Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA

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Tobacco plants were transformed with constructs in which the transgene was a cDNA of replicating potato virus X (PVX) RNA. The constructs, referred to here as amplicons, were the intact genome of PVX and PVX constructs modified to carry the β-glucuronidase (GUS) reporter gene either as an additional gene or as a replacement for the coat protein gene (PVX/GUS/CP and PVX/GUS respectively). Transformed plants carrying these constructs displayed several phenotypes that we attribute to post-transcriptional gene silencing. These phenotypes include the absence of viral symptoms, low accumulation of transgene-derived RNA, extreme strain-specific resistance against PVX, low and non-uniform GUS expression (in the PVX/GUS and PVX/GUS/CP plants) and suppression of transiently expressed RNA sharing homology with the transgene. Importantly, the amplicon-mediated gene silencing was exhibited in all lines tested. There was no evidence of gene silencing in seven lines expressing a PVX RNA that was unable to replicate. From these data we conclude that the replicating viral RNA is a potent trigger of gene silencing. Moreover, amplicon-mediated gene silencing provides an important new strategy for the consistent activation of gene silencing in transgenic plants.

Keywords: gene silencing/potato virus X/transformed tobacco plants/virus replication/virus resistance

Introduction

The conventional approach to achieve high level expression of a foreign gene in plants involves transformation of a plant with the gene of interest coupled to a suitable promoter. Although this strategy has been successful there is often variable expression of the transgene in different plant lines produced with the same construct (Hobbs et al., 1990; Peach and Velten, 1991; Longstaff et al., 1993). Consequently, to analyse the phenotype of a transgene it may be necessary to generate many transgenic lines, either to average out the noise in the experimental data, or to identify a rare line in which the transgene is stably expressed at the required high level.

Much of the variation in transgene expression is probably due to chromosomal effects. For example, the transgene could have integrated in a domain lacking features of the DNA or chromatin that are necessary for high level expression. These features could facilitate transcription or protect the transgene from any inhibitory influence of the surrounding region of the chromosome (Lewin, 1994; Mlynarova et al., 1994, 1995; Allen et al., 1996). Alternatively, if there are multiple copies of the transgene there could be ectopic pairing of homologous DNA leading to transcriptional or post-transcriptional gene silencing (recently reviewed by Matzke and Matzke, 1995; Baulcombe and English, 1996). To reduce the variability in transgene expression it would be necessary to develop a procedure that is not affected by, or which can overcome these chromosomal effects. One such approach relied on the presence of nuclear scaffold attachment regions in the transgene constructs (Allen et al., 1993, 1996). However, this strategy did not significantly reduce the between-line variation in transgene expression (Allen et al., 1993, 1996).

As an alternative approach we have combined the use of a virus vector and transgene technology. We use the term ‘amplicon’ to describe transgene constructs in which the transgene is a cDNA of replicating potato virus X (PVX) RNA into which foreign genes may have been inserted. Transgenic expression of the amplicon would initiate infection of the transformed cell by the sequence of events shown in Figure 1. First, the transgene would be transcribed from the cauliflower mosaic virus (CaMV) 35S promoter in the normal way (Figure 1, step 1). The RNA product would be transported from the nucleus to the cytoplasm (Figure 1, step 2) where it would serve as the mRNA template for translation of the first open reading frame (ORF) in the PVX genome (Figure 1, step 3). The product of this ORF is an RNA-dependent RNA polymerase (RdRp) which is required for PVX replication. Using the transgene-derived RNA as a template, the RdRp would synthesize negative strand RNA (Figure 1, step 4). The RdRp would then use this negative strand RNA as a template to synthesize various species of positive strand RNAs (Figure 1, step 5). These positive strand RNAs would include the full-length viral genome RNA and subgenomic RNAs. The subgenomic RNAs would all be 3′ co-terminal but with different 5′ ends, each produced from RNA promoters located internally in the viral genome (Figure 1). The synthesis of subgenomic RNAs is a viral strategy to permit translation of ORFs located downstream of ORF1 (Figure 1, step 6). Steps 3 to 6 of the scheme described in Figure 1 represent the normal RNA-based replication cycle of PVX. Step 5 in the scheme represents an RNA amplification step in the replication cycle of the virus so that, if a foreign gene is inserted into the PVX amplicon as part of a subgenomic RNA, it would be expressed at much higher levels than if expressed directly from a transgene promoter. Moreover, since the contribution of viral replication to the steady state level of the transgene-derived RNA would mask the variation in direct transcription we predicted that foreign gene expression would be reproducibly high in every plant.
Fig. 2. Constructs expressed in transgenic plants. Schematic representation of the constructs introduced into tobacco plants via binary vectors carried in Agrobacterium tumefaciens LBA4404. Each construct was inserted between the CaMV 35S promoter and the nos terminator sequence. The various PVX ORFs are labelled: RdRp, RNA-dependent RNA polymerase; 25, 12, 8, the ‘triple gene block’ genes (encoding proteins of 25, 12 and 8 kDa respectively); CP, coat protein gene. The GUS sequence, shown as a hatched box, was inserted as an additional ORF in PVX/GUS/CP and was expressed from a duplicated CP subgenomic promoter, or as a direct replacement for the CP in PVX/GUS. PVX/ΔREP/CP was essentially PVX/GUS but with a 1.7 kb deletion within the RdRp ORF to prevent the initiation of viral replication.

of the transgene in the amplicon plants initiated the PVX replication cycle and resulted in the accumulation of GUS, as predicted. However, the expression of the amplicon constructs did not result in consistently high levels of GUS activity. In fact, rather than achieving high-level transgene expression, the amplicons produced a high level of post-transcriptional gene silencing in every plant of every line tested.

Results

PVX constructs for transgenic expression

Figure 2 illustrates the PVX cDNA amplicon constructs that were assembled and introduced into Nicotiana tabacum cv Petite Havana by Agrobacterium tumefaciens-mediated transformation. One construct had the PVXUK3 genome intact (PVX; Figure 2). The other constructs were also based on the PVXUK3 genome but were modified to include the GUS gene inserted either as an additional ORF in the viral genome (PVX/GUS/CP; Figure 2) or as a replacement for the coat protein (PVX/GUS; Figure 2). Control plants were also generated which expressed a defective PVX/GUS construct encoding a non-functional RNA-dependent RNA polymerase (PVX/ΔREP/GUS; Figure 2). The transgene-derived RNA from this construct would not initiate the viral replication cycle. Other control plants expressed GUS directly from the 35S promoter (35S/GUS; Figure 2).

To simplify subsequent analyses we selected lines in which there was a single transgene insert. These plant lines (five to seven independent lines for each construct) were identified by DNA gel blot analysis of the primary transformants (T0 plants) and by the segregation ratios for GUS and/or the nptII gene in the T0 selfed progeny (T1 plants) (data not shown).

Expression of the PVX transgenes

Surprisingly, none of the amplicon plants exhibited symptoms of PVX infection. However, as expected, there were...
Consistent gene silencing in transgenic plants

Table I. MUG assay data on transgenic plants

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Line number</th>
<th>Mean GUS activity ± SE (number of plants assayed)</th>
<th>Range</th>
<th>Highest to lowest ratio</th>
</tr>
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<tbody>
<tr>
<td>35S/GUS</td>
<td>SA94055</td>
<td>270.0 ± 15.70 (18)</td>
<td>187–428</td>
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</tr>
<tr>
<td></td>
<td>SA94082</td>
<td>157.1 ± 9.53 (14)</td>
<td>112–223</td>
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</tr>
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<td></td>
<td>SA94085</td>
<td>310.7 ± 17.16 (14)</td>
<td>222–438</td>
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<td></td>
<td>SA94088</td>
<td>37.5 ± 6.68 (13)</td>
<td>6–76</td>
<td>13</td>
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<td>SA93025</td>
<td>150.8 ± 11.51 (13)</td>
<td>102–228</td>
<td>2</td>
</tr>
<tr>
<td>PVX/GUS</td>
<td>SA93008</td>
<td>211.1 ± 29.91 (10)</td>
<td>89–385</td>
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<tr>
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<td>SA93012</td>
<td>82.2 ± 10.17 (15)</td>
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<td></td>
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<td>7–243</td>
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<td>4–117</td>
<td>29</td>
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<td></td>
<td>SA94004</td>
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<td></td>
<td>SA94016</td>
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<td>1.6–2.2</td>
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<td></td>
<td>SA94057</td>
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<td>0.5–1.4</td>
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<td></td>
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<td>&lt;0.05 (10)</td>
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<td>&lt;0.05 (10)</td>
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<td>–</td>
</tr>
<tr>
<td></td>
<td>SA94037</td>
<td>&lt;0.05 (8)</td>
<td>–</td>
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</table>

Units of GUS activity are expressed as nmol of 4-methylumbelliferone produced per min per mg total leaf disc protein.

Southern analyses and segregation ratios of 3:1 for the GUS positive trait indicated that the transgene had integrated at a single locus. Segregants without the transgene (confirmed by PCR analysis for NPTII sequence) all had <0.05 units of activity.

Virus particles in the amplicon plants expressing the PVX or PVX/GUS/CP transgene. It could be ruled out that the lack of PVX symptoms in the amplicon plants was due to a mutation in the viral genome because inoculation of non-transformed plants with virions extracted from the PVX amplicon plants led to symptoms of PVX infection within 7 days post inoculation. The yield of virions from the amplicon plants was 50–75% lower than from non-transformed plants that were manually inoculated with PVX (at 12 days post inoculation). Nevertheless, the presence of virions in the amplicon plants indicated that the transgene-derived RNA had been replicated and that subgenomic RNAs had been produced.

Expression of GUS from the amplicon constructs also suggested that there was replication of the transgene-derived RNA and production of subgenomic RNAs. There was GUS activity in every plant expressing the PVX/GUS transgene but not in any of the PVX/REP/GUS plants which expressed viral RNA unable to initiate the PVX replication cycle (Table I). The levels of GUS in the PVX/GUS plants were similar to those in the 35S/GUS control plants (Table I) and not higher, as would be expected if every cell was supporting high level replication of the transgene-derived RNA (Figure 1). Also unexpected was the degree of variation in the levels of GUS activity, between and within lines, which was as great in the PVX/GUS lines as in the 35S/GUS lines (Table I). In plants expressing the PVX/GUS/CP transgene the levels of GUS activity were also variable but were up to one hundred-fold lower than in the PVX/GUS plants (Table I).

In the PVX/GUS and PVX/GUS/CP plants the GUS activity was restricted to single cells or small groups of cells. These groups of GUS-producing cells were evident in every plant expressing the PVX/GUS/CP or PVX/GUS transgene and in many tissue types of these plants including true leaves, stems, cotyledons and roots (Figure 3).

Virus resistance in the amplicon lines

The absence of GUS activity in some cells of the amplicon plants could reflect the suppression of either transcription from the transgene or the accumulation of viral RNAs. To test for the latter possibility we inoculated amplicon lines with various strains of PVX. We assumed that a mechanism able to suppress accumulation of the transgene-derived viral RNA would also suppress an inoculated PVX and the plants would be resistant to PVX.

Resistance to virus infection in the amplicon lines was assessed first by monitoring the development of symptoms after manual inoculation of different strains of PVX. The amplicon plants and controls (PVX/REP/GUS and non-transformed plants) were inoculated with the PVX UK3, PVXNL1, PVXCP2, PVXNL4 and PVXCP4 isolates of PVX. The PVX UK3 isolate was the original source of the viral transgenomes. The other isolates differed from PVX UK3 by up to 20% at the nucleotide sequence level (Skryabin et al., 1988; Huisman et al., 1989; Orman et al., 1990; Kavanagh et al., 1992; W.De Jong, unpublished).

In the amplicon T1 plants inoculated with PVX UK3 the symptoms of viral infection developed in 25% of the plants at the same time as in the plants expressing the PVX/REP/GUS transgene or in the non-transformed plants. The remaining 75% of the amplicon plants failed to develop symptoms by 14 days post inoculation whereas all of the control plants showed symptoms within 9 days post inoculation (Figure 4A). This 3:1 segregation ratio of the resistance trait indicated that resistance was expressed in most, if not all, of the transformed progeny of the amplicon lines. In contrast, symptoms developed...
on every amplicon and control plant inoculated with PVX<sub>CP2</sub>, PVX<sub>CP4</sub>, PVX<sub>NL1</sub> and PVX<sub>NL4</sub>, although there was a slight delay in the appearance of symptoms on the plants expressing the PVX or PVX/GUS/CP transgene compared to the PVX/GUS and control plants (Figure 4B). The data shown in Figure 4 are combined from several independent experiments and are particularly notable because every line generated with the PVX, PVX/GUS or PVX/GUS/CP construct showed resistance to PVX<sub>UK3</sub>.

From these data we conclude that the amplicon plants displayed two distinct types of resistance. There was a strain-specific resistance and a weaker resistance effective against a broader spectrum of PVX isolates manifested as a slight delay in symptom expression (Figure 4). The strain-specific resistance, displayed by all of the amplicon lines, was dependent on replication of the transgene-derived RNA since it was not produced in the PVX<sub>ΔREP/GUS</sub> plants. The weaker, broad spectrum resistance was associated with coat protein production and is probably related to coat protein-mediated resistance conferred by ‘conventional’ coat protein transgenes (Hemenway et al., 1988; C.Spillane, J.Verchot, T.Kavanagh and D.Baulcombe, submitted). This weak resistance in the amplicon plants compared with conventional coat protein-mediated resistance may indicate that the coat protein in virions confers resistance less effectively than free coat protein.

To further analyse the strain-specific resistance, a PVX.GUS construct was inoculated to non-transformed control plants and to the PVX<sub>ΔREP/GUS</sub> and PVX plants. PVX.GUS is a PVX<sub>UK3</sub> vector expressing the GUS reporter...
gene (Chapman et al., 1992), which produced large GUS-positive foci of infection on the non-transformed plants and the PVX/ΔREP/GUS plants (Figure 5A). In contrast, on the PVX plants there were no PVX.GUS foci (Figure 5B). Infrequently, and after prolonged histochemical staining, there were three to six small spots of GUS activity visible on the leaves of PVX plants inoculated with PVX.GUS (Figure 5C). Based on these reporter gene data we concluded that the absence of symptoms following inoculation of the amplicon plants with PVXUK3 was due to resistance to initial infection rather than tolerance of PVX. This conclusion was supported by the absence of detectable green fluorescence on the leaves of PVX, PVX/GUS or PVX/GUS/CP plants inoculated with a PVXUK3 vector carrying the green fluorescent protein reporter gene (Baulcombe et al., 1995) (data not shown).

The conclusion that there was PVX resistance in the initially inoculated cell was confirmed by inoculation of PVX to protoplasts prepared from the transgenic plants. The inocula for these protoplast assays were in vitro synthesized transcripts of the full-length cDNA clone of PVXUK3 (Kavanagh et al., 1992) or PVXCp2 (S. Santa-Cruz, unpublished). RNA gel blot analysis of the protoplast extracts at 1 day post inoculation revealed that the genomic and subgenomic RNAs of both strains accumulated at high levels in the protoplasts of the non-transformed (Figure 6) or the PVX/ΔREP/GUS lines (data not shown). The PVXCp2 RNA accumulated to the same extent in protoplasts of the amplicon and non-transformed lines (Figure 6). In contrast, the protoplasts of the amplicon lines displayed extreme resistance to PVXUK3 (Figure 6). There was a low level of PVX RNA in the protoplasts expressing the PVX or PVX/GUS/CP transgene (Figure 6). This RNA was present at the same level in both the mock- and the transcript-inoculated samples, suggesting that it was transgene-derived RNA that had become encapsidated. No transgene-derived RNA was detected in the protoplasts expressing the PVX/GUS transgene (Figure 6).

The strain-specific resistance against PVX indicates the presence of an activity in the amplicon lines that inhibits accumulation of the transgene-derived RNAs by suppression of virus accumulation. This activity could account for the low and variable pattern of amplicon-mediated GUS production in addition to the strain-specific resistance to PVX. In many respects this activity is similar to homology-dependent resistance which is an RNA-mediated mechanism associated with post-transcriptional gene silencing in transgenic plants (Mueller et al., 1995; Smith et al., 1995; Swaney et al., 1995; Goodwin et al., 1996; Pang et al., 1996).

**Post-transcriptional gene silencing in the amplicon plants**

To test whether gene silencing was activated in the amplicon plants, we performed a transient expression assay similar to that used previously by Hobbs et al. (1993) to investigate silencing of GUS transgenes. Our assay involved particle bombardment of GUS reporter constructs into leaf discs excised from the plants expressing the PVX transgene. To provide homology between the transiently expressed GUS mRNA and the PVX transgene, the GUS reporter construct was modified to include part

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**Fig. 5.** Histochemical staining for GUS activity in PVX.GUS-inoculated transgenic plants. PVX.GUS virions were manually inoculated to plants expressing (A) the PVX/ΔREP/GUS transgene (a leaf from line SA93019 is shown although similar results were obtained with all of the PVX/ΔREP/GUS lines listed in Table I), (B) and (C) the PVX transgene (leaves from line SA94104 are shown although similar results were obtained with lines SA94091 and SA94092). The inoculated leaves at 5 days post inoculation are shown. PVX.GUS accumulated on the PVX/ΔREP/GUS plants but not on the PVX plants. Non-transformed plants inoculated with PVX.GUS virions showed the same pattern of GUS staining as PVX/ΔREP/GUS plants (data not shown). The leaves A and B were stained for 2–3 h. Leaf C was stained overnight, after which small spots of GUS activity were visible, as indicated by arrows.
of the PVX sequence on the 3′ side of the GUS ORF. If gene silencing was active, we predicted that it would also suppress the accumulation of the transiently expressed GUS mRNA because of the homology with the amplicon transgene. The constructs used in the transient assay were based on a plasmid expressing GUS RNA directly from the 35S promoter (pSLJ4D4; Figure 7). Parts of the PVXUK3 cDNA clone were inserted on the 3′ side of the GUS ORF in pSLJ4D4, in both sense and antisense orientations (Figure 7). Each plasmid construct was coated onto gold particles and electrostatically bombarded into the leaves of PVX, PVX/AREP/GUS, or non-transformed plants. Histochemical staining for GUS activity of the bombarded leaves revealed that there was substantial and statistically significant suppression of GUS expression in the leaves of the PVX amplicon plants, but only when the plasmid constructs contained a region of the PVX genome (Figure 8). Suppression was manifested as a reduced number of blue spots on the PVX amplicon leaves compared with the number on the non-transformed control plants (Figure 8). Based on the number of blue spots in this transient assay, the GUS expression in the PVX plants was 50–90% less than that observed in the non-transformed plants. Expression of GUS was suppressed from plasmids with the PVX sequences transcribed in either the sense or the antisense orientation. No suppression of GUS expression in the transient assay was observed in the plants expressing the PVX/AREP/GUS transgene (Figure 8).

The control construct, pSLJ4D4 (GUS RNA expressed from the 35S promoter; Figure 7), produced a similar number of spots on the PVX, PVX/AREP/GUS and non-transformed plants (Figure 8). Thus, these transient assay data indicated that a post-transcriptional gene silencing mechanism was active in the PVX amplicon plants. Unfortunately, we could not perform similar analyses on the PVX/GUS or PVX/GUS/CP plants because it was not possible to distinguish between the transgene-derived GUS transgene and the transiently expressed GUS.

Discussion
We had expected that the phenotype of the plants expressing the amplicon constructs would be influenced by virus replication. Thus, in the lines with the PVX and the PVX/GUS/CP constructs we predicted that there would be virus particles and the mild mosaic symptoms of PVX infection, as in infected plants. In plants with the PVX/GUS and the PVX/GUS/CP amplicons we had expected that there would be GUS accumulation and that the spatial distribution of GUS would be uniform. We had also expected that the replication of the transgene-derived PVX RNA would be immune to the chromosomal factors that account for the between-line variation in transgene expression. In fact, the phenotype of the amplicon plants differed from these expectations in several respects. First, virions formed in the PVX and PVX/GUS/CP plants but their accumulation was at much lower levels than in infected plants. Secondly, none of the amplicon plants showed symptoms of virus infection. Thirdly, there was GUS activity in the PVX/GUS and PVX/GUS/CP plants, but the levels varied considerably between plants (Table I) and the activity was distributed non-uniformly (Figure 3).

The initial analyses of the amplicon plants indicated that these unexpected phenotypes could be accounted for by a virus resistance mechanism that is similar to the homology-dependent resistance mechanism observed in...
plants expressing part of a viral genome (Mueller et al., 1995; Smith et al., 1995; Swaney et al., 1995; Dawson, 1996; Goodwin et al., 1996; Pang et al., 1996). This homology-dependent resistance, like the resistance in the amplicon plants, is both highly strain specific (Figures 4, 5 and 6) and associated with low accumulation of transgene-derived RNA (Figure 6). Homology-dependent resistance in transgenic plants is related to post-transcriptional gene silencing (Mueller et al., 1995; Smith et al., 1995; Swaney et al., 1995; Goodwin et al., 1996; Pang et al., 1996). Because of this, we considered it likely that gene silencing would be active in the amplicon plants and that there would be suppression of nuclear genes as well as viral genes. The outcome of the transient assay for post-transcriptional gene silencing (Figure 8) was completely consistent with this prediction and, in the main part of this discussion, we compare the amplicon phenotype to other examples of post-transcriptional gene silencing in transgenic plants. However, before this broader assessment of post-transcriptional gene silencing we consider some notable features of the amplicon phenotype.

The first of these features concerns the spotted pattern of GUS expression in the amplicon plants (Figure 3). Our interpretation of this phenotype is based on the assumption that the transgenically expressed viral RNA, being produced incessantly in most cells, would provide an intense pressure for infection. From time to time this intense infection pressure would overcome the resistance to give virus accumulation and, with PVX/GUS and PVX/GUS/CP plants, lead to transient expression of the GUS gene carried in the amplicon construct. An alternative explanation in which the GUS spots are attributed to somatic instability of the resistance mechanism is less likely because the pattern of GUS expression did not correspond to developmental sectors in the leaves. Moreover, no PVX-susceptible leaf sectors were observed after manual inoculation of PVX/GUS to the amplicon plants (Figure 5).

A second notable feature of the amplicon phenotypes is the 100-fold difference in the level of GUS in the PVX/GUS and PVX/GUS/CP lines. In principle this difference could have been due to substantially more effective gene silencing in the PVX/GUS/CP lines in which the GUS levels were low (Table I). However, a more likely explanation is based on the role of the PVX coat protein as a negative regulator of subgenomic RNA production (Chapman et al., 1992). According to this idea, the PVX coat protein in the PVX/GUS/CP lines would have suppressed production of the GUS mRNA from the amplicon construct and thereby suppressed GUS synthesis. An additional factor may be the position of the promoter for GUS mRNA in the two constructs. In the PVX/GUS construct, the promoter for the GUS mRNA is closer to the 3′ end of the PVX genome than the corresponding promoter in the PVX/GUS/CP lines. In the main part of this discussion, we compare the amplicon phenotype to other examples of post-transcriptional gene silencing in transgenic plants. However, before this broader assessment of post-transcriptional gene silencing we consider some notable features of the amplicon phenotype.
Although there are other reports describing transgenic expression of plant viral genomes, the phenotypes of these plants did not clearly indicate that gene silencing was activated. For example, as with the amplicon plants, there was virus resistance in plants expressing a mild strain of tobacco mosaic virus (TMV) (Yamaya et al., 1988b). However, this resistance was not observed in plants expressing a virulent strain of TMV (Yamaya et al., 1988a).

In plants expressing cucumber mosaic virus (CMV) RNAs 1 and 2 there was resistance to challenge inoculation with CMV. This resistance was more effective against RNA rather than virion inocula (Suzuki et al., 1996). However, unlike the amplicon plants, the CMV resistance was not strain-specific and was associated with a high level accumulation of the transgene-derived RNA (Suzuki et al., 1996). In a further example, Nicotiana tabacum plants were transformed with replicating RNAs 1 and 2 of brome mosaic virus (BMV). Protoplasts from these plants were resistant to BMV but not to CMV, and showed a low level accumulation of the transgene-derived RNAs (Kaido et al., 1995). Also, transgenic plants expressing BMV RNAs carrying the human gamma interferon (IFN-γ) gene failed to accumulate the predicted high levels of IFN-γ (Mori et al., 1993). In the light of the data presented here, in particular those from the transient expression assay, it seems likely that these phenotypes of the BMV transgenic plants can be explained by gene silencing.

Consistent with this proposed activation of gene silencing by the transgenic expression of replicable viral genomes, there are reports of other virus-mediated effects that may also be due to gene silencing. These include the photobleaching symptoms induced by modified TMV vectors carrying part of a phytone desaturase gene that were inoculated to Nicotiana benthamiana (Kumagai et al., 1995). The viral vector induced suppression of the host phytone desaturase gene in the infected tissue which consequently lacked carotenoid protection and became photobleached (Kumagai et al., 1995). In a second example, transgenes based on the coat protein ORF of potyviruses became silenced following infection of the transgenic plants with the corresponding potyviruses (Lindbo et al., 1993; Dougherty et al., 1994; Smith et al., 1994). Associated with this silencing of the transgene there was also virus resistance operating at the RNA level which was manifested as recovery of the plant from a diseased to a healthy condition (Lindbo et al., 1993; Dougherty et al., 1994; Smith et al., 1994).

Why do some inoculated or transgenically expressed viruses activate gene silencing? It could be in part because their RNAs accumulate at a very high level and activate a sequence-specific mechanism of RNA degradation. This threshold model has been invoked previously to account for the recovery phenomenon in transgenic plants (Lindbo et al., 1993; Dougherty et al., 1994; Smith et al., 1994) and the requirement for viral replication to activate gene silencing in our plants is consistent with this. However, we do not consider that a high level of an RNA species is sufficient to activate gene silencing and the associated homology-dependent resistance. If a high level accumulation of an RNA was all that was needed, the accumulation of an inoculated virus should lead to recovery as in the plants with potyvirus transgenes, even in non-transformed plants. Common observation reveals that recovery is not a usual response to virus infection of non-transformed plants. Therefore, activation of gene silencing requires more than just high level accumulation of an RNA species.

A second factor that may be involved in virus-induced gene silencing is sequence homology of the virus with either a transgene or an endogenous gene. As a result of this homology there could be an interaction of the viral genome with the nuclear DNA. A precedent for an RNA–DNA interaction that could be relevant to virus-induced gene silencing is in transgenic tobacco plants expressing viroid cDNAs. The presence of replicating viroid RNA in these plants caused sequence-specific methylation of the viroid transgene (Wassenegger et al., 1994). If there is RNA-directed DNA methylation in the plants displaying virus-induced gene silencing it could initiate a sequence of events leading to post-transcriptional gene silencing. In several examples of gene silencing induced by conventional transgenes it has been suggested that DNA methylation could be implicated (Hobbs et al., 1990, 1993; Ingelbrecht et al., 1994; Smith et al., 1994; English et al., 1996). However, as for the RNA threshold, this proposed RNA–DNA interaction leading to DNA methylation cannot be a single factor that is sufficient for activation of gene silencing. Our PVX/REP/GUS plants and all other transgenic plants that do not display gene silencing illustrate that homology of an RNA with a transgene will not necessarily lead to gene silencing.

A third factor that has been invoked as an initiator of post-transcriptional gene silencing in transgenic plants is the presence of aberrant RNA. ‘Aberrancy’ in this scenario is a feature of the RNA that somehow differs from RNAs that are encountered by cells in situations that do not lead to gene silencing. The gene silencing in the amplicon plants could fit into this aberrant RNA model if the viral RNA transcribed from the transgene represents a modified (i.e. aberrant RNA) template for the viral replicase. Alternatively the aberrancy could be concerned with the site or timing of viral RNA synthesis rather than a structural feature.

Our current work is directed towards understanding the mechanism of virus-induced gene silencing in infected and transgenic plants. Meanwhile, even without understanding the underlying mechanism, the analyses presented here suggest that amplicon constructs may represent a more reproducible approach for the activation of gene silencing in transgenic plants than is currently available. In 17 lines tested there was remarkably little variation in the level of gene silencing as indicated by the virus resistance and transient expression assays (three lines tested). None of the other approaches to gene silencing, including those involving increased transgene dosage, high-level transgene expression or the presence of direct or inverted repeats in the transgene construct, produces an effect that is as reproducible from line to line as the amplicon-mediated gene silencing described here. Future experiments will test the potential of the amplicon constructs to silence a range of viruses and nuclear genes in plants. In addition it would be interesting to find out whether gene silencing in organisms other than plants can be activated by replicable viral RNAs.

**Materials and methods**

**Constructs and plant transformation**

All of the viral cDNA constructs were inserted between the CaMV 35S promoter and the transcriptional terminator of the nopaline synthase.
(nos) gene and transferred into the binary vector pSL7291. PVX was the full-length cDNA clone of PVX (Figure 2). PVX/GUS was the PVX cDNA clone carrying the GUS gene as a direct replacement for the coat protein gene (Figure 2). PVX/AREP/GUS was generated by digestion of PVX/GUS with BglII restriction enzyme and religation (Figure 2). PVX/GUS/CP was the PVX cDNA clone carrying GUS as an additional gene in the viral genome, inserted between the ‘triple gene block’ and the coat protein ORF (Figure 2). A duplicated coat protein promoter sequence upstream of the GUS gene allowed GUS to be expressed as gene expression in stably transformed plant cells.

**Analysis of GUS activity**

Histochemical staining for GUS activity was performed on 3T and T1 plants, as described by Angell and Baulcombe (1995). 4-Methyl umbelliferyl glucuronic acid (MUG) assays on T1 plants (5 weeks old) were performed as described by Chapman et al. (1992). For each plant, leaf discs were excised from three different leaves and the sap extract pooled.

**Preparation of PVX virions and virion RNA**

The preparation of virions and virion RNA was as described previously (Baulcombe et al., 1984).

**Transcription in vitro, preparation and electroporation of protoplasts, and RNA gel blot analyses**

The plasmids ptXS and pcP2 were linearized with Sphi restriction enzyme and transcribed in vitro as described by Chapman et al. (1992). The reactions were performed as described previously (Davies et al., 1992). The RNA products were digested with BamHI and ligated with BamHI-digested pSL4J4D to generate the plasmids 437, 438, 439 and 441. The 1.7 kb BglII fragment from ptXS was ligated with BamHI-digested pSL4J4D to generate the plasmids 437, 438, 439 and 441. The orientation of the PVX sequence in pSL4J4D was determined by restriction enzyme analyses. The PVX fragments used for constructs 437, 438 and 439 were individually cloned in both sense and antisense orientations (Figure 7).

**Leaf disc bombardment**

Leaf discs were excised from fully expanded leaves from 6-week-old plants, placed onto Murashige and Skoog medium containing 3% sucrose and immediately bombarded with gold particles coated with plasmid DNAs. Plasmid DNAs were prepared using a QIAGEN-tip 100 column (Qiagen Inc. Dorking, Surrey) and were precipitated onto 0.95 μm diameter gold particles as described by McCabe et al. (1988), but with a further 20-fold dilution of the preparation. Particle acceleration was performed using the ACCCELL gene delivery system (Agracetus Inc.) under partial vacuum using a helium pressure of 10,000 kPa. The leaf discs were incubated at room temperature in the dark for 24 h and then stained for GUS activity.

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**References**


