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Specific degradation of 3' regions of GUS mRNA in posttranscriptionally silenced tobacco lines may be related to 5'-3' spreading of silencing

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ABSTRACT

Target regions for posttranscriptional silencing of transgenes often reside in the 3' region of the coding sequence, although there are exceptions. To resolve if the target region is determined by the gene undergoing silencing rather than by the structure of the transgene loci or the plant genetic background, we have performed detailed analyses of target regions in three spontaneously β -glucuronidase (GUS) silencing tobacco lines of different origin. From quantitative cosuppression experiments, we show that the main target region in all three tobacco lines is found within the 3' half of the GUS coding region but upstream of the last 200 nt. The quantities of small (21–25 nt) RNAs homologous to 5' or 3' regions of the GUS coding sequence were found to correlate approximately with the target strength of the corresponding regions. These results suggest that transgene locus structure and plant genetic background are not major determinants of silencing target regions. We also show that virus-induced gene silencing (VIGS) of GUS in *Nicotiana benthamiana* is induced equally effectively with *Potato virus X* carrying either the 5' or 3' third of the GUS coding region. This indicates that both regions can act as efficient inducers as well as targets of posttranscriptional silencing, although the 3' region is the predominant target region in the spontaneously silencing transgenic plant lines examined. Finally, we investigated spreading of the target region in the *N. benthamiana* plants undergoing VIGS. Surprisingly, only evidence for spreading of the target region in the 5'-3' direction was obtained. This finding may help explain why the majority of target regions examined to date lie within the 3' region of transgenes.

Keywords: β -glucuronidase; gene silencing; posttranscriptional; small RNAs; virus-induced gene silencing

INTRODUCTION

Posttranscriptional gene silencing (PTGS) in plants, and its counterpart in animals, RNA interference (RNAi), is a specific RNA-degrading mechanism(s) that lately has been recognized as a defense system against invading nucleic acids. Several studies have shown that plant viruses are targeted by this mechanism (Anandalakshmi et al., 1998; Brigneti et al., 1998; Li et al., 1999), and there are strong indications that PTGS/RNAi also protects the genome from transposon damage (Jensen et al., 1999; Elbashir et al., 2001b). Introduction of double-stranded RNA (dsRNA) into cells, either directly or via transcription of transgenes with internal inverted repeats, induces the PTGS/RNAi mechanism and

causes silencing of endogenous genes or transgenes with homology to the introduced dsRNA (Montgomery et al., 1998; Waterhouse et al., 1998; Levin et al., 2000; Li et al., 2000; Smith et al., 2000). Also transformation with genes without internal repeats can trigger the PTGS reaction; this has often but not always been found to coincide with the presence of multiple copies of the transformation cassette integrated as inverted repeats (Muskens et al., 2000).

PTGS in plants is associated with the production of 21–23-nt RNA fragments (small RNAs; Hamilton & Baulcombe, 1999; Hutvagner et al., 2000). Similar small interfering RNAs have been found to mediate RNAi in *Drosophila* and cultured mammalian cells (Elbashir et al., 2001a, 2001b). In *Drosophila*, the small RNAs are produced from dsRNA by the enzyme Dicer, a member of the RNase III family of dsRNA-specific endonucleases (Bernstein et al., 2001). Also in *Drosophila*, in vitro studies have shown that the small RNAs can serve as

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guides for a ribonuclease complex (RISC) that cleaves single-stranded RNA (ssRNA) complementary to the small RNAs (Hammond et al., 2000; Elbashir et al., 2001a). Recent data from *Drosophila* and *Caenorhabditis elegans* suggest that the small RNAs can also serve as primers for an RNA-dependent RNA polymerase (RdRP) synthesizing dsRNA from ssRNA complementary to the small RNAs; the resulting dsRNA is subsequently degraded into more 21–23-nt RNAs (Lipardi et al., 2001; Sijen et al., 2001a).

Several investigations have been carried out to define the PTGS target regions of posttranscriptionally silenced genes in transformed plants (English et al., 1996; Sijen et al., 1996; Marano & Baulcombe, 1998; Jacobs et al., 1999; Sonoda et al., 1999; Hutvagner et al., 2000; Han & Grierson, 2002). The most widely used method for determining PTGS target regions is to inoculate silenced plants with chimeric viruses containing parts of the silenced genes and measure virus accumulation. If the transgene fragment carried by the virus is targeted by PTGS, the chimeric virus genome will also be degraded. A study of transgenic *Nicotiana benthamiana* plants silencing the *Cowpea mosaic virus* movement protein gene showed the 3' region of the gene to be the main target for degradation (Sijen et al., 1996). Similarly in the T4 and T7 tobacco lines, which exhibit PTGS of the exogenous *Uida* gene (encoding β -glucuronidase (GUS)), the target region was found to be confined to the 3' region (English et al., 1996). Examination of *N. benthamiana* plants transformed with the coat protein gene of *Sweet potato feathery mottle virus* showed that the virus resistant lines could be divided into two groups with either the 3' region or the entire transgene mRNA as PTGS target regions (Sonoda et al., 1999). In the case of plants transformed with the basic β -1,3-glucanase gene, the target region was dispersed throughout the entire coding region (Jacobs et al., 1999) and in plants transformed with the 54K gene of *Tobacco mosaic virus*, the target region was situated in the central region of the gene (Marano & Baulcombe, 1998). Using a different approach, the target region in a GUS-silencing tobacco line was mapped to the 3' two-thirds of the gene by the presence of small RNAs homologous to this region (Hutvagner et al., 2000). Similarly, in a tomato line showing PTGS of the endogenous polygalacturonase gene due to the presence of a 3'-truncated homologous transgene, the small RNAs were found to be derived predominantly from the 3' end of the transgene (Han & Grierson, 2002).

To test whether the silencing target is determined by the transgene sequence or linked to transformational events, vectors, or plant genotypes, we have characterized and compared the target regions in three different transgenic tobacco lines showing spontaneous silencing of the *Uida* gene. The T4 line (Hobbs et al., 1990) has previously been characterized with respect

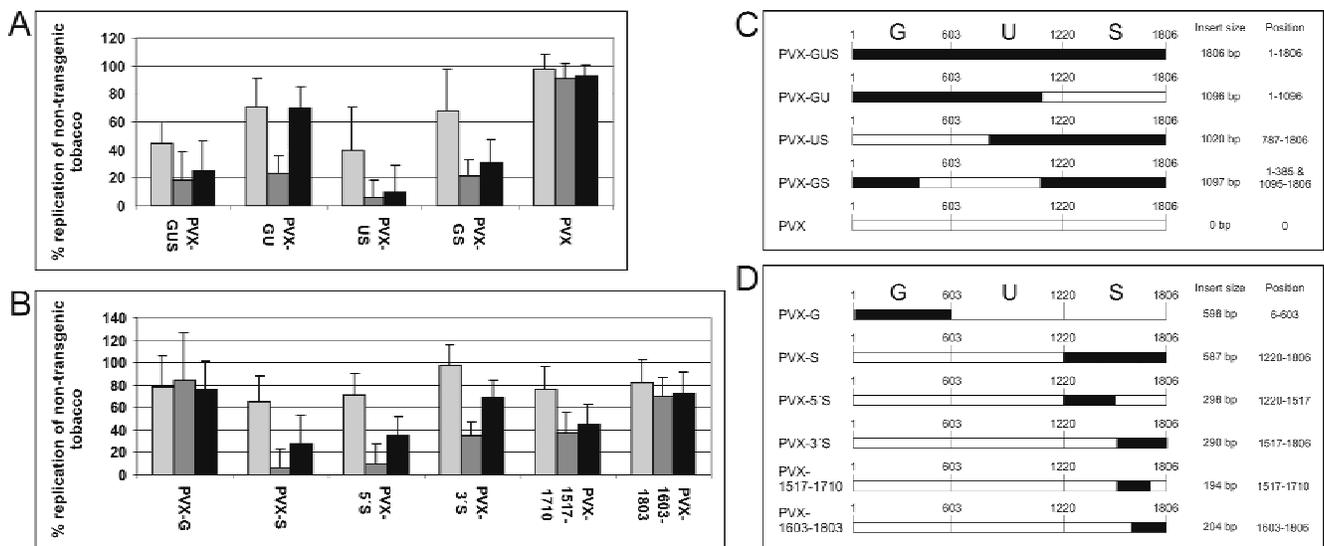
to silencing target region (English et al., 1996; see above). This line is homozygous for a single transgene locus containing an inverted repeat of the T-DNA, with the 35S-*Uida* cassette situated at and being transcribed towards the center of the repeat (Hobbs et al., 1990). The 6b5Ho line (Elmayan & Vaucheret, 1996) is also homozygous for one locus, which probably contains more than one copy of the *Uida* gene. The 8226 line is homozygous for three loci, each containing at least one copy of the *Uida* gene (Bucherna et al., 1999). In all three lines, transcription of the *Uida* gene is controlled by the 35S-promoter and two of them (T4 and 8226) share the nopaline synthase terminator (nos-ter). However, these plant lines differ with respect to the binary vectors used, transgene integration pattern, and genetic backgrounds.

Delineation of the target regions was mainly performed by measuring replication of derivatives of *Potato virus X* (PVX) carrying selected parts of the *Uida* coding region. For all three tobacco lines, the main silencing target region was found to be within the 3' half of the gene whereas the 5' third and the last 200 nt of the coding region were very weakly targeted. Virus-induced gene silencing (VIGS) of *Uida* expression in *N. benthamiana* was equally efficient with PVX carrying the 5' or 3' part of *Uida* (designated PVX-G and PVX-S, respectively). Small RNAs from plants in which VIGS was induced by PVX-G exhibited homology to both the 5' and 3' regions of *Uida* whereas plants induced with PVX-S only contained small RNAs with homology to the 3' region, suggesting that target regions of a silenced (trans)gene can only spread or spread more readily in the 5'-3' direction. This may help to explain why the 3' region is the predominant silencing target in the spontaneously silencing GUS plants.

RESULTS

Characterization of PTGS target regions

To compare the silencing targets in the three tobacco lines T4, 6b5Ho, and 8226, 5-week-old plants of each line as well as wild-type tobacco were biolistically inoculated with PVX or derivatives carrying different parts of the *Uida* gene (Fig. 1). One week after inoculation, the bombarded leaf areas were collected and PVX accumulation was measured by ELISA. The first set of experiments utilized the same PVX derivatives used by English et al. (1996). These carry either the complete *Uida* gene or *Uida* derivatives with deletions in the 5', central, or 3' part (PVX-US, PVX-GS, and PVX-GU; Fig. 1C). In all three lines, the PVX chimera that carries both the central and 3' *Uida* region was the most suppressed, indicating that the main target region(s) in all lines lies within the 3' half of the coding sequence (Fig. 1A).



To define the target regions more precisely, shorter fragments of *Uida* were inserted into PVX (see Fig. 1D). As seen from Figure 1B, the 5' third was confirmed to be a weak target in all three lines, as PVX-G replicated to around 80% of replication in nontransgenic tobacco. PVX-S was a strong target in 6b5Ho and 8226 but only moderate in T4. The same overall picture was seen for PVX-5'S indicating that the main target region of the S part lies within the 5' half. This is supported by the PVX-3'S construct, which was much less cosuppressed. The most 3' region of *Uida* was analyzed using two regions of approximately 200 nt. The last 200 nt of the gene (PVX-1603–1803) was a very weak target in all three tobaccos whereas a region approximately 100 nt upstream (PVX-1514–1710) was moderately suppressed in 6b5Ho and 8226. This latter result shows that the lack of suppression of PVX-1603–1803 was not due to the small size of the insert.

The overall levels of silencing as determined from replication levels of all PVX-GUS chimeras were significantly different between the three transgenic lines, with T4 showing the weakest and 6b5Ho the strongest silencing. When considering the PVX-GUS chimeras individually, statistically significant differences between the transgenic lines were observed with some PVX chimeras and not with others, suggesting that the localization of strong and weak target regions differed somewhat between the lines. In particular, PVX-GU was significantly more cosuppressed in 6b5Ho than in the other two lines, whereas PVX-GS and PVX-US were significantly more cosuppressed in both 6b5Ho and 8226 than in T4, indicating that the relative contributions of 5', central, and 3' regions differed between lines.

The size of the inserted transgene segment in chimeric viruses has previously been shown to negatively

correlate with viral accumulation in silenced plants (Sijen et al., 1996; Jacobs et al., 1999). We also found a significant correlation between the mean accumulation of PVX chimeras in transgenic lines and the size of the insert ($R = -0.306$, $P < 0.0001$). Linear regression of viral accumulation against the size of the inserts in the PVX constructs was performed and the residues of this regression were analyzed by ANOVA. Significant differences between the PVX constructs were also detected in this analysis showing that the insert size alone does not explain all the observed differences in accumulation of the PVX constructs. The accumulation of the constructs PVX-US, PVX-S, PVX-5'S, and PVX-3'S was more reduced than expected by the insert size whereas the accumulation of the constructs PVX-GU, PVX-G, and PVX-1603–1803 was larger than expected by the insert size (data not shown). The complete statistical analysis can be found at <http://www.dias.kvl.dk/statistics.html>.

Effect of plant age

In a previous study, target regions in the T4 tobacco line were examined in 5- to 7-week-old plants (English et al., 1996). In the study describing the 6b5Ho plants, GUS activity was carefully monitored and found to be suppressed to a level <1% of initial activity at day 15 after germination (Elmayan & Vaucheret, 1996). The third genotype used here, 8226, has been found to silence GUS after less than 5 weeks (Bucherna et al., 1999). To determine the effect of plant age on the analysis, cosuppression of three PVX chimeras was compared in 5- and 8-week-old plants (eight plants for each chimera). Cosuppression of PVX-S was similar in 5- and 8-week-old plants for all three genotypes. Repli-

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cation of PVX-GUS was further suppressed in 8-week-old T4 plants compared to those 5 weeks old (replication being 25% and 40% of nontransgenic plants, respectively), whereas replication of PVX-GUS in 6b5Ho and 8226 was almost unchanged. Only PVX-G showed a significant reduction in replication from approximately 80% to approximately 50% of replication in nontransgenic plants for all three genotypes in 8-week-old plants (data not shown).

Effect of target position in PVX

It has been proposed that the position of the target sequence in the PVX genome may influence cosuppression of virus replication (English et al., 1997). To address this question, we constructed two PVX derivatives carrying either a weak target region (the G-region, nt 6–603) followed by a strong target region (S, nt 1220–1806) (PVX-GS'), or the same sequences in the reverse order (PVX-SG; note that PVX-GS' is different from PVX-GS used in the previous experiments). As seen in Figure 2, cosuppression of PVX-GS' and PVX-SG was found to be similar for all three tobacco genotypes, showing that the positional effect, if any, is negligible. This was confirmed by a statistical ANOVA analysis (not shown; see <http://www.dias.kvl.dk/statistics.html>).

Small RNAs derive from regions eliciting cosuppression

In the experiments described so far we used PVX-GUS chimeras to delineate PTGS target regions. To validate this method, we repeated part of the analysis using the level of small RNAs as a measure of target strength. Two plants of each transgenic line and two nontransgenic tobacco plants were analyzed by Northern blot-

ting using probes complementary to either the G- or S-region of the GUS mRNA coding region (Fig. 3). The 8226 plants showed no small-RNA signal from the G-region whereas a considerable amount of small RNAs from the S-region could be detected. The 6b5Ho plants showed detectable amounts of G-region small RNAs and a very strong signal from the S-region. The T4 plants exhibited no G-region signal and a low signal from the S-region. No signal was obtained from wild-type plants of cultivars Wisconsin 38 (W38) or Xanthi. PhosphorImager quantification of binding of the two probes to 30 pg full-length GUS DNA applied to each gel (Fig. 3, FL GUS) revealed that the G-probe was approximately 6 times stronger than the S-probe. From this we calculate that the S/G ratio for the two 8226 plants is approximately 70 and 30, respectively. For the 6b5Ho plants, this ratio is 8.2 and 8.8, and for the T4 plants, 17 and 58. The amounts of small RNAs from the S-region in T4 and from the G-region in 6b5Ho plants are similar.

Both weak and strong target regions induce silencing efficiently by VIGS

In all three silenced tobacco lines, the G-region was found to be very weakly targeted, which raised the question of whether this sequence was intrinsically a poor inducer and/or target for PTGS. We used a GUS-transformed line of *N. benthamiana* homozygous for *Uida* (O. Voinnet, pers. comm.) to address this question. To compare the efficiency of VIGS induced by different parts of the GUS coding region we bombarded the plants with the following constructs: PVX, PVX-G, PVX-S, or PVX-GUS. As can be seen from Figure 4A, GUS activity in plants infected with PVX-G or PVX-S was silenced to approximately 30% of plants

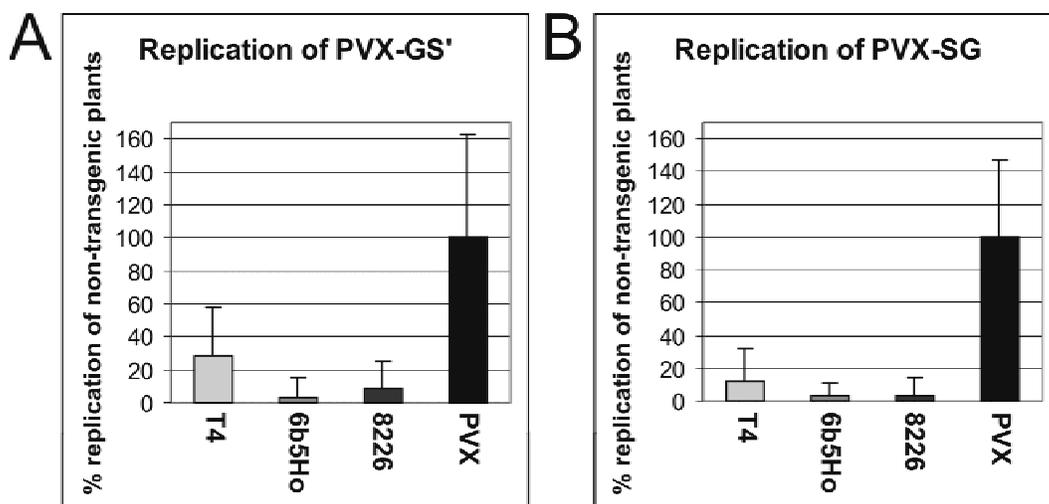


FIGURE 2. Effect of the position of a strong target region in PVX. **A, B:** Replication of PVX-GS' and PVX-SG, respectively, in the three silenced tobacco genotypes is shown as percentage + SD of replication in nontransgenic tobaccos.

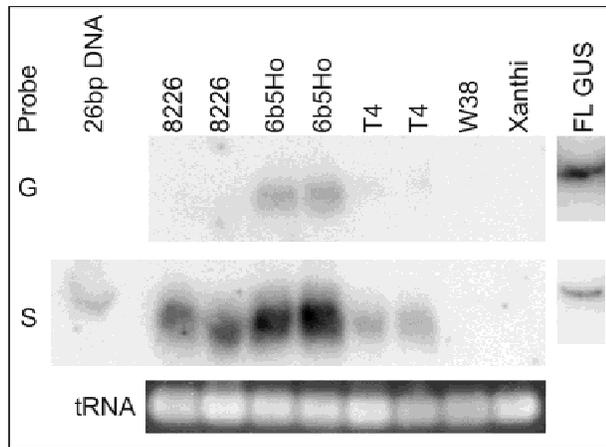


FIGURE 3. Small RNAs derive from regions eliciting cosuppression. RNA from the three silenced tobacco genotypes (8226, 6b5Ho, and T4) or from wild-type tobacco (cultivars Wisconsin 38 and Xanthi) was probed with radioactive antisense probes for either the G region (upper panel) or the S region (lower panel). A 26-mer oligonucleotide from the S region of GUS was included as a size marker. As a control for loading, the ethidium bromide-stained tRNA fraction is shown below. FL GUS: 30-pg full-length GUS DNA.

infected with wild-type PVX at 19 days postinfection (dpi), whereas plants infected with PVX-GUS were fully silenced. One week later, plants infected with PVX-G or -S were also fully silenced. This time/size dependency was confirmed in the second experiment (Fig. 4B) in which the plants were examined at an earlier stage of infection. At 11 dpi, the plants infected with PVX-G or -S showed rather weak silencing whereas the PVX-GUS infected plants had only 20% of the GUS activity of PVX infected plants. Subsequently all three groups of plants became fully silenced. These experiments demonstrate that the 5' and 3' regions of the GUS coding sequence are equally efficient inducers of silencing through VIGS.

Virus-induced gene silencing of GUS is accompanied by spreading in the 5'-3' direction

The presence of small RNAs in the transgenic *N. benthamiana* plants undergoing VIGS was investi-

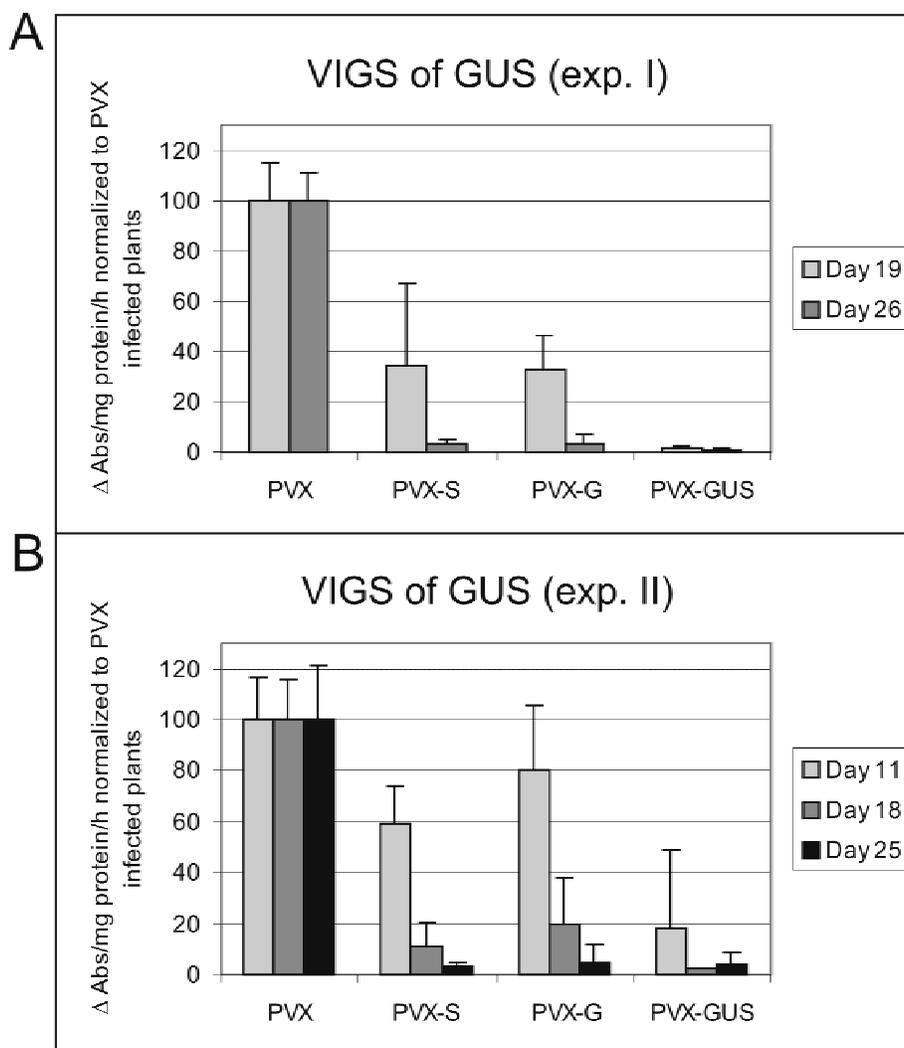


FIGURE 4. Both weak and strong natural transgene targets can induce silencing efficiently. **A, B:** GUS-expressing *N. benthamiana* plants were bombarded with PVX, PVX-S, PVX-G, or PVX-GUS. GUS activity was measured at different time points after bombardment (experiment I: days 19 and 26; experiment II: days 11, 18, and 25). GUS activity is shown as Δ Abs/mg protein/h normalized to expression in PVX-bombarded plants (percentage + SD).

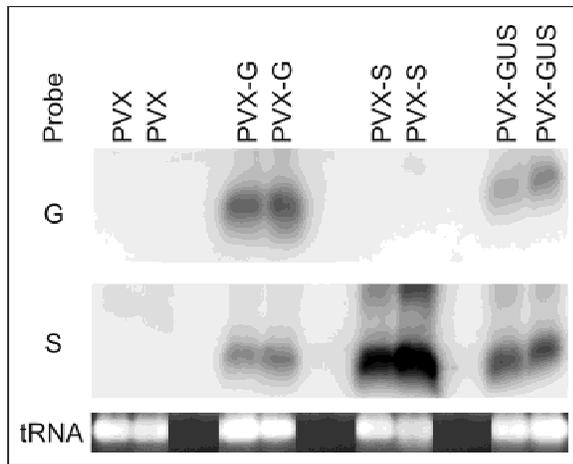


FIGURE 5. Spreading of silencing is observed only in the 5'-3' direction. RNA from GUS-expressing *N. benthamiana* bombarded with PVX, PVX-G, PVX-S, or PVX-GUS was probed with radioactive anti-sense probes for either the G- or the S-region of GUS mRNA (Fig. 5). Plants infected with PVX-G contained small RNAs from both the G- and S-regions whereas plants infected with PVX-S only contained small RNAs from the S-region. Plants infected with PVX-GUS contained small RNAs from both the G- and the S-region. No signal was obtained from plants infected with wild-type PVX. These results suggest that the silencing target region is able to spread in the 5'-3' direction but not, or less readily, in the 3'-5' direction.

gated. On 30 dpi of VIGS experiment II (Fig. 4B), RNA was extracted from two randomly chosen plants from each group and analyzed by northern blotting using probes complementary to either the G- or the S-region of GUS mRNA (Fig. 5). Plants infected with PVX-G contained small RNAs from both the G- and S-regions whereas plants infected with PVX-S only contained small RNAs from the S-region. Plants infected with PVX-GUS contained small RNAs from both the G- and the S-region. No signal was obtained from plants infected with wild-type PVX. These results suggest that the silencing target region is able to spread in the 5'-3' direction but not, or less readily, in the 3'-5' direction.

In several nonplant systems, spreading of silencing has been shown to take place only in the 3'-5' direction (see Discussion). Hypothetically, if the 35S-GUS *N. benthamiana* line used in this study contains a rearranged GUS gene with a G region positioned downstream of the S region, the observed strong G-to-S spreading of silencing could happen via a 3'-5' spreading mechanism. To exclude this possibility, the genotype of the 35S-GUS line was explored in some detail. Sequence analysis of several independently produced RT-PCR products extending from primers covering different parts of the S region of GUS and into the poly(A) tail revealed no unexpected nucleotides. Genomic DNA was digested with *HincII* (which cuts in the U region of GUS and 1,470 nt further downstream near the end of the octopine synthase terminator sequence) and hybridized with a G region probe. The resulting two bands suggested that the transgene locus contains at least two copies of the T-DNA. Thus the sequenced RT-PCR products might derive from an intact copy of the T-DNA,

whereas another copy might be rearranged. However, the southern blot of *HincII*-digested genomic DNA was reprobbed with an S-region probe producing only the expected band of approximately 1,500 nt. Together these data argue strongly against the presence of a rearranged transgene producing transcripts with G region-derived sequences downstream of the S region. We therefore conclude that the observed results are caused by 5'-3' spreading of the silencing target region.

DISCUSSION

We have examined three GUS-silencing tobacco lines for cosuppression of PVX-GUS chimeras. All PVX chimeras carrying sequences from nt 1220–1517 in the GUS coding sequence were strongly cosuppressed; cosuppression was further increased if the sequence between nt 1096 and 1220 was also included. From this, we conclude that the main silencing target in all three GUS-silencing tobacco lines lies within the 3' half of the GUS coding sequence. The last 200 nt of the coding sequence were, however, very weakly targeted. Our results are in basic agreement with a previous study of the T4 line by English et al. (1996), where the silencing target was concluded to lie within the 3' third of the coding sequence. The cosuppression results also agree well with our data from Northern blots of small RNAs present in the GUS-silencing plants. First, in all three tobacco lines we found considerably more small RNAs homologous to the 3' third than to the 5' third of the GUS sequence. Second, the 6b5Ho line showed the strongest cosuppression of PVX-GUS chimeras and also had the highest amounts of GUS-homologous small RNAs per microgram of RNA. Hamilton and Baulcombe (1999) likewise found much larger amounts of GUS-homologous small RNA in 6b5Ho plants than in T4 plants. The presence of significant amounts of small RNA homologous to the 5' GUS region in 6b5Ho plants is somewhat surprising, considering that this region appears to be very weakly targeted for degradation. One possible explanation is that detection of small RNAs is a more sensitive assay for silencing than cosuppression of PVX chimeras. Another possibility is that the small RNAs detected with the 5'-specific probe are not actually associated with cytoplasmic RNA degradation. For example, recent data suggest that small RNAs may be produced from dsRNA in the nucleus as well as in the cytoplasm (Mette et al., 2001). The 5'-specific small RNAs found in 6b5Ho plants could conceivably be a product of a nucleus-confined PTGS reaction not affecting cytoplasmically replicating viruses.

The similarity in target region localization between the three lines could be caused by similarities in transgene integration patterns. The T4 line is homozygous for a single transgene locus containing an inverted repeat of the T-DNA, with the 35S-*Uida* cassette situated

at and being transcribed towards the center of the repeat (Hobbs et al., 1990). The 6b5Ho line is also homozygous for one locus, which probably contains more than one copy of the *UidA* gene, but the exact structure of this locus is not known (Elmayan & Vaucheret, 1996). The 8226 line is homozygous for three loci each containing at least one intact copy of the *UidA* gene (Bucherna et al., 1999). From southern blot data, one locus appears to be an inverted repeat with the two 35S-*UidA* genes situated at the outer borders of the repeat and being transcribed outwards, whereas the other two loci appear to contain only one *UidA* copy (Bucherna et al., 1999; our unpubl. data). 3'-centered inverted repeats such as found in the T4 line have been suggested to induce silencing due to dsRNA formed by transcription continuing through the center of the repeat (Muskens et al., 2000). Such a mechanism might predominantly target the 3' region of the gene, if the transcription only continued for a limited stretch in the antisense direction. However the 8226 line appears to contain only a 5'-centered repeat of the *UidA* gene. Similarly a fourth GUS-silencing tobacco line, the T7 line, which contains the same construct as T4 but integrated as a 5'-centered inverted repeat (Hobbs et al., 1990), has also been shown to target the 3' region of GUS (English et al., 1996). Together these observations suggest that although inverted repeat integrations may be involved in triggering the PTGS reaction, the direction of the repeat is not the main determinant of PTGS target regions.

Transgene antisense RNA may also be generated by transcription from plant promoters situated downstream of the integration site, or from promoter sequence-containing fragments of the transformation construct integrated downstream of the transgene. Integration of short fragments of the transformation construct next to intact copies may occur quite frequently (Krizkova & Hrouda, 1998) and would be overlooked in most southern blot analyses. Thus the possibility remains that generation of truncated antisense transcripts from the 3' end of the transgene determine the preferential degradation of 3' mRNA regions.

Although the overall distribution of strong and weak PTGS target regions was found to be common to all three tobacco lines, minor but statistically significant differences in relative target strength of different regions were observed between the lines. This may mean that differences in transgene integration patterns and/or in organization of the transformation cassette may subtly influence the development of PTGS.

The distribution of strong and weak PTGS target regions could also be determined by the transgene sequence. For example, small RNAs derived from GC-rich regions could form more stable hybrids with complementary sequences, or the enzymes in the PTGS pathway may have sequence preferences. All the different GUS sequences used in this study (see Fig. 1C

and D) have similar total GC contents around 50%, but (other) local sequence characteristics may be of importance. Some support for the influence of the transgene sequence on PTGS targeting comes from our observation that the extreme 3' end of the coding region is very weakly targeted. Similarly, Hutvagner et al. (2000) found no small RNAs homologous to the 3' untranslated region of GUS mRNA in the GUS-silencing tobacco line NLG4-He. That the localization of the main target region(s) in the 3' half of the gene is not a function of transgene sequence is, however, suggested by similar findings in several other systems (see Introduction). Furthermore, we found 5' and 3' regions of the GUS coding sequence to be equally effective inducers of GUS silencing using VIGS, demonstrating that both sequences can be both efficient inducers and targets of PTGS.

The unexpected observation that virus-induced silencing appeared to spread more readily to transgene sequences downstream of the inducer region than to sequences upstream may point to an entirely different explanation for the predominance of 3' regions as PTGS targets. Mechanistically spreading of the target region in the 3'-5' direction is more easily explained, as a host RdRP (Dalmay et al., 2000; Mourrain et al., 2000) could use antisense RNA from the double-stranded inducer region as primer for synthesis of cRNA on a transgene mRNA template (Sijen et al., 2001b). Indeed, experiments in *Drosophila* embryo extracts have shown that small RNAs can serve as primers to transform target mRNA into dsRNA, which is subsequently degraded into new small RNAs (Lipardi et al., 2001). Similarly, small RNAs corresponding to target mRNA sequences upstream of the dsRNA inducer were produced in *C. elegans* in a reaction requiring a cellular RdRP (Sijen et al., 2001a). Because RNA synthesis only occurs by 3' addition of nucleotides, the use of small RNAs as primers can only generate new dsRNA from the mRNA region upstream of the initial inducer sequence. Indeed, only spreading of silencing in the 3'-5' direction was observed in the *Drosophila* and *C. elegans* experiments (Lipardi et al., 2001; Sijen et al., 2001a).

However, several observations in plants have suggested that PTGS can spread to transgene regions downstream as well as upstream of the primary target. Jones et al. (1999) used PVX carrying 5' or 3' regions of the green fluorescent protein (GFP) coding region to initiate silencing in GFP-expressing *N. benthamiana* plants. The virus-induced DNA methylation of the entire coding region of the GFP transgene occurred irrespectively of whether 5' or 3' GFP sequences were carried by the virus. However, a causal connection between DNA methylation and RNA degradation in PTGS has not been demonstrated. Hamilton et al. (1998) transformed tobacco with extra copies of the tobacco ACC-oxidase gene (*ACO1*) carrying an inverted repeat of the 5' UTR. Transformants showed posttranscriptional

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silencing of both the transgenic and endogenous *ACO1* gene and in addition also the endogenous *ACO2* gene was silenced. The *ACO2* gene only exhibits significant similarity to *ACO1* in the coding region. This implies that the PTGS target region spread along the transgenic DNA or transcript from the inverted repeat in the 5' UTR to the downstream coding sequence. Using a transgenic GFP-expressing *N. benthamiana* line, Voinet et al. (1998) demonstrated cosuppression of sequences in the 5' half of the GFP coding region after biolistic introduction of DNA carrying the 3' half of the GFP coding region, and vice versa. These data demonstrate spreading of the PTGS target region in both 5'-3' and 3'-5' directions. Hypermethylation of regions up to 1,400 bp downstream of the polyadenylation signal of a posttranscriptionally silenced neomycin phosphotransferase II gene could also be an example of 5'-3' spreading of silencing (Van Houdt et al., 1997).

The mechanism behind 5'-3' spreading of silencing is at present unclear, but unprimed synthesis by a plant RdRP of RNA complementary to target mRNA sequences may be involved. Such unprimed transcription could, in principle, generate new small RNAs from regions both upstream and downstream of the initial target area.

The fact that we observed small RNAs from the 3' region of GUS after VIGS induction with PVX carrying a 5' GUS sequence, but not vice versa, could be because unprimed transcription of sequences downstream of the initial target area in plants are favored over primed or unprimed transcription of sequences upstream of the initial target. In vitro experiments with a tomato RdRP showed that unprimed transcription preferentially commenced at the 3' terminal of the template (Schiebel et al., 1993a, 1993b). This was put forward by Han and Grierson (2002) as a possible explanation for the preferential production of small RNAs from the 3' end of a posttranscriptionally silenced polygalacturonase transgene. However the fact that the extreme 3' end of GUS mRNAs undergoing PTGS was found to be poorly targeted (Hutvagner et al., 2000; this study) argues against this as a general rule. Instead, the plant RdRP may prefer a polyadenylated RNA as template, or the plant RdRP may associate with the small RNA-containing ribonuclease complex that cleaves the target mRNA, followed by postcleavage association of the RdRP with the 3' cleavage product. Alternatively, some mechanism may exist in plants that actively inhibits the generation of dsRNA and/or small RNAs from regions upstream of the initial target region. Although there are at present no data to suggest how such a mechanism might operate, its existence is also suggested by results obtained by Sonoda et al. (1999) in a study of silencing induction over a graft union. When scions of *N. benthamiana* lines expressing a *Sweet potato feathery mottle virus* coat protein (SPFMV CP) transgene were grafted onto other lines exhibiting silencing tar-

geted against either the entire SPFMV CP transgene or only the 3' region, PTGS induced in the scions were targeted against the same region as in the rootstock. However, scions exhibiting spontaneous silencing targeted against only the 3' region of the CP gene did not change this pattern of silencing after being grafted onto rootstocks showing silencing targeted against the entire CP gene. Perhaps a similar mechanism may be inhibiting the 3'-5' spread of PTGS target in these plants and in our VIGS experiment.

While this manuscript was under review, Baulcombe and coworkers published data demonstrating spreading both 5'-3' and 3'-5' of virus-induced silencing of a green fluorescent protein (GFP) transgene in *N. benthamiana* (Vaistij et al., 2002). Based on the presence of small RNAs corresponding to the 3' region of the GFP mRNA after induction of silencing with the 5' region, and vice versa, these authors conclude that silencing can spread more than 300 nt in both directions. The apparent discrepancy between these results and our data may be reconciled if the RdRP, shown by Vaistij et al. (2002) to be essential for both 3'-5' and 5'-3' spreading, initiates cRNA synthesis at or near the 3' end of targeted mRNAs but proceeds for only a limited number of nucleotides. As pointed out by Vaistij et al. (2002), the GUS coding region is considerably larger (~1,800 nt) than the GFP coding region (~700 nt), and a bias for cRNA synthesis in 3' regions over 5' regions may only be apparent in larger mRNAs. In agreement with this interpretation, we have preliminary data indicating spreading of silencing into the central U region of GUS after virus induction of silencing with either 5' (G) or 3' (S) sequences (data not shown).

Although 5'-3' spreading of silencing has previously been observed in plants, we believe this study is the first to demonstrate a predominance of 5'-3' spreading over 3'-5' spreading. Further studies are needed to clarify the underlying mechanisms, but our data does suggest a link between the commonly observed preferential targeting of 3' regions in silenced transgenes, and the RdRP-mediated spread of silencing.

MATERIALS AND METHODS

Plant material

Five- and 8-week-old tobacco plants (6 and 9 weeks after sowing) of the five genotypes *Nicotiana tabacum* L. cv. Xanthi, *Nicotiana tabacum* L. cv. Wisconsin 38, T4 (Hobbs et al., 1990; *Nicotiana tabacum* L. cv. Xanthi transformed with pBI121 (35S-*Uida*-nos-ter)), 6b5Ho (Elmayan & Vaucheret, 1996; *Nicotiana tabacum* L. cv. Paraguay PBD6 transformed with pKYLX71-35S² containing *Uida* and the *rbcS* 3'-region), and 8226 (Bucherna et al., 1999; *Nicotiana tabacum* L. cv. Wisconsin 38 transformed with pMON9749 containing 35S-*Uida* nos-ter) were used for the experiments.

Biolistics and ELISA

We coated $25 \pm 3 \mu\text{g}$ gold particles ($0.9 \mu\text{m}$ diameter) with DNA (approximately $1 \mu\text{g}/\text{shot}$) according to the manufacturer's recommendations (Bio-Rad). Plants were inoculated using a handheld gene gun (Helios, Bio-Rad) at approximately 150 psi, each plant at two different leaves. Both inoculated areas from each plant were assayed together 7 dpi by ELISA against the coat protein of PVX. Each construct was tested in eight plants and each experiment was repeated once (with a new coating). Every experiment included all five genotypes. Tests were performed to ensure that the samples were diluted enough to lie within the linear range of the ELISA.

Constructs

35S-promoter versions of the PVX-GUS, -GU, -US, and -GS constructs previously used by English et al. (1996) were created by exchanging the *Apal/Spel* fragment from pPVX204 (Baulcombe et al., 1995) with the same fragment from the respective PVX chimeras. Other PVX chimeras were constructed by inserting PCR-amplified GUS fragments into the vector pPVX.CES. This is a modified pPVX204 vector in which the *Apal/Spel* fragment was exchanged with the corresponding fragment from pPC2S (Baulcombe et al., 1995), thereby substituting the *Clal/EcoRV/SalI* cloning site for the GFP gene. Unless otherwise stated, all primers contained a 5' nonspecific 6-nt sequence (ACTGGT) to allow efficient restriction enzyme digestion, and either a *Clal* site (sense) or a *SalI* site (antisense) for insertion into pPVX.CES. PVX-SG was cloned by opening PVX-S with *SalI* and inserting a PCR-amplified G-fragment containing *SalI* sites at both ends. The PVX-GS' was cloned as a three-part ligation using a PCR-generated G-fragment (nt 6–603) containing *Clal/EagI* sites and an S-fragment (nt 1220–1803) containing *EagI/SalI* sites into the *Clal/SalI* of PVX.CES. PCR amplifications were done using the proofreading *pfu* DNA-polymerase (Stratagene) and pGC3 (Chapman et al., 1992) as template. The short constructs PVX-1517–1806, PVX-1603–1806, and PVX-1220–1517 were sequenced, and all other PVX-constructs were checked by restriction enzyme analysis.

Detection of small RNAs

RNA extraction

RNA from 2 g of leaf tissue was extracted in 100 mM Tris-HCl, pH 8.5, 100 mM NaCl, 50 mM EDTA, pH 8.0, 2% (w/v) SDS. The RNA was phenol/chloroform extracted twice, chloroform extracted, and ethanol precipitated. The RNA was redissolved in $300 \mu\text{L}$ H_2O , precipitated on ice using PEG 8000 (5% (w/v)) and 0.5 M NaCl, and redissolved in $100 \mu\text{L}$ formamide.

Northern blot

Ten microliters RNA in formamide were heated to 65°C , $5 \mu\text{L}$ loading buffer were added ($2 \times$ TBE, 40% (w/v) sucrose, 0.1% bromophenol blue), and samples were run on 15% TBE/urea acrylamide gels (Novex). RNA was transferred to Hybond N+ membranes (Amersham) in a semidry blotting

apparatus using $0.1 \times$ TBE, $3 \text{ mA}/\text{cm}^2$ for 25 min. The membrane was equilibrated in $20 \times$ SSC for 30 min at room temperature and dried at 37°C . The filter was prehybridized in Church–Gilbert solution for 2.5 h at 60°C , riboprobes were added, and hybridization continued overnight. The filter was washed $2 \times 15 \text{ min}$ at room temperature in $2 \times$ SSC/0.1% SDS, $2 \times 15 \text{ min}$ at 55°C in $0.2 \times$ SSC/0.1% SDS and exposed to a PhosphorImager screen.

Riboprobes

The G (nt 6–603) and S (nt 1220–1806) regions from GUS were inserted in pBluescript SK- in the *EcoRI* site. Clones with inserts in antisense orientation relative to the T7 promoter were linearized with *BamHI* and phenol/chloroform extracted. After T7 transcription in the presence of $\alpha\text{-}^{32}\text{P}$ -CTP, the template was digested with RNase-free DNase and hydrolyzed in carbonate buffer to an average length of 50 nt. After neutralization with 3 M sodium acetate, pH 5.0, the probe was added to the prehybridization buffer.

VIGS and GUS analysis

35S-GUS *N. benthamiana* (provided by Olivier Voinnet) were bombarded with PVX, PVX-G, PVX-S, or PVX-GUS, 10 plants for each construct. Each plant received two shots of approximately $1 \mu\text{g}$ DNA per shot. Samples for GUS assays were taken at 19 and 26 dpi (experiment I) and 11, 18, and 25 dpi (experiment II). The leaf sample (pinched out with an eppendorf lid) was homogenized in $100 \mu\text{L}$ GUS extraction buffer (Jefferson, 1987). The supernatant was diluted 1:20, and $10 \mu\text{L}$ were used for both analysis of GUS activity (the *p*-nitrophenyl glucuronide assay (Jefferson, 1987), performed in duplicate) and total protein content (Bio-Rad Protein Reagent, bovine serum albumin as standard, performed in triplicate). Samples for analysis of small RNAs were taken at 30 dpi in experiment II. RT-PCR analysis was performed on total RNA extracted from uninfected 35S-GUS *N. benthamiana* plants. An oligo-dT primer was used for the RT reaction and as downstream primer for the PCR reactions. Upstream primers for the PCR reaction covered nt 1220–1237 or 1514–1530 of the GUS coding sequence, respectively.

Statistical analysis

Analyses of variance (ANOVA) and linear regressions of the accumulation of the virus detected by ELISA (as a percentage of the mean accumulation in the two nontransgenic tobacco genotypes, W38 and Xanthi) were performed using the General Linear Model procedure of the SAS software (SAS Institute, 1988). Unless indicated, hypotheses for ANOVA were confirmed by the Shapiro and Wilk's test (residues show a normal distribution if the probability is above 0.05) and the Bartlett's test (variances are homogeneous if the probability is above 0.05). When ANOVA results showed significant effects (*F*, probability of the Fisher's test, below 0.05), means were compared with a Newman–Keuls test. Bravais–Pearson's coefficients of correlation (Dagnélie, 1998) were calculated and their significance evaluated after Fisher's *z* transformation (Dagnélie, 1998).

Additional statistical data

A complete statistical analysis of the data in this article is available on the Internet at <http://www.dias.kvl.dk/statistics.html>.

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