Supporting Information

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SI Materials and Methods

Virus Isolates. The isolates of SPFMV (SPFMV-Piu) and SPCSV (SPCSV m2–47) used for plant inoculation in this study were originally obtained from sweet potato plants infected in the field in Peru (1, 2). Both isolates belong to the EA strains of their respective viruses. They were maintained in *Ipomoea setosa* plants in an insect-proof greenhouse at the International Potato Center (CIP). The isolates SPCSV-Ug and SPCSV-Is have been described (2). They belong to the EA strain and WA strain of SPCSV, respectively (2, 3, 4). The entire genomes of SPFMV-Piu (FJ155666) and SPCSV-Ug (RNA1, AJ428554; RNA2, AJ428555) (3) have been sequenced. *RNase3* and *p22* of SPCSV-Ug and *RNase3* of SPCSV-Is and SPCSV m2–47 used in this study have been cloned (2, 4). Isolates of SPMMV and SPCFV were obtained from the CIP Virology Unit (1) and maintained in *Ipomoea nil*.

Agroinfiltration Assay. The cloning strategy and vector plasmids used in the agroinfiltration assays in this study have been described (4, 5). In brief, virus genes were amplified by PCR and cloned using the pCR-Blunt cloning system (Invitrogen) in *Escherichia coli* DH5α cells. From this cloning vector the genes were moved to an intermediary vector using the *Not*I site and the *Fse*I site added to the 5′ end and 3′ end, respectively, of the amplified fragments using the PCR primers. This intermediary cloning vector contained the *Cauliflower mosaic virus* 35S promoter and the 3′-terminator region of the nopaline synthase gene (3′-nos). The cassette 35S-(viral gene)-3′nos was transferred to the binary vector pKOH200 for agroinfiltration (5). Constructs were verified by sequencing at the DNA sequencing unit of the Haartman Institute, University of Helsinki, Helsinki, Finland. The binary vectors for expression of the RNase3 genes of the different isolates were designated as pA-R3-Ug, pA-R3-Is, and pA-R3-m2–47. The vector for expression of *p22* of SPCSV-Ug was designated as pA-p22.

A mutant of the SPCSV-Ug RNase3 gene (RNase3-Ala) described in a previous study (4) was used. The E37A and D44A mutations of RNase3-Ala are located in the highly conserved RNase III signature motif (6–13) and are required for the dsRNA endonuclease activity of RNase III enzymes (Fig. S4). The pKOH200-based binary vectors described in a previous study (4) contain the ß-glucuronidase (GUS) gene with a plant intron to prevent GUS expression in *Agrobacterium* (14) (pA-GUS), the "cycle 3" GFP gene (Clontech) (pBIN35S-GFP) and the helper component proteinase (HC-Pro)-encoding sequence of *Potato virus A* (PVA; genus *Potyvirus*) (pA-HC-Pro).

Competent cells of *Agrobacterium tumefaciens* strain C58C1 with Ti plasmid pGV3850 were transformed with the binary vectors using the freeze-thaw method (15). Agroinfiltration (4, 16, 17) was performed using different *Agrobacterium* cultures that were diluted with induction medium (10 mM $MgCl_2$, 150 μM acetosyringone) so that a final optical density (600 nm) of 0.5 was obtained. Subsequently, the cultures of the strain carrying pBIN35S-GFP, and strains carrying other constructs were combined in a 1:3 ratio (vol/vol) for infiltration. For co-infiltration treatments that included fewer constructs than others, the missing volume was replaced by the *Agrobacterium* strain expressing GUS. Infiltrations were carried out on the *Nicotiana benthamiana* line 16c genetically transformed to constitutively express the *Aequoria victoriae* GFP (18). Seeds of this line were kindly provided by Prof. D. Baulcombe, Sainsbury Laboratory, John Innes Center, Norwich, U.K. Plants were grown from seeds in a controlled growth chamber (temperature 18–22 °C, relative humidity 70%; photoperiod of 16 h, 200 μE $m^{-2}s^{-1}$ with illumination by fluorescent lamps) at the University of Helsinki and watered with a solution containing 1.0% (N:P:K = 16:9:22) fertilizer. Leaves of 3-week-old plants were agroinfiltrated using a 2-mL syringe to the underside of the leaf. Infiltrated tissues were monitored daily for GFP fluorescence using a hand-held UV lamp (B-100 AP; UVP) at 2–7 days post-infiltration (d.p.i.). Photographs were taken with a FUJI-Film pix4200 digital camera (FujiFilm Corporation) and processed using Adobe Photoshop version 7.0.1 (Adobe Systems).

Transgenic Sweet Potato Lines Expressing SPCSV RNase3. Pathogen-free plants of the Peruvian sweet potato landrace 'Huachano' (accession no. CIP420065) maintained under in vitro conditions were obtained from the germplasm collection of CIP and grown for 4 weeks in an incubation room (25–27 °C, photoperiod 16 h, relative humidity 70%, 3,000 lux). Leaves with a petiole of about 1.5 to 2 cm were cut from the top third of the plantlets and used as explants in transformation experiments. Single bacterial colonies of the *A. tumefaciens* strain EHA105 harboring plasmid pA-R3-Ug were used for plant transformations using the protocol described (19). Regeneration of putative transformed explants was subsequently achieved by following a somatic embryogenesis protocol (19). Regenerated plantlets were multiplied in vitro on MPB propagation medium [MS salts (Gibco BRL, Invitrogen) 4.3 g/L, sucrose 30 g/L, ascorbic acid 0.2 g/L, arginine 0.1 g/L, 1,4-diaminobutane 0.02 g/L, gibberellic acid 0.01 mg/L, calcium pantothenate 2 mg/L, and Phytagel (Sigma-Aldrich Chemical Co.) 3 g/L, pH 5.8]. For testing with viruses, plantlets were transferred to soil in a biosafety greenhouse at CIP and grown under natural daylight during the summer season (photoperiod 14 h; solar irradiation 12–20 MWs/m^2/day; day temperature 27 °C and night temperature 23 °C; relative humidity 60%) and watered and fertilized as needed.

RNA Isolation. Total RNA was isolated from 400 mg fresh leaf material using TRIzol (Invitrogen) following the manufacturer's instructions. Total RNA was resuspended in 250 μl sterile MilliQ water (Sigma-Aldrich). Low molecular weight (LMW) RNA was obtained by adding an equal volume of 4 M LiCl to the total RNA preparation and incubating the mixture on ice overnight. After centrifugation for 20 min at 12,000 $\times$ *g* (4 °C) the pellet containing high molecular weight (HMW) RNA was stored in 70% ethanol, and the supernatant containing LMW RNA was transferred to a new tube containing an equal volume of isopropanol and incubated for 1–2 h at −20 °C. RNA pellets were washed with 70% ethanol and resuspended in 25 μL (for LMW RNA) or 50 μL (for HMW RNA) nuclease-free water (Sigma). The amount and quality of the RNA were checked using a spectrophotometer (8543 UV-Visible, Agilent Technologies) and agarose gel electrophoresis.

Plant Inoculation and Virus Detection. SPFMV-Piu, SPMMV, SPCFV, and SPCSV m2–47 were transferred to test plants via grafting. SPFMV was detected from the youngest fully opened leaves by double antibody sandwich ELISA (DAS-ELISA), as follows. Two leaf discs (150 mg) were ground in a 1.5 mL Eppendorf tube with 1 mL extraction buffer [PBS (PBS) containing 5% (vol/vol) Tween 20 and 2% (wt/vol) polyvinylpyrrolidone (MW 40,000)] using a small plastic pestle and centrifuged at 6,000 $\times$ *g* for 2 min. Two aliquots of the supernatant (100 μL

each) were transferred to an ELISA microtiter plate (Greiner Bio-One) previously coated with rabbit polyclonal antibodies to SPFMV coat protein (provided by CIP) and incubated at 4 °C overnight. After washing for 3 times for 3 min with PBS containing 5% (vol/vol) Tween 20, 100 μL of alkaline phosphatase-conjugated anti-SPFMV antibodies (provided by CIP) diluted as recommended by CIP staff using extraction buffer was added to each well; the plate was then incubated at 37 °C for 3 h and washed as before. The color reaction was developed using *p*-nitrophenyl phosphate (Sigma) as a substrate. Absorbance (405 nm) was recorded using a Benchmark Microplate reader (Bio-Rad Laboratories). Detection of SPMMV, SPCFV, and SPCSV was carried out using nitrocellulose membrane ELISA (NCM-ELISA) as described (1). Polyclonal antibodies specific to the viral coat protein (produced at CIP) and goat anti-rabbit antibodies conjugated with alkaline phosphatase (Bio-Rad Laboratories) were used diluted 1:1,000 (vol/vol).

Northern Blot Hybridization. HMW RNA (5 μg) was separated in a 5.5% formaldehyde-containing denaturing 1% agarose gel and blotted onto a Hybond-NX membrane (Amersham Biosciences) overnight by capillary transfer. RNA was fixed to the membrane by exposure to UV light for 1 min and prehybridized in a solution containing 50% formamide (Sigma), 5× sodium chloride/sodium phosphate-EDTA (SSPE), 5% SDS (SDS), 2.5× Denhart's solution, and 1 mg/mL herring sperm DNA (Sigma). A probe complementary to *gfp* was prepared and labeled with [γ-^{32}P]UTP (Amersham) by in vitro transcription of *gfp* cloned into pCR-Blunt (Invitrogen) behind the T7 promoter. Hybridizations were carried out in freshly prepared prehybridization solution containing 25 μL of the in vitro transcription reaction at 55 °C overnight. The next day, membranes were washed at 68 °C 3 times in 5× sodium chloride/sodium citrate buffer + 0.5% SDS. Membranes were exposed to X-ray film (Kodak) for 4, 16, and 48 h and developed using an X-Omat 1000 automated developer (Kodak).

For siRNA detection, LMW RNA (15 μg) was analyzed in a 15% polyacrylamide gel containing 8 M urea (ATTO Corporation) and electroblotted to Hybond-NX membrane using a TransBlot Semidry Electrophoretic Transfer Cell (Bio-Rad) for 30 min following the manufacturer's instructions. RNA was fixed to the membranes by exposure to UV light. The aforementioned [γ-^{32}P]UTP-labeled probe was used to detect the *gfp*-derived siRNA. SPFMV-specific siRNAs were detected with a probe prepared from the 3′-proximal part of the SPFMV EA strain genome (including the 3′-end of the NIb-encoding region and the whole CP-encoding region) as above. The probe was heated in carbonate buffer (120 mM Na_2CO_3, 80 mM $NaHCO_3$, pH 10.2) at 60 °C for 170 min for fragmentation to obtain an average size probe of 50 nucleotides (20) and added to the hybridization solution. Washing and detection were performed as above except that they were carried out at 35 °C.

Western Blot Analysis. Leaf material (200 mg) was crushed in liquid nitrogen and boiled in 200 μL SDS-polyacrylamide gel electrophoresis sample buffer (20). Proteins were separated in a denaturing 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Hybond-P, GE Healthcare) by electroblotting as above. The RNase3 protein was detected with a specific rabbit antiserum raised against SPCSV RNase3, as described (4). Antibodies were diluted 1:500 with Tris-buffered saline (pH 7.4) containing 2.5% (wt/vol) fat-free milk powder and 1% sap from a healthy, non-transgenic sweet potato leaf to prevent any cross-reaction with host proteins. Anti-rabbit monoclonal mouse antibodies conjugated with horseradish peroxidase (Amersham), the Supersignal West Pico chemiluminescent substrate (Pierce Biotechnology), and exposure to an X-ray film were used to detect signals by the ECL method according to the manufacturer's instructions (Amersham).

RNA Cleavage Assays with RNase3. *RNase3* of SPCSV-Ug and the mutated gene, *RNase3-Ala*, were cloned in the pET11d vector (Stratagene) and used to transform *E. coli* BL21(DE3) cells for expression and purification of the proteins according to standard procedures (20). Bacteria were grown under selection with ampicillin at 37 °C. Samples (1 mL) of the bacterial culture (total volume 1.0 l) were taken at intervals of 1 h following induction with isopropyl-β-d-thiogalactoside. Purification of the 6× His-tagged proteins was accomplished using Ni-NTA agarose columns according to the manufacturer's instructions (Expressionist, Qiagen). The final eluates (containing 300 mM imidazole) were dialyzed overnight at 4 °C using cassettes with a size exclusion limit of 10,000 Da (Slide-A-Lyzer Dialysis Cassette 10,000 MWCO, Pierce) against a 2× protein storage buffer containing 30 mM Tris-HCl (pH 8.0), 500 mM KCl, 0.1 mM ethylene diamine tetraacetic acid, and 0.1 mM DTT. Protein concentrations were measured using the Bradford assay including known amounts of BSA for comparison. The 2× storage buffer was diluted by adding an equal volume of glycerol, and the protein samples were stored at −20 °C for use.

The siRNAs were ordered as single-stranded sense and antisense RNA oligonucleotides (21, 22, and 24 nucleotides). The sense oligonucleotides (10 pmol) were labeled by phosphorylation with [γ-^{32}P]ATP for 30 min using T4 polynucleotide kinase (Fermentas). Unincorporated label was removed using a Micro Spin G-25 column (GE Healthcare). The oligos were further purified by gel extraction as described (21). Duplexes of the oligonucleotides (dsRNA) were obtained by heating a mixture of labeled sense and unlabeled antisense oligonucleotides at 98 °C for 4 min and cooling at room temperature for 1 h. Each double-stranded siRNA substrate (4 pmol) was incubated in a reaction mix (20 μL) containing 9 μM RNase3 or RNase3-Ala, or 1 U of *E. coli* RNase III (New England Biolabs) in a reaction buffer [30 mM Tris-HCl, pH 7.5; 10 mM $MgCl_2$, 5% glycerol (vol/vol)] for 1 h at 37 °C. The reaction on synthetic siRNAs was stopped by boiling for 10 min in TBE-urea sample buffer (Bio-Rad). However, the reaction on unlabeled siRNA isolated from sweetpotato was stopped by adding EDTA (final concentration 10 mM) and incubating at 65 °C for 5 min. The reactions were analyzed by electrophoresis on a 20% polyacrylamide gel (1× TBE, 7.5 M urea) for 2 h at 60 mA. The labeled RNAs were visualized using a PhosphorImager (Fuji FLA-5010) following exposures for 4 h and 16 h. Unlabeled siRNAs were visualized by staining the gel with ethidium bromide.

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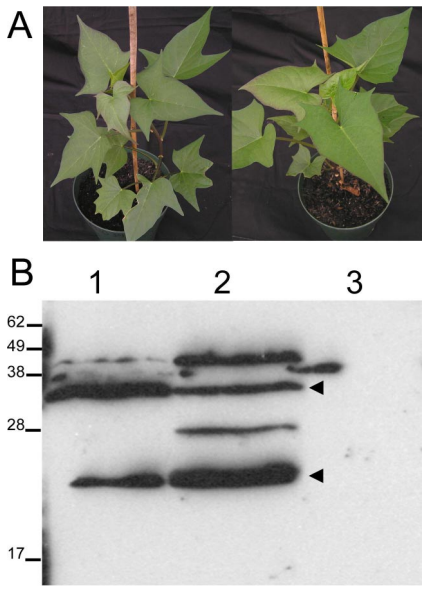

Fig. S1. Expression of *Sweet potato chlorotic stunt virus* (SPCSV) RNase3 and phenotype of *RNase3* transgenic sweet potato plants. (*A*) *RNase3* transgenic (*Left*) and non-transgenic (*Right*) sweet potato plants are phenotypically indistinguishable. (*B*) Western blot analysis using antibodies specific to RNase3 showing the presence of the RNase3 protein in the leaf lysate of the *RNase3*-transgenic sweet potato plant (lane 2); no signal is observed in the non-transgenic sweet potato plant lysate (lane 3). Purified recombinant RNase3 of SPCSV was analyzed in lane 1 as a control. Arrowheads point to the monomeric and dimeric forms of RNase3. The 2 additional RNase3 bands in the transgenic line (lane 2) might be due to post-translational modification of the protein.

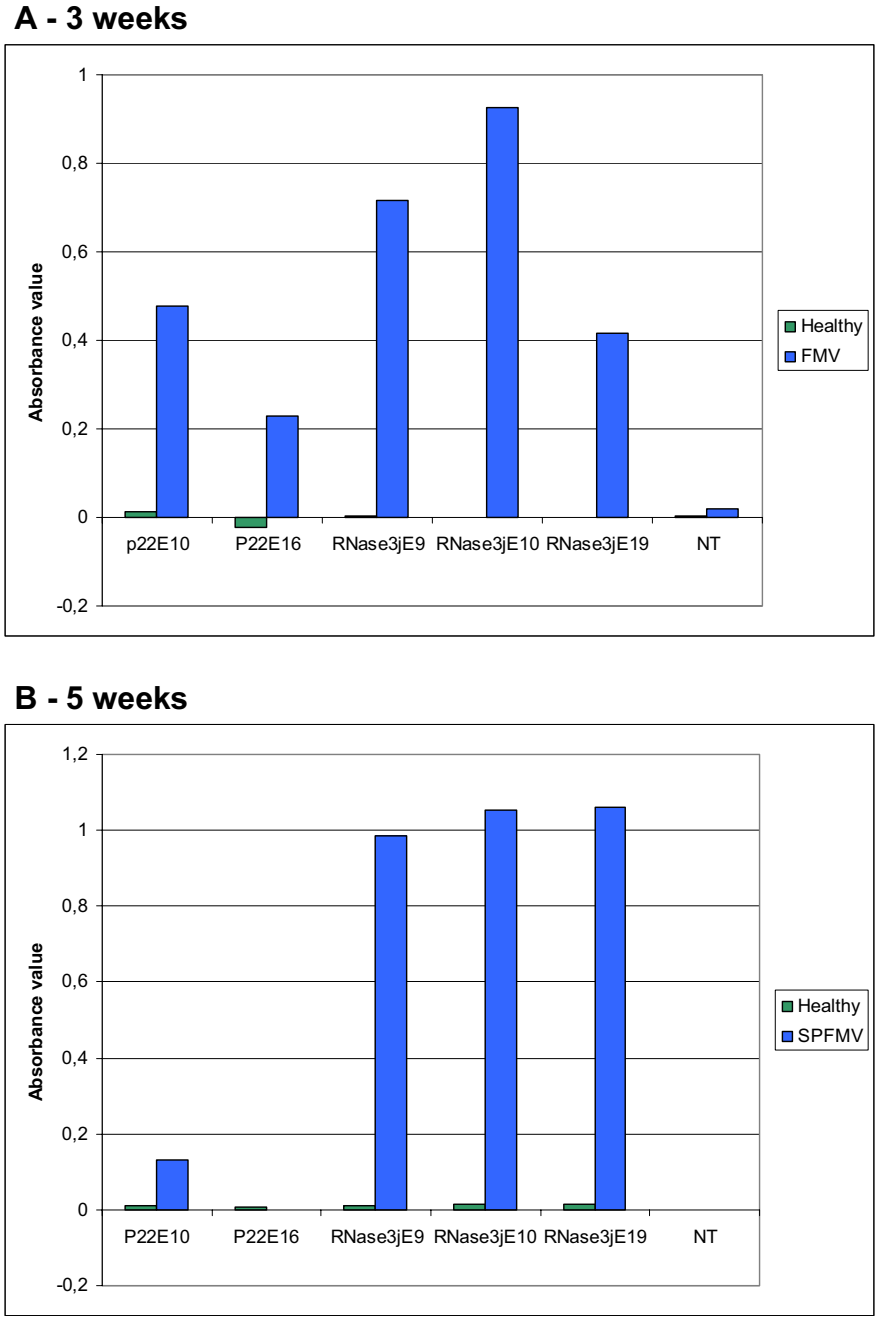

Fig. S2. Relative amounts of *Sweet potato feathery mottle virus* (FMV/SPFMV) coat protein antigen in the systemically infected leaves of p22-transgenic (2 independent lines), RNase3-transgenic (3 lines) and non-transgenic (NT) sweet potato cv. 'Huachano' (*A*) 3 weeks and (*B*) 5 weeks post-inoculation as detected by double antibody sandwich ELISA. Only RNase3-transgenic plants developed the severe symptoms of sweet potato virus disease (SPVD) following infection with SPFMV. The p22-transgenic and NT plants remained symptomless or expressed mild mottling 3 weeks post-inoculation and recovered from infection by 5 weeks post-inoculation.

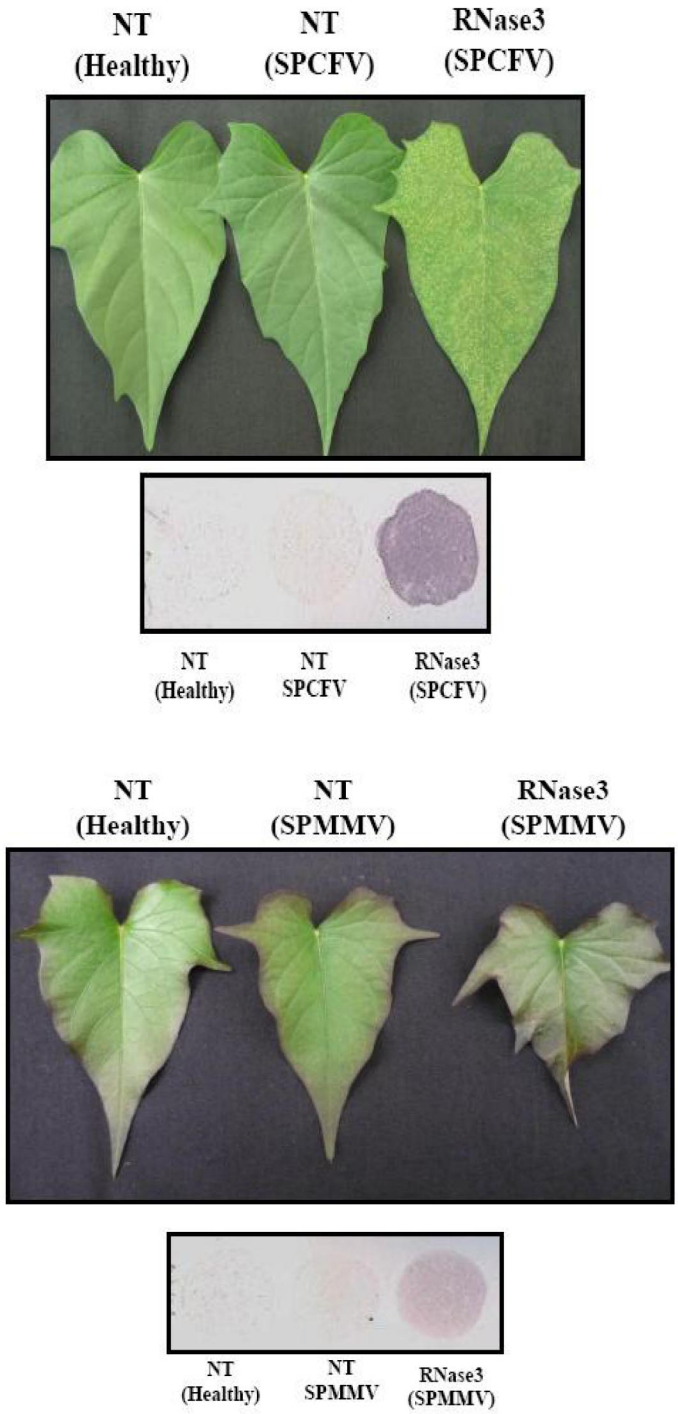

Fig. S3. Systemic infection of non-transgenic (NT) and RNase3-transgenic sweet potato cv. 'Huachano' with *Sweet potato chlorotic fleck virus* (SPCFV; *Top*) and *Sweet potato mild mottle virus* (SPMMV; *Bottom*). Both viruses are barely detectable in the non-inoculated upper leaves of the inoculated NT plants (results shown from nitrocellulose membrane ELISA) and no symptoms are observed. However, in RNase3-transgenic plants, virus concentrations are elevated and readily detected. SPCFV-infected plants develop apparent symptoms of chlorosis (chlorotic flecks). SPMMV-infected plants display leaf malformation and stunting.

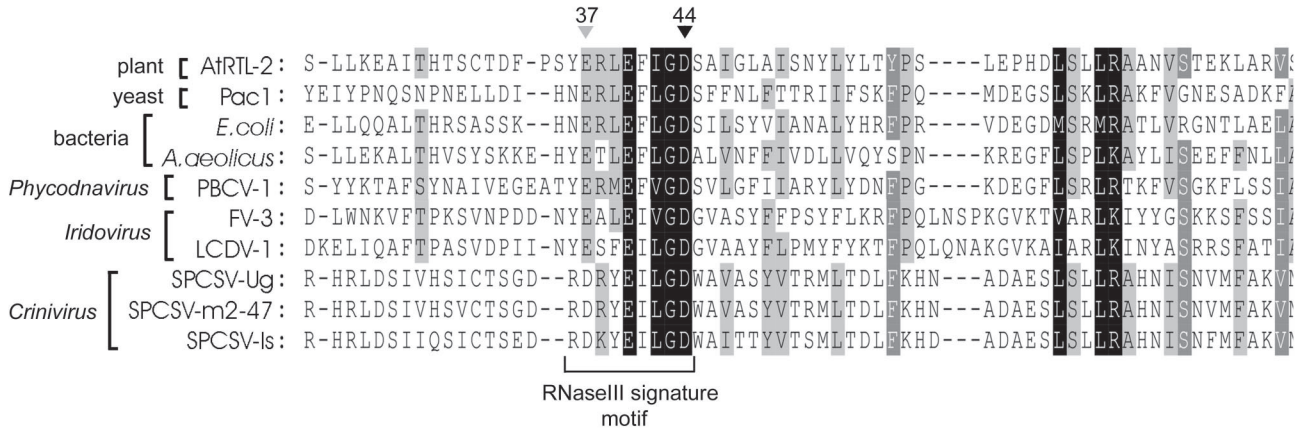

Fig. S4. Class 1 RNase III−like proteins encoded by different organisms. Sequences were analyzed using the VectorNTI-v9 program package (Invitrogen) and the PSI-BLAST program available from the National Center for Biotechnology Information (NCBI). Only the amino acid sequences corresponding to the endonuclease domain region are shown. Numbers 37 and 44 at the top of the alignment indicate the positions of the residues necessary for dsRNA cleavage (RNase III signature motif) (6) based on SPCSV RNase3 (3, 4). AtRTL-2, RNase III from *Arabidopsis thaliana* (7); PacI, RNase III from *Schizosaccharomyces pombe* (8); '*E. coli* ' and '*A. aeolicus* ' indicate RNase III from *Escherichia coli* (9) and *Aquifex aeolicus* (10), respectively. PBCV-1, RNase III from *Paramecium bursaria chlorella virus* (11) (a DNA virus; *Phycodnaviridae*); FV-3, RNase III from *Frog virus 3* (*Iridoviridae*, genus *Ranavirus*, which includes DNA viruses that infect fish and amphibians) (12); and LCDV-1, RNase III from *Lymphocystis disease virus 1* (*Iridoviridae*, genus *Lymphocystivirus*, which includes DNA viruses that infect fish) (13). SPCSV-Ug, SPCSV-m2–47 and SPCSV-Is refer to RNase III proteins of the 3 isolates of SPCSV used in this study.

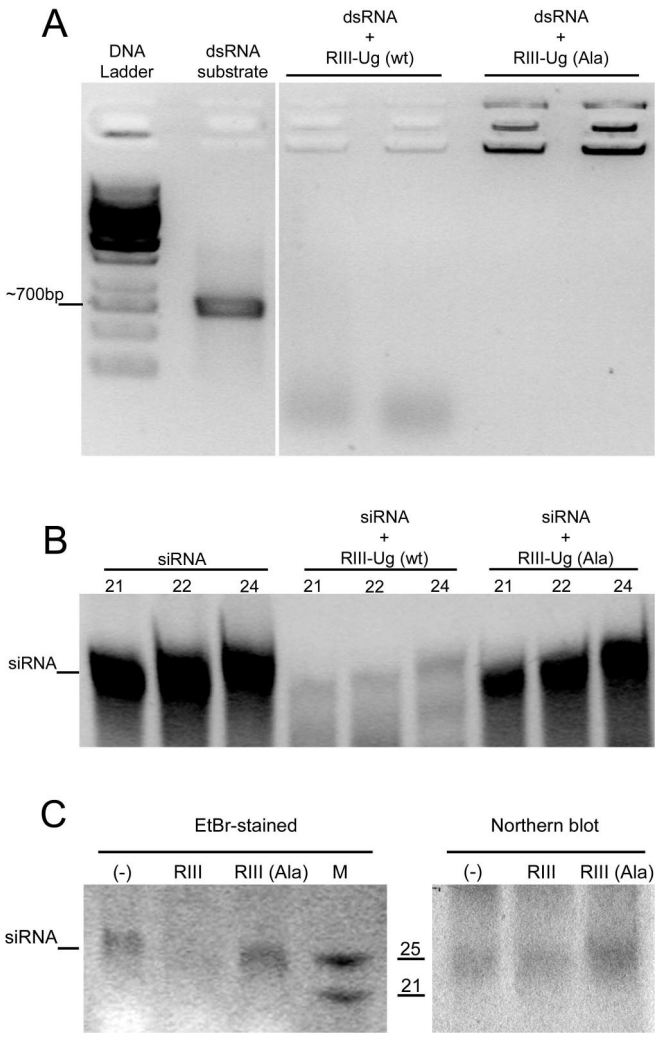

Fig. S5. The RNase3-Ala mutant does not cleave dsRNA. (*A*) A dsRNA substrate (≈700 bp) was incubated in the presence of RNase3 [RIII-Ug (wt)] and its mutant RNase3-Ala [RIII-Ug (Ala)] in which amino acids 37 and 44 in the signature motif were substituted with alanine (see Fig. S4). The 700-bp dsRNA substrate was synthesized by in vitro transcription of *gfp* flanked by T7 and T3 promoters for 1 h at 37 °C, heated to 95 °C for 5 min, and then cooled for 1 h at room temperature. The dsRNA (200 ng) in each reaction was analyzed by mixing 20 μL of the reaction with 20 μL of 2× RNA loading dye (Fermentas UAB) and separating by electrophoresis in a 2% agarose gel. Cleavage by wild-type RNase3 was efficient, whereas the mutant RNase3-Ala could not cleave the dsRNA substrate. However, RNase3-Ala could still bind dsRNA, as indicated by retardation of dsRNA in the wells. Two samples from 2 independent assays were analyzed in adjacent wells. (*B*) RNase3 [RIII-Ug (wt)] cleaved synthetic double-stranded siRNAs of 21, 22, and 24 bp, in contrast to RNase3-Ala [RIII-Ug (Ala)] in a 1-h incubation at 37 °C. Samples were analyzed by electrophoresis using a 15% polyacrylamide TBE-urea gel. For cleavage analysis of siRNA, see *SI Materials and Methods*. (*C*) A portion of double-stranded siRNA in the pool of siRNAs isolated from an SPFMV-infected sweetpotato plant was cleaved by RIII-Ug (lane RIII), as revealed by comparison to the same amount of siRNA that was not treated (-) or was treated with mutant RNase3-Ala. The small RNAs were analyzed by 20% polyacrylamide TBE-urea gel and stained with ethidium bromide (*Left*). Subsequently, RNA was transferred to Hybond NX membrane by semidry blotting and detected by Northern blot hybridization using an SPFMV-specific radioactive RNA probe (*Right*) (for details, see *SI Materials and Methods*). M, siRNA size marker.