A Calmodulin-Related Protein That Suppresses Posttranscriptional Gene Silencing in Plants

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Posttranscriptional gene silencing (PTGS) is an ancient eukaryotic regulatory mechanism in which a particular RNA sequence is targeted and destroyed. The helper component–proteinase (HC-Pro) of plant potyviruses suppresses PTGS in plants. Using a yeast two-hybrid system, we identified a calmodulin-related protein (termed rgs-CaM) that interacts with HC-Pro. Here we report that rgs-CaM, like HC-Pro itself, suppresses gene silencing. Our work is the first report identifying a cellular suppressor of PTGS.

Although PTGS was first identified in plant systems, similar pathways appear to operate in filamentous fungi, nematodes, and a variety of animal systems where it is referred to as RNA interference (*1–4*). Double-stranded RNA (dsRNA) induces PTGS in many systems (*5–7*) and, in plants, it can also be triggered by cytoplasmically replicating viruses, many of which produce dsRNA replication intermediates (*8*, *9*). Once the mechanism is activated, any homologous RNA is degraded, whether it is transcribed from the transgene, the endogenous gene, or the viral RNA. The fact that plant viruses can act both as inducers and as targets of posttranscriptional gene silencing (*8–12*) has led to the idea that PTGS evolved as a defense mechanism against viruses in plants. Our work and that of others identified the first reported suppressor of PTGS, the HC-Pro of plant potyviruses (*13–15*). Additional viral suppressors have been reported since (*16*, *17*). The finding that viral proteins suppress PTGS supports the idea that silencing is a natural antiviral defense pathway and provides an approach to understanding the mechanism of PTGS in plants.

Because the effect of HC-Pro on suppression of PTGS is clearly mediated by protein, rather than RNA (*13*, *14*), we used the yeast two-hybrid system (*18*) to search for plant proteins that interact with HC-Pro and that may, therefore, be involved in silencing. Eight tobacco proteins were identified by their interaction with tobacco etch viral (TEV) HC-Pro in this assay (*19*), including a calmodulin-related protein termed rgs-CaM (regulator of gene silencing–calmodulin-like protein). The features of rgs-CaM include a C-terminal domain containing three EF-hand calcium binding motifs with high similarity to plant calmodulins and calmodulin-related proteins, as well as an N-terminal domain of 40 to 50 amino acids that may specify the intracellular location or the regulatory properties of rgs-CaM (Fig. 1A). rgs-CaM mRNA is present at low levels in leaves and flowers (Fig. 1B, lanes 1 and 2) and at higher levels in stem and root (Fig. 1B, lanes 3 and 4). Expression of rgs-CaM can be induced in leaves of *Nicotiana tabacum* (cv. Havana 425) when HC-Pro is also expressed, either from a transgene (Fig. 1B, compare lanes 5 and 6) or from infection with a virus that encodes HC-Pro (Fig. 1B, compare lanes 7 and 8 with lanes 9 and 10), indicating that HC-Pro either directly or indirectly controls rgs-CaM mRNA levels.

Our initial approach to determine whether rgs-CaM plays a role in gene silencing was to overexpress it in transgenic plants (*20*). Nearly one-third (10/34) of the rgs-CaM primary transformants in *N. benthamiana* developed an unusual phenotype that was also observed in plants expressing the potyviral HC-Pro suppressor of gene silencing: a small differentiated tumor that forms at the junction of the stem and the root (Fig. 2, A, E, and G, compare with C). Like tumors in the HC-Pro transgenic lines, the rgs-CaM tumors are composed of cells that are properly differentiated but aberrantly arranged (compare the arrangement of cells in the tumors shown in Fig. 2, F and H, with the normal arrangement of cells at the stem-root junction shown in Fig. 2D). Northern analysis of RNA from an rgs-CaM line with the tumor phenotype shows a very high level of rgs-CaM expression as compared with nontransgenic control plants (Fig. 2B, compare lanes 1 and 2). Because transgenic lines that overexpress rgs-CaM have a phenotype similar to that seen in transgenic lines that express HC-Pro, we expected that rgs-CaM might also be a suppressor of PTGS.

To test whether a transgenic line that overexpresses rgs-CaM is altered in PTGS, we used a virus-induced gene-silencing (VIGS) assay (*11*, *13*, *21*). In the VIGS assay, a plant line transgenic for a reporter gene is infected with a viral vector carrying the same reporter gene. The viral vector initially replicates and moves systemically through the plant, expressing high levels of the reporter gene. Later in infection, VIGS is triggered, silencing the transgene and, ultimately, the viral vector as well. We have previously shown that HC-Pro interferes with VIGS in a green fluorescent protein (GFP) transgenic line (*N. benthamiana* line 16C) infected with a potato virus X (PVX) vector carrying GFP (PVX-GFP) (*13*). To test the effect of rgs-CaM in the same system, we crossed an rgs-CaM–expressing transgenic line with the GFP-ex-

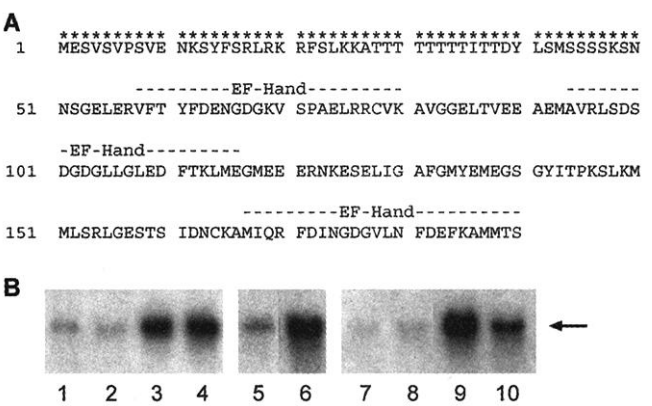

Fig. 1. Sequence and expression profile of rgs-CaM. **(A)** Amino acid sequence of rgs-CaM showing the location of three EF-hands in the calmodulin-like C-terminal domain. Asterisks appear above amino acids comprising the N-terminal extension on rgs-CaM. **(B)** Northern analysis showing the level of rgs-CaM mRNA in nontransformed *N. tabacum* (cv. Xanthi) flowers (lane 1), leaves (lane 2), stems (lane 3), roots (lane 4), or in leaves of nontransformed *N. tabacum* (cv. Havana 425) (lane 5), or transgenic line U-6B (*29*) expressing high levels of HC-Pro (lane 6), or in mock-inoculated and upper leaves of *N. tabacum* (cv. Havana 425) (lanes 7 and 8, respectively), and TEV-inoculated and upper leaves of the same tobacco variety (lanes 9 and 10, respectively). Northern analyses (*23*) used 10 μg of total nucleic acid per lane and were probed with ^{32}P-labeled, randomly primed rgs-CaM cDNA. Ethidium bromide staining of 18*S* rRNA was used to confirm equal loading of RNA in all lanes. The arrow marks the location of the rgs-CaM mRNA.

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pressing line 16C to produce plants hemizygous for both the GFP and rgs-CaM transgenes. These plants were infected with PVX-GFP. Plants expressing high levels of rgs-CaM (Fig. 3D, lane 2) either failed to silence or showed only delayed and reduced silencing when infected with PVX-GFP. Thus, plants that overexpress rgs-CaM continued to fluoresce green under long-wave ultraviolet (UV) light 10 days after inoculation with PVX-GFP (Fig. 3B) and accumulated high levels of both the GFP-transgene mRNA and the PVX-GFP viral RNAs (Fig. 3C, lane 2). In contrast, hemizygous 16C control plants (without the rgs-CaM transgene) became completely silenced for the GFP transgene at about 10 days after inoculation with PVX-GFP, as demonstrated by failure to produce GFP (Fig. 3A), as well as by a dramatic reduction in both GFP-transgene RNA and PVX-GFP viral RNA (Fig. 3C, lane 3). These results show that a transgenic line that overexpresses rgs-CaM is defective in silencing, supporting the idea that rgs-CaM is a cellular suppressor of PTGS.

To further test the role of rgs-CaM in gene silencing, we used a reversal of silencing assay (*14*). In this approach, a transgenic line (line 16C) expressing high levels of GFP is induced for silencing by infiltrating its leaves with *Agrobacterium tumifaciens* carrying a plasmid that also expresses GFP. The *Agrobacterium* induces a localized PTGS of the GFP transgene, which subsequently spreads systemically throughout the plant. The silencing of GFP in these plants can be reversed by infection with a PVX vector that expresses a viral suppressor of silencing, such as the potyviral HC-Pro (*14*). In our experiments (*22*), we silenced the GFP transgene by infiltration with *Agrobacterium* and then infected the silenced plants with PVX vectors carrying wild-type P1/HC-Pro [PVX-5′TEV (*23*)] from the potyvirus TEV, an untranslatable version of the TEV HC-Pro [PVX-noHC (*23*)], or the rgs-CaM cDNA sequence (PVX-rgsCaM). Plants infected with each of these viruses developed symptoms typical of the particular PVX vector (a mild mosaic pattern on leaves for PVX-rgsCaM and PVX-noHC or a systemic necrosis for PVX-5′TEV). As previously shown (*13*, *14*), the plants infected with PVX-noHC remained silenced for the GFP transgene (and, therefore, red under UV light, Fig. 4A). In contrast, plants infected with PVX-rgsCaM showed reversal of the GFP silencing in the systemically infected portion of the previously silenced plant similar to that induced by PVX-5′TEV (Fig. 4B, areas of green in the red background). RNA blot analyses showed that GFP mRNA is present in almost the amounts found before silencing when PTGS is suppressed by either HC-Pro or rgs-CaM (Fig. 4C). This result is consistent with that

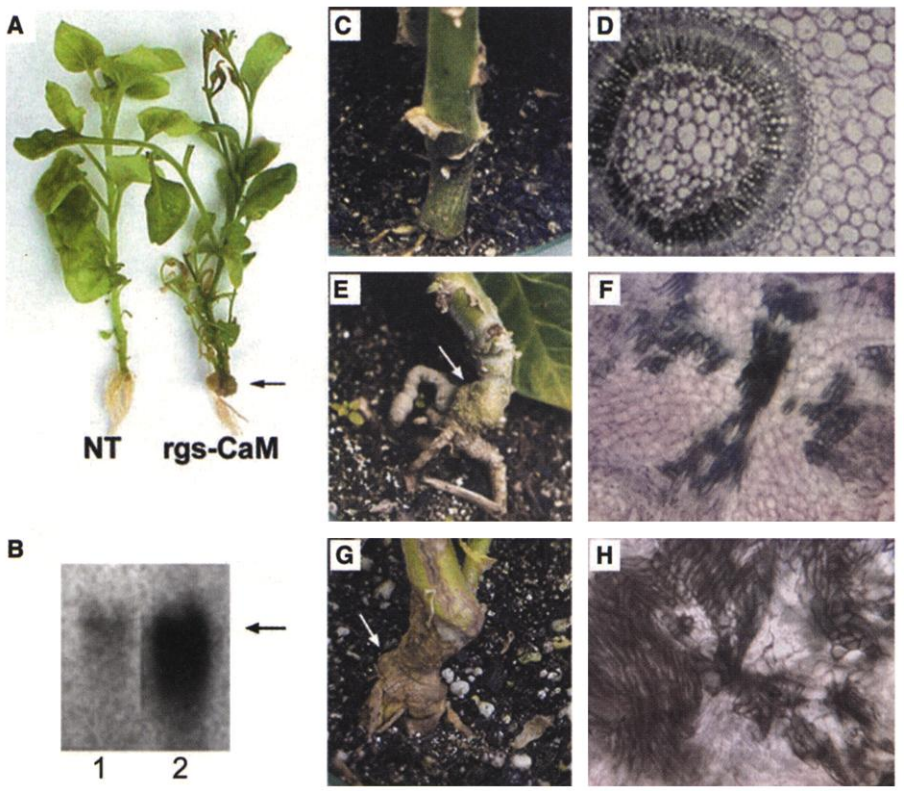

Fig. 2. A differentiated tumor forms at the stem-root junction of tobacco plants transgenic for either HC-Pro (E and F) or rgs-CaM (A, G, and H). (**A**) Phenotype of nontransformed *N. benthamiana* plant (NT) compared with that of an rgs-CaM transgenic line (rgs-CaM). (**B**) Northern analysis showing level of rgs-CaM mRNA from leaves of nontransformed control plants (lane 1) or the equivalent leaves of the rgs-CaM line shown in (A) (lane 2). (**C**) Stem-root junction in nontransformed control tobacco plant. (**D**) Cross section through the stem-root junction in control plants. (**E** and **G**) Tumors at the stem-root junction of HC-Pro or rgs-CaM transgenic lines, respectively. (**F** and **H**) Cross sections showing aberrant arrangement of differentiated cells in HC-Pro and rgs-CaM transgenic lines, respectively. The arrows (in A, E, and G) point to the tumors at the stem-root junction in the rgs-CaM and HC-Pro transgenic plants.

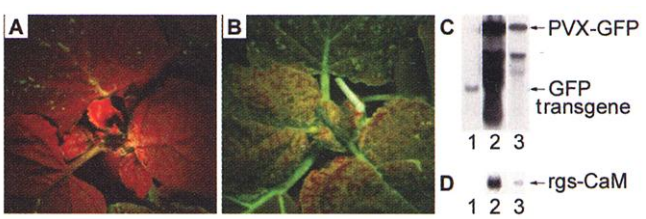

Fig. 3. A transgenic line that overexpresses rgs-CaM is deficient in VIGS. (**A**) A hemizygous GFP-expressing transgenic line infected with PVX-GFP showing VIGS of GFP. (**B**) A hemizygous transgenic line expressing both GFP and rgs-CaM infected with PVX-GFP showing continued expression of GFP. (**C**) RNA gel blots showing levels of RNA from PVX-GFP and GFP transgene in the uninoculated hemizygous GFP line (lane 1); in the hemizygous GFP, rgs-CaM line infected with PVX-GFP (lane 2); or in the hemizygous GFP line infected with PVX-GFP (lane 3). (**D**) RNA gel blot showing level of rgs-CaM mRNA in the same plants listed in panel (C).

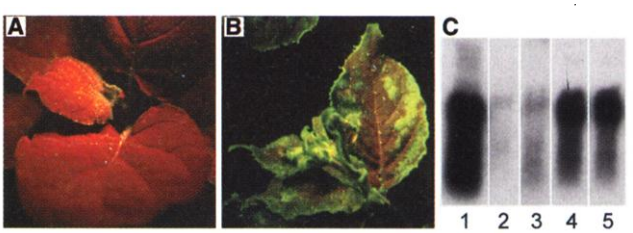

Fig. 4. Reversal of PTGS by infection with a PVX vector expressing rgs-CaM. (**A**) A plant silenced for the GFP transgene and infected with a PVX vector expressing an untranslatable-HC-Pro remains silenced for GFP. (**B**) A plant silenced for the GFP transgene and infected with a PVX vector expressing rgs-CaM shows reversal of GFP silencing. (**C**) Northern analysis showing GFP mRNA in the GFP transgenic line 16C before (lane 1) and after (lane 2) infiltration with *Agrobacterium* to induce PTGS of the GFP transgene. *Agrobacterium*-silenced plants were infected with PVX carrying a nontranslatable HC-Pro (lane 3), or with PVX expressing either wild-type TEV HC-Pro (lane 4) or rgs-CaM (lane 5).

from the VIGS assay described above and is a direct demonstration that rgs-CaM is a cellular suppressor of PTGS.

Gene silencing is an important genetic mechanism with both theoretical and practical implications. Mutant studies have recently led to the identification of several eukaryotic proteins that are required for PTGS and are likely components of the silencing machinery (*4*, *24*, *25*). Viral suppressors of PTGS provide a powerful tool for finding additional cellular proteins involved in PTGS, including ones that might not be easily found by using mutants. Because calmodulin and related proteins normally act by binding calcium and subsequent activation of target proteins (*26*, *27*), this finding points to a role for calcium in regulating the activity of the PTGS pathway and predicts that the downstream target protein of rgs-CaM will also be a suppressor of PTGS. Given that rgs-CaM was found by its interaction with HC-Pro and that its expression can be induced by HC-Pro, it is tempting to speculate that the suppression of PTGS by HC-Pro is mediated, at least in part, by the activation of rgs-CaM and subsequent amplification of an endogenous pathway that negatively regulates gene-silencing activity.

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19. A cDNA library prepared from a mixture of RNAs isolated from a tobacco suspension culture at various time points (0, 2, and 5 hours) after treatment with parasiticein, an elicitor of the hypersensitive-response secreted by the fungus *Phytophthora*, was cloned into the HybriZAP activation domain vector (Stratagene, La Jolla, CA). Two million colonies from this library were screened by using the pGBT9 binding domain vector with the TEV HC-Pro cloned in at the Bam HI and Pst I sites.
20. The cDNA encoding rgs-CaM was subcloned from pBluescript (Stratagene) into the binary vector pGA482, transformed into *A. tumifaciens* strain GV3101R, and used for leaf-disc transformation of *N. benthamiana* (*28*). pGA482 does not carry a promoter, thus rgs-CaM in these transgenic lines is expressed from a natural plant promoter.
21. An *N. benthamiana* transgenic line hemizygous for rgs-CaM was crossed with transgenic line 16C, generating offspring hemizygous for the GFP transgene, half of which were also hemizygous for the rgs-CaM transgene. Plants were infected with PVX-GFP and assayed for GFP expression by using long-wave UV light (*13*). RNA for Northern analysis was isolated 12 days after inoculation.
22. Seedlings of *N. benthamiana* line 16C, homozygous for the GFP transgene, were infiltrated with *A. tumifaciens* (strain C58C1) carrying a binary plasmid with GFP under control of the 35S-cauliflower mosaic virus promoter. PTGS of the GFP transgene spread systemically, and plants were silenced by 3 weeks after infiltration and then inoculated with PVX vectors as described in the text. The PVX-rgsCaM construct was made by PCR amplification of rgs-CaM cDNA and cloning into an infectious PVX-cDNA just upstream of the gene for coat protein.
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30. We thank T. Smith for help with figures and D. Baulcombe for providing line 16C and GFP–*A. tumifaciens*. C.M. provided the yeast two-hybrid cDNA library. The work was supported by grants to V.B.V. from U.S. Department of Agriculture, National Research Initiative, Genetic Mechanisms Program (number 9802199) and Akkadix Corporation, La Jolla, CA.

8 May 2000; accepted 21 August 2000

Regulation of STAT3 by Direct Binding to the Rac1 GTPase

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The signal transducers and activators of transcription (STAT) transcription factors become phosphorylated on tyrosine and translocate to the nucleus after stimulation of cells with growth factors or cytokines. We show that the Rac1 guanosine triphosphatase can bind to and regulate STAT3 activity. Dominant negative Rac1 inhibited STAT3 activation by growth factors, whereas activated Rac1 stimulated STAT3 phosphorylation on both tyrosine and serine residues. Moreover, activated Rac1 formed a complex with STAT3 in mammalian cells. Yeast two-hybrid analysis indicated that STAT3 binds directly to active but not inactive Rac1 and that the interaction occurs via the effector domain. Rac1 may serve as an alternate mechanism for targeting STAT3 to tyrosine kinase signaling complexes.

Members of the STAT family of transcription factors are unusual among transcription factors in that they have characteristics of cytoplasmic signaling molecules such as an Src-homology 2 (SH2) domain and tyrosine phosphorylation sites (*1*). The SH2 domain targets the STATs to cytokine receptors such as the interleukin-6 (IL-6) and interferon-γ receptors (*2*). Upon tyrosine phosphorylation in response to receptor activation, the STATs dimerize through interaction of the SH2 domain with phosphorylated tyrosine residues and translocate to the nucleus. There, they directly bind DNA and form complexes with other transcription factors through protein-protein interactions (*3*). Several of the STATs are also activated by growth factor receptors (*4*, *5*). Receptor kinases, Jak family kinases, and Src family kinases have been implicated in this process (*6*–*10*). Reactive oxygen species (ROS) activate the Jak2 and TYK2 kinases as well as STAT3 and participate in growth factor signaling to the STATs (*11*). Because the small guanosine triphosphatase (GTPase) Rac has been implicated in the generation of ROS in response to growth factors (*12*), we investigated whether Rac1 has a role in STAT3 activation by growth factors.

Dominant negative N17Rac was cotransfected with STAT3 into COS-1 cells and phosphorylation of STAT3 in response to epidermal growth factor (EGF) stimulation was examined with phospho-specific antibodies. EGF treatment resulted in stimulation of both tyrosine and serine phosphorylation of STAT3 (Fig. 1A). However, dominant negative Rac1 completely abolished STAT3 phosphorylation on both tyrosine and serine residues induced by EGF, but had no effect on STAT3 expression (COS-1 cells express very little endogenous STAT3). In contrast, cotransfection with wild-type Rac1 did not

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