Single-Strand Conformation Polymorphism Analysis of Apple Stem Grooving Capillovirus Sequence Variants

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ABSTRACT

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In an earlier study, we demonstrated that isolates of apple stem grooving capillovirus (ASGV) from fruit trees comprise at least two to four sequence variants that differ considerably from each other in nucleotide sequence. In order to characterize the population of sequence variants within a single tree, we applied a combination of an immunocapture reverse transcription-polymerase chain reaction (IC-RT-PCR) and a single-strand conformation polymorphism (SSCP) analysis of a nested

asymmetric PCR product. In the SSCP analysis of the PCR products from ASGV-infected apple, Japanese pear, or European pear trees, two to four bands were detected in samples from all trees, indicating that ASGV exists as a mixture of sequence variants. The composition of sequence variants (the number of bands and their relative quantity) differed among leaf samples from different branches, showing that each sequence variant is distributed unevenly within an individual tree. The SSCP analysis of isolates after serial passage in *Chenopodium quinoa* plants indicated that passages changed the composition of sequence variants originally contained in ASGV isolates; i.e., some sequence variants dominated and others decreased to undetectable levels.

Apple stem grooving capillovirus (ASGV) is the type species of the genus *Capillovirus* and is distributed worldwide in apple (8), European pear (12), Japanese pear (21), Japanese apricot (15), and cherry (4). Recently, capilloviruses from citrus and lilies that had been classified as citrus tatter leaf virus (3,13) were found to be isolates of ASGV (7,21).

ASGV has very flexuous filamentous particles, 600 to 700 nm in length (6), and contains a polyadenylated, positive-sense RNA of 6,496 nt, excluding the 3' poly(A) tail, and a single 27-kDa coat protein (CP) (20,22). So far, the nucleotide sequences of the genomes of three ASGV isolates, P-209 from apple and Li-23 and L from lilies, have been completely determined (9,16,20). The genome of ASGV has two overlapping open reading frames (ORF1 and ORF2). ORF1 encodes a 241- to 242-kDa polypeptide (2,105 amino acids [aa]) containing consensus motifs of methyltransferase, papainlike protease, nucleotide triphosphate-binding helicase, RNA polymerase, and CP in the C-terminal region. ORF2 encodes a 36-kDa putative movement protein (320 aa). A region (aa positions 1585 to 1868) of the ORF1-encoded protein between the polymerase and CP that encodes ORF2 in another frame does not have any functional motifs found in plant virus genomes and shows high variabilities among isolates and sequence variants. This region has been designated the variable region (V-region) (7).

In a previous report (7), we demonstrated that the ASGV isolates from apple, European pear, and Japanese pear comprise at least two to four sequence variants that differ considerably from each other in nucleotide sequence. ASGV was isolated from infected fruit trees, inoculated in the herbaceous plants *Nicotiana occidentalis* and *Chenopodium quinoa*, and used for reverse transcription-polymerase chain reaction (RT-PCR). The PCR products were then cloned, and selected clones were sequenced. Because

they represent only a small subsample, cloned DNA molecules do not always represent the true composition of the sequence variants within a sample.

To clarify the composition and distribution of the sequence variants within a single tree, we directly amplified fragments of the ASGV genome present in infected fruit tree leaves by immunocapture (IC)-RT-PCR (17) and then analyzed them by single-strand conformation polymorphism (SSCP) analysis (10). We also investigated the effect of serial passage by mechanical inoculation in *C. quinoa* plants on the composition of sequence variants of ASGV isolates by IC-RT-PCR and SSCP analysis.

MATERIALS AND METHODS

Virus and plant materials. Young leaf samples were collected from apple (F38), Japanese pear (N4), and European pear (Bartlett and La France) trees in May 1997. In a previous study, these trees were found to be infected with ASGV isolates containing several sequence variants (7). Leaf samples were stored at -80°C until used for IC-RT-PCR.

C. quinoa leaf samples infected with ASGV isolates P-209 or Li-23 and inoculated with an infectious cDNA clone of isolate Li-23 (pCT35SF) (16) were collected at 14 days after inoculation and used for IC-RT-PCR and SSCP analysis.

Isolates AAK from apple and JP3 from Japanese pear were transmitted to *N. occidentalis* plants (7). Leaves of infected *N. occidentalis* plants were ground with 0.1 M phosphate buffer (pH 7.0) and inoculated to *C. quinoa* plants. After 2 to 3 weeks, leaves with symptoms were homogenized in the same manner and inoculated onto *C. quinoa* plants again. This serial passage in *C. quinoa* was repeated 15 times.

IC-RT-PCR and nested asymmetric PCR. Three primers were designed to amplify a part of the V-region that had a nucleotide sequence highly variable among isolates and sequence variants. These primers are CTLV-2(+) (CCACAGTATGAACAGGACAC, homologous to nucleotide positions 5283 to 5302 of the Li-23 genome); ASCT-PC(-) (TCAGAAGTTCCTTTGCCGGG, complementary to nucleotide positions 5715 to 5734 of both P-

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209 and Li-23 genomes), and SGOP2(-) (GCTGAGAGGGAC-CTAAATCC, complementary to nucleotide positions 5598 to 5617 of the Li-23 genome) (Fig. 1). Annealing sites of these primers were highly conserved among the isolates and sequence variants examined to date.

IC-RT-PCR was performed according to the procedure of Candresse et al. (1) with minor modification. Leaf tissue (0.1 g) was ground in 1 ml of 0.02 M phosphate, pH 7.4, 2.68 mM KCl, 0.137 M NaCl, and 0.2% Tween 20 (PBS-T) containing 2% polyvinylpyrrolidone, and the extract was centrifuged at $1,4000 \times g$ for 10 min. The clarified extract (100 μ l), previously diluted fivefold

with PBS-T, was placed into a 0.5-ml microtube precoated with anti-ASGV serum and incubated overnight at 4°C. After it was washed five times with PBS-T buffer, the tube was dried. We then added 10 μ l of a reaction mixture containing $1 \times$ first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, and 10 mM dithiothreitol), 10 pmol of ASCT-PC primer, 0.5 mM deoxynucleoside triphosphate (dNTP) mix, 0.3% Triton X-100, and 100 units of M-MLV reverse transcriptase RNase H minus (United States Biochemical Corp., Cleveland, OH). After incubation at 37°C for 1 h, cDNA was precipitated with ethanol, dried, and then dissolved in 10 μ l of distilled water. In the first PCR, cDNA solution (1 μ l) was added to a final volume of 20 μ l of PCR mixture containing $1 \times$ EX Taq buffer, 0.25 mM dNTP mix, a primer pair (CTLV2(+) and ASCTPC(-), 10 pmol each) (Fig. 1), and 1.25 units of EX Taq (Takara Shuzo Co., Otsu, Japan). PCR was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and polymerization at 72°C for 1 min. A nested asymmetric PCR (second PCR) (2) was conducted as follows. The first PCR product (0.2 μ l) was added to the PCR mixture as above (final volume, 20 μ l) with an asymmetric primer pair (2 pmol of CTLV2(+) and 10 pmol of SGOP2(-)), and amplification was then performed for 40 cycles under the same conditions as in the first PCR, except that the annealing temperature was 57°C. To check for a false positive reaction caused by contamination, a negative (no template) control was included in each PCR experiment.

SSCP analysis. The second asymmetric PCR product (5 μ l) was mixed with 10 μ l of loading buffer (95% formamide, 20 mM

Fig. 1. Genome map and locations of primer pairs used in this study and the expected products amplified by primer pairs in the first and second polymerase chain reactions of the apple stem grooving virus (ASGV) genome. The V-region and coat protein (CP) region in open reading frame (ORF) 1 are shaded.

P-209	5303	TGAGTTGTTTCTCTTGACATTGGAGTTGCATACAGATGCGTCAACTCTGCAAGGTTTTTGGAAACAAAATGGCGATTGAGGATGGGCTTCACAGGCA
V3-1		-----
OKITSU-WASE		C-----C-----T-----T-----C-----T-----G-----
Li-23		-----T-G-C-----T-T-T-A-TC-T-C-----G-----G-----A-C-----G-----A-C-G-----T
V3-2		A-C-----T-C-G-T-----T-A-TC-T-C-----G-----G-----A-C-----G-----C-G-----T
JP3-CO		A-C-----T-C-G-T-----G-----T-A-TC-T-C-----G-----G-----A-C-----G-----C-G-----T
EPBL-CO		C-C-----T-C-G-C-----T-A-TC-T-C-----G-----G-----A-C-----G-----C-G-----T
EPLF-CO		C-C-----T-C-G-C-----T-A-TC-T-CC-----G-----G-----A-C-----G-----C-G-----T
AF38-3		A-AC-----C-CT-G-----G-----T-T-----T-----T-A-----CC-T-----G-----T-----G-----C-----G
AAK-CO		A-AC-----C-CT-G-----G-----T-T-----T-----T-A-----CC-T-----G-----T-----G-----C-----G
JP4-CO		A-C-----CT-G-----G-----T-T-----T-----T-A-----CC-T-----G-----T-----G-----C-----G
BUC-neg.No.3		A-AC-----C-CT-G-----G-----T-T-----T-----T-G-----A-C-T-----G-----T-----G-----C-----G
JP4-3-1		A-C-A-----C-----C-----G-----T-----A-T-A-C-----G-----A-T-----G-----C-----G
AAK-3		A-C-----G-----T-----A-T-A-----G-----A-T-----G-----C-----G
P-209	5403	ATCAGCGGCTGTGAAGCACTTAAATTCAATGAGGAATCAAGATGGCATCCTGGATCGCAGATCCCCGCTGTTCTCTGGAAGAAGGTGACCAAAACGTGC
V3-1		-----A-----
OKITSU-WASE		-----G-----A-----T-----T-----G-----
Li-23		--T-T-----G-----C-G-T-----A-----T-A-----A-TT-----AT-AG-T-A-A-----G-----TT-----T-T
V3-2		--T-----C-G-----C-G-----A-----A-----A-T-----A-AG-T-A-----G-----T-----T-A
JP3-CO		--T-----C-G-----C-G-----A-----A-----A-T-----A-AG-----A-----G-----T-----T-A
EPBL-CO		--T-----A-----G-----C-----A-G-----G-----T-----AT-AG-T-A-----G-----T-----T-A
EPLF-CO		--T-----G-----C-----A-G-----G-----T-----AT-AG-T-A-----G-----T-----T-A
AF38-3		-----T-A-----G-----T-----A-----A-----T-----A-----G-----G-----T-----T-A
AAK-CO		-----T-A-----G-----T-----A-----A-----T-----A-----A-----T-----G-----T-----T
JP4-CO		-----T-A-----G-----C-----T-----A-----T-----T-----A-----A-----T-----G-----T-----T
BUC-neg.No.3		-----T-A-----G-----C-G-T-----A-----T-----T-----A-----A-----T-----G-----T-----T
JP4-3-1		-----T-A-----G-----A-----A-G-----A-----A-----A-----T-----G-----T-----T
AAK-3		-----A-----A-G-----A-----A-----A-----A-----T-----G-----T-----T
P-209	5503	ACATTGAAAAGAGATTGTTTCAGAGGTGACAAGGTTAGAGGTCACGCTCAATTTCCGCTAAAAGGGGGCCAAACTCAAGGGTGCAAGAAAAGAGA 5597
V3-1		-----G-A-----A-----C-----AA-----G-T-T-----T-----T-----G-AATC-----G-----
OKITSU-WASE		-----G-C-----A-----A-----T-----T-----A-----G-AACC-----G-----
Li-23		-----G-C-----A-----A-----T-----T-----A-----G-AACC-----G-----A-----
V3-2		-----G-C-----A-----A-----T-----T-----A-----G-AACC-----G-----A-----
JP3-CO		-----G-C-----A-----A-----T-----T-----A-----G-AACC-----G-----A-----
EPBL-CO		-TG-----G-C-----A-----T-----A-----G-AACC-----G-----
EPLF-CO		-TG-----G-C-----A-----T-----A-----G-AACC-----G-----
AF38-3		-----G-A-C-----T-----AA-C-GC-A-----T-----A-A-G-----GGA-CCA-G-----G-----
AAK-CO		-----G-A-C-----T-----AA-GC-A-----T-----A-A-G-----GGA-CCA-G-----G-----
JP4-CO		-----G-A-C-----T-----A-C-GC-A-----T-----A-A-G-----GGA-CCA-G-----G-----
BUC-neg.No.3		-----A-----A-C-----T-----A-C-GC-A-----C-----A-A-A-G-----A-CCA-G-----G-----
JP4-3-1		-T-----A-----A-----G-----A-C-G-----G-----T-----C-T-C-----TCAG-----C-----
AAK-3		-T-----A-----A-----GA-----A-C-----A-G-----T-----T-C-T-C-----TCAA-----C-----

Fig. 2. Nucleotide sequence alignment of the regions corresponding to the products amplified by the second nested asymmetric polymerase chain reaction of apple stem grooving virus (ASGV) sequence variants and isolates described previously (7). Isolates are from apple (P-209, AF38-3, AAK-CO, and AAK-3), Japanese pear (V3-1, V3-2, JP3-CO, JP4-CO, and JP4-3-1), European pear (EPBL-CO and EPLF-CO), *Citrus* spp. (OKITSU-WASE and BUC-neg. No. 3), and lily (Li-23). Nucleotides that differ from those of ASGV isolate P-209 (20) are shown below the P-209 sequence.

EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) and electrophoresed in a 12% polyacrylamide plus bis-acrylamide (49:1) gel with 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA). Electrophoresis was carried out at 17°C at 250 V for 13 h, and then the gel was stained with ethidium bromide and detected as described by Yap and McGee (19).

Sensitivity of SSCP analysis. Two plasmids containing cDNA amplified by a primer pair (CTLV2(+)) and ASCTPC(−) from isolates P-209 and Li-23 were mixed at various ratios (10 ng:10 ng to 10 ng:0.05 ng per µl). The mixtures (1 µl) were then used as templates for asymmetric PCR and SSCP analyses as described above.

RESULTS

Optimum conditions for distinguishing ASGV sequence variants by SSCP. In order to determine the optimum conditions for SSCP analysis, electrophoresis conditions (acrylamide gel concentration and running temperature) were tested by using asymmetric PCR products from 14 cDNA clones of isolates or sequence variants with determined nucleotide sequences (Fig. 2). Among these clones, there were one to 64 nucleotide differences within the amplified region (295 nt), excluding primer sequences. When acrylamide gel concentration and running temperature were 12% and 17°C, respectively, most clones were distinguishable from each other as single bands with different electrophoretic mobilities, except for the clones P-209, V3-1, and AAK-3, which had similar mobilities (Fig. 3). The nucleotide sequences of P-209 and V3-1 differed at only one position, whereas those of P-209 and AAK-3 differed at 39 bases in the amplified region. On the

Fig. 3. Single-strand conformation polymorphism analysis of the nested asymmetric polymerase chain reaction products from 14 cDNA clones of isolates and sequence variants shown in Figure 2.

Fig. 4. Single-strand conformation polymorphism analysis of the products from nested asymmetric polymerase chain reaction (PCR) from apple stem grooving virus-infected *Chenopodium quinoa*. Lane 1, a mixture of the PCR products from P-209 and Li-23 cDNA clones; lane 2, P-209-infected *C. quinoa*; lane 3, Li-23-infected *C. quinoa*; lane 4, an infectious cDNA clone of Li-23 (pCT35SF)-inoculated *C. quinoa*.

other hand, SSCP analysis of EPBL-CO and EPLF-CO, which differ by only two bases, showed clear differences in band mobility. Nucleotide sequences of isolates P-209 and Li-23 differed at 56 base positions in the region, and these were well separated in the SSCP analysis. Therefore, P-209 and Li-23 were used for markers in the following experiments.

After two DNA clones (P-209 and Li-23) were mixed, SSCP analysis with dilutions up to 0.5 ng:10 ng showed that minor subpopulations (in either of the clones) were readily detectable (data not shown), consistent with the report by Enomoto et al. (2). The results indicated that the SSCP analysis used in this study can be used to detect minor sequence variants that represent as little as 5% of the genomic population.

Detection of ASGV sequence variants in vivo by SSCP analysis. At first, IC-RT-PCR and SSCP analysis were conducted with *C. quinoa* leaves infected with P-209 and Li-23. As shown in Figure 4, a single band was detected in P-209- or Li-23-infected leaves, and the mobility of each band coincided with that of a band from each cDNA clone. In pCT35SF-inoculated leaves, a band with the same mobility as those from the Li-23 cDNA clone and the Li-23-infected *C. quinoa* leaves was also detected.

Single leaf samples (a, b, c, and d) were collected from each of four different branches from each tree (apple, F38; Japanese pear, N4; and European pear, Bartlett and La France) and subjected to IC-RT-PCR and SSCP analysis. In the F38 tree, four distinct bands (i, ii, iii, and iv) were readily detected (Fig. 5). Three bands (ii, iii, and iv) were detected in sample a, two bands (i and iii) in

Fig. 5. Single-strand conformation polymorphism analysis of the products from nested asymmetric polymerase chain reaction (PCR) of leaf samples from apple stem grooving virus-infected apple (F38), Japanese pear (N4), and European pear (Bartlett and La France). Lane M contains a mixture of PCR products from P-209 and Li-23 cDNA clones; lanes a, b, c, and d contain products of single-leaf samples from four different branches within individual trees. The differentiated bands are indicated by Roman numerals.

sample b, two bands (ii and iii) in sample c, and three bands (i, ii, and iii) in sample d. Only band iii was found in all four leaves examined. In the N4 tree, two bands (i and ii) were detected in sample a, whereas only band ii was detected in samples b, c, and d (Fig. 5). In the Bartlett tree, two bands (i and ii) were detected in samples b and d. In samples a and c, only band ii was detected (Fig. 5). Three bands (i, ii, and iii) were detected in samples from all branches in the La France tree (Fig. 5). However, the intensity of the bands differed among branches. For example, band i was strongest in all samples, whereas the intensity of band iii in sample b was low compared with the intensity in the other three samples.

Effect of serial passage of ASGV isolates in *C. quinoa* on the composition of sequence variants. In general, ASGV isolates from original fruit trees have been maintained and propagated in suitable herbaceous plants (*C. quinoa*) by serial mechanical inoculation. We investigated by SSCP analysis whether the composition of sequence variants in an isolate changes after passage in *C. quinoa* plants. In isolate AAK, four distinct bands (i, ii, iii, iv) were detected in infected *N. occidentalis* (designated passage 0) (Fig. 6). Band i had the highest relative intensity of the four bands. In samples after serial passage in *C. quinoa*, the intensities of bands i and ii were almost constant after each passage. However, band iii decreased in the first passage sample and could not be detected after the fifth passage. In contrast to band iii, band iv increased in the first passage and was detected with the same intensity as band i after the fifth, tenth, and fifteenth passages. No change in band composition was observed after the fifth passage. In isolate JP3, four bands (i, ii, iii, and iv) of almost equal intensities were detected in infected *N. occidentalis* (Fig. 6). After the first passage in *C. quinoa*, bands ii and iv could not be detected. Band i disappeared in the fifth passage. Only band iii could be detected in samples after the fifth, tenth, and fifteenth passages.

DISCUSSION

In this study, we have shown that the composition of the population of sequence variants within an ASGV-infected sample can be analyzed by a combination of IC-RT-PCR and SSCP analysis. SSCP analyses of several plant viruses, e.g., beet necrotic yellow vein furovirus (5), rice ragged stunt oryzavirus (14), and citrus

tristeza closterovirus (11), have been reported previously. In those studies, SSCP analysis was used for genotyping the variants or strains. We believe the present report is the first on the use of SSCP analysis to examine plant virus sequence variation within an infected plant.

We amplified a part of the V-region of the ASGV genome for SSCP analysis because the high variability of the nucleotide sequence of this region among isolates and sequence variants was expected to differentiate each sequence variant. The SSCP analysis, in which the PCR products from cDNA clones with known sequences were used, showed that 11 of 14 isolates or sequence variants have distinct band mobilities. There are reports that band mobilities in the SSCP analysis are difficult to predict from the nucleotide changes (5,10). In our results, P-209 and AAK-3 showed almost the same mobilities in the SSCP analysis in spite of the presence of 35 nucleotide changes between the two isolates. This suggests that a single band may be composed of DNA molecules with different sequences, although we think this is a rare phenomenon.

In the SSCP analysis of ASGV in leaf samples from apple (F38), Japanese pear (N4), and European pear (Bartlett and La France), at least two to four sequence variants were found to be present within each tree. These results are consistent with those of our previous study in which sequencing analysis was used to study the PCR products of several capillovirus isolates (7). The composition of sequence variants in an individual tree differed among samples (Fig. 5), i.e., among samples a, b, and c and d in F38, between a and b, c, and d in N4, and between a and c and b and d in Bartlett, although there are common sequence variants in all four samples in each tree. These results indicate that the ASGV in a single infected plant is a mixture of sequence variants and that sequence variants are not necessarily distributed evenly among branches or leaves within a tree. Since ASGV is spread only by graft transmission, sequence variation within a single tree may occur by means of successive accumulation of nucleotide substitutions into the preexisting genome and/or by mixed infection of each sequence variant originally contained in a rootstock or scion by graft transmission (7).

On the contrary, the SSCP analyses of P-209- and Li-23-infected *C. quinoa* leaves showed a single band in each isolate, indicating that the genomes of these isolates appeared to be homogenous. P-209 and Li-23 used in this study were originally isolated from fields about 20 years ago (3,18) and since then have been maintained in *C. quinoa* plants. We previously speculated that serial passages of isolates in indicator plants may have resulted in the predominance of a specific sequence variant resulting from selective multiplication (7). This speculation is supported by the fact that the composition of sequence variants in isolates AAK and JP3 has changed after serial passages in *C. quinoa* plants. After five passages, only one band (sequence variant) was detected in isolate JP3, which originally consisted of four sequence variants. The disappearance of bands in the SSCP analysis, however, does not necessarily mean the absence of sequence variants. It would be very interesting to test whether the generation of sequence variants depends on host species, because sequence variants occur frequently in fruit trees but not in *C. quinoa*. This could be tested by the IC-RT-PCR and SSCP analyses of fruit tree seedlings inoculated with an infectious pCT35SF clone.

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Fig. 6. Single-strand conformation polymorphism (SSCP) analysis of apple stem grooving virus (ASGV) isolates AAK and JP3 after serial passages in *Chenopodium quinoa* plants. Lane M contains a mixture of the polymerase chain reaction products from P-209 (upper band) and Li-23 (lower band) cDNA clones. Lane 0 contains ASGV-infected *Nicotiana occidentalis* directly inoculated with sap of fruit tree leaves. Lanes 1, 5, 10, and 15 contain infected *C. quinoa* samples after 1, 5, 10, and 15 serial passages, respectively. The differentiated SSCP bands are indicated by Roman numerals.

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