]	5'-GAACGAGCAGAACCTGT-3'
Tnt1 4489+	5'-CTCTATGTACCATGCAAGG-3'
Tnt1 TR1-	5'-GCTACCAACCAAACCAAGTCAAC-3'
EFf	5'-GCACTGTCATTGATGCTCC-3'
EFr	5'-GTCAAGAGCCTCAAGGAGAG-3'

[1] To genotype the *c*, *h* and *l* Tnt1 insertions, the following combinations of primers were used:
- Tnt1 625-/c#1 and c#1/c#2
- Tnt1 625-/h#1 and h#1/h#2
- Tnt1 625-/l#1 and l#1/l#2