Corrections

APPLIED MATHEMATICS
Correction for “On the behavior of a capillary surface in a wedge,” by Paul Concus and Robert Finn, which appeared in issue 2, June 1, 1969, of Proc Natl Acad Sci USA (63:292–299).
Owing to a printer’s error, the initial sentence of the abstract appeared as “Estimates above and below are obtained for the height of the equilibrium-free surface of a liquid” rather than the intended “Estimates above and below are obtained for the height of the equilibrium free surface of a liquid.” Also, the first sentence of the introduction, “Consider a volume of liquid that partially fills a cylindrical container Z, forming an equilibrium-free surface S, as determined by surface and gravitational forces” should have read “Consider a volume of liquid that partially fills a cylindrical container Z, forming an equilibrium free surface S, as determined by surface and gravitational forces.” These errors can lead to basic misunderstanding as to the nature of the problem addressed in the article. The online version of this article has been corrected.

www.pnas.org/cgi/doi/10.1073/pnas.0901567106

CELL BIOLOGY
The authors note that author name Moojin Suh should have appeared as Moo-Jin Suh. The online version has been corrected. The corrected author line and author contributions footnote appear below.


www.pnas.org/cgi/doi/10.1073/pnas.0900927106

www.pnas.org
Interaction with host SGS3 is required for suppression of RNA silencing by tomato yellow leaf curl virus V2 protein


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The V2 protein of tomato yellow leaf curl geminivirus (TYLCV) functions as an RNA-silencing suppressor that counteracts the innate immune response of the host plant. The host-cell target of V2, however, remains unknown. Here we show that V2 interacts directly with SGS3, the tomato homolog of the Arabidopsis SGS3 protein (AtSGS3), which is known to be involved in the RNA-silencing pathway. SGS3 genetically complemented an AtSGS3 mutation and restored RNA silencing, indicating that SGS3 is indeed a functional homolog of AtSGS3. A point mutant of V2 that is unable to bind SGS3 also lost its ability to suppress RNA silencing, suggesting a correlation between the V2–SGS3 interaction in planta and the suppressor activity of V2.

Plants have evolved an innate immune response to invading viruses that is based on posttranscriptional RNA silencing (1–4). RNA silencing induced by transcripts of sense transgenes and viral DNA genomes is thought to involve conversion of single-stranded (ss) RNA species into double-stranded (ds) RNA by RDR6, with the help of SGS3 (5–7). Alternatively, SGS3 may be involved in the transport of the RNA-silencing signal (8). dsRNAs are processed by the cellular machinery to produce siRNAs that are incorporated into RNA-induced silencing complexes (RISCs), within which siRNAs direct the cleavage of the complementary viral transcripts (9, 10).

To counteract the plant antiviral response, many viruses produce suppressor proteins that block the host RNA silencing by targeting different steps of the silencing pathway (11–14). For example, the potyviral HcPro most likely inhibits unwinding of RNA by targeting different steps of the silencing pathway (11–14). In addition, suppressor proteins that block the host RNA silencing pathway. SGS3 genetically complemented an AtSGS3 mutation and restored RNA silencing, indicating that SGS3 is indeed a functional homolog of AtSGS3. A point mutant of V2 that is unable to bind SGS3 also lost its ability to suppress RNA silencing, suggesting a correlation between the V2–SGS3 interaction in planta and the suppressor activity of V2.

Results

V2 Interacts with Tomato and Arabidopsis SGS3. To identify a host-cell target of V2, we used a two-hybrid screen (24, 25) with a tomato (Solanum lycopersicum) cDNA library and the V2 protein as bait. Screening of ~1 × 10⁶ transformants resulted in the identification and isolation of four independent cDNA clones producing V2 interactors, two of which represented the same cDNA clone. Coexpression of V2 and one of its interactors, designated SGS3 (GenBank accession no. EF590136), activated the LEU2 reporter gene and enabled yeast transformants to grow on a leucine dropout medium. This interaction was specific because the cells that were cotransformed with either the V2-expressing construct and the empty library vector pJG4-5, or with the SGS3-expressing construct and pEG202-expressing Bcoid homedomain (pRFHM1), which is often used to detect nonspecific two-hybrid interactions (26), were unable to survive in the absence of leucine (Fig. 1A). Cells expressing all three combinations of proteins grew to the same extent in the presence of leucine (data not shown), indicating that the tested proteins did not adversely and nonspecifically affect yeast-cell physiology. Amino acid sequence analysis revealed significant homology between the identified V2 interactors, SGS3, and Arabidopsis SGS3 (AtSGS3) (Fig. 1B). As is typical for members of the SGS3 protein family (27), both SGS3 and AtSGS3 contained a conserved XS domain with an as-yet-unknown function (Fig. 1B) (27).

Next, we examined the subcellular localization patterns of YFP-tagged SGS3 or AtSGS3 coexpressed with CFP-tagged V2. Fig. 2 shows that, in tobacco protoplasts, V2-CFP coexpressed with YFP-SGS3 (Fig. 2A) or YFP-AtSGS3 (Fig. 2B) accumulates in distinct microbodies throughout the cell cytoplasm, and that the accumulated SGS3 or AtSGS3 largely colocalizes with V2. These observations were confirmed in leaf tissues of tomato, the native TYLCV host (Fig. 2C). SGS3/V2 colocalization was then quantified by counting the corresponding microbodies that overlapped within the coexpressing cell. These calculations showed that, on average, 97% of V2 colocalized with SGS3 and 86% of SGS3 colocalized with V2, suggesting that most of these protein microbodies overlap within the coexpressing cells. This colocalization of SGS3/AtSGS3 and V2 is consistent with their ability to interact with one another.

Interestingly, the colocalization experiments also suggested that neither V2 nor SGS3 can move between plant cells. Because agroinfiltration is performed with a mixture of two bacterial strains, each carrying one of the tested genes, the target cells are sometimes transformed by only one bacterial strain, resulting in cells that express a single tagged protein (i.e., either V2 or SGS3). This finding is exemplified in Fig. 2C, which shows two adjacent cells (1 and 2). Cell 1 expressed both SGS3 and V2, and cell 2 expressed only SGS3. Fig. 2D shows the reciprocal situation, with cell 1 expressing both SGS3 and V2.

Reference

1. This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. EF590136 and AF234296).

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YFP and CFP did not generate detectable FRET (Fig. 3), which is indicative of FRET (30, 31). As expected, free coexpressed YFP-SlSGS3/V2-CFP and YFP-AtSGS3/V2-CFP, respectively, indicated interaction between these proteins, whereas the absence of cell growth in two negative controls indicated the specificity of this interaction. The indicated cell inocula were plated with a determination of the subcellular localization of the interacting proteins (28, 29). YFP-SlSGS3 or YFP-AtSGS3 was transiently expressed with V2-CFP in tobacco protoplasts. FRET between these proteins was detected by acceptor photobleaching (30, 31). Fig. 3 indicates colocalization and energy transfer of V2 or SISGS3 from the expressing cell to the adjacent cell that does not express these proteins.

Quantification of the CFP signal after photobleaching of YFP revealed an increase in the intensity of the donor fluorescence with a FRET efficiency (EF) of 32.2 ± 8.0% and 32.6 ± 5.2% for YFP-SlSGS3/V2-CFP and YFP-AtSGS3/V2-CFP, respectively, which is indicative of FRET (30, 31). As expected, free coexpressed YFP and CFP did not generate detectable FRET (Fig. 3C).

**SISSG3 Functionally Complements an Arabidopsis sgs3 Mutant.** To directly demonstrate that SlSGS3 in fact represents a functional homolog of AtSGS3, we examined whether the SlSGS3 gene can complement the known RNA-silencing-deficient phenotype of the L1/sgs3-1 Arabidopsis line (8), in which a chemically induced sgs3-1 mutation of AtSGS3 abrogates RNA silencing of a tandem arrangement of the β-glucuronidase (GUS) transgene in the parental L1 transgenic line (32). We produced double-transgenic plants that constitutively expressed the SiSSG3 cDNA (L1/sgs3-1/SiSSG3) or AtSSG3 cDNA (L1/sgs3-1/AtSSG3). The resultant plants were first examined for the presence and expression of the SiSSG3 or AtSSG3 transgenes. PCR-based analysis by using SiSSG3- and AtSSG3-specific primers revealed the presence of the SiSSG3 sequences in L1/sgs3-1/SiSSG3, but not in L1/sgs3-1 parental control plants (Fig. 4A). Both L1/sgs3-1/AtSSG3 and L1/sgs3-1 plants contained the endogenous AtSSG3 gene, distinguished by its larger size because of the presence of the intron, but only L1/sgs3-1/AtSSG3 contained the AtSSG3 cDNA transgene (Fig. 4A). Our RT-PCR analysis by using primers specific for the SiSSG3 and AtSSG3 transgenes, but not for the endogenous AtSSG3 gene, detected the corresponding transcripts only in L1/sgs3-1/SiSSG3 and L1/sgs3-1/AtSSG3 plants, respectively. In control experiments, analysis of actin-specific transcripts generated similar amounts of PCR products in all samples, indicating equal efficiencies of the RT-PCRs (Fig. 4B).

Next, four independent lines of L1/sgs3-1/SiSSG3 and 10 independent lines of L1/sgs3-1/AtSSG3, as well as the parental plants L1/sgs3-1 and L1, were examined for their ability to silence the GUS reporter transgene. Fig. 4C shows that, although the L1/sgs3-1 plants exhibited significant levels of GUS expression, both L1/sgs3-1/SiSSG3 and L1/sgs3-1/AtSSG3 plants efficiently silenced the transgene. This silencing was comparable to that observed in the original L1 silenced line (Fig. 4C). These observations indicate that the SiSSG3 cDNA genetically complements the sgs3-1 mutation, suggesting that SiSSG3 represents a functional homolog of AtSSG3.

**The V2–SISSG3 Interaction Is Required for RNA Silencing.** To correlate between V2–SiSSG3 binding and the RNA-silencing-suppressor
activity of V2, we used a substitution mutant of V2, C84S/C86S. First, consistent with previous observations (23), we showed that the C84S/C86S mutant was indeed compromised in its ability to suppress RNA silencing. RNA silencing was detected by a visible increase in GFP expression after coinfiltration of a wild-type *Nicotiana benthamiana* plant with two strains of *Agrobacterium*, one that carries V2 or its mutant and the other that contains the RNA-silencing initiator and reporter gene, GFP (23). GFP expression, which could be observed 2 days postinfiltration (dpi) (data not shown), was almost completely silenced at 7 dpi. This silencing was efficiently suppressed by the coexpressed V2, resulting in easily detectable GFP fluorescence (Fig. 5A). The C84S/C86S mutant of V2 lost its RNA-silencing-suppression activity, failing to restore GFP expression in the inoculated leaves (Fig. 5A). These results were confirmed by RT-PCR analyses of GFP transcripts (data not shown).

We then examined the effects of the C84S/C86S mutation on V2’s subcellular localization and interaction with SISGS3. Fig. 5B shows that the C84S/C86S mutant exhibited the same microbody-associated localization pattern as the wild-type V2 (compare with Fig. 2A), indicating that the mutation did not significantly alter the subcellular distribution of the protein. The FRET studies, however, showed that the mutant V2 lost most of its ability to interact with SISGS3 (Fig. 5C). These results suggest that disrupting the V2–SISGS3 interaction substantially impairs the suppressor function of V2, but does not interfere with the overall accumulation and subcellular distribution of the protein, effectively uncoupling between colocalization and proteint–protein interaction.

**Discussion**

TYLCV is a member of the Geminiviridae family of plant viruses characterized by their ssDNA genome, which replicates and transcribes in the host-cell nucleus. Although TYLCV is a DNA virus that replicates by a dsDNA intermediate, it is capable of inducing RNA silencing in infected plants (33, 34). This antiviral reaction is counteracted by RNA-silencing suppressors encoded by several geminiviruses (14), as well as by many other plant

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**Fig. 3.** Interaction between V2 and SISGS3 or AtSGS3 in planta. (A and B) Protein–protein interaction was monitored by FRET microscopy of living *N. tabacum* protoplasts coexpressing V2-CFP and either YFP-SlSGS3 (A) or YFP-AtSGS3 (B). Representative acceptor photobleaching images show CFP (donor) and YFP (acceptor) channels before and after bleaching. After bleaching, CFP fluorescence increases and YFP fluorescence decreases in the bleached areas indicated by rectangles. All images are projections of several confocal sections. (C) Quantification of donor fluorescent intensity in representative samples. Data represent average values of three independent experiments, with 10 protoplasts each, with indicated standard deviation values.

**Fig. 4.** Genetic complementation of the *Arabidopsis* *L1/sgs3-1* phenotype by *SISGS3* and restoration of RNA silencing. (A) PCR-based identification of *L1/sgs3-1/SlSGS3* and *L1/sgs3-1/AtSGS3* transgenic plants by detection of the *SISGS3* (Left) and *AtSGS3* (Right) transgenes, respectively. Lane M, molecular size markers (indicated in kilobase pairs [kbp]); lane 1, *L1/sgs3-1* parental line showing no *SISGS3* cDNA-specific PCR product; lane 2, *L1/sgs3-1/SlSGS3* line producing the *SISGS3* cDNA-specific 786-bp PCR product; lane 3, *L1/sgs3-1* parental line showing a 1,191-bp PCR product specific for the endogenous AtSGS3 gene with its intron, but no AtSGS3 cDNA-specific 888-bp band; lane 4, *L1/sgs3-1/AtSGS3* plants yielding 1,191- and 888-bp PCR products specific for the intron-containing endogenous AtSGS3 gene and the AtSGS3 cDNA transgene, respectively. (B) RT-PCR-based detection of transcripts derived from the *SISGS3* and *AtSGS3* cDNA transgenes. Lane M, molecular size markers (kbp); lane 1, *L1/sgs3-1* parental line; lane 2, *L1/sgs3-1/SlSGS3* plants; lane 3, *L1/sgs3-1/AtSGS3* plants. *L1/sgs3-1/SlSGS3* and *L1/sgs3-1/AtSGS3* plants yielded RT-PCR products specific for the *SISGS3* and *AtSGS3* transgenes, respectively, whereas all plants contained transcripts of the constitutively expressed *Actin* gene. (C) Silencing of the GUS reporter in the indicated plant lines. Note that expression of *SISGS3* or *AtSGS3* transgenes restored RNA silencing of GUS in *L1/sgs3-1* plants to levels comparable to those of the original silenced *L1* line. Data represent average values of three independent experiments with indicated standard deviations.
viruses (11–13). Of these viral proteins, TYLCV V2 is only the second reported RNA-silencing suppressor, after CMV 2b (21), that is likely to target a protein component of the host RNA-silencing machinery directly. Unlike 2b, however, which interacts with the AGO1 endonuclease component of the RISC (21), V2 binds SGS3, whose function in the silencing pathway remains unclear, but may include production of dsRNA (5), transport of the RNA-silencing signal (8), or protection of transcript fragments from degradation, allowing RDR6 to generate duplex RNA (35).

Because TYLCV is a geminivirus, it is especially interesting that SGS3 is specifically required for the RNA-silencing defense against geminiviruses (6). Thus, interaction of V2 with SGS3 makes biological sense. We suggest that V2 binding may inactivate SGS3, thereby blocking the silencing pathway. Because SGS3 most likely performs a stoichiometric, rather than an enzymatic, function (5, 8, 35), binding to and potentially disabling a large proportion of cellular SGS3 by V2 produced by the invading virus would substantially impair SGS3’s function during RNA silencing. This finding is consistent with our observations that the overexpression of V2 from a strong 35S promoter in cells that express the SGS3 gene from the weaker native promoter results in strong suppression most likely because of more efficient binding V2. Furthermore, the fact that the V2 mutant, which is unable to bind SGS3, loses its ability to suppress silencing supports our notion that the V2–SGS3 interaction may represent one of the key events in V2-induced RNA-silencing suppression in TYLCV-infected plant cells.

Potential inactivation of SGS3 by V2 also may affect the development of TYLCV disease symptoms. TYLCV-infected tomatoes are distinguished by curling of their leaves (36), and tomato plants carrying a mutation within the SGS3 locus exhibit an aberrant leaf phenotype (Y. Eshed, personal communication). Consistent with this effect on leaf morphology, SGS3 also has been implicated in determining the adaxial identity of the leaf (37). Besides helping to better understand the complex nature of the TYLCV–host interaction, our observations may be useful in designing new approaches to combating viral infection, for example, by disrupting the V2–SGS3 interaction.

Materials and Methods

Plasmid Construction. To produce a bait construct, the PCR-amplified ORF of TYLCV V2 (23) was inserted into the EcoRI-Xhol sites of pEG202 (38), resulting in pEG202-V2.

For subcellular localization studies, TYLCV-V2 was tagged at its C terminus with CFP by cloning a PCR-amplified V2 ORF into the BglII-EcoRI sites of pSAT6EYFP-C1 (39). PCR-amplified SGS3 cDNAs were inserted from pCAMBIA1300 into the HindIII-SacI sites of pSAT6EYFP-C1 (39), resulting in pSAT6EYFP-SGS3 and pSAT6EYFP-AtSGS3. The expression cassette containing SGS3 or AtSGS3 cDNAs was then inserted into the HindIII-SacI sites of pSAT6EYFP-SGS3 and pSAT6EYFP-AtSGS3, respectively.

For genetic complementation studies, the SGS3 or AtSGS3 cDNAs were first cloned into the EcoRI-Sall sites of pSAT6A-ECFP or pCAMBIA1300 (38), resulting in pSAT6A-ECFP-SGS3 and pCAMBIA1300-ECFP-SGS3.

For RNA-silencing-suppression assays, all proteins were expressed from binary vectors as described in ref. 23. The C84S/C86S amino acid substitution...
Detection of Protein-Protein Interactions by FRET Microscopy. The FRET procedure was performed by using the acceptor photobleaching method (30). All other conditions were as described for confocal imaging. The microscope was configured with a 458–515-nm dichroic mirror for dual excitation and a 515-nm beam splitter to help separate CFP and YFP fluorescence. 

Quantification of GUS Expression. Arabidopsis mature rosette leaves were ground and assayed for GUS activity by using the fluorescent substrate 4-methylumbelliferyl β-D-galactoside as described in ref. 44. The enzymatic activity was expressed as nanomolar concentration of the fluorescent product 4-methylumbelliferone per microgram of total plant protein. All experiments were performed in triplicate, and the resulting data represent average values with indicated standard deviations.

ACKNOWLEDGMENTS. We thank Hervé Vaucheret (Institut National de la Recherche Agronomique, Versailles) for providing seeds of A. thaliana L-sgs3-1 EMS mutant line (6) (a kind gift from H. Vaucheret, Institut National de la Recherche Agronomique Centre de Versailles, Versailles, France) which express high levels of GFP, allowing its visualization at low magnification. For intracellular localization studies performed at higher magnification, we used young leaves of tomato, the native TYLCV host.

For confocal imaging, we used an Leica TCS SP2 confocal microscope equipped with a 63×1.4 NA oil objective. GFP and YFP were excited at 488 and 515 nm and imaged by using BA480–495 and BAS535–565-nm emission filters, respectively. For chlorophyll autofluorescence imaging, a 560-nm IF emission filter was used. Transmitted light images were obtained by using Nomarski differential interference contrast. Visual detection of GFP fluorescence in plant leaves was performed by using a Leica MZFLIII fluorescence stereomicroscope with a Leica DC200 camera. GFP was detected under a mercury lamp light by using a 450–490-nm excitation filter and a 500–550-nm emission filter. Photographic images were prepared by using Adobe Photoshop version 10.0 (Adobe Systems).

PCR and RT-PCR. To detect the SGS3 and AtSGS3 transgenes, DNA was extracted from leaf tissue and assayed by PCR, which was designed to produce 786-bp- and 888-bp-specific fragments, respectively. Under these conditions, the endogenous AtSGS3 gene yielded a 1,191-bp PCR product because of the presence of an intron.

For RT-PCR, 1 μg of RNA was extracted from frozen leaves with a TRI reagent (Sigma–Aldrich) according to the manufacturer’s instructions. The RNA was reverse-transcribed by using a Reverse-ITTM 1st Strand Synthesis Kit (ABgene) and PCR-amplified to produce 992-bp, 921-bp, and 600-bp fragments specific for SGS3, AtSGS3, and Actin, respectively. PCR and RT-PCR products were resolved on a 1% agarose gel and detected by ethidium-bromide staining. The specificity of the amplification products was verified by DNA sequencing.