Characterization of host-dependent mutations of apple fruit crinkle viroid replicating in newly identified experimental hosts suggests maintenance of stem–loop structures in the left-hand half of the molecule is important for replication

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Abstract

Apple fruit crinkle viroid (AFCVd) is a tentative member of the genus Apscaviroid, family Pospiviroidae. AFCVd has a narrow host range and is known to infect apple, hop and persimmon as natural hosts. In this study, tomato, cucumber and wild hop have been identified as new experimental herbaceous hosts. Foliar symptoms were very mild or virtually undetectable, but fruits of infected tomato were small, cracked and distorted. These symptoms resemble those observed on some AFCVd-sensitive apple cultivars. After transfer to tomato, cucumber and wild hop, sequence changes were detected in a natural AFCVd isolate from hop, and major variants in tomato, cucumber and wild hop differed in 10, 8 or 2 nucleotides, respectively, from the predominant one in the inoculum. The major variants in tomato and cucumber were almost identical, and the one in wild hop was very similar to the one in cultivated hop. Detailed analyses of the host-dependent sequence changes that appear in a naturally occurring AFCVd isolate from hop after transfer to tomato using small RNA deep sequence data and infectivity studies with dimeric RNA transcripts followed by progeny analysis indicate that the major AFCVd variant in tomato emerged by selection of a minor variant present in the inoculum (i.e. hop) followed by one to two host-dependent de novo mutations. Comparison of the secondary structures of major variants in hop, tomato and persimmon after transfer to tomato suggested that maintenance of stem–loop structures in the left-hand half of the molecule is critical for infection.

INTRODUCTION

The genus Apscaviroid in the family Pospiviroidae contains 10 species and four tentative members whose molecular sizes range from ca. 300 to 370 nt [1]. Members infect mainly woody species such as Malus, Pyrus, Citrus (Rutaceae) and Vitis. Viroids in the family Pospiviroidae are classified based on nucleotide sequence similarity and the presence or absence of several conserved sequences, e.g. a central conserved region and terminal conserved region or terminal conserved hairpin. An overall nucleotide sequence identity of greater than 90% is employed to demarcate individual species, but biological properties such as host specificity, symptoms and pathogenicity must also be taken into consideration [1]. In this respect, information on the biological properties of members of the genus Apscaviroid is still insufficient when compared to viroids in other genera such as Pospiviroid and Hostuviroid. This is mainly because suitable herbaceous hosts have not been identified, and it is sometimes necessary to spend several months or years to analyse the biological properties using such woody indicator species as apple cultivars Starkrimson and Braeburn for apple dimple fruit viroid [2], Orin and NY58-22 for apple fruit crinkle viroid (AFCVd) [3] or Red Delicious for apple scar skin viroid (ASSVd) [4].

Although their natural hosts are restricted mainly to woody species, some apscaviroids have been reported to infect herbaceous hosts experimentally; for example, Australian grapevine viroid (AGVd) infects cucumber (Cucumis sativus) and...
tomato (*Solanum lycopersicum*) [5, 6], and pear blister canker viroid can be transmitted to cucumber [7]. In addition, ASSVd has recently been reported to infect cucumber, tomato, *Phaseolus vulgaris*, *Nicotiana tabacum*, *Nicotiana glutinosa*, *Nicotiana benthamiana*, *Solanum melongena* and *Chenopodium quinoa* [8], although these results do not correspond with those in a previous report [9].

AFCVd is a tentative member of the genus *Apscaviroid* and was first identified as the causal agent of apple fruit crinkle disease [10, 11]. It was later detected in cultivated hops (*Humulus lupulus*) showing severe stunting and leaf curling similar to the hop stunt disease caused by hop stunt viroid [12], and a distinct isolate of AFCVd was identified more recently in Japanese persimmon (*Diospyros kaki*) [13]. As such, and despite its high economic impact, AFCVd remains a tentative member of the genus *Apscaviroid*, partly because its detailed biological properties have not yet been reported and also because possible differences with AGVd are not clear, i.e. the two viroids share ca. 85% overall nucleotide sequence identity.

Here, we have identified tomato and cucumber as symptomatic hosts for AFCVd and characterized them as experimental hosts that can be used to analyse AFCVd biology based on the molecular structure. We describe specific symptoms on selected tomato cultivars and compare AFCVd-specific small RNA (sRNA) profiles in infected tomato fruits and leaves. Detailed analysis of host-dependent mutations appearing after infection in tomato based on deep sequencing, as well as sequencing of full-length AFCVd cDNA clones, suggests that maintenance of stem-loop structures in the left-hand half of the molecule is critical for infection.

**RESULTS**

**Infectivity of a natural AFCVd isolate from hop in tomato, cucumber and wild hop**

**Characterization of symptoms on tomato and cucumber as herbaceous assay plants for AFCVd**

Because AGVd, a close relative of AFCVd, is known to infect cucumber and tomato, AFCVd-hopAK was challenge inoculated to four tomato cultivars (Rutgers, Momotaro, Red Olé and Red Ruby) and one cucumber cultivar (Sujo), as well as a vegetatively propagated wild hop clone. Three of the five cuttings of wild hop became AFCVd positive at 2 months post-inoculation (mpi) and showed stunting and curling of the upper leaves (Fig. 1a). Although no symptoms were visible on the whole plants or leaves of tomato or cucumber, flowers on infected cucumbers became wrinkled around 2 mpi. All cucumber and tomato plants, except for one Red Ruby tomato plant, were AFCVd positive by Northern blotting at 2 mpi. Note that the progeny recovered from tomato and cucumber appeared to be a little larger in size than those from hop plant (Fig. 1e).

All five plants from each of the four tomato cultivars were transplanted to an open plot in order to observe their growth and productivity under the natural growing conditions. By the end of the growing season, the Rutgers plants were mildly stunted, and their fruits were abnormal in shape and colour, i.e. fruits were small and cracked/distorted (Fig. 1b). Momotaro, a large fruit-size tomato variety, grew normally but produced some crinkled fruits (Fig. 1c). Red Olé and Red Ruby, medium and mini fruit-size varieties, respectively, were virtually normal, both in the size of plant and in the shape and colour of fruit. As shown in Fig. 1(d), comparisons using Welch’s t-test revealed a statistically significant (at the 5% level) reduction in mean fruit weight associated with AFCVd infection for varieties Rutgers and Red Olé.

These results demonstrated that AFCVd is infectious to tomato and cucumber, as well as to wild hop. Plants growing at 25°C (16 h day-length) required 1–2 months to become AFCVd positive by Northern blotting, and foliar symptoms were generally very mild or virtually invisible. Flowers on infected cucumbers, in contrast, became wrinkled, and the fruits on some tomato varieties were

![Fig. 1. Disease symptoms associated with AFCVd infection. (a) Stunting and leaf curling symptoms on wild hop (right). Left, healthy control. (b) Abnormal fruit shape, colour and size on Rutgers tomato during the early (left), middle (centre) and ripening (right) stages of infection (upper row). Lower row, fruit from healthy control plants. (c) Tomato fruit crinkle on Momotaro tomato (right). Left, fruit from healthy plant. (d) Comparison of fruit weight for healthy (blue) and infected (red) Momotaro, Rutgers, Red Olé and Red Ruby tomatoes. Differences for Rutgers and Red Olé were statistically significant (an asterisk indicates 5% significance value by Welch’s t-test). (e) RNA gel blot hybridization analysis of circular and linear forms of AFCVd isolated from wild hop, tomato and cucumber. Note that the progeny isolated from tomato and cucumber were a little larger than those from wild hop.](image-url)
misshapen and/or discoloured. These symptoms are somewhat similar to those observed on certain AFCVd-sensitive apple cultivars.

Changes in the nucleotide sequence of AFCVd after infection in tomato, cucumber and wild hop

A total of 45 (15 each) full-length cDNA clones of AFCVd-hopAK progenies recovered from infected tomato, cucumber and wild hop were sequenced, resulting in 15, 13 and 14 full-length sequences, respectively. The major variant in tomato differed in 10 nt positions compared to the major sequence in the inoculum (AFCVd-hopAK6-2, accession AB104532) [12], i.e. G23A, 71/72+A, G77A, U82A, G178A, U206A, A291U, 304/305+A, A308U and 319/320+U (Fig. 2a). The major variant in cucumber differed at eight positions (G23A, 71/72+A, G77A, U82A, G178A, A291U, 304/305+U and A308U), which were very similar to those in tomato (Fig. 2a). In contrast, the major variant in wild hop differed at only two positions (G178A and A308U; Fig. 2a). Note that, consistent with the observations from RNA gel blot hybridization analysis, the major AFCVd variants present in tomato and cucumber were 373 nt in length, 3 nt larger than either that in wild hop or the major variant in the inoculum (i.e. AFCVd-hop-AK6-2).

Because evidence of host-specific mutation was found after infection in tomato and cucumber, the analysis was repeated by infecting a new group of tomato plants (cv. Rutgers) with variant AFCVd-hopAK6-2. Eleven full-length sequences of the resulting progeny were obtained, and a major variant accounting for 8 of the 11 clones was identified. The sequence was different in eight mutations compared to the major variant in the inoculum, and changes were identified at eight positions, i.e. G23A, 71/72+A, G77A, U82A, G178A, A291U, 304/305+U and A308U (Fig. 2b, solid arrows). Thus, results from the two assays in tomato were consistent. When RNA extracted from this second set of plants was transferred to a third group of tomato seedlings and maintained for 2 months, the major variant detected (accounting for 11 of the 12 clones sequenced) contained three additional mutations, i.e. A173-, U206A and 319/320+U (Fig. 2b, dotted arrow with parentheses). Although U (9 of 11 clones) predominated over A (2 of 11 clones) at position 206 in the first propagation in tomato, it became predominant (12 of 12 clones) in the succeeding propagation. The same tendency was observed for the U insertion between positions 319 and 320, i.e. an additional A residue was present in only 2 of 11 clones recovered after the first propagation in tomato, but this number increased to 12 of 12 clones in the succeeding propagation.

Fine mapping of nucleotide changes appearing after infection using AFCVd-specific sRNA data sets

As described above, sequence analysis of full-length cDNA clones identified a number of mutations in AFCVd-hopAK.
associated with host adaptation or selection in tomato. It is, however, costly and laborious to sequence large numbers of cDNA clones, and it can be difficult to decide how many clones are required and sufficient to detect all significant mutations in the population. Our initial analyses involved only a limited number of cDNA clones (i.e. 15 and 11, a total of 26), and therefore, we attempted to confirm our results using AFCVd-specific sRNA data sets obtained by deep sequencing analysis of AFCVd-hopAK-infected tomato leaves and fruit.

The data sets from healthy and AFCVd-infected leaf samples contained approximately 2.6 million and 2.4 million reads of sRNA ranging in size from 16 to 36 nt, respectively. The corresponding sets from healthy and AFCVd-infected fruit samples contained 4.1 million and 4.0 million reads. Analysis of the raw data revealed a total of 264,503 reads in AFCVd-infected leaves [genomic [(+)-sense]/anti-genomic [(-)-sense] ratio=2.19] that either were perfect matches to AFCVd or contained a single mismatch, 778,334 reads (1.79) in AFCVd-infected fruits, and only 19 or 21 reads in healthy leaves and fruits. The number of AFCVd-sRNA reads per million was 110,114 (11.0 %) in AFCVd-infected leaves and 192,664 (19.3 %) in AFCVd-infected fruits, indicating that concentration of AFCVd-sRNA in fruit is almost twice that in leaves. In terms of size, AFCVd-sRNAs containing 22 nt were most abundant, followed by 21, 24 and 23 nt. The first two species represented ca. 90 % of the total AFCVd-sRNA reads. With respect to orientation, AFCVd-sRNA derived from positive strand constituted ca. 70 % in leaves and 65 % in fruits.

Mapping AFCVd-sRNAs to the viroid genome revealed major hotspots near positions 90–110 for the positive strand and 100–120 for the negative strand, both included in the upper central conserved region (Fig. 3, red arrows). An additional six or seven minor hotspots were also detected elsewhere in the positive and negative strands, respectively (Fig. 3, blue arrows). In spite of their uneven distribution, AFCVd-sRNAs were derived from more or less the entire AFCVd genome (and its complement), the number of reads at each positions ranging from 200–300 in the least abundant region to more than 100,000 in the major hotspot regions (Fig. 3b).

Using this large-scale sequencing data, nucleotide polymorphisms (i.e. mutations) were detected at 11, 10, 13 and 13 positions in the data sets constructed from (+)-sense/fruit, (+)-sense/fruit, (+)-sense/leaf and (-)-sense/leaf, respectively (Table 1). When the nucleotide positions showing polymorphism were plotted on the secondary structure of the AFCVd genome (AFCVd-hop-AK6-2,
accession AB104532), all the mutations detected by clone sequencing analysis except for A173– were represented (Figs 2b and 4a). They included two of the three additional mutations (i.e. U206A and 319/320+U) appearing after an additional passage in tomato (Fig. 2b, those in parentheses). Yet another mutation that was not detected by clone sequencing analysis was found, i.e. G136A was found in (+)- and (–)-sense fruit data sets with 35.1 % of the population (Fig. 4a).

Other novel mutations were also found in the leaf data sets, i.e. three new mutations (G130A, G136U and U232C) were detected in both (+)-sense and (–)-sense data (Fig. 4b, arrow with star), and the other mutation, U51A (for a total of four), was detected in (–)-sense data (Fig. 4b, dotted arrow with M). This U-to-A substitution found at position 51, however, was limited only to (–)-sense leaf data with low frequency and did not seem to be inherited in (+)-sense populations; therefore, it could be a non-viable mutation. The ratio of mutant to wild-type nucleotide at each position varied between 6.6 % (342 of 5175) and 100 % (2376 of 2376) (Table 1). The ratio tended to be lower for mutations not detected by clone-sequencing analysis.

**Infectivity of in vitro RNA transcripts derived from selected AFCVd variants from hop, tomato and persimmon in tomato plant**

In the infection assays described above, the major AFCVd sequence variants recovered from infected tomato and cucumber showed 8–10 nt differences from AFCVd-hopAK, the isolate from the original hop plant used for inoculum.

### Table 1. Heterogeneous nucleotide positions detected using AFCVd-specific sRNA data sets (21–24 nt) obtained by deep sequencing analysis of AFCVd-infected tomato fruit and leaf tissue

| Nucleotide numbers in (–)-sense were reversed to make them correspond to the numbers in (+)-sense, i.e. G23A in (–)-sense in the table corresponds to C348U in (+)-sense. |

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Were these ‘new’ variants already present in the inoculum or did they emerge de novo after infection in the new hosts? To answer this question, dimeric cDNA clones of major AFCVd variants from hop (hop-AK6-2), tomato (hop-AK6-T2, accession AB795265) and persimmon (persimmon-HS1, accession LC169594) were constructed and then groups of five tomato seedlings (cv. Rutgers) were inoculated with the corresponding in vitro RNA transcripts by particle bombardment. Two months post-infection, none of the inoculated plants showed any visible symptoms. Nevertheless, Northern blotting revealed that four, one and five of the plants inoculated with hop-AK6-2, hop-AK6-T2 and persimmon-HS1 had become AFCVd positive. All three variants tested were infectious to tomato. One infected plant from each group, a total of three, was selected and used for RNA extraction, amplification and cloning of full-length AFCVd cDNA, and nucleotide sequence analysis of the respective progeny. A total of 15 cDNA clones from each group were analysed, and 14, 10 and 15 full-length sequences were obtained from hop-AK6-2, hop-AK6-T2 and persimmon-HS1, respectively. As shown in Fig. 5(a–c), the major variants recovered from tomato plants infected with hop-AK6-2, hop-AK6-T2 and persimmon-HS1 differed from the original sequence used for inoculation in two positions [G178A (mutant percentage 92.9 %) and A308U (71.4 %)], two positions [U82A (100 %) and U305– (100 %)] and one position [C92U (100 %)], respectively. The progeny of hop-AK6-2 contained another three minor mutations, i.e. G77A (28.6 %), U78G (14.3 %) and A308C (14.3 %). Those of hop-AK6-T2 contained two additional minor mutations, i.e. G67A (20 %) and A68G (20 %).

It should be noted here that only 3 (i.e. G77A, G178A and A308U) of the 10 mutations found after inoculation of tomato seedlings with a natural AFCVd isolate (AFCVd-hopAK) (Figs 2 and 4) were detected in the progenies of clonal hop-AK6-2 dimeric RNA, indicating that major sequence variants appearing in tomato after inoculation with the natural hop isolate arise mainly by selection from natural variants pre-existing in the inoculum followed by the addition of one or two spontaneous mutations favouring replication in tomato. Like hop-AK6-2, hop-AK6-T2 and persimmon-HS1 also generated a small number of host-dependent mutations during adaptation to tomato, but these were not identical, indicating that different AFCVd variants adapted differently and did not converge to the same sequence.

**DISCUSSION**

AFCVd is known to infect apple, hop and persimmon as natural hosts, and in these studies, we have identified three additional symptomatic experimental hosts, tomato, cucumber and wild hop. Furthermore, bioassays on four tomato cultivars were used to analyse AFCVd pathogenicity. When these cultivars were inoculated with a natural AFCVd isolate from cultivated hop, they all became infected, but only Rutgers showed very mild stunting. Two
large fruit-size tomato cultivars exhibited fruit symptoms resembling those of fruit crinkle disease of apples. Although obvious symptoms were not observed on Red Olé (a middle fruit-size tomato cultivar), differences in fruit weight were statistically significant. For these three varieties, AFCVd infection could affect tomato production, altering fruit shape, size and/or weight. It is important to note, however, that Momotaro, another large fruit-size cultivar, did not show statistically significant difference in fruit weight. These differences in symptom expression are likely caused by differences in the genetic makeup of the various tomato cultivars, as has previously been shown for certain potato spindle tuber viroid isolates and tomato cultivars [14]. An appropriate herbaceous assay host(s) to analyse AFCVd pathogenicity is now available, and it will become possible to work on comparative studies of biological properties of AFCVd with AGVd and some other members in the genus Apscaviroid to clarify their taxonomic relationship.

After infection in tomato and cucumber, major variants detected in the progeny of a natural AFCVd-hopAK isolate showed a similar number of nucleotide sequence differences (10 and 8, respectively) from the predominant variant in the inoculum. As natural AFCVd-hop isolates have been shown to contain a mixture of variants [12, 15], this result suggested that the variants adapted to tomato or cucumber were quickly selected from those already present in the inoculum and became predominant soon after infecting in tomato. Actually, the fact that one of the predominant variants in the infected tomato (hop-AK6-T2) differed from the predominant variant in the inoculum. (10 and 8, respectively) from the predominant variant in the inoculum. (10 and 8, respectively) from the predominant variant in the inoculum.
selection of specific variant(s) were also observed in HSVd in Etrog citron and cucumber [16], HSVd in grapevine and hop [17], citrus exocortis viroid in natural and indicator citrus hosts [18], citrus dwarfing viroid in different citrus host species [19], and peach latent mosaic viroid in GF-305 indicator peach cultivar [20, 21]. In line with these results, the data presented here on AFCVd in hop and tomato also supported the notion that composition and structure of viroid population are determined by the host in which they replicate.

To further examine this point, three dimeric cDNA clones were constructed based on a major variant of a natural AFCVd-hopAK isolate (i.e. hop-AK6-2), a randomly selected variant from tomato derived from a natural isolate AFCVd-hopAK (i.e. hop-AK6-T2) and a natural isolate from persimmon (i.e. persimmon-HS1). Their in vitro dimeric RNA transcripts were then individually inoculated to tomato. Characterization of the resulting progeny revealed that persimmon-HS1 was the most stable, gaining only 1 nt substitution at position 92. Hop-AK6-2 and hop-AK6-T2 gained two additional mutations at positions 178 and 308, and 82 and 305, respectively. In contrast, inoculation of tomato with AFCVd-hopAK, a naturally occurring isolate of AFCVd from hop, yielded progeny that differed at 10 positions compared to the major variant in the inoculum. The relative stability of the progeny derived from cloned cDNAs is consistent with our hypothesis that the major variant recovered from tomato inoculated with the naturally occurring isolate AFCVd-hopAK emerged by selection of a minor variant pre-existing in the inoculum followed by incorporation of one or more host-dependent de novo mutations.

To identify possible common features shared by the different combinations of sequence changes, we used mfold [22] to compare their predicted effects on the secondary structure of AFCVd. The results of these calculations revealed that the rod-like structures of all starting variants and their respective progenies, except for hop-AK6-2, contain 36 loops. The A308U mutation in the progeny of hop-AK6-2 changes loop 15 from an AU-bulge loop into C-bulge loop (Fig. 5a, boxed). Similarly, the mutation 305–U in the progeny of hop-AK2-T2 is predicted to change the same loop from a UU-bulge loop into C-bulge loop (Fig. 5b, boxed). In contrast, the C/U substitution at position 92 in the progeny of persimmon-HS1 has no effect on the predicted structure (Fig. 5c). Loop 15 (a C-bulge loop) was already present in the parental persimmon-HS1, and the same C-bulge loop structure can be seen in the progenies of all three variants.

Fig. 6. Comparison of nucleotide sequences and secondary structures of (a) AFCVd-AK6-2-T and (b) AFCVd-persimmon-HS1-T, the major variants derived from cloned dimeric RNA transcripts of AFCVd-hop and AFCVd-persimmon after infection in tomato. Differences from AK6-2-T were plotted on the secondary structure of persimmon-HS1-T. Stem–loop structures in the boxed area were conserved in the two variants. Dashed box indicates the central conserved region present in all members of the genus Apscaviroid.
after replication in tomato despite the fact that their nucleotide compositions are not the same (Fig. 5a–c). This suggests that loop 15 (i.e. stem–loop structure containing C-bulge loop; boxed structure in Fig. 5) is important for AFCVd replication/accumulation in tomato; furthermore, one possible reason for the appearance of the de novo mutations observed in the progeny arising from in vitro RNA transcripts of hop-AK6-2 and hop-AK6-T2 may reflect constraints on the tertiary structure of loop 15.

Comparison of the predicted secondary structures for the progeny of hop-AK6-2 (i.e. hop-AK6-2-T, accession LC199970) and persimmon-HS1 (i.e. persimmon-HS1-T, accession LC199972) after infection in tomato was extremely interesting. Although the progeny differed at a total of 25 positions, prediction of their secondary structures by mfold suggested that nine of these mutations cause changes in six of the eight loops on the extreme right side of the molecule (Fig. 6). Two of the eight loops on the left side of the molecule (i.e. loops 6 and 8) also seem to tolerate changes. In contrast, none of the 11 mutations in the central part of the molecule seems to cause structural changes, i.e. the predicted secondary structures of the two variants were highly conserved between the two variants in this region (Fig. 6, boxed region). These results suggest that, as discussed by Elena et al. [23], viroid evolution is constrained by the need to maintain the highly base-paired secondary structure (including certain loop structures) in the central part of the molecule.

Sequence analysis of full-length AFCVd cDNA clones indicated that all progeny preparations from tomato contained just a single major variant. These variants must be the predominant ones in their respective populations, but it is difficult to decide how many cDNA clones are required to identify all biologically significant mutations in the AFCVd genome. Fortunately, because of the recent progress in next-generation sequencing, a large amount of nucleotide sequence information is now available from a variety of virus- and viroid-infected plants. Access to this wider and deeper range of sequence-based information provides a powerful new way to study the mutations of virus and viroid populations in the infected plant. We have used AFCVd-sRNA data obtained by deep sequencing to analyse the patterns of mutations arising in AFCVd progenies replicating in tomato.

The deep sequence data obtained enabled us to identify heterogeneous nucleotide positions in AFCVd genome from an unprecedented number of data points, i.e. 274 to 32,355 nucleotides at each sequence position. As expected, all the mutations identified by sequencing cloned cDNAs were also detected by this approach. In addition, several other mutations that were not detected by cDNA sequencing were also identified. Mutations such as the one at position 230, which occur at low frequencies as low as 6.6% and had previously escaped detection, can now be detected and quantified. Consequently, mutational analysis based on viroid-specific sRNA data obtained by deep sequencing analysis, in combination with cDNA clone sequencing, provided a powerful tool to detect substantially all the mutation positions in viroid genome precisely and quantitatively. Meanwhile, it is necessary to consider the limitations of this approach. First, this approach does not allow us to establish which of these mutations are simultaneously admitted in the same viroid molecule. Second, deep sequencing data are always associated with certain sequencing errors produced in the process used perform sequencing and/or analyse huge data sets by bioinformatiics tools such as adaptor trimming, which should be taken into consideration especially for those nucleotide changes identified at a very low frequency. These limitations can be overcome by the integration of deep sequencing results with analyses by cDNA clone sequencing and bioassay using an appropriate host plant. Fine mapping data of mutations in viroid genomes will contribute to clearer understanding of AFCVd pathogenicity to the host plants such as tomato, apple, hop or persimmon based on various molecular structures. This approach can also be applicable to the other viroid–host interactions.

**METHODS**

**AFCVd infectivity assay – viroid extraction and molecular hybridization**

A hop isolate of AFCVd (AFCVd-hopAK) was maintained in the infected hop plants (H. lupulus, cv. Kirin II) [12]. Low molecular weight RNA was extracted from infected plants, dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 % bentonite at a concentration of 500 ng µl⁻¹ and used for infectivity assays. Five seedlings each of four tomato cultivars at the four-leaf stage, one cucumber cultivar at the cotyledon stage and vegetatively propagated plants of a wild hop clone (H. lupulus var. cordifolius, Souma 1) were dusted with carborundum (600 mesh) and rub inoculated with 10 µl inoculum per plant [12]. Plants were kept in a growth chamber controlled at 25 °C (16 h day-length) for 2 months.

One and 2 mpi nucleic acids soluble in 2 M LiCl were extracted using the 2× CTAB method [24] and examined for the presence of AFCVd by Northern blot hybridization using DIG-labelled AFCVd cRNA probe [12]. Infected tomato plants were transplanted to a plot in an open field and further examined for growth and fruit productivity from early July to the end of September. Fruits were harvested after they ripened in August to September and weighed to analyse the effect of AFCVd infection on fruit development. Difference in the average weight of fruit from early July to the end of September. Fruits were harvested after they ripened in August to September and weighed to analyse the effect of AFCVd infection on fruit development. Difference in the average weight of fruit from early July to the end of September. Fruits were harvested after they ripened in August to September and weighed to analyse the effect of AFCVd infection on fruit development.

**Reverse transcriptase-PCR**

Nucleic acids soluble in 2 M LiCl extracted from tomato (cv. Rutgers), cucumber (cv. Suyo) and wild hop at 2 mpi of
AFCVd-hopAK were further used for reverse transcriptase (RT)-PCR amplification of full-length cDNA copies of AFCVd progeny. Reverse transcription was performed using MuLV Reverse Transcriptase (Invitrogen) and AFC-SalI-R (5′-AACGATCGAGCTACGAGCAG-3′) as primer. PCR was performed with LA-Taq polymerase (Takara Bio) using AFC-Sall-F (5′-AAGTCGACGAAGGG TCCTCGAC-3′) and AFC-Sall-R as primers. RT-PCR products were fractionated in 7.5 % polyacrylamide gels containing 1× Tris/acetate/EDTA buffer. Full-length AFCVd cDNAs with size ca. 370 nt were recovered from the gel and ligated into pGEM-T vector (Promega) for cloning and sequence analysis [26].

**Construction of dimeric cDNA clones and biotic inoculation**

Because all AFCVd variants contain a unique SalI restriction enzyme site, dimeric cDNA clones with SalI termini were constructed from the major variant AFCVd-hopAK-62 (accession AB104532) in a hop plant infected with AFCVd-hopAK, the randomly selected variant AFCVd-hop-T2 (accession AB795265) in tomato plants infected with AFCVd-hopAK and the variant AFCVd-persimmon-HS1 (accession LC169594) in a persimmon fruit infected with natural AFCVd-persimmon isolate. The resulting constructs were inserted into SalI site of pBluescript II SK(−) (Stratagene) according to the protocol by Adkar-Purushothama et al. [26] under the control of T7 RNA polymerase promoter in order to produce (+)-sense dimeric transcript in vitro. For biotic inoculation, dimeric (+)-sense RNA transcripts were immobilized on gold microcarriers (1 µm) using a calcium-mediated precipitation protocol [27]. The Helios Gene Gun System from Bio-Rad was used for biotic inoculation at a pressure of 100–150 p.s.i. at a distance of approximately 5 cm from the Gene Gun spacer. Tomato (cv. Rutgers) seedlings at the two or three true-leaf stages were inoculated from the Gene Gun spacer. Tomato (cv. Rutgers) infected with AFCVd-hopAK at 3 mpi.

**Isolation and deep sequencing analysis of sRNA populations**

Samples of newly expanded leaf and premature fruit tissue (−1 g) were separately collected from tomato plants (cv. Rutgers) infected with AFCVd-hopAK at 3 mpi. Equivalent samples were also collected from healthy tomato plants as a control. Samples were frozen in liquid nitrogen and ground into a fine powder using sterilized mortars and pestles. RNA was extracted using TRI REAGENT (Molecular Research Center) according to the manufacturer’s instructions, quantified photometrically and sent to Hokkaido System Science Co. for quality check using an Agilent 2100 Bioanalyzer (Agilent Technologies). All four samples (−50 ng) were processed simultaneously to construct sRNA libraries using TruSeq Small RNA Sample Prep kit (Illumina) for analysis on an Illumina HiSeq using an index-sequencing strategy. Indexes were CGATGT for healthy leaf, TGACCA for healthy fruit, ACAGTG for AFCVd-infected leaf and GCCAT for AFCVd-infected fruit. Adapter sequences were removed from the ends of the resulting raw short-read data based on the presence of an exact 10 nt match with the termini of the respective adapters, and identical short reads were grouped according to read size (15–37 nt). In this way, adapter-trimmed short read data were converted to both a non-redundant and a redundant (allowing one mismatch per short-read sequence) format. These non-redundant and redundant data were then mapped to either the (+)-sense or (−)-sense strand of AFCVd using hssmap, a specially written C language program that processes data from circular molecules by adding an appropriate extension to the 3′-terminus of a linear reference sequence.

**Funding information**

This work was supported in part by the Japan Society for the Promotion of Science (JSPS) KAKENHI grant nos 24380026 and 15H04455 and by JSPS under the Bilateral Joint Research Projects between Japan-Czech (MSMT Kontakt II LH14255) Research Cooperative Program (April 2014 to March 2016).

**Acknowledgements**

We thank Dr Robert A. Owens (USDA/ARS, MPPL, USA) for critical reading and valuable suggestions on the manuscript.

**Conflicts of interest**

The funders of this work had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**References**


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