Mixed Virus Infections of Garlic Determined by a Multivalent Polyclonal Antiserum and Virus Effects on Disease Symptoms

Miyuki Takaichi, Takayuki Nagakubo, and Kenji Oeda, Biotechnology Laboratory, Sumitomo Chemical Co., Ltd., 4-2-1 Takatsukasa, Takarazuka, Hyogo 665-8555, Japan

ABSTRACT

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A garlic virus-specific polyclonal antiserum was developed against a mixture of flexuous rod-shaped virus particles isolated from mosaic-diseased garlic plants (15). This antiserum was used in Western blot analysis against tissues from mosaic-diseased garlic plants, at least seven viral coat protein (CP) bands (from 38 to 32 kDa) were identified. Using Western blot analysis with *Potyvirus*-specific antibodies and reverse transcription-polymerase chain reaction (RT-PCR) analysis, we concluded that three of the seven bands corresponded to CPs of *Leek yellow stripe virus* (LYSV) (38 kDa) and two different *Onion yellow dwarf virus* (OYDV) strains (35.5 or 34 kDa). The 35 kDa band corresponded to the CP of GV1-*Carlavirus*, and the other four bands, 36, 35 (not GV1), 33, and 32 kDa, were identified as the CPs of four mite-borne viruses, based on RT-PCR analysis. Based on the molecular weights of CP, mixed infections of *Potyvirus*, *Carlavirus*, and mite-borne viruses were characterized. LYSV causes apparent disease symptoms in garlic plants, however, little reduction in bulb weights. Conversely, garlic plants infected with three different mite-borne viruses expressed weak symptoms and yield losses. Mixed infections of OYDV, the mite-borne viruses, and LYSV caused severe disease symptoms and considerable reduction of bulb weights.

Additional keywords: *Allium sativum*, cDNA, meristem-tip

Viruses of *Allium* species have been well studied, as these crops are cultivated throughout the world. Viruses of garlic (*Allium sativum* L.) accumulate in bulbs and are spread by vegetative propagation (27,28). Reduction in yield and quality due to virus infection presents a serious economic problem (5,8,16,27,28). A reliable virus indexing method is required to identify these viruses. In particular, a simple and efficient method is indispensable for virus indexing of seed bulbs produced from meristem-tips.

Aphid-borne *Potyvirus*es can cause distinct symptoms and are one of the major viruses in *Allium* crops. At least two *Potyvirus*es, *Leek yellow stripe virus* (LYSV) and *Onion yellow dwarf virus* (OYDV), have been detected in mosaic-diseased garlic plants (4,24). Both viruses have been reported in garlic in other countries (2,3,11,13,14,19,20). OYDV was found at a higher frequency than was LYSV. Other viruses have been identified based on physical and serological evidence. *Carlaviruses*, *Shallot latent virus* (SLV) (23), and *Garlic common latent virus* (GCLV) (3), as

well as several mite-borne viruses (6,22,26,29) also infect garlic plants. Often, these viruses occur as mixed infections in garlic plants (1,12). Most of these viruses cannot be propagated in other plant species (25). Therefore, an individual virus is difficult to purify using standard techniques so that the effects of each virus on disease symptom and bulb weight are not well understood.

The cDNA clones coding for the coat protein (CP) of garlic viruses were isolated, and the nucleotide sequences of CP genes of one *Carlavirus* (GV1) and one *Potyvirus* (GV2/LYSV) were determined (15). Four independent nucleotide sequences exhibiting a genomic organization of *Shallot virus X* (ShVX) were also determined (9,18). These viruses have a close serological relationship to mite-borne viruses (18,26,29). The nucleotide sequence of cDNA that codes for the CP of OYDV has also been determined (10). Thus, at least four garlic viruses, GV1-*Carlavirus*, GV2 (LYSV), OYDV, and four mite-borne viruses could be differentiated, using reverse transcription-polymerase chain reaction (RT-PCR) (21). Nevertheless, effects of each virus on disease symptoms and yield loss are not clear.

The purpose of this research was to identify mixed-virus infections of garlic, using a garlic virus-specific polyclonal antiserum. In addition, the symptoms and yield losses affected by mixed virus infection were evaluated.

MATERIALS AND METHODS

Sources of viruses. Virus-diseased plants of garlic cv. Howaito-roppen derived from local clones and from meristem tip-derived clones were collected in fields in Aomori prefecture in Northern Japan and labeled A, B, D, or J to represent collection sites. These clonal plants were cultivated in isolated field in Misawa. Small leaves of garlic plants were collected three or four weeks after germination for Western blot or RT-PCR analysis.

Western blot. Four different antibodies were used in this study. The *Potyvirus*-specific IgG (Sigma, St. Louis, MO), LYSV-specific and OYDV-specific IgG were purchased (IPO-DLO, Wageningen, Netherlands). The garlic virus-specific polyclonal antiserum was developed by injection of rabbits with a mixture of virus particles prepared from mosaic-diseased garlic plants (15).

Leaves of garlic plants (approximately 100 mg) were homogenized in 200 ml of borate buffer (0.1 M Na₂B₄O₇, pH 8.0), and centrifuged at 5,500 × g for 10 min at 4°C to separate the crude extract. The supernatant (about 10 µg protein) was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the resolved proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Membranes were incubated with virus antibody diluted at 1:1,000 for 30 min. These membranes were washed and then incubated with alkaline phosphate conjugated with anti-rabbit or anti-mouse goat IgG (Jackson Immuno Research Laboratories, West Grove, Pa.) diluted at 1:5,000 for 30 min. The membrane was developed by incubation with 5-bromo-4-chloro-3-indoryle phosphate (BCIP) and nitro blue tetrazolium (NBT) (15).

RT-PCR experiments. Leaves of virus-infected garlic plants were crushed in liquid nitrogen. Total RNA was extracted with a ISOGEN RNA extraction kit (Nippon Gene, Toyama, Japan). First strand cDNA was synthesized from total RNA, using a First Strand cDNA Synthesis Kit (Amersham International, Little Chalfont, UK). The oligonucleotide primers were designed to amplify CP genes of LYSV, OYDV, GV1-*Carlavirus*, and mite-borne viruses as previously described (21). The PCR reaction mixture containing the cDNA templates and primers was subjected to 27 cycles of PCR, denatured at

Corresponding author: Kenji Oeda
E-mail: oeda@sc.sumitomo-chem.co.jp

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95°C for 1 min, annealed at 57°C for 1 min, and extended at 72°C for 1 min. The resulting reaction products were analyzed by 1% agarose gel electrophoresis, as described (21).

Evaluation of productivity of garlic bulbs. Seed bulbs derived from meristem-tips were propagated in three farmers' fields (near the towns of Rokunohe, Tenmabayashi, and Takko). Twenty bulbs were collected from each field after 1, 2, and 5 consecutive cultivation years, and culti-

vated in an isolated field in Misawa to monitor disease symptoms and bulb weights caused by re-infection.

In order to determine viruses that were responsible for disease symptoms and yield losses, we selected 10 different garlic clones (clone 1 to 10). These clones were infected either with a single virus or with several viruses in various combinations.

Forty cloves of each clone weighing 8 to 10 g were planted in an isolated field in Misawa in October of 1998. The develop-

ment of disease symptoms in these plants was monitored in May 1999, and the weight of each bulb was taken in July.

RESULTS

Indexing of *Potyvirus*. Several viruses were identified in mixed infection in mosaic-diseased garlic plants. A *Potyvirus*-specific monoclonal antibody was used to characterize the infecting viruses in diseased garlic plants (Fig. 1A). Three specific protein bands, with molecular masses of 38, 35.5, and 34 kDa, were detected in samples J-5, B-17, and B-15. LYSV-specific and OYDV-specific antibodies were used to identify viruses in the same plants because both LYSV and OYDV are *Potyvirus*s. LYSV-specific antibody reacted only with the 38-kDa band (Fig. 1B), whereas the 35.5- and 34-kDa bands were recognized by the OYDV-specific antibody (Fig. 1C). These results indicate that the 38 kDa protein band corresponds to the CP of LYSV, whereas the 35.5- and 34-kDa bands are derived from the CPs of OYDV.

RT-PCR primers for LYSV were used, which amplify full length LYSV (GV2) CP, and 1,023 bp of LYSV-specific DNA band was detected from J-5, B-17, and B-15 plants from which 38-kDa protein band was detected (Fig. 1A and D). When primers for OYDV that amplify the central region of OYDV CP were used, the 601 bp of OYDV-specific DNA band was amplified in J-5 and B-17 plants from which a 34- or 35.5-kDa band was detected, respectively (Fig. 1C). The 35.5-kDa protein band detected in B-17 plants was tentatively named OYDV 1, and the 34-kDa protein band in J-5 plants was tentatively named OYDV 2.

Indexing garlic viruses based on prepared polyclonal antiserum. In our earlier work (15), we developed antiserum against mixed viral particles purified from mosaic-diseased garlic plants cultivated in a farm field in Aomori prefecture. A variety of diseased plants were analyzed using this antiserum.

The prepared garlic virus-specific polyclonal antiserum identified the 36-, 35-, 33-, and 32-kDa protein bands in addition to 38-, 35.5-, and 34-kDa bands (Fig. 2A). Of these identified protein bands, the 36-, 35-, 33-, and 32-kDa protein bands did not react with the *Potyvirus* antibodies. Therefore, viruses with these protein bands were analyzed using RT-PCR (Fig. 2B) which revealed that the LYSV-specific DNA band was amplified in plants producing a 38 kDa protein band (B-10 and B-16), but not in plants without the 38-kDa band (A-9, A-18, and D-8). Conversely, OYDV-specific bands were amplified in B-10 and B-16 plants with 34- or 35.5-kDa protein bands, respectively. These results again confirmed that the 38-kDa protein band detected on Western blots was derived from LYSV, and the 35.5- and 34-kDa protein bands were from OYDV.

Fig. 1. Characterization of garlic-infecting *Potyvirus* using Western blot analysis. **A**, Using *Potyvirus*-specific monoclonal antibody in Western blots, three protein bands, 38, 35.5, and 34 kDa, were detected. **B**, Using *Leek yellow stripe virus* (LYSV)-specific antibody, the 38 kDa protein band was identified. **C**, Using *Onion yellow dwarf virus* (OYDV)-specific antibody, the 35.5 and 34 kDa protein bands were identified. **D**, Indexing for LYSV and OYDV by reverse transcription-polymerase chain reaction.

We analyzed infection patterns of mite-borne viruses, using PCR primers of the conserved central region of CPs of four mite-borne viruses (22). The mite-borne virus-specific DNA bands were amplified from B-10, A-9, A-18, and D-8 plants (Fig. 2B), from which protein bands with a molecular mass of 36, 35, 33, and 32 kDa was detected. The digestion patterns of amplified DNA after RT-PCR (22) were unique for four different types of mite-borne viruses (Fig. 2C). Accordingly, based on the digestion patterns, three mite-borne viruses, Mite 2/35 kDa, Mite 3/33 kDa and Mite 4/32 kDa, corresponded to as A, B, and D types, respectively, of the reported mite-borne viruses (22). Since the digestion pattern of amplified DNA of Mite 1 of

A-9 plant has not been reported, the 36-kDa band of A-9 plant could be coded by a new mite-borne virus.

GV1-*Carlavirus* was detected from B-15 and A-18 plants (Table 1). Both LYSV and GV1-*Carlavirus* specific DNA bands were detected in the B-15 plant, from which the 38- and 35-kDa protein bands were detected in Western blot analysis. Since the 38-kDa band was determined to be the CP of LYSV, the 35-kDa band was thought to be the CP of GV1-*Carlavirus*. Similarly, the 35-kDa band of A-18 plant was derived from GV1-*Carlavirus*, but the 35-kDa protein band of D-6 plant was thought to be CP of Mite 2 (Table 1).

Virus infection patterns in the field: Disease symptom development through

consecutive cultivation. Seed bulbs derived from meristem-tips were propagated in three fields for 1 to 5 consecutive cultivation years. Twenty bulbs, randomly collected from each field, were grown in an isolated field in Misawa to determine virus re-infection (Fig. 3). Disease symptoms appeared in many of the plants in all three fields. In the samples from Rokunohe, the symptom rate was about 15% in the first year, and 65% after 5 years cultivation (Fig. 3A). Similarly, the rates were 20 and 42% in samples from Tenmabayashi (Fig. 3B), and 25 and 68% in samples from Takko (Fig. 3C), in the first and second years, respectively. Infecting viruses were identified using the prepared polyclonal antiserum. The resulting symptom rates indicated good correlation to re-infection rates of LYSV (21). There was, however, no apparent reduction in mean bulb weight of the samples (Fig. 3D–F).

Disease symptom development and yield loss of mixed infection. To determine viruses responsible for disease symptoms and yield losses we selected 10 different garlic clones. They were infected with either a single virus or several viruses in various combinations. Forty cloves weighing 8 to 10 g each were planted in an isolated field in the town of Misawa, and symptom development in mature plants was investigated by visual examination. At least three different mite-borne viruses were detected in clones 2, 3, 4, and 5. One and two mite-borne viruses were found in 2 and 3, respectively. Obvious disease symptoms, however, were not observed in these clones (Fig. 4A). In clones 4 and 5, three different mite-borne viruses were detected, and 20% of the analyzed plants developed symptoms (Fig. 4A). Mean bulb weight after harvest was 44 g in the case of virus-free clone 1 (control). Both 2 and 3 were infected with one or two mite-borne viruses, and were almost the same bulb weight as the control. In contrast, the mean bulb weight of clone 5, in which three different mite-borne viruses were identified was 30 g, a loss of 33.5%. A 14% loss of bulb weight was observed in clone 4,

Fig. 2. Characterization of seven coat protein bands from 38 to 32 kDa in Western blots. **A**, Using prepared garlic virus-specific polyclonal antiserum in Western blot, seven protein bands were detected. **B**, Indexing for four viruses, *Leek yellow stripe virus* (LYSV), *Onion yellow dwarf virus* (OYDV), GV1-*Carlavirus*, and mite-borne viruses (Mite) by reverse transcription-polymerase chain reaction. **C**, Characterized coat protein bands represented schematically.

Table 1. Viral coat proteins detected in Western blot analysis using prepared garlic virus-specific polyclonal antiserum and four viruses detected by reverse transcription-polymerase chain reaction (RT-PCR) analysis

Analysis	Bands detected	Virus name	Plant number ^a									
			J-5	B-17	B-15	B-10	B-16	A-9	A-18	D-8	D-6	D-13
Western blot	38 (kDa)	LYSV	+	+	+	+	+					
	36	Mite 1 ^b						+				
	35.5	OYDV1		+			+					
	35	GV1 or Mite 2 ^b			+				+		+	
	34	OYDV2	+			+						
	33	Mite 3 ^b				+	+		+	+	+	
RT-PCR	32	Mite 4 ^b				+	+		+	+	+	
	1,023 (bp)	LYSV	+	+	+	+	+					
	601	OYDV	+	+		+	+					
	773	GV1 <i>carlavirus</i>			+				+			
	180	Mite 1-4 ^b				+	+	+	+	+	+	

^a Blank spaces = not detected.

^b Mite-borne viruses.

Fig. 3. Disease symptom development and bulb weight of meristem-tips derived clones cultivated in open fields. **A** to **C**, Percentages of plants with disease symptoms. **D** to **F**, mean bulb weights. Garlic plants derived from meristem-tips culture were cultivated in three fields, Rokunohe, Tenmabayashi, and Takko. These *Leek yellow stripe virus*-infected plants were collected after 1, 2, and 5 consecutive cultivation years. Infection by other viruses was negligible.

Fig. 4. Effects of mixed virus infections on disease symptom development and bulb weight. **A**, Percentage of disease symptoms. **B**, Percentage of mean bulb weight. **C**, Clone 1 is virus free and derived from meristem-tips (control). Clones 2 to 10 were infected by various combinations of viruses.

which also proved to be infected with three different mite-borne viruses (Fig. 4B).

Clone 6 was infected only with LYSV and disease symptoms were observed in 65% of the plants examined, but bulb weight was not affected. Both LYSV and a mite-borne virus were found in clone 7, and obvious disease symptoms were observed in most of the plants tested (Fig. 4A), but yield loss was estimated to be only 9% (Fig. 4B). By contrast, mixed infection of LYSV and OYDV (clones 8 and 9) resulted in severe symptoms in all plants (Fig. 4A), with a yield loss of 33%. Clone 10, infected with three viruses, LYSV, OYDV, and a mite-borne virus, showed the most severe disease symptoms and a yield loss of 44%.

DISCUSSION

Seven classes of protein bands were detected and identified as LYSV, OYDV, GV1-*Carlavirus*, and mite-borne viruses. Proteolytic degradation was not evident, and each protein band in the Western blots indicated one specific virus CP band, except for 35-kDa protein band which was identified from both the mite-borne virus (Mite 2) and the GV1-*Carlavirus*. Fortunately, RT-PCR techniques discriminated between these two CPs.

OYDV-O and OYDV-G were isolated from onion and garlic plants, respectively. DNA sequences of CP genes of OYDV-O and OYDV-G shared an 88% homology (10). We designed RT-PCR primers to amplify the common central region of the CP genes of both OYDV strains (21). The RT-PCR data obtained using OYDV-specific primers correlated with the presence of 35.5- and 34-kDa protein bands. Kobayashi et al. (10) detected OYDV-G and OYDV-O CP bands with Western blot tests using OYDV-G antibody, the molecular weight of OYDV-G was larger than that of OYDV-O. Therefore, the OYDV-1 35.5-kDa and OYDV-2 34-kDa protein bands in this work may correspond to be OYDV-G and OYDV-O, respectively. It is also possible that both OYDV 1 and OYDV 2 are subtypes of OYDV-G, if the OYDV-O is the onion-specific virus (24). If differences in apparent molecular mass reflect the amino-terminal variation of the amino-acid sequence of their CPs, one may be a new strain of OYDV-G.

Meristem-tip derived virus-free clones dramatically improve garlic production. Peiwen et al. (17) and Walkey and Antil (27) reported that yields of virus-free garlic increased 25 to 80% and 35 to 89%, respectively, compared with infected garlic. However, re-infection in plants in the field is the major factor that discourages use of virus-free clones. LYSV was found at the highest frequency in all the different tested fields in this study. Disease symptoms were observed in proportion to the infection rate of LYSV, but the apparent reduction in bulb

weight was not directly related to the infection of LYSV.

A mixed infection of LYSV and OYDV led to severe disease symptoms and yield reduction. In France, a 17 to 54% reduction of bulb weight was due to LYSV, and a 39 to 60% reduction was due to OYDV (7). Taken together, OYDV is thought to exhibit more severe disease symptoms and yield reductions than LYSV.

Four types of mite-borne viruses considered to be closely related to *Shallot virus X* (ShVX) (18,22). They were classified as a latent virus, but their disease symptoms and yield reductions were not described (25,26). Our data are consistent with the report that infection by a single mite-borne virus does not cause apparent symptoms. Infections with three different mite-borne viruses, however, exhibited weak mosaic symptoms in April in Aomori and resulted in reduced bulb weights. Accurate indexing for individual infecting viruses is needed to fully understand the damage effects of each virus on both disease symptoms and bulb production.

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