Characterization of *Melon necrotic spot virus* Occurring on Watermelon in Korea

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*Melon necrotic spot virus* (MNSV) was recently identified on watermelon (*Citrullus vulgaris*) in Korea, displaying as large necrotic spots and vein necrosis on the leaves and stems. The average occurrence of MNSV on watermelon was found to be 30–65% in Hapcheon and Andong City, respectively. Four isolates of the virus (MNSV-HW, MNSV-AW, MNSV-YW, and MNSV-SW) obtained from watermelon plants in different areas were non-pathogenic on ten general indicator plants, including *Chenopodium quinoa*, while they infected systematically six varieties of *Cucurbitaceae*. The virus particles purified by 10–40% sucrose density gradient centrifugation had a typical ultraviolet spectrum, with a minimum at 245 nm and a maximum at 260 nm. The morphology of the virus was spherical with a diameter of 28–30 nm. Virus particles were observed scattered throughout the cytoplasm of watermelon cells, but no crystals were detected. An ELISA was conducted using antiserum against MNSV-HW; the optimum concentrations of IgG and conjugated IgG for the assay were 1 μl/ml and a 1:8,000–1:10,000 dilutions, respectively. Antiserum against MNSV-HW could capture specifically both MNSV-MN from melon and MNSV-HW from watermelon by IC/RT-PCR, and they were effectively detected with the same specific primer to produce product of 1,172 bp. The dsRNA of MNSV-HW had the same profile (4.5, 1.8, and 1.6 kb) as that of MNSV-MN from melon. The nucleotide sequence of the coat protein of MNSV-HW gave a different phylogenetic tree, having 17.2% difference in nucleotide sequence compared with MNSV isolates from melon.

**Keywords**: *Melon necrotic spot virus*, purification, phylogenetic tree, watermelon, symptom

*Melon necrotic spot virus* (MNSV) is a species of the genus *Carmovirus* in the family *Tombusviridae*. MNSV was originally reported in *Cucumis melo* and *Cucumis sativus* in Japan (Kishi, 1966), and in *Citrullus vulgaris* (Avgelis, 1989). Experimentally, MNSV has been shown to be virulent in host plants of the *Cucurbitaceae* family, including *C. melo*, *C. sativus*, *Cucumis lanatus*, *Cucurbita moschata*, *Lagenaria siceraria*, *Vigna unguiculata*, and *Vigna sesquipedalis* (Hibi, 1986). However, MNSV has a much narrower host range, and it is transmitted through causal agents such as seed and the soil fungus *Olpidium radicale* (Gonzalez-Garza et al., 1979). MNSV, which has been reported worldwide, including in Asia, Europe, and Latin America (Herrera et al., 2010), typically causes necrotic spots on the leaves, stems, and fruits of cucurbits, causing serious economic damage with losses reaching 100%. A few necrotic spots on leaves and stems can enlarge over time and form on the outer skin of watermelon at the fruit ripening stage. Watermelon fruit showing necrosis can become decayed on the red-colored inner flesh. Symptoms occur suddenly in watermelon at the fruit ripening stage, and, for that reason, simple control methods such as containing and eradicating diseased plants cannot be used at
the early growth stage to diminish economic losses.

MNSV first occurred on melon cultivated in a plastic house in southern Naju in Jeollanam-do Province of Korea, in 2001 (Choi et al., 2003). The primary source of the MNSV detected on the melon plants in Naju was infested seeds imported from Japan. After the first occurrence of MNSV on melon in 2001, it spread continuously through the major melon cultivation areas in Korea and occurred nationwide within 5–6 years. Severe symptoms, including necrotic spots on the leaves of watermelon plants, arose suddenly in Hapcheon County, Kyeongsangnamdo Province, in 2005. Following its initial occurrence on watermelon plants in Hapcheon County, MNSV occurred in Andong, Kyeongsangbukdo Province, in 2006, and continuously in different areas of Yanggu County in Kangwondo Province, Gochang County, and Iksan in North Jeolla Province in 2007, with an incidence rate of 2–90% throughout Korea (Kim et al., 2008).

In this study, we characterized the MNSV Korean isolates from watermelon based on biological, serological, cytopathological and molecular properties.

Materials and Methods

**Biological testing.** Leaves or fruits of watermelon plants exhibiting necrotic spots were macerated in 4 volumes of 0.01 M sodium phosphate buffer, pH 7.0, with a chilled mortar and pestle. The sap was filtered through a membrane (0.2 μm) and then inoculated to watermelon using powdered (600-mesh) carborundum. A single portion of the inoculated leaves was inoculated to healthy seedlings of watermelon with three transfers and the biologically infected tissues were homogenized. The crude sap was centrifuged at 8,000 × g for 20 min. Next, 8% PEG6000 was added with 200 mM NaCl to the supernatant and stirred for 1 h on ice. After centrifugation at 8,000 × g for 20 min, the pellets were suspended in 0.2 M sodium acetate, pH 5.0. The supernatant was layered on 20% sucrose in 0.01 M Tris-HCl, pH 7.3, for molecular sieve filtering and then centrifuged at 35,000 rpm for 1 h. The pellets were suspended in 0.01 M Tris-HCl, pH 7.3, and then ultracentrifuged at 25,000 rpm for 2 h after centrifugation at 8,000 rpm for 10 min. The milky virus band was diluted with 0.01 M Tris-HCl, pH 7.3, and centrifuged at 35,000 rpm for 50 min. The pellets were suspended in 0.01 M Tris-HCl, pH 7.3, and used for electron microscopy and antiserum production.

**Antiserum production and ELISA.** For the production of antiserum against MNSV-HW, the purified virus (1 μg/ml) was injected intravenously twice and intramuscularly three times, alternating at weekly intervals into a young rabbit. Total bleeding was done at 6 weeks after the first injection. For ELISA, IgG was purified using a commercial protein A-IgG purification kit. Infected leaves were homogenized in phosphate buffer, pH 7.4, containing 0.05% Tween 20 and 2% polyvinylpyrrolidone using a chilled mortar. Crude sap from the infected leaves was diluted 10–1,000 times. The concentrations of IgG used were 1 and 2 μg/ml. The dilutions of conjugated IgG used were 2,000–10,000 with five steps. Next, 200 μl of crude sap were added to the wells of microplates after coating with gamma globulin (IgG). The plate was incubated overnight in a refrigerator. After washing, 20 μl of enzyme-labeled IgG were added. P-Nitophenyl phosphate was used as a substrate.

**dsRNA analysis.** dsRNA was extracted from leaves of watermelon showing necrotic spots (Morris and Dodds, 1979). Electrophoresis of dsRNA from watermelon infected with MNSV-HW was carried out using dsRNA of *Cucumber mosaic virus* as a marker on the gel plate of 6% polyacrylamide in 1 M Tris-EDTA buffer, pH 8.0, containing 0.83 M boric acid and 10 mM EDTA. The dsRNA bands were stained with a Bio-Rad silver stain kit (Hercules, CA).
**Immuno-capture (IC)/RT-PCR and RT/PCR.** Infection with MNSV was confirmed serologically and genetically by IC/RT-PCR and RT-PCR, respectively (Cho et al., 2006). For IC/RT-PCR, 50 µl of IgG were added to a microtube, which was then incubated for 120 min at 37°C. After washing with 0.01 M PBS-T, pH 7.0, 50 µl of crude sap from watermelon showing necrotic spots were added per tube and the mixture was incubated for 120 min at 37°C. Total RNA from watermelon showing viral symptoms was extracted using a commercial kit from Qiagen (Hilden, Germany). The primer set used to detect MNSV by IC/RT-PCR and RT-PCR was designed to detect the coat protein (CP) gene of the virus: I (5’-ATGCGTTTAACCATCGCCAT-3’) and II (5’-TAGGCGAGGTAGGCGGTTTCA-3’). cDNA amplification was conducted using the Access Quick RT-PCR system (Promega, Madison, WI). The RT-PCR conditions were: 48°C for 45 min, followed by 94°C for 2 min and 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s, with a final hold at 72°C for 7 min (Choi et al., 2003).

**Results and Discussion**

**Disease occurrence.** The first occurrence of MNSV in Korea occurred on watermelon plants grown in five plastic houses in Hapcheon County, Gyeongsangnamdo Province, in April of 2005 with an average infection rate of 30.0% (Table 1). But, through the investigation of virus disease incidence in the same house in the following year, we confirmed that the incidence could be reduced effectively by up to 2.0% using paddy rice cultivation. In June 2006 in another area of Andong, MNSV also occurred on watermelon plants with an average infection rate of 60.0% in eight plastic houses; almost none of the watermelon fruits grown in four of the houses could be used commercially due to an infection rate of 100%. MNSV can be transmitted by infected seeds and by the soil fungus *O. radical* (Gonzalez-Garza, 1979). Even when healthy seeds of watermelon are used, fungal vectors can be controlled using the cultural practice of grafting with gourd stocks. In addition, soil sterilization using methyl bromide can raise yields by 58–115% (Huitron et al., 2009). The incidence of MNSV on watermelon could be reduced effectively from 20–100% to 2.0% by crop rotation with paddy rice (Table 1). Thus, to reduce the virus incidence caused by continuous cropping, rice crop rotation and soil sterilization have been used and reported (Park et al., 2010).

| Area investigated | Date investigated | Average incidence rate (%) | Symptom occurrence |
|-------------------|-------------------|-----------------------------|--------------------|
| Hapcheon          | April 12, 2005    | 30.0                        | NS, FN             |
|                   | April 8, 2006     | 2.0                         | NS, FN             |
| Andong            | June 13, 2006     | 65.0                         | NS, FN             |

*NS, necrotic spots on leaves and stems; FN, fruit necrosis at the inner flesh. Virus infection was judged by visual inspection, serological testing, and RT-PCR.

*The incidence decreased after rice cultivation in a paddy field.

*Watermelon could not be harvested from four houses out of eight with 100% infection.

**Symptoms of MNSV infection in field-grown watermelon.** The symptoms produced by MNSV on watermelon were necrotic spots on the leaves and stems (Fig. 1A) that spread to the main stem of the fruit (Fig. 1B). Cross-sections revealed necrotic spots on all parts of the infected watermelons during the early growth stage (Fig. 1C), while at later stages of infection, the necrotic spots on the watermelon fruits seemed to be preferentially induced on the outer fleshy rind (Fig. 1D). Seeds were collected from mature watermelons showing signs of necrosis on the flesh due to infection by MNSV-AW (Fig. 1E), and seeds were collected from watermelons of the same cultivar but without symptoms on the flesh (Fig. 1F). The seeds from the infected watermelons had an unevenly colored skin, large discolored spots, and, occasionally, raised spots. The discolored spots mingled into an enlarged spot on the seed coat. No difference in seed shape or size was detected between the infected and healthy seeds.

**Host range and symptoms.** Because the necrotic spots on the watermelons were similar to those caused by the bacterial disease watermelon blotch, the crude sap of watermelon fruits showing evidence of necrotic spots was filtered through a sterilized bacteria-proof Millipore membrane (Billerica, MA) and a large volume syringe. Necrotic spots and vein necrosis were produced on watermelon seedlings by mechanical inoculation with filtered sap. The necrotic spots were purified biologically using three transfers to watermelon seedlings and used as a virus source. The virulence of four MNSV isolates (MNSV-HW, MNSV-AW, MNSV-YW, and MNSV-SW) from watermelon was compared with that of MNSV-MN from melon (Table 2). The four isolates from watermelon could not produce any symptoms on ten indicator plants, including *Chenopodium*...
amaranticolor, Gomphrena globosa, and Nicotiana rustica. The MNSV Korean isolates from watermelon have a narrow host range outside of cucurbits compared with other isolates occurring in Japan and Europe (Avgelis, 1989).

However, the MNSV Korean isolates from watermelon have varied in virulence by the broader pathogenicity within the Cucurbitaceae family, which includes melon, cucumber, squash, and gourd (Table 2 and Fig. 2). All four MNSV isolates induced large necrotic spots on inoculated cotyledons of C. vulgaris (Fig. 2A) and L. leucantha (Fig. 2F), and this was followed by plant death. On Cucurbita pepo, MNSV-HW and MNSV-AW produced large necrotic spots (Fig. 2E1 and E2), while MNSV-YW and MNSV-SW induced small necrotic spots (Fig. 2E3 and E4), on inoculated cotyledons. Pinpoint necrotic spots were produced on inoculated cotyledons of C. sativus (Fig. 2C) and C. moschata (Fig. 2D) by all four isolates of MNSV from watermelon. The MNSV from watermelon displayed the weakest virulence on inoculated cotyledons of C. melo; only a few pinpoint necrotic spots were produced by MNSV-HW (Fig. 2B1) and MNSV-SW (Fig. 2B4), and a few by MNSV-AW (Fig. 2B2) and MNSV-YW (Fig. 2B3). The pinpoint necrotic regions observed on the inoculated leaves of C. melo were of the same severity as those caused by watermelon isolates from Greece (Avgelis, 1989) and Japan (Ohki et al., 2008), as well as a melon isolate (Choi et al., 2003).

According to the previous reports about the pathogenicity of MNSV occurring naturally on three cucurbits (watermelon, melon, and cucumber), it was found that MNSV
isolated from watermelon could systemically infect watermelon but not melon or cucumber, and that MNSV from cucumber could systemically infect both melon and cucumber but not watermelon (Avgelis, 1989; Bos et al., 1984; Kishi, 1966; Ohki et al., 2008; Tomlinson and Thomas, 1986). Korean MNSV isolates from watermelon systemically infected melon and cucumber with weak virulence and caused a few necrotic spots on the inoculated and upper leaves (Fig. 3A and B). The weak symptoms on melon caused by MNSV from watermelon at 25–28°C in this study seemed not to be affected by the relatively high temperature used for mechanical inoculation compared to the enhanced systemic infection detected at 20 or 25°C during the resistance screening of melon seedlings (Mallor et al., 2003). The critical temperature for symptom expression in melon seedlings inoculated mechanically with MNSV is still in doubt. Nevertheless, at relatively high temperatures of 25–28°C, Korean MNSV from watermelon could produce systemic symptoms in melon seedlings with a few necrotic spots (Fig. 3A and B) and severe symptoms in watermelon seedlings, including leaf and stem necrosis (Fig. 3C and D). In addition, necrotic spots on watermelon grafted onto rootstock of gourd, L. siceraria, were observed in Korea in this study, but the causal agent of MNSV could not be detected by passing to melon in Japan (Ohki et al., 2008). MNSV could be controlled by grafting using rootstock of the gourds ‘RS841’ and ‘Shintosa Cameloforce,’ resulting in yields of 58–115% without chemical soil fumigation (Huitron et al., 2009). The control of MNSV in Korea by the grafting of watermelon scions onto gourd stock seems to be inadequate because the MNSV occurring on watermelon displayed strong virulence, including systemic symptoms such as stem necrosis (Fig. 3E).

Based on the symptoms observed on cucurbits, the pathogenic mutation of Korean MNSV from watermelon seems to be ongoing.

**Virus purification and particle morphology.** Purified MNSV-HW could be obtained from the milky virus band using ultracentrifugation with 10–40% sucrose density gradients (Fig. 4A). The purified virus had a typical ultraviolet absorption spectrum, with a minimum at 245 nm and a maximum at 260 nm (Fig. 4B). The rate at 260/280 was approximately 1.14.

The morphology of the MNSV-HW particles was iso-
metric, with a diameter of 28‒30 nm (Fig. 4C). Virus particles were detected in the cytoplasm of the host cells, but viral crystals were not observed (Fig. 4D).

ELISA. Antiserum against MNSV-HW was produced and its IgG purified using a commercial protein purification kit. The optimum dilution of purified IgG and conjugated IgG for ELISA was 1 µl/ml and a 1:8,000 dilution, respectively (Fig. 5). No reaction or yellow color occurred in the negative controls of healthy plant sap and PBS-Tween 20. An ELISA for MNSV-HW could be conducted specifically from test samples (i.e., crude sap from infected watermelon leaves) diluted up to 1,000 times.

dsRNA pattern, IC/RT-PCR and RT-PCR. The electrophoretic migration of dsRNA from MNSV-HW revealed three genomes, approximately 4.5, 1.8, and 1.6 kb in length (Fig. 6A). The molecular sizes of the three dsRNAs of MNSV-HW were identical to those of MNSV-MN from melon (Choi et al., 2003), a German isolate (Riviere et al., 1989), and Japanese isolate (Matsuo et al., 1991). Antiserum against MNSV-HW from watermelon could capture specifically both MNSV-MN from melon (Fig. 6B) and MNSV-HW from watermelon (Fig. 6C) by IC/RT-PCR. A 1.172-kb fragment was produced using the MNSV primer by IC/RTPCR and RT-PCR (Fig. 6D).

Phylogenetic relationship of the CP gene. Four isolates of the virus (MNSV-HW, MNSV-AW, MNSV-YW, and MNSV-SW) collected from watermelon plants showed over 96% nucleotide sequence identity in coat protein region (data not shown). Among them, the nucleotide sequence of the CP gene (p42) from MNSV-HW was compared with those of seven isolates of MNSV from melon uploaded from the NCBI database (Fig. 7). A phylogenetic analysis revealed 82.8% homology between the MNSV-
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Divergence in the nucleic acid sequences encoding p42 placed MNSV-HW from watermelon in a different phylogenetic tree branch than the MNSV isolates from melon. Among the MNSV isolates from melon, the European isolates from Spain and The Netherlands were separated from the

Fig. 3. A few necrotic spots on the inoculated leaf (arrow in A) and the upper leaves of melon (arrow in B) were induced by MNSV-HW. Necrotic spots and vein necrosis were produced on the inoculated leaves of watermelon (C), necrosis on the upper leaves was followed by plant death (D), and stem necrosis on gourd (E) after mechanical inoculation with MNSV-HW.

Fig. 4. A milky virus band of MNSV-HW was formed after 10–40% sucrose density gradient centrifugation at 25,000 rpm (A). Purified MNSV-HW diluted 10 fold had the typical ultraviolet absorption spectrum, with a maximum at 260 nm and a minimum at 245 nm (B). Purified virus particles of MNSV-HW were isometric, with a diameter of 28–30 nm (A). The isometric virus particles were present in the cytoplasm of watermelon cells (B). Black bar = 200 nm. White bar = 1,000 nm.

Fig. 5. ELISAs using antiserum against MNSV-HW. The optimum concentrations of IgG and conjugated IgG were 1 µl/ml and a 1:8,000–10,000 dilution, respectively.

HW from watermelon and MNSV from melon. Divergence in the nucleic acid sequences encoding p42 placed MNSV-HW from watermelon in a different phylogenetic tree branch than the MNSV isolates from melon. Among the MNSV isolates from melon, the European isolates from Spain and The Netherlands were separated from the
Korean and Japanese isolates.

The percent homology with the p42 nucleotide sequence from European and Latin American isolates was 93% (Herrera et al., 2006) and 98% (Herrera et al., 2010), respectively. The percent homology of the European and Latin American isolates and Japanese isolates was relatively low at 75% (Herrera et al., 2010). The genotype of MNSV could be divided geographically into European and Latin American isolates and Japanese isolates with a homology of 74% regardless of whether it was originally isolated from melon or watermelon. However, MNSV from watermelon in Japan was grouped differently in the phylogenetic tree (Herrera et al., 2010).

The difference in p42 nucleotide sequence between Korean watermelon isolates of MNSV-HW and melon isolates from Europe, Japan, and Korea was relatively low at 17.2% compared to 36% between the European and Latin American isolates and Japanese isolates. The percent identity between p42 of MNSV-HW from watermelon and p42 of MNSV from melon was 73.5% for Korean melon, 72.0–74.6% for Japanese melon, and 75.5–75.6% for European melon (data not shown). The genetic diversity based on complete genome sequences among the isolates of MNSV from watermelon should be studied further in relation to their virulence in Cucurbitaceae.

In the present study, we analyzed the characteristics of the Korean MNSV isolates collected from watermelon plants. Our findings revealed that Korean MNSV isolates from watermelon were the serologically closely related to the MNSV isolate from melon using ELISA and IC/RT-PCR, and had the same dsRNA pattern with MNSV isolate from melon. However, they are distantly from the MNSV isolate from melon in pathogenicities on Cucurbitaceae family and phylogenetic tree based on nucleotide sequence of CP region. These results suggested that Korean MNSV might have introduced via the different routes or undergone divergence on infecting different host plants.

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