Virology 442 (2013) 180–188

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

The 2b protein of *Asparagus virus 2* functions as an RNA silencing suppressor against systemic silencing to prove functional synteny with related cucumoviruses

Hanako Shimura*, Chikara Masuta, Naoto Yoshida, Kae Sueda, Masahiko Suzuki

Research Faculty of Agriculture, Hokkaido University, Kita 9 Nishi9, Kita-ku, Sapporo 0608589, Japan

ARTICLE INFO

Article history:
Received 31 October 2012
Returned to author for revisions
5 April 2013
Accepted 18 April 2013
Available online 13 May 2013

Keywords:
Asparagus virus 2
Ilarvirus
Genetic variability
2b protein
RNA silencing suppressor

ABSTRACT

Asparagus virus 2 (AV-2) is a member of the genus *Ilarvirus* in the family *Bromoviridae*. We cloned the coat protein (CP) and the 2b protein (2b) genes of AV-2 isolates from asparagus plants from various regions and found that the sequence for CP and for 2b was highly conserved among the isolates, suggesting that AV-2 from around the world is almost identical. We then made an AV-2 infectious clone by simultaneous inoculation with in vitro transcripts of RNAs 1–3 of AV-2 and in vitro-synthesized CP, which is necessary for initial infection. Because 2b of cucumoviruses in *Bromoviridae* can suppress systemic silencing as well as local silencing, we analyzed whether there is functional synteny of 2b between AV-2 and cucumovirus. Using the AV-2 infectious clone, we here provided first evidence that *Ilarvirus* 2b functions as an RNA silencing suppressor; AV-2 2b has suppressor activity against systemic silencing but not local silencing.

Introduction

Asparagus virus 2 (AV-2) is a member of the genus *Ilarvirus* and belongs to ilarvirus subgroup 2. Asparagus plants infected with AV-2 have no obvious symptoms, but AV-2 infection reduces vigor and productivity (Elmer et al., 1996; Jaspers et al., 1999; Tomassoli et al., 2012). AV-2 is transmitted not only through seeds collected from infected asparagus, but also through seeds collected from healthy plants fertilized by AV-2-infected pollens; AV-2 is not transmitted by aphids (Elmer et al., 1996; Jaspers and Pearson, 1997). In addition, AV-2 is mechanically transmitted mainly through infected sap on cutting knives and probably through feeding thrips as shown for other ilarviruses (Greber et al., 1991).

AV-2 infection of asparagus has been reported in Europe, North America and Japan (Elmer et al., 1996; Uyeda and Mink, 1981). Fujisawa et al. (1983) first identified AV-2 in the asparagus fields of Hokkaido, the largest production area in Japan, and named the Japanese isolate AV-2-J. In spite of the serious distribution, AV-2 infection has not been surveyed for the last few decades in Hokkaido as well as in other areas in Japan. In addition, the production of virus-free asparagus has not been attempted until now because AV-2 infection reduces the rooting and survival of asparagus explants in vitro, suggesting that it is not easy to prepare AV-2-free asparagus in vitro culture for field production (De Vries-Paterson et al., 1992). To develop a strategy to eliminate AV-2 from asparagus, we need to elucidate the molecular characteristics involved in such mechanisms as seed transmission and viral RNA silencing suppression for AV-2. If we can grow AV-2-free asparagus in fields, we would be able to increase the yield and quality of asparagus. Toward this endeavor, we here investigated whether AV-2 was still present in asparagus fields in Japan and then initiated the molecular study of AV-2.

The genus *Ilarvirus* is included in the family *Bromoviride*, which also includes cucumoviruses and bromoviruses, some of the most extensively studied plant viruses. All members have a tripartite RNA genome (RNAs 1–3). RNA 1 encodes the 1a protein with methyltransferase and helicase motifs. RNA 2 encodes the 2a protein, which is an RNA-dependent-RNA polymerase (RdRp). RNA 2 of some members (i.e., cucumoviruses and subgroup 1 and 2 in ilarviruses) has a second gene encoding the 2b protein (2b) (Xin et al., 1998), which is expressed through a subgenomic RNA, RNA4A. Neither alfamoviruses nor bromoviruses in the *Bromoviride* have been found to encode the 2b protein. In cucumoviruses, 2b has been shown to be an RNA silencing suppressor (RSS) (Burgyán and Havelda, 2011), but whether the 2b of ilarvirus functions as an RSS has not been known. RNA 3 encodes the movement protein (MP) in the 5′ half and the coat protein (CP) in the 3′ half. The CP, expressed by subgenomic RNA4, is necessary for genome activation for alfamovirus and ilarviruses (Jaspars, 1999; Neeleman et al., 2004), suggesting the possibility that replication of ilarviruses is

* Corresponding author. Fax: +81 11 706 3634.
E-mail address: hana@res.agr.hokudai.ac.jp (H. Shimura).

0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.04.015

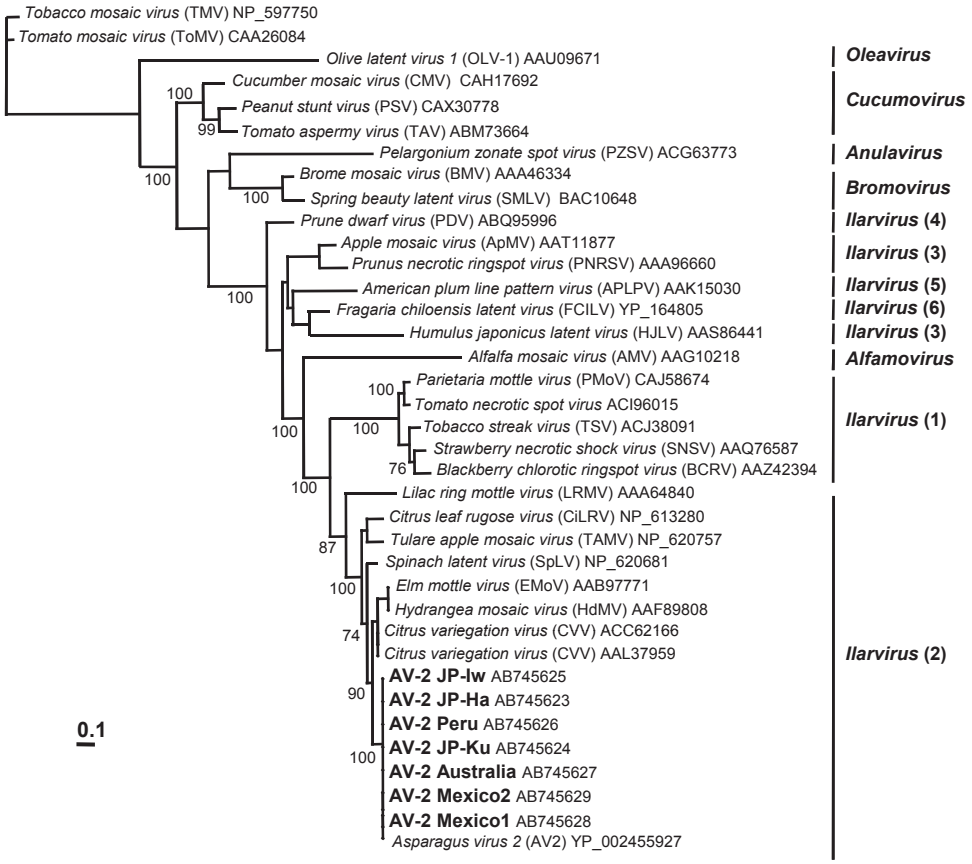

Fig. 1. Phylogenetic relationships among the nucleotide sequences of the CP genes from representative ilarviruses and related viruses in the *Bromoviridae*. The trees were constructed by the maximum-likelihood method implemented in the PAUP 4.0 software. Bootstrap values (1000 replicates) are given at branch points (only values >70 are shown). The evolutionary-distance scale is the number of substitutions per site. *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV) were used as outgroups. AV-2 isolates from Japan (JP-Ku, JP-Ha and JP-Iw), Australia, Mexico and Peru in bold face were newly sequenced in this study.

closely related to that of alfamoviruses. In fact, functional similarity of CPs of *Alfalfa mosaic virus* (AMV, alfamovirus) and *Tobacco streak virus* (TSV, ilarvirus) has been demonstrated; the two viral CPs share a limited, unique consensus sequence (Ansel-McKinney et al., 1996; Swanson et al., 1998).

Cucumber mosaic virus (CMV) isolates, which belong to *Bromoviridae*, are divided into two subgroups I and II based on the nucleotide and amino acid sequence of the viral genes. The 2b genes in subgroups I and II functionally differ in silencing suppressor activity; 2b of subgroup I has a stronger RSS activity than that of subgroup II. Because among the viruses in *Bromoviridae*, only cucumoviruses and ilarviruses have a 2b gene, the function of 2b of AV-2 is of our particular interest: there is the possibility that AV-2 isolates might be also divided into some subgroups just like CMV, and AV-2 2b thus shows functional synteny to the 2b of cucumoviruses. For AV-2 isolates, two serotypes (AV-2-P and AV-2-S) that vary in their infectivity on *Chenopodium quinoa* have been reported for AV-2 (Uyeda and Mink, 1981), suggesting that variability in the nucleotide sequences for the viral genes could explain serological and symptomatic differences. Because only one sequence for the CP and the 2b genes of AV-2 are available in the database, there is no molecular evidence for the genetic variability of AV-2 (Rafael-Martín and Rivera-Bustamante, 1999; Scott and Zimmerman, 2009; Tomassoli et al., 2012). Therefore, we first examined the sequence variability of AV-2 CP and 2b from different isolates and geographic origins. Based on the results, we created an infectious clone from one AV-2 isolate and tested whether AV-2 2b works as an RSS using the AV-2 infectious clone.

In this paper, we found unexpectedly low genetic variability among the AV-2 isolates from different geographic locations. In addition, we developed an efficient method to synthesize infectious transcripts via genome activation by in vitro-synthesized CP, and we also found that AV-2 2b functioned as a systemic RSS. This is the first demonstration that Ilarvirus 2b functions as an RSS similar to the 2b of cucumoviruses in *Bromoviridae*. We discuss the implications of AV-2 2b as an RSS against systemic silencing and contributions to unique AV-2 characteristics such as latent infection and seed transmission.

Results

Genetic variability of AV-2 isolates

In preliminary RT-PCR experiments, we detected AV-2 from the commercial asparagus from different asparagus-growing areas in Japan and other countries. For our phylogenetic analysis, we here cloned the CP (654 nt) and 2b (582 nt) genes from infected asparagus spears obtained from different geographic locations: Japanese asparaguses (from Kuriyama [Hokkaido], Hakodate [Hokkaido] and Iwate [Honshu]) and imported asparagus (from Peru, Mexico and Australia). The CP and the 2b genes from each isolate (at least 5 clones) were sequenced to avoid nucleotide changes due to PCR errors. Multiple alignments of the CP and 2b genes showed that both genes were highly conserved with more than 99% identity among the isolates examined (Fig. S1). The genetic variability of the CP and 2b genes, which was estimated by dividing the number of variant nucleotides relative to the most frequent nucleotide sequence by the total number of nucleotides sequenced, was very low (0.0069 and 0.0091, respectively). Maximum likelihood (ML) phylogenetic trees based on the nucleotide

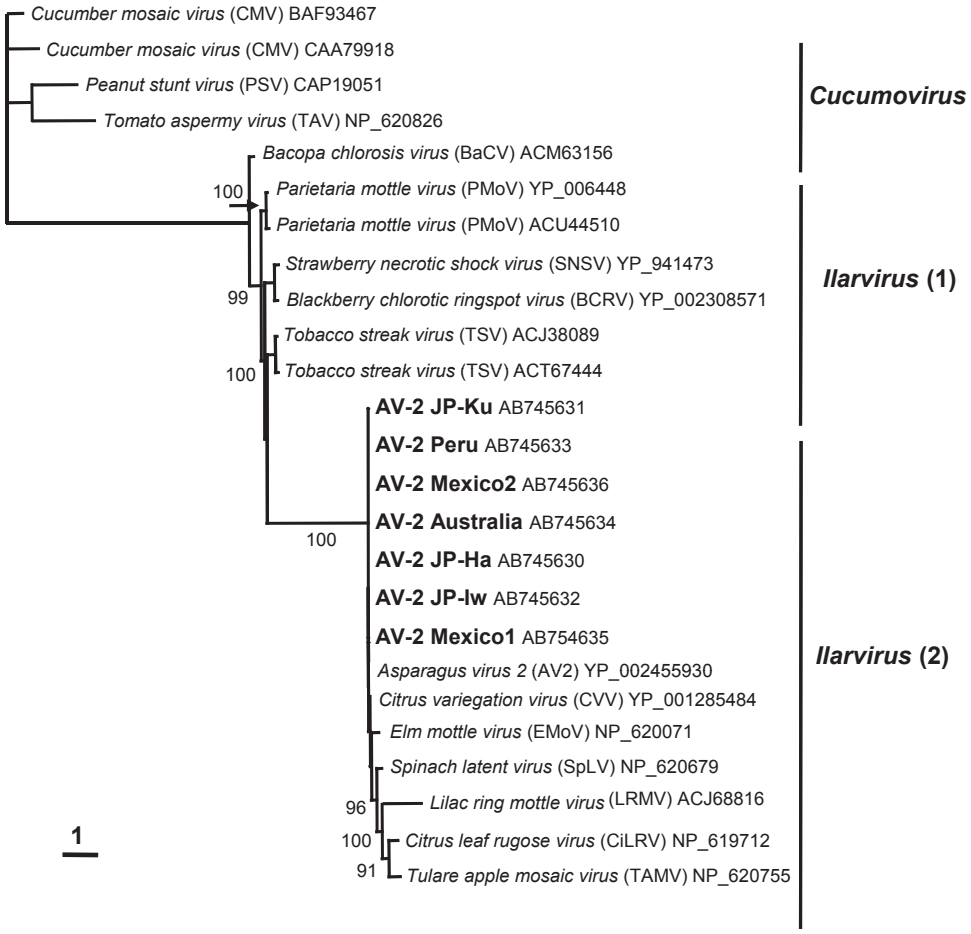

Fig. 2. Phylogenetic relationships among the nucleotide sequences of the 2b genes from representative ilarviruses and related viruses in the *Bromoviridae*. The trees were constructed by the maximum-likelihood method implemented in the PAUP 4.0 software. Bootstrap values (1000 replicates) are given at branch points (only values >70 are shown). The evolutionary-distance scale is the number of substitutions per site. Cucumoviruses were used as outgroups. AV-2 isolates from Japan (JP-Ku, JP-Ha and JP-Iw), Australia, Mexico and Peru in bold face were newly sequenced in this study.

sequences of CP (Fig. 1) and 2b (Fig. 2) showed essentially similar topologies; all AV-2 isolates cluster with the members of ilarvirus subgroup 2. In addition, phylogenetic tree for the CP revealed that AMV had a close relationship with ilarvirus subgroups 1 and 2; AMV first branched off and then the ilarviruses divided into subgroups 1 and 2.

Construction of infectious clones and genome activation of AV-2

Our sequence analysis revealed that the CP and 2b genes are highly conserved irrespective of geographic origin, and their genetic variability was rather low. Considering the low variability among the AV-2 isolates, we initiated a molecular study using one set of infectious clones from the Japanese strain, AV-2-J (Fujisawa et al., 1983). The CP and the 2b sequences of AV-2-J were almost identical to those of the other isolates.

Full-length cDNA clones of AV-2 RNAs 1–3 were constructed in the pGEM-T easy vector; they are referred to as AV-2-1, -2 and -3, respectively. These clones were all sequenced and confirmed to be almost identical to those in the database; especially the 5′ and 3′ sequences of the viral RNAs were the same as the published sequences (Rafael-Martín and Rivera-Bustamante, 1999; Scott and Zimmerman, 2009). When we used in vitro transcripts from the three RNAs to inoculate leaves on plants of *Nicotiana benthamiana*, the transcripts failed to establish AV-2 infection (Table S1). We then added in vitro-transcribed RNA 4 to the inoculum but we were not able to detect AV-2 in the inoculated plants, suggesting that RNA 4 does not necessarily activate the genome for AV-2 RNAs (Table S1). We then tested an idea from a study on genome activation for AMV (Guogas et al., 2005), a virus that is related to ilarviruses and cannot replicate in the absence of its CP. We first synthesized the AV-2 CP using the in vitro wheat germ translation system, which can yield up to several hundreds of micrograms of target proteins in one reaction depending on the proteins produced (Fig. 3A). We added the in vitro-synthesized AV-2 CP (at 5- to 10-fold molar excess of the in vitro transcripts) to the inoculum and inoculated *N. benthamiana* and *N. tabacum*. At 7 days postinoculation (dpi) in *N. benthamiana*, we detected AV-2 symptoms in the upper non-inoculated leaves, which had faint vein-clearing, and by 14 dpi leaves had developed mottling (Fig. 3B, Table S1). We observed tiny necrotic spots in *N. tabacum* at 7 dpi (Fig. 3B). In addition, we prepared sap from the infected leaves of *N. benthamiana* and inoculated *C. quinoa* with the sap; the plants eventually developed chlorotic spots (Fig. 3B) as reported for AV-2-J by Fujisawa et al. (1983).

RNA silencing suppression by AV-2

Because we could now utilize the AV-2 infectious clones as a tool to analyze the functions of the viral proteins, we first investigated whether AV-2 could suppress host RNA silencing. We used the agroinfiltration assay using the *N. benthamiana* 16c transgenic plants that constitutively express the GFP gene (Ruiz et al., 1998). To trigger RNA silencing against GFP, we used the

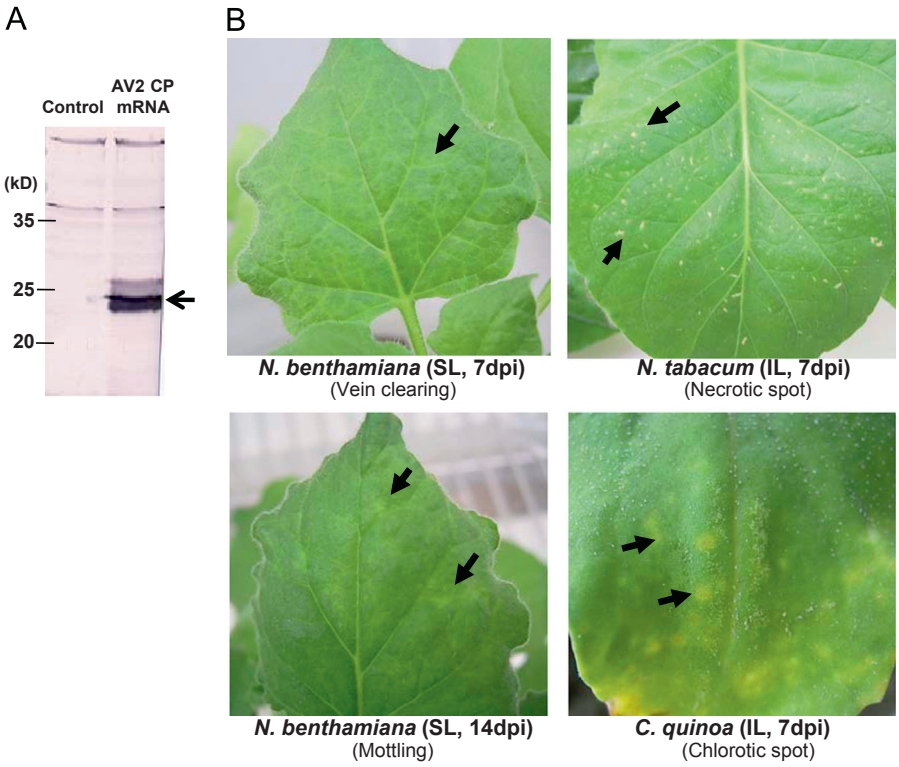

Fig. 3. Western blot analysis of in vitro-translated AV-2 CP in the wheat germ system and symptoms on plants inoculated with the infectious clones of AV-2. (A) In vitro translated AV-2 CP. AV-2 CP mRNA (15 μg) was used in a 16 h incubation at 20 °C. 4 μL of 200 μL was separated on a 15% SDS-PAGE gel and analyzed in a Western blot. Control does not include mRNA. (B) Symptoms on *Nicotiana tabacum* and *N. benthamiana* after inoculation with the in vitro-transcribed AV-2 RNAs plus the in vitro-translated AV-2 CP, and symptoms on *Chenopodium quinoa* after inoculation with sap from infected *N. benthamiana*. Arrows indicate the typical symptoms. IL: inoculated leaves, SL: systemic leaves.

inverted-repeat construct of GFP in the plant expression vector pBE2113 (pBE2113:GFP-IR). *Agrobacterium* containing pBE2113:GFP-IR was infiltrated into a leaf of AV-2-infected *N. benthamiana* 16c plants 7 days after AV-2 inoculation, and non-infiltrated upper leaves were monitored for red veins induced by systemic spread of RNA silencing against GFP. In the mock-inoculated 16c plants, systemic silencing against the GFP gene was clearly induced, but we found no evidence of GFP silencing in AV-2-infected plants (Fig. 4, Table 1), suggesting that AV-2 can suppress RNA silencing.

Because 2b of cucumoviruses can suppress systemic silencing as well as local silencing (Brigneti et al., 1998; Goto et al., 2007), we then examined whether AV-2 2b can also function as a local RSS. We tested whether AV-2 2b and AV-2 CP can suppress local RNA silencing by two methods, a transient silencing suppression assay using agrobacterium co-infiltration method and a protoplast assay. In the agrobacterium infiltration, neither AV-2 2b nor AV-2 CP inhibited local RNA silencing, while CMV 2b had strong RSS activity against local silencing (Fig. 5A). The accumulation of viral proteins in the leaves was confirmed by Western blots. Similarly, with the protoplast method, we found that AV-2 2b and AV-2 CP had little local RSS activity in *N. benthamiana* (Fig. 5B).

We next tested whether AV-2 2b and AV-2 CP can suppress systemic RNA silencing using *N. benthamiana* 16c plants. Using our previous method (Goto et al., 2007), we infiltrated leaves of young 16c plants with suspensions of *Agrobacterium* carrying plasmid constructs to express the GFP gene, GFP inverted-repeat (GFP-IR) and AV-2 2b (or AV-2 CP). When uninfiltrated upper leaves were evaluated under UV light at 10 dpi, the upper leaves of plants infiltrated with a mixture of GFP+GFP-IR+AV-2 CP showed systemic spread of RNA silencing, but plants infiltrated with a mixture of GFP+GFP-IR+AV-2 2b showed a 2–4 days delay of systemic RNA silencing in the upper leaves (data not shown). To confirm this delay in systemic RNA silencing by AV-2 2b, we then used the *Potato virus X* (PVX) vector (PVX:AV-2-2b) to express AV-2 2b in the entire 16c plant. We also created PVX:AV-2-CP to express AV-2 CP in the entire 16c plant. Four days after inoculation with PVX:AV-2-2b (or PVX:AV-2-CP), *Agrobacterium* suspensions carrying GFP+GFP-IR constructs were used to infiltrate the upper leaves to induce GFP silencing (Fig. 6A). In this experiment, AV-2 2b clearly suppressed spread of systemic silencing of GFP while AV-2 CP did not cause delay of systemic silencing (Fig. 6B, Table 2). These results suggest that the 2b protein of AV-2 but not the CP has RSS activity against systemic silencing but not local silencing. Ten days after agroinfiltration, we observed more severe symptoms in PVX:AV-2-2b-infected plants than those in the control PVX-infected plants, suggesting that AV-2 2b may have enhanced the viral symptoms through its RSS activity (Fig. 7). However, AV-2 CP also caused severe symptoms, which were even more severe than those induced by AV-2 2b (Fig. 7). Because AV-2 CP did not affect spread of systemic silencing, it may have another function to enhance the viral pathogenicity.

Discussion

As far as we know, a comparative study of AV-2 isolates in various asparagus cultivars from different geographic regions has never been done. In the present study, we found that asparagus spears grown in various countries (Australia, Peru and Mexico and three locations in Japan) were frequently infected with AV-2. In addition, we found that the sequences of 2b and CP were highly conserved among the isolates, suggesting that AV-2 from around the world is almost identical. In agreement with our observations, the low variability in CP sequences has been previously described between Italian and Mexican isolates (Tomassoli et al., 2012). Although we had not expected that the CP and 2b genes would

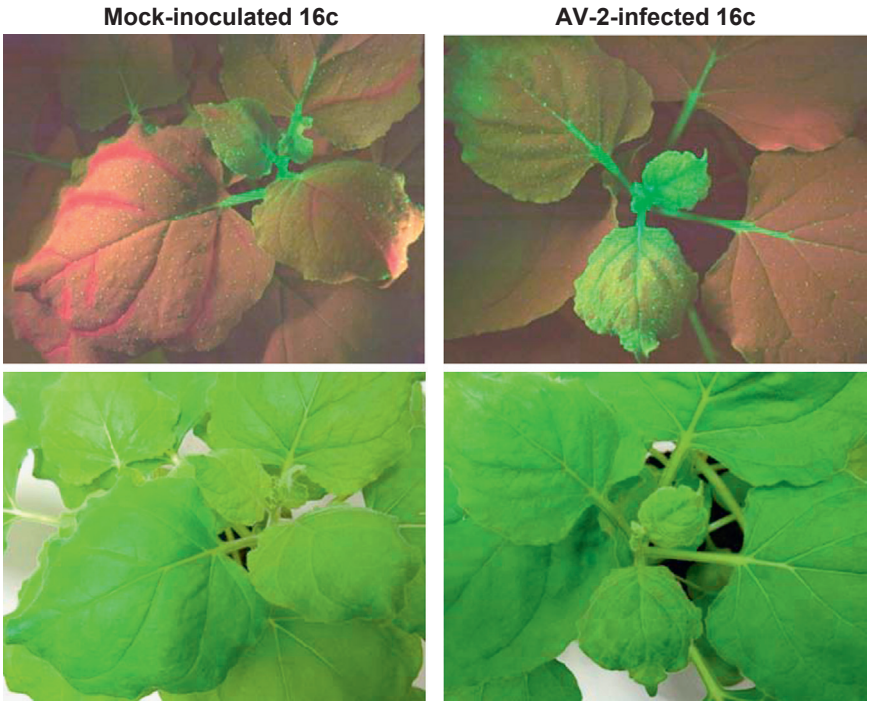

Fig. 4. Suppression of RNA silencing by AV-2. *Agrobacterium* cultures carrying the pBE2113:GFP-IR construct were infiltrated into AV-2-infected *N. benthamiana* 16c plants 7 days after AV-2 inoculation. As a control, the same *Agrobacterium* cultures were infiltrated into healthy *N. benthamiana* 16c plants (Mock-inoculated 16c), and then non-infiltrated upper leaves were evaluated under UV light at 10 dpi. Note that in the mock-inoculated 16c plant, systemic silencing against the GFP gene was clearly induced, but GFP silencing was not observed in the AV-2-infected 16c plant. The lower images show the symptoms of AV-2-infected 16c plant; there was little difference between mock-inoculated and AV-2-infected 16c plants.

be both highly conserved among AV-2 isolates, it could happen because AV-2 is seed-transmissible and commercially available asparagus seeds often contain AV-2 and are easily exchanged worldwide.

The genome of ilarviruses (subgroups 1 and 2) contains a 2b gene at a position similar to that of the cucumovirus 2b gene. However, the ilarvirus 2b gene is twice as large as the cucumovirus counterpart, and there is little similarity between the two gene sequences. The cucumovirus 2b gene has been identified as a strong RSS for both local and systemic silencing (Goto et al., 2007; Qi et al., 2004), but a possible role of the 2b gene in ilarviruses has not yet been investigated. Because the function of ilarvirus proteins has not been characterized, unlike other members in *Bromoviridae*, we do not know whether any proteins encoded by AV-2 have RSS activity. Considering that AV-2 does not normally cause any severe symptoms in infected plants, we speculated that AV-2 might not possess strong RSS. However, in this study, we demonstrated that the 2b protein of AV-2 actually has RSS activity against systemic silencing but the suppression is not obvious against local silencing. For seed (pollen)-transmitted viruses like AV-2, we believe that RSS activity against systemic silencing would be more important than that against local silencing for virus when invading reproductive/growing cells (pollens and apical meristem tissue). It has been demonstrated that seed transmission is related to the ability of virus to infect into meristem tissues (Amari et al., 2009), and thus seed-transmitted virus needs to counteract the host RNA silencing in the meristem. Because host RNA silencing should be ready before viral invasion, systemic silencing must play a major role in a meristem to achieve effective inhibition of the viral invasion. In contrast, if the invader virus produces an RSS protein against systemic silencing, the virus can overcome the host defense machinery in the meristem. The 2bs of other ilarviruses may share the same property. For example, *Spinach latent virus* (SpLV, ilarvirus subgroup 2) and viruses in ilarvirus subgroup 1 also spread to seeds (Bos et al., 1980; Sharman et al., 2009), indicating that those ilarviruses likely invade and propagate in meristems. We therefore believe that the ilarvirus 2bs may have an important role in the ability of ilarviruses to systemically infect their host.

Table 1
Effect of AV-2 infection on systemic silencing.

Inoculated plant	4 dpi[a]	5 dpi	7 dpi	10 dpi
Control	1/4 [b]	2/4	3/4	4/4
AV-2 infected plant	0/4	0/4	0/4	0/4

[a] Days post-infiltration (dpi) with Agrobacterium containing pBE2113:GFP-IR.
[b] No. of GFP-silenced plants/no. of infiltrated plants.

As we showed in our phylogenetic analyses, ilarviruses are most closely related to AMV, which does not have a 2b gene, but are more distantly related to cucumoviruses, which have a 2b gene. The 2b gene is speculated to be either an ancestral gene of *Bromoviridae* that had been lost in bromoviruses, AMV and some ilarviruses or it may have been independently created in cucumoviruses and ilarviruses sometime during their evolution. Because we now know that two types of 2b genes have a similar function as an RSS, it is hard to imagine that cucumoviruses and ilarviruses coincidentally acquired the gene at the same genomic position and that the gene also happened to have the same function. Taken together, we consider that our finding of the function of AV-2 2b supports the former hypothesis; the ancestral virus must have once had a 2b gene conferring RSS activity. Our observation that the genetic variability of the AV-2 genes is very low, and the genomes of AV-2 have changed little does not contradict the idea that AV-2 2b has maintained a 2b sequence similar to that of ancestral virus. However, the question why the 2b genes remain in cucumoviruses and some ilarviruses but not in bromoviruses and AMV remains unanswered.

To test for suppression of RNA silencing in AV-2-infected plants, we developed a system for AV-2 infectious clones because without infectious clones, our AV-2-J, which had been isolated about 30

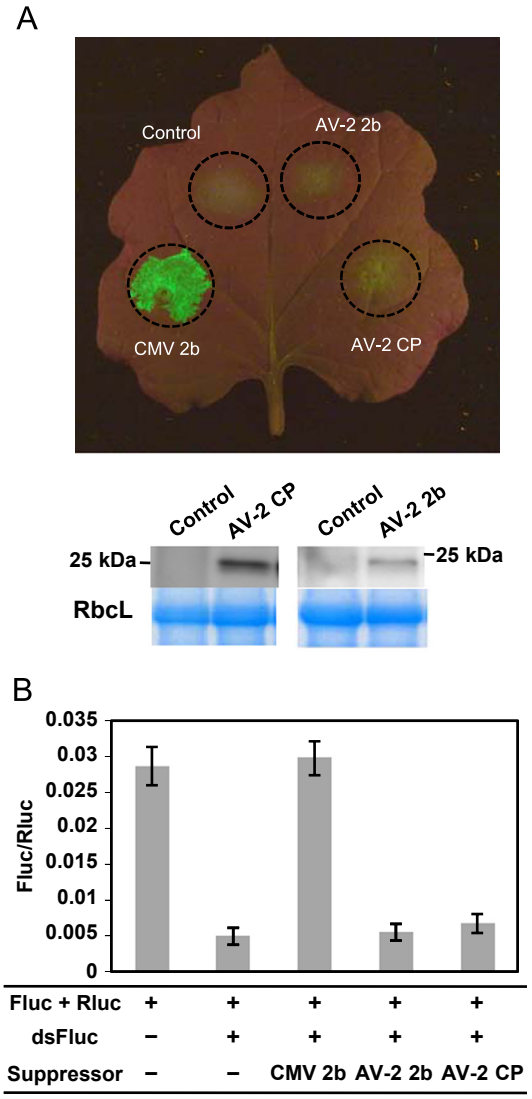

Fig. 5. Effect of AV-2 2b and AV-2 CP on local RNA silencing. (A) Inoculum containing *Agrobacterium* cultures carrying the constructs of pBE2113:GFP, pBE2113:GFP-IR with the viral gene constructs (AV-2 2b or AV-2 CP) was used to infiltrate a leaf of *N. benthamiana*. The *Cucumber mosaic virus* (CMV) 2b construct (CMV 2b) was used as a positive control. *Agrobacterium* cultures carrying the constructs of pBE2113:GFP, pBE2113:GFP-IR were also prepared as a negative control (Control). Photographs were taken 3 dpi. The lower panel is Western blots of the AV-2 2b or CP produced in the infiltrated patches. The CBB-stained Rubisco large subunit (RbcL) was used as a loading control. (B) Effect of AV-2 2b and AV-2 CP on RNA silencing suppressor (RSS) activity in the protoplast assay at 24 h after transfection. Results are means with standard deviations from at least 3 independent replicates. Note that CMV 2b showed strong suppression activity against Fluc silencing, but there was little RSS activity by AV-2 2b and AV-2 CP.

years ago, may have been a mixture of quasi-species or even contaminated with other asparagus viruses. Using this system, we are now examining the functional genomics of AV-2 to devise a strategy for producing virus-free asparagus.

Materials and methods

RNA extraction and primers for cDNA synthesis

Asparagus spears, produced in different asparagus-growing areas, were purchased in a market. Total nucleic acids were extracted from asparagus tissues by a conventional phenol–chloroform method. cDNA clones of the CP and 2b genes of AV-2 for phylogenetic analysis were directly synthesized by RT-PCR using a Takara RNA LA PCR kit (Takara Bio, Japan). AV-2-specific primers were designed based on the genome sequences obtained from the GenBank database (AV-2 RNA2, EU919667; AV-2 RNA3, X86352). The obtained CP and 2b sequences have been submitted to DDBJ (AB745623-AB745636).

Phylogenetic analysis

Multiple sequence alignments were performed using the Clustal W program (Thompson et al., 1994) and then adjusted by eye using the BioEdit software (Hall, 1999). Phylogenetic relationships were calculated using the program of PAUP* 4.0 (Swofford, 1999), and trees were constructed by maximum likelihood (ML). One thousand bootstrap replications were calculated after the appropriate model was selected.

Construction of infectious clones

For construction of an infectious clone of AV-2, AV-2-J was kindly provided by Dr. I. Fujisawa and Dr. S. Tsuda (NARO Agricultural Research Center, Tsukuba, Japan). Full-length cDNA clones of AV-2 RNAs 1–3 were amplified from AV-2-J-infected leaves and cloned in the pGEM-T easy vector. For in vitro transcription, RNA 1 and RNA 2 were amplified from the cDNA clones in the corresponding plasmids by long PCR with LA Taq polymerase (Takara Bio, Japan) using primer pair T7-AV-2-12-5G (5′-GCGTAATACGACTCACTATAGGTATTGTGCAG-TATTAT-3′) and AV-2-12-3 (5′-GCATCTCCTTTGGAGGCATCTA-3′). Similarly, RNA 3 was amplified using primer pair T7-AV-2-3-5G (5′-GCGTAATACGACTCACTATAGGCTTTCGAGACTAATAG-3′) and AV-2-3-3 (5′-AGCATCTCCTTTGGAGGCATC-3′). The 5′ end primers contained the T7 promoter sequence. Infectious RNAs were then in vitro-transcribed from the purified PCR products. In vitro transcription for viral RNAs was performed as described by Suzuki et al. (1991).

In vitro translation of AV-2 CP and inoculation

The cDNA clone of CP was inserted downstream of the tobacco mosaic virus Ω sequence in the in vitro transcription vector (pEU3-N II) with T7 RNA polymerase to create pEU3-CP for the subsequent in vitro wheat germ translation (WEPRO7240 Expression kit) (CellFree Science, Yokohama, Japan). The in vitro-transcribed RNA (15 μg) from pEU3-CP was added to the translation cocktail (200 μL volume) and incubated for 16 h at 20 °C as described by the manufacturer. The translation product was confirmed by Western blot analysis using anti-AV-2 CP antibodies. For inoculation with infectious clones of AV-2, the in vitro-synthesized transcripts of the three genomic RNAs 1–3 of AV-2 (~10 μg total) were mixed with a 5- to 10-fold molar excess of in vitro-translated CP. An equal volume of 0.1 M phosphate buffer was then added to the mixture to create the inoculum for rub-inoculation of *N. benthamiana* leaves using carborundum.

Assay for RNA silencing suppression

To construct an assay system for RNA silencing suppression of AV-2, we used *N. benthamiana* 16c transgenic plants that constitutively express the GFP gene (Ruiz et al., 1998). To trigger RNA silencing against GFP, we created the inverted-repeat construct of GFP in the plant expression vector pBE2113 (pBE2113:GFP-IR).

To assess RNA silencing suppression of AV-2 2b and AV-2 CP, we conducted agroinfiltration of leaves or transfection of protoplasts of *N. benthamiana* (wild type). To express AV-2 2b and AV-2 CP in a leaf of *N. benthamiana*, cDNA clones of AV-2 2b and AV-2 CP were inserted in the pBE2113 vector to create pBE2113: AV-2 2b and pBE2113: AV-2 CP, respectively. To express GFP in a leaf of *N. benthamiana*, we also created the GFP-sense expression

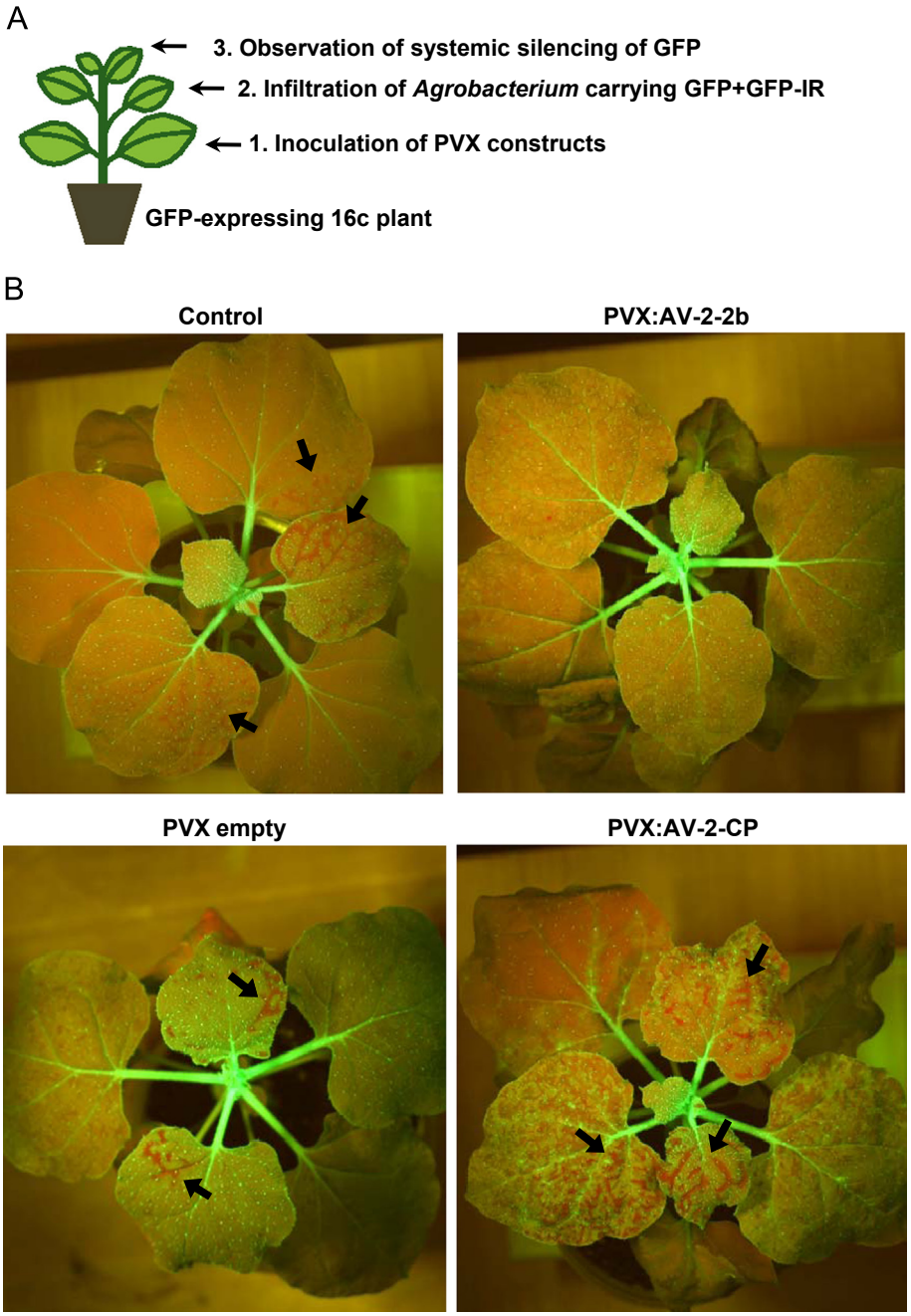

Fig. 6. Effect of AV-2 2b and AV-2 CP on systemic RNA silencing. (A) Schematic methods of the PVX vector-used systemic silencing assay. *N. benthamiana* 16c plants were inoculated with the PVX vector constructs containing AV-2 2b or AV-2 CP. Four days after PVX inoculation, GFP silencing was induced by agroinfiltration with *Agrobacterium* carrying pBE2113:GFP and pBE2113:GFP-IR; plants were then observed for systemic silencing of GFP in non-infiltrated upper leaves. (B) Systemic GFP silencing 10 days after agroinfiltration. Note that AV-2 2b clearly suppressed spread of systemic silencing of GFP. Control is the plant that was agroinfiltrated with pBE2113:GFP and pBE2113:GFP-IR but not inoculated with the viruses. Arrows indicate red veins induced by systemic silencing against GFP.

Table 2
Effects of AV-2 2b and AV-2 CP on systemic silencing.

Inoculum	4 dpi[a]	5 dpi	6 dpi	10 dpi
Control (Mock)	1/3 [b]	2/3	3/3	3/3
PVX	0/3	1/3	3/3	3/3
PVX:AV-2-2b	0/3	0/3	0/3	1/3
PVX:AV-2-CP	0/3	2/3	3/3	3/3

[a] Days post-infiltration (dpi) with Agrobacterium containing pBE2113:GFP and pBE2113:GFP-IR.
[b] No. of GFP-silenced plants/no. of infiltrated plants.

construct, pBE2113:GFP. The Ti-plasmid construct was then introduced into *Agrobacterium tumefaciens* KYRT1 strain, which was supplied by Dr. G. B. Collins (University of Kentucky, USA). *Agrobacterium* suspensions were prepared to an optical density at 600 nm of 1.0. Inocula were prepared by mixing the *Agrobacterium* cultures containing the various constructs (pBE2113:GFP, pBE2113:GFP-IR and a viral gene construct) in the ratio of 5:1:5 and used to infiltrate a leaf of *N. benthamiana*. To confirm the accumulation of AV-2 2b or CP produced in the infiltrated patches by Western blots, total protein (50 μg) extracted from patches was separated by SDS-PAGE, and AV-2 2b and CP were detected with

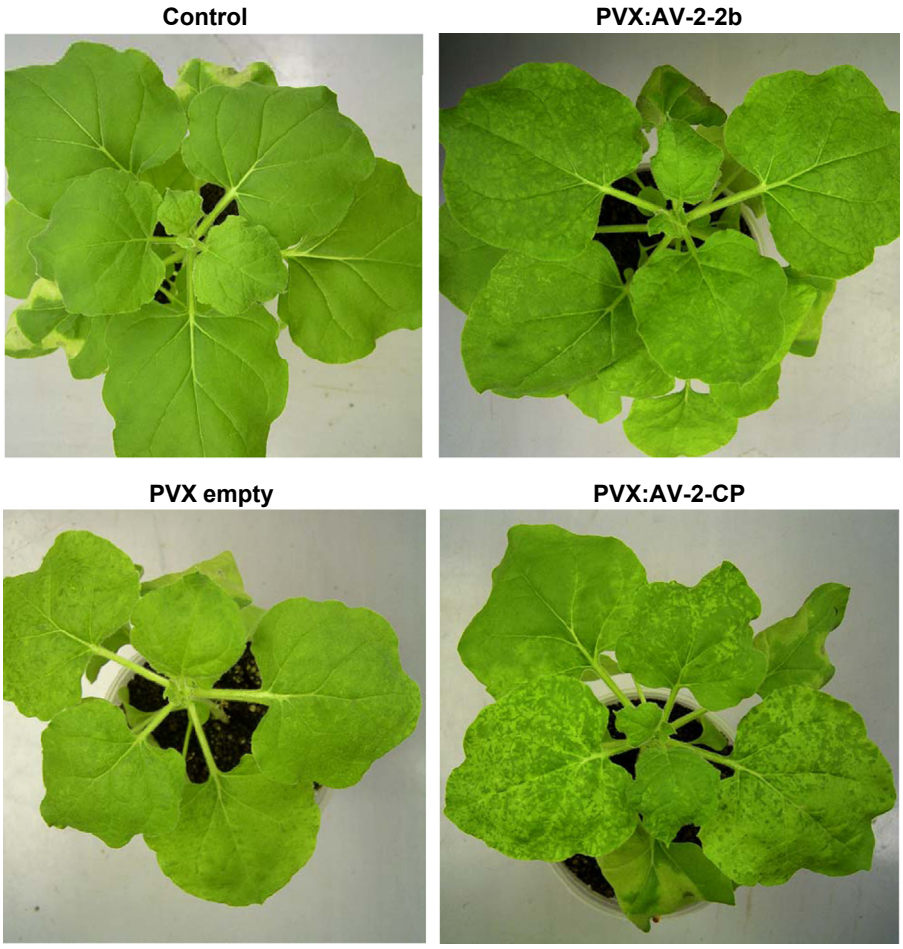

Fig. 7. Symptoms on the *N. benthamiana* 16c plants inoculated with PVX:AV-2-2b or PVX:AV-2-CP. Note that PVX:AV-2b or PVX:AV-2-CP induced more severe symptoms than the PVX vector (empty). Control is the plants that were agroinfiltrated with pBE2113:GFP and pBE2113:GFP-IR but not inoculated with the viruses.

anti-FLAG antibodies. The FLAG peptide had been fused to the C-terminus of the AV-2 CP and 2b for detection by Western analysis.

For the protoplasts assay, protoplasts of *N. benthamiana* were prepared and transfected essentially as described in Shimura et al. (2008). Briefly, we used the luciferase gene as a reporter gene; the firefly luciferase (Fluc) was the target of RNA silencing and the Renilla luciferase (Rluc) was an internal control for transfection (Shimura et al., 2008). As a silencing inducer, dsRNA of the Fluc gene (dsFluc) was prepared by in vitro transcription using a PCR-amplified fragment containing the T7 promoter sequence at both 5′ and 3′ ends. The AV-2 2b and AV-2 CP were cloned in the pE2113 vector (Mitsuhara et al., 1996). RNA silencing was induced in protoplasts by co-transfecting with the reporter genes and dsFluc with or without 3 μg of the plasmids containing a viral gene. Transfected protoplasts were then incubated for 24 h in the dark at 25 °C. After incubation, protoplasts were harvested and analyzed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

PVX vector construct

The PVX vector was obtained from Dr. D.C. Baulcombe (The Sainsbury Laboratory, Norwich, UK), and AV-2 2b and AV-2 CP were cloned into the PVX vector. *N. benthamiana* leaves were inoculated with in vitro transcripts of the recombinant constructs to make inocula for further inoculation experiments (Chapman et al., 1992).

Acknowledgments

We thank Dr. D.C. Baulcombe (The Sainsbury Laboratory, Norwich, UK) for providing the PVX vector. We also thank Dr. G.B. Collins (University of Kentucky, USA) for providing *Agrobacterium tumefaciens* KYRT1 strain. We are grateful to Dr. I. Fujisawa and Dr. S. Tsuda (NARO Agricultural Research Center, Tsukuba, Japan) for providing AV-2-J. This work was supported in part by a Grant-in-Aid for Research and Development Projects for Application in Promoting New Policy of Agriculture Forestry and Fisheries from the Ministry of Agriculture, Forestry and Fisheries (MAFF) (Japan).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.04.015.

References

Amari, K., Burgos, L., Pallás, V., Sánchez-Pina, M.A., 2009. Vertical transmission of *Prunus* necrotic ringspot virus: hitch-hiking from gametes to seeding. J. Gen. Virol. 90, 1767–1774.

Ansel-McKinney, P., Scott, S.W., Swanson, M., Ge, X., Gehrke, L., 1996. A plant viral coat protein RNA binding consensus sequence contains a crucial arginine. EMBO J. 15, 5077–5084.

Bos, L., Huttinga, H., Maat, D.Z., 1980. *Spinach latent virus*, a new ilarvirus seed-borne in *Spinacia oleracea*. Eur. J. Plant Pathol. 86, 79–98.
Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W., Baulcombe, D.C., 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J. 17, 6739–6746.
Burgyán, J., Havelda, Z., 2011. Viral suppressors of RNA silencing. Trends Plant Sci. 16, 265–272.
Chapman, S., Kavanagh, T., Baulcombe, D., 1992. *Potato virus X* as a vector for gene expression in plants. Plant J. 2, 549–557.
De Vries-Paterson, R.M., Evans, T.A., Stephens, C.T., 1992. The effect of asparagus virus infection on asparagus tissue culture. Plant Cell Tiss. Org. Cult. 31, 31–35.
Elmer, W.H., Johnson, D.A., Mink, G.I., 1996. Epidemiology and management of the diseases causal to asparagus decline. Plant Dis. 80, 117–125.
Fujisawa, I., Goto, T., Tsuchizaki, T., 1983. Some properties of asparagus virus II isolated from *Asparagus officinalis* in Japan. Ann. Phytopath. Soc. Jpn. 49, 683–688.
Goto, K., Kobori, T., Kosaka, Y., Natsuaki, T., Masuta, C., 2007. Characterization of silencing suppressor 2b of cucumber mosaic virus based on examination of its small RNA-binding abilities. Plant Cell Physiol. 48, 1050–1060.
Greber, R.S., Klose, M.J., Milne, J.R., Teakle, D.S., 1991. Transmission of prunus necrotic ringspot virus using plum pollen and thrips. Ann. Appl. Biol. 118, 589–593.
Guogas, L.M., Laforest, S.M., Gehrke, L., 2005. Coat protein activation of alfalfa mosaic virus replication is concentration dependent. J. Virol. 79, 5752–5761.
Hall, T.A., 1999. BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for WINDOWS 95/98/NT. Nucl. Acids Symp. 41, 95–98.
Jaspars, E.M., 1999. Genome activation in alfamo- and ilarviruses. Arch. Virol. 144, 843–863.
Jaspers, M.V., Falloon, P.G., Pearson, M.N., 1999. Long-term effects of asparagus virus 2 infection on growth and productivity in asparagus. Ann. Appl. Biol. 135, 379–384.
Jaspers, M.V., Pearson, M.N., 1997. Transmission of asparagus virus 2 in an asparagus crop. Proceedings of the 50th New Zealand Plant Protection Conference. pp. 84–88.
Mitsuhara, I., Ugaki, M., Hirochika, H., Ohshima, M., Murakami, T., Gotoh, Y., Katayose, Y., Nakamura, S., Honkura, R., Nishimiya, S., Ueno, K., Mochizuki, A., Tanimoto, H., Tsugawa, H., Otsuki, Y., Ohashi, Y., 1996. Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant Cell Physiol. 37, 49–59.
Neeleman, L., Linthorst, H.J., Bol, J.F., 2004. Efficient translation of alfamovirus RNAs requires the binding of coat protein dimmers to the 3′ termini of the viral RNAs. J. Gen. Virol. 85, 231–240.
Qi, Y., Zhong, X., Itaya, A., Ding, B., 2004. Dissecting RNA silencing in protoplasts uncovers novel effects of viral suppressors on the silencing pathway at the cellular level. Nucl. Acids Res. 32, e179.
Rafael-Martín, M., Rivera-Bustamante, R.F., 1999. Molecular characterization of the RNA 3 of asparagus virus 2. Arch. Virol. 144, 185–192.
Ruiz, M.T., Voinnet, O., Baulcombe, D.C., 1998. Initiation and maintenance of virus-induced gene silencing. Plant Cell 10, 937–946.
Scott, S.W., Zimmerman, M.T., 2009. The nucleotide sequences of the RNA 1 and RNA 2 of asparagus virus 2 show a close relationship to citrus variegation virus. Arch. Virol. 154, 719–722.
Sharman, M., Persley, D.M., Thomas, J.E., 2009. Distribution in Australia and seed transmission of Tobacco streak virus in *Parthenium hysterophorus*. Plant Dis. 93, 708–712.
Shimura, H., Fukagawa, T., Meguro, A., Yamada, H., Oh-Hira, M., Sano, S., Masuta, C., 2008. A strategy for screening an inhibitor of viral silencing suppressors, which attenuates symptom development of plant viruses. FEBS Lett. 582, 4047–4052.
Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N., Takanami, Y., 1991. Functional analysis of deletion mutants of cucumber mosaic virus RNA 3 unsing an in vitro transcription system. Virology 183, 106–113.
Swanson, M.M., Ansel-McKinney, P., Houser-Scott, F., Yusibov, V., Loesch-Fries, L.S., Gehrke, L., 1998. Viral coat protein peptides with limited sequence homology bind similar domains of alfalfa mosaic virus and tobacco streak virus RNAs. J. Virol. 72, 3227–3234.
Swofford, D.L., 1999. PAUP*. Phylogenetic analysis using parsimony (* and other methods). Version 4. Sinauer, Sunderland, MA.
Thompson, J.D., Higgins, D.G., Bibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673–4680.
Tomassoli, L., Tiberini, A., Vetten, H.J., 2012. Viruses of *Asparagus*. Adv. Virus Res. 84, 345–365.
Uyeda, I., Mink, G.I., 1981. Properties of asparagus virus II, a new member of the ilarvirus group. Phytopathology 71, 1264–1269.
Xin, H.W., Ji, L.H., Scott, S.W., Symons, R.H., Ding, S.W., 1998. Ilarviruses encode a cucumovirus-like 2b gene that is absent in other genera within the *Bromoviridae*. J. Virol. 72, 6956–6959.