Soybean antiviral immunity conferred by dsRNase targets the viral replication complex

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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 Rs4, which encodes an RNase H family protein with dsRNA-degrading activity. An active-site mutant of Rs4 is incapable of inhibiting virus multiplication and is associated with an active viral RNA polymerase complex in infected cells. These results suggest that Rs4 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.
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 silencing. 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 siRNA. To counteract RNA silencing by the host, viruses encode suppressors of RNA silencing, many of which bind siRNA and prevent RISC formation.

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 pathogen. Other virus resistance genes encode structurally unrelated proteins with various functions. 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 proteins. To protect from potyviruses, resistance genes have been introduced into many crop cultivars such as potato, pepper, papaya, plum, Brassica crops, and legumes.

**Soybean mosaic virus** (SMV) is a potyvirus that reduces soybean yields and seed quality worldwide. 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. In contrast, RsV4 confers broad-spectrum resistance to SMV strains through an atypical mechanism that delays SMV multiplication. 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. 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 plants (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. 4 and 6). 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 H family protein is RsV4. The predicted RsV4 protein sequences of Beeson (PI548510) and PI88788 were different, and without the Rnase H family gene and found that three of them conferred recessive susceptibility to strain SMV-C. We further classified 63 soybean accessions that carried the 3.6-kbp deletion into 16 types based on amino acid sequence (Supplementary Fig. 4). We further classified 63 soybean accessions that carried the 3.6-kbp deletion into 16 types based on amino acid sequence (Supplementary Figs. 4 and 6). 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 H family protein is RsV4. The predicted RsV4 protein sequences of Beeson (PI548510) and PI88788 were different, and without the Rnase H family gene and found that three of them conferred recessive susceptibility to strain SMV-C. We further classified 63 soybean accessions that carried the 3.6-kbp deletion into 16 types based on amino acid sequence (Supplementary Fig. 4). We further classified 63 soybean accessions that carried the 3.6-kbp deletion into 16 types based on amino acid sequence (Supplementary Fig. 4).

**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 proteins is changed from aspartate to asparagine (Fig. 1b). We introduced a mutation into RsV4 to substitute the DEDD motif with N (D99N). Transient expression of RsV4 by agroinfiltration inhibited SMV replication in Nicotiana benthamiana but the RsV4(D99N) mutant did not (Fig. 2e), suggesting that resistance depends on an activity of the RNase H 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 replication. We thus investigated the substrate preference of RsV4. Purified FLAG-tagged RsV4 protein degraded neither single-strand RNA nor a
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 cytoplasm26,27. 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 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 protein22,28–30, 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 Nib polymerase31,32, 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 Nib 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 Nib 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 OD$_{600}$ = 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.
Agriculturally important viruses worldwide. 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 *Tobaviridae*), 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 dsRNA 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 dsRNA protein, RTL2, 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 ToMV, TIP1 for cucumber mosaic virus (CMV), and elf (iso)4E for TuMV (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-kbp 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.
resistance gene Tm-1 inhibits multiplication of viruses that are not adapted to tomato. Most dominant virus resistance genes in plants encode NB-LRR proteins that elicit a defense reaction upon recognition of the corresponding viruses. Several others encode a variety of proteins functionally divided into two groups: inhibitors of viral propagation that target RNA replication or systemic transport, and modulators of plant immunity that include an enzyme sulfonylating salicylic acid and RNA-dependent RNA polymerases. We found that Rsv4 encodes a dsRNase that associates with the SMV replication machinery. Although sequestering dsRNA is a universal feature of eukaryotic positive-strand RNA viruses and functions as a countermeasure to host antiviral immunity, Rsv4 is able to contact P3 and/or other viral proteins during replication complex assembly and enter the viral replication compartment, where it degrades viral dsRNA formed during replication.

Rsv4 interacts, whether directly or indirectly, with the SMV P3 protein (Fig. 4b). A key amino acid residue of SMV P3 that is recognized by Rsv4, Q1033, is predicted to reside within the transmembrane region. Thus, Rsv4 and P3 may interact via transmembrane helix–helix interactions. It is possible that even in the presence of Rsv4, SMV makes Rsv4-free replication complexes at a low frequency, enabling viral genomic RNA to be continuously produced. The second-generation genomes could also form functional replication complexes at a low frequency. When progeny SMV genomes move to neighboring uninfected cells, they would again form Rsv4-free replication complexes at a low frequency. We propose that these processes could explain the slow SMV spread observed even in Rsv4 plants. SMV mutants whose P3 proteins have lower affinity to Rsv4 would form Rsv4-free replication complexes more frequently than wild-type strains. SMV-L(Q1033K) only partially breaks Rsv4-mediated resistance (Fig. 2f), and its P3 protein still had a detectable affinity to Rsv4 in the immunoprecipitation assay (Fig. 4c). Considering that SMV mutants that show virulence on Rsv4 plants had reduced fitness in susceptible hosts, P3 mutations that abolish affinity to Rsv4 may be deleterious to SMV. Future studies for quantitative measurements of the affinity between Rsv4 and P3, and their derivatives, would provide further support for our model.

Fig. 5 Rsv4 confers broad-spectrum resistance against potyviruses. a Accumulation of viral RNA in inoculated plants. Turnip mosaic virus (TuMV), potato virus Y (PVY), bean common mosaic virus (BCMV), peanut mottle virus (PeMoV), potato virus A (PVA), plum pox virus (PPV), or tomato mosaic virus (ToMV) were inoculated onto N. benthamiana leaves expressing Rsv4 or Rsv4(D99N), and accumulation of viral RNA in systemic leaves was detected by RT-PCR at 7–14 dpi. rRNAs were used as loading controls. b Symptoms of TuMV-inoculated N. benthamiana expressing Rsv4 or Rsv4(D99N) at 12 dpi. The source data of Fig. 5a are provided as a Source Data file.

Fig. 6 Targeted inhibition of virus multiplication by dsRNases fused with host factors involved in viral RNA replication. a Working model for targeted inhibition. By fusing a dsRNase (dR) with host factors (HF) included in the viral replication complex, dsRNase is delivered into the replication compartment. b Inhibition of multiplication of three different viruses by dsRNases designed to target the replication complexes of each virus. Accumulation of coat proteins (CP) in inoculated leaves was examined by Coomassie staining (ToMV and CMV) or western blotting (TuMV) at 2–4 dpi. The RTL2(D100A) constructs contain a catalytic mutation in RTL2. The source data of Fig. 6b are provided as a Source Data file.
As a complement to traditional breeding, biotechnological engineering has been an alternative approach to producing virus-resistant crops. Abe et al. showed that transgenic tobacco plants expressing the coat protein (CP) gene of tobacco mosaic virus are resistant to the virus. This method, referred to as pathogen-derived resistance, also includes RNA interference against viral genome sequences. Pathogen-derived resistance has been used to produce many virus-resistant crops; 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 countries. Another way to engineer virus-resistant crops is to knockout/knockdown of host genes that are essential for virus multiplication. 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 F2 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. Twenty-nine additional accessions known to have Rsv4 were 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). PVY was a generous gift from Drs. S. Namba, Y. Yamaji, and K. Mamei (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 parameters based on the sequence of soybean chromosomes (cultivar Williams 82, version Gmax_1980) obtained from the Phytome FTP site (https://phytome.jgi.doe.gov/pz/portal.html). To ensure specificity, primer sequences were searched against Gmax_189 on the number of potential binding sites, amplicon size, and location by using Genome Seeder with default parameters. Total genomic DNA was extracted from young fresh leaves (0.3 g) or seed powder (50 mg) bits using guanidine hydrochloride and proteinase K according to Khosla et al. 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 mixes (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; 1 and 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 ABI3730 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.4.056 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_A46 and Rsv4_320 (Supplementary Data 2), and the SMV-C resistance phenotype was examined among 62 F2 progeny 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_A46 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 sequencing method. Bioinformatic and genotyping methods were designed to amplify 600–1000-bp fragments based on the procedure described above. These primer pairs are listed as Rsv4-aa00-01, Rsv4-aa00-06-35, Rsv4-aa00-05, and Rsv4-aa00-06-34 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 v3.1 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 Sequence 4.2 (Geneious Corporation). Accession number is KF210402.

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 mixes (10 µl) contained 0.2 µl of template DNA (30 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 classed as SPAD values (10 kbp for Rsv4-Seq03; 10 kbp for Rsv4-Seq04) and 6.3 kbp (Enrei type) for Rsv4-Seq04.

Virus inoculation and detection. SMV-C and SMV-L (Q1033K) cDNAs were cloned into pBBL21 for agroinfection into N. benthamiana leaves. For inoculation of soybean plants, viruses were propagated in soybean cultivar ‘Tsuruno Tamago’ (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 motor and pestle. Inoculation was performed before the trifoliate leaves emerged. Uninoculated 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

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inoculated to confirm phenotype classifications when necessary. Inoculation by potyviruses other than SMV into N. benthamiana leaves was performed by mechanical inoculation using a needleless syringe. Anti-SMV-CP rabbit antisera was raised against an E. coli-expressed SMV-C protein. For quantification of SMV accumulation, the SMV CP bands of Western blotting were measured by ImageJ. Anti-SMV-P3 and -NIb rabbit antisera were raised against synthetic peptides: TAVSMGTQEQQVQMGQVGYKKKQDQYFSGMD, respectively. Anti-TiMV-CP antisera 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 pairs as described above. Anti-TiMV-CP rabbit antisera was raised against an E. coli-expressed TiMV-C protein. For quantification of SMV accumulation, the SMV CP bands of Western blotting were measured by ImageJ. Anti-SMV-P3 and -NIb rabbit antisera were raised against synthetic peptides: TAVSMGTQEQQVQMGQVGYKKKQDQYFSGMD, respectively. Anti-TiMV-CP antisera 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.

Isolation of Peking Rsv4 mutants. A mutant library was developed for Peking, and Rsv4 mutants were identified as in Tsuda et al. Briefly, seeds of Peking were treated with 50 μg/mL of the chemical mutagen, and M2 seeds 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 the delimited 2.5-kbp fragment of the Rsv4 region by a 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 (AAC). Rsv4-FLAG and D99N-FLAG were created by inserting 5'-GGAGGTGGAGATTATAAGGATGATGATGATA and 5'-GGAAGATCTGAGCAGAAGCTTATTTCTGAGGAGGATCTTTGAG-3' before the stop codon of the respective 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 10 s at 68 °C. The DNA fragment was double-digested by restriction enzymes (Hin dIII and Xho I) and cloned into the CaMV 35 S promoter, by Nishizawa et al. T0 plants derived from hygromycin-resistant and red-fluorescent embryogenic cells were grown at 28 °C day/23 °C night in a growth chamber.

Interaction analyses of Rsv4 and SMV P3. Rsv4-FLAG or D99N-FLAG were harvested 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 Complete EDTA-free Protease Inhibitor [Roche]) by using a grinder. After clarification by centrifugation (800 × g), the membrane fraction was pelleted by centrifugation at 16,000 × 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 3X FLAG peptide in 30 mM HEPES-KOH pH 7.4, 100 mM KCl, 2 mM DTT, 0.2% Triton X-100, 3 μg per ml BSA, 1 tablet per 50 ml Complete EDTA-free Protease Inhibitor (Roche). The elute was mixed with an equal volume of glycogen and stored at −30 °C. For substrate RNA production, pGEM-7-MV-7610-7890 was constructed by inserting a SMV-C cDNA fragment amplified by PCR using primers SMV7610F and SMV7960R (Supplementary Data 2) into pGEM-Zf(+) between the ClaI and BamHI sites. Substrate RNA was transcribed from BamHI-linearized pGEM-7-MV-7610-7890 using T7 RNA polymerase in the presence of [α-32P]UTP (800 × g). A synthetic RNA (5'-GGAAGATCTGAGCAGAAGCTTATTTCTGAGGAGGATCTTTGAG-3') or RNA transcribed from Cla-linearized pGEM-7-MV-7610-7890 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 in Fig. 4a. (a) c DNA. RNase T1 and S1 nucleases 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.

R-Flag 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 μl in vitro-transcribed SMV full-length genomic RNA) and 5X R-Flag reaction solution (1 μl of reaction mixture: 10 mM GTP; 2 mM MgCl2; 25 μM MgAc2; 50 mM DTT, 0.5 mg per ml actinomycin D, 4 μl per μl RNasin Plus [Promega], 1 mg per ml creatine kinase, 100 mM creatine phosphate, 370 μM per ml [α-32P]CTP) at 37 °C for 1 h. Formamide loading buffer was added to the reaction mixture, and samples were heated at 94 °C for 5 min before loading onto a 6% denaturing PAGE gel. The gel was stained with an ethidium bromide solution, and bands were visualized with a Typhoon FLA 9500 (GE Healthcare) and quantified with ImageJ.
25 °C for 90 min. Following phenol extraction and ethanol precipitation, single-stranded RNA was digested with T1 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 elf(isoE) 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 pMHL17133. As a negative control, 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 pMHL17133. A mixture of Agrobacterium strains that express p19 and one of the fusion proteins (ODmax = 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 tool 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’ genetic distances for nucleotide and protein sequences, respectively. Genome assemblies of azuki bean (Vigna angularis [Willd.] Ohwi and Ohashi), V. angularis var. pinnata (Cajanus cajan [L.]. Millsp.) ICPL119; v1; and common bean (Phaseolus vulgaris L.) Pvilguris_218_v1.0, were obtained from the Vigna Genome Server (VigGS; http://viggs.dna.affrc.go.jp), Legume Information System (LIS; https://legumeinfo.org/organism/Cajanus/cajan), and PhytoZome FTP site, respectively. Dot plot analysis 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.

**Finding a source.** 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 SMV-C cDNA were deposited in DDBJ under accession numbers LC323107 and LC325439, respectively. The genomic DNA sequences for the Rsv4 regions of different soybean germplasms are deposited under accession numbers DDBJ LC325366–LC325438 and GenBank accession numbers MN178650–MN178656. The source data used in generating Figs. 2b, 3a, 3b, and 4b and Supplementary Figs. 3c, 6a, and 6b are provided as a Source Data file.

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