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34. We thank J. Doyle, P. Endress, L. Thein, P. Soltis, S. Graham, Y.-L. Qiu, J. Palmer, and C. dePamphilis for helpful discussions and for sharing unpublished data, and C. Soohoo and C. Davis for technical support. Financial support was provided by NSF grant DEB-9806937.

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# A Species of Small Antisense RNA in Posttranscriptional Gene Silencing in Plants

**Andrew J. Hamilton and David C. Baulcombe***

**Posttranscriptional gene silencing (PTGS) is a nucleotide sequence–specific defense mechanism that can target both cellular and viral mRNAs. Here, three types of transgene-induced PTGS and one example of virus-induced PTGS were analyzed in plants. In each case, antisense RNA complementary to the targeted mRNA was detected. These RNA molecules were of a uniform length, estimated at 25 nucleotides, and their accumulation required either transgene sense transcription or RNA virus replication. Thus, the 25-nucleotide antisense RNA is likely synthesized from an RNA template and may represent the specificity determinant of PTGS.**

Posttranscriptional gene silencing occurs in plants and fungi transformed with foreign or endogenous DNA and results in the reduced accumulation of RNA molecules with sequence similarity to the introduced nucleic acid (*1*, *2*). Double-stranded RNA induces a similar effect in nematodes (*3*), insects (*4*), and protozoa (*5*). PTGS can be suppressed by several virus-encoded proteins (*6*) and is closely related to RNA-mediated virus resistance and cross-protection in plants (*7*, *8*). Therefore, PTGS may represent a natural antiviral defense mechanism and transgenes might be targeted because they, or their RNA, are perceived as viruses. PTGS could also represent a defense system against transposable elements and may function in plant development (*9*–*11*).

To account for the sequence specificity and posttranscriptional nature of PTGS, it has been proposed that antisense RNA forms a duplex with the target RNA, thereby promoting its degradation or interfering with its translation (*12*). If these hypothetical antisense RNA molecules are of a similar size to typical mRNAs, they would have been readily detected by routine RNA analyses. However, there have been no reports of such antisense RNA that is detected exclusively in plants or animals exhibiting PTGS. Nevertheless, PTGS-specific antisense RNA may exist, but may be too short for easy detection. We carried out analyses specifically to detect low molecular weight antisense RNA in four classes of PTGS in plants (*13*). The first class tested was transgene-induced PTGS of an endogenous gene ("cosuppression"). We used five tomato lines (T1.1, T1.2, T5.1, T5.2, and T5.3), each transformed with a tomato 1-aminocyclopropane-1-carboxylate oxidase (ACO) cDNA sequence placed downstream of the cauliflower mosaic virus 35*S* promoter (35*S*). Two lines (T5.2 and T5.3) exhibited PTGS of the endogenous ACO mRNA (Fig. 1A). Low molecular weight nucleic acids purified from the five lines were separated by denaturing polyacrylamide gel electrophoresis, blotted, and hybridized to an ACO sense (antisense-specific) RNA probe (Fig. 1B). A discrete, ACO antisense RNA (*14*) of 25 nucleotides (nt) was present in both PTGS lines but absent from the nonsilencing lines. Twenty-five–nucleotide ACO RNA of sense polarity and at the same abundance as the 25-nt ACO antisense RNA was also present only in the PTGS lines (Fig. 1C).

PTGS induced by transgenes can also occur when a transgene does not have homology to an endogenous gene (*1*). Therefore, we tested whether this type of PTGS was also associated with small antisense RNA. We analyzed three tobacco lines carrying 35*S*-β-glucuronidase (GUS) transgenes. Two of these lines, T4 (*15*) and 6b5 (*16*), exhibited PTGS of GUS. The third line (6b5×271) tested was produced by crossing 6b5 with line 271 (*17*), in which there is a transgene suppressor of the 35*S* promoter in 6b5. There was no PTGS of GUS in 6b5×271 because of the transcriptional suppression of the 35*S* GUS transgene (*18*). Hybridization with a GUS-specific probe revealed that low molecular weight GUS antisense RNA was present in T4 and 6b5 (Fig. 2, lanes 1 and 2) but absent from line 6b5×271 (Fig. 2, lane 3). The amount of antisense RNA correlated with the extent of PTGS: Line 6b5 has stronger PTGS of GUS than line T4 (*18*) and had more GUS antisense RNA (Fig. 2).

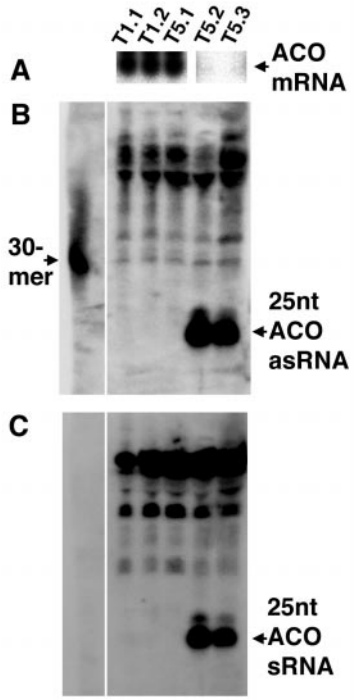


**Fig. 1.** Twenty-five–nucleotide ACO antisense and sense RNA in PTGS lines. (**A**) Endogenous ACO mRNA abundance in five tomato lines containing 35*S*-ACO transgenes. ACO mRNA was amplified by reverse transcriptase–polymerase chain reaction and detected by hybridization with labeled ACO cDNA. (**B** and **C**) Low molecular weight RNA from the same five lines and a 30-nt ACO antisense RNA were fractionated, blotted, and hybridized with either ACO sense RNA (**B**) or antisense RNA (**C**) transcribed from full-length ACO cDNA. The low hybridization temperature permitted some nonspecific hybridization to tRNA and small ribosomal RNA species, which constitute most of the RNA mass in these fractions. The oligonucleotide hybridized only to the antisense-specific probe (**B**). Twenty-five–nucleotide, PTGS-specific RNA is indicated.

Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK.

*To whom correspondence should be addressed. E-mail: david.baulcombe@bbsrc.ac.uk

As for PTGS of ACO in tomato, the GUS antisense RNA was a discrete species of ~25 nt.

In some examples of PTGS, silencing is initiated in a localized region of the plant. A signal molecule is produced at the site of initiation and mediates systemic spread of silencing to other tissues of the plant (*19*, *20*). We investigated whether systemic PTGS of a transgene encoding the green fluorescent protein (GFP) is associated with 25-nt GFP antisense RNA. PTGS was initiated in *Nicotiana benthamiana* expressing a GFP transgene by infiltration of a single leaf with *Agrobacterium tumefaciens* containing GFP sequences in a binary plant transformation vector (*19*). Two to 3 weeks after this infiltration, the GFP fluorescence disappeared owing to systemic spread of PTGS as described (*11*, *20*). We detected 25-nt GFP antisense RNA in systemic tissues exhibiting PTGS of GFP. It was not detected in equivalent leaves of plants that had not been infiltrated or in nontransformed plants that had been infiltrated with *A. tumefaciens* (Fig. 3).

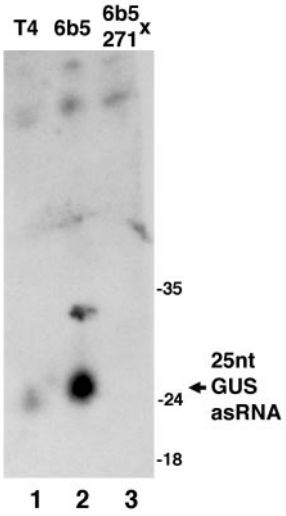


**Fig. 2.** Twenty-five–nucleotide antisense GUS RNA is dependent on transcription from the 35*S* promoter. Twenty-five–nucleotide GUS antisense RNA was detected by hybridization with hydrolyzed GUS sense RNA transcribed from the 3′ 700 base pairs of the GUS cDNA.

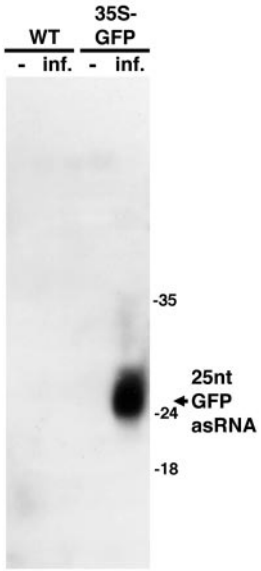


**Fig. 3.** Twenty-five–nucleotide antisense GFP RNA in systemically silenced tissue. Lower leaves of untransformed *N. benthamiana* (WT) and *N. benthamiana* carrying an active 35*S*-GFP transgene (35*S*-GFP) were infiltrated with *A. tumefaciens* containing the same 35*S*-GFP transgene in a binary vector. RNA from upper, noninfiltrated leaves of these plants (inf.) and from equivalent leaves of noninfiltrated plants (−) was hybridized with GFP sense RNA transcribed from a full-length GFP cDNA. Only the transgenic *N. benthamiana* infiltrated with the *A. tumefaciens* accumulated 25-nt GFP antisense RNA.

A natural manifestation of PTGS is the RNA-mediated defense induced in virus-infected cells (*8*). Therefore we investigated whether virus-specific, 25-nt RNA could be detected in a virus-infected plant. Twenty-five–nucleotide RNA complementary to the positive strand (genomic) of potato virus X (PVX) was detected 4 days after inoculation of *N. benthamiana* and continued to accumulate for at least another 6 days in the inoculated leaf (Fig. 4). Twenty-five–nucleotide PVX RNA accumulated to a similar extent in systemically infected leaves but was not detected in mock-inoculated leaves.

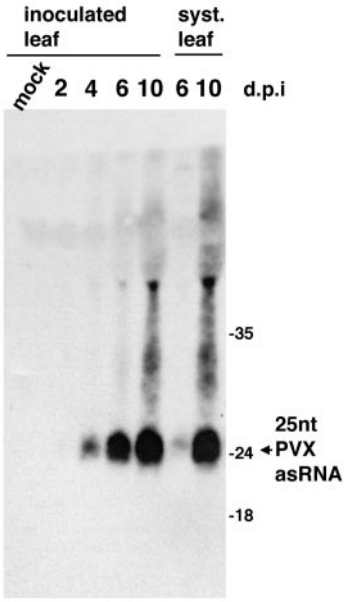


**Fig. 4.** Twenty-five–nucleotide antisense PVX RNA accumulates during virus replication. RNA was extracted from inoculated leaves after 2, 4, 6, and 10 days and from systemic (syst.) leaves after 6 and 10 days (d.p.i.: days post inoculation). RNA was extracted from mock-inoculated leaves after 2 days. Twenty-five–nucleotide PVX antisense RNA was detected by hybridization with PVX sense RNA transcribed from a full-length PVX cDNA.

Thus, 25-nt antisense RNA, complementary to targeted mRNAs, accumulates in four types of PTGS. We have detected 25-nt RNA in other examples of PTGS (*22*), and never detected 25-nt RNA in the absence of PTGS. This correlation and the properties of 25-nt RNA are consistent with a direct role for 25-nt RNA in PTGS induced by transgenes or viruses (*12*). Twenty-five–nucleotide RNA species also serve as molecular markers for PTGS. Their presence could be used to confirm other examples of transgene- or virus-induced PTGS and perhaps also to identify endogenous genes that are targeted by PTGS in nontransgenic plants.

The 25-nt antisense RNA species are not degradation products of the target RNA because they have antisense polarity. A more likely source of these RNAs is the transcription of an RNA template. This is consistent with the presence of the 25-nt PVX RNA in PVX-infected cells that do not contain a DNA template (Fig 4, "syst. leaf"). The dependency of 25-nt GUS antisense RNA accumulation on sense transcription of a GUS transgene also supports the RNA template model (Fig. 2). An RNA-dependent RNA polymerase, as required by this model, is required for PTGS in *Neurospora crassa* (*23*). With the present data, we cannot distinguish whether the antisense RNA is made directly as a 25-nt species or as longer molecules that are subsequently processed. The precise role of 25-nt RNA in PTGS remains to be determined. However, because they are long enough to convey sequence specificity yet small enough to move through plasmodesmata, it is possible that they are components of the systemic signal and specificity determinants of PTGS.

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13. Total RNA was extracted from leaves of tomato, tobacco, and *N. benthamiana* as described [E. Mueller, J. E. Gilbert, G. Davenport, G. Brigneti, D. C. Baulcombe, *Plant J.* **7**, 1001 (1995)]. From these preparations, low molecular weight RNA was enriched by ion-exchange chromatography on Qiagen columns after removal of high molecular weight species by precipitation with 5% polyethylene glycol 8000–0.5 M NaCl (for tobacco and *N. benthamiana*) or by filtration through Centricon 100 concentrators (Amicon) (for tomato). Low molecular weight RNA was separated by electrophoresis through 15% polyacrylamide–7 M urea–0.5× tris-borate EDTA gels, transferred onto Hybond Nx filters (Amersham), and fixed by ultraviolet cross-linking. Prehybridization was in 45% formamide, 7% SDS, 0.3 M NaCl, 0.05 M $Na_2HPO_4$–$NaH_2PO_4$ (pH 7), 1× Denhardt's solution, and sheared, denatured, salmon sperm DNA (100 mg/ml) at between 30° and 40°C. Hybridization was in the same solution with single-stranded RNA probes transcribed with $\alpha$-$^{32}$P-labeled uridine triphosphate. Before addition to the filters in the prehybridization solution, probes were hydrolyzed to lengths averaging 50 nt. Hybridization was for 16 hours at 30°C (ACO probes), 35°C (GUS probe), or 40°C (GFP and PVX probes). Sizes of RNA molecules were estimated by comparison with $^{33}$P-phosphorylated DNA oligonucleotides run on the same gels but imaged separately. Additionally, samples from different types of PTGS, including those shown, were frequently run on the same gel. Alignment of the filters after hybridization with different specific probes confirmed that the PTGS-specific signals were identical in size. The probes used are in each case sequence specific. We have observed no cross-hybridization between 25-nt signals in different PTGS systems using either filter hybridization or RNAase protection (www.sciencemag.org/feature/data/1042575.shl). We do not have an exact measurement of the amount of 25-nt RNA per cell, but given the short exposure times routinely used to detect these molecules and taking into account their size, they are likely to be abundant in cells exhibiting PTGS.
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21. A high-titer, synchronized PVX infection on leaves of untransformed *N. benthamiana* was initiated by infiltration of single leaves with *A. tumefaciens* containing a binary plasmid incorporating a 35*S*-PVX-GFP sequence. Once transcribed, the PVX RNA replicon is independent of the 35*S*-PVX-GFP DNA, replicates to high levels, and moves systemically through the plant. The *A. tumefaciens* does not spread beyond the infiltrated patch and is not present in systemic leaves (*20*). The GFP reporter in the virus was used to allow visual monitoring of infection progress. We have obtained similar signals with wild-type PVX inoculated as virions in sap taken from an infected plant.
22. The other examples of PTGS tested were in *N. benthamiana* (spontaneous silencing of a 35*S*-GFP transgene), tomato (35*S*-ACO containing an internal direct and inverted repeat), petunia (cosuppression of chalcone synthase transgenes and endogenes), and *Arabidopsis thaliana* (PTGS of 35*S*-GFP by a 35*S*-PVX-GFP transgene).
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# TCR-Mediated Internalization of Peptide-MHC Complexes Acquired by T Cells

**Jing-Feng Huang,[1] Young Yang,[1] Homero Sepulveda,[1] Weixing Shi,[1] Inkyu Hwang,[2] Per A. Peterson,[1] Michael R. Jackson,[1] Jonathan Sprent,[2] Zeling Cai[1]***

**Peptide–major histocompatibility complex protein complexes (pMHCs) on antigen-presenting cells (APCs) are central to T cell activation. Within minutes of peptide-specific T cells interacting with APCs, pMHCs on APCs formed clusters at the site of T cell contact. Thereafter, these clusters were acquired by T cells and internalized through T cell receptor–mediated endocytosis. During this process, T cells became sensitive to peptide-specific lysis by neighboring T cells (fratricide). This form of immunoregulation could explain the "exhaustion" of T cell responses that is induced by high viral loads and may serve to down-regulate immune responses.**

T cell responses are initiated by T cell receptor (TCR) recognition of pMHCs on APCs (*1*). Upon specific interaction of T cells with APCs, TCR and MHC molecules are assembled at the center of supramolecular activation clusters (*2*). The fate of these TCR-MHC clusters at the T cell–APC contact site is unclear. However, it is known that interaction of TCRs with pMHC complexes is followed by TCR down-regulation (*3*) and that T cell–APC interaction can cause APC-derived surface molecules to adhere to the surface of T cells (*4*).

To investigate the fate of MHC clusters at the T cell–APC contact sites, we used *Drosophila*, RMA-S, and dendritic cell lines expressing MHC class I ($L^d$)–green fluorescent protein fusion molecules ($L^d$-GFP) (*5*) as APCs to activate CD8 T cells from the 2C TCR transgenic mouse line (*6*, *7*). 2C T cells recognize $L^d$ plus either QL9 peptide or a closely related peptide, p2Ca (*8*). Empty $L^d$-GFP molecules expressed on the surface of RMA-S and *Drosophila* cells can be efficiently loaded with exogenous peptides (*7*, *9*). Within 5 min of 2C T cells interacting with either *Drosophila* cells (Fly.$L^d$-GFP) or RMA-S cells (RMA-S.$L^d$-GFP) plus the QL9 peptide, $L^d$-GFP molecules formed large clusters at the T cell–APC contact site [Fig. 1A and Web figure 1 (*10*)]. After 30 min, the $L^d$-GFP clusters at the T cell–APC interface decreased in size, and small aggregates of $L^d$-GFP appeared concomitantly within 2C T cells at sites distal to the contact site [Fig. 1, B and C, Web figure 2 (*10*)]. This process was not seen with P1A (*11*) (Fig. 1D), a control peptide that binds to $L^d$ but is not recognized by 2C TCR (*7*). Aggregates of $L^d$-GFP were also detected inside 2C T cells after interaction with the $L^d$-GFP–transfected dendritic cell line DC2.4 (*12*) (Fig. 1E), which presents endogenous p2Ca peptide. To exclude the possibility that GFP itself could mediate the acquisition of $L^d$ by T cells, we used *Drosophila* cells expressing untagged $L^d$ (no GFP) loaded with QL9 peptide as APCs. After culture for 1 hour, multiple aggregates of $L^d$ molecules were detected in 2C T cells by intracellular staining with monoclonal antibody (mAb) to $L^d$ (*13*) (Fig. 1F). Thus, in the presence of antigenic peptides, $L^d$ molecules can be acquired from APCs by T cells, and this acquisition is independent of GFP.

Acquisition of APC-derived $L^d$ molecules by 2C T cells was further demonstrated by immunoprecipitation (Fig. 1, G and H). After culture with $^{35}$S-methionine–labeled fibroblasts (L cells) transfected with $L^d$ (L-$L^d$) (*14*), 2C T cells were highly purified and immunoprecipitation was performed with mAbs to class I molecules. Immunoprecipitation of $L^d$ from 2C T cells cultured with L-$L^d$ plus QL9 peptide was prominent (Fig. 1G). In the presence of the control P1A peptide (*11*), precipitation of $L^d$ was limited, but clearly detectable, presumably due to the presentation of endogenous p2Ca peptide in L-$L^d$ cells. In contrast, other MHC class I molecules ($K^k$ and $D^k$) expressed by L cells were not detectable in 2C T cells by immunoprecipitation (Fig. 1G). The peptide-

[1]R. W. Johnson Pharmaceutical Research Institute, 3210 Merryfield Row, San Diego, CA 92121, USA. [2]Department of Immunology, IMM4, Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

*To whom correspondence should be addressed. E-mail: zcai@prius.jnj.com