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Soybean antiviral immunity conferred by dsRNase targets the viral replication complex

Kazuhiro Ishibashi¹, Masayasu Saruta^{2,8}, Takehiko Shimizu^{3,9}, Miao Shu¹, Toyoaki Anai⁴, Kunihiro Komatsu^{5,10}, Naohiro Yamada⁶, Yuichi Katayose^{7,11}, Masayuki Ishikawa¹, Masao Ishimoto^{3,12} & Akito Kaga³

Eukaryotic positive-strand RNA viruses replicate their genomes in membranous compartments formed in a host cell, which sequesters the dsRNA replication intermediate from antiviral immune surveillance. Here, we find that soybean has developed a way to overcome this sequestration. We report the positional cloning of the broad-spectrum soybean mosaic virus resistance gene *Rsv4*, which encodes an RNase H family protein with dsRNA-degrading activity. An active-site mutant of *Rsv4* is incapable of inhibiting virus multiplication and is associated with an active viral RNA polymerase complex in infected cells. These results suggest that *Rsv4* enters the viral replication compartment and degrades viral dsRNA. Inspired by this model, we design three plant-gene-derived dsRNases that can inhibit the multiplication of the respective target viruses. These findings suggest a method for developing crops resistant to any target positive-strand RNA virus by fusion of endogenous host genes.

¹Plant and Microbial Research Unit, Division of Plant and Microbial Sciences, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan. ²Crop Breeding and Food Functional Components Division, Western Region Agricultural Research Center, National Agriculture and Food Research Organization, 1-3-1 Senyu-cho, Zentsuji-shi, Kagawa 765-8508, Japan. ³Soybean and Field Crop Applied Genomics Research Unit, Institute of Crop Science, National Agriculture and Food Research Organization, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8518, Japan. ⁴Faculty of Agriculture, Saga University, 1 Honjo-machi, Saga 840-8502, Japan. ⁵Research Team for Crop Cold Tolerance, Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Hitsujigaoka 1, Toyohira, Sapporo, Hokkaido 062-8555, Japan. ⁶Nagano Vegetable and Ornamental Crops Experiment Station, 1066-1, Soga, Shiojiri, Nagano 399-6461, Japan. ⁷Advanced Genomics Breeding Section, Institute of Crop Science, National Agriculture and Food Research Organization, 1-2 Ohwashi, Tsukuba, Ibaraki 305-8634, Japan. ⁸Present address: Soybean Breeding Unit, Institute of Crop Science, National Agriculture and Food Research Organization, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8518, Japan. ⁹Present address: Advanced Genomics Breeding Section, Institute of Crop Science, National Agriculture and Food Research Organization, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8518, Japan. ¹⁰Present address: Crop Breeding and Food Functional Components Division, Western Region Agricultural Research Center, National Agriculture and Food Research Organization, 1-3-1 Senyu-cho, Zentsuji-shi, Kagawa 765-8508, Japan. ¹¹Present address: Department of Planning and Coordination, National Agriculture and Food Research Organization, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8517, Japan. ¹²Present address: Division of Basic Research, Institute of Crop Science, National Agriculture and Food Research Organization, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8517, Japan. Correspondence and requests for materials should be addressed to A.K. (email: kaga@affrc.go.jp)

Eukaryotes have evolved several layers of antiviral defense systems. Double-stranded (ds) RNA is a virus-specific molecular pattern recognized by hosts that triggers innate immunity or RNA silencing¹. In plants, dsRNAs produced by either viral or plant endogenous RNA-dependent RNA polymerases (RdRp) are processed by the Dicer-like RNase III proteins to generate 21-nt to 24-nt small interfering (si) RNAs. siRNAs are incorporated into Argonaute proteins to make an RNA-induced silencing complex (RISC), which cleaves RNAs having sequences complementary to the siRNA². To counteract RNA silencing by the host, viruses encode suppressors of RNA silencing, many of which bind siRNA and prevent RISC formation².

In addition to the RNA-silencing-mediated basal resistance, plants have genes that confer resistance to specific viruses. Most virus resistance genes in plants encode nucleotide binding site-leucine-rich repeat (NB-LRR) proteins that elicit a defense reaction upon recognition of pathogens³. Other virus resistance genes encode structurally unrelated proteins with various functions³. Resistance genes have been introduced into crops to protect them from viral diseases; however, the available resistance genes cover only a limited range of viruses.

The genus *Potyvirus* in the family *Potyviridae*, a family of positive-strand RNA viruses, includes many economically important crop viruses such as *Potato virus Y* (PVY), *Turnip mosaic virus* (TuMV), and *Plum pox virus* (PPV). Potyviruses are transmitted by aphids, and their genome encodes a large polyprotein that is processed into ten or eleven proteins⁴. To protect from potyviruses, resistance genes have been introduced into many crop cultivars such as potato⁵, pepper⁶, papaya⁷, plum⁸, *Brassica* crops⁹, and legumes¹⁰.

Soybean mosaic virus (SMV) is a potyvirus that reduces soybean yields and seed quality worldwide^{11,12}. Four dominant SMV resistance genes have been found in soybean accessions and introduced into commercial cultivars. *Rsv1*, *Rsv3*, and *Rsv5* are strain-specific and are suggested to encode NB-LRR proteins^{13–17}. In contrast, *Rsv4* confers broad-spectrum resistance to SMV strains through an atypical mechanism that delays SMV multiplication¹⁸. In this study, we aim to clone the *Rsv4* gene and elucidate the resistance mechanism. The mechanism of *Rsv4*-mediated SMV resistance deepens our knowledge of the virus–host arms race and inspire us to develop a method to engineer virus-resistant organisms by fusion of endogenous host genes.

Results

Positional cloning of the SMV resistance gene *Rsv4*. Here, we report positional cloning of *Rsv4* (Fig. 1a, Supplementary Fig. 1). Using 9320 progenies of a cross between soybean cultivars Peking (*Rsv4*; resistant) and Enrei (*rsv4*; susceptible), we mapped the gene within a 9.8-kbp region on soybean chromosome 2, adjacent to the *Rsv4* region previously mapped by other groups^{19–21}. In this region of Enrei and the SMV-susceptible cultivar Williams 82, two tandemly repeated homologous open reading frames (ORFs; NM_001249088 and NM_001253944) were found (Fig. 1a). Both ORFs contain an intron and encode RNase H-like proteins (Supplementary Fig. 1c, d). In contrast, a 3.6-kbp deletion was found in this region of the Peking genome, and only a single ORF that encodes a 259-amino-acid protein was found (Fig. 1a, Supplementary Fig. 1). The predicted protein from the Peking ORF has an RNase H-like domain and a transmembrane helix (Fig. 1b). Transgenic soybean plants overexpressing this RNase H family protein from Peking showed resistance to all tested SMV strains except for SMV-L(Q1033K), a mutant SMV that has an amino acid substitution in the P3 cistron and can

multiply more rapidly than its parental strain in *Rsv4* plants²² (Fig. 2a–c). Thus, this RNase H family protein inhibits SMV multiplication with the same virus specificity seen in *Rsv4* soybean lines. Following ethyl methanesulfonate (EMS) mutagenesis of Peking, we isolated four independent mutant alleles of this RNase H family gene and found that three of them conferred recessive susceptibility to strain SMV-C (Fig. 2d, Supplementary Table 1, Supplementary Fig. 2). We conclude that the *Rsv4* gene encodes this RNase H family protein.

The 3.6-kbp deletion identified in resistant cultivar Peking was rare in cultivated soybean but was found in Chinese and Korean soybean (*Glycine max*) landraces and more frequently in *Glycine soja*, the wild progenitor of soybean (Fig. 3). All of the resistant accessions we examined contained the 3.6-kbp deletion. On the other hand, there were susceptible accessions both with and without the 3.6-kbp deletion, indicating that it did not always confer SMV resistance (Supplementary Fig. 3). We further classified 63 soybean accessions that carried the 3.6-kbp deletion into 16 types based on amino acid sequence (Supplementary Figs. 3 and 4). A close relationship was observed between amino acid sequence and a degree of SMV CP accumulations, providing supporting evidence that the gene encoding the RNase family protein is *Rsv4*. The predicted *Rsv4* protein sequences of Beeson (PI548510)²³ and PI88788¹⁸, which are both resistant to SMV but reported to have different *Rsv4* alleles, differed at 3 and 16 positions, respectively, from that of Peking and V94-5152 (PI596752)¹⁹ (Supplementary Fig. 4). The SMV-C resistance of Fukusennari was mapped to the *Rsv4* region by using progenies of a cross with Enrei, but its sequence is different from those of Peking, Beeson, and PI88788 (Supplementary Figs. 3 and 4). The syntenic regions in other legume genomes contain complex arrangements of RNase H genes, suggesting the prevalence of genomic rearrangements at this locus (Supplementary Fig. 5). While RNase H proteins are universally encoded in plant genomes, the N-terminal transmembrane helix, such as found in *Rsv4*, is unique to legume RNase H proteins.

Rsv4 is a dsRNase interacting with SMV replication machinery.

The *Rsv4* protein contains a DEDN motif in which the fourth residue of the DEDD motif in the active site of many RNase H proteins²⁴ is changed from aspartate to asparagine (Fig. 1b). We introduced a mutation into *Rsv4* to substitute the first D of the DEDN motif with N (D99N). Transient expression of *Rsv4* by agroinfiltration inhibited SMV multiplication in *Nicotiana benthamiana* but the *Rsv4*(D99N) mutant did not (Fig. 2e), suggesting that resistance depends on an activity of the RNase H-like domain. When *Agrobacterium* containing a wild-type *Rsv4* construct was infiltrated at high concentrations, virus multiplication was inhibited even for SMV-L(Q1033K) (Fig. 2f), indicating that overexpression of *Rsv4* can increase the durability of the resistance. In the *N. benthamiana* transient overexpression system, one of the two ORFs from Jack (susceptible), NM_001249088, inhibited SMV multiplication while the inhibitory activity was weaker than *Rsv4* when *Agrobacterium* concentration was low (Supplementary Fig. 6a). NM_001249088 mRNA was expressed at similar or slightly higher levels to *Rsv4* in soybean leaves (Supplementary Fig. 6b). These results suggest that NM_001249088 encodes a functional antiviral protein but that the effects of its endogenous expression in soybean have been overcome by SMV.

Although the canonical function of RNase H is to degrade RNA strands of DNA–RNA hybrids, no DNA–RNA hybrid is formed during positive-strand RNA virus replication²⁵. We thus investigated the substrate preference of *Rsv4*. Purified FLAG-tagged *Rsv4* protein degraded neither single-strand RNA nor a

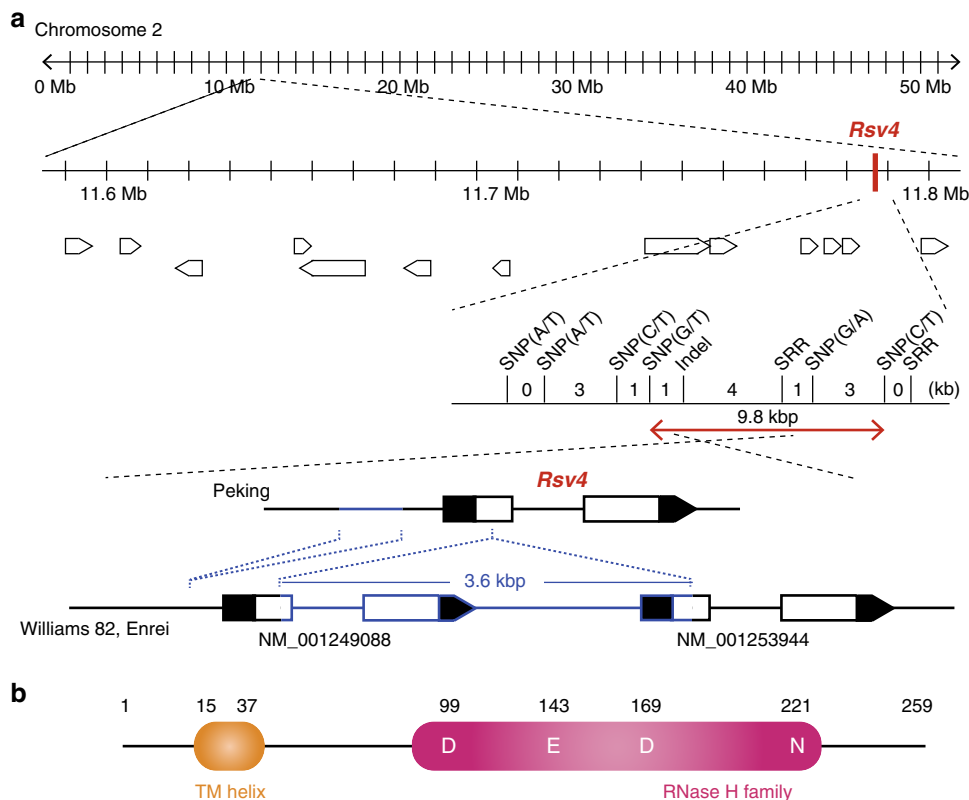


Fig. 1 Positional cloning of *Rsv4*. **a** Fine mapping of *Rsv4*. The gene structures in the Peking, Williams 82, and Enrei genomes are shown at the bottom. Indels are indicated in blue. Black boxes are untranslated regions. **b** Predicted *Rsv4* protein structure. Amino acid positions of the transmembrane (TM) helix and active-site residues of the RNase H family domain are shown

DNA–RNA hybrid, but it showed manganese-dependent dsRNase activity (Fig. 4a). Magnesium ion did not activate the dsRNase activity of *Rsv4* even at 10 mM. Thus, *Rsv4* is a member of the RNase H family that degrades only dsRNA. The D99N mutation abolished dsRNase activity (Fig. 4a), suggesting that this activity is essential for SMV resistance. Positive-strand RNA viruses replicate their genomes via a complementary (negative-strand) RNA, which leads to the formation of dsRNA. Thus, a plausible mechanism of action of *Rsv4* is to degrade viral dsRNA formed during replication.

Positive-strand RNA viruses replicate their genomes in a membranous replication compartment that is isolated from the cytoplasm^{26,27}. Viruses thereby hide their dsRNA replicative intermediates from cytoplasmic surveillance mechanisms. Thus, *Rsv4* must somehow enter the site of SMV RNA replication to be able to efficiently inhibit the replication of the virus by degrading viral dsRNA. To test this possibility, we examined whether *Rsv4* physically associates with the SMV replication machinery. Since SMV-L(Q1033K) has an amino acid substitution in the P3 protein^{22,28–30}, we first examined the interaction of *Rsv4* with P3. Currently, the function of P3 protein of potyviruses remains obscure; however, it has transmembrane regions and interacts with viral N1b polymerase^{31,32}, and it is suggested to be an essential component of the viral replication complex³³. When Myc-tagged SMV P3 protein was co-expressed with FLAG-tagged *Rsv4* protein in *N. benthamiana* leaves, P3-Myc was co-immunoprecipitated with *Rsv4*-FLAG from detergent-solubilized membranes of the leaf extracts (Fig. 4b). The tagged *Rsv4*(D99N) mutant protein (D99N-FLAG) retained the ability to bind P3-Myc (Fig. 4b), indicating that binding ability is not sufficient for resistance. We did not observe any difference between Myc-fused P3 proteins of SMV-C (wild-type) and SMV-

L(Q1033K) in their ability to bind *Rsv4*-FLAG in the immunoprecipitation experiment (Fig. 4c). This is consistent with the above result that *Rsv4* could inhibit SMV-L(Q1033K) multiplication when expressed at high levels (Fig. 2f).

Since D99N-FLAG interacted with P3 but did not inhibit SMV multiplication, we speculated that the *Rsv4*(D99N) mutant protein associates with the viral replication complex without affecting viral replication. We thus further examined whether a viral RdRp is co-purified with D99N-FLAG from solubilized membranes of SMV-infected leaves. Before solubilization, RdRp activity was detected in membrane fractions of D99N-FLAG-expressing and GUS (negative control)-expressing leaves inoculated with SMV, but not in those of SMV-inoculated *Rsv4*-FLAG-expressing or non-inoculated GUS-expressing leaves (Fig. 4d). After solubilization and immunoprecipitation with anti-FLAG antibody, RdRp activity was detected in the D99N-FLAG-immunoprecipitated fraction (Fig. 4d). SMV N1b protein (the catalytic subunit of RdRp) and P3 protein were detected in the D99N-FLAG-immunoprecipitated fraction. Thus, D99N-FLAG physically associates with a replication-catalyzing viral protein complex. These results strongly suggest that in *Rsv4*-expressing cells, *Rsv4* enters membrane-surrounded viral replication compartments through its interaction with replication machinery; in the replication compartments, it degrades viral dsRNA replication intermediates. The results also suggest that an active potyviral RdRp contains P3 protein in addition to the N1b protein.

***Rsv4* confers broad-spectrum resistance against potyviruses.** The genus *Potyvirus* consists of over 150 species, accounting for ~15% of all named plant virus species, and includes many

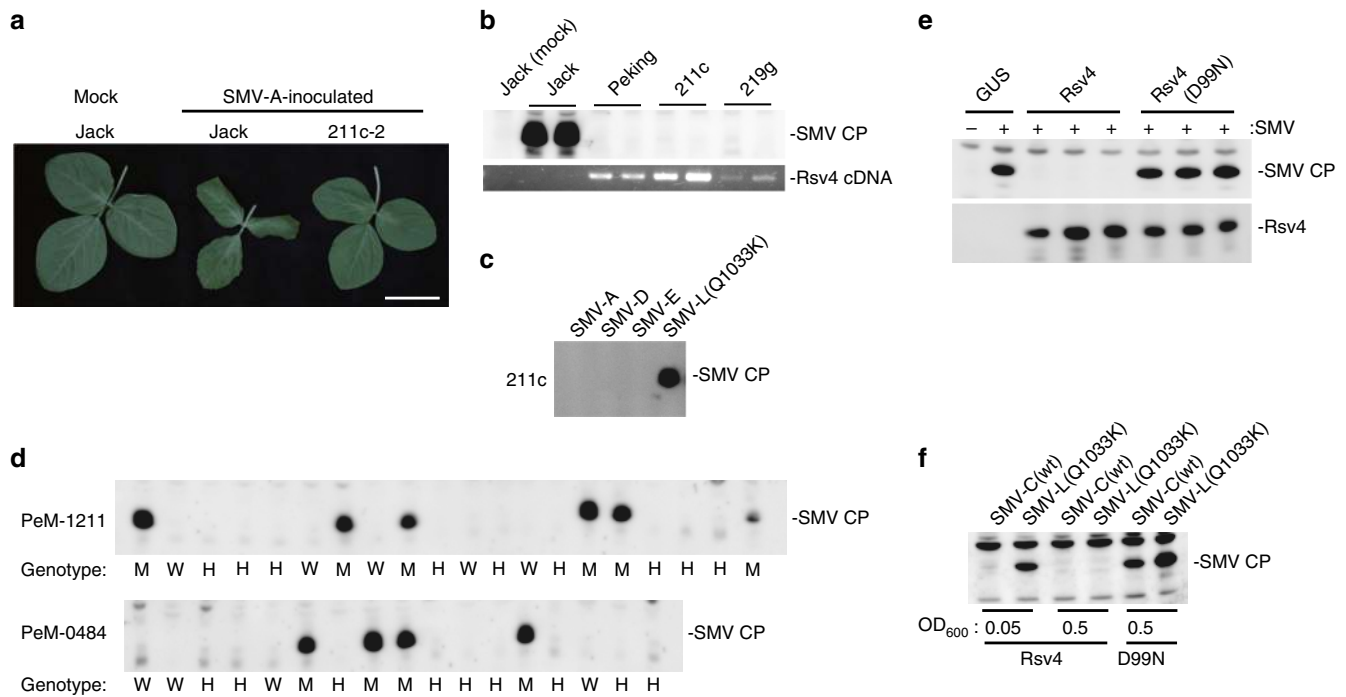


Fig. 2 *Rsv4* encodes an RNase H family protein. **a** Symptoms of SMV-inoculated soybean plants. 211c-2 is a transgenic line derived from susceptible cv. Jack, overexpressing *Rsv4* from Peking. Bar = 5 cm. **b** SMV coat protein (CP) accumulation in control and transgenic soybean plants. 211c expresses *Rsv4* cDNA driven by the cauliflower mosaic virus 35 S promoter, while 219 g carries a genomic DNA fragment of Peking containing the putative native promoter of *Rsv4*. SMV CP accumulation in non-inoculated upper leaves was detected by western blotting at 10 days post-inoculation (dpi) (upper panel). *Rsv4* mRNA was detected by RT-PCR (lower panel). Lanes represent individual plants. **c** SMV strains SMV-A, -D, -E, and -L(Q1033K) were used to inoculate 211c, and CP accumulation was analyzed as in panel **b**. **d** Co-segregation of PeM-1211 and PeM-0484 (identical nonsense mutations in Peking *Rsv4* gene) and SMV-susceptible phenotype. W wild-type, M mutant-type, H heterozygous. **e** *Rsv4*, but not the *Rsv4*(D99N) mutant protein, inhibits SMV multiplication in *N. benthamiana* leaves. SMV CP accumulation was detected by western blotting at 5 dpi. **f** SMV-L(Q1033K) is less sensitive to *Rsv4* than SMV-C. *Agrobacterium* strains containing constructs to express *Rsv4* or *Rsv4*(D99N) were infiltrated at the indicated concentrations. 1 day after infiltration, *Agrobacterium* harboring SMV cDNA was infiltrated at OD₆₀₀ = 0.005. CP accumulation was detected at 7 dpi. The source data of Fig. 2b-f are provided as a Source Data file

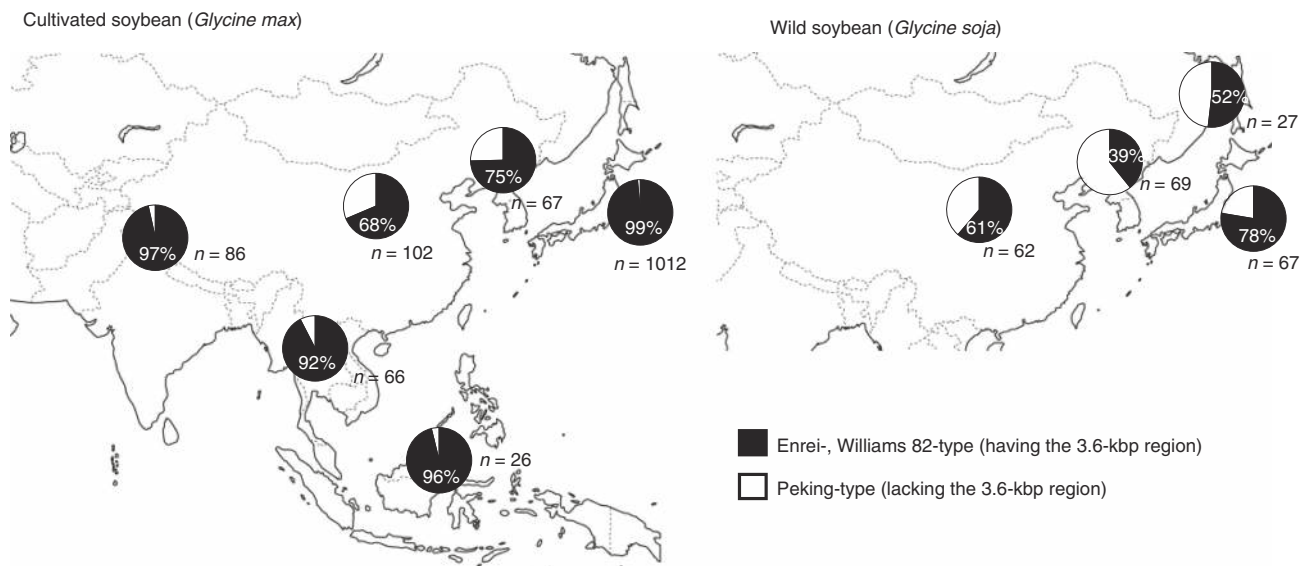


Fig. 3 Geographical distribution of genetic diversity of the *Rsv4* locus. The presence or absence of the 3.6-kbp region in *Rsv4* in cultivated soybean (*Glycine max*) and wild soybean (*G. soja*) are shown in black or white, respectively. The number of investigated plants and percentage of plants having the 3.6-kbp region are indicated for each geographical area. See also Supplementary Data 1

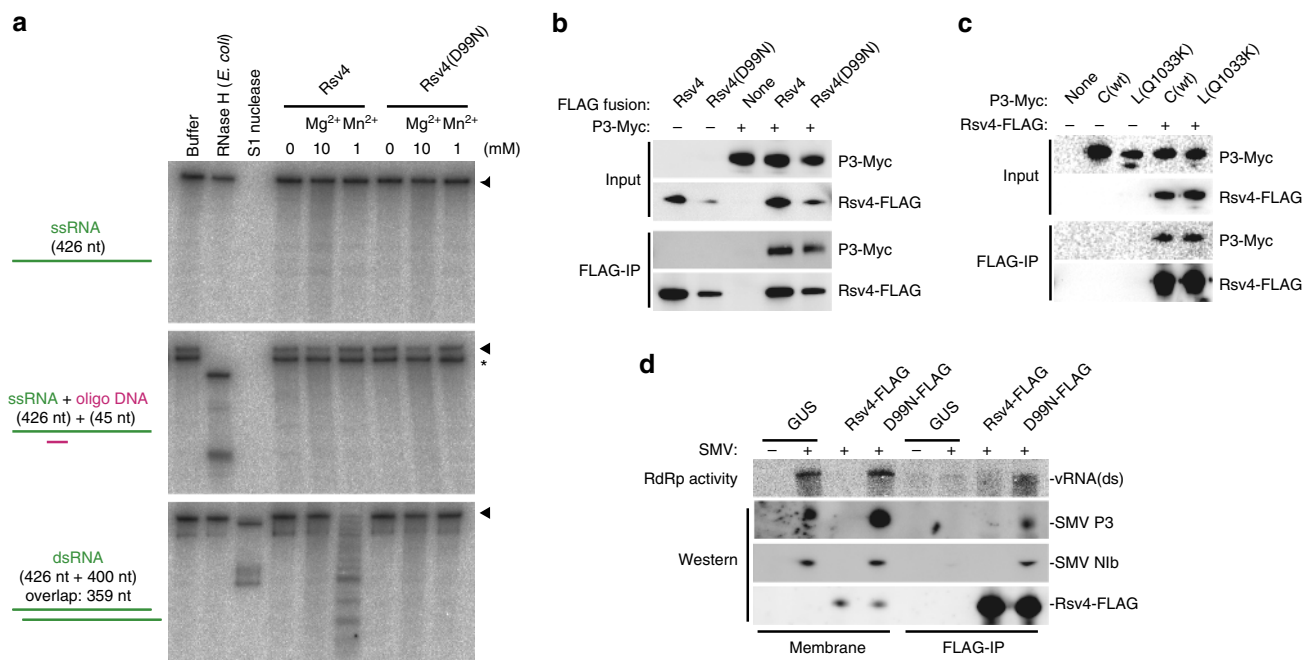


Fig. 4 Rsv4 is a Mn²⁺-dependent dsRNase that associates with SMV replication machinery. **a** Activity of Rsv4. Purified Rsv4 protein or Rsv4 with a D99N substitution was incubated with ³²P-labeled ssRNA (top), ssRNA hybridized with oligo DNA (middle), or dsRNA (bottom). Divalent cation was supplied as indicated. As controls, *E. coli* RNase H and S1 nuclease were used. RNA was detected by urea-PAGE followed by autoradiography. Positions of the substrate RNAs are indicated to the right by arrowheads. The bands marked by an asterisk migrated at the same speed as the ssRNA. **b** SMV P3-Myc is co-immunoprecipitated with Rsv4-FLAG. Proteins were detected by anti-FLAG and anti-Myc antibodies. **c** P3-Myc from SMV-L(Q1033K) is co-immunoprecipitated with Rsv4-FLAG. C(wt) indicates strain SMV-C. **d** Rsv4(D99N) associates with SMV RdRp in infected leaves. Membrane fractions of leaf homogenates (Membrane) and immunoprecipitates using anti-FLAG antibody after solubilization (FLAG-IP) were analyzed by ³²P incorporation (RdRp activity) and western blotting (P3 and N1b). The source data of Fig. 4a-d are provided as a Source Data file

agriculturally important viruses worldwide³⁴. We tested whether Rsv4 inhibits multiplication of other potyviruses by using agroinfiltration-mediated transient expression in *N. benthamiana*. Remarkably, multiplication of TuMV, PVY, PPV, bean common mosaic virus (BCMV), potato virus A (PVA), and peanut mottle virus (PeMoV) was inhibited by Rsv4 (Fig. 5a). TuMV causes severe symptoms in *N. benthamiana* but did not induce any symptoms when inoculated onto Rsv4-expressing leaves (Fig. 5b). Thus, Rsv4 can confer broad-spectrum resistance against potyviruses. Accumulated levels of the genomic RNA of tomato mosaic virus (ToMV; genus *Tobamovirus*, family *Virgaviridae*), a positive-strand RNA virus, were comparable in the presence of Rsv4 and Rsv4(D99N) (Fig. 5a). This result disproves the possibility that Rsv4 nonspecifically degrades dsRNAs of all positive-strand RNA viruses, and provides further evidence for the importance of the interaction of Rsv4 with potyvirus replication machinery for resistance.

Engineering dsRNases targeting viral replication sites. Virtually all eukaryotic positive-strand RNA viruses replicate in membrane-bound replication complexes, and the dsRNA replication intermediates are formed only within the membrane compartments. The model for Rsv4 action we describe here suggests that dsRNase can confer resistance against any positive-strand RNA virus if the enzyme is delivered into the replication compartment (Fig. 6a). To examine this antiviral activity, we fused a dsRNase protein, RTL2³⁵ from *Arabidopsis thaliana* (chosen because RTL2 does not have a transmembrane region), with three host proteins, each of which has been proposed to be associated with the replication complex of a specific virus: TOM1 for ToMV³⁶, TIP1 for cucumber mosaic virus (CMV)³⁷, and eIF (iso)4E for TuMV³⁸ (genus *Tobamovirus*, *Cucumovirus*, and

Potyvirus, respectively). When transiently expressed in *N. benthamiana* leaves, these fusion proteins greatly suppressed the multiplication of the corresponding viruses (Fig. 6b). Neither RTL2 itself nor fusion proteins with a catalytic mutation in RTL2 (D100A) inhibited multiplication of the viruses. These results reinforce the validity of the model for the virus resistance mechanism used by Rsv4, i.e., penetration of dsRNases into viral replication compartments, and suggest its applicability to designing resistance genes against viruses of interest.

Discussion

In the present study, we identified the broad-spectrum SMV resistance gene *Rsv4* of soybean by positional cloning and demonstrated its function by transgenic expression in SMV-susceptible soybean, mutagenesis in SMV-resistant soybean, transient expression in *N. benthamiana*, and biochemical analyses. Genomic rearrangements likely caused the 3.6-kbp insertion/deletion in the *Rsv4* locus, but it is unclear exactly how the locus evolved. The geographical distribution of the 3.6-kbp insertion/deletion in soybean and *Glycine soja* shown in Fig. 3 suggests that genetic variation of this chromosome region was reduced during expansion of soybean outside its probable areas of domestication (China and Korea). Tandemly repeated SMV-susceptible *Rsv4* alleles with the 3.6-kb region also encode RNase H proteins with the DEDD(N) motif (Supplementary Fig. 5), and NM_001249088 (present in susceptible cultivars containing the 3.6-kbp insertion) inhibited SMV multiplication when overexpressed in *N. benthamiana* (Supplementary Fig. 6a). Thus, it is possible that the SMV-susceptible *Rsv4* alleles confer resistance to viruses that are not adapted to soybean, as in the case of *Tm-1/tm-1* of tomato: a susceptible allele of ToMV

As a complement to traditional breeding, biotechnological engineering has been an alternative approach to producing virus-resistant crops. Abel et al. showed that transgenic tobacco plants expressing the coat protein (CP) gene of tobacco mosaic virus are resistant to the virus⁵⁰. This method, referred to as pathogen-derived resistance, also includes RNA interference against viral genome sequences⁵¹. Pathogen-derived resistance has been used to produce many virus-resistant crops⁵²; however, only a handful of these transgenic plants having viral genes have been commercialized because of problems including a lack of regulatory framework in developing countries⁵³. Another way to engineer virus-resistant crops is knockout/knockdown of host genes that are essential for virus multiplication⁵⁴. This strategy does not need transgenic techniques when the knockout/knockdown is achieved using mutagenesis or genome editing, but few examples are available since loss of function of host factor genes most often affects plant growth.

We could successfully imitate *Rsv4* to design custom-made antiviral proteins by fusion of a dsRNase with host proteins used by viruses for their replication. In contrast to the genetically modified virus-resistant crops created by introduction of viral genes, dsRNase-mediated resistance only requires a fusion of endogenous host genes. Thus, using this method in combination with a knock-in technology, it may be possible to create transgene-free virus-resistant crops. The resultant virus-resistant plants would grow normally if the original functions of the host factors are unaffected by fusion with dsRNase. Although transient expression of the dsRNase–host factor fusion proteins did not completely inhibit multiplication of the target viruses (Fig. 6b), knock-in plants would be expected to show higher resistance because the endogenous dsRNase-free host factors would no longer be available for the viruses. An additional advantage of this method is that plants resistant to multiple viruses may be produced by fusing dsRNase to factors commonly used by diverse viruses.

Methods

Plant materials. A genetic mapping population for *Rsv4* was developed from a cross between SMV-C-resistant cultivar ‘Peking’ (*Rsv4*) and SMV-C-susceptible cultivar ‘Enrei’ (*rsv4*). Both soybeans were obtained from Nagano Vegetable and Ornamental Crops Experiment Station, Shiojiri, Japan, and registered as accession numbers GmWMC084 (Peking) and GmJMC025 (Enrei) in the NIAS soybean mini core collection of the Genetic Resources Center, NARO, Tsukuba, Japan. An F₂ mapping population consisting of 190 plants was initially used to delimit the *Rsv4* locus by using simple sequence repeat (SSR) markers. For fine mapping, five backcross populations consisting of 9320 individuals in total were further developed from F₁ plants between ‘Enrei’ and ‘Peking’ using ‘Enrei’ as a female recurrent parent in the cross to obtain materials having recombination around *Rsv4* (Supplementary Fig. 1). Seeds with recombination detected by marker analysis were selectively grown to obtain progenies in the field or vinyl greenhouse at the Institute of Crop Sciences, Tsukuba, Japan (36°2′N, 140°8′E), from June to November. The progenies of the recombinants were used to examine resistance to SMV.

A total of 1383 cultivated (*Glycine max* [L.] Merr.) and 261 wild (*G. soja* Sieb. & Zucc.) soybean accessions from the Genetic Resources Center, NARO, in Japan and the United States Department of Agriculture (USDA) were used for diversity analyses (Supplementary Data 1). The detailed genetic relationship of the materials was reported by Kaga et al.⁵⁵. Twenty-nine additional accessions known to have *Rsv4*^{18,56} or derived from collection with a name ‘Peking’ were obtained from USDA.

Viruses. The following viruses were obtained from the Genetic Resources Center, NARO: SMV-A (MAFF 104063), SMV-C (104065), SMV-D (104066), SMV-E (104067), TuMV (715062), PVY (307024), BCMV (715049), PVA (307028), and PeMoV (307044). PPV was a generous gift from Drs. S. Namba, Y. Yamaji, and K. Maejima (University of Tokyo). Full-length SMV-L(Q1033K) cDNA was synthesized according to the deposited nucleotide sequence (EU871724) for construction of the infectious clone.

Fine mapping of *Rsv4*. Publicly available SSR markers (Supplementary Data 2) were initially used to construct a linkage map around *Rsv4* using the F₂ population

as described above. Based on these results, 25 SSR markers were designed to SSR core motifs around *Rsv4* by using Primer3 with default parameters⁵⁷ based on the reference sequence of soybean chromosomes (cultivar Williams 87, version Gmax_189⁵⁸) obtained from the Phytosome FTP site (<https://phytosome.jgi.doe.gov/pz/portal.html>). To ensure specificity, primer sequences were searched against Gmax_189 to examine the number of potential binding sites, amplicon size, and location by using Genome Tester⁵⁹ with default parameters. Total genomic DNA was extracted from young fresh leaves (0.3 g) or seed powder (50 mg) by using guanidine hydrochloride and proteinase K according to Khosla et al.⁶⁰ with several modifications. Primers fluorescently labeled with three kinds of dye (6-FAM, HEX, and NED) were used to analyze SSR markers. Multiplex PCR consisting of several markers with different dyes was performed using a QIAGEN Multiplex PCR Kit (QIAGEN). PCR mixtures (5 µl) contained 0.2 µl of template DNA (50 ng per µl), 2.5 µl of 2 × Multiplex PCR Mix, 1 µl of 5 × Q solution, and 0.1 µl of 10 µM each primer. Touchdown PCR was programmed on a GeneAmp 9700 (Applied Biosystems) as follows: 1 cycle of initial denaturation at 95 °C for 15 min; 3 cycles of 94 °C for 30 s and 68 °C for 3 min; 3 cycles of 94 °C for 30 s and 66 °C for 3 min; 3 cycles of 94 °C for 30 s and 64 °C for 3 min; 3 cycles of 94 °C for 30 s, 62 °C for 3 min, and 1 min at 72 °C; 3 cycles of 94 °C for 30 s, 60 °C for 3 min, and 1 min at 72 °C; 3 cycles of 94 °C for 30 s, 58 °C for 3 min, and 1 min at 72 °C; 40 cycles of 30 s at 94 °C, 3 min at 55 °C, and 1 min at 72 °C; and final extension for 10 min at 72 °C. The PCR product was diluted 1/10 with water, and 1 µl was denatured in 10 µl of Hi-Di formamide with 0.2 µl of GeneScan 400HD ROX size standard and separated on an ABI 3730 capillary sequencer (Applied Biosystems). SSR marker genotypes were determined by using ABI GeneMapper ver.4.0 software (Applied Biosystems). The linkage map of *Rsv4* was constructed in JoinMap ver.4.0⁶¹ and the marker order of the F₂ linkage map was determined using the maximum-likelihood mapping algorithm. The recombination frequency was converted into genetic distance (cM) using the Haldane mapping function. In addition, the linkage between the genotypes of two SSR markers, *Rsv4_446* and *Rsv4_320* (Supplementary Data 2), and the SMV-C resistance phenotype was examined among 68 F₂ progenies between Tosan155 (derived from a cross between Fukusennari and Enrei) and Enrei to test whether the SMV-C resistance of Fukusennari maps to the *Rsv4* region.

For fine mapping, the genotypes of markers *Rsv4_446* and *Rsv4_320* were screened to obtain recombinants in the *Rsv4* region in the backcross populations. Single-nucleotide polymorphisms (SNPs) were genotyped for the recombinants by the direct-sequencing method. Primer pairs were designed around the SNPs to amplify 600–1000-bp fragments based on the procedure described above. These primer pairs are listed as *Rsv4-s04-07_1*, *Rsv4-s035-6_71*, *Rsv4-86105snp_42*, *Rsv4-RC-1_93*, and *Rsv4-RC-3_55* in Supplementary Data 2. Reaction mixtures consisted of 5 µl of 2 × GoTaq[®] Colorless Master Mix (Promega, Madison, WI, USA), 0.2 µl of 10 µM each forward and reverse primers, and 0.2 µl of the template DNA in a total volume of 10 µl. The PCR was performed as follows: 1 cycle of initial denaturation at 95 °C for 2 min; 40 cycles of denaturation for 15 s at 95 °C, annealing for 30 s at 53 °C, and extension for 1.5 min at 60 °C. The PCR products were cleaned with ExoSAP-IT (USB Corporation, Cleveland, OH, USA). Sequencing was performed with an ABI Prism BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) and 5 pmol of one of the primers used to amplify the PCR product on an ABI 3730xl automated DNA analyzer (Applied Biosystems) according to the manufacturer’s manual. The sequence chromatograms were aligned with the reference sequence to determine genotypes with Sequencher 5.2 (Gene Codes Corporation, Ann Arbor, MI, USA).

Two sequence-tagged site (STS) markers, detected by the *Rsv4*-Seq03 and *Rsv4*-Seq04 primer pairs (Supplementary Data 2), were designed to amplify 6-kbp and 6.3-kbp regions, respectively, near *Rsv4*. PCR mixtures (10 µl) contained 0.2 µl of template DNA (50 ng per µl), 2 µl of 5 × PrimeSTAR GXL Buffer (Takara Bio, Shiga, Japan), 1.0 µl of PrimeSTAR GXL DNA Polymerase (1.25 U per µl), 0.8 µl of 2.5 mM dNTPs, and 0.1 µl of 20 µM each forward and reverse primers. PCR was performed on a GeneAmp PCR System 9700 (Applied Biosystems) with the following program: initial denaturation for 5 s at 98 °C; 35 cycles of denaturation for 10 s at 98 °C, annealing and extension for 30 s at 68 °C; and final extension for 30 s at 68 °C. Genotypes for the two STS markers were visually classified based on fragment size by 1% agarose gel electrophoresis: 2.5 kbp (Peking type) and 6 kbp (Enrei type) for *Rsv4*-Seq03; 10 kbp (Peking type) and 6.3 kbp (Enrei type) for *Rsv4*-Seq04.

Virus inoculation and detection. SMV-C and SMV-L(Q1033K) cDNAs were cloned into pBI121 for agroinfection into *N. benthamiana* leaves. For inoculation of soybean plants, viruses were propagated in soybean cultivar ‘Tsuruno Tamagoi’ (SMV-C, D, and E) or ‘Jack’ (SMV-A, C, and E). Each inoculum was prepared from 1 g of infected leaf tissue, which was homogenized in 10 ml of 0.1 M sodium phosphate buffer, pH 7.0, by using a mortar and pestle. Inoculation was performed before the trifoliolate leaves emerged. Unifoliolate soybean leaves were dusted with carborundum before inoculation, then rubbed softly with a cotton puff to distribute the inoculum, and finally rinsed with tap water. Inoculated plants were grown in a greenhouse at 18 to 25 °C for 2 to 3 weeks and classified into three phenotype classes based on symptoms of 15 to 30 plants per line: resistant (all plants were symptomless), susceptible (more than 80% of plants had mosaic symptoms), and segregating (about 25% of plants had mosaic symptoms). Additional plants were

inoculated to confirm phenotype classifications when necessary. Inoculation of potyviruses other than SMV into *N. benthamiana* leaves was performed mechanically using homogenates of infected leaves as described above. Anti-SMV-CP rabbit antiserum was raised against an *E. coli*-expressed SMV-C CP. For quantification of SMV accumulation, the SMV CP bands of Western blotting were measured by ImageJ⁶². Anti-SMV-P3 and -N1b rabbit antisera were raised against synthetic peptides CFPVAVSMGTQSESDVSAQ and CVGA-QYKGGKQDYFSGMD, respectively. Anti-TuMV-CP antiserum was purchased from the Japan Plant Protection Association (Tokyo, Japan). All antisera used for Western blotting were diluted at 1:1000 in TBST containing 5% (w/v) skim milk. Primers used for detection of viral RNA by RT-PCR are listed in Supplementary Data 2.

Sequencing analysis of the Rsv4 region. Long-range PCR was conducted to amplify a 16-kbp genomic fragment including the delimited 9.8-kbp fragment of the *Rsv4* region from 'Peking' using primer pair rsv4L15_L_4957 (Supplementary Data 2). PCR mixtures (10 μ l) contained 0.2 μ l of template DNA (50 ng per μ l), 2 μ l of 5 \times PrimeSTAR GXL Buffer (Takara Bio), 0.2 μ l of PrimeSTAR GXL DNA Polymerase (1.25 U per μ l), 0.8 μ l of 2.5 mM dNTPs, and 0.1 μ l of 20 μ M each forward and reverse primers. PCR was performed on a GeneAmp PCR System 9700 (Applied Biosystems) with the following program: initial denaturation for 5 s at 98 °C; 30 cycles of denaturation for 10 s at 98 °C, annealing and extension for 7 min 50 s at 68 °C; and final extension for 30 s at 68 °C. The sequence of the 16-kbp fragment was determined by the construction of random transposon insertion clones according to the protocol supplied with the EZ-Tn5™ <KAN-2> Insertion Kit (Epicentre). Sequencing was performed with an ABI Prism BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems) and 5 pmol of the sequencing primers provided with the EZ-Tn5™ kit on an ABI-3730xl automated DNA analyzer (Applied Biosystems) according to the manufacturer's manual. The bidirectional sequence chromatograms generated from the 470 clones were assembled into a 16,159-bp contig using Sequencher 5.2.

RNA analysis. Total RNA was isolated from young trifoliolate leaves by using an RNeasy® Plant Mini Kit following the on-column DNase protocol (QIAGEN). RT-PCR was conducted by using a PrimeScript One Step RT-PCR Kit (Takara Bio) and the primer pair ORF2 (Supplementary Data 2), designed to the presumed *Rsv4* ORFs. PCR was performed on a GeneAmp PCR System 9700 (Applied Biosystems) with the following program: initial denaturation for 5 s at 98 °C; 30 cycles of denaturation for 10 s at 98 °C, annealing and extension for 7 min 50 s at 68 °C; and final extension for 30 s at 68 °C. mRNA was purified from total RNA using a MagExtractor™ kit (TOYOBO, Japan). Full-length cDNA sequence was obtained by using a GeneRacer™ Kit (Invitrogen) and nested primer sets for 5' amplification (rsv4-5-1 and rsv4-5-2) or 3' amplification (rsv4-3-1 and rsv4-3-2; Supplementary Data 2) according to the manufacturer's manual.

Transformation of soybean plants with Rsv4. The *Rsv4* RT-PCR product amplified by primer pair ORF2 was re-amplified using an adaptor primer pair, OEatg-1 and OETga-1 (Supplementary Data 2). The fragment was double-digested by restriction enzymes *SpeI* and *XhoI* and cloned into the *SacI*-*XbaI* sites of the pMDC123-GFP vector⁶³. Then, a fragment containing the cauliflower mosaic virus (CaMV) 35S promoter, *Rsv4* cDNA, and the NOS terminator of *Agrobacterium tumefaciens* was amplified using the primer pair P35SF1spe and TNOSR1xho (Supplementary Data 2), and the DNA fragment was double-digested by *SpeI* and *XhoI* and cloned into the *SpeI*-*XhoI* sites of the pUHR SK plasmid vector.

The genomic DNA construct for transformation was obtained as follows. PCR mixtures (50 μ l) contained 2 μ l of template DNA (50 ng per μ l), 10 μ l of 5 \times PrimeSTAR HS Buffer (Takara Bio), 1 μ l of PrimeSTAR HS DNA Polymerase (2.5 U per μ l), 4 μ l of 2.5 mM dNTPs, and 2.5 μ l of 20 μ M *Rsv4*-Seq04 primer pair (Supplementary Data 2). PCR was programmed as follows: initial denaturation for 2 s at 98 °C; 30 cycles of denaturation for 10 s at 98 °C, annealing and extension for 5 min at 68 °C; and final extension for 10 s at 68 °C. The DNA fragment was double-digested by restriction enzymes *SpeI* and *HindIII* and cloned into the *SpeI*-*HindIII* sites of the pUHR SK plasmid vector. The sequences of several clones were confirmed in advance of transformation.

The construct was introduced by biolistic transformation into cultured cells derived from immature embryos of the SMV-susceptible cultivar 'Jack' as described by Nishizawa et al.⁶⁴. T0 plants derived from hygromycin-resistant and red-fluorescent embryogenic cells were grown at 28 °C day/23 °C night in a temperature-controlled glasshouse. Genomic DNA from regenerated T0 plants and T1 plants was used to confirm transformation by the constructs. Two primer pairs, pDsRed2_135 and hpt (Supplementary Data 2), were used to amplify the *DsRed* and *HPT* genes, respectively. In addition, RT-PCR for total RNA from young leaves was conducted to confirm expression of *Rsv4* in each T1 and T2 plant by using the primer pair ORF2 (Supplementary Data 2).

Long-range PCR was performed to identify germplasm with a 3.6-kbp deletion (as found in the Peking genome) using the primer pair *Rsv4*-Seq03 as described above. Based on the fragment sizes, germplasm was classified for the absence (PCR product size is 2.5 kbp) or presence (6 kbp) of the 3.6-kbp fragment by 1% agarose gel electrophoresis.

Isolation of Peking Rsv4 mutants. A mutant library was developed for Peking, and *Rsv4* mutants were identified as in Tsuda et al.⁶⁵. Briefly, seeds of Peking were treated with the chemical mutagen EMS, and M2 seeds produced by M1 plants were treated with EMS once again to increase the mutation density. The resultant mutant library, which consisted of DNA and seeds from 1536 M2 plants, was screened by using indexed amplicon sequencing to retrieve *Rsv4* mutants. The primer pair Gm02-Rsv4-1n-5000_F and Gm02-Rsv4-1n-5001_R (Supplementary Data 2) was used to amplify a 3.6-kbp region covering *Rsv4* using the long-range PCR method described above and used for indexed amplicon sequencing. Only base changes leading to stop codon or amino acid substitutions observed in more than 2% of the aligned amplicon reads on the 3.6-kbp Peking *Rsv4* sequence were selected to isolate mutants from the library by direct sequencing using the primer pair ORF2 (Supplementary Data 2). After identification of mutants, genotypes of their progenies were confirmed by the same procedure.

Transient expression in N. benthamiana. *Rsv4* cDNA was cloned into pMLH7133⁶⁶. The D99N mutant was created by changing the 99th codon, for aspartic acid (GAT), to that for asparagine (AAT). *Rsv4*-FLAG and D99N-FLAG were created by inserting 5'-GGAGGTGGAGATTATAAGGATGATGATGATA AG-3' before the stop codon of the respective *Rsv4* gene. P3-Myc was created by inserting the SMV-C cDNA sequence encoding the Gly766 to Gln1112 residues of the polyprotein between the initiation codon (ATG) and the Myc-tag coding sequence (5'-GGAAGATCTGAGCAGAAGCTTATTCTGAGGAGATCTTT GAGCTC-3') and cloned into pMLH7133. A synthetic p19 gene of tomato bushy stunt virus was cloned into pRI101-AN (TaKaRa Bio). Unless otherwise noted, a mixture of *Agrobacterium* strains that express the proteins of interest and p19 was infiltrated into *N. benthamiana* leaves using a needleless syringe at OD₆₀₀ = 0.5 each. 1 day after infiltration, *Agrobacterium* harboring SMV cDNA was infiltrated at OD₆₀₀ = 0.1 for SMV inoculation.

RNase assay. *N. benthamiana* leaves expressing *Rsv4*-FLAG or D99N-FLAG were homogenized in an ice-cold buffer (30 mM HEPES-KOH pH 7.4, 100 mM KCl, 2 mM EDTA, 2 mM DTT, 1 tablet per 50 ml cComplete EDTA-free Protease Inhibitor [Roche]) by using a grinder. After clarification by centrifugation (800 \times g), the membrane fraction was pelleted by centrifugation at 16,000 \times g for 15 min. The pellet was suspended in homogenization buffer containing 1% Triton X-100, and the solubilized proteins were purified using anti-FLAG antibody-conjugated agarose (SIGMA). FLAG-tagged proteins were eluted with 0.1 mg per ml 3 \times FLAG peptide in 30 mM HEPES-KOH pH 7.4, 100 mM KCl, 2 mM DTT, 0.2% Triton X-100, 3 μ g per ml BSA, 1 tablet per 50 ml cComplete EDTA-free Protease Inhibitor (Roche). The eluate was mixed with an equal volume of glycerol and stored at -30 °C. For substrate RNA production, pGEM-SMV-7610-7960 was constructed by inserting a SMV-C cDNA fragment amplified by PCR using primers SMV7610F and SMV7960R (Supplementary Data 2) into pGEM-7Zf(+) between the *Clal* and *BamHI* sites. Substrate RNA was transcribed from *BamHI*-linearized pGEM-SMV-7610-7960 using T7 RNA polymerase in the presence of [α -³²P]CTP. A synthetic DNA (5'-GGTTCTCTAACATTTCTTCCAAACCAC CAGTCTTCCATGAAAA-3') or RNA transcribed from *Clal*-linearized pGEM-SMV-7610-7960 using SP6 RNA polymerase was annealed to the substrate. Digestion was performed in 30 mM HEPES-KOH pH 7.4, 50 mM KCl, 2 mM DTT, 0.1% Triton X-100 at 25 °C for 30 min. MgCl₂ or MnCl₂ was supplemented as indicated (Fig. 4a). *E. coli* RNase H and S1 nuclease were purchased from TaKaRa Bio, and digestion was performed in 30 mM HEPES-KOH pH 7.4, 50 mM KCl, 4 mM MgCl₂, 2 mM DTT, 0.1% Triton X-100 for RNase H or in the supplied buffer for S1 nuclease. After the reactions, RNA was purified and analyzed by 7 M urea-10% PAGE followed by autoradiography.

Interaction analyses of Rsv4 and SMV P3. *Rsv4*-FLAG and P3-Myc were co-expressed in *N. benthamiana* leaves by agroinfiltration. 3 days after infiltration, the leaves were homogenized in TR buffer (30 mM HEPES-KOH pH 7.4, 80 mM KOAc, 1.8 mM Mg(OAc)₂, 2 mM DTT, 1 tablet per 50 ml cComplete EDTA-free Protease Inhibitor [Roche]). After clarification by centrifugation (800 \times g), membranes were pelleted by 16,000 \times g for 15 min. The pellet was suspended in TR buffer containing 1% Triton X-100, incubated with anti-FLAG antibody at 4 °C for 2 h, washed four times with wash buffer (30 mM HEPES-KOH pH 7.4, 100 mM NaCl, 2 mM DTT, 0.2% Triton X-100, 1 tablet per 50 ml cComplete EDTA-free Protease Inhibitor [Roche]), and eluted with wash buffer containing 0.1 mg per ml 3 \times FLAG peptide. The eluted samples were analyzed by western blotting using anti-Myc (Cat#16286-1-AP; Cosmo Bio, Tokyo, Japan) and anti-DYKDDDDK (Cat#018-22381; Wako Pure Chemical, Osaka, Japan) antibodies diluted at 1:1000 in TBST containing 5% skim milk.

Viral RdRp assay. Membrane fractions of SMV-inoculated leaf extracts (6 days post-inoculation) and FLAG-purified fractions after solubilization that were prepared as described above were incubated with a template RNA (20 ng per μ l in vitro-transcribed SMV full-length genomic RNA) and 5 \times RdRp reaction solution (5 mM ATP, 5 mM GTP, 5 mM UTP, 62.5 μ M CTP, 25 mM Mg(OAc)₂, 50 mM DTT, 0.5 mg per ml actinomycin D, 4 U per μ l RNasin Plus [Promega], 1 mg per ml creatine kinase, 100 mM creatine phosphate, 370 MBq per ml [α -³²P]CTP) at

25 °C for 90 min. Following phenol extraction and ethanol precipitation, single-stranded RNA was digested with S1 nuclease (TaKaRa Bio), and the remaining dsRNA was separated by 8 M urea–2.4% PAGE and detected by autoradiography.

Construction of RTL2-fusion proteins. *RTL2*, *TIP1*, and *eIF(iso4E)* cDNAs were amplified from *Arabidopsis thaliana* Col-0 RNA and *TOM1* cDNA was amplified from *Nicotiana tabacum* cv. Samsun RNA using the primers listed in Supplementary Data 2. cDNAs for target proteins and RTL2 were fused by overlap PCR with ten glycine codons (GGT×10) as a linker and cloned into pMLH7133. As negative controls, a mutation causing D100A (GAT to GCT) of RTL2 was introduced into each fusion protein by site-directed mutagenesis, and RTL2 alone was cloned into pMLH7133. A mixture of *Agrobacterium* strains that express p19 and one of the fusion proteins (OD₆₀₀ = 0.2 and 0.5, respectively) was infiltrated into *N. benthamiana* leaves using a needleless syringe. Two days after infiltration, ToMV, CMV, or TuMV was mechanically inoculated.

Diversity analysis. Multiple alignments of nucleotide and protein sequences were constructed using a progressive sequence alignment tool⁶⁷ implemented in the CLC Genomics Workbench with default parameter values. A BLAST (blastp) search to find sequences similar to Rsv4 was performed against the nonredundant (nr) protein database maintained by NCBI with an *E*-value cutoff of 3e⁻¹⁰. A phylogenetic tree was constructed by neighbor joining with 1000 bootstrap replications based on ‘Kimura 80’ and ‘Kimura protein’⁶⁸ genetic distances for nucleotide and protein sequences, respectively. Genome assemblies of azuki bean (*Vigna angularis* [Willd.] Ohwi and Ohashi), *V. angularis_v1*; pigeon pea (*Cajanus cajan* [L.] Millsp.), ICPL87119_v1; and common bean (*Phaseolus vulgaris* L.), Pvulgaris_218_v1.0, were obtained from the *Vigna* Genome Server (VigGS; <https://viggs.dna.affrc.go.jp>), Legume Information System (LIS; <https://legumeinfo.org/organism/Cajanus/cajan>), and Phytosome FTP site, respectively. Dot plot analysis⁶⁹ implemented in the CLC Genomics Workbench was used to find similar regions on the same chromosome, among orthologous soybean chromosomes, and between genomes of different species using default parameters and a window size of 9.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this Article is available as a Supplementary Information file. The datasets generated and analyzed during the current study are available from the corresponding author upon request. The nucleotide sequences of SMV-C cDNA and Peking *Rsv4* mRNA are deposited in DDBJ under accession numbers [LC323107](#) and [LC325439](#), respectively. The genomic DNA sequences for the *Rsv4* regions of different soybean germplasm are deposited under accession numbers DDBJ [LC325364](#)–[LC325438](#) and GenBank accession numbers [MN178650](#)–[MN178656](#). The source data underlying Figs. 2b–f, 4a–d, 5a, and 6b and Supplementary Figs. 3c, 6a, and 6b are provided as a Source Data file.

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References

- DeWitte-Orr, S. J. & Mossman, K. L. dsRNA and the innate antiviral immune response. *Future Virol.* **5**, 325–341 (2010).
- Pumplin, N. & Voynet, O. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. *Nat. Rev. Micro.* **11**, 745–760 (2013).
- De Ronde, D., Butterbach, P. & Kormelink, R. Dominant resistance against plant viruses. *Front Plant Sci.* **5**, 307 (2014).
- Revers, F. & Garcia, J. A. In *Advances in Virus Research* Vol. 92 (eds Maramorosch, K. & Mettenleiter, T. C.) 101–199 (Academic Press, 2015).
- Solomon-Blackburn, R. M. & Barker, H. Breeding virus resistant potatoes (*Solanum tuberosum*): a review of traditional and molecular approaches. *Heredity* **86**, 17–35 (2001).
- Kyle, M. M. & Palloix, A. Proposed revision of nomenclature for potyvirus resistance genes in Capsicum. *Euphytica* **97**, 183–188 (1997).
- Sharma, S. K. & Tripathi, S. In *Plant Virus-Host Interaction* (eds Gaur, R. K., Hohn, T. & Sharma, P.) 177–194 (Academic Press, Boston, 2014).
- Hartmann, W. & Neumüller, M. Breeding for resistance: breeding for Plum pox virus resistant plums (*Prunus domestica* L.) in Germany. *EPPO Bull.* **36**, 332–336 (2006).
- Li, G. et al. TuMV management for brassica crops through host resistance: retrospect and prospects. *Plant Pathol.* **68**, 1035–1044 (2019).
- Jain, S., McPhee, K., Kumar, A., Rouf Mir, R. & Singh, R. In *Agricultural Sustainability* (eds Bhullar, G. S. & Bhullar, N. K.) 221–244 (Academic Press, San Diego, 2013).
- Ross, J. P. Effect of aphid-transmitted soybean mosaic virus on yields of closely related resistant and susceptible soybean lines. *Crop Sci.* **17**, 869–872 (1977).
- Hajimorad, M. R., Domier, L. L., Tolin, S. A., Whitham, S. A. & Saghai Maroof, M. A. *Soybean mosaic virus*: a successful potyvirus with a wide distribution but restricted natural host range. *Mol. Plant Pathol.* **19**, 1563–1579 (2018).
- Hajimorad, M. R. & Hill, J. H. *Rsv1*-mediated resistance against *Soybean mosaic virus*-N is hypersensitive response-independent at inoculation site, but has the potential to initiate a hypersensitive response-like mechanism. *Mol. Plant Microbe Interact.* **14**, 587–598 (2001).
- Suh, S. J. et al. The *Rsv3* locus conferring resistance to *Soybean mosaic virus* is associated with a cluster of coiled-coil nucleotide-binding leucine-rich repeat genes. *Plant Gen.* **4**, 55–64 (2011).
- Cho, E.-K. & Goodman, R. M. Strains of soybean mosaic virus: classification based on virulence in resistant soybean cultivars. *Phytopathology* **69**, 467–470 (1979).
- Klepado, M. et al. Two tightly linked genes for soybean mosaic virus resistance in soybean. *Crop Sci.* **57**, 1844–1853 (2017).
- Tran, P.-T., Widyasari, K., Seo, J.-K. & Kim, K.-H. Isolation and validation of a candidate *Rsv3* gene from a soybean genotype that confers strain-specific resistance to soybean mosaic virus. *Virology* **513**, 153–159 (2018).
- Gunduz, I., Buss, G. R., Chen, P. & Tolin, S. A. Genetic and phenotypic analysis of *Soybean mosaic virus* resistance in PI 88788 soybean. *Phytopathology* **94**, 687–692 (2004).
- Saghai Maroof, M. A. et al. Fine mapping and candidate gene discovery of the soybean mosaic virus resistance gene, *Rsv4*. *Plant Gen.* **3**, 14–22 (2010).
- Wang, D. et al. Fine mapping and analyses of *R_{SC8}* resistance candidate genes to soybean mosaic virus in soybean. *Theor. Appl. Genet.* **122**, 555–565 (2011).
- Ilut, D. C. et al. Identification of haplotypes at the *Rsv4* genomic region in soybean associated with durable resistance to soybean mosaic virus. *Theor. Appl. Genet.* **129**, 453–468 (2016).
- Chowda-Reddy, R. V. et al. Mutations in the P3 protein of *Soybean mosaic virus* G2 isolates determine virulence on *Rsv4*-genotype soybean. *Mol. Plant Microbe Interact.* **24**, 37–43 (2011).
- Shakiba, E. et al. Inheritance and allelic relationships of resistance genes for *Soybean mosaic virus* in ‘Corsica’ and ‘Beeson’ soybean. *Crop Sci.* **53**, 1455–1463 (2013).
- Nowotny, M., Gaidamakov, S. A., Crouch, R. J. & Yang, W. Crystal structures of RNase H bound to an RNA/DNA hybrid: substrate specificity and metal-dependent catalysis. *Cell* **121**, 1005–1016 (2005).
- Buck, K. W. In *Advances in Virus Research* (eds Maramorosch, K., Murphy, F. A. & Shatkin, A. J.) 159–251 (Academic Press, 1996).
- den Boon, J. A. & Ahlquist, P. Organelle-like membrane compartmentalization of positive-strand RNA virus replication factories. *Annu. Rev. Microbiol.* **64**, 241–256 (2010).
- Paul, D. & Bartenschlager, R. Architecture and biogenesis of plus-strand RNA virus replication factories. *World J. Virol.* **2**, 32–48 (2013).
- Ahangaran, A., Habibi, M. K., Mohammadi, G.-H. M., Winter, S. & García-Arenal, F. Analysis of *Soybean mosaic virus* genetic diversity in Iran allows the characterization of a new mutation resulting in overcoming *Rsv4*-resistance. *J. Gen. Virol.* **94**, 2557–2568 (2013).
- Khatibi, B., Fajolu, O. L., Wen, R. H. & Hajimorad, M. R. Evaluation of North American isolates of *Soybean mosaic virus* for gain of virulence on *Rsv*-genotype soybeans with special emphasis on resistance-breaking determinants on *Rsv4*. *Mol. Plant Pathol.* **13**, 1077–1088 (2012).
- Wang, Y., Khatibi, B. & Hajimorad, M. R. Amino acid substitution in P3 of *Soybean mosaic virus* to convert avirulence to virulence on *Rsv4*-genotype soybean is influenced by the genetic composition of P3. *Mol. Plant Pathol.* **16**, 301–307 (2015).
- Merits, A., Guo, D., Järvekülg, L. & Saarma, M. Biochemical and genetic evidence for interactions between potato A potyvirus-encoded proteins P1 and P3 and proteins of the putative replication complex. *Virology* **263**, 15–22 (1999).
- Guo, D., Rajamäki, M.-L., Saarma, M. & Valkonen, J. P. T. Towards a protein interaction map of potyviruses: protein interaction matrixes of two potyviruses based on the yeast two-hybrid system. *J. Gen. Virol.* **82**, 935–939 (2001).
- Cui, X. et al. The C-terminal region of the Turnip mosaic virus P3 protein is essential for viral infection via targeting P3 to the viral replication complex. *Virology* **510**, 147–155 (2017).
- Wylie, S. J. et al. ICTV virus taxonomy profile: *Potyviridae*. *J. Gen. Virol.* **98**, 352–354 (2017).
- Kiyota, E. et al. An Arabidopsis RNase III-like protein, AtRTL2, cleaves double-stranded RNA in vitro. *J. Plant Res.* **124**, 405–414 (2011).

36. Yamanaka, T. et al. *TOM1*, an *Arabidopsis* gene required for efficient multiplication of a tobamovirus, encodes a putative transmembrane protein. *Proc. Natl Acad. Sci. USA* **97**, 10107–10112 (2000).
37. Kim, M. J., Kim, H. R. & Paek, K.-H. Arabidopsis tonoplast proteins TIP1 and TIP2 interact with the cucumber mosaic virus 1a replication protein. *J. Gen. Virol.* **87**, 3425–3431 (2006).
38. Beauchemin, C., Boutet, N. & Laliberté, J.-F. Visualization of the interaction between the precursors of VPg, the viral protein linked to the genome of *Turnip mosaic virus*, and the translation eukaryotic initiation factor iso 4E in planta. *J. Virol.* **81**, 775–782 (2007).
39. Ishibashi, K., Naito, S., Meshi, T. & Ishikawa, M. An inhibitory interaction between viral and cellular proteins underlies the resistance of tomato to nonadapted tobamoviruses. *Proc. Natl Acad. Sci. USA* **106**, 8778–8783 (2009).
40. Ishibashi, K., Masuda, K., Naito, S., Meshi, T. & Ishikawa, M. An inhibitor of viral RNA replication is encoded by a plant resistance gene. *Proc. Natl Acad. Sci. USA* **104**, 13833–13838 (2007).
41. Yoshida, T. et al. The plant non-canonical antiviral resistance protein JAX1 inhibits potyviral replication by targeting the viral RNA-dependent RNA polymerase. *J. Virol.* **93**, e01506–e01518 (2018).
42. Liu, Q. et al. An atypical thioredoxin imparts early resistance to *Sugarcane mosaic virus* in maize. *Mol. Plant* **10**, 483–497 (2017).
43. Chisholm, S. T., Parra, M. A., Anderberg, R. J. & Carrington, J. C. Arabidopsis *RTM1* and *RTM2* genes function in phloem to restrict long-distance movement of tobacco etch virus. *Plant Physiol.* **127**, 1667–1675 (2001).
44. Whitham, S. A., Anderberg, R. J., Chisholm, S. T. & Carrington, J. C. Arabidopsis *RTM2* gene is necessary for specific restriction of tobacco etch virus and encodes an unusual small heat shock-like protein. *Plant Cell* **12**, 569–582 (2000).
45. Cosson, P. et al. *RTM3*, which controls long-distance movement of potyviruses, is a member of a new plant gene family encoding a meprin and TRAF homology domain-containing protein. *Plant Physiol.* **154**, 222–232 (2010).
46. Wang, Q. et al. *STV11* encodes a sulphotransferase and confers durable resistance to rice stripe virus. *Nat. Commun.* **5**, 4768 (2014).
47. Verlaan, M. G. et al. The tomato yellow leaf curl virus resistance genes *Ty-1* and *Ty-3* are allelic and code for DFDGD-class RNA-dependent RNA polymerases. *PLoS Genet.* **9**, e1003399 (2013).
48. Butterbach, P. et al. Tomato yellow leaf curl virus resistance by *Ty-1* involves increased cytosine methylation of viral genomes and is compromised by cucumber mosaic virus infection. *Proc. Natl Acad. Sci. USA* **111**, 12942–12947 (2014).
49. Wang, Y. & Hajimorad, M. R. Gain of virulence by *Soybean mosaic virus* on *Rsv4*-genotype soybeans is associated with a relative fitness loss in a susceptible host. *Mol. Plant Pathol.* **17**, 1154–1159 (2016).
50. Abel, P. et al. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738–743 (1986).
51. Pooggin, M. M. RNAi-mediated resistance to viruses: a critical assessment of methodologies. *Curr. Opin. Virol.* **26**, 28–35 (2017).
52. Lindbo, J. A. & Falk, B. W. The impact of “coat protein-mediated virus resistance” in applied plant pathology and basic research. *Phytopathology* **107**, 624–634 (2017).
53. Kreuze, J. F. & Valkonen, J. P. T. Utilization of engineered resistance to viruses in crops of the developing world, with emphasis on sub-Saharan Africa. *Curr. Opin. Virol.* **26**, 90–97 (2017).
54. Gal-On, A., Fuchs, M. & Gray, S. Generation of novel resistance genes using mutation and targeted gene editing. *Curr. Opin. Virol.* **26**, 98–103 (2017).
55. Kaga, A. et al. Evaluation of soybean germplasm conserved in NIAS genbank and development of mini core collections. *Breed. Sci.* **61**, 566–592 (2012).
56. Shi, A., Chen, P., Zheng, C., Hou, A. & Zhang, B. A PCR-based marker for the *Rsv1* locus conferring resistance to *Soybean mosaic virus*. *Crop Sci.* **48**, 262–268 (2008).
57. Rozen, S. & Skaletsky, H. In *Bioinformatics Methods and Protocols* (eds Misener, S. & Krawetz, S. A.) 365–386 (Humana Press, Totowa, 1999).
58. Schmutz, J. et al. Genome sequence of the paleopolyploid soybean. *Nature* **463**, 178–183 (2010).
59. Anderson, R., Reppo, E., Kaplinski, L. & Remm, M. GENOMEMASKER package for designing unique genomic PCR primers. *BMC Bioinformatics* **7**, 172 (2006).
60. Khosla, S., Augustus, M. & Brahmachari, V. Sex-specific organisation of middle repetitive DNA sequences in the mealybug *Planococcus lilacinus*. *Nucleic Acids Res.* **27**, 3745–3751 (1999).
61. Ooijen, J. W. *JoinMap 4.0: software for the calculation of genetic linkage maps in experimental populations* (Kyazma BV, Wageningen, Netherlands, 2006).
62. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
63. Liu, B. et al. The soybean stem growth habit gene *Dt1* is an ortholog of arabidopsis *TERMINAL FLOWER1*. *Plant Physiol.* **153**, 198–210 (2010).
64. Nishizawa, K., Kita, Y., Kitayama, M. & Ishimoto, M. A red fluorescent protein, DsRed2, as a visual reporter for transient expression and stable transformation in soybean. *Plant Cell Rep.* **25**, 1355–1361 (2006).
65. Tsuda, M. et al. Construction of a high-density mutant library in soybean and development of a mutant retrieval method using amplicon sequencing. *BMC Genomics* **16**, 1014 (2015).
66. Mitsuhashi, I. et al. Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol.* **37**, 49–59 (1996).
67. Feng, D.-F. & Doolittle, R. F. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. *J. Mol. Evol.* **25**, 351–360 (1987).
68. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111–120 (1980).
69. Maizel, J. V. & Lenk, R. P. Enhanced graphic matrix analysis of nucleic acid and protein sequences. *Proc. Natl Acad. Sci. USA* **78**, 7665–7669 (1981).

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Author contributions

K.I. and A.K. designed the work. M. Saruta, T.S., N.Y., Y.K., and A.K. performed positional cloning of *Rsv4*. M. Ishimoto and K.K. developed transgenic soybeans expressing *Rsv4*. T.A. and A.K. identified *Rsv4* mutant lines. K.I. and M. Shu performed agroinfiltration-mediated *N. benthamiana* transient assays. K.I. assayed the activities of *Rsv4* and potyviral RdRp. K.I., M. Ishikawa, M. Ishimoto, and A.K. coordinated and supervised the work and analyzed the data. K.I., M. Ishikawa, and A.K. wrote the paper.

Additional information

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Competing interests: Patent applications have been filed by the National Agriculture and Food Research Organization relating to work in this study. Patent number [WO/2016/148074](https://doi.org/10.1038/s41467-019-12052-5) is claimed by K.I., M. Saruta, T.S., M. Ishikawa, M. Ishimoto, and A.K. Patent number [JP,2018-191622.A](https://doi.org/10.1038/s41467-019-12052-5) is claimed by K.I.

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