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Functional degeneration of the resistance gene *nsv* against *Melon necrotic spot virus* at low temperature

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Abstract The single recessive gene, *nsv*, which confers resistance against *Melon necrotic spot virus* (MNSV), has recently been used to develop virus-resistant melon cultivars in Japan. However, the Chiba isolate of MNSV, a common isolate in Japan, infected resistant cultivars when inoculated melon plants were grown at 15°C. Viral RNAs accumulated in protoplasts from resistant cultivars at both 15 and 20°C. Mechanical inoculation of the cotyledons caused MNSV to spread throughout the leaves at 15°C, but not at 20°C. These results support our novel hypothesis that a temperature-sensitive inactivation of disease resistance genes occurs at the *nsv* locus in melon cultivars with the resistance gene grown at temperatures below 20°C.

Keywords Cell-to-cell movement · Methyl bromide · *Olpidium bornovanus* · Protoplast · Recessive resistance · Viral replication

Abbreviations

DAS-ELISA	double-antibody sandwich enzyme-linked immunosorbent assay
dpi	days post-inoculation
eIF4E	eukaryotic initiation factor 4E
hpi	hours post-inoculation
IgG	immunoglobulin G
MNSV	*Melon necrotic spot virus*
PBS	phosphate-buffered saline
USDA-ARS	United States Department of Agriculture Agricultural Research Station
UTR	untranslated region

Melon necrotic spot virus (MNSV) infection of melons (*Cucumis melo*) causes a necrotic spot disease, which leads to worldwide economic losses in melon production (Kishi 1966; Yosida et al. 1980; Avegelis 1985; Ryden and Person 1986). Though different types of symptoms are observed at each

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growth stage of melon plants, the most common symptom of MNSV infection is necrosis of the large mesophyll and veins of true leaves (Kishi 1966; Furuki 1981).

MNSV has spherical particles, 30 nm diam, and belongs to the genus *Carmovirus* in the family *Tombusviridae*. It is transmitted by contaminated seeds or underground by the soil-borne obligate fungus *Olpidium bornovanus* (Furuki 1981; Campbell and Sim 1994; Campbell et al. 1996). Soil fumigation by methyl bromide, used between cultivations, is the only chemical treatment that effectively prevents MNSV from spreading. However, since 2005 the Montreal protocol forbids the use of this chemical in developed countries for environmental reasons, and consequently, alternative strategies to control spread of the virus disease are required.

Gonzalez-Garza et al. (1979) found that several melon cultivars were not infected by MNSV, which led to the identification of the single recessive resistance gene, *nsv* (Coudriet et al. 1981). The function of this resistance gene is to prevent the accumulation of viral RNAs at the single-cell level (Díaz et al. 2004). Recently, the cloning and molecular characterization of the *nsv* gene has been reported by Nieto et al. (2006). They found that the *nsv* gene is a variant of the eukaryotic initiation factor 4E (eIF4E), which is involved in translation. The *nsv* gene has been effectively used to develop MNSV-resistant melon cultivars through conventional crossings by commercial companies. However, a new strain, MNSV-264, has recently appeared in Spain that overcomes the resistance of melon plants carrying the *nsv* gene (Díaz et al. 2002).

We carried out cultivation tests in the winter season to detect newly-bred resistant melon plants in soils infested with MNSV and *O. bornovanus*. Serological diagnosis detected MNSV in these melon fruits despite a lack of visible symptoms on plants. This finding suggested two possibilities: the appearance of a new MNSV strain similar to MNSV-264, or the loss of resistance in melon plants affected by the low temperature or other environmental conditions. An isolate of MNSV from the fields failed to infect resistant melon cultivars at 25°C after mechanical inoculation of the cotyledons. Moreover, the genomic RNA sequence of the isolate was similar to a common Japanese isolate of MNSV. Therefore, to determine whether resistance was affected by low temperatures, we examined the infectivity of a common MNSV isolate in resistant melon plants carrying the *nsv* gene under two different temperatures (15 and 20°C). Our analysis focused on replication in melon protoplasts and local spreading through cotyledons.

First, we confirmed the influence of growth temperature on MNSV infection in resistant melon cultivars. The Chiba isolate of MNSV (MNSV-Chiba; Accession No. AB250684), a common isolate in Japan, was obtained from infected melon plants in Chiba prefecture, Japan. *Cucumis melo* cv. Miyabi-syunjyuukei (Yokohamaueki Co. Ltd., Kanagawa, Japan) was used as the susceptible melon (*Nsv*/- genotype). Resistant melons (*nsv*/*nsv* genotype) Perlita (NSL34588), PMR-5 (Ames26809) and Gulfstream (NSL60241) were provided by the United States Department of Agriculture–Agricultural Research Station (USDA–ARS) North Central Regional Plant Introduction Station. Cotyledons of seven day-old seedlings were mechanically inoculated with sap from MNSV-Chiba-infected melons, and inoculated seedlings were grown in growth chambers at two different temperatures (15 and 20°C) with a 16-h light/8-h dark regime. At 21 days post-inoculation (dpi), four parts of the plant (the roots, hypocotyls, cotyledons and first true leaf) were separately ground in phosphate-buffered saline (PBS; pH 7.0) containing 0.05% Tween 20 (PBST) at 1:20 (w/v) dilution. Infection of MNSV in each part of the plant was analyzed by a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA; Clark and Adams 1977) using an anti-MNSV immunoglobulin G (IgG) primary antibody and an alkaline phosphatase-conjugated anti-MNSV IgG secondary antibody (Japan Plant Protection Association, Tokyo, Japan). The enzyme reaction was carried out for 30 min and absorbance at 405 nm was then measured with an ELISA plate reader (Model 550, Bio-Rad, Hercules, CA). An absorbance value >0.1, which represented the average readings of healthy plants as negative controls plus triple the standard deviation, was considered indicative of a positive MNSV infection.

MNSV inoculation of susceptible melon plants resulted in systemic infection of all parts of the plants, regardless of growth temperature. In resistant melons, by contrast, MNSV was detected in all inoculated cotyledons tested at 15°C, but not at 20°C, supporting previous observations (Gonzalez-Garza et al. 1979). In PMR-5 melons, MNSV invaded the roots of one

Table 1 MNSV infection in parts of resistant or susceptible melon plants grown at two different temperatures

	15°C			20°C		
	Resistant		Susceptible	Resistant		Susceptible
Tissue	Perlita	PMR-5	Miyabi-syunjyuukei	Perlita	PMR-5	Miyabi-syunjyuukei
Upper leaf	0/10[a]	0/10	8/10	0/10	0/10	9/10
Cotyledon	15/15	15/15	15/15	0/15	0/15	15/15
Hypocotyl	0/10	0/10	9/10	0/10	0/10	9/10
Root	0/15	1/15	15/15	0/15	0/15	15/15

[a] Number of MNSV positives/inoculated melon plants.

plant out of 15, but did not infect the hypocotyls or the true leaves of the plant. These results showed that MNSV-Chiba could accumulate in the cotyledons of resistant melons inoculated at low temperature, allowing an infrequent systemic infection (Table 1).

We next analyzed replication of the virus in melon protoplasts from the susceptible cv. Miyabi-syunjyuukei and the resistant cv. Perlita at 15 and 20°C using northern blotting. Preparation of protoplasts, inoculation with in vitro transcript RNAs from the MNSV-Chiba complementary DNA infectious clone pTMNW1 (Mochizuki et al. 2008), extraction of total RNAs and RNA electrophoresis were performed according to the procedures previously described by Kido et al. (2008). Because of fine detection of RNA bands from a susceptible melon in northern blot analysis, total RNA extracted from 5×10^4 protoplasts of a susceptible melon was electrophoresed in per lane of a gel, but that from 2.5×10^5 protoplasts of a resistant melon was applied. The membrane with RNAs transferred from the gel was stained once with 0.02% methylene blue solution for visualization of 28S ribosomal RNA (rRNA). Viral genomic and subgenomic RNAs on the membrane were then detected by an α-^{32}P-labelled riboprobe specific to the coat protein gene, which was synthesized in vitro from pMNCP1 (Kido et al. 2008). Hybridization procedures were performed as previously described by Hamilton and Baulcombe (1999). Detection was carried out using the VersaDoc 5000 (Bio-Rad Laboratories).

In susceptible melon protoplasts, large amounts of MNSV RNAs accumulated at all time points at both temperatures (Fig. 1). Unexpectedly, MNSV RNAs were also detected in resistant melon protoplasts at both temperatures, although at a lower quantity than in susceptible melons. Previously, viral RNAs of a Spanish isolate, MNSV-Mα5, were unable to accumulate in the protoplasts of resistant melons at 27/25°C, suggesting that *nsv* gene resistance provides immunity at the single-cell level (Díaz et al. 2004). To confirm this inconsistency between the two isolates, MNSV-Chiba was inoculated into resistance melon protoplast at 25°C; viral RNAs were then detected in the same manner as at 20°C (data not shown), indicating that replication of MNSV-Chiba occurred intracellularly in resistant melon, regardless of temperature. Previously, it was reported that the viral genomic determinant for overcoming *nsv* resistance was located at the 3′-

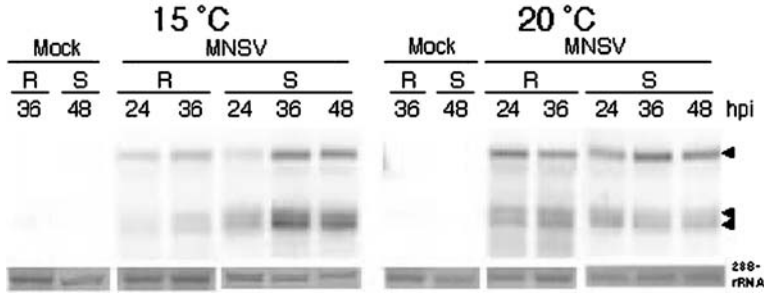

Fig. 1 Northern blot analysis of MNSV RNA extracted from resistant (R: Perlita) or susceptible (S: Miyabi-syunjyuukei) melon protoplasts infected with MNSV-Chiba. Protoplasts were maintained at 15 or 20°C, in the dark, and then sampled at 24, 36 and 48 hpi. 28S ribosomal RNA was stained with 0.02% methylene blue. The MNSV genome and subgenomic RNAs (*black arrowheads*) were detected by an α-^{32}P-labelled riboprobe specific to the coat protein gene

untranslated region (UTR) as the nucleotide sequence (Díaz et al. 2004). When the nucleotide sequence of 5′- and 3′-UTRs between MNSV-Mα5 (Accession No. AY122286; Díaz et al. 2003) and MNSV-Chiba was compared, these identities were approximately 75 and 85%, respectively, even though each protein coding region was >85% similar in nucleotide sequence and 90% in amino acid sequence, respectively (data not shown). Therefore, the contrast in the ability to replicate between the two viruses in resistant melon cultivars may be due to the difference in 3′-UTR sequence of the MNSV genome. Perhaps the 3′-UTR of MNSV-Chiba interacts with mutated eIF4E, but that of MNSV-Mα5 does not.

Next, we investigated MNSV local infection and its spread into the inoculated cotyledons of resistant melons at different temperatures. Cotyledons of the cvs Miyabi-syunjyuukei and Perlita were inoculated with MNSV-Chiba, and symptom development was observed at different temperatures (Fig. 2a, left panels). In Miyabi-syunjyuukei melons, necrotic spots appeared at 5 and 7 dpi, at 15 and 20°C, respectively. In Perlita melons, tiny chlorotic spots began to develop 7 dpi at 15°C, and continued to expand until 21 dpi (Fig. 2a, white arrowheads). This was also observed in the other resistant cvs PMR-5 and Gulfstream (data not shown). No visible symptoms developed on the cotyledons of these melons at 20°C.

To compare the distribution of MNSV in cotyledons at two different temperatures, leaf vacuum blot analysis was carried out using ten leaves per experiment (Kido et al. 2008; Fig. 2a, right panels). In Miyabi-syunjyuukei melons, MNSV was detected in the areas that overlapped the necrotic spots at both

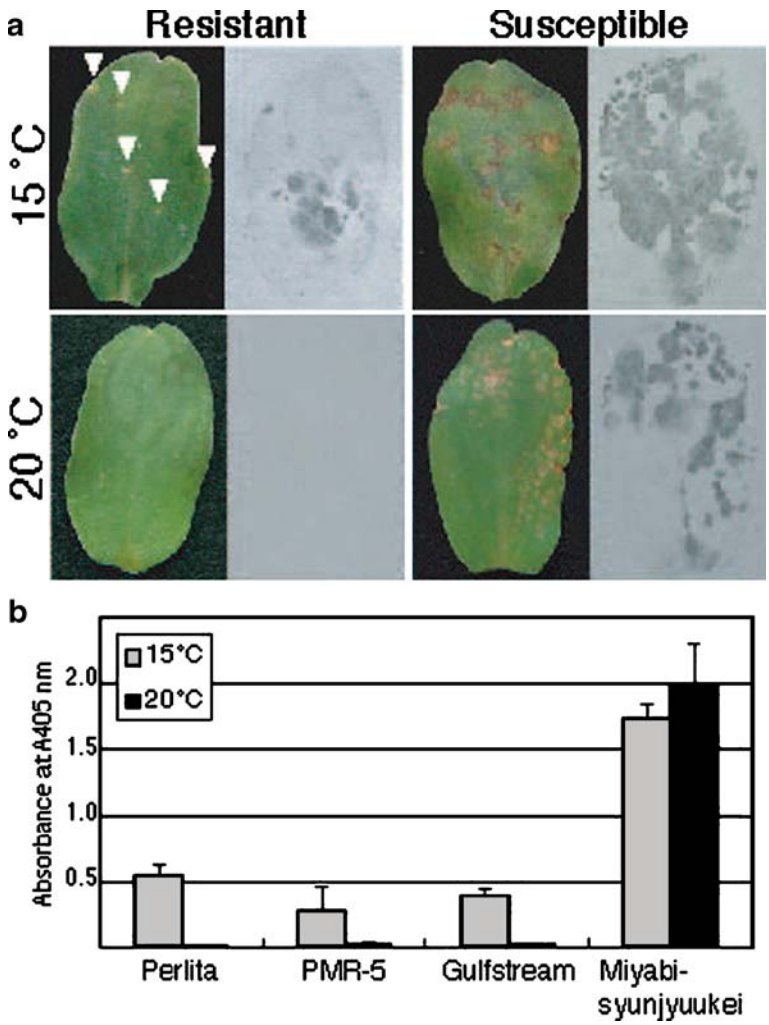

Fig. 2 **a** Formation of the local lesion and distribution of MNSV-Chiba in inoculated cotyledons of resistant (Perlita) or susceptible (Miyabi-syunjyuukei) melons grown at 15 or 20°C. The distribution of MNSV at 21 dpi was detected by vacuum blot analysis. The panels on the left show the local lesion of the cotyledons before the immuno-detection of MNSV. The *white arrowheads* show chlorotic spots on an inoculated cotyledon at 15°C. The panels on the right show MNSV immuno-detection corresponding to the leaf in the panel on the left (the positive areas are darkly stained). **b** Accumulation of MNSV in inoculated cotyledons of resistant or susceptible melons at 15 or 20°C. The absorbance value of the DAS-ELISA was averaged across ten inoculated cotyledons at 21 dpi

15 and 20°C. In Perlita melons at 15°C, MNSV infection was also observed around the chlorotic spots in the cotyledons, although the area of virus accumulation was smaller than that of susceptible melon leaves. At 20°C, MNSV infection was not detected in the resistant cotyledons. DAS-ELISA was used to quantitatively determine the amount of MNSV in cotyledons using ten inoculated cotyledons per cultivar (Fig. 2b). MNSV was abundant in the cotyledons of Miyabi-syunjyuukei melons at both 15 and 20°C. By contrast, MNSV was present at low levels in Perlita melon cotyledons at 15°C, and was undetectable at 20°C, supporting the results of the leaf vacuum blot analysis. The nucleotide sequence of MNSV obtained from chlorotic spots on the cotyledons of resistant melons at 15°C was identical to the original sequence of the parental virus, MNSV-Chiba (data not shown), indicating that MNSV infection and spread did not occur as a mutated viral strain.

Recently, the *nsv* gene has been identified as the eIF4E (Nieto et al. 2006). The difference between resistance or susceptibility is governed by a single amino-acid substitution in the protein (Nieto et al. 2006, 2007). Until now, many recessive resistance genes against plant viruses have been identified as eukaryotic initiation factors or their relatives (Robaglia and Caranta 2006), and the function of those genes was estimated to be deficient in viral replication in a single cell level (Sato et al. 2003; Kanyuka et al. 2005; Stein et al. 2005; Albar et al. 2006). Among them, however, some eukaryotic initiation factors have been involved with viral cell-to-cell movement in *Pea seed-borne mosaic virus* and *Cucumber mosaic virus* (CMV) (Gao et al. 2004; Yoshii et al. 1998, 2004). The cell-to-cell movement of CMV in *Arabidopsis cum1* mutant, which is an eIF4E mutated gene, decreased by inefficient production of CMV 3a movement protein (Yoshii et al. 1998, 2004). In the case of the *nsv* gene, MNSV-Chiba could replicate in a resistant melon cell possessing the gene as shown in Fig. 1, whereas the virus could not spread beyond the inoculated cotyledon at 20°C (Fig. 2a), suggesting that the *nsv* gene restricts a cell-to-cell movement of MNSV. Recently, it has been reported that cell-to-cell movement of MNSV is managed by the double gene block, 7A and 7B proteins, based on subgenomic viral RNA expression (Genovés et al. 2006; Navarro et al. 2006). The *cum1* gene, mentioned above as a variant of eIF4E, is responsible for the inefficient translation of RNA3, rather than that of RNA4 for CP. The mechanism for this may be related to the cell-to-cell movement protein CMV 3a from RNA3 which is inefficient due to a misidentification of the specific secondary structure formed by its 5′-UTR, maybe together with its 3′-UTR, leading to resistance in *Arabidopsis* (Yoshii et al. 2004). Accordingly, being an eIF4E variant, the *nsv* gene might restrict local movement of MNSV-Chiba upon the translational control of the subgenomic RNA for the double gene block.

It has previously been demonstrated that binding of human recombinant eIF4E to cap analogue is temperature-dependent, meaning that eIF4E might be more tightly connected with the cap structure under relatively low-temperatures (Friedland et al. 2005). Therefore, it is reasonable that the *nsv* gene interacts more efficiently with the MNSV genome at low temperatures, which explains how MNSV-Chiba invades resistant melons at 15°C in our findings.

It is still unclear whether the *nsv* resistance gene restricts MNSV replication, cell-to-cell movement or both. Further detailed studies are needed to uncover the mechanisms behind the *nsv* resistance to MNSV in melon plants.

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