Further Evidence that Shallot Yellow Stripe Virus (SYSV) Is a Distinct Potyvirus and Reidentification of Welsh Onion Yellow Stripe Virus as a SYSV Strain

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ABSTRACT

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An antiserum to shallot yellow stripe virus (SYSV) was raised and used in combination with a range of other antisera to potyviruses of *Allium* spp. in electron microscopic decoration experiments. The serological results corroborated an earlier finding that the type isolates of SYSV and Welsh onion yellow stripe virus (WoYSV) are closely related to each other and only distantly related to onion yellow dwarf (OYDV) and leek yellow stripe (LYSV) viruses, the two other major potyviruses infecting *Allium* spp. Moreover, the decoration results indicated that Japanese potyviruses

named OYDV and Wakegi yellow dwarf virus are isolates of SYSV. Sequence analysis of the 3'-terminal regions of the SYSV and WoYSV genomes revealed coat protein (CP) amino acid and 3'-nontranslated region (3'-NTR) nucleotide sequence identities of 95 and 89%, respectively. The CP amino acid and 3'-NTR nucleotide sequences of these viruses differed from those of OYDV and LYSV by >25 and >67%, respectively. The serological and molecular studies showed that SYSV and WoYSV are different strains of a potyvirus distinct from OYDV and LYSV. For priority reasons, we propose that these strains together with the Wakegi-type isolates of OYDV described in Japan be referred to as SYSV and that SYSV isolates from *Allium* spp. other than shallot be designated as the Welsh onion strain of SYSV (SYSV-Wo).

Viruses of *Allium* spp. (7,36) have attracted considerable attention in recent years. Those of garlic and shallot are especially topical internationally, because these crops are of great economic importance and their vegetative propagules are distributed worldwide. Several of the viruses that infect *Allium* spp., such as certain carlaviruses (29) and mite-borne filamentous viruses (32,33), generally induce no or only mild symptoms. However, the aphid-borne potyviruses (28) usually cause distinct symptoms and are considered economically important in *Allium* spp. crops. Onion yellow dwarf (OYDV) and leek yellow stripe (LYSV) viruses have been characterized earlier as distinct potyviruses because of their specialization on different natural hosts (onion and leek, respectively), characteristic symptoms on experimental hosts, and relatively distant serological relationships (8). Investigations on potyvirus isolates from numerous samples of *Allium* spp. of botanically and geographically diverse origin led Van Dijk (28) to distinguish two new aphid-borne potyviruses, namely shallot yellow stripe (SYSV) and Welsh onion yellow stripe (WoYSV) viruses, specialized on shallot (*A. cepa* var. *ascalonicum* Backer) and Welsh onion (*A. fistulosum* L.), respectively.

SYSV was isolated from shallot samples originating from southeast Asia (Indonesia, China, and Thailand), where it appears to be common. It did not infect leek (*A. ampeloprasum* L. var. *porrum* (L.) Gay), Welsh onion, *Chenopodium* spp., and *Nicotiana* spp.; was infectious to onion 'Crystal Wax', which is resistant to most isolates of OYDV; and did not react with the Dutch antisera to OYDV and LYSV. WoYSV was found to be serologically related to SYSV and distantly related to OYDV, but different from these

and other potyviruses in its pathogenicity to rakkyo (*A. chinense* G. Don) and Welsh onion (28). Since no antisera to formally described isolates of SYSV and WoYSV were available in the former studies, further serological tests were suggested (28) to clarify the taxonomic difference between SYSV and WoYSV.

Based on sequence data for potyviruses present in botanically and geographically diverse samples of *Allium* spp., Tsuneyoshi et al. (24) have recently provided evidence for the occurrence of three distinct groups of potyvirus isolates from *Allium* spp. Two of them were assigned to OYDV and LYSV based on high levels of coat protein (CP) amino acid sequence identities to OYDV and LYSV isolates studied elsewhere. They proposed the name Wakegi yellow dwarf virus (WYDV) for a group of closely related potyvirus isolates from shallot, rakkyo, Welsh onion, and Wakegi onion (*A. wakegi* Araki) in Japan and Indonesia, although they pointed to the similarity of the WYDV isolates to Indonesian isolates of SYSV and WoYSV described earlier by Van Dijk (28). No attempts were made to characterize the WYDV isolates biologically and serologically or to compare them with Van Dijk's SYSV and WoYSV isolates.

We have now purified virions of SYSV and produced a specific antiserum to serologically characterize this virus as a distinct potyvirus. The relationships between SYSV, WoYSV, and other potyviruses of *Allium* spp. were also studied at the molecular level by determining and comparing the nucleotide sequences of the CP gene and 3'-nontranslated region (3'-NTR) of their RNAs.

MATERIALS AND METHODS

Virus isolates. The virulent SYSV isolate Ac127I originated from a shallot sample from Sumenep, Java (28), and the WoYSV isolate Af19I from a severely diseased crop of Welsh onion 'Papak' at Ciwidey, Java, Indonesia (28,30,31). The SYSV isolates 642Sh

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and 318Sh, originating from shallot plants in Thailand and Taiwan, respectively, and the WoYSV isolates 7WO and 198WO, both collected from Welsh onion in Taiwan, were maintained in the original host plant by vegetative propagation. The OYDV isolate Ac41, previously described by Bos et al. (8), was from the DLO Research Institute for Plant Protection (IPO-DLO) plant virus collection. With the exception of the LYSV isolate 510L, all OYDV and LYSV isolates from the plant virus collections at IPO-DLO and Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA) were maintained by sap inoculation onto onion ('Stuttgarter Riesen') and leek ('Jolant') seedlings, respectively, under aphid-free conditions in the glasshouse at 22°C as previously described (28). The LYSV isolate from leek in Singapore (510L) was maintained in *Chenopodium quinoa* by mechanical inoculation.

Virion purification. Virions of SYSV Ac127I were purified from sap-inoculated 'Stuttgarter Riesen' onion plants showing typical yellow stripe symptoms (28). After homogenization of frozen leaf material in McIlvaine's sodium citrate buffer (0.18 M, pH 7) containing 0.01 M sodium thioglycolate and 0.02 M sodium diethyldithiocarbamate, the homogenate was stirred overnight at 4°C with 1% Triton X-100, filtered through cheesecloth, and differentially centrifuged at 10,000 × g for 10 min, 77,000 × g for 1.5 h, 10,000 × g for 10 min, and 35,000 × g for 1.5 h. After resuspension of the pellet and low-speed centrifugation, the supernatant was subjected to sucrose density-gradient centrifugation (10 to 40%; 2 h at 83,000 × g). Virion-containing fractions were concentrated by high-speed centrifugation (3 h at 126,000 × g) followed by two cycles of quasi-isopycnic centrifugation in 397 mg of CsCl per ml (22 h at 111,000 × g). Resulting virion-containing fractions were dialyzed against McIlvaine's buffer mixed with glycerol (1:1) and stored at -20°C.

Preparation of antiserum. Rabbits were immunized by administering, at 4-week intervals, three consecutive subcutaneous injections containing purified virions (0.5 mg) emulsified in Freund's incomplete adjuvant. A final booster injection of purified virions (0.25 mg) emulsified in the same adjuvant was given subcutaneously 3 months after the last injection. Rabbits were bled before and after the final booster injection. The immunoglobulin G (IgG) fraction was purified according to standard methods.

Electron microscopic decoration experiments and sources of antibodies. Decoration tests were performed at BBA, Braunschweig, essentially as described by Vetten et al. (35). The monoclonal antibody (MAb) 1H12 raised against SYSV Ac127I as well as the antisera to OYDV 510 and LYSV isolates from leek (L-BBA) and garlic (512G) were from the stock of BBA, Braunschweig. Antisera to SYSV Ac127I (described above), an onion isolate (Ac41) of OYDV, and a leek (Aa22) isolate of LYSV were from the stock of IPO-DLO, Wageningen. Antisera to an onion isolate of OYDV, OYDV-Fukami, OYDV-O, and WYDV, were provided by H. Lot (Montfavet, France), M. Fukami (Chiba, Japan), S. T. Ohki (Osaka, Japan) (18), and S. Sumi (Hiroshima, Japan), respectively.

Cloning and sequencing of SYSV Ac127I at BBA, Braunschweig. For cloning and sequencing of the 3' end of the SYSV genomic RNA, viral RNA was extracted from purified virions of SYSV isolate Ac127I and used for oligo(deoxyribosylthymine [dT])-primed cDNA synthesis and cloning into a Bluescript pSK⁻ vector essentially as described by Maiss et al. (16). This resulted in a number of clones with inserts of up to 2,000 bp whose terminal parts were sequenced for obtaining tentative sequence data. Only clones containing a poly(A) tail were further analyzed by primer-walking to obtain overlapping sequence information for the 3' end of the SYSV genome. All recombinant DNA techniques were performed as specified by Sambrook et al. (19). Sequencing of selected clones was done on double-stranded DNA templates according to Sanger et al. (20). Sequence data were compiled and analyzed with UW-GCG (version 8; Genetic Computer Group, Inc., Madison, WI).

Immunocapture reverse transcriptase polymerase chain reaction (RT-PCR). Eppendorf tubes (0.7 ml) were coated with 100 µl of anti-SYSV IgG (2 µg/ml) in phosphate-buffered saline (PBS) for 3 h at 37°C, followed by three washings with PBS. Thereafter, virions were trapped by overnight incubation of tubes at 4°C with 200 µl of leaf extracts (100 mg/ml of PBS) from 'Stuttgarter Riesen' onion when showing clear SYSV symptoms ~3 weeks after sap inoculation. Unbound leaf material was removed by washing the tubes three times with PBS. Excess liquid was carefully removed after the last washing step. Prior to cDNA synthesis, trapped virus particles were denatured by adding 8 µl of 5× First Strand Buffer (Life Technologies, Inc., Gaithersburg, MD), 1 µl of first-strand primer (100 ng/µl), and 21 µl of H₂O, followed by incubation of the tube at 65°C for 5 min. Thereafter, cDNA was synthesized by adding 4 µl of H₂O, 4.5 µl of 0.1 M dithiothreitol (Life Technologies, Inc.), 1 µl of deoxynucleoside triphosphate (dNTP) mix (2.5 mM each), and 0.5 µl of Moloney murine leukemia virus reverse transcriptase (200 U/µl; Life Technologies, Inc.). The reaction mixture was incubated at 37°C for at least 1 h.

The resulting cDNA was amplified by PCR using either degenerate or specific upstream and downstream primers. A total of 5 µl of cDNA was mixed with 27 µl of H₂O, 4 µl of 10× Taq polymerase buffer (HT-Technologies Ltd., Cambridge, United Kingdom), 1 µl of each primer (100 ng/µl), 1 µl of dNTPs (2.5 mM each), and 1 µl of Taq polymerase (0.5 U/µl; HT-Technologies Ltd.). Incubation was in a PTC 200 thermocycler (MJ Research Inc., Watertown, MA): 5 min at 95°C, followed by 35 cycles of 30 s at 92°C, 30 s at 52°C, and 45 s at 72°C, and a final extension of amplification products for 10 min at 72°C. Amplification products were analyzed for size and yield by electrophoresis on a 1% Tris-acetate EDTA (TAE) agarose gel.

PCR primers. Initial cDNA synthesis and PCR amplification of the 3' end of the SYSV, WoYSV, and OYDV genomic RNAs was done using P9502 as downstream primer (Table 1) and CPUP as a degenerate upstream primer (Table 1) derived from a highly conserved domain of the potyviral CP gene. This primer pair had previously been used successfully to amplify the 3'-terminal region of lettuce mosaic virus isolates (25) and other potyviruses (26).

The N-terminal CP-encoding region of SYSV and WoYSV RNAs was amplified using upstream primer SYS-UP and downstream primer SYS-DW (Table 1) derived from an alignment of the OYDV sequence (D73378) (23) and a sequence of the 3' end of the RNA of SYSV Ac127I as determined at BBA, Braunschweig. The N-terminal region of OYDV was amplified using upstream primer OYD-UP and downstream primer OYD-DW (Table 1), both derived from OYDV isolates sequenced at BBA, Braunschweig (34).

Cloning and sequencing of amplification products. Amplification products were purified from the reaction mixture using a PCR cleanup kit (Boehringer GmbH, Mannheim, Germany). The purified PCR fragment was ligated into a pGEM-T PCR cloning vector (Promega Corp., Madison, WI), and the ligation mixture was transformed to *Escherichia coli* JM109 highly competent cells (Promega Corp.), according to the manufacturer's instructions. Plasmids were isolated from recombinant *E. coli* by alkaline lysis and analyzed for the presence of the expected insert by restriction analysis using *Bam*HI.

TABLE 1. Primers used for cDNA synthesis and polymerase chain reaction amplification of the 3' end of the shallot yellow stripe virus, Welsh onion yellow stripe virus, and onion yellow dwarf virus genomic RNAs

Primer name	Primer sequence ^a
P9502	5'-GCGGATCCTTTTTTTTTTTTTTTT-3'
CPUP	5'-TGAGGATCCTGGTGYATHGARAAYGG-3'
SYS-UP	5'-GCAGGATCCAACACCRAGTTATGTGTC-3'
SYS-DW	5'-TTCGGATCCATRTGAGCTTCCTTCGC-3'
OYD-UP	5'-CTGGATCCGAGTKCGATAYCAAG-3'
OYD-DW	5'-CACGGATCCGYGCCATWATYTGTCTG-3'

^a R = A or G, Y = C or T, W = A or T, K = T or G, and H = A or T or C.

Plasmids containing an insert of the expected size were isolated from overnight cultures of recombinant bacteria using the Wizard miniprep DNA purification system (Promega Corp.) according to the manufacturer's instructions. Several purified plasmid DNAs were sequenced on an ABI automatic sequencer (Applied Biosystems, Inc., Foster City, CA) using standard M13 forward and reverse primers. Sequence data were compiled and analyzed using the UW-GCG (version 8) and the Lasergene (DNASTAR Inc., Madison, WI) software packages.

RESULTS

Serological properties. Purified preparations of SYSV Ac127I virions contained numerous flexuous, filamentous particles of ~750 nm, often in end-to-end aggregation. The antiserum raised against SYSV had a homologous titer of 1:1,024 in microprecipitin tests. To determine the serological relationships between SYSV Ac127I and WoYSV Af19I, as well as their serological affinities to other potyvirus isolates from shallot and Welsh onion and to the type and other isolates of OYDV and LYSV, decoration experiments were conducted by testing a range of potyvirus isolates with 10 antisera and one MAb to SYSV in all possible combinations (Table 2). All isolates from shallot and Welsh onion tentatively assigned to SYSV and WoYSV, respectively, reacted intermediately to strongly with the MAb and antiserum to SYSV Ac127I as well as with the three antisera supplied from Japan. However, these four antisera did not decorate or only weakly decorated particles of the OYDV and LYSV isolates, whereas MAb 1H12 failed to decorate particles of any OYDV and LYSV isolate. On the other hand, the three OYDV antisera gave stronger decorations in homologous combinations, but did not decorate or only weakly decorated SYSV and WoYSV particles and did not decorate the particles of the LYSV isolates. The three LYSV antisera used showed an intermediate to strong decoration of LYSV particles, but failed to decorate particles of SYSV, WoYSV, and OYDV isolates, except in one case in which a weak reaction was obtained.

Molecular properties. *Sequencing of SYSV at BBA.* The 3' region of the SYSV Ac127I RNA was cloned using conventional oligo(dT)-primed cDNA synthesis and cloning methods. A total of 1,145 nucleotides (nt) (without the poly(A) tail) of the 3' end of the SYSV Ac127I genome were determined (EMBL accession #AJ223826).

It contained one large open reading frame (ORF) coding for a protein of 314 amino acids followed by a 3'-NTR of 197 nt. Alignment of the amino acid sequence of the putative protein with polyproteins of potyviruses revealed that the predicted sequence had high similarity with corresponding portions of the polyprotein of other potyviruses, starting at the C-terminal end of the nuclear inclusion protein b (NIb) and containing the entire CP. The putative cleavage site of the SYSV NIa protease appeared to be Q56/A57 on the basis of the alignment. This prediction was supported by the presence of a DAAN motif, a variation of the DAGK motif (2), at six residues downstream from the putative cleavage site. The predicted CP is 258 amino acids with a calculated molecular mass of 29.5 kDa.

Restriction fragment length polymorphism (RFLP) and sequence analysis of SYSV and WoYSV at IPO-DLO. For elucidating the correct taxonomic position of WoYSV, the sequences of the CP-encoding regions and the 3'-NTR of SYSV isolate Ac127I and WoYSV isolate Af19I were determined. Virus particles of SYSV Ac127I or WoYSV Af19I were trapped by immunocapture using the SYSV Ac127I antiserum. Subsequent RT-PCR experiments with primers P9502 and CPUP resulted in DNA fragments of approximately 650 bp for both SYSV and WoYSV. To assess the possible relationship between SYSV and WoYSV, PCR fragments were subjected to RFLP analysis using several frequently cutting endonucleases (*RsaI*, *MspI*, *AluI*, and *MseI*). This analysis revealed clear differences in RFLP patterns for PCR products obtained for SYSV and WoYSV (data not shown).

PCR fragments obtained for the two virus isolates were cloned, and plasmid DNAs from recombinant *E. coli* were analyzed for the presence of the desired insert by digestion with *Bam*HI, a site present at the 5' end of the PCR primers used for amplification. For both SYSV and WoYSV, at least 10 recombinant plasmids were used as templates in a PCR experiment with primers P9502 and CPUP. Resulting DNA fragments were again subjected to RFLP analysis with the same enzymes used before. For all recombinant plasmids of SYSV and WoYSV, RFLP analysis of the PCR fragments gave RFLP patterns typical of each virus.

For SYSV and WoYSV, one clone each was selected for sequencing. For each clone, the largely overlapping DNA sequences resulting from the forward and reverse primers were combined. This resulted in DNA sequences of 645 nt for SYSV and 643 nt

TABLE 2. Serological relationships among potyvirus isolates from *Allium* spp. as assessed by decoration experiments

Isolates and origins ^a	Anti-SYSV MAb 1H12	Antisera to SYSV ^b				Antisera to OYDV ^b			Antisera to LYSV ^b		
		Ac127I Netherlands	WYDV Japan	OYDV Fukami	OYDV-O Sako	51O Germany	Ac41 Netherlands	France	L-BBA Germany	512G Taiwan	Aa22 Netherlands
SYSV											
Ac127I, Indonesia (type)	+++* ^c	+++*	+++	++	+++	+	+	+	+	—	—
642Sh, Thailand	++	+++	++	++	++	—	+	+	—	—	—
318Sh, Taiwan	+++	+++	+	++	+++	+	+	—	—	—	—
WoYSV											
Af19I, Indonesia (type)	++	+++	++	+++	+++	—	++	+	—	—	—
7WO, Taiwan	+++	+++	++	+++	+++	—	+	+	—	—	—
198WO, Taiwan	+++	+++	++	+++	+++	+	+	—	—	—	—
OYDV											
Ac41, Netherlands (type)	—	+	+	+	+	+++	+++*	+++	—	—	—
51O, Germany	—	—	—	+	+	+++*	+++	+++	—	—	—
747O, Sudan	—	+	—	—	+	+++	+++	++	—	—	—
LYSV											
Aa22, Netherlands (type)	—	—	—	—	—	—	—	—	+++	++	++
L-BBA, Germany	—	—	—	—	—	—	—	+	++*	++	++
510L, Singapore	—	+	—	—	—	—	—	—	+++	++	++

^a Virus isolates maintained at IPO-DLO and BBA are designated in two different ways. Isolate codes used at IPO-DLO consist of the original host (AC = *Allium cepa*, Af = *A. fistulosum*, and Aa = *A. ampeloprasum*) and a number, whereas those used at BBA consist of a number and the original host (L = leek, O = onion, Sh = shallot, and WO = Welsh onion).

^b All antisera and the monoclonal antibody (MAb)-containing culture supernatant were used at a dilution of 1:50. SYSV = shallot yellow stripe virus, OYDV = onion yellow dwarf virus, LYSV = leek yellow stripe virus, and WoYSV = Welsh onion yellow stripe virus.

^c Decoration was scored as strong (+++), intermediate (++), weak (+), and nondetectable (-); * = homologous reactions.

for WoYSV, consisting of 447 nt of the CP-encoding sequence and a 3'-NTR of 197 and 195 nt, respectively (excluding the poly(A) tail).

Alignment of the partial CP amino acid and complete 3'-NTR nucleotide sequences of SYSV Ac127I determined at IPO-DLO with those determined at BBA revealed, as expected, high levels of sequence identity (100 and 99.5%, respectively). Surprisingly, however, a very high level of identity was also observed between SYSV and WoYSV for their partial CP amino acid and 3'-NTR sequences of (98 and 89%, respectively). This was unexpected, because SYSV and WoYSV seemed to be distinct potyviruses based on the RFLP analysis of their PCR fragments. Closer examination

of the sequence alignments revealed that, in the amplified fragments, a total of eight sites, present for the four restriction enzymes selected for the RFLP analysis, revealed nucleotide differences in the recognition sequences (Fig. 1), resulting in different RFLP patterns for SYSV and WoYSV.

Sequence comparison of SYSV and WoYSV with OYDV. SYSV and WoYSV sequences were compared with sequences available in the EMBL database. Surprisingly, a striking identity was found with a potyvirus sequence from Wakegi onion deposited as a Japanese OYDV isolate under accession #D73378. The C-terminal portion of the CP sequences of SYSV and WoYSV showed amino acid sequence identities of 97 and 98%, respectively. For the 3'-NTR

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ccaacaccgagttatgtgtcatatcaagcgacagaatcagaagacgctgcgaatgtgaacactgacaagcaagtcgga 80
      a      c g tt      a      t      t      g t
P T P S Y V S Y Q ↑ A T E S E D A A N V N T D K Q V G K
                      S      T

      TaqI      RsaI      RsaI
gaacaaggataaggacagagatgttgatgttggcacatcaggtgagttttcagttccgaaggtgaaaatgttatcagata 160
      t      t ag      c c      t      g      a      a c      g g
N K D K D R D V D V G T S G E F S V P K V K M L S D
      K                      A

      AluI      MboI
aaatgcgtttgccacgaattggaagaaagtgttactcaacggcaaacatcttttatcttataaacctgaccaaattgat 240
      g      g      aa t      t a      g      c g      t      g
K M R L P R I G K K V V L N G K H L L S Y K P D Q I D
E      V      I      A      V

      ttgtataacacacgagccacacgacagtttaagacgtggtacgagcggtgaaactagaatatgaattgacggatga 320
      a c      g a g      c a      t      c a      t      c
L Y N T R A T H A Q F K T W Y E A V K L E Y E L T D E
      D

      acaaatgaaaatagtcgatgaatgggttgatggtatggtgcattgagaacggcacatcacaaaatttgacgggggtttgga 400
      g      g      a      c a      c a      a
Q M K I V M N G L M V W C I E N G T S Q N L T G V W
      MseI

      RsaI      DdeI
cgatgatggatggcgacaaccaaattggagtacccactcagtcctattattgacaatgctaaccctacattcagacaaata 480
      t t      a t      a c      c a a a
T M M D G D N Q M E Y P L S P I I D N A K P T F R Q I

      MspI
atggcacatttcagtgcagcagctgaagcgtacattgaatataggaatgccacagaaaaatacatgcctcggtatggact 560
      c      c
M A H F S D A A E A Y I E Y R N A T E K Y M P R Y G L

      AluI AluI/DdeI
tcagcgaaacttaagagaatacagcctagcacgttacgcattcgcactttttatgagatgaattcaaagacgcgatccgcg 640
      t      t      g
Q R N L R E Y S L A R Y A F D F Y E M N S K T P I R

      cgaaggaagctcacatgcagatgaaagcggcagcggtagaggggcagctaaccgtatgtttggcttgatggttaacata 720
      t      a      t
A K E A H M Q M K A A A V R G A A N R M F G L D G N I
      V

      RsaI
ggcaccgatgatgagaacacagaacgacatacagcagctgatgtgaataaggatcatcacacgctgctcgggtcttcgtat 800
a      t
G T D D E N T E R H T A A D V N K D H H T L L G L R M
S      V

      MseI
gtaacaaacatatatatatatttaatatatagtgctgcgtgattatctttcgttatatttgaatatgagcaaacatagtgtgg 880
      . tt
*      c

      ttacaccaccgtttgtggcatagtatatgatataacatcgctagcactatttgcaccatgatactcagtgtggtgtacc 960
      ct      t      t      cg      t      cac

      HinfI      RsaI
acgatgaaagagttgagtggttgcacgttttgttagggagac 1001
      --g      ca      t

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Fig. 1. Nucleotide (lower case letters) and deduced amino acid sequences (upper case letters) of the 3' end of the shallot yellow stripe virus (SYSV) Ac127I genome. Nucleotides and deduced amino acid residues of Welsh onion yellow stripe virus (WoYSV) Af19I differing from those of SYSV Ac127I are indicated below the sequences. The putative cleavage site between the nuclear inclusion protein b and coat protein is marked by an arrow. The annealing site of the degenerate potyvirus primer CPUP is in bold. Recognition sites for common restriction endonucleases that differ between the sequences of SYSV and WoYSV due to differences in nucleotide sequence are underlined.

region, nucleotide sequence identities were 87 and 93%, respectively. These high levels of sequence identities among SYSV, WoYSV, and the Japanese OYDV sequences suggested a close relationship among these viruses.

To gain more insight into the relationship of SYSV and WoYSV to the Japanese OYDV isolate, the region encoding the CP N-termini of SYSV Ac127I and WoYSV Af19I was amplified using the primer pair SYS-UP/SYS-DW. In addition, the entire CP-encoding region and 3'-NTR of the RNA of the type isolate of OYDV Ac41 (8) was amplified using the primer combinations P9502-CPUP and OYD-UP-OYD-DW. PCR experiments with these primers on cDNA generated with P9502 for both SYSV and WoYSV resulted in DNA fragments of approximately 700 bp, and for OYDV, in DNA fragments of 480 and 650 bp. All fragments were purified, cloned in pGEM-T vectors, and sequenced. Sequence data were combined with data previously obtained. This resulted in a total of 1,011 nt for SYSV Ac127I (EMBL accession #Y11747), 1,009 nt for WoYSV Af19I (EMBL accession #Y11748) and 1,003 nt for OYDV Ac41 (EMBL accession #Y11826), all of which contained the complete CP gene and 3'-NTR of these viruses.

The sequences of SYSV and WoYSV both encode a CP of 258 amino acids. The cleavage site between the Nib and CP gene is most likely located at the Q/A dipeptide for SYSV and WoYSV (Fig. 1). Alignment of complete CP amino acid and 3'-NTR nucleotide sequences of SYSV, WoYSV, and Japanese OYDV showed identity levels of ~95% for the CP and 88% for the 3'-NTR. An alignment of these sequences with OYDV Ac41 and a LYSV isolate from leek (deposited by J. Schubert, EMBL accession #X89711) revealed much lower levels of identities averaging 73 and 68 % for the CP and 32 and 27% for the 3'-NTR, respectively. Results of the alignments are summarized in Table 3.

Sequence comparisons of SYSV isolates. The CP and 3'-NTR sequences of the type isolates of SYSV and WoYSV were compared with those of the Wakegi-type isolates reported by Tsuneyoshi et al. (24). Multiple alignments of these sequences showed that their CP amino acid and 3'-NTR nucleotide sequence identities varied from 95 to 100% and from 85 to 100%, respectively. Moreover, both the CP amino acid and 3'-NTR nucleotide sequences of the three shallot isolates were more closely related to one another and different from those of the five isolates originating from nonshallot hosts such as Welsh onion, Wakegi onion, and rakkyo.

Detailed comparison of the CP amino acid sequences revealed that the CP core domain contained six amino acid positions that were identical in the three shallot isolates but distinct from the corresponding residues in the five nonshallot isolates (Fig. 2A). With the exception of CP amino acid position 2, there were no isolate group-specific differences in the 24 amino-terminal amino acids of the SYSV isolates.

Alignment of the 3'-NTR sequences also revealed a number of nucleotide positions in which the shallot isolates differed from the nonshallot isolates (Fig. 2B). Using the Structure Analysis of RNA (STAR) program of Abrahams et al. (1), a search for potential secondary structures in the 3'-NTR of all SYSV isolates suggested

the presence of three possible hairpin-loop structures (Fig 2B). Only the 3'-proximal potential hairpin-loop structure was located in a region where major differences between shallot and nonshallot isolates occurred (Fig. 2B). In particular, the three nucleotides C/TAC of the nonshallot isolates, located in the loop of this potential hairpin-loop structure, were replaced by GGT in the shallot isolates that, in addition, had an insertion of two nucleotides (TA) just 3' of this potential stem-loop structure.

DISCUSSION

Results of detailed host range and serological studies by Van Dijk (28) on isolates from a large number of *Allium* spp. suggested that, in addition to OYDV and LYSV, two distinct new potyviruses might exist for which the names WoYSV and SYSV were proposed. WoYSV differed from SYSV in infecting rakkyo (*A. chinense*) and Welsh onion (*A. fistulosum*), in inducing local necrotic reactions in *C. amaranticolor* and *C. quinoa*, and in its weak reaction with a Dutch antiserum to OYDV (1:3 to 1:10 dilution). The production of an antiserum and MAb to SYSV has now permitted a further serological comparison of the type isolates of SYSV and WoYSV with other potyvirus isolates from shallot and Welsh onion as well as with the type isolates of OYDV and LYSV (Table 2).

Based on the serological data presented, the shallot and Welsh onion potyvirus isolates studied appear to be serologically closely related to one another, as they were serologically indistinguishable under the conditions of the decoration experiments using antisera diluted 1:50. This observation, and the frequently observed weak decoration reactions obtained in heterologous combinations between the OYDV antisera and the isolates from shallot and Welsh onion as well as in reciprocal combinations, suggested that SYSV and WoYSV isolates are serologically closely related to, but clearly distinct from, OYDV. The SYSV MAb 1H12 reacted only with the shallot and Welsh onion isolates, not with any of the OYDV and LYSV isolates, corroborating the serological similarity of SYSV and WoYSV and their discrimination from OYDV and LYSV. Since the LYSV antisera and isolates generally did not react in heterologous combinations with OYDV, SYSV, and WoYSV, the serological relationship of these isolates to LYSV is distant. These results indicate that SYSV and WoYSV are strains of the same virus that, in accordance with the earlier conclusion by van Dijk (28), is clearly distinct from LYSV and OYDV. Most interestingly, the three Japanese antisera to Welsh onion isolates of potyviruses referred to as OYDV (Fukami), OYDV-O, and WYDV reacted strongly with SYSV and WoYSV isolates only and showed no or only weak reactions with OYDV isolates, including the type isolate Ac41 (8). This suggested that the virus isolates used to raise the Japanese antisera were inappropriately identified and, in fact, were isolates of SYSV and WoYSV.

For potyviruses, the sequences of the 3'-NTR and the N-terminal region of the CP are considered virus specific, and these sequences have been extensively used for the classification of potyviruses and potyvirus strains (11,22). The SYSV Ac127I sequence determined independently both at IPO-DLO and BBA differed over 1,001 nt only in three nucleotides: one each in the Nib, CP, and 3'-NTR regions. Only one of these resulted in an amino acid exchange (T/K) at position 96 in the CP. When the 3' end of the WoYSV Af19I RNA was compared with that of SYSV Ac127I, CP amino acid, and 3'-NTR, sequence identities of 95 and 89%, respectively, were observed, confirming their classification as two strains of the same virus as already revealed by the decoration results. The observed levels of identity clearly met the criteria for considering SYSV and WoYSV as distinct strains of the same potyvirus species (22). Surprisingly, the CP amino acid sequences of SYSV and WoYSV also showed identities of 95 and 97%, respectively, with an OYDV sequence (EMBL accession #D73378) that Tsuneyoshi et al. (24) later referred to as WYDV. Since the IPO-DLO plant virus collection holds type isolates of all potyviruses from *Allium*

TABLE 3. Pairwise percent identities among coat protein amino acid sequences (above diagonal) and 3'-nontranslated region nucleotide sequences (below diagonal) of shallot yellow stripe virus (SYSV) Ac127I, Welsh onion yellow stripe virus (WoYSV) Af19I, Wakegi yellow dwarf virus (WYDV), onion yellow dwarf virus (OYDV) Ac41, and leek yellow stripe virus (LYSV)

	SYSV Ac127I	WoYSV Af19I	WYDV ^a	OYDV Ac41	LYSV (X89711)
SYSV Ac127I	...	95	95	74	69
WoYSV Af19I	89	...	97	73	68
WYDV ^a	86	93	...	74	57
OYDV Ac41	33	31	31	...	58
LYSV (X89711)	27	27	28	25	...

^a Sequence (accession #D73378) erroneously assigned to onion yellow dwarf virus, but later referred to as Wakegi yellow dwarf virus.

Comparison of the CP amino acid and 3'-NTR nucleotide sequences of SYSV and WoYSV with those of the Wakegi-type isolates of OYDV (24) revealed a grouping of isolates according to plant species from which the SYSV sequence was obtained. The differences between the two groups resulted chiefly in six amino acid residues of the CP core and in a strikingly distinct putative hairpin-loop present at the 3' end of the 3'-NTR (Fig. 2A and B). For potato virus Y, the classification of isolates as strain groups based on biological and CP properties was supported by differences in loop sequences of potential hairpin-loop structures in the 3'-NTR (27). Additional circumstantial and experimental evidence indicated that differences in CP amino acid and 3'-NTR nucleotide sequences of potyvirus isolates may be biologically significant (17, 21, 22, 27). Therefore, it is tempting to speculate that at least some of the observed differences in the amino acid sequences of the CP core domain and in the nucleotide sequences of the 3'-NTR might be associated with the proposed grouping of SYSV isolates. However, more host range and sequence data on further SYSV isolates, both from shallot and nonshallot hosts, will be required to substantiate the proposed grouping of host-specialized strains of SYSV.

Virus identification in *Allium* spp., especially the vegetatively propagated species, is complicated by the frequency of mixed infections and infections by several members of the genera *Potyvirus* and *Carlavirus* and the yet-unclassified mite-borne filamentous viruses, as well as by host-adapted and serologically deviating strains of these viruses (4, 5, 9, 28, 29, 32, 37). With the advent of molecular plant virology, many researchers produced sequence information and proposed several new virus names without proper identification of their virus isolates, using available type isolates of, and antisera to, the viruses already formally described. This also applies to recent research on potyviruses of *Allium* spp., although type isolates of and antisera to OYDV and LYSV, the two major potyviruses infecting onion, shallot, and garlic in many parts of the world, have been available for many years (6, 8, 10). Proper biological and serological identification of virus isolates is a prerequisite to molecular characterization. Only this allows identification of the molecular determinants of the biological properties of the virus isolates.

In conclusion, the results of our serological and molecular studies suggest that East Asian potyvirus isolates from shallot (*A. cepa* var. *ascalonicum*), rakkyo (*A. chinense*), Welsh onion (*A. fistulosum*), and Wakegi onion (*A. wakegi* Araki, a hybrid of *A. cepa* × *A. fistulosum*) (13) are predominantly isolates of one virus. For priority reasons, all of these isolates including the Wakegi-type isolates reported by Tsuneyoshi et al. (24) should be referred to as SYSV. The SYSV isolates from *Allium* spp. other than shallot should be designated as the Welsh onion strain of SYSV (SYSV-Wo).

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