

Molecular Characterization of *Apple stem grooving virus* Isolated from *Talaromyces flavus*

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Talaromyces flavus mediates the transmission of *Apple stem grooving virus* (ASGV) to several host plants. The ASGV-F carried by *T. flavus* was partially purified from the fungus. Based on sequence analysis and homology searches, this is closely related to other ASGV strains isolated from host plants. The partially purified viral coat protein (CP) was separated on a 12% SDS-polyacrylamide gel and analyzed by Western blotting with an ASGV anti-serum. A single band at 28 kDa reacted with the ASGV anti-serum. The deduced amino acid sequence of the ORF-1 showed conserved domains, including an NTP-binding helicase motif, GFAGSGKT. The amino acid sequences of the helicase and CP showed strong homology to other ASGV strains (98%). All ASGV isolated from plants and fungi had salt bridges composed of the CP and the GFAGSGKT motif of the helicase, which are commonly conserved in plant viruses. These results suggest that ASGV-F is one of ASGV strains isolated from *T. flavus* based on sequence similarity as well as the serological analysis of CP.

Keywords : *Apple stem grooving virus*, coat protein, helicase, *Talaromyces flavus*

In the previous studies, we reported that *Talaromyces flavus* served as a vector of *Apple stem grooving virus* (ASGV), and transmitted virus was also infectious to the host plants. ASGV usually latently infects Rosaceae fruit trees such as apple, pear, apricot and cherry (Yoshikawa et al., 1996). ASGV caused apple top working disease in Japan and induced decline syndrome in Mitsuba Kaido (*Malus sieboldii*), a rootstock of apple trees (Lister, 1970). ASGV

is transmitted by grafting and mechanical inoculation into susceptible hosts such as *Phaseolus vulgaris*, *Chenopodium quinoa* and *Nicotiana glutinosa* (Lister, 1970). ASGV has a monopartite genome consisting of a 6.5 kb, single stranded, plus-sense RNA that is 5' capped and 3' polyadenylated. ASGV RNA contains two overlapping open reading frames: ORF1 (6.3 kb) and ORF2 (1.0 kb), which encode proteins of 241 and 36 kDa in molecular mass, respectively (Yoshikawa et al., 1992; Ohira et al., 1995). The larger ORF1 encodes an apparently chimeric polyprotein containing at least two conserved regions: the replicase (Rep) region contains several domains (methyltransferase, papain-like protease, NTP-binding helicase and RNA-dependent RNA polymerase domains), which was characteristics of viral Rep. The coat protein (CP) region is located at the C-terminal end and contains typical motifs of CP (Yoshikawa et al., 1992; Ohira et al., 1995). ORF2 encodes a protein with conserved motifs for both movement proteins (MP) and viral proteases (Yoshikawa et al., 1992; Ohira et al., 1995). The expression strategies of these ORFs are not yet well understood.

Most mycoviruses have dsRNA genomes, either encapsidated in particles (Buck, 1986), present as unencapsidated forms in the cytoplasm (Ahn and Lee, 2001), or found in the mitochondrial fraction (Hong et al., 1998; Lakshman et al., 1998). Rarely, single-stranded RNA (ssRNA) genomes have been reported in mycoviruses: only four ssRNA mycoviruses have been identified to date. The *Mushroom bacilliform virus* (MBV) was found in *Agaricus bisporus* (Tavantzis et al., 1980; Revill et al., 1994), and the *Scelerophthora macrospora Virus B* (SmVB) was isolated from *S. macrospora*, the pathogenic fungus responsible for downy mildew in gramineous plants (Yokoi et al., 1999). *Botrytis virus F* (BVF) infects *Botrytis cinerea*, an impor-

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tant pathogen affecting a large number of economically important vegetables, flowers, and fruit crops (Robyn et al., 2001). *Oyster mushroom spherical virus* (OMSV) was isolated from a cultivated oyster mushroom, *Pleurotus ostreatus*. This virus was associated with the recent epidemic of oyster mushroom die-back disease in Korea (Yu et al., 2003).

Here, an ASGV-F was purified from *T. flavus*. We confirmed that this strain possesses the highly conserved helicase, RNA-dependent RNA polymerase (RdRp), and coat protein (CP) domains found in other strains of ASGV. Phylogenetic analysis showed that this ASGV-F was indeed a strain of ASGV. This strain also showed relatively high sequence homology to the filamentous form of BVF when the coat protein sequences were aligned with those of the ssRNA mycoviruses.

The strains of *T. flavus* used in this study were 2-3 IO-A as an ASGV harboring fungus strain and 16-6 VF as a virus-free *T. flavus*. Both ASGV-F harboring *T. flavus* and virus-free strains were maintained on a solid medium [3.9% w/v potato dextrose agar (PDA), Difco, USA]. Preparative cultures for RNA analysis were grown for 20 days at room temperature under a 16 h light/8 h dark cycle, with cellophane on the agar surface to permit mycelial harvesting. The virus was purified based on the method of Kim et al. (2003) with some modifications. For purification of the virus, the mycelia grown in 250 mL of potato dextrose broth (PDB) at room temperature for 20 days were collected on 0.45 µm filter paper (Whatman) via vacuum filtration, followed by two washes with distilled water. Using a mortar and pestle containing liquid nitrogen, 100 g of the fungal mycelia were finely ground, and was transferred to a centrifuge tube, and added with 0.1 M sodium phosphate buffer (pH 7.0). Cell debris was removed by centrifugation at 12,000 rpm for 30 min in a Sorvall GSA rotor. The virus was precipitated with 6% polyethylene glycol (MW, 8000) and 0.15 M sodium chloride on ice for 12 h, and then centrifuged at 10,000 g for 20 min. The pellet was resuspended in the same buffer. Following another low-speed centrifugation, the supernatant was centrifuged in a Beckman 70 Ti rotor at 30,000 rpm for 3 h. The pellet was resuspended in the same buffer, and then subjected to 20% sucrose density gradient centrifugation using a SW-27 rotor at 24,000 rpm for 3 h. The virus band was collected and concentrated by centrifugation at 30,000 rpm for 3 h in a Beckman 70 Ti rotor. The virus preparation was stored at -70°C until use.

For viral coat protein analysis, the purified virus was mixed with an equal volume of 1x sample buffer (50 mM Tris-Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and heated for 5 min at 100°C. Further analysis followed previously published

methods (Shim et al., 2004). In order to characterize the viral cDNA, RNA was extracted from cultures of virus-infected *T. flavus* strain 2-3 IO-A grown in PDB. Primer pairs for the amplification of ASGV cDNA were designed based on the nucleotide sequence of the reported ASGV strain (D14995). The following primer pairs were used for the amplification of helicase (5'-AGC GGC ATA CAA TGG GCT AA-3' & 5'-TGG CAC TGG CAA CCT CAA A-3'), RdRp (5'-ACT TTT TCT GCC TGG ATA GCT-3' & 5'-CTG TCT TTC ACT CAT CAT CTT G-3'), and CP (5'-CAT ATG AGT TTG GAA GAC GTG CTT C-3', and 5'-CTC GAG ACC CTC CAG TTC CAA GTT A-3'). The single PCR products from each amplification were subcloned into the pGEM-T Easy vector (Promega), according to the manufacturer's instructions. cDNA clones were sequenced in both forward and reverse directions. Sequence alignment was performed using the CLUSTAL W program (version 1.8) (Thompson et al., 1994). Helicase, RdRp, and CP regions were aligned independently of the entire replicase alignment. Unrooted neighbor-joining distance trees were constructed using the NEIGHBOR program. Sequence identity and similarity analyses were carried out using BLAST 2 and CLUSTAL W. The 'tymo-like' virus group, composed of isometric particles, included genera *Tymovirus* and *Marafivirus*. The 'potex-like' virus group, composed of flexuous rod-shaped particles, included genera *Capillovirus*, *Foveavirus*, and *Trichovirus*. The ssRNA genome mycovirus group includes Mushroom bacilliform virus, *Botrytis virus F*, and *Scelerophthora macrospora virus B*.

In previous studies, we found that *Talaromyces flavus* could transmit ASGV to uninfected host plants. This study represents the first molecular characterization of the ASGV-F harbored by *T. flavus*. The virion, which was present at very low titers in the fungus, was isolated and partially purified. The results of the cDNA sequence analysis and homology searches indicate that the ASGV -F is closely related to other ASGV strains.

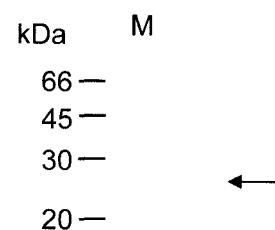


Fig. 1. Western blot of viral proteins separated by a 12% SDS-polyacrylamide gel electrophoresis. M, low range standard markers (Gibco). The viral CP recognized by the anti-ASGV serum is noted by the arrow.

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ASGV-M -----RDKRPNYSTWMTQTFKSSKQIRFLSKSPKAMQWQTCENVQVKENG--
ASGV-K MAFTYRNPLEIAINKLPSKQDQLLSLTTDEIEKLEVTNRNPFSSITPEDQLLTKHGL
      * * * * * : : : : : * * * * * : : : : :
ASGV-M -----VASAKMNSPPFIARYGLPDNGENILDSIRPMPVT
ASGV-K TLAPIGFKAHSHVPSKMIENHLLYICVPSLLSSFKSVAFFSLRESKANSFLKMHSAFSGH
      * * * * * : : : : : * * * * * : : : : :
ASGV-M NVPKRSMPYTLQISKR----HSVAYENYNSNPVASYTKLCAQ-----IVYLHH
ASGV-K KIKSMGMYNAILDGDKRYGVDVSTSPRERVIGLRDQCLTRNFKPIIFMHDELHFLSP
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M LCLDFFQD-----GGRQPKTAVLAPWTKRDLGICFAVGFLELSCV
ASGV-K FDIAYLFTIPEIDRVIATTVFPIELLFGDKVSKPRVYTYKVVHSSFSFYPDGVASECY
      : : : : : * * * * * : : : : : * * * * * : : : : :
ASGV-M LGIMR-----IRNLIVSFDGRACNDFNHFNKPGCLLSE
ASGV-K EQNLANSKWPFPTCNGIQWANRRVRVTKLQSLFAHHVFSFDRGRACNDFNHFNKPGCLLSE
      : : : : : * * * * * : : : : : * * * * * : : : : :
ASGV-M EMRLLTTRKFDKAVINRSTVSSSLTYMACLKTANAASAVAKLRQLEKRDLYPDELNFVYSF
ASGV-K EMRLLTTRKFDKAVINRSTVSSSLTYMACLKTANAASAVAKLRQLEKRDLYPDELNFVYSF
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M GEHFKNFMRDDDFDVSVLQVVKDKFCQVMHPHISASFPEPTEFHLNMRKLLNLDLATKIE
ASGV-K GEHFKNFMRDDDFDVSVLQVVKDKFCQVMHPHISASFPEPTEFHLNMRKLLNLDLATKIE
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M VPLPTIIVLDKVNFIETRFHARMEVASAR
ASGV-K VPLPTIIVLDKVNFIETRFHARMEVASAIGVNLDDLKGRPDFENESEBEYFSENGYLFMPS
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M -----FELRKITSSREFFSILIQHADIQTN-----
ASGV-K KHTNDRNWI LNSCPVRVDYSKLVRARRFLKRDVFEIILKRRPPTQLFLEFNKDFGGPV
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M ---NSTEAVPMAGTAIKHMAHRP-----
ASGV-K SFEKKEVKKGEAATRVDEIKAPHEPSSCNLSTEDGQGFEGSLPLDLTISCFPEQIEIRPKR
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M -----
ASGV-K KRKNCVFKATAAHLGIETQDLNLFVNEDI SDELLDCIEEDKGLSHEMIEEVLVTKGLS
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M -----SRLGDMSGYICVHVGRSITITPYWTVVYQTSAREPEMNQRSIQAL
ASGV-K MVTYSDPKREMAVLRKRYGVNGKMYCTIKGNHCELSSEKCFIRLLKEGGNAQMSNENLNAD
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M PLHDG---LRCPRRRLSHYPHPRGPT-----RL
ASGV-K SMFDLGFVHNKERAVLAKSMARGTGLLNSFDNFCKEVVGLELFPDNPSSVVGRLR
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M GFAGSGKTHKVL
ASGV-K GFAGSGKTHKVLQWYINYPNVKRMFISPRMLAEVEGRKGTACQVHTWETALRKIDGT
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M --EVFVDEIGLYPPRRNMRNRFALHVMVNG-ADRNGTAAALRPSGNCQPELRCFGDPLQM
ASGV-K FMEVFDVEIGLYPPGYLTLQMCAPRRTVKQSERFLKSKLAELSKTCLSLRCFGDPLQM
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M RYDRGFG-LVDAEHMVGILLR-----SLGCWY
ASGV-K RYSAEDTNLDDKTHELDLMIKTIKHYLLQGVRFGEWQSLINMPTRIDSEISIKFFA
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M -----LPIGHLKIASKPSG-----VYKEMQVPEG----
ASGV-K DMSVKSSEYGLVLRARREDIGVFAGRIPVATVSESQGMTIDKRVLTCLDQNLFAGGANA
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M -----VEGPKGVGICCLKG-----
ASGV-K AIVAITRSRVGPDFVLKGNLTKIQRMSQRTIQWFIEKKRIPMERIVNMPGASPFYESP
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M -----ICAMYFVQCDFKVLQG-----QWFOARQNLQKQQLMRA
ASGV-K LDVGNSSIQDKASHDVPFIMPFINLAEBEVDPEICGDVVKPVEWFKCHVFPVDFDPLMAE
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M ISRKSQTKG-----EAQCGVDELRES-----KIPNSGTGLANGGH
ASGV-K IFDKVAAKEKREPFQSI LGMNSQFLDMEKNGCKIDILPFARQNVFPHQASDDVTFWAGVQ
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M KWGHYCIWKIQTQKQFSLQTFNSEFIFLS-----DEPLPSQLKLFPTPQLKAC
ASGV-K KRIRKSNWREKTKPEPEIQGRELLSEFLSMLPCEFKVNIKIDIEEGEKSPLEKRLKISE
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M MLFVWVRSQGIIDIGLGRGYVTLIENSER-----RHACVSP
ASGV-K KMWANHSERSDIDWKLDFVFLPMKSOYCTKEGKMFTEAKAGQTLACFOHIVLFRFGMLR
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M PVRCFFNNQLGASRPLPYQK-----DKSAPCFRFFSIP-----
ASGV-K AIESAFLRSCGDSYIYHSGKNFFCLDSFVTKNAEVEFDGFSIESDYTAFFDSQDHVILAFE
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M MSLEDVLQLARRHRVGVYLWKTQIDPGKEILLTVPPPEPKEGESFEGRELYLLLCNHYCK
ASGV-K MSLEDVLQLARRHRVGVYLWKTQIDPGKEILLTVPPPEPKEGESFEGRELYLLLCNHYCK
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M YLFGNIAVFGSSDKTQFPAVGFDTPPVHYNLTTPREGTEEOKKAREGMSGEKTKVWRI
ASGV-K YLFGNIAVFGSSDKTQFPAVGFDTPPVHYNLTTPREGTEEOKKAREGMSGEKTKVWRI
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M DLSNVVPELKTFAATSQRQNSLNECTFRKLCPEFADLAREFLHERWSNIYKWPKAFKSP
ASGV-K DLSNVVPELKTFAATSQRQNSLNECTFRKLCPEFADLAREFLHERWSNIYKWPKAFKSP
      * * * * * : : * * * * * : : * * * * * : : * * * * *
ASGV-M WVAFDPATGLKMNKLTPEKQVIDRMTKRLFRTEGQKGVFEAGSESNLELE
ASGV-K WVAFDPATGLKMNKLTPEKQVIDRMTKRLFRTEGQKGVFEAGSESNLELE
      * * * * * : : * * * * * : : * * * * * : : * * * * *
    
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Fig. 2. Comparison of amino acid sequences of ORF 1 between ASGV-F and ASGV-K (AY596172). Identical residues are indicated by asterisks and similar residues are indicated by colons. The conserved helicase motif is shown in the shaded box. The alignment was generated using CLUSTAL W.

The partially purified viral particles were separated on a 12% SDS-polyacrylamide gel and analyzed by Western blotting using an ASGV anti-serum provided by the

National Horticultural Research Institute and National Cheju Agricultural Experiment Station (Fig. 1). One band at 28 kDa reacted with the ASGV anti-serum, consistent with the mobility of the viral CP from purified ASGV isolated from pears.

The ASGV ORF1 encodes a 241 kDa polyprotein containing a replicase region, in which all of the characteristic motifs for methyltransferase (Rozanov et al., 1992), papain-like proteinase (Rozanov et al., 1992), NTP-binding helicase (Gorbalenya and Koonin, 1989), RdRp (Koonin, 1991), and CP (Yoshikawa et al., 1992; Ohira et al., 1995) are located. Specific primers for helicase, RdRp, and CP were designed according to the genome sequences and organization of ASGV strains previously isolated from plants (Shim et al., 2004). The deduced amino acid sequence of ASGV-F showed conserved domains, which contain an NTP-binding helicase motif, GFAGSGKT (Fig. 2). But a complete comparison of the RdRp amino acid sequence has not been conducted since a full length cDNA of RdRp was not amplified. However, when partial sequences of ASGV-F were analyzed, these sequences were similar to those of ASGV-K (AY596172), which is associated with PBNLS. The predicted amino acid sequences of ASGV-F showed 83% similarity to ASGV-K (AY596172) and 72% similarity to the ASGV-Japan strain (D14995). A BLAST search revealed that the ASGV-F CP has a relatively high homology to all ASGV strains (98%) (Fig. 2 and Fig. 4).

We were not able to detect virus particles in the fungus, but we assume that ASGV-F has a structure similar to ASGV-K because the ASGV-F CP has a high degree of amino acid homology to the CP of other strains, as well as conserved residues that have been associated with the putative salt bridge and corresponding hydrophobic core found in flexuous rod-shaped ssRNA plant viruses (Fig. 3) (Dolja et al., 1991).

To compare the phylogeny and genetic characteristics of

ASGV-BEAN	(155)	CTF R KLCEPFAD	(24)	AFEKSPWVAF F FAT	(32)
ASGV-FUNGI	(155)	CTF R KLCEPFAD	(24)	AFEKSPWVAF F FAT	(32)
ASGV-CP	(2012)	CTF R KLCEPFAD	(24)	AFEKSPWVAF F FAT	(32)
ASGV-PEAR	(2012)	CTF R KLCEPFAD	(24)	AFEKSPWVAF F FAT	(32)
CVA	(2250)	LTF R QLCEPFAE	(22)	AYFNCPAVV F FNK	(45)
PVT	(119)	KTL R QMCMPFAK	(21)	LCRKAKV V FAS	(48)
ACL SV	(156)	MTF R QVCEAFAP	(24)	VGSKYPELM F FNK	(45)
ASPV	(320)	CTLRQYCAFYAK	(24)	TRYAAFDFF F GVES	(52)
BVF	(116)	QTL R QFARPYAP	(24)	SVGAPPHVC i FND	(139)
MBV	(155)	NCLGAHSV K VFA	(24)	VVPANKQLSS P SS	(40)
SmV8	(285)	NI V QQT S VSFVA	(24)	VGVLEVELV H LEG	(48)

Fig. 3. Amino acid sequence alignments of coat proteins from ASGV-F, flexuous rod-shaped plant viruses and ssRNA mycoviruses. The conserved positively- and negatively-charged residues (Arg and Asp, respectively), which are proposed to form a salt bridge crucial to the tertiary structure of the protein, are shown in bold (modified from Dolja et al., 1991).

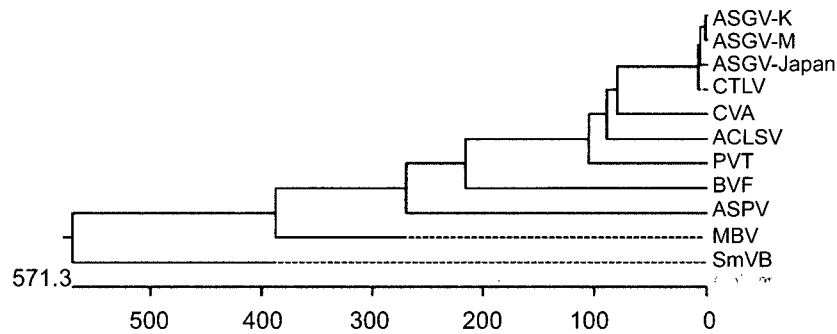


Fig. 4. Phylogenetic tree constructed from alignment of the putative salt bridge region of the ASGV-F coat protein. Numbers indicate the percentage of bootstrap replicates (N=100) that support each branch node (only values >50% are shown). A bar represents 10 substitutions per 100 amino acids. Viruses used in the analysis and their NCBI GenBank accession numbers are: ASGV-K (AY596172), ASGV-Japan (D14995), CTLV (D14455), CVA (X82547), ACLSV (P27737), PVT (D10172), BVF (NP068550), ASPV (D21829), MBV (NP042511), and SmVB (NP821131).

ASGV-F, the amino acids of the 3' terminus were aligned with other RNA viruses of the Flexiviridae, the family to which ASGV belongs. Flexiviridae comprises *Capillovirus*, *Allexivirus*, *Carlavirus*, *Foveavirus*, *Mandarivirus*, *Potexvirus*, *Trichovirus*, and *Vitivirus*. *Cherry virus A* (CVA, belonging to the same genus as ASGV), *Apple stem pitting virus* (ASPV), *Apple chlorotic leaf spot virus* (ACLSV, which is infectious to a number of fruit trees) and *Potato virus T* (PVT, which is serologically similar to ASGV) were chosen for comparison. ASGV-F was most closely related to the ASGV-Korea strain (ASGV-K) which appears to cause PBNLS disease, and less closely related to the ASGV-Japan strain which does not cause PBLNS (Fig. 4). ASGV-F showed a high degree of homology to ACLSV, which is capable of infecting the same host plants, including apples. Serologically, PVT showed a similarity to ASGV. Interestingly, ASGV-F was more similar to BVF, a flexuous rod-shaped ssRNA mycovirus, than to ASPV.

To clarify the characteristics and biology of ASGV-F, electron microscopy (EM) and a complete genome sequence analysis are needed. Additionally, it will be important to isolate an infectious clone in future studies, so that the mechanisms of virus replication in both fungi and plants, and virus transmission from plant to fungus, fungus to plant, and fungus to fungus can be better understood.

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