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Lower levels of transgene silencing in roots is associated with reduced DNA methylation levels at non-symmetrical sites but not at symmetrical sites

Ida Bagus Andika, Hideki Kondo, Muhammad Danial Rahim and Tetsuo Tamada*
*Research Institute for Bioresources, Okayama University, Kurashiki Okayama 710-0046, Japan (*author for correspondence; e-mail ttamada@rib.okayama-u.ac.jp)*

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Abstract

Transgene transcripts were recently shown to accumulate at higher levels in roots, relative to leaves, of silenced-transgenic *Nicotiana benthamiana* plants and to be inversely related with the accumulation of small interfering RNAs (siRNAs), suggesting that RNA silencing is less active in roots than in leaves (Andika *et al*., 2005. Mol. Plant–Microbe Interact. 18: 194). Here we show that the lower transgene RNA silencing activity in roots was associated with lower transgene methylation levels at non-symmetrical CpNpN context but not at symmetrical CpG or CpNpG context in three sets of transformant plants with different exogenous genes. In contrast, such a difference between roots and leaves was not observed for the Tnt1 retrotransposon: no Tnt1 transcript was detected in roots or in leaves of *N. benthamiana*, while equal levels of Tnt1-derived siRNA accumulation and Tnt1 methylation were found. From our data and previously reported information, we suggest that roots have less of an activity that acts at the step of generation of siRNAs.

Abbreviations: BNYVV, *Beet necrotic yellow vein virus*; C, cytosine; DIG, digoxigenin; dsRNA, double-stranded RNA; GFP, green fluorescent protein; LTR, long terminal repeat; Nos, nopaline synthase; ORF, open reading frame; PTGS, post-transcriptional gene silencing; RdDM, RNA-directed DNA methylation; RdRP, RNA-dependent RNA polymerase; RNAi, RNA interfering; RTD, readthrough domain; siRNA, small interfering RNA; VIGS, virus-induced gene silencing

Introduction

RNA silencing [known as quelling in fungi, post-transcriptional gene silencing (PTGS) and virus-induced gene silencing (VIGS) in plants or RNA interfering (RNAi) in animals] is a sequence-specific RNA degradation pathway highly conserved in a broad range of eukaryotes (Vance and Vaucheret, 2001; Hannon, 2002). RNA silencing is believed to operate against molecular parasites that include RNA/DNA viruses (Covey *et al*., 1997; Ratcliff *et al*., 1997), transgenes (Napoli *et al*., 1990; Lindbo *et al*., 1993) and mobile DNA elements (transposons) (Ketting *et al*., 1999; Wu-Scharf *et al*., 2000), or in developmental regulation of gene expression (Grishok *et al*., 2001; Ketting *et al*., 2001). It is triggered by double-stranded RNA (dsRNA) (Waterhouse *et al*., 1998; Bass, 2000) which is then cut by a Dicer (RNaseIII-like protein) into small dsRNA of 21–25 nucleotides (nt) termed small interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999; Bernstein *et al*., 2001; Tang *et al*., 2003; Xie *et al*., 2004). The siRNA is then incorporated into a multi-subunit endonuclease, the RNA-induced silencing complex, where it serves as a

guide to target complementary RNA molecules for degradation (Hammond *et al*., 2000; Nykänen *et al*., 2001).

Molecular analyses in plants show a close association between transgene RNA silencing or transposon silencing and methylation of the transcribed region of the silenced sequence, suggesting that DNA methylation may have a role in triggering and/or maintenance of the pathways (Bender, 2001). For example, introduction of methylation-deficient mutations into *Arabidopsis* results in a partial loss of transgene RNA silencing and reactivation of transposons (Morel *et al*., 2000; Miura *et al*., 2001). Likewise, mutations in genes required for RNA silencing in plants result in a reduction of transgene and transposon methylation (Dalmay *et al*., 2000; Fagard *et al*., 2000; Mourrain *et al*., 2000; Xie *et al*., 2004).

The RNA silencing event observed in single cells may be universal among various eukaryotes; nevertheless, different kingdoms have unique RNA silencing features based on their organ systems. Plants consist of leaf, stem and root organs which differ in their metabolism and development. Silencing signals can spread between cells through plasmodesmata, through a plant via phloem and even between plants by grafting of unsilenced scions onto the silenced rootstocks (Palauqui *et al*., 1997; Voinnet *et al*., 1997). Two distinct short (21–22 nt) and long classes (24–26 nt) of siRNAs accumulate in plant, which is in contrast to the accumulation of only short class of siRNAs in animal (Hamilton *et al*., 2002; Tang *et al*., 2003). The short class is thought to be involved in target RNA degradation, while the long class is likely to be involved in systemic silencing and DNA methylation (Hamilton *et al*., 2002).

Recent comparative analysis of two organs, roots and leaves, revealed an interesting phenomenon; levels of RNA silencing activity in roots were lower than in leaves (Andika *et al*., 2005). Transgenic *Nicotiana benthamiana* plants silenced for the 54-kDa readthrough domain (RTD)-open reading frame (ORF) of *Beet necrotic yellow vein virus* (BNYVV), a single-stranded positive-sense RNA virus, exhibited higher accumulation of the transgene transcripts in roots than in leaves, indicating a lower degree of RNA degradation in roots. Moreover, siRNAs accumulated at very low levels in roots, in contrast to the abundant accumulation of siRNAs in leaves of the same plants. Leaves of the silenced plants were immune to foliar rub-inoculation with BNYVV, whereas their roots could be infected by viruliferous zoospores of the fungal vector, although virus multiplication was limited (Andika *et al*., 2005).

Here, using three sets of transformants with different exogenous genes, we present evidence that the lower transgene RNA silencing activity in roots is a general phenomenon in plants and is correlated with reduced levels of DNA methylation at non-symmetrical cytosine (C) residues (CpNpN) but not at symmetrical C residues (CpG and CpNpG). By contrast, silencing of Tnt1, a retroviral-like transposon, operates similarly in roots and leaves as demonstrated by equal levels of transposon-derived long siRNAs and equal methylation levels of non-symmetrical C residues.

Materials and methods

Plant materials

Transgenic *N. benthamiana* lines G5 and G10, carrying the 35S promoter (35S-pro):synthetic green fluorescent protein (sGFP) transgenes and transgenic *N. benthamiana* lines RT8-2 and RT24-2, carrying the 35S-pro:54-kDa RTD-ORF transgene were previously described (Andika *et al*., 2005). Transgenic *N. benthamiana* line 16c carrying a single 35S-pro:GFP transgene was previously described (Ruiz *et al*., 1998). The transgenic and non-transgenic *N. benthamiana* plants were grown as described previously (Andika *et al*., 2005).

Agroinfiltration-mediated induction of transgene RNA silencing

Leaves of 4-week-old 16c seedlings were infiltrated with *Agrobacterium* strain C58 carrying a binary Ti plasmid into which the 35S-pro:GFP cassette had been inserted (Voinnet and Baulcombe, 1997). Infiltration was performed as described by English *et al*. (1997).

Plant tissue culture

Leaves of 16c plants showing silencing of GFP transgene 2 weeks post-infiltration were harvested and sterilized. Leaf disks were plated onto Murashige–Skoog medium complemented with

1 mg/l 6-benzylaminopurine. Culturing was carried out as described previously (Topping, 1998).

DNA and RNA gel blot analyses

Genomic DNA was isolated from root and leaf tissues collected from four plants as described previously (Andika *et al*., 2005). Total DNA (10–20 μg) was digested with appropriate restriction enzymes (75 units) overnight. DNA gel electrophoresis and DNA blot analysis were performed as described previously (Andika *et al*., 2005). The blots were hybridized with digoxigenin (DIG)-labelled DNA probes corresponding to the full length sGFP ORF, 54-kDa RTD ORF or GFP ORF prepared by using a PCR DIG probe synthesis kit (Roche, Mannheim, Germany).

Total RNA was extracted from root, stem or leaf tissue collected from four plants as described previously (Andika *et al*., 2005). Poly $(A)^+$ mRNAs were purified with an mRNA Isolation Kit (Roche), according to the protocols supplied by the manufacturer. RNA gel electrophoresis and gel blot analysis were performed as described previously (Andika *et al*., 2005). For detection of sGFP and GFP transgene transcripts, the probes used for DNA blot analysis were used. For detection of α-tubulin transcripts, a DIG-labelled DNA probe corresponded to the 861 bp cDNA of *N. benthamiana* α-tubulin was used.

Extraction of low molecular weight RNAs and gel blot analyses were performed as described previously (Andika *et al*., 2005). The blots were hybridized with the ^{32}P-labelled antisense RNA probes corresponding to the full length GFP ORF, 307 nt of Tnt1 long terminal repeat (LTR) sequence or 169 nt of the TS SINE element sequence. These RNA probes were generated by *in vitro* transcription using SP6 or T7 RNA polymerases (Promega, Madison, WI, USA). Tnt1 and TS SINE elements were PCR-amplified from *N. benthamiana* genomic DNA and cloned into pGEM-T vector (Promega). Sequence analysis showed that Tnt1 and TS SINE clones corresponded most closely to accession number AJ228064 and D17455, respectively.

RT-PCR analysis

Total RNA was extracted from root and leaf tissues by using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) and DNA contamination was removed by DNaseI treatment. 0.5 μg of total RNA was used in 20 μl reverse transcription reaction with M-MLV reverse transcriptase (Invitrogen, San Diego, CA, USA) according to the manufacturer's recommendations. Two reverse primers specific for Tnt1 LTR sequence: Tnt1-r (5′-TCACTCTTTCTTTCTCTCTT-3′) and α-tubulin gene: Tub-r (5′-GATAACTGTACTGGTCTTCA-3′) were used for the reaction. An aliquot (2 μl) of the RT reaction was used for two separate PCR amplifications (each 25 μl reaction). The same reverse primers were used as for the RT reaction, in addition to the following forward primers: Tnt1-f (5′-GGAGGGGGAGATTGATGATG-3′) for amplification of Tnt1 transcripts and Tub-f (5′-CATACCCTCACCAACATACC-3′) for amplification of α-tubulin transcripts.

Bisulphite sequence analysis

Bisulphite treatment was performed in the presence of urea essentially as described (Paulin *et al*., 1998). Before treatment, DNA samples were digested with *Hin*dIII. Desalting of DNA was performed by using GENECLEAN II Kit (Qbiogene, Carlsbab, CA, USA). The following primers were used to amplify the top strand of the 54-kDa RTD-ORF-nopaline synthase (Nos) terminator target: the forward primer RT-f (5′-GGGGTTAATAATAATATTAG-3′) and the reverse primer Nos-r (5′-AAACCCATCTCATAAATAAC-3′); Tnt1 LTR target: the forward primer Tnt1-f and the reverse primer Tnt1-r. PCR amplification was performed with a 2 min denaturation at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 45 °C and 60 s at 72 °C and a final extension at 72 °C for 7 min. PCR products were cloned into pGEM-T vector before sequence analysis.

Results

Transgene methylation in roots and leaves of transgenic plants

We previously showed that in a silenced line (RT24-2) of transgenic plants containing the BNYVV 54-kDa RTD-ORF, transgene transcripts accumulated at higher levels in roots than in leaves and this was inversely related with

the accumulation of siRNAs (Andika *et al*., 2005). To further characterize the transgene transcripts detected in roots of the silenced plants, we carried out an RNA gel blot analysis using the poly $(A)^+$ fraction of mRNAs extracted from the transgenic plants. The results showed that in the RT24-2 plants, the transgene mRNA accumulated at low levels in the roots, but did not accumulate in the leaves (Figure 1A, lanes 3 and 4). In contrast, the transgene mRNA accumulated to high levels in roots and leaves of the 54-kDa RTD-ORF-expressing plants (RT8-2) (Figure 1A, lanes 1 and 2). These results indicate that the low amount of transgene transcripts detected in roots of the silenced plants was the normal mRNA containing poly $(A)^+$ tails.

To examine whether the lower RNA silencing activity observed in roots is associated with the levels of transgene methylation, total genomic DNA was extracted from roots and leaves of RT24-2 and RT8-2 plants and were subjected to methylation analysis using the methylation-sensitive restriction enzymes, followed by DNA gel blot analyses. DNA samples were digested with the restriction enzymes *Sau*3AI, *Sau*96I and *Hpa*II. *Sau*3AI and *Sau*96I, which have recognition sequences GATC and GGNCC, respectively, are sensitive to methylation of non-symmetrical C residues or symmetrical C residues when the nucleotide 3′ to recognition sequence is a G residue. *Hpa*II, which has a recognition sequence CCGG, is sensitive to cytosine methylation at symmetrical sites. Figure 1B shows the organization of the 54-kDa RTD-ORF transgene, the location of restriction sites, the sequence context of C residues in the restriction sites and the sizes of digestion products of a non-methylated transgene.

The DNA samples from roots and leaves of the RT8-2 plants were almost completely digested with the *Sau*3AI, *Sau*96I and *Hpa*II (Figure 1C, lanes 1, 2, 5, 6, 9 and 10), indicating that the transgene in non-silenced plants was not methylated at any of these sequence contexts. In contrast, the digestion of DNA samples from leaves and roots of the RT24-2 plants gave additional larger hybridizing fragments (Figure 1C, lanes 3, 4, 7, 8, 11 and 12), indicating that the transgene in the silenced plants was methylated at these sites. However, different fragment patterns between roots and leaves were observed in digestion with *Sau*3AI and *Sau*96I,

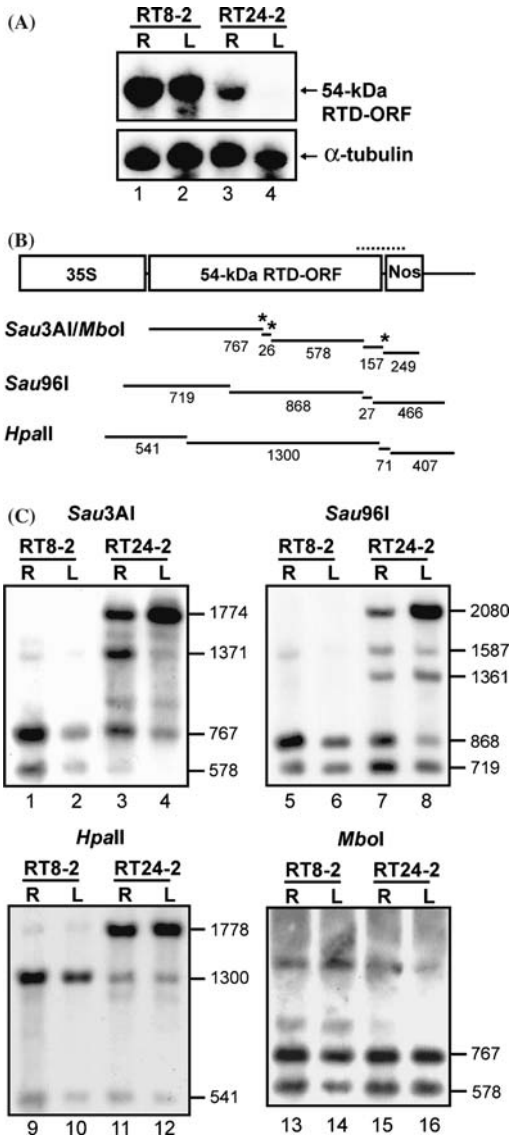

Figure 1. Transgene mRNA accumulation and transgene methylation in BNYVV 54-kDa RTD-ORF-transgenic *N. benthamiana* plants. (A) RNA gel blot analysis of transgene mRNAs in roots (R) and leaves (L) of 54-kDa RTD-ORF-expressing (RT8-2) and 54-kDa RTD-ORF-silenced plants (RT24-2). As a loading control, the expression level of the α-tubulin gene is shown. (B) Structure of the 54-kDa RTD-ORF transgene, including the 35S promoter (35S), the 54-kDa RTD-ORF, the Nos terminator (Nos), and the size of expected digestion products in base pairs. Restriction sites marked by asterisks contain cytosine residues in a symmetrical CpG context. A dashed line indicates the region examined by bisulphite sequence analysis (372 bases). (C) DNA gel blot analysis of restriction enzyme-digested DNA samples from roots and leaves of RT8-2 and RT24-2 plants. DNA samples were digested with *Sau*3AI, *Sau*96I, *Hpa*II or *Mbo*I as indicated. Sizes (base pairs) of relevant DNA fragments are indicated.

for which the recognition sites contain C residues at non-symmetrical sites in the transgene sequence (Figure 1B). Thus, in DNA samples from the RT24-2 plants digested with *Sau*3AI, a fragment of 1371 bp was detected from the roots, but not from the leaves (Figure 1C, lanes 3 and 4). Similarly, in digestion with *Sau*96I, a fragment of 2080 bp was found as a strong band in the sample from leaves, whereas in the DNA samples from roots, the signals for the fragments of 719 and 868 bp were strong (Figure 1C, lanes 7 and 8). These results indicate that cytosine methylation levels at non-symmetrical sites were lower in roots than in leaves. No difference of the fragment patterns was observed between DNA samples of roots and leaves digested with *Hpa*II (Figure 1C, lanes 11 and 12), indicating that cytosine methylation levels at symmetrical sites were not different in the two tissues.

To rule out the possibility that the quality or nature of the DNA extracted from roots and leaves affects the extent of the enzyme digestion, DNA samples were digested with *Mbo*I, an enzyme that is not sensitive to cytosine methylation. The same extent of digestion was observed between DNA samples from roots and leaves of the RT8-2 and RT24-2 plants (Figure 1C, lanes 13–16). This indicates that the different fragment patterns observed in digestion with the methylation-sensitive enzymes were not due to the quality or nature of DNA samples.

To confirm that the phenomenon observed in silenced (RT24-2) plants occurs for other silenced transgenic plants, we examined transgenic plants carrying the sGFP gene (Andika *et al*., 2005). Our previous RNA blot analyses showed that low levels of transgene transcripts were detected in roots but not in leaves of sGFP-silenced plants (G10) (Andika *et al*., 2005). In this experiment, we compared levels of transgene transcripts in roots, stems and leaves of the sGFP-expressing (G5) and sGFP-silenced (G10) plants. The results showed that, in the G10 plants, low amounts of transgene transcripts were detected in the roots, but not in the stems and leaves (Figure 2A, lanes 4–6). In contrast, the transgene transcripts accumulated to high levels in roots, stems and leaves of the G5 plants (Figure 2A, lanes 1–3). These results confirm that the extent of RNA degradation in roots was lower than that in stems and leaves.

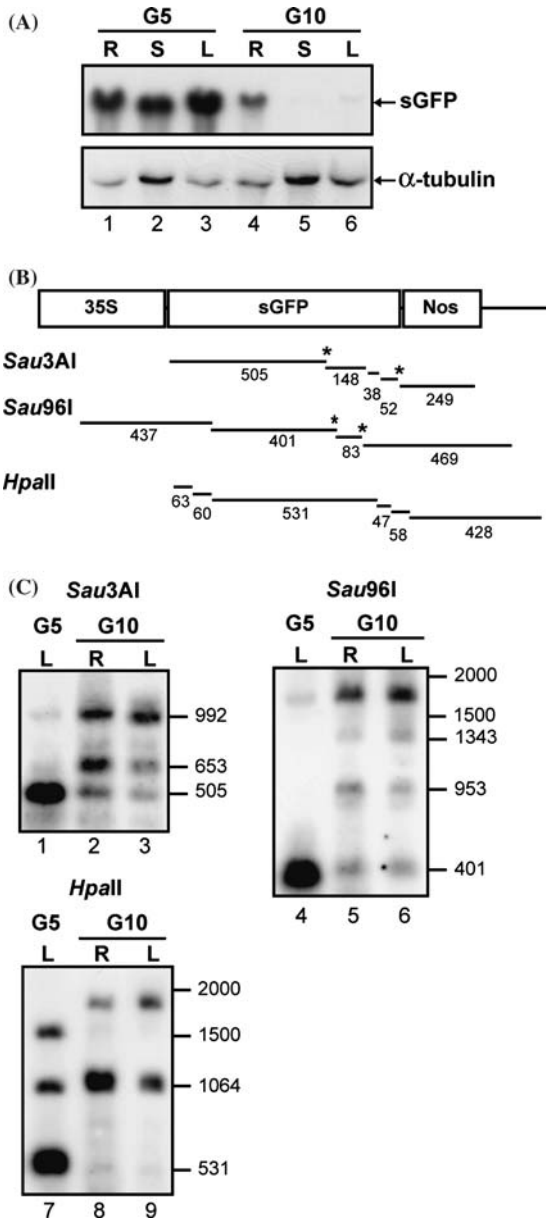

Figure 2. Transgene mRNA accumulation and transgene methylation in sGFP-transgenic *N. benthamiana* plants. (A) RNA gel blot analysis of transgene mRNAs in roots (R), stems (S) and leaves (L) of sGFP-expressing plants (G5) and sGFP-silenced plants (G10). (B) Structure of the sGFP transgene, including the 35S promoter (35S), the sGFP, the Nos terminator (Nos), and the size of expected digestion products in base pairs. Restriction sites marked by asterisks contain cytosine residues in a symmetrical CpNpG context. (C) DNA gel blot analysis of restriction enzyme-digested DNA samples from leaves of G5 plants and roots and leaves of G10 plants. DNA samples were digested with *Sau*3AI, *Sau*96I or *Hpa*II as indicated. Sizes (base pairs) of relevant DNA fragments are indicated.

In the next experiment, DNA samples from roots and leaves of the G5 and G10 plants were subjected to methylation analysis as described above. Figure 2B shows the organization of the sGFP transgene, the location of restriction sites, the sequence context of C residues in the restriction sites and the sizes of digestion products of a non-methylated transgene. The digestion of the DNA samples from leaves of the G5 plants with *Sau3*AI and *Sau*96I gave hybridizing major fragments of 505 and 401 bp, respectively (Figure 2C, lanes 1 and 4), while in digestion with *Hpa*II, there were hybridizing a major fragment of 531 bp and two additional larger fragments, 1064 bp and a fragments of between 1500 and 2000 bp (Figure 2C, lane 7). These data indicate that the transgene of the G5 plants was not methylated in the CpNpN or CpNpG context but was partially methylated in a CpG context (Figure 2B). There were consistently stronger hybridization signals for additional larger fragments in DNA samples from roots and leaves of the G10 plants digested with those three enzymes, indicating that the transgene was highly methylated at all sequence contexts. However, in digestion with *Sau*3AI, a slightly different fragment pattern was observed between those tissues. A fragment of 653 bp showed a stronger signal in the DNA sample from roots than from leaves (Figure 2C, lanes 2 and 3). This fragment is produced by failure to cleave at a *Sau*3AI site containing a C residue at symmetrical site separating the two adjacent fragments of 505 and 148 bp of the non-methylated transgene (Figure 2B). This result indicates that the levels of cytosine methylation at non-symmetrical sites were lower in roots than in leaves, whereas the levels of symmetrical cytosine methylation were similar. No difference of the fragment patterns was observed between those tissues in digestion with *Sau*96I (Figure 2C, lanes 5 and 6) and no hybridizing fragment of 531 bp was detected in digestion with *Hpa*II (Figure 2C, lanes 8 and 9), further indicating that the levels of cytosine methylation at symmetrical sites were not different. In the digestion with *Sau*96I and *Hpa*I, the hybridizing fragments between 1500 and 2000 bp were observed in DNA samples from roots and leaves of the G10 plants (Figure 2C, lanes 5, 6, 8 and 9). These fragments were likely due to the methylation of the region outside of the sGFP and Nos termination sequences.

Transgene methylation in roots and leaves of the GFP transgenic plants in which silencing was induced by agroinfiltration

When a 35S-pro:GFP transgene is agro-infiltrated into the leaves of GFP transgenic 16c plants, local silencing of GFP expression is induced in the infiltrated area by 4–6 days post-infiltration, followed by systemic silencing, in which the GFP expression is suppressed in newly developing leaves (Voinnet and Baulcombe, 1997). In this experiment, we examined RNA silencing activity and DNA methylation levels in GFP transgenic plants in which silencing was ectopically induced. The total RNA was extracted from roots and upper leaves of 30 day post-infiltrated plants and subjected to an RNA gel blot analysis. As shown in Figure 3A, the levels of GFP mRNA accumulation were greatly reduced in roots and leaves of the infiltrated plants, compared with the accumulation levels in the non-infiltrated GFP-expressing plants. However, transgene transcripts accumulated to higher levels in roots than in leaves of the infiltrated plants (Figure 3A, lanes 3 and 4), similar to previous observations for the silenced transgenic plants.

In general, silencing is thought to be induced by the spread of signal from the infiltrated leaves, and therefore it is possible that the spread of the silencing signal to roots may affect the silencing efficiency. To examine this possibility, we cultured leaf explants from GFP-silenced plant by agroinfiltration. The shoots derived from leaf explants were grown into mature plants with well-developed roots and subjected to analysis as described above. The results showed that in the plants generated from the leaves of the silenced plant, higher levels of transgene transcripts accumulated in roots than in leaves and siRNAs accumulated to very low level in roots, whereas they accumulated to high levels in leaves (Figure 3B, lanes 3 and 4). These results suggest that the higher levels of transgene transcript accumulation in roots of the silenced plants is not due to ineffective spread of silencing signal to roots.

Methylation analysis was carried out on DNA samples from roots and leaves of the GFP-expressing and silenced plants by agroinfiltration

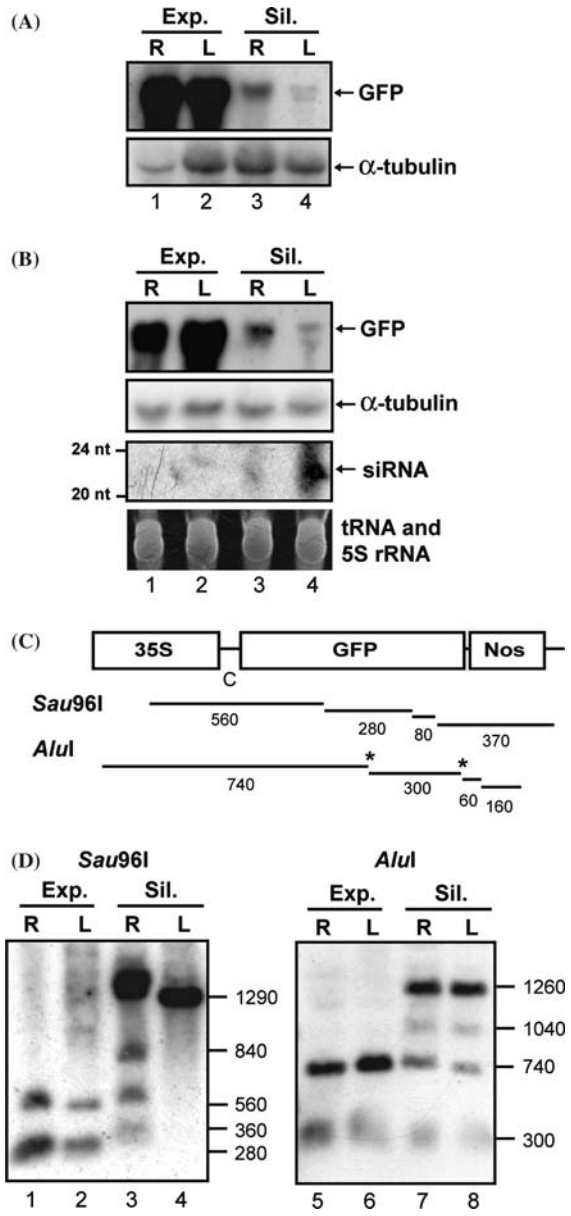

Figure 3. Transgene mRNA accumulation, siRNA accumulation and transgene methylation in roots (R) and in leaves (L) of *N. benthamiana* GFP-transgenic 16c line. (A) RNA gel blot analysis of transgene mRNAs in roots and leaves of expressing (Exp.) and silenced plants (Sil.). (B) RNA gel blot analysis of transgene mRNAs and siRNAs in roots and leaves of plants cultured from leaves of expressing or silenced plants. The positions for 20- and 24-nucleotide DNA oligomers are indicated. Ethidium bromide-stained tRNA and 5S rRNA are shown (bottom panel). (C) Structure of the GFP transgene, including the 35S promoter (35S), the chitinase endoplasmic reticulum targeting signal sequence (C), the GFP, the Nos terminator (Nos), and the size of expected digestion products in base pairs. Restriction sites marked by asterisks contain cytosines residues in a symmetrical CpNpG context. (D) DNA gel blot analysis of restriction enzyme-digested DNA samples from roots and leaves of expressing and silenced plants. DNA samples were digested with *Sau*96I or *Alu*I as indicated. Sizes (base pairs) of relevant DNA fragments are indicated.

using the restriction enzymes *Sau*96I and *Alu*I. Within the transcribed region of the 35S-pro:GFP transgene, all of the *Sau*96I restriction sites contain cytosine residues at non-symmetrical sites, while two out of four *Alu*I restriction sites contain cytosine residues at symmetrical sites (Figure 3C). In digestion with *Sau*96I, the smaller fragments of 840, 560 and 360 bp were detected in addition to a major fragment of 1290 bp in the DNA samples from roots of the silenced plants, whereas these fragments were not present in the samples from the leaves (Figure 3D, lanes 3 and 4), indicating that cytosine methylation levels at non-symmetrical sites were lower in roots than in leaves. No difference was observed between roots and leaves in digestion with *Alu*I (Figure 3D, lanes 7 and 8), indicating that cytosine methylation levels at symmetrical sites were similar in roots and leaves. The methylation analysis of DNA samples from roots and leaves of plants regenerated from leaves of the silenced plants also showed similar results (data not shown). Thus, this and previous analyses indicate that tissue-specific differences in silencing occurs generally in transgenic plants, irrespective to the types of induction of transgene RNA silencing.

Bisulphite sequence analysis of methylation in roots and leaves of the BNYVV 54 kDa RTD-ORF transgenic plants

Methylation analyses using methylation-sensitive enzyme digestion as described above only provide data on the methylation status of a limited number of cytosine residues. Therefore we employed the bisulphite sequence method which makes use of conversion of non-methylated cytosine into uracil by sodium bisulphite to sample other cytosine residues for methylation. DNA samples from roots and leaves of 54-kDa RTD-ORF-silenced plants (RT24-2) and RTD-ORF-expressing plant (RT8-2) were treated with bisulphite, and the bisulphite-treated DNA samples were PCR-amplified with a primer set

designed to amplify the upper strand of the region of 372 bases corresponding to the 3′ junction between the 54-kDa RTD-ORF and the Nos terminator sequence (Figure 1B). The examined region contains 63 cytosine residues, consisting of 31, 14 and 18 cytosine residues at CpG, CpNpG and CpNpN sequence contexts, respectively. Total sequences of 44 independent clones were obtained, consisting of 16 clones from roots and leaves of the RT24-2 plants and 12 clones derived from leaves of the RT8-2 plants. Sequence analyses of the clones from leaves of the RT8-2 plants showed that cytosine residues were efficiently converted to uracil except for some clones which contained a few unconverted cytosine residues mainly in the symmetrical CG context. The percent cytosine methylation was around 7% of total cytosines. This result indicates that the bisulphite treatment performed in this experiment efficiently converts non-methylated cytosine residues to uracil. In contrast, sequence analyses of clones from roots and leaves of the silenced plants showed high numbers of unconverted cytosine residues (Figure 4A). However, it was apparent that the number of unconverted cytosine residues at non-symmetrical sites was higher in leaves than in roots, whereas no significant difference was observed for cytosine residues at symmetrical sites, indicating a different distribution of cytosine methylation between the roots and leaves. For example, in the three restriction recognition sites (*Sau*3AI, *Sau*96I and *Sau*96I) containing non-symmetrical C residues (Figure 4A, upper panel), the percentage of methylated strands for leaves was 88%, 88% and 63%, respectively and for roots 63%, 44% and 31%, respectively, whereas in the three recognition sites (*Hpa*II, *Sau*3AI and *Hpa*II) containing symmetrical C residues (Figure 4A, lower panel), the percentage of methylated strands for leaves was 88%, 88% and 88%, respectively and for roots 75%, 88% and 88%, respectively. The percent cytosine methylation at CpG, CpNpG and CpNpN contexts calculated from total cytosine residues contained in the clones revealed that the levels of cytosine methylation at non-symmetrical CpNpN context were significantly lower in roots than in leaves, whereas cytosine methylation at symmetrical CpG and CpNpN contexts was at the same levels (Figure 4B). Thus, the cytosine methylation analyses using bisulphite sequencing were consistent with the analyses using methylation-sensitive restriction enzyme digestion.

Silencing of Tnt1 retrotransposon in roots and leaves of N. benthamiana

There are several reports supporting the idea that a natural role of RNA silencing is to restrict transposons. Mutations in genetic factors that are required for transgene RNA silencing in Algae and in Arabidopsis also result in reactivation of transposable elements (Wu-Scharf *et al.*, 2000; Miura *et al.*, 2001). Transposon-derived long siRNAs (24–26 nt) accumulate in plants and it was suggested that the long size of siRNAs is involved in transcriptional silencing and DNA methylation of transposons (Hamilton *et al.*, 2002). To compare the extent of transposon silencing in roots and leaves, we studied the transcriptional status, the accumulation of siRNAs and the methylation levels of Tnt1 retrotransposon of tobacco plants (Grandbastien *et al.*, 1989). First, we analyzed the transcriptional status of the Tnt1 in roots and leaves of *N. benthamiana* plants by RT-PCR using a primer set specific for a LTR. No Tnt1 transcript accumulation was detected either in roots and leaves (Figure 5A, upper panel), whereas α-tubulin transcripts were detected in the same cDNA reaction (Figure 5A, lower panel). This result indicated that Tnt1 is silenced in both tissues.

Second, we analyzed the accumulation of Tnt1-derived siRNAs using a probe specific for the LTR sequence. Figure 5B shows that Tnt1-derived long siRNAs accumulated at similar levels in roots and leaves of *N. benthamiana* plants (lanes 2 and 3, upper panel), whereas in the RNA samples from leaves of *Beta vulgaris* (beet) plants there was no detection of siRNAs (lane 1, upper panel), indicating that the signal was specific for *N. benthamiana*. The membrane was reprobed with a probe specific for a TS SINE element of tobacco (Yoshioka *et al.*, 1993) which has also been shown to accumulate long siRNAs (Hamilton *et al.*, 2002). RNA gel blot showed that the TS SINE element-derived siRNAs accumulated at the same level in roots and leaves (Figure 5B, lanes 2 and 3, middle panel).

Finally, we analyzed the methylation status of Tnt1 in roots and leaves by bisulphite sequence.

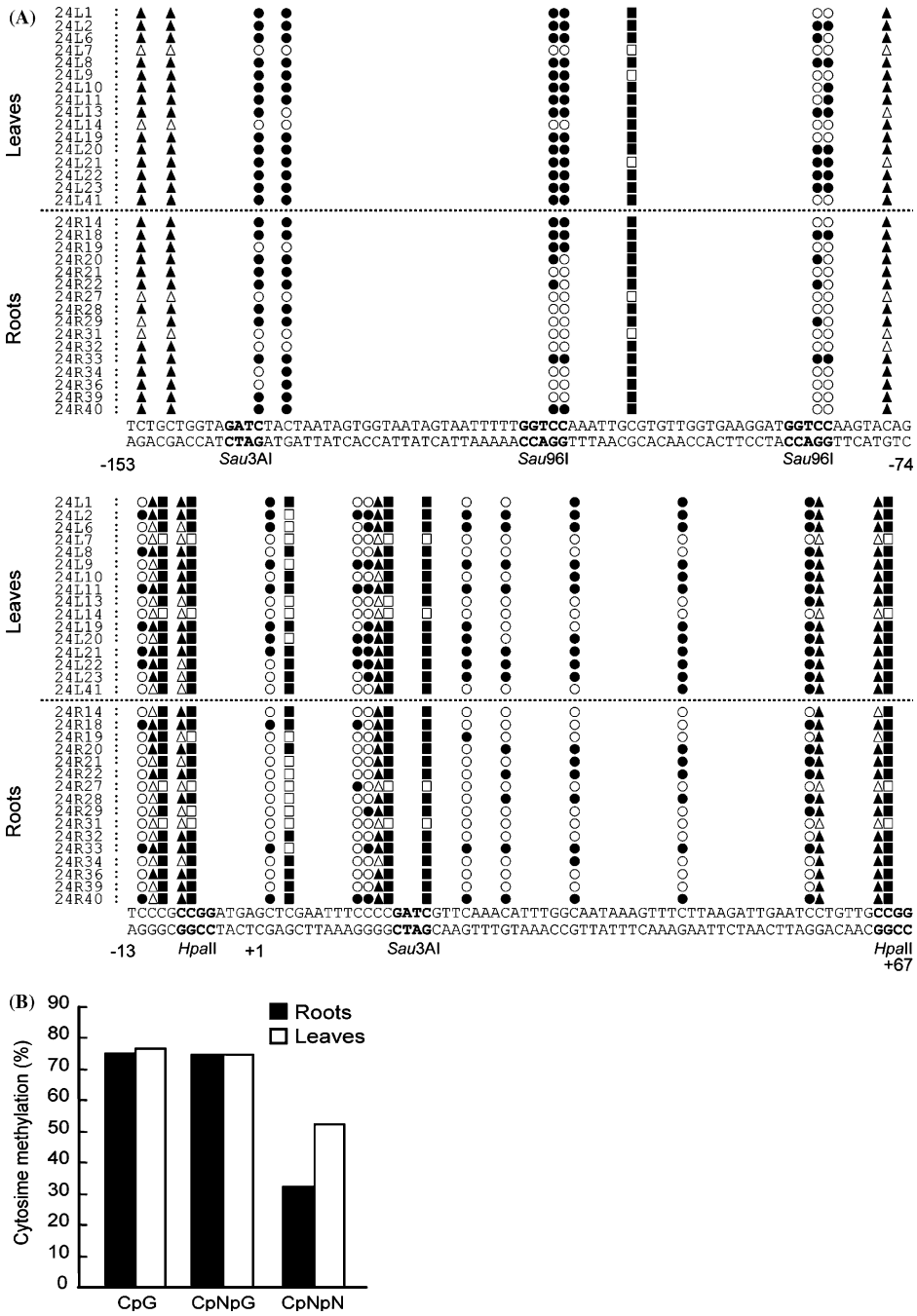

Figure 4. Bisulphite sequencing analysis of transgene methylation in roots and leaves of 54-kDa RTD-ORF-silenced plants (RT 24-2). A region of 372 bases of the upper strand of the junction between the 54-kDa RTD-ORF and Nos termination sequences ranging from −213 to +159, relative to the boundary between the 54-kDa RTD-ORF and Nos terminator sequences, was examined (Figure 1B). (A) The distribution of cytosine methylation in roots and in leaves (16 clones each) is partially presented, from −153 to −74 (upper panel) and from −13 to +67 (lower panel). The location of restriction sites of *Sau*3AI, *Sau*96I and *Hpa*II are shown (bold fonts). Squares, triangles and circles indicate cytosine residues in CpG, CpNpG and CpNpN contexts, respectively. Open and filled symbols indicate non-methylated and methylated cytosine residues, respectively. (B) Percent methylation at CpG, CpNpG and CpNpN contexts in roots and in leaves calculated from total cytosine residues present in the clones.

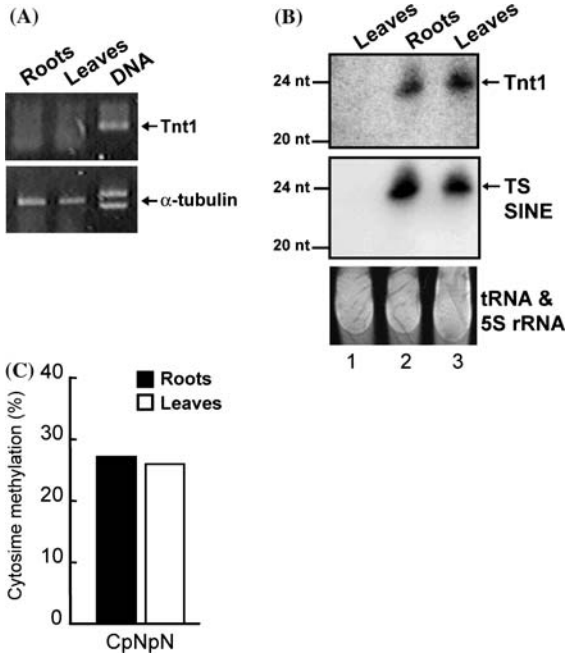

Figure 5. The silencing of Tnt1 retrotransposon in roots and leaves of *N. benthamiana*. (A) RT-PCR analysis of Tnt1 transcripts in roots and leaves. Genomic DNA extracted from leaves was included as a control in the PCR amplifications. Length of predicted PCR products: Tnt1, 307 bp; (α-tubulin, 861 bp. (B) RNA gel blot analysis of siRNAs specific for Tnt1 and TS SINE element in roots (lane 2) and leaves (lane 3). Low molecular weight RNA samples extracted from leaves of *Beta vulgaris* (beet) was included as a negative control in the blot analysis (lane 1). The membrane was reprobed with a TS SINE specific probe (middle panel). The positions for 20- and 24-nucleotide DNA oligomers are indicated. Ethidium bromide-stained tRNA and 5S rRNA are shown (bottom panel). (C) Bisulphite sequencing analysis of Tnt1 methylation in roots and leaves. Percent methylation at CpNpN context in roots and in leaves calculated from total cytosine residues present in the clones.

We selected the LTR region for analysis, because only the sequence of this region is available for *N. benthamiana* (accession no. AJ228064). Each of 12 independent clones from roots and leaves was sequenced. Unfortunately, the LTR region contains only cytosine residues at non-symmetrical CpNpN context (37 residues). Therefore we could not determine the cytosine methylation status at symmetrical sites. The bisulphite sequencing analysis revealed that the Tnt1 methylation was at similar levels in roots and leaves (Figure 5C). These results indicated that Tnt1 silencing operates to similar levels in roots and leaves of *N. benthamiana* plants.

Discussion

Our previous and present studies demonstrate that RNA silencing is less active in roots than in leaves of transgenic *N. benthamiana* plants silenced for three different exogenous genes: less degradation of target RNA and lower accumulation of siRNAs in roots (Andika *et al.*, 2005). The RT24-2 (BNYVV 54-kDa RTD ORF) and G10 (sGFP) lines show strong autonomous transgene RNA silencing, while the 16c (GFP) line (Ruiz *et al.*, 1998) shows systemic RNA silencing induced by agroinfitration. This finding is also supported by the other recent reports that an exogenous gene encoding Fab antibody fragment and an endogenous endoplasmic reticulum ω-3 fatty acid desturase (NtFAD) gene are suppressed at lower levels in roots than in leaves of *Arabidopsis thaliana* (De Wilde *et al.*, 2001) and *Nicotiana tabacum* (Tomita *et al.*, 2004). It also has been shown that, in *Agrobacterium rhizogenes*-mediated root transformation, the silencing signal did not spread systemically to non-transformed roots and only inefficiently to the non-transgenic shoots (Kumagai and Kouchi, 2003; Limpens *et al.*, 2004). In addition, we observed that roots of non-transgenic plants show lower RNA silencing activity against BNYVV, an RNA virus which does not involve DNA in its replication (Andika *et al.*, 2005). In both transgene- and virus-induced RNA silencing, the most remarkable feature is a low accumulation of siRNAs in roots. Together, these observations suggest that a lower silencing level in roots of plants is a widespread or universal phenomenon.

In this study, we have provided additional evidence that the lower transgene RNA silencing activity in roots in three sets of transformant plants was associated with lower transgene methylation levels at non-symmetrical CpNpN context but not at symmetrical CpG and CpNpG contexts. The qualitative change in methylation was shown by both DNA gel blot and bisulphite sequence analyses. In contrast to the transgene silencing, however, no differences in the silencing of the Tnt1 retrotransposon were found between two tissues in which transposon-derived long siRNAs were detected and transposon DNA was methylated at equal levels. In *Arabidopsis*, production of dsRNA from aberrant transgene transcripts requires RDR6, an RNA-dependent RNA polymerase

(RdRP) (Dalmay *et al*., 2000; Mourrain *et al*., 2000), while transposon silencing requires RDR2, a paralog of RDR6 (Xie *et al*., 2004). Transgene- and virus-derived siRNAs are mainly of the shorter class (21–22 nt), whereas transposon-derived siRNAs are of the longer class (24–26 nt), suggesting that they are produced by distinct Dicers (Hamilton *et al*., 2002; Tang *et al*., 2003; Xie *et al*., 2004). Thus, transgene RNA silencing and transposon silencing are regulated differently, although they share some genetic components (Hamilton *et al*., 2002: Herr *et al*., 2005).

RNA-directed DNA methylation (RdDM) is closely related to silencing in plants and is thought to be mediated by a siRNA-guided effector complex (Mathieu and Bender, 2004). RdDM involves two separated steps of *de novo* methylation and maintenance methylation. It is known that siRNA-guided *de novo* methylation in symmetrical and non-symmetrical sites are accomplished by DRM methyltransferase activity (Cao *et al*., 2002). Symmetrical CpG and CpNpG methylation can be maintained in some cases in the absence of the RNA trigger, whereas symmetrical CpNpN methylation requires the continuous presence of RNA (Aufsatz *et al*., 2002). Based on these observations, lower accumulation of transgene-derived siRNAs in roots suggests the possibility that roots produce less RNA signals for *de novo* methylation, so that levels of only non-symmetrical methylation would be reduced. Thus, a low steady state level of guide RNA would lead to the observed bias in methylation in roots tissues. This also would account for our observation that no difference in non-symmetrical CpNpN transposon methylation was found between roots and leaves, because continuous production of transposon-derived siRNAs would lead to an efficient transposon *de novo* methylation.

Our previous data indicate that roots of non-transgenic plants infected with BNYVV display a lower accumulation of virus-derived siRNAs (Andika *et al*., 2005). RNA viruses do not require cellular silencing pathways to produce dsRNA (Vance and Vaucheret, 2001). Taken together, we suggest that roots have less of an activity that acts in the processing dsRNA into siRNAs. The lower siRNA levels in turn lead to a lower level of non-symmetrical methylation. However, it cannot be ruled out that the conversion of transgene RNA to dsRNA by an RdRp would be much less efficient in roots than in leaves.

Roots and shoots are two morphologically different organs whose patterning and growth are regulated by different genetic pathways (Byrne *et al*., 2003). There has been little information about the expression levels and potential tissue and cell types specific pattern of various factors involved in RNA silencing. We observed that silencing was suppressed in meristematic tissues of roots of autonomously GFP-silenced plants (line G10) or systemically silenced plants (line 16c) induced by agroinfitration (Voinnet *et al*., 1998; I.B. Andika and T. Tamada, unpublished data). It is not clear whether the differences of silencing activity between roots and leaves are due to the presence of apical tissues or other specific cells in which the silencing does not occur or less active. Further work is needed to examine the differences of activity of RNA silencing-related components (e.g. RdRP or Dicer-like proteins) in roots and shoots.

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