COMPARATIVE STUDY ON GENOMES OF TWO JAPANESE MELON NECROTIC SPOT VIRUS ISOLATES

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Summary. – Nucleotide sequences of the genomes of two Japanese Melon necrotic spot virus (MNSV) isolates, NH and NK were determined. The open reading frames (ORFs) in both genomes encode five proteins: p29 (the pre-readthrough domain of p89), p89 (the readthrough domain of p89 identified as the putative RNA-dependent RNA polymerase), p14 (the pre-readthrough domain of p7A), p7A (the putative movement protein), and p42 (coat protein, CP). Nucleotide and amino acid sequence identities of the five proteins of NH and NK isolates were estimated at 97.4– 99.5% and 97.7–100 %, respectively. NK isolate but not NH isolate infected systemically leaves of *Cucumis melo* plants. When deduced amino acid sequences of p7A proteins of NH and NK isolates were compared, only one difference at position 16 (serine in NH isolate and isoleucine in NK isolate) was observed. p7A protein is considered the putative movement protein. The serine of p7A protein of NH isolates may be involved in systemic infection. In addition, phylogenetic relationships of genes based on nucleotide sequences revealed that NH and NK isolates might form a group, and S isolate, serologically different from NH and NK isolates, might represent a distinct isolate not belonging to this group.

Key words: Melon necrotic spot virus; NH isolate; NK isolate; movement protein; phylogenetic relationship

Introduction

MNSV, a member of the genus *Carmovirus* of the family *Tombusviridae* (Murphy *et al.*, 1995), has been found in melon and cucumber grown in greenhouse (Hibi and Furuki, 1985). The virus is transmissible mechanically and by the soil-inhabiting fungus *Olpidium radicale*.

MNSV is an isometric virus of approx. 30-nm diameter with a single-stranded, positive-sense RNA of 4.3 kb (Riviere and Rochon, 1990). Sequence analysis of the genome of MNSV Dutch (D) isolate (Riviere and Rochon, 1990) revealed that it contains five ORFs. An ORF near the 5-terminus of the genome is terminated with an amber codon. Its translation would yield a 29 K protein, and an 89 K read-through product (the putative RNA-dependent RNA polymerase). A small centrally located ORF encodes a 7A protein, the putative movement and nucleic acid-binding protein of carmoviruses (Marcos *et al.*, 1999). A 14K protein might represent a readthrough product of this ORF. The 3'-proximal ORF encodes the 42K CP (Riviere *et al.*, 1989; Ohshima *et al.*, 1994).

Several isolates of MNSV originate from the USA, the Netherlands, United Kingdom and Japan (Bos *et al.*, 1984; Hibi and Furuki, 1985; Riviere *et al.*, 1989; Matsuo *et al.*, 1991; Sano *et al.*, 1999). It has been reported that NH and NK isolates collected in Japan differed in the frequency with which they induced systemic infection of *Cucumis melo* L. (Matsuo *et al.*, 1991). Whereas NH isolate produced necrotic spots on inoculated and uninoculated upper leaves of cultivars Makuwa and Coromon, NK isolate produced necrotic spots on inoculated leaves only. S isolate was serologically distinct from NH and NK isolates (Matsuo, 1993) but similar to NK isolate in the frequency with which

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Abbreviations: aa = amino acid; BYDV = Barley yellow dwarf virus; CMV = Carnation mottle virus; CNV = Cucumber necrosis virus; CP = coat protein; ds = double-stranded; MNSV = Melon necrotic spot virus; MMLV = Moloney murine leukemia virus; nt = nucleotide; ORF = open reading frame; RT-PCR = reverse transcription–polymerase chain reaction; TCV = Turnip crinkle virus

it induced systemic infection of *Cucumis melo* L. (Matsuo *et al.*, 1991). In addition, virions of three above mentioned isolates contained one to three different subgenomic RNAs (Matsuo *et al.*, 1991).

In this work, we report the nucleotide sequences of genomes of NH and NK isolates of MNSV and compare the deduced amino acid sequences of proteins to identify which amino acid might be involved in movement of MNSV. Moreover, we describe phylogenetic relationships of genes of several MNSV isolates.

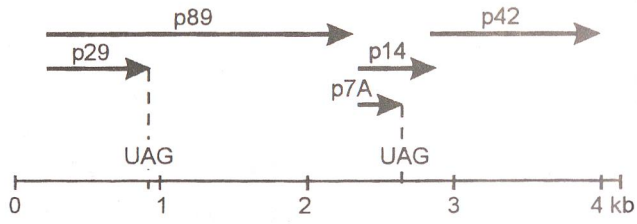

Fig. 1
Genomic map of MNSV NH and NK isolates
p89, p7A and p42 proteins are the putative RNA-dependent RNA polymerase (replicase), the putative movement protein, and CP, respectively.

Materials and Methods

Virus. MNSV NH and NK isolates were propagated on *Cucumis melo* L. cv. Arususeinu-natsukei 2 in greenhouse and purified according to Hibi and Furuki (1985). The genomic RNA of MNSV was extracted from the purified virus by phenol/chloroform.

Cloning. Two procedures were employed for the cDNA cloning of MNSV genomes. (1) First-strand cDNAs were synthesized from MNSV RNA with minus-strand primers using Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL) (Ohshima *et al.*, 1994). Second-strand cDNAs were synthesized from the first-strand cDNAs and dsDNA products were amplified with pairs of primers by PCR (Ohshima *et al.*, 1994). The primers were designed from the sequences of the MNSV Dutch (D) isolate genomic RNA (Riviere and Rochon, 1990) and the MNSV NH isolate p42 gene (Ohshima *et al.*, 1994). The amplified dsDNAs were digested with *Not*I and cloned into the *Not*I site of pBluescript II SK$^-$ vector (Stratagene). (2) First- and second-strand cDNAs were synthesized from MNSV RNA according to Gubler and Hoffman (1983) using the cDNA Synthesis System Plus (Amersham). For the first-strand cDNA synthesis, random hexanucleotide primers were used. The termini of dsDNAs were blunt-ended by T4 DNA polymerase (Nippon Gene). The 5′-termini of dsDNAs were phosphorylated by T4 polynucleotide kinase (Nippon Gene) and the dsDNAs were cloned into the *Sma*I site of dephosphorylated pBluescript II SK$^-$. The recombinant vectors were introduced into and multiplied in *Escherichia coli* XL1-Blue and then extracted by the boiling method according to Holms and Quigley (1981).

Sequencing. Nucleotide sequences of genomes of MNSV isolates were determined from several overlapping clones. ds DNAs were sequenced by the Dye Primer Cycle Sequencing Kit (Amersham). The M13 forward and reverse primers, or primers designed from the nucleotide sequence of genome of MNSV D (Riviere and Rochon, 1990), NH and NK isolates were used. Nucleotide sequences of genes of each isolate were determined using at least three cDNA clones.

Phylogenetic analysis. Multiple alignments of nucleotide sequences of genes were done using the Clustal W Program (Thomson *et al.*, 1994). Phylogenetic relationships of genes were determined by distance methods implemented in the PHYLIP package (Felsenstein, 1989). Distance matrices were calculated by DNADIST with the Kimura two-parameter option, and distance trees were constructed from these matrices by NEIGHBOR with implemented neighbor-joining method (Saitou and Nei, 1987). The obtained trees were displayed by DRAWGRAM to optimize the fitting of branch lengths to the Kimura's distance (Kimura, 1980). A bootstrap value for each internal node was calculated by performing 100 random resamplings using SEQBOOT (Felsenstein, 1985) and by synthesizing the resulting set of trees using CONSENSE.

Results and Discussion

Genomic map

The genomic map for MNSV NH and NK isolates based on ORFs deduced from nucleotide sequences is shown in Fig. 1. It contains five ORFs encoding p29 protein (the pre-readthrough domain of p89), p89 protein (the readthrough protein of p29), p7A protein (the pre-readthrough domain of p14), p14 protein (the readthrough protein of p7A) , and p42 protein (coat protein). The genomic map of NH and NK isolates corresponds well to that of MNSV D isolate reported earlier (Riviere and Rochon, 1990).

p29 protein

The nucleotide sequences of p29 genes of NH and NK isolates were 804 nt long and encoded 268 aa (Fig. 2). NH and NK p29 proteins shared a high degree of amino acid sequence similarity (98.5%, Table 1). The actual function of p29 protein is unknown.

Table 1. Nucleotide (above diagonal) and amino acid sequence identities (%) of p29 and p89 proteins of MNSV isolates NH, NK and D

	NH	NK	D
NH		98.1/98.2	93.2/93.6
NK	98.5/97.7		93.1/93.7
D	96.6/97.5	96.6/97.9	

p29/p89 values are shown.

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                10         20         30         40         50         60         70         80
NH      MDTGLKFLVS GGLATSSVIR KVSAVSSLDS SLPSSSILSA IHGSWTSAIS HDCSKIAKVA TVIGIGYLGV RIGAAWCRRA    80
NK      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------T    80
D       ---------- ---------- ---------- ---------- ---------- ---------- AIV------- ---------T    80

              90         100        110        120        130        140        150        160
NH      PGITNSIITY GEEVVEQVKV DIDEEAEEES DTGEEIVVGT IGIGIHTNVN PEVRAKRRHR SRPFIKKIVN LTKNHFGGCP   160
NK      ---------- ---------- ----D----- ---------- ---------- ---------- ---------- ----------   160
D       ---------- ---------- ----D----- -I-------- ---------- ---------- ---------- ----------   160

              170        180        190        200        210        220        230        240
NH      DSSKSNVMAV SKFVYEQCKQ HNCLPHQTRL IMSVAVPLVL SPDMYDISSK ALLNSEILTE NRATLDRLKT LDGWLSHLVC   240
NK      ---------- ---------- ---------- ---------F N--------- ---------- ---------- ----------   240
D       ---------- ---------- ---------- ---I------ ---------- ---------- ---------- -----T----   240

              250        260        270        280        290        300        310        320
NH      HPLSTKAWRR AIDNLCGLPD WKAFKLVN*G CLEELAGFCT SVRRGTHPDM TEFPQDRPIK TRKLYCLGGV GTSVKFNVHN   320
NK      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   320
D       ----A----- ---------- ---------- ---------- ---------- ---------- ---------- ----------   320

              330        340        350        360        370        380        390        400
NH      NSLANLRRGL IERVFFVEND KKELEPAPKP LSGVFDRLTW FRRKLHNIVG THSSISPGKS LDFYTGRRRT IYEGAVKSLE   400
NK      ---------- ---------- --D------- ---------- ---------- --------QF ---------- ----------   400
D       ---------- V--------- ---------- ---A------ ------S--- --------QF ---------- ----------   400

              410        420        430        440        450        460        470        480
NH      GLSVQRRDAY LKTFVKAEKI NTTKKPDPAP RVIQPRNVRY NVEVGRYLRR FEHYLYRGID EIWNGPTIIK GYTVEQIGKI   480
NK      ---------- ---------- ---R------ ---------- ---------- -------R-- ---------- ----------   480
D       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   480

              490        500        510        520        530        540        550        560
NH      ARDAWDSFVS PVAIGFDMKR VRPHVSSDAL KWEHSVYLDA LGHDSYLAEL LEWQLVNKGV GYASDGMIKY KVDGCRMSGD   560
NK      ---------- ---------- FDQ------- ---------- FC-------- ---------- ---------- ----------   560
D       ---------- ---------- FDQ------- ---------- FC-------- -K-------- ---------- ----------   560

              570        580        590  +++   600        610        620        630        640
NH      MNTAMGNCLI ACAITHDFFR SRGIRARLMN NGDDCVVICE KECAAVVKAD MVRHWRQFGF QCELECDAEV FEQIEFCQMR   640
NK      ---------- ---------- -P-------- ---------- ---------- ---------- ---------- ----------   640
D       ---------- ---------- ---------- ---------- ---------- ---------- ---------I ----------   640

              650        660        670        680        690        700        710        720
NH      PVYDGEKYVM VRNPLVSLSK DSYSVGPWNG VNHARKWVNA VGLCGLSLTG GIPVVQSYYN MMIRNTQSVN SSGILRDVSF   720
NK      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------   720
D       ---------- ---------- ---------- I--------- ---------- ---------- ---------- ----------   720

              730        740        750        760        770        780        790
NH      ASGFRELARL GNRKSGAISE DARFSFYLAF GITPDLQRAM ESDYDAHTIE WGFVPQGNPR IQPISWTLNE L            791
NK      ---------- ----N----- ---------- ---------- ---------- ---------- ---------- -            791
D       ---------- ---------- ---------- ---------- ---------- ---------- ---------- -            791
```

Fig. 2

Amino acid sequence alignment of p29 and p89 proteins of various MNSV isolates

The sequences are numbered from the p29 start codon. GDD motif is marked by (+) above the sequence of NH isolate. Dash indicates identical amino acid in comparison to NH isolate. Asterisk indicates the amber stop codon of p29 protein.

p89 protein

The nucleotide sequences of p89 genes of NH and NK isolates were 2373 nt long, contained an amber codon for the aa at position 269, and encoded 522 aa (Fig. 2). p89 protein of NH isolate shared a high amino acid sequence similarities with those of NK (97.7%) and D (97.5%) isolates (Table 1). P89 proteins of NH and NK isolates contained a GDD motif and surrounding conserved amino acids, characteristic of most viral RNA-dependent RNA polymerase (Kamer and Argos, 1984; Riviere and Rochon, 1990). It has been reported by Riviere and Rochon (1990) that MNSV p89 shares a high amino acid sequence similarity with the putative polymerases of Carnation mottle virus (CMV, genus *Carmovirus*) (Guilley *et al.*, 1985) and Turnip crinkle virus (TCV, genus *Carmovirus*) (Carrington *et al.*, 1989), Cucumber necrosis virus (CNV, genus *Tombusvirus*) (Rochon and Tremaine, 1989), and Barley yellow dwarf virus (BYDV, family *Luteoviridae*) (Miller *et al.*, 1988).

p14 and p7A proteins

The nucleotide sequences of p7A genes of NH and NK isolates were 195 nt long and encoded 65 aa (Fig. 3). p7A protein, the pre-readthrough domain of p14 protein of NH and NK isolates shared a very high degree of amino acid sequence similarity (98.5%, Table 2). There was only one amino acid difference between NH and NK p7A proteins:

```
                10         20         30         40         50         60         70         80
NH     MDSQRTVEQT NPRGRSKERG DSGGKQKNSM GRKIANDAIS ESKQGVMGAS TYIADKIKVT INFNF*CMAC YRCDSSPGDY     80
NK     ---------- -----I---- ---------- ---------- ---------- ---------- ---------- ----------     80
D      --------L- ---------- ---------- ---------- ---------- ---------- ---------- C----------     80

                90         100        110        120        130
NH     SGALLILFIS FVFFYITSLS PQGNTYVHHF DNSSVKTQYV GISTNGDG                                      128
NK     ---------- ---------- ---------- ---------- --------                                      128
D      ---------- ---------- ---------- -S-------- --------                                      128
```

Fig. 3

Amino acid sequence alignment of p14 and p7A proteins of various MNSV isolates

The sequences are numbered from the p7A start codon. Dash indicates identical amino acid in comparison to NH isolate. Asterisk indicates the amber stop codon of p7A protein.

NH p7A protein had serine at position 16 while NK p7A had isoleucine there. It has been suggested that this protein might be involved in the transport ("movement protein") of carmoviruses (Guilley *et al.*, 1985; Carrington *et al.*, 1989; Riviere and Rochon, 1990; Marcos *et al.*, 1999). NH and NK isolates differed in the frequency with which they induced systemic infections of *Cucumis melo* L. (Matsuo *et al.*, 1991). NK isolate produced necrotic spots on inoculated leaves of *Cucumis melo* cvs. Makuwa and Coromon, while NH isolate produced necrotic spots not only on inoculated leaves but also on uninoculated upper leaves. Thus the serine at position 16 in p7A protein may be involved in transport of MNSV (in this case in systemic infection), but we cannot exclude CP, because CMV CP is also considered to have the transport function (Francki *et al.*, 1991; Murphy *et al.*, 1995). It has been reported that the frequency of systemic infection of *Cucumis melo* L. with NK isolate was similar to that with S isolate but not with NH isolate (Matsuo *et al.*, 1991). E.g., leucine at position 28 and glycine at position 301 in CP (Fig. 4) may be involved in systemic infection of MNSV. The function of the readthrough p14 protein is unknown.

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                10         20         30         40         50         60         70         80
NH     MAMVKRINNL PTVKLAKQAL PLLTNPKIVN KAIDVVPLVV QGGQKLSKAA KRLLGAYGGN ISYTEGARPG AISAPVAISR     80
NK     ---------- ---------- -------L-- ---------- ---------- ---------- -------K-- ----------     80
H      ---------- ---------- ---------- ---------- ---------- ---------- -------K-- ----------     80
D      ---------- ---------- ---A---L-- ---------- ---R------ ---------- -------K-- ----------     80
S      ---------- ---------- ---A---L-- ---------- -S-------- ---------- ---------- ----------     80

                90         100        110        120        130        140        150        160
NH     RVAGMKPRFV RSEGSVKIVH REFIASVLPS NDLTVNNGDV NIGKYRVNPS NNALFTWLQG QAQLYDMYRF TRLRFTYIPT    160
NK     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------    160
H      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------    160
D      ---------- ---------- ---------- S--------- ---------- ---------- ---------- ----I-----    160
S      ---------- ---------- ---------- ---------- -V-------- ---------- ---------- ----------    160

                170        180        190        200        210        220        230        240
NH     TGSTSTGRVS ILWDRDSQDP LPIDRAAISS YAHYADSTPW AENVLVVPCD NTWRYMNDTN AVDRKLVDFG QFLFATYSGA    240
NK     ---------- ---------- ---------- -------A-- ---------- ---------- ---------- ----------    240
H      ---------- ---------- ---------- -------A-- ---------- ---------- ---------- ---L------    240
D      ---------- L--------- ---------- ---S---A-- ---------- ---------- ---------- ----------    240
S      ---------- ---------- ---------- ---C---S-- ---------- ---------- ---------- ----------    240

                250        260        270        280        290        300        310        320
NH     GATAHGDLYV EYAVEFKDPQ PIAGMVCMFD RLVSFSEVGS TIKGVNYIAD RDVITTGGNI SVNINIPGTY LVTIVLNATS    320
NK     ---------- ---------- ---------- ---------- ---------- ---------- G--------- -------V--    320
H      ---------- ---------- ---------- ---------- ---------- ---------- G--------- -----F----    320
D      -S-------- ---------- ---------- ----L----- ---------- ---------- G--------- ----------    320
S      ---------- ---------- ---------- -S-------- ---------- ---------- G--------- ----------    320

                330        340        350        360        370        380        390
NH     IGSLTFTGNS KLVGNSLNVT SSGASALTFT LNSTGVPNST NSSFSVGTVV ALTRVRMTIT RCSPETAYLA               390
NK     ---------- ---------- ---------- ---------- ---------- ---------- ----------               390
H      ---------- ---------- ---------- ---------- ---------- ---------- ----------               390
D      --P------- --------L- ---------- ---------S D--------- ---------- ----------               390
S      ---------- ---------- GG-------- -----I--NS D--------- G--------- ----------               390
```

Fig. 4

Amino acid sequence alignment of p42 coat protein of various MNSV isolates

The sequences are numbered from the p42 start codon. Dash indicates identical amino acid in comparison to NH isolate.

Table 2. Nucleotide (above diagonal) amino acid sequence (below diagonal) identities (%) of p14 and p7A proteins of MNSV isolates NH, NK and D

	NH	NK	D
NH		99.0/98.5	96.9/97.0
NK	99.2/98.5		96.4/96.5
D	97.6/98.5	96.9/96.9	

P14/p7A values are shown.

p42 protein

The nucleotide sequences of p42 (CP) genes of NH and NK isolates were 1170 nt long and encoded 390 aa (Fig. 3). To date, nucleotide sequences of p42 genes of four Japanese isolates (NH, NK, S, and H) and a Dutch isolate have been determined (Riviere *et al.*, 1989; Ohshima *et al.*, 1994; Matsuo *et al.*, 1998; Sano *et al.*, 1998). They are compared in Table 3. The p42 genes shared 95.1–99.0% amino acid sequence identity.

Phylogenetic relationship

Phylogenetic relationships of various MNSV isolates were analyzed on the basis of the nucleotide sequences of all genes, p89 gene and p42 gene (Fig. 4). All three phylogenies show that NH isolate is closest to NK isolate. It has been reported that the low frequency of systemic infection of *Cucumis melo* L. with NK isolate is similar to that of S isolate but not that of NH isolate (Matsuo *et al.*, 1991). On the other hand, NH and NK isolates were found serologically related to each other but distinct to S isolate (Matsuo *et al.*, 1991). Thus, biological characteristic does not correspond to phylogenetic characteristics of these three Japanese isolates. The p42 (CP) phylogeny shows that NH, NK, H and probably also D isolates but not S isolate form a group in the MNSV species. In spite of the fact that the D isolate collected in the Netherlands is most geographically from the four Japanese isolates, D isolate is located near

Table 3. Nucleotide (above diagonal) and amino acid sequence (below diagonal) identities (%) of p42 proteins of MNSV isolates NH, NK, D, and S

	NH	NK	H	D	S
NH		97.4	95.6	88.7	84.9
NK	98.7		96.8	90.0	85.2
H	98.7	99.0		88.8	85.1
D	95.9	96.2	96.2		84.7
S	96.2	96.2	95.6	95.1	

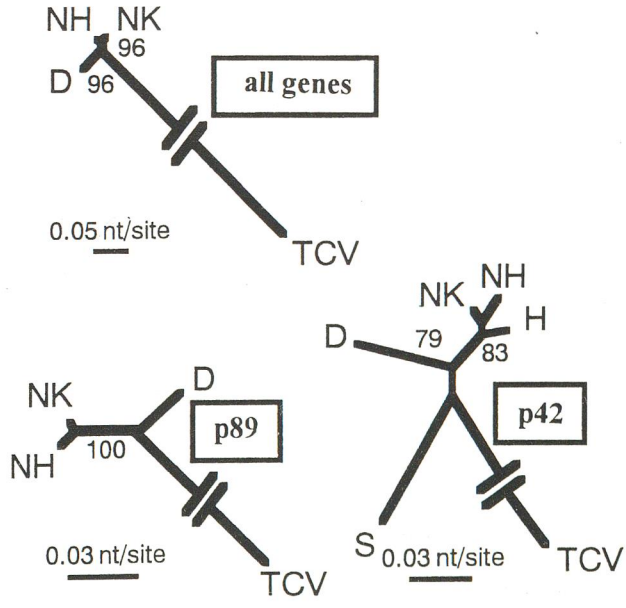

Fig. 5
Phylogenetic trees based on the nucleotide sequences of all genes, p89 gene and p42 gene of various MNSV isolates
The percentage of bootstrap replications (confidence) in which each node was recovered is given when above 70%. The scale bar represents the Kimura distance for each branch length. The phylogenetic trees were constructed using Turnip crinkle virus (TCV) as the out group.

NH and NK isolates in this phylogeny, suggesting that S isolate may be considered a distinct one differing from the others.

Footnote. The nucleotide sequence data of MNSV NH and NK isolates reported in this paper are deposited at the DDBJ, EMBL and GenBank databases under Acc. Nos. AB044291 and AB044292, respectively.

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