De novo methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus

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The relationship between co-suppression and DNA methylation was explored in transgenic plants showing inducible co-suppression following infection with a cytoplasmically replicating RNA virus. Induction resulted in a loss of transgene mRNA and resistance to further infection, factors typical of post-transcriptional gene silencing. In infected plants, *de novo* methylation of the transgene appeared to precede the onset of resistance and only occurred in plants where the outcome was co-suppression. The methylation was limited to sequences homologous to the viral RNA and occurred at both symmetric and non-symmetric sites on the DNA. Although methylation is predicted to occur in mitotic cells, the virus was found not to access the meristem. A diffusible sequence-specific signal may account for the epigenetic changes in those tissues.

Keywords: co-suppression/diffusible signal/meristem/methylation/RNA virus

Introduction

Co-suppression is a term that has been used to describe transcriptional silencing and post-transcriptional gene silencing (PTGS) in plants, fungi and animals (Bingham, 1997). The term encompasses examples of the repression of gene expression when multiple copies of the gene (often as transgenes) were present in the genome, or when homologous sequences were added ectopically (as DNA or RNA). Bingham (1997) also suggested that co-suppression could be an ancient development in evolution that has been conserved through the different kingdoms by strong selection pressure from pathogens, and by genetic redundancy created by mobile genetic elements and gene duplication events.

In plants, cytoplasmically replicating RNA viruses have the ability to affect nuclear-derived gene expression in a homology-dependent manner (Lindbo *et al.*, 1993; Kumagai *et al.*, 1995; Ruiz *et al.*, 1998). In this case, the alteration of gene expression is in the form of PTGS, where there is homology-dependent degradation of RNA in the cytoplasm. PTGS can repress the expression of introduced transgenes or nuclear endogenes and, additionally, can result in degradation of the triggering viral RNA, leading to resistance. Introduced RNA has been shown to function in the down-regulation of endogenes in *Caenorhabditis elegans* (Fire *et al.*, 1998).

Despite extensive studies, the mechanism behind PTGS is still incompletely understood. Various models have implicated aberrant RNA molecules (Baulcombe and English, 1996; English *et al.*, 1996; Sijen *et al.*, 1996; Wasseneger and Pélissier, 1998) or double-stranded RNA (Metzlaff *et al.*, 1997; Montgomery and Fire, 1998) in triggering PTGS. It was proposed that these molecules could be a consequence of excessive (more than a threshold) transgene expression (Lindbo *et al.*, 1993; Dougherty and Parks, 1995; Meins and Kunz, 1995), ectopic pairing of DNA or DNA methylation (Baulcombe and English, 1996; English *et al.*, 1996; Sijen *et al.*, 1996). Nevertheless, sufficient correlative evidence has accumulated for it to be suggested that transcriptional and PTGS may be fundamentally linked (Matzke and Matzke, 1995; Bingham, 1997; Wasseneger and Pélissier, 1998). The processes could have in common modifications to chromatin structure and/or DNA methylation, provoked by specialized RNA products (i.e. aberrant RNAs).

Methylation of cytosine residues in DNA, in particular, has been examined for its role in transcriptional gene silencing (reviewed in Flavell, 1994; Martienssen and Richards, 1995; Kass *et al.*, 1997) where methylation of cytosines in promoter sequences and transcriptional start sites has been reported. The silencing may be linked to interference with the binding of transcription factors and/or due to localized changes in chromatin structure. A role for methylation in PTGS has also been implicated. However, this area has not been fully explored and the evidence is often conflicting. Ingelbrecht *et al.* (1994) demonstrated a correlation between PTGS of reporter transgenes and methylation in tobacco and, in another study, a silenced *uidA* transgene was found to be methylated in the 3' coding region, which was also the target for PTGS (English *et al.*, 1996). However, some studies found there to be no correlation between PTGS and methylation (van Blokland *et al.*, 1994; Goodwin *et al.*, 1996). Methylation and the commonality between transcriptional silencing and PTGS have been brought together recently in a detailed model (Wasseneger and Pélissier, 1998) which proposes that both silencing mechanisms result from the action of small antisense RNAs made on aberrant RNA templates in the cytoplasm.

One problem for the verification of previous functional models of co-suppression is that experimentation has been based upon plant lines with transgenes at different loci and with varying copy number, and where co-suppression was established very early in development. In virus-induced PTGS, virus replication triggers homology-dependent co-suppression of expressed genes such that it can be monitored in a single transgenic line and related to the multiplication and location of the virus. Both plant DNA (Kjemtrup *et al.*, 1998) and RNA viruses (Lindbo *et al.*, 1993; Kumagai *et al.*, 1995; Guo and Garcia, 1997;

Jones *et al.*, 1998; Ruiz *et al.*, 1998) have been shown to promote silencing. Since all plant RNA viruses replicate in the cytoplasm, an important question arises as to whether there is communication between cytoplasmic RNA and homologous nuclear DNA. It is also unclear what the consequences of the communication might be in terms of genome modification that would lead to the triggering and long-term maintenance of the silenced state. Although sequence-specific RNA-directed *de novo* methylation of homologous transgenes has been reported following viroid replication in the nucleus of transgenic plants, no evidence for induced co-suppression was described (Wasseneger *et al.*, 1994).

We have used transgenic peas showing inducible PTGS by the potyvirus, pea seed-borne mosaic virus (PSbMV; Jones *et al.*, 1998), to investigate whether changes in transgene methylation are associated with the onset of silencing, and whether the spatial distribution of infection would permit this to be directed by a physical interaction between the virus-specific RNA and the nuclear transgene.

Results

Transgenic pea plants show co-suppression after virus infection

Five transgenic pea lines (#2, #4, #5, #31 and #35) with single-copy transgenes were generated previously (Jones *et al.*, 1998). The pea lines expressed the PSbMV replicase (Nlb) gene sequence downstream of the PSbMV 5'-untranslated region (UTR) between a double cauliflower mosaic virus (CaMV) 35S promoter and a CaMV poly(A) terminator. Two (#2 and #31) of the five lines exhibited an inducible resistance based upon PTGS when challenged with the homologous PSbMV isolate DPD1. This resistance was inducible in plants either hemi- or homozygous for the transgene. The features of this resistance are that, although the virus replicates in the inoculated leaf (Figure 1A, leaf 0) and spreads systemically to the first new leaves in parallel with infections in non-transgenic plants, the virus becomes increasingly restricted in its distribution in subsequently developing leaves by the onset of resistance. This phenomenon has been termed 'recovery' (Lindbo *et al.*, 1993). The recovered tissues were virus-free, and were resistant to challenge inoculation (Jones *et al.*, 1998). Typical for plant virus infections that exploit the phloem for systemic translocation, symptoms were absent from the first leaf above the inoculation site (Figure 1A, leaf +1), a leaf that had already made the developmental transition from photosynthetic sink to source. At 7 days post-inoculation, symptoms were seen first on the second (+2) pair of leaves above the inoculated leaf. In the resistant plants, the next two pairs of leaves (+3 and +4) above the inoculated leaf showed only partial symptom coverage (restricted to the distal portion of the leaf) after their emergence from the shoot whorl between 12 and 15 days post-inoculation. The fifth pair of leaves above the inoculated leaf in recovered plants were always symptom-free, as were all subsequently emerging leaves (Figure 1A). The phylotactic and spatial organization of the virus-free areas was indicative of the development of resistance in parallel with leaf development and therefore suggested that resistance was established at or close to the shoot apical meristem.

Fig. 1. Co-suppression-mediated virus resistance in transgenic plants. (A) Transgenic (Nib) and non-transgenic (NT) plants were inoculated on the first expanded leaf (0) which later showed small chlorotic lesions. Leaf +1 remained uninfected and was asymptomatic. Leaf +2 was completely infected and showed a complete coverage with chlorosis (shaded portion). Leaves +3 and +4 of the transgenic lines #2 and #31 showed the beginning of the recovery phenomenon as a restriction of symptoms and virus accumulation to the distal portion of the leaf. Leaf +5 (and all subsequent leaves) was fully resistant. (B) Northern blot analysis of duplicate RNA samples from separate uninfected #2 plants (lanes 1), PSbMV-infected non-transformed pea (lanes 2), PSbMV infected +2 leaves of line #2 plants (lanes 3), chlorotic portions of +3 leaves of line #2 plants (lanes 4) and symptomless portions of +3 leaves of line #2 plants (lanes 5). A probe specific for the entire Nib coding region was used, and detects both PSbMV viral RNA and transgene RNA. PSbMV RNA is visible as a high-molecular-weight virus genomic RNA (PSbMV) and several smaller bands (PSbMV*); transgene mRNA is indicated (Nib). An equal amount of RNA (10 µg) [illustrated by the abundance of rRNAs in (C)] was loaded per lane.

To monitor the effect of resistance on the accumulation of the viral RNA and transgene mRNA, total RNA samples isolated from uninfected, PSbMV DPD1-infected and the chlorotic and symptomless areas of partially recovered leaves were subjected to Northern blot analysis with a PSbMV Nib probe (Figure 1B). In the +2 leaves of line #2 plants (lanes 3), PSbMV RNA accumulated to the same level as in non-transgenic plants (lanes 2). In the same leaves, the level of transgene RNA accumulation was similar to that seen in non-infected line #2 plants (compare lanes 1 and 3). However, in partially recovered leaves (+3 and +4), the chlorotic area showed a slightly lower level of virus replication and reduced transgene accumulation (lanes 4). The symptomless areas in all the leaves showed no viral RNA accumulation (lanes 5), although prolonged exposure of the autoradiograph revealed a very low level of transgene mRNA (data not shown).

Methylation only occurs in plants that exhibit co-suppression

To assess whether co-suppression following virus infection is associated with an alteration in the methylation status of the transgene, genomic DNAs from the five transgenic pea lines were analysed using methylation-sensitive restriction enzymes. These lines differed in the expression of the transgene and in their ability to show induced PTGS. We showed previously (Jones *et al.*, 1998) that the triggering of resistance was independent of the level of transgene expression. DNA samples from +5/non-silenced (lines #4, #5 and #35) and +5/silenced (lines #2 and #31) leaves from infected plants, and uninfected leaves from all lines, were analysed using methylation-sensitive restriction enzymes. Three restriction enzyme digests using *AluI*, *HindIII* or *DraI* were performed and the results analysed using a probe specific for the entire Nib coding sequence. *AluI* is sensitive to methylation on both symmetrical and non-symmetrical cytosines, *HindIII* is sensitive to non-symmetrical cytosine methylation and *DraI* is not affected by cytosine methylation.

Pea lines #31 and #35 differ from lines #2, #4 and #5 in the structure of the transgene. In addition to the UTR and Nib sequence in lines #2, #4 and #5 (Figure 2D), lines #31 and #35 have a direct downstream repeat of the 3' half of the Nib gene (Figure 2E). The locations of *AluI*, *HindIII* and *DraI* sites within and around the Nib gene and the sizes of expected digestion products are shown in Figure 2D and E. The locations of sites for *AvaI*, another enzyme sensitive to methylation on symmetrical cytosines, are also shown.

Following digestion, identical patterns of hybridization were obtained for uninfected and infected samples of each line using the methylation-insensitive enzyme *DraI* (fragments of 0.45 and 1.68 kbp for lines #2, #4 and #5, and 0.45 and 2.5 kbp for lines #31 and #35; Figure 2C). In contrast, lines #2, and #31 showed a difference between the uninfected and infected/silenced samples following digestion with *AluI* (Figure 2A) or *HindIII* (Figure 2B). Digestion products accountable for only by methylation of sites within the UTR + Nib sequence were observed. Hence, in silenced samples, *AluI* fragments of 1.7 and 2.4 kbp and several smaller fragments were detected for lines #2 and #31, respectively. *HindIII* digestion of line #2 DNA should produce fragments of 0.98 and 1.48 kbp. However, an additional fragment of 2.46 kbp was detected in samples from silenced tissues. Similarly, *HindIII* digestion of silenced line #31 showed fragments of 2.3, 4.22 and 5.7 kbp in addition to the fragments of 0.82, 1.48 and 3.4 kbp detected in samples from uninfected tissues (Figure 2B). In both cases, the larger fragments can only be accounted for if the *HindIII* sites within the Nib sequence were methylated while the site within the *Agrobacterium tumefaciens* T-DNA downstream of the Nib sequence and that at the 3' end of the promoter remained unmethylated (Figure 2D and E). The other lines (#4, #5 and #35), which do not undergo PTGS, had identical hybridization patterns for samples from uninfected and infected tissue with all three enzymes (Figure 2). Thus, *de novo* methylation occurs only in plants that show inducible co-suppression of the transgene and only after virus infection.

Infected/silenced plants set seed normally. Progeny

Fig. 2. Methylation associated with co-suppressing plants. Southern blot analysis of DNA samples from mock-inoculated (–) and silenced/infected (+) leaves of transgenic lines #2, #31, #5, #35 and #4. DNA samples from +5 leaves were digested with (A) *AluI*, (B) *HindIII* and (C) *DraI* and probed with the Nib-specific probe. Only lines #2 and #31 show PTGS following infection. Sizes (in kbp) of relevant fragments are indicated. The transgene structure in lines #2, #4 and #5, and lines #31 and #35 differ; the structures are illustrated in (D) and (E), respectively. Right border (RB), double 35S promoter (2×35S), 5' UTR (5'), Nib coding region (Nib), direct repeat of the 3' half of the Nib gene (Ib) and CaMV poly(A) terminator (CaMV polyA). Restriction sites for *AluI* (AI), *AvaI* (Av), *DraI* (D) and *HindIII* (H) and expected digestion products are shown; sites have asterisks if they are subject to methylation. *AluI* restriction sites subject to methylation are shown by asterisks only. The size of the largest methylated *AluI* fragment (2.56 kbp) and fragments obtained from the digestion of unmethylated DNA by other enzymes are shown.

Fig. 3. Methylation of non-homologous sequences? Southern blot analysis of DNA samples from mock-inoculated (–) and silenced leaves (+) of line #2 plants. Samples were digested with *Ava*I, *Hind*III, *Dra*I and *Alu*I, and were from the same pool of digested DNA as those used in Figure 2. Probes used were chlorophyll *a/b*-binding (*cab*) protein cDNA and the 35S promoter, as indicated. *Alu*I digestion of *cab* results in products too small to resolve on this gel but showed no high molecular weight fragments after infection. The equivalent range of fragments for *Dra*I- and *Hind*III-digested DNA indicates that the *cab* gene was not methylated extensively before infection. *Alu*I digestion of the 2× 35S promoter results in fragments of 0.33 and 0.29 kbp.

seedlings express the NIB transgene again and are no longer resistant to PSbMV infection. As was the case for the parent plants, they will recover from infection. Thus gene silencing and induced resistance are not passed to the next generation (Jones *et al.*, 1998). To determine if this was reflected in a reversion of the methylation status to that seen in uninfected and unsilenced plants, DNA samples from the progeny of infected/silenced parental line #2 plants were compared with DNA samples from the progeny of uninfected parental plants and samples from silenced progeny tissue. *Alu*I digestion resulted in an identical unmethylated *Alu*I profile for samples from uninfected plants and from the progeny of silenced plants (not shown). As previously (Figure 2), *Alu*I digestion of DNA from silenced tissues showed extensive methylation within the transgenic PSbMV sequences (not shown).

Methylation is restricted to sequences homologous to PSbMV

To investigate whether increased methylation was a general feature of genes within silenced tissues and to determine whether the increased methylation extended beyond the homologous transgenic sequences, the same digested line #2 DNA samples from uninfected plants and recovered +5 leaves from infected plants (Figure 2), and a further sample digested with *Ava*I, were analysed using a host gene, *cab* (chlorophyll *a/b*-binding protein; Cashmore, 1984), the double 35S promoter or CaMV poly(A) terminator as probes. The *cab* gene is one of a gene family, which is highly expressed in photosynthetic tissues. Identical patterns of hybridization were observed for DNA samples from mock-inoculated and silenced leaves of line #2 plants after digestion with *Ava*I, *Hind*III and *Dra*I, and using the *cab* gene as a probe (Figure 3). *Alu*I digestion products of *cab* were difficult to detect as there are numerous *Alu*I recognition sites within the *cab* sequence which resulted in very small DNA fragments. However, in contrast to

Fig. 4. Methylation in relation to virus infection and PTGS. Southern blot analysis of line #2 plants using DNA samples prepared from (1) mock-inoculated unsilenced leaves, (2) infected +2 leaves and (3) fully recovered +5 leaves; a representative of four replicate experiments is shown. Samples were digested with *Alu*I, *Ava*I, *Hind*III and *Dra*I, as indicated. The probe used was specific to the NIB coding region. Sizes (in kbp) of relevant fragments are indicated. Partial digestion was a feature of all *Ava*I digests and gave rise to larger products that were accountable for by incomplete digestion at the *Ava*I site within the T-DNA just within the right border. It is possible that this *Ava*I site is methylated to some degree in all the samples.

the result obtained when the same samples were analysed using NIB as a probe (Figure 2A), no high molecular weight *Alu*I bands were observed (data not shown). Although the methylation status of the *cab* gene family before inoculation was not known, the absence of any increase in size of the many *Hind*III and *Alu*I fragments suggests that virus infection did not induce a widespread methylation of heterologous coding sequences. Identical patterns of hybridization for the two samples were also observed following *Alu*I digestion when the 35S promoter (Figure 3) or CaMV poly(A) sequence (data not shown) were used as probes. In digests of either DNA sample, the 35S probe detected fragments of 0.33 and 0.29 kbp, i.e. those expected if neither of the two sites within the promoter region (Figure 2D) were methylated. Hence, the extent of methylation appeared to be restricted to the UTR and NIB sequence, and did not extend into the double 35S promoter or the CaMV poly(A) terminator sequence.

Transgene methylation precedes the onset of co-suppression

The inducibility of PTGS in our transgenic pea plants provided the opportunity to relate transgene methylation spatially/temporally to silencing. In four replicated experiments, genomic DNA from plants of line #2 was prepared from uninfected (mock-inoculated), infected (prior to silencing; +2 leaves) or fully silenced (+5) leaves. The uninfected and silenced leaves were from separate, yet genetically identical plants at the same developmental stage. As before, four restriction enzyme digests using *Alu*I, *Ava*I, *Hind*III or *Dra*I were performed and the results analysed using a probe specific for the entire NIB coding sequence. The predicted hybridization pattern was observed for all three samples when digested with *Dra*I, and for all digests when using DNA from uninfected tissue (Figure 4). However, bands that could only be accounted for by incomplete digestion of the DNA with *Alu*I, *Ava*I and *Hind*III were present in the samples from +5 leaves. Surprisingly, incomplete digestion was also seen in DNA samples from +2 leaves (Figure 4, compare samples 1 with samples 2 and 3), albeit to a lesser extent.

These are leaves that do not show reduced viral RNA and transgene mRNA accumulation characteristic of PTGS (Figure 1). Hence, infection-induced methylation of the homologous DNA sequences was apparent prior to the onset of PTGS.

Virus indirectly triggers PTGS in the meristem

Methylation occurs during the S phase of mitosis by the action of DNA methyltransferases (Leonhardt *et al.*, 1992; discussed in Adams, 1995). At mitosis, the dissolution of the nuclear membrane provides the possibility for the direct physical interaction between an RNA usually restricted to the cytoplasm and homologous genomic sequences. Since PTGS is absolutely dependent upon an initial virus infection and is associated with newly emerging leaves, we wished to determine whether PSbMV spreads through the plant to reach the sites of highest mitotic activity where it could induce methylation of the transgene. These are the meristematic tissues and, possibly, parts of the developing dicot leaf where cell division continues until ~50% leaf expansion is achieved (Poethig and Sussex, 1985).

To obtain information about the location of the virus during infection and recovery, *in situ* hybridization experiments were performed. The particular questions we addressed were whether or not the virus enters the meristematic region and whether the alteration in transgene expression correlated with the distribution of PSbMV accumulation. Shoot apices were harvested 7 and 16 days post-inoculation from PSbMV-inoculated transgenic line #2, non-transgenic line #2 F₂ segregants and non-transformed pea plants. Apices were also harvested from non-inoculated plants of the same line at identical time points. Tissues were fixed, embedded, sectioned longitudinally and subjected to *in situ* hybridization. Hybridization probes were chosen such that the accumulation of viral RNA could be distinguished from transgene mRNA; an Nlb-specific probe was used to detect both viral and transgene RNA, and a virus-specific probe (cylindrical inclusion; CI gene) was used to detect viral RNA alone. By probing near consecutive sections from the same tissue with the Nlb and CI probes, the location of the virus could be distinguished on a comparative basis from the location of the transgene mRNA alone.

The distribution of PSbMV during a normal infection of non-transformed pea was shown with either the Nlb or CI probes in comparison with the background labelling obtained with uninfected plants (Figure 5). At 7 days post-inoculation, the virus was present in the outer expanding leaves and the vasculature of the stem. No virus was detectable in the meristematic regions or youngest developing leaves (Figure 5C and D). The pattern of hybridization (which indicates the location of virus accumulation) was almost identical for the two probes; the slight differences are likely to be due to the probing of near, but not strictly consecutive, sections. At 16 days post-inoculation (Figure 5E and F), virus distribution was essentially similar to that at 7 days. The virus, although present over a greater leaf area than at 7 days, was still restricted to tissue with a developed vasculature and was not detected in meristematic tissue or youngest developing leaves. Our failure to detect viral RNA by *in situ* hybridization accords with our inability to detect viral RNA by sensitive RT-PCR techniques in silenced leaves or shoot apices (Jones

Fig. 5. Distribution of viral RNA during infection of non-transgenic plants. Sections shown in (A and B), (C and D) and (E and F) were from apices of mock-inoculated, 7 day-infected (+7d) and 16 day-infected (+16d) plants, respectively. Sections were subjected to *in situ* hybridization with probes to detect positive sense Nlb (A, C and E) and CI (B, D and F) RNA. Outer leaves (O) and the region of the shoot meristem (M) are illustrated in (A) and (B). Note that all the sections were processed for the same period of time and are directly comparable. Bar = 2 mm.

et al., 1998; data not shown). Experiments performed on infected non-transgenic segregants of line #2 gave an equivalent pattern of virus accumulation to that recorded for non-transformed pea (data not shown).

In line with the constitutive expression of the CaMV 35S promoter (Odell *et al.*, 1985), Nlb transgene mRNA accumulation in an uninfected line #2 plant was seen throughout the tissue, although a stronger hybridization signal was associated with the vasculature and regions with densely packed cells, i.e. developing leaves (compare

Fig. 6. Distribution of viral RNA and transgene RNA during infection of co-suppressing plants. Sections shown in (A and B), (C and D) and (E and F) were from apices of mock-inoculated, 7 day-infected and 16-day infected line #2 plants, respectively. Sections were subjected to *in situ* hybridization with probes to detect positive sense Nlb (A, C and E) and CI (B, D and F) RNA. Small spots of non-specific signal (B, compare with equivalent section in Figure 5B) were seen occasionally but were easily distinguished from the specific signal. Detail of (A) and (C) is shown in Figure 7. Note that all the sections were processed for the same period of time and are directly comparable. Bar = 2 mm.

Figure 6A with Figure 5A). As expected, only background hybridization was obtained for parallel sections analysed with the CI probe (Figure 6B). At 7 days post-inoculation, virus was detected in the stem and older leaves of line #2

Fig. 7. Expanded view of the relative distribution of transgene and viral RNA. Panels are magnified images from Figure 6A and C. Arrows indicate axillary buds in which silencing of the Nlb transgene is less pronounced. The locations of the shoot apical meristem (M), the youngest three leaf primordia (* in A) and the vasculature (V) are indicated. Bar = 1 mm.

transgenic plants. The strength and pattern of hybridization obtained for the Nlb (Figure 6C) and CI probes (Figure 6D) was similar, and suggested that the majority of signal was due to presence of the virus. It also suggested that as early as 7 days post-inoculation there had been a reduction in transgene mRNA accumulation (compare Figure 7A and B in which both tissues were analysed with the Nlb probe), at least in uninfected cells. In two axillary meristems (Figure 7B, arrows), there appeared to be some transgene expression remaining. However, all other regions had substantially reduced levels of Nlb expression. This included the shoot apical meristem. At 16 days post-inoculation, the virus was very localized and only detected in the tip of one of the older leaves (Figure 6E and F). Newly emerging leaves contained no virus and had very reduced levels of Nlb mRNA.

Discussion

We have used the induction of gene silencing by a plant virus to study the relationship between the onset of the PTGS in leaves and *de novo* DNA methylation of a homologous transgene. The induction of PTGS, as evidenced by the development of virus resistance and a substantial reduction in the steady-state levels of transgene mRNA, was monitored such that a single transgene locus could be assessed prior to, and following the onset of, silencing. By comparing transgenic lines that do and do not show inducible virus resistance, *de novo* methylation was found to be correlated with the ability to show PTGS rather than with virus replication *per se*.

Information concerning the control and creation of patterns of methylation in eukaryotes is limited (Bestor and Tycko, 1996), although more is known concerning the maintenance of existing patterns rather than of *de novo* methylation events. Sequences CpG and CpNpG have strand symmetry and it is this symmetry that is thought to allow maintenance of cytosine methylation patterns after DNA replication (reviewed in Adams, 1995). Methylation of non-symmetrical sites in plants, as observed here and in a few other species (Ingelbrecht *et al.*, 1994; Meyer *et al.*, 1994; Jacobsen and Meyerowitz, 1997), must involve *de novo* methylation events at each round of DNA replication.

We have detected methylation on all the symmetrical and non-symmetrical sites tested and shown that it was restricted to the region of homology between virus and transgene. It was distributed throughout the region of homology, was extensive in silenced leaves and was also detected to a lesser extent in infected leaves of the same plants. In some of the analyses, <100% methylation of homologous sequences in silenced tissues was observed. This could be accounted for by the imperfect maintenance of patterns of methylation by DNA methyltransferases (Silva *et al.*, 1993; Goyon *et al.*, 1994).

RNA-directed *de novo* methylation

How could methylation be initiated following infection by a cytoplasmically replicating virus? We provide evidence of *de novo* methylation occurring as a consequence of the presence of homologous RNA that is normally restricted to the cytoplasm. The implication of our results is that sequence-specific communication between cytoplasmic RNA and nuclear DNA is possible, and that it affects both methylation and gene expression. It has been shown previously that RNA-directed *de novo* methylation of transgenic viroid sequences in plants can occur (Wasseneger *et al.*, 1994), although viroid replication in the nucleus means that there is a clear potential for direct RNA–DNA interaction. For PSbMV, either viral RNA (single- or double-stranded, or fragments thereof) must enter the nucleus in somatic cells, or a direct interaction with homologous DNA could be potentiated in mitotic cells following dissolution of the nuclear membrane. Wasseneger and Péliissier (1998) proposed a model for co-suppression whereby methylation may be directed by the diffusion or transport into the nucleus of short antisense RNAs formed in the cytoplasm by the action of a host-encoded RNA-dependent RNA polymerase on template aberrant RNAs (e.g. viral or transgene mRNA). The

patterns of methylation, PTGS and PSbMV resistance we have described point to the shoot apex, and probably mitotic cells, as the site for such an interaction. It is known that DNA methyltransferases recognize unusual DNA structures (Bestor, 1987; Smith *et al.*, 1991; Chen *et al.*, 1995), and in RNA-directed *de novo* methylation these may be DNA–RNA hybrids or triple helices.

Methylation as a cause or consequence of gene silencing

A key question is whether methylation represents a cause or consequence of the change in gene expression, or whether methylation and PTGS represent separate pathways triggered by a common event. This has been complicated recently by the suggestion that PTGS can be divided into discrete triggering and maintenance phases (Ruiz *et al.*, 1998). In addition to the strong correlation between PTGS and methylation in virus-resistant leaves, the observation that methylation was detected in +2 leaves before PTGS points to a causal relationship. This conclusion is based upon the absence of any detectable reduction in virus accumulation in +2 leaves. It is conceivable, however, that methylation and silencing occur in parallel in a few isolated cells that would not have been identified in these experiments. In a causal relationship, we can speculate that the methylation might result in locally altered chromatin structure and the formation of aberrant RNAs identifiable as initiators of PTGS by as yet unknown host factors.

Although different organisms have different mechanisms to maintain patterns of gene expression (reviewed in Wolffe, 1994), evidence from *Neurospora crassa* strain dim2 (Cogoni *et al.*, 1996) argues, to the contrary, that methylation may not be a cause of silencing. This strain shows quelling (a phenomenon related to PTGS), although it is defective in cytosine methylation. Ideally, plants unable to methylate cytosines at symmetrical and non-symmetrical sites on the target sequence should be tested in order to answer more directly the question of cause or consequence.

Evidence for remote triggering of virus-induced gene silencing

Methylation probably occurs immediately after DNA replication when the DNA is accessible by the DNA methyltransferase (Leonhardt *et al.*, 1992; Adams, 1995). In plants, this would be within the shoot apex and newly emerging leaves. However, we found that PSbMV was unable to invade the shoot meristem, being tightly restricted to the developed vasculature from where it invaded new leaves during development. Hence, it was surprising that there was a marked reduction in the transgene mRNA throughout the shoot meristem even though the virus could not enter this tissue. The best interpretation of this is that in non-infected cells of shoot apex, PTGS is directed by a sequence-specific diffusible signal generated initially in infected cells. Since silencing occurs in the shoot meristem where the methylation pattern is likely to be set, methylation could actually be directed by the signal rather than by the virus. Evidence for a systemic signal for gene silencing has been shown in other systems that give rise to PTGS (Palaqui *et al.*, 1997; Voinnet and Baulcombe, 1997; Kjemtrup *et al.*, 1998), but in none of these examples is silencing triggered by a

cytoplasmic infectious agent or does the signal have the capacity to act in the tissues of the shoot apex. The nature of this signal is the subject of much attention at present and will probably be crucial to the understanding of silencing in plants, animals and other organisms.

The relationship between cytoplasmic RNA, gene silencing and methylation

Our data strongly suggest that cytoplasmic RNA is able to induce epigenetic changes. Through these studies, a relationship between the RNA-mediated induction of PTGS, *de novo* methylation of DNA and cell division can be envisaged. In the uninfected plants, the transgene is unmethylated. Following inoculation, the virus replicates, then moves through the vasculature and into young expanding leaves. Cell division probably continues to occur in the young leaf until it reaches 50% of full expansion. Therefore, there would be occasional potential for direct interaction between the viral RNA (or fragments thereof) and the transgene DNA, and thereby the induction of methylation. The production of a diffusible signal that moves from cell to cell, presumably through plasmodesmata, could be a consequence of this interaction. The action of the signal sets PTGS and, potentially, transgene methylation in the shoot apex (meristem and leaf primordia not accessed by the virus). All new tissues would then show DNA methylation and PTGS, seen in our case as virus resistance. Subsequent generations derived from the silenced plants do not show PTGS or DNA methylation. This is not unexpected since meiotic resetting of patterns of gene expression and methylation has been observed previously for other epigenetic phenomena in plants (Dehio and Schell, 1994).

The fundamental difference between the work reported here and other studies of PTGS and methylation (Ingelbrecht *et al.*, 1994; English *et al.*, 1996; Sijen *et al.*, 1996) is that here the silenced state is not pre-set but can be induced and studied in a reproducible way in tissues that are amenable to both molecular and cell biological studies. If, as is being popularly proposed (Bingham, 1997; Wasseneger and Pelissier, 1998), transcriptional and PTGS in higher organisms are mechanistically related, and if the processes are fundamental to genetic control of endogenes and transgenes, then inducible phenomena may be essential to dissection of the underlying processes. In plants, induction may be achieved by viruses, as described here, or by the localized introduction of ectopic homologous sequences (Voinnet *et al.*, 1998). Our observation that cytoplasmic viral RNA can affect methylation of homologous nuclear sequences may have implications for feedback mechanisms between the cytoplasm and the nucleus to control the expression of endogenous genes.

Materials and methods

Plant lines and viral isolates

The transgenic pea lines carrying the Nib gene of PSbMV DPD1 and virus inoculation have been described previously (Jones *et al.*, 1998).

RNA extraction and Northern blotting

Extraction of total RNA was achieved using RNA Isolator (Genosys) according to the manufacturer's instructions. RNA gel electrophoresis and Northern blotting were performed as described previously (Jones *et al.*, 1998). A probe produced from the PSbMV Nib 1.56 kbp PCR

fragment (described below) was used to detect both viral and Nib transgene RNA.

DNA extraction and Southern blotting

Genomic DNA was extracted from pea leaves according to Ellis (1994). Southern blotting was performed as described previously (Jones *et al.*, 1998). Random-primed [³²P]DNA probes were produced from the double 35S promoter and CaMV poly(A) terminator sequence derived from pJIT60 (Guerineau *et al.*, 1992), a 1.56 kbp PCR product consisting of the entire PSbMV Nib gene and a 1.0 kbp cDNA clone of cab protein (Cashmore, 1984).

Generation of probes for *in situ* hybridization

Standard molecular biology techniques were used throughout. A plasmid construct carrying the Nib gene of PSbMV DPD1 (nucleotides 7328–8888; Johansen *et al.*, 1991) was made by ligation of *Bam*HI-digested pBluescript SK+ (Stratagene, La Jolla, CA) with a *Bam*HI-digested PCR fragment generated using primers A: 5'-TCATGGATCCATGCGGGATGACGCATGGCTAGAG-3' and B: 5'-TAATCGGATCCAA-TCGAACCTTGATTGATCCATC-3' (*Bam*HI sites underlined) and template pPS13 (Johansen *et al.*, 1991). A construct carrying the CI gene (nucleotides 3941–5849; Johansen *et al.*, 1991) of PSbMV was made by ligating *Bam*HI-*Xba*I-digested pBluescript SK+ with a *Bam*HI-*Xba*I-digested PCR fragment generated using primers C: 5'-GTTGG-ATCCATGAGTATTGATGATATTCATGATG-3' and D: 5'-AACTT-CTAGATTTACTGACAACGCACTGTCTCAGC-3' (restriction sites underlined).

For the transcription reactions, plasmids were linearized downstream of the inserted sequence, and positive and negative sense probes were generated by standard *in vitro* transcription conditions (Jackson, 1991) using digoxigenin-11-UTP, and T3 and T7 RNA polymerase.

***In situ* hybridization**

Methods for fixing, embedding and processing of pea shoot apices for *in situ* hybridization used techniques described previously (Wang and Maule, 1994, 1995). Experiments were repeated three times with 4–6 apices; results were always consistent. Under the conditions used, positive sense probes gave only background signals for either infected, uninfected, transgenic or non-transgenic tissues.

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